Phorbolester 12-Myristate 13-Acetate Up-regulates the Transcription of MUC2 Intestinal Mucin via Ras, ERK, and NF-κB*

Hae-Wan Lee,a–e Dae-Ho Ahn,a,b,d Suzanne C. Crawley,e–k Jian-Dong Li,f James R. Gum, Jr.,e–k Carol B. Basbaum,a Nancy Q. Fan,b David E. Szymkowski,h Sang-Young Han,a–e Bong H. Lee,a–j Marvin H. Sleisenger,e–k and Young S. Kim,a–e,k

From the *Gastrointestinal Research Laboratory, Veterans Affairs Medical Center, San Francisco, California 94121, the †Departments of Medicine and Anatomy, University of California, San Francisco, California 94143, the ‡Gonda Department of Cell and Molecular Biology, House Ear Institute, and the Department of Otolaryngology, University of Southern California, Los Angeles, California 90057, and the Roche Bioscience, Palo Alto, California 94304.

MUC2 is a secretory mucin normally expressed by goblet cells of the intestinal epithelium. It is overexpressed in mucinous type colorectal cancers but down-regulated in colorectal adenocarcinoma. Phorbolester 12-myristate 13-acetate (PMA) treatment of colon cancer cell lines increases MUC2 expression, so we have undertaken a detailed analysis of the effects of PMA on the promoter activity of the 5′-flanking region of the MUC2 gene using stably and transiently transfected promoter reporter vectors. Protein kinase C inhibitors (bisindolylmaleimide, calphostin C) and inhibitors of mitogen-activated protein/extracellular signal regulated kinase kinase (MEK) (PD98059 and U0126) suppressed up-regulation of MUC2. Src tyrosine kinase inhibitor PP2, a protein kinase A inhibitor (KT5720), and a p38 inhibitor (SB 203580) did not affect transcription. Western blotting and reverse transcription-PCR analysis confirmed these results. In addition, co-transfections with mutants of Ras, Raf, and MEK showed that the induction of MUC2 promoter activity by PMA required these three signaling proteins. Our results demonstrate that PMA activates protein kinase C, stimulating MAP kinase through a Ras- and Raf-dependent mechanism. An important role for nuclear factor κB (NF-κB) was also demonstrated using the inhibitor caffeic acid phenethyl ester and electrophoretic mobility shift assays. Such identification of pathways involved in MUC2 up-regulation by PMA in the H3M3 colon cancer cell line may serve as a model for the effects of cytokines and growth factors, which regulate MUC2 expression during the progression of colorectal cancer.

Mucins are very large proteins featuring O-glycosylated, tandemly repeated serine- and threonine-rich regions. They are synthesized by the epithelial cells lining the gastrointestinal, respiratory, and genitourinary tracts. Genomic and cDNA sequencing has identified at least fifteen different mucin genes, which encode either secretory or membrane-associated proteins (1–3). Mucins are expressed in a characteristic tissue- and cell type-specific manner. MUC2 is one of four structurally related but differentially expressed secretory mucins located on chromosome 11p15. Within the intestinal epithelium, MUC2 is highly expressed in goblet cells but absent from the absorptive cell type (4–7). Altered expression of mucin genes occurs in many epithelial cancers. Specifically, low expression of MUC2 has been reported in colorectal adenocarcinoma, whereas a very high level of MUC2 expression is observed in mucinous colorectal carcinomas, a distinct histological type of colorectal cancer (4, 5, 7, 8). However, relatively little is known about the mechanisms responsible for regulation of MUC2 gene expression in vivo.

Phorbolesters such as PMA1 function as tumor promoters and have been reported to modulate diverse cellular responses such as gene transcription, cellular growth and differentiation, programmed cell death, the immune response, and receptor desensitization through protein kinase C (PKC) signaling pathways. PMA can substitute for diacylglycerol, the endogenous activator of PKC, and it has been used as a model agent to study the mechanisms utilized by growth factors, hormones, and cytokines to regulate growth and differentiation of cells (9–11). Phorbolesters, as well as cytokines and bacterial lipopolysaccharides, have been shown to up-regulate mucin genes (12–17). We recently reported that PMA up-regulates several mucin genes, including MUC2, in colon cancer cell lines (18). However, detailed analysis of the downstream signaling pathways involved in PMA/PKC-induced up-regulation of MUC2 has not been done. One well studied mode of PKC-mediated signaling involves transmission of signals from PKC to mitogen-activated protein kinases (MAPKs). MAPK activation by PMA has been reported to occur via both Ras-dependent and Ras-independent pathways: PC-12 rat adrenal pheochromocytoma (19, 20), Jurkat leukemic T cells (21), and primary rat ventricular myocytes (22) have exhibited Ras-dependent...
activation of MAPK by PMA; but in NIH3T3 mouse myeloma cells (23), COS-1 (24), and 293 embryonic kidney cell lines (25), this activation appears to be Ras-independent. Thus, the involvement of Ras in signaling processes initiated by PMA appears to be cell type-dependent and specifically determined by which signaling pathways have been activated and/or the cell's repertoire of kinases.

HM3 human colon cancer cell line contains the most common K-ras mutation type found in colorectal cancers, the glycine to aspartate mutation at codon 12. Because many growth factors and cytokines utilize Ras-dependent signaling pathways, this cell line serves as a model system for studying alterations in gene expression that occur in the progression of colorectal cancers. In this study we show that PMA stimulates expression of the MUC2 gene in HM3 cells through activation of PKC. Such MUC2 up-regulation is Ras- and Raf-dependent, requiring activation of the MEK/ERK signaling pathway, and ultimately involves activation of a nuclear factor, NF-κB.

**EXPERIMENTAL PROCEDURES**

**Materials**—TriReagent and PMA were obtained from Sigma, Bisindolylmaleimide I from Calbiochem, calphostin C, MT320, and SB203580 were purchased from Calbiochem. Antibodies for ERK1/2 (p44/p42 MAPK), SAPK/JNK, and p38 were purchased from New England Biolabs/Cell Signaling. Secondary antibodies were purchased from Zymed Laboratories Inc., South San Francisco, CA. Oligonucleotides were synthesized by Operon, Alameda, CA.

**Tissue Culture**—HM3, a subclone of the LS174T adenocarcinoma cell line, was maintained at 37 °C in 5% CO₂ atmosphere in Dulbecco's modified Eagle's minimum medium containing 10% heat-inactivated fetal bovine serum with penicillin and streptomycin. HM3 cells stably transfected with MUC2 promoter (−2864/19) pGL2 (Promega) luciferase construct (HM3M2) were maintained in medium containing 600 μg/ml G418 (Geneticin). Inhibitor Assays—The HM3M2 cells were serum-starved overnight and then pretreated with inhibitors for 1 h before exposure to 0.25 μM PMA for 4 h. Calphostin C was used under a fluorescent lamp of 15 watts located 15 cm above the plates.

**RNA Isolation and RT-PCR**—Total RNA was isolated using TriReagent (Molecular Research Center Inc.), and 3 μg was primed with random hexamers and reverse transcribed using Superscript II (Invitrogen) in a final volume of 50 μl. One microliter of this mixture was PCR-amplified in a 10-μl reaction using AmpliTaq DNA polymerase (Applied Biosystems) with the addition of 5% dimethyl sulfoxide. Primers for MUC2 were (forward) 5'TGC CTG CCC GCT TGG TTG GTG 3 and (reverse) 5' CAG CTC CAG CAT GAG TGC C-3'. 18S rRNA was simultaneously amplified as an internal standard, using a 9:1 ratio of 3' blocked/unblocked (alternate) primers from Ambion QuantumRNA™ 18S rRNA kit. The PCR reaction mixture was denatured at 94 °C for 5 min followed by 30 cycles at 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s. Alternatively, blocked and unblocked primers for β-actin (forward) 5' ATC TGG CAC CAC ACC TTC TAC AAT GAG CTG C-3' and (reverse) 5' CCG CAT ACT CCT GGT TGA TCC ACA GTG G-3') were used to amplify this message as an internal control. All PCR products were separated on ethidium bromide-stained gels, and band intensities were integrated using NIH Image software.

**Plasmids**—Transfected Transfection, and Luciferase Reporter Assays—Plasmids were prepared using the Plasmid MAXI Prep Kit from Qiagen. The expression vectors for Ha-Ras, dominant-negative N17Ras, and constitutively activated v-Ha-Ras were a gift from Geoffrey Cooper (Boston University, Boston, MA). HMEK1(K97R) was a gift from Alan Saltiel (Parke-Davis Pharmaceutical Research Division, Ann Arbor, MI). Expression vectors for wild type and dominant-negative pp90rsk (pp90rskΔC) were a gift from Warner Greene, University of California San Francisco. MUC2 promoter reporter assays employed pGL2 vector (Promega) containing various regions of the MUC2 gene 5'-flanking sequence described previously (26, 27). In addition, the MUC2 promoter region −1528/−907, subcloned upstream of the thymidylate kinase promoter, served as the expression construct for variations in transcription efficiency. Inhibitor experiments using stably transfected HM3M2 cells were carried out similarly with overnight serum starvation, 1 h of inhibitor pretreatment, and then 4 h of PMA treatment. Promoter activity was assessed using the Luciferase Assay Kit from Promega. Data is presented graphically as the average of four replicates from a representative experiment with standard deviation provided by error bars and statistical significance determined by Student's t test with confidence levels indicated in the figure legends.

**Western Blotting**—After various treatments, total cell lysates were prepared in 10 mM Tris-HCl, pH 8.5, 0.4 mM EDTA, 2% SDS, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM phenethylisulfonfyl fluoride, 10 mM sodium fluoride, 0.4 mM sodium orthovanadate, and 10 mM pyrophosphate. The protein concentration of supernatant was determined by using the bichinchonic acid-based BCA Protein Assay Kit (Pierce). Equal amounts of protein were subjected to 10% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose (28), which was blocked with 3% bovine serum albumin in Tris-buffered saline (TBS); 10 mM Tris-HCl with 150 mM NaCl, pH 7.4), probed with specific primary antibodies, washed with TBS containing 0.1% Tween, and then probed with secondary antibodies conjugated to horseradish peroxidase. Immunoreactive bands were visualized by chemiluminescence using the Renaissance kit (PerkinElmer Life Sciences).

**Electrophoretic Mobility Shift Assays (EMSA)**—Nuclear extracts were prepared according to Ref. 29. Protein concentrations were determined using the Bradford assay method (Bio-Rad). A double-stranded oligonucleotide probe corresponding to the human MUC2 promoter region from −1458 to −1430 (5'-CGCTTTGTTTGCTTCCCCAGGCTAGTC-3') was used as a radiolabeled probe for 20 min in a solution containing 3 μg of protein in 10 mM HEPES-KOH at pH 7.9, 210 mM NaCl, 0.75 mM MgCl₂, 0.1 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM phenethylisulfonfyl fluoride, and 12.5% glycerol before separation on a 4% (19:1 acrylamide/bisacrylamide) polyacrylamide gel in Tris acetate-EDTA buffer. Specificity of protein binding to radiolabeled oligonucleotide was determined by a modification of 10-fold excess of unlabelled competing oligonucleotide. For supershift assays, antibodies specific for p50, p65, or c-Rel subunits of NF-κB (Santa Cruz Biotechnology) were preincubated with nuclear protein for 30 min on ice.

**RESULTS**

**PMA Up-regulates MUC2 mRNA Level and Induces MUC2 Transcriptional Activity**—PMA increased MUC2 mRNA in a time- and dose-dependent manner with the peak effect at 4 h (Fig. 1A). Densitometric analysis revealed that PMA caused a 4-fold increase in mRNA levels after 4 h at 0.5 μM concentration (Fig. 1B). In addition, PMA up-regulated transcriptional activity of the MUC2 luciferase reporter in HM3M2 cells in a dose-dependent manner up to 0.5 μM (Fig. 1C).

**PMA Increases the Transcriptional Activity of MUC2 Promoter Reporter Constructs**—Stably transfected MUC2 promoter deletion constructs all showed significant (p < 0.001) increases in activity upon treatment with PMA (Fig. 2). As reported previously (26), constructs containing sequence up-stream of base −1308 were significantly more active than smaller constructs. The −1628/−19 construct was chosen for further experiments as it exhibited the highest level of PMA responsiveness.

**PKC but Not PKA Mediates the Activation of MUC2 Promoter Activity by PMA**—Bisindolylmaleimide I is a highly selective cell-impermeable PKC inhibitor that acts as a competitive inhibitor for the ATP-binding site of PKC. 0.1 μM bisindolylmaleimide inhibited PMA induction of MUC2 promoter activity by 80% (Fig. 3A). Another specific inhibitor of PKC, calphostin C, also inhibited PMA-induced MUC2 promoter activation by ~80%. RT-PCR was used to confirm that bisindolylmaleimide and calphostin C also caused a reduction in endogenous MUC2
transcript levels (Fig. 3B). It should be noted that in these experiments RT-PCR provides a less sensitive measurement of the effects of PMA and inhibitors due to the relatively high levels of MUC2 in untreated cells, resulting from the stable nature of the MUC2 transcript (26). KT5720, a potent specific inhibitor of PKA, did not inhibit the induced up-regulation of MUC2 promoter activity or message (Fig. 3). Similarly, Src-tyrosine kinase inhibitor PP2 (0.5 μM) had no significant effect \( (p < 0.05) \) on MUC2 promoter activity. PP3, a negative control for PP2, and epidermal growth factor (EGF) receptor kinase inhibitor also failed to inhibit PMA-induced MUC2 up-regulation. Thus, PKC inhibitors were able to block the up-regulation of MUC2 promoter activity, suggesting that this kinase is responsible for PMA-induced MUC2 up-regulation.

Activation of MEK and ERK1/2 but Not p38 Mediates MUC2 Promoter Activation by PMA.—To investigate the role of MEK, we used PD98059 and U0126 to selectively block the activity of MEK. These inhibitors completely blocked MUC2 induction by PMA (Fig. 4A) and reduced the level of endogenous MUC2 transcript, estimated using RT-PCR (Fig. 4B). SB203580, a highly specific and cell-permeable inhibitor of p38 MAP kinase, failed to inhibit the MUC2 promoter activity induced by PMA (Fig. 4A). AG126, an inhibitor of ERK2 tyrosine phosphorylation also significantly inhibited PMA-induced MUC2 up-regulation in a dose-dependent manner (Fig. 4C). Because pp90rsk is a possible downstream effector of ERK1/2 signaling, domi-
Involvement of MEK, Raf, and Ras in Induction of MUC2 Promoter Activity by PMA—Transient transfections with dominant-negative MEK significantly (p < 0.001) inhibited basal and PMA-stimulated activity of the MUC2 promoter. On the other hand, transfection with wild-type MEK activated the MUC2 promoter in the absence of PMA, and this activation was not significantly augmented by the addition of PMA (Fig. 6A). Transient co-transfections with dominant-negative Raf completely blocked PMA-mediated MUC2 induction in a dose-dependent manner (Fig. 6B). MUC2 promoter reporter co-transfections using wild-type Ha-Ras did not affect transcriptional activities, with or without addition of PMA. On the other hand, dominant-negative N17Ras inhibited both MUC2 promoter activation by PMA and basal promoter activity as well. v-Ha-Ras induced a 5-fold increase in MUC2 transcription rate, similar to levels observed upon PMA treatment. When HM3 cells transfected with v-Ha-Ras were also treated with PMA, a significant (p < 0.001) synergistic effect was observed (Fig. 6C).

**NF-κB Is Involved in the Induction of MUC2 Promoter Activity by PMA**—CAPE, an inhibitor of NF-κB activation, completely blocked the induction of MUC2 by PMA (Fig. 7A). This result was confirmed using RT-PCR to estimate changes in endogenous MUC2 transcript levels (Fig. 7A, inset). In EMSA, nuclear extracts from untreated HM3 cells failed to exhibit binding of the transcription factor to the NF-κB oligonucleotide probe. However, nuclear extracts from PMA-treated cells contained a protein that bound to the probe and was displaced by the addition of unlabeled competitor oligonucleotide. Incorporation of a mutation within the NF-κB consensus site (MT3) produced an oligonucleotide that failed to block binding of the transcription factor to the probe.

**FIG. 4.** MUC2 promoter activity is reduced by inhibitors of MEK and ERK. A, HM3M2 cells were treated with PMA or vehicle after 1 h of pretreatment with various inhibitors at increasing concentrations. Inhibitor concentrations were: 0, 50, and 100 μM PD98059; 0, 0.5, 2, 5, and 10 μM UO126; and 0, 0.5, 2, 5, and 10 μM SB203580. Luminometer readings were normalized with respect to values obtained from untreated cells. Values for activities are the average of four replicates with standard deviation represented by error bars. (**, p < 0.001, compared with assays without added inhibitor). B, HM3M2 cells were treated as above, and then total RNA was analyzed by RT-PCR. Inhibitor concentrations were: 75 μM PD98059; 5 μM UO126; 2 μM SB203580. Results shown are representative of at least two separate experiments. C, HM3M2 cells were pretreated with indicated concentrations of AG126, exposed to PMA for 4 h, and then assayed for luciferase activity. Values were normalized with respect to untreated controls and are given as the average of four assays with the standard deviation shown by error bars. (**, p < 0.001, compared with assays without added inhibitor). D, HM3 cells were co-transfected with 4 μg each of -1626/+19 MUC2 promoter construct and expression vector for wild-type (WT) or dominant-negative (DN) pp90rsk. After 2 days, cells were treated with PMA and assayed as usual. Activities were normalized to readings for Renilla luciferase internal control and are shown as the average of four assays with standard deviation shown as error bars.

**FIG. 5.** ERK1/2 is activated by PMA. HM3 cells were treated with PMA (0.25 μM) for the indicated periods of time; 20 μg of total cell lysate protein was subjected to electrophoresis. Nitrocellulose blots were probed with antibodies to phosphorylated and non-phosphorylated forms of ERK1/2, JNK (p54/p46), and p38 as indicated. In one experiment (20 μg, right lane) cells were pretreated for 1 h with PD98059 inhibitor (75 μM) before addition of PMA for 20 min as a control.
binding to the labeled oligonucleotide, indicating the specificity of the interaction of the transcription factor. Addition of the p65 antibody caused this band to supershift (Fig. 7B). Addition of p50 antibody seemed to have little effect in this experiment, but in other experiments a slight decrease in the intensity of the band was observed upon addition of the p50 antibody, indicating a possible interaction. Thus it appears that the p65 subunit is involved with MUC2 up-regulation by PMA, but involvement of the p50 subunit remains to be demonstrated. Antibody to c-Rel failed to cause a supershift. A luciferase reporter vector (TK-LUC) containing the thymidylate kinase minimal promoter inserted downstream from the NF-κB site of MUC2 (−1528/−1307) was activated by PMA (Fig. 7C), indicating that the NF-κB binding site in this region functions as an enhancer of MUC2 transcription.

**DISCUSSION**

In this study, we first determined that PMA increased MUC2 message levels in HM3 cells. The transcriptional activity of a
MUC2 -2864/+19 promoter/reporter construct stably transfected into HM3 human colon cancer cell line HM3M2 was similarly increased by PMA, indicating that PMA-induced up-regulation occurs at the transcriptional level. We also determined that PMA treatment was able to significantly up-regulate transcription from all MUC2 deletion constructs containing the previously identified proximal Sp1/CACC box elements(s). The greatest promoter activities, however, were observed for larger constructs (−1308/+19, −1628/+19, and −2864/+19). The −1628/+19 construct was chosen for further experiments because of its high basal activity and because it contained two cis elements previously determined to be functional, namely the Sp1/CACC box and the NF-κB sites (26, 27).

To identify the signaling pathways involved in PMA-mediated MUC2 up-regulation, the effects of various chemical inhibitors and dominant-negative expression vectors on PMA-stimulated MUC2 transcription were measured. A schematic summary of our findings is shown in Fig. 8. As expected, PKC inhibitors bisindolylmaleimide I and calphostin C blocked MUC2 up-regulation by PMA, indicating that PKC activation by phorbol ester is directly responsible for the induction of MUC2 promoter activity. The induction of MUC2 transcription by PMA was not suppressed by either PKA inhibitor KT5720 or by Src type tyrosine kinase inhibitor PP2. The slight, significant up-regulation by both KT5720 and EGF receptor kinase inhibitor PP3 are consistent with relief of feedback inhibition of Raf by PKA (30). PP3 is used as a negative control for PP2; however, the failure of PP3 to inhibit PMA-stimulated up-regulation also indicates that EGF receptor transactivation via PMA-induced shedding of EGF likely does not occur. The apparent lack of involvement of Src type tyrosine kinases is in contrast to previous results obtained using the same cell line, which showed that lipopolysaccharide-induced MUC2 up-regulation was Src-dependent. This indicates that PKC phosphorylation of Raf circumvents the requirement for Src-mediated phosphorylation of Raf.

PKC-mediated activation of the Ras/Raf/MEK/ERK signaling pathway has been well studied, but the exact nature and order of events leading to Raf activation remain to be fully elucidated, involving phosphorylation reactions catalyzed by several kinases, conformational changes, and translocation to the plasma membrane by Ras (30). Dominant-negative N17Ras is a membrane-associated H-Ras with an asparagine-to-serine substitution at codon 17, yielding a Ras protein with a high affinity for GDP. The resulting N17Ras-GDP complex is thought to function as a dominant-negative inhibitor of Ras activation through sequestration of the guanine nucleotide exchange factor(s) required for normal GDP release from the (inactive) Ras-GDP complex (31, 32). The ability of N17Ras to inhibit PMA-mediated Raf activation has been reported to be cell line-dependent (19–25). In the case of HM3 cells, co-transfection with the dominant-negative mutant N17Ras was able to inhibit basal as well as PMA-stimulated MUC2 transcription. Thus Raf activation requires interaction with activated Ras and phosphorylation by PMA-activated PKC. In addition, dominant-negative Raf very effectively inhibited MUC2 transcriptional activity induced by PMA. This inhibition occurred in a dose-dependent fashion, demonstrating that Raf, along with Ras, participates in MUC2 transcriptional activation by PMA and that both are required.

Ras is thought to activate a number of signaling pathways, including the Raf/MEK/ERK MAP kinase pathway, the MEKK/SEK/JNK pathway, a phosphatidylinositol 3-kinase/Akt/NF-κB pathway, a p120GAP/p190Rac/NF-κB pathway, and a Raf/MEKK1/IκK/IκB/NF-κB pathway (30, 33–35). To identify relevant events downstream from Raf and, in particular, to identify which MAP kinase pathways were involved in MUC2 expression, various kinase inhibitors were tested for their ability to reduce MUC2 transcription. Specific inhibitors of MEK (PD98059, U0126) completely inhibited basal as well as PMA-induced MUC2. To confirm the involvement of MEK in mediating the effects of PMA, we co-transfected wild-type and dominant-negative forms of MEK with the −1628/+19 MUC2 promoter construct and showed that PMA induction of MUC2 transcriptional activity was inhibited by dominant-negative MEK. These findings indicate that the induction of MUC2 transcriptional activity by PMA is MEK-dependent. Additionally, AG126, which is an inhibitor of ERK2 phosphorylation, caused a significant decrease in MUC2 transcriptional activity. Western blots using antibodies specific for phosphorylated forms of ERK1/2, p38, and JNK showed that ERK1/2 is rapidly phosphorylated in response to PMA treatment.

We were not able to demonstrate any PMA effect on SAPK/JNK, p38 was shown to be constitutively activated in this cell line, and it showed a small increase in phosphorylation upon PMA treatment in some experiments. However, the SB203580 inhibitor of p38 did not affect MUC2 transcriptional activity, indicating that p38 is not required for stimulation of MUC2 transcription. This is an important observation because it has been shown that p38 is a downstream kinase responsible for Raf-independent Ras activation of NF-κB (36).

Inhibition of basal MUC2 transcription by N17Ras indicates that there is some basal activation of Ras and downstream effectors, which may derive from the fact that HM3 cells express mutant G12D K-Ras. This glycine-to-aspartate mutant form of K-Ras is the most common form found in colorectal carcinomas, occurring more frequently than the more intensely studied G12V mutant forms. The G12D K-Ras has been re-
ported to be less active than the G12V forms and, consequently, has biological and biochemical properties intermediate to the wild-type and G12V isoforms (37). It has also been reported that N17Ras will only inhibit wild-type but not oncogenic Ras forms; the latter, because of their very low GTPase activity, remain in the active GTP-bound form and thus do not rely on exchange factors for reactivation. However, the ability of N17Ras to inhibit basal MUC2 transcription suggests that the G12D K-Ras form, because of its 4-fold higher GTPase activity (37), is susceptible to inhibition by N17Ras.

The occurrence of the G12D K-Ras mutation in HM3 cells may be expected to produce a low-level activation of the Raf/MEK/ERK pathway and the resulting basal MUC2 expression. Dominant-negative mutants of both Raf and MEK, as well as MEK inhibitors PD98059 and U0126, negatively affected basal as well as PMA-up-regulated MUC2 transcription, consistent with low-level constitutive activation of the Raf/MEK/ERK pathway. Lending further support for this possibility is the observation of low-level phosphorylation of ERK1/2 in Western blots of lysates from untreated HM3 cells. Transfection with activated (G12V) v-H-Ras produced an up-regulation of MUC2 transcription. The ability of PMA to further up-regulate MUC2 transcription in the presence of v-H-Ras suggests that there may be further activation of the endogenous, functionally distinct K- and N-Ras isoforms or that oncogenic v-H-Ras is capable of activating MUC2-responsive pathways that are not activated by wild-type Ras.

Many (>50) different proteins, including transcription factors and kinases, have been identified as downstream targets of ERK1/2, including S6 kinase pp90rsk and EGF receptor, as well as transcription factors such as Elk-1, Ets1, c-Myc, and signal transducer and activator of transcription proteins (30, 33). Whereas dominant-negative pp90rsk was previously shown to inhibit MUC2 transcription stimulated by lipopolysaccharide (27), we were unable to demonstrate the same effect on PMA-induced transcription. This indicates that events downstream of ERK1/2 may be different for lipopolysaccharide versus PMA. Thus, whereas pp90rsk was proposed to mediate lipopolysaccharide-stimulated NF-κB activation (27), it appears that pp90rsk is not responsible for PMA-mediated activation of NF-κB. Further experiments are required to confirm and elucidate this point. Recently, ERK2 has been reported to phosphorylate Sp1 (38). A functional Sp1 binding site was previously identified in the proximal region of the MUC2 promoter between bases −94 and −65 (26). In our analysis of the effect of PMA on MUC2 deletion constructs, this region also appears to confer PMA responsiveness. As well as Sp1, a number of other unidentified proteins also bound to this −94/−65 oligonucleotide, giving a complex pattern in EMSA (26). However, using this same oligonucleotide we were unable to detect any differences between PMA-treated and -untreated cells.2 It is possible that this region of the promoter is important but that PMA-induced up-regulation involves increased transcriptional activity of Sp1 or other nuclear factors that are already bound to this proximal region of the MUC2 promoter. Further experiments are required to identify the PMA-responsive, ERK-activated MUC2 promoter elements.

Transcription factor NF-κB plays a crucial role in the regulation of numerous genes involved in the inflammatory response and control of cell death. Activation of NF-κB is mediated through phosphorylation, ubiquination, and subsequent degradation of inhibitor IκB. This enables the free NF-κB dimer (p50/p65) to translocate to the nucleus and activate target genes (39). The ultimate upstream mediators of NF-κB activation, for example the IκB kinase complex and its activating kinases, are still in the process of being identified. The transcriptional activity of NF-κB is also enhanced directly by phosphorylation at various sites on both subunits. The kinases responsible for these phosphorylations may include Ras, PKA, and PKC. These enzymes and the consequences of phosphorylations at multiple sites are currently being elucidated (39, 40). MUC2 was previously shown to be up-regulated by lipopolysaccharide via Ras/Raf/MEK/ERK/pp90rsk/NF-κB in lung epithelial cells (27). CAPE, an inhibitor of NF-κB translocation, reduced endogenous as well as PMA-up-regulated MUC2 transcription, indicating that NF-κB is also involved in MUC2 transcription induced by PMA. Furthermore, EMSAs using an oligonucleotide containing the previously identified MUC2 NF-κB site demonstrated that nuclear extracts from PMA-treated HM3 cells gave more intense bands than nuclear extracts from untreated cells. Finally, the transcriptional competence of the NF-κB cis element was demonstrated using promoter-reporter constructs containing the same region of the MUC2 promoter from bases −1528 to −1307 inserted upstream of the minimal thymidylate kinase promoter. Because pp90rsk was not shown to be involved in MUC2 up-regulation, NF-κB is likely activated via another Ras-activated pathway, such as Raf/MEKK1/IκBα or Ras/phosphatidylinositol-3-kinase/IκBα.

In conclusion, we demonstrate that MUC2 is regulated by PMA results in activation of PKC, stimulating the ERK MAP kinase pathway and up-regulating MUC2 in a Ras- and Raf-dependent manner. NF-κB was also shown to be directly involved in MUC2 up-regulation by PMA. In contrast to previous work on MUC2 transcription using lipopolysaccharide as an inducer, the effects of PMA are independent of Src and pp90rsk and are complicated by the involvement of other, proximal cis elements within the MUC2 promoter.

Acknowledgments—We thank Drs. Roger H. Erickson and Guoren Deng for helpful discussions, Drs. Geoffrey Cooper, Warner Greene, and Alan Saltiel for plasmids, and James Hicks, Roy Lai, and Stacey Yang for technical assistance.

REFERENCES

1. Moniaux, N., Escande, F., Perchot, N., Aubert, J. P., and Batra, S. K. (2001) Front. Biosci. 6, D1192–D1206
2. Williams, S. J., Wreschner, D. H., Tran, M., Eyre, H. J., Sutherland, G. R., and McGucken, M. A. (2001) J. Biol. Chem. 276, 18327–18336
3. Yang, D. H., and Lloyd, K. O. (2001) J. Biol. Chem. 276, 27371–27375
4. Ho, S. B., Niehans, G. A., Li, Y., Czarnetzki, B., Lecuit, M., and Solomon, E. T. (1993) Cancer Res. 53, 641–651
5. Audie, J. P., Janin, A., Pochet, N., Copin, M. C., Gosselin, B., and Aubert, J. P. (1993) J. Histochem. Cytochem. 41, 1479–1485
6. Chang, S.-Y., Dohrmann, A. F., Basbaum, C., Ho, S. B., Tsuda, T., Toribara, N.-W., Gum, J. R., and Kim, Y. S. (1994) Cancer Res. 54, 17277–17307
7. Sylvester, P. A., Myerscough, N., Warren, B. F., Carles, I., and McFarland, A. P. (2001) J. Biol. Chem. 276, 3277–3283
8. Hanski, C., Riede, E., Gratchev, A., Wlox, M. G., Blau, H., Blau, M., Stein, B., Kim, H. R., Surn, J. M., and Hahn, S. Y. (1995) J. Histochem. Cytochem. 43, 1727–1737
9. Riecken, E. O. (1997) FEBS Lett. 416, 172–176
10. Ho, S. B., Niehans, G. A., Li, Y., Czarnetzki, B., Lecuit, M., and Solomon, E. T. (1993) Cancer Res. 53, 641–651
11. Leibowitz, H. J., Stein, B., Kim, H. R., Surn, J. M., and Hahn, S. Y. (1995) J. Histochem. Cytochem. 43, 1727–1737
12. Sylvester, P. A., Myerscough, N., Warren, B. F., Carles, I., and McFarland, A. P. (2001) J. Biol. Chem. 276, 3277–3283
13. Hanski, C., Riede, E., Gratchev, A., Wlox, M. G., Blau, H., Blau, M., Stein, B., Kim, H. R., Surn, J. M., and Hahn, S. Y. (1995) J. Histochem. Cytochem. 43, 1727–1737
14. Levinsky, N., and Leverve, C. L., Lough, C. W., Angus, C. S., Ogbine, F. P., and Shelhamer, J. H. (1995) Am. J. Respir. Cell Mol. Biol. 12, 196–204
15. Yoon, J.-H., Gray, T., Gunn, I., Koo, J. S., and Nettleship, P. (1997) Am. J. Respir. Cell Mol. Biol. 16, 724–731
16. Koo, J. S., Jett, M. A., Belloni, P., Yoon, J.-H., Kim, Y.-D., and Nettleship, P. (1995) Biochem. J. 328, 351–357
17. Basbaum, C., Leimbahar, H., Longhore, M., Li, D., Gensch, E., and McNamara, N. N. (1999) Am. J. Respir. Crit. Care Med. 160, 844–848
18. Han, S. Y., Lee, M., Kim, H. R., Baek, S. H., Ahn, D. H., Chae, H. S., Erickson, R. H., Sleisinger, M. H., and Kim, Y. S. (2000) Int. J. Oncol. 17, 487–494
19. Thomas, S. M., DeMarco, M., D’Arcangelo, G., Hagle, S., and Brugge, J. S. (1992) Cell 68, 1031–1040

2 H.-W. Lee, D.-H. Ahn, S. C. Crawley, J.-D. Li, J. R. Gum, Jr., C. B. Basbaum, N. Q. Fan, D. E. Szymkowski, S.-Y. Han, B. H. Lee, M. H. Sleisinger, and Y. S. Kim, unpublished results.
20. Wood, K. W., Sarnecki, C., Roberts, T. M., and Blenis, J. (1992) Cell 68, 1041–1050
21. Li, Y. Q., Hi, C. S. T., Costabile, M., Goh, D., Der, C. J., and Ferrante, A. (1999) J. Immunol. 162, 3316–3320
22. Chioochees, A., Paterson, H. F., Marais, R., Clerk, A., Marshall, C. J., and Sugen, P. H. (1999) J. Biol. Chem. 274, 19762–19770
23. DeVries-Smits, A. M., Burgering, B. M., Leevers, S. J., Marshall, C. J., and Bos, J. L. (1992) Nature 357, 602–604
24. Howe, L. R., Leevers, S. J., Gomez, M., Kakielny, S., Cohen, P., and Marshall, C. J. (1992) Cell 71, 335–342
25. Ming, X. F., Burgering, B. M. T., Wennstrom, S., Claesson-Welsh, L., Heldin, C. H., Bos, J. L., Korma, S. C., and Thomas, G. (1994) Nature 371, 426–429
26. Gum, J. R., Hicks, J. W., and Kim, Y. S. (1997) Biochem. J. 325, 259–267
27. Li, J.-D., Feng, W., Gallup, M., Kim, J.-H., Gum, J., Kim, Y., and Basbaum, C. (1998) Proc. Natl. Acad. Sci. 95, 5718–5723
28. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
29. Andrews, N. C., and Faller, D. V. (1991) Nucleic Acids Res. 19, 2499
30. Kolch, W. (2000) Biochem. J. 351, 289–305
31. Feig, L. A., and Cooper, G. M. (1988) Mol. Cell. Biol. 8, 3235–3243
32. Marais, R., Light, Y., Mason, C., Paterson, H., Olson, M. F., and Marshall, C. J. (1998) Science 280, 109–112
33. Widmann, C., Gibson, S., Jarpe, M. B., and Johnson, G. L. (1999) Physiol. Rev. 79, 143–180
34. Shields, J. M., Pruit, K., McFall, A., Shaub, A., and Der, C. J. (2000) Trends Cell Biol. 10, 147–154
35. Rebollo A., and Martinez-A, C. (1999) Blood 94, 2971–2980
36. Norris, J. L., and Baldwin, A. S. (1999) J. Biol. Chem. 274, 13841–13846
37. Al-Mulla, F., Milner-White, E. J., Geing, J. J., and Birnie, G. D. (1999) J. Pathol. 187, 433–438
38. Merchant, J. L., Du, M., and Todisco, A. (1999) Biochem. Biophys. Res. Comm. 254, 454–461
39. Karin, M., and Ben-Neriah, Y. (2000) Ann. Rev. Immunol. 18, 621–663
40. Mercurio, F., and Manning, A. M. (1999) Curr. Opin. Cell Biol. 11, 226–232