MUC5AC mucin gene regulation in pancreatic cancer cells

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Abstract. MUC5AC is a secretory mucin normally expressed by the surface mucous cells of the human stomach and in the bronchial tract. It is absent from normal pancreas, but *de novo* expression of this mucin occurs in early-stage pancreatic intraepithelial neoplasias and in the invasive ductal adenocarcinoma of the pancreas, prompting this study of MUC5AC gene regulation in pancreatic cancer cells. Promoter deletion constructs and EMSA studies revealed that transcription factors Sp1 and AP-1 are both involved in basal transcription of the MUC5AC gene. Phorbol 12-myrisate 13-acetate (PMA) increased MUC5AC mRNA expression and transcriptional activities of MUC5AC promoter-reporter deletion constructs containing AP-1 consensus sites. EMSA studies showed that Fos/Jun binding to putative AP-1 sites is increased by PMA treatment. Western blot analysis showed that ERK, JNK and p38 are all activated by PMA treatment in SW1990 cells. Inhibitors of mitogen-activated protein/extracellular signal regulated kinase (MEK), such as ERK inhibitor PD98059 and JNK inhibitors dicumarol and SP60015, but not p38 inhibitor SB203580, inhibited PMA-induced MUC5AC reporter activity. Our studies indicate that Sp1 is involved in basal MUC5AC promoter activity while AP-1 is involved in basal and PMA-induced MUC5AC promoter activation in pancreatic cancer cells. Furthermore, PMA-induced MUC5AC gene transcription appears to be mediated by activating Sp1, PKC/ERK/AP-1 and PKC/JNK/AP-1 pathways.

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Introduction

Pancreatic cancer tends to have a very poor prognosis, with a 5-year survival rate of only 3% and median survival rate of only 6 months (1). This is due to the aggressiveness of pancreatic cancers as well as to the lack of both early-stage diagnostic methods and effective therapies. Invasive ductal adenocarcinoma accounts for 85% of pancreatic cancers and are generally thought to arise from pancreatic ductal cells. A progression model for pancreatic adenocarcinoma has been proposed in which specific molecular alterations occur as intraepithelial neoplasias (PanIN) and develop into carcinomas (2,3). The most common and earliest known genetic alteration involves K-ras mutation, which has been detected in early PanIN lesions. Recent mouse models of pancreatic cancer have been generated by directing expression of mutant K-ras in pancreatic ductal epithelia, providing strong evidence that this mutation is sufficient to initiate the formation of PanIN lesions (4). Other molecular changes shown to occur and likely contributing to the progression to cancer include p21 WAF1/CIP1 overexpression, HER-2/neu overexpression, and inactivation of p16 (INK4A/ARF) in early PanIN lesions, and inactivation of p53, SMAD/DPC4, and BRCA2 gene mutations in late PanIN lesions (2,3).

Mucins are large, highly O-glycosylated proteins expressed in a tissue and cell type specific manner by normal epithelial cells in either secreted or membrane-associated form (5,6). These molecules provide a protective but permeable luminal barrier for the airways and for the digestive and reproductive tracts. By contrast, considerable alterations in the level and the pattern of mucin gene expression occur with malignant transformation. The epithelium lining normal pancreatic ducts express MUC1, with very low or no expression of other mucins (7). However, *de novo* expression of MUC5AC, which is a secretory mucin normally expressed in gastric pit cells and airways, but not in normal pancreas, occurs in the PanIN lesions and in invasive ductal adenocarcinomas of the pancreas.

MUC5AC gene regulation has been studied mostly in the context of airway epithelial response to inflammatory stimuli and bacterial pathogens (8,9). MUC5AC expression has been shown to be responsive to retinoic acid in normal human tracheobronchial epithelial cells, and this response seems to

be mediated by a sequence element located approximately 1.2 kb upstream of the start of transcription on the gene promoter (10). Cytoplasmic proteins from non-typeable *Haemophilus influenzae* were shown to up-regulate MUC5AC transcriptional regulation in HM3 colon cancer cells via the p38 MAP kinase signaling pathway, with negative cross-talk by the phosphoinositide 3-kinase/Akt signaling pathway (11,12). *Pseudomonas aeruginosa* has been reported to induce MUC5AC transcription via activation of the EGFR signaling pathway and ERK1/2 in airway epithelial cell line NCI-H292 (13,14).

IL-1b and TNFα have also been reported to induce MUC5AC overexpression in human airway epithelial cells (15) mediated by ERK and p38 MAP kinase signaling pathways acting on downstream effectors mitogen and stress activated protein kinase 1 (MSK1) and cAMP response element binding protein (CREB). More recently, it was reported that the -3752/-3426 region of the MUC5AC promoter was able to confer TGF-ß-mediated repression of reporter transcription, via the Smad 3/4 signaling pathway (11). However, at the present time, little is known about the molecular mechanisms involved in the *de novo* expression of the MUC5AC gene in pancreatic cancer cells.

Therefore, we sought to identify the mechanisms responsible for ectopic expression of MUC5AC in PanIN lesions and invasive ductal adenocarcinomas of the pancreas by characterizing some of the regulatory pathways and the factors responsible for MUC5AC up-regulation in pancreatic cancer cells. In the present study, we examined the transcriptional activity of the MUC5AC promoter region in pancreatic cancer cell lines, SW1990 and HPAF, and identified Sp1 and AP-1 as key factors responsible for MUC5AC transcription.

Materials and methods

Materials. TriReagent and phorbol 12-myristate 13-acetate (PMA) were obtained from Sigma. The Dual-Luciferase™ reporter assay system was from Promega (Madison, WI). Bisindolylmaleimide I, PD98059, SB203580, and SP600125 were purchased from Calbiochem. Antibodies for Sp1 (PEP2), panFos (sc-253X), pan-Jun (sc-44X), c-Fos (sc-52X) and c-Jun (sc-45X) were purchased from Santa Cruz Biotechnology, Inc., Santa Cruz, CA. Antibodies for ERK1/2, phospho-ERK1/2, SAPK/JNK, phospho-JNK, and p38 and phospho-p38 were purchased from Cell Signaling Technology (Beverly, MA). Immunohistochemical reagents were purchased from Zymed Laboratories Inc. (South San Francisco, CA). Operon/Qiagen (Alameda, CA) synthesized the oligonucleotides. Monoclonal antibody (21M1) to MUC5AC was a gift from Dr J. Bara (INSERM, Paris, France).

Immunohistochemistry and in situ hybridization. Five micron sections of paraffin-embedded tissue were stained as described previously (7). Monoclonal antibody 21M1 to MUC5AC was used.

Plasmid construction. Segments of the 5'-flanking region of the human MUC5AC mucin gene containing bases -3759/+68 and -2892/+68 were previously sub-cloned into firefly luciferase reporter vector pGL3 (16). -1978/+68, -1679/+68, -1384/+68 and -1029/+68 deletion constructs were made using 5'-primers which annealed at the indicated positions and included extra 5' bases encoding a Mlul site. The reverse primer annealed at a unique Bsu36l site and ligated into the parental -3759/+68 vector cut by the same enzymes. Constructs -2164/+68 (Mlul-Nsil), -529/+68 (Mlul-BstEll), -323/+68 (Mlul-AfLll) and -63/+68 (Mlul-BbrPI) were prepared by deleting fragments with the indicated restriction enzyme pairs, generating a blunt end by incubation with polymerase (±dNTPs), followed by re-ligation. Plasmids were prepared using the EndoFree Plasmid DNA Prep kit from Qiagen.

Cell culture. MUC5AC-positive SW1990 and HPAF cells were maintained at 37˚C in a 5% CO_2 atmosphere in Dulbecco's modified Eagle's minimum medium (DMEM) containing 10% heat-inactivated fetal bovine serum with penicillin and streptomycin.

Inhibitor assays. Treatment with 0.5 μm PMA was performed on SW1990 cells at 70-80% confluence. The time periods of PMA treatment were 4 and 8 h after serum-starvation overnight. For some experiments, the SW1990 cells were pre-incubated with inhibitors for 1 h before exposure to 0.5 μm PMA for 4 h.

RNA isolation and RT-PCR. Total RNA was extracted using TriReagent. Total RNA (1.5 μg) was reverse transcribed using SuperScript II (Invitrogen) primed with random hexamers, in a final volume of 25 μl. Two μl of this mixture was used for PCR amplification in a 20-μl reaction using AmpliTaq DNA polymerase (Applied Biosystems) with the addition of 5% dimethylsulfoxide (DMSO). The primers for MUC5AC were (forward) 5'-TGCACCTGTGACAGCAGGAT-3' and (reverse) 5'-ACCTCCACCTTCCTATGGCT-3'. 18S rRNA was simultaneously amplified as an internal standard, using a 9:1 ratio of 3' blocked/unblocked primers from Ambion's Quantum RNA™ 18S (Alternate) Internal Standard kit. The PCR reaction mixture was denatured at 94˚C for 30 sec, 58˚C for 30 sec and 72˚C for 30 sec. PCR products were separated by agarose gel electrophoresis in the presence of ethidium bromide.

Transient transfection and luciferase reporter assays. Cells were transfected using Superfect (Qiagen) as indicated in the figure legends, using pRLO (a promoterless Renilla luciferase vector, Promega) as internal control. Transfected cells were serum-starved overnight before treatment. Promoter/reporter luciferase activity was measured using the Dual-Luciferase™ reporter assay system (Promega). Firefly luciferase activity was normalized with respect to pRLO Renilla luciferase internal control activity. Data is presented graphically as the average of triplicates from a representative experiment, with standard deviation provided by error bars.

Electrophoretic mobility shift assays (EMSA). Nuclear protein extracts were prepared as described (17), with protein concentrations determined using the Bradford Assay method (Bio-Rad). Five μg of protein were used for Sp1 EMSA studies and 10 μg per AP-1 EMSA. Double-stranded oligonucleotide probe Sp1/CACCC included a sequence from -92 to -56 (5'-GAA CCA CAG GCC CCG CCC TGC CCA CCC

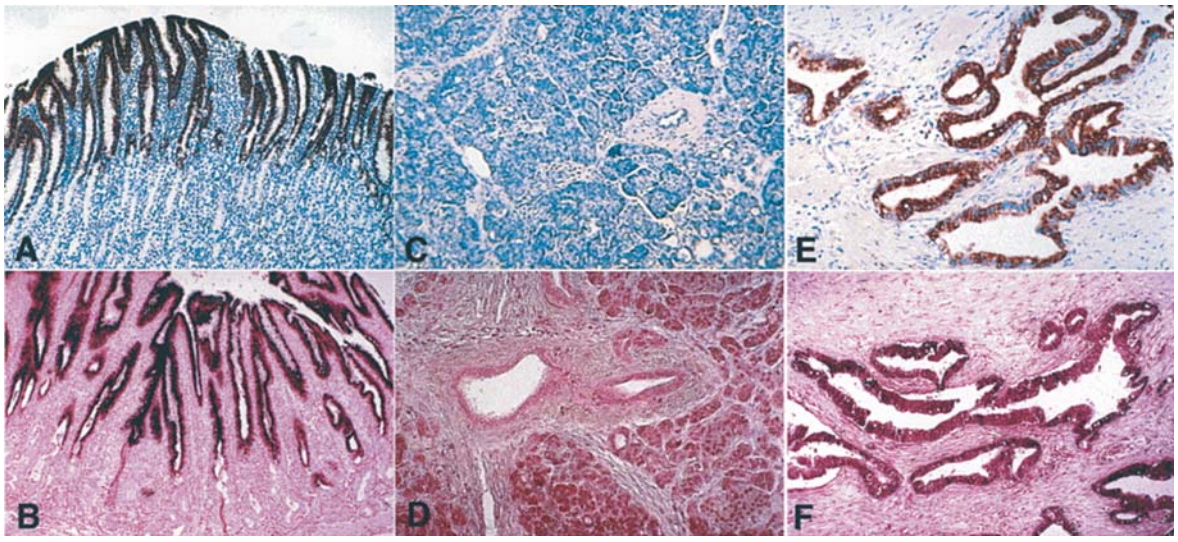

Figure 1. MUC5AC is expressed in normal stomach and *de novo*-expressed in pancreatic cancer. Immunohistochemical detection of MUC5AC mucin (A, C and E) and *in situ* detection of MUC5AC message (B, D and F) in normal human stomach (A and B) and invasive ductal adenocarcinoma of the pancreas (E and F) but not in normal pancreatic tissue (C and D).

ACG TGA AGC A-3'). Sp1 concensus oligo had sequence 5'-ATT CGA TCG GGG CGG GGC GAG C-3'. MUC5AC AP-1 oligos were designed to include a sequence from -2074 to -2045 (5'-TCA CTC ACT GAC TCA TTG ACT CAT TCC CTC-3') or from (-1921 to -1899), chosen at random from among multiple putative AP-1 binding sites. AP-1 concensus oligo had sequence 5'-CGC TTG ATG ACT CAG CCG GAA-3'. Equimolar amounts of reverse complementary oligonucleotides were annealed to give 3' overhangs, which were filled, radiolabeled by incubation with Klenow fragment and [α-^{32}P]-dCTP and dNTP(-C). Unreacted label was removed using QlAquick™ nucleotide removal kit (Qiagen). Nuclear proteins (5 μg) were incubated with radiolabeled probe (~1,000,000 c.p.m./reaction) for 20 min in solution with 3 μg of poly(dl-dC) in 10 mM HEPES-KOH at pH 7.9, 210 mM NaCl, 0.75 mM $MgCl_2$, 0.1 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM PMSF, and 12.5% glycerol. For supershift analysis, antibodies specific to Sp1, pan-Fos, c-Fos, pan-Jun and c-Jun were pre-incubated with nuclear extracts for 30 min at room temperature. Following electrophoresis for 3 h at 160 V on a 4% non-denaturing polyacrylamide gel, the gel was vacuum dried and autoradiographed overnight at -80˚C.

Western blotting. After various treatments, total cell lysates were prepared in 10 mM Tris-HCl, pH 6.8, 0.4 mM EDTA, 2% SDS, 10 μg/ml leuptin, 10 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 10 mM sodium fluoride, 0.4 mM sodium orthovandate and 10 mM pyrophosphate. The protein concentration of supernatant was determined by using the bicinchoninic acid based BCA protein assay kit (Pierce Chemicals, Rockford, IL). Equal amounts of protein were subjected to 10% SDS polyacrylamide gel electrophoresis, and transferred to nitrocellulose (18) 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 the indicated antibodies, and visualized using horseradish peroxidase conjugated secondary antibodies and chemiluminescent substrates (Renaissance kit; NEW Life Science Products).

Results

Tissue distribution of MUC5AC. MUC5AC message and protein are normally detected in foveolar epithelial cells of the stomach (Fig. 1A and B). MUC5AC is absent from all cell types of the normal pancreas (Fig. 1C and D) but then arises *de novo* in invasive ductal adenocarcinoma of the pancreas, as detailed elsewhere (7). Pancreatic cancer epithelial cell lines, SW1990 and HPAF, also stained positive for MUC5AC and thus were chosen for studies of MUC5AC transcriptional regulation.

MUC5AC promoter activity in SW1990 and HPAF cell lines. A total of 3.8 kb of sequence from the MUC5AC 5'-flanking region was analyzed in these experiments (GenBank accession number, AF016834). As reported previously (16), a TATA-like box exists at -32/-26 upstream of the translational start site, and there are two putative Sp1 binding sites (at -140 and -70) and a CACCC box sequence at -70 (Fig. 2). Two imperfect long tandem repeats, devoid of guanine residues, occur from -3010 to -2360 and -2360 to -1660. The tetranucleotide sequence, CTCA, occurs 152 times within this region. The TGACTCA recognition sequence for Fas-Jun (AP-1) binding occurs 20 times between base -3759 and -1384, and a second AP-1 binding sequence, TAACTCA, occurs 7 times (Fig. 2).

SW1990 and HPAF cell lines were transfected with various lengths of the MUC5AC 5'-flanking sequence cloned upstream of a luciferase reporter gene in pGL3-Basic. Fig. 2 shows the result of this transfection study. Promoter activity decreased in a step-wise fashion with sequential truncations from -3759 to -1384. As noted above, this region contains multiple AP-1 sites and this data suggests that they may be important for activity. A second region important for activity exists between bases -323 and -63. The two Sp1 binding sites and the CACCC box sequence are present in this region (Fig. 2). Deletion constructs -1384/+68, -1029/+64, -526/+64, -323/+64 have much the same activities in both cell lines, suggesting that

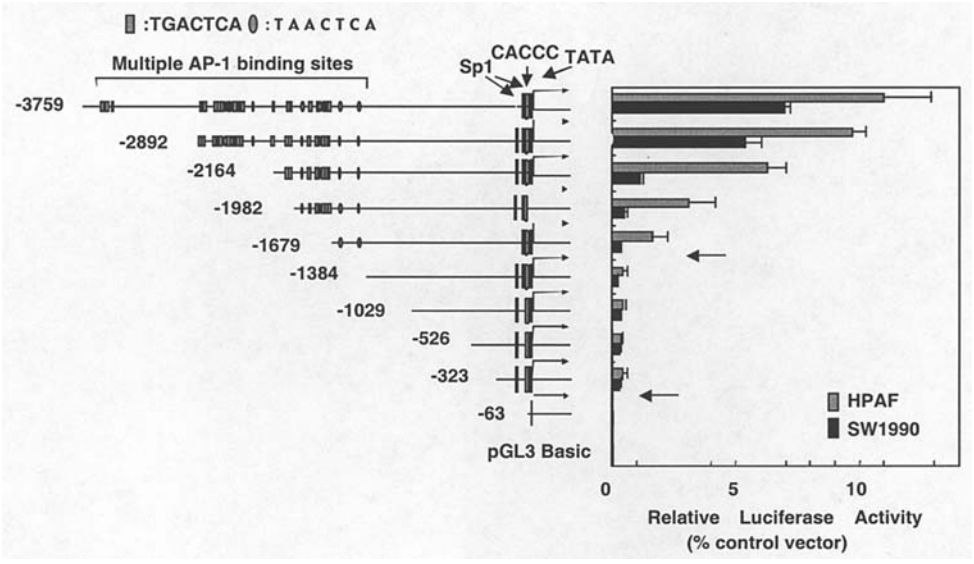

Figure 2. Activity of different MUC5AC promoter-reporter deletion constructs in SW1990 and HPAF cell lines. Transcriptional activities are expressed relative to SV40 promoter driven pGL2 control vector. Arrows indicate significant changes in transcriptional activity from the next shortest constructs.

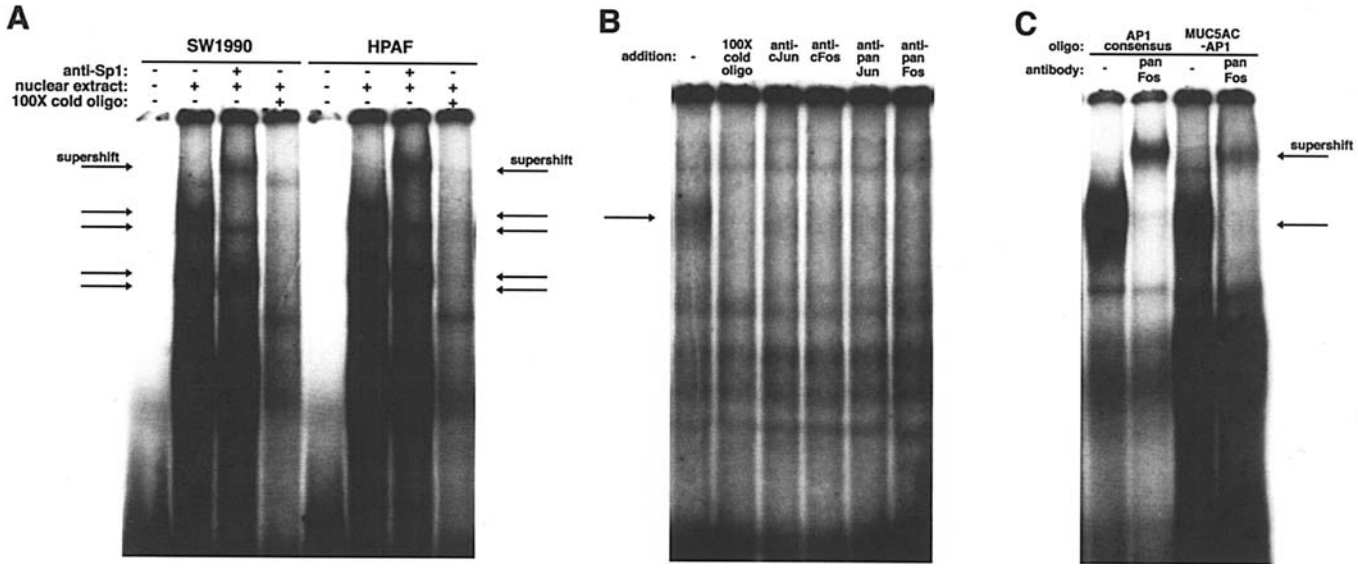

Figure 3. EMSA and supershift analysis demonstrate nuclear protein binding to putative Sp1 and AP-1 sites. A double-stranded oligonucleotide probe corresponding to the human MUC5AC Sp1/CACCC box site from -92 to -56 was incubated with 5 μg of nuclear protein from SW1990 cells (A). An oligonucleotide encompassing the MUC5AC AP-1 recognition site from -2074 to -2045 was incubated with 10 μg nuclear protein from SW1990 (B) or HPAF (C). EMSA using a concensus AP-1 oligonucleotide is also shown for comparison. 100-fold concentration of unlabeled oligonucleotides were added to demonstrate specificity by competitively inhibiting the binding to radiolabeled oligonucleotide. Antibodies used for supershifts are indicated and an arrow indicates supershifted bands.

important cis-elements lie upstream of this region. The loss of promoter activity which occurs upon elimination of the sequence between -323 and -64 probably indicates the removal of sequence elements necessary for basal promoter activity. Considering the above, we hypothesized that the AP-1 sites, Sp1 sites and CACCC box are important elements regulating MUC5AC transcription.

EMSA supershift analysis of SW1990 cell nuclear proteins with Sp1, Fos and Jun antibodies. Fig. 3A shows results of EMSA that was performed using an oligonucleotide probe encompassing the proximal Sp1/CACCC site from -92 to -56. Binding by SW1990 nuclear proteins resulted in several intense bands, which were diminished by the addition of an excess of non-radioactive Sp1/CACCC box or Sp1 consensus oligonucleotide. The addition of anti-Sp1 antibody caused the upper band to supershift, indicating that Sp1 and other transcription factors may be bound at this site. Although not shown, a similar analysis of the more distal Sp1 site at base -140 and of putative NF-κB sites at -950 and -220 showed protein binding by nuclear proteins, but no supershift by relevant antibodies. In addition, a double stranded MUC5AC Sp1 oligonucleotide extending from -99 to -70 and thus lacking the CACCC box gave results similar to the Sp1-CACCC

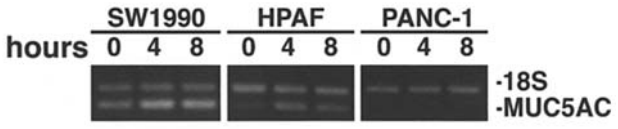

Figure 4. RT-PCR analysis shows increased MUC5AC mRNA levels in SW1990, HPAF and PANC-1 cells treated with PMA. Cells were serum-starved overnight, and then harvested 0, 4 and 8 h after 0.5 μM PMA treatment. 18S rRNA was co-amplified with the MUC5AC message, as an internal control.

probe, indicating that the CACCC box sequence was not required for binding of nuclear factors. On the other hand, an EMSA probe designed from bases -76 to -47, thus containing the CACCC box but excluding the Sp1 site, failed to bind nuclear proteins (data not shown). Sp1 is one of the Sp-family transcription factors that exist as a constitutive active form in the nucleus (19,20). Sp3 is another Sp-family transcriptional factor that represses Sp1 function (21). Sp1-specific antisera supershifted a band in the EMSA shown in Fig. 3A. But other bands were not supershifted by Sp1 antibody, indicating that other transcription factors (e.g. Sp3) are probably involved.

Because of the high number of potential AP-1 binding sites, and the lack of strong evidence showing which particular sites might be involved in MUC5AC transcription, two different sites were chosen at random for design of double-stranded probes for EMSA analysis, one centered between bases -1921 and -1899 and one located between bases -2074 and -2045. Both probes gave similar results. Fig. 3B shows EMSA results that were obtained using a labeled probe containing the putative AP-1 recognition site from -2074 to -2045, incubated with nuclear extracts from SW1990. A broad band was obtained (lane 1) and the addition of excess non-radioactive AP-1 oligonucleotide eliminated the band (lane 2). All antibodies, anti-Jun, anti-Fos, anti-pan-Jun and anti-pan-Fos, caused a marked decrease in the intensity of the band, but no clear-cut supershifted band was visible. EMSA using HPAF nuclear proteins gave a more intense band, possible reflecting a greater abundance of AP-1 in this cell line. A supershifted band was visible with HPAF nuclear extract. These results suggest that the AP-1 complex may be involved in the transcription of the MUC5AC gene.

PMA increased MUC5AC mRNA levels and promoter activity. Since PMA is a well-known activator of AP-1 (22,23), the ability of PMA to up-regulate MUC5AC expression was investigated. SW1990, HPAF and PANC-1 cell lines were serum-starved overnight, and then treated for 0, 4, or 8 h with 0.5 μm PMA. Total RNA was isolated and reverse transcribed, and MUC5AC cDNA was amplified by PCR. 18S rRNA was co-amplified to serve as an internal control. Fig. 4 illustrates the time-course increase in MUC5AC PCR product after PMA treatment, showing that MUC5AC mRNA levels increased in SW1990 and HPAF cell lines upon PMA treatment, with maximum levels observed after 4 h. PANC-1 cells had barely detectable basal MUC5AC RNA levels which did not increase upon PMA treatment.

The effect of PMA on MUC5AC promoter-reporter activity was analyzed in SW1990 cells using deletion constructs (Fig. 5). PMA enhanced promoter-reporter activity in constructs containing the region between -3759 and -1384. This region contains multiple AP-1 sites. Constructs shorter than -1679/+68, which do not contain AP-1 binding sites, showed less response to PMA. These results suggest that AP-1 binding sites mediate MUC5AC gene transcription.

EMSA supershift analysis of PMA-treated SW1990 nuclear proteins. AP-1 is known to be induced by PMA in several cell types (22,23). To test this possibility, nuclear proteins were isolated from PMA-treated and untreated SW1990 cells and EMSA assays were performed using the MUC5AC AP-1 nucleotide. A faint band was typically observed using nuclear proteins from untreated cells, and the intensity of this band was increased using nuclear proteins from PMA-stimulated cells (Fig. 6). The AP-1 transcription factor is a

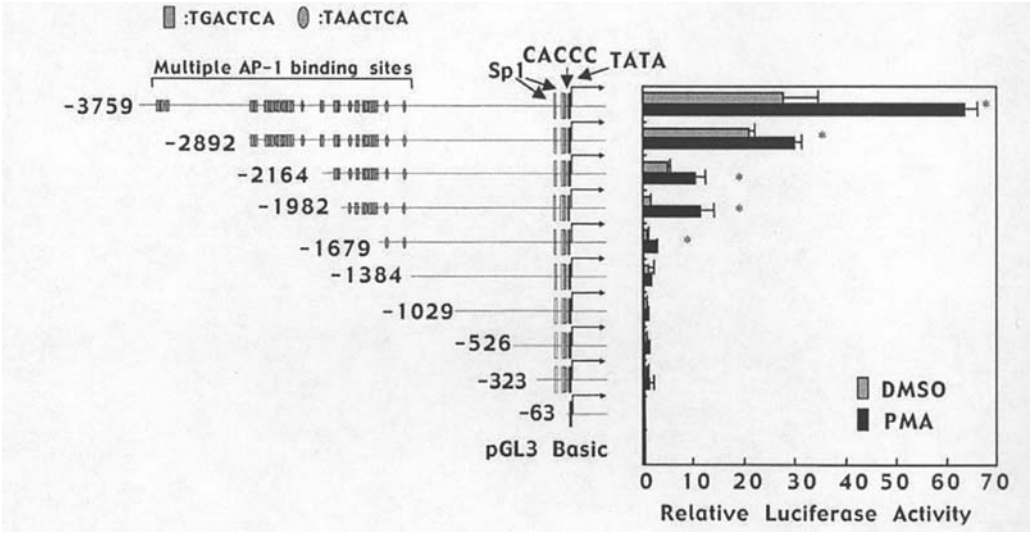

Figure 5. PMA increases the promoter activity of MUC5AC deletion constructs in SW1990 cell lines. SW1990 cells transfected with a series of MUC5AC promoter deletion constructs were treated with PMA (0.5 μM) for 4 h before harvesting for luciferase assays. *Significant differences in transcriptional activity between PMA-treated and non-treated SW1990 cells.

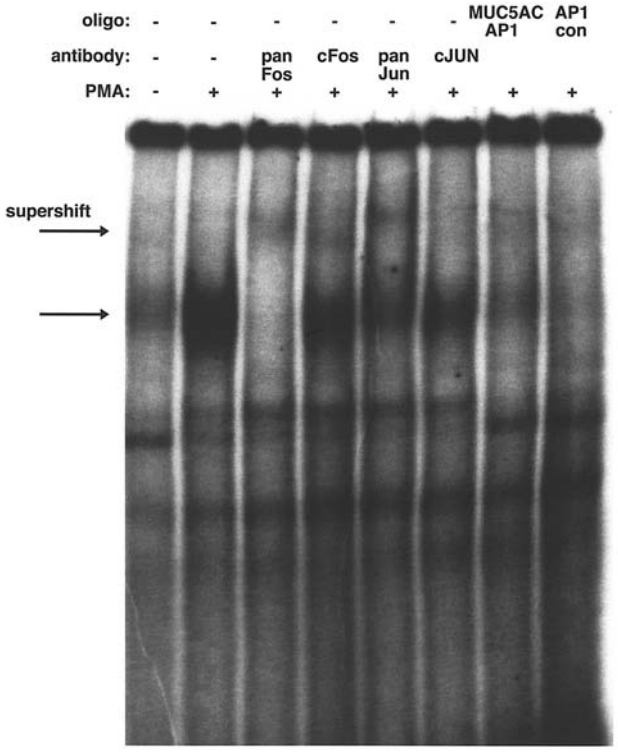

Figure 6. PMA increases the binding of nuclear proteins to oligonucleotides containing MUC5AC Sp1 and AP-1 binding sites. Double-stranded oligonucleotide probes corresponding to human MUC5AC AP-1 binding sites were incubated with nuclear proteins (10 μg) from SW1990 cells. Cells were treated with PMA (0.5 μM) for 4 h before harvesting. Antibodies used for supershift are indicated. 100-fold non-labeled oligonucleotides were used as cold competitors.

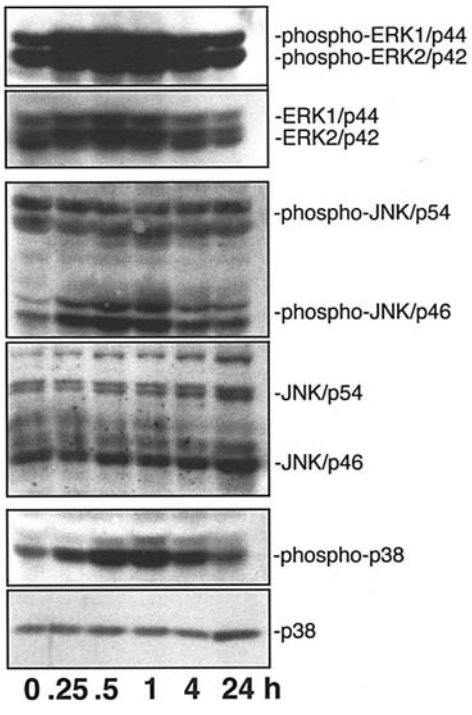

Figure 7. PMA treatment activates ERK, JNK and p38 kinases in SW1990 cells. Cells were treated with PMA (0.5 μM) for indicated periods of time. Twenty μg/lane of total cell lysate protein was subjected to SDS-PAGE electrophoresis. Nitrocellulose blots were probed with antibodies to phosphorylated and non-phosphorylated forms of ERK1/2, JNK (p54/p46) and p38, as indicated.

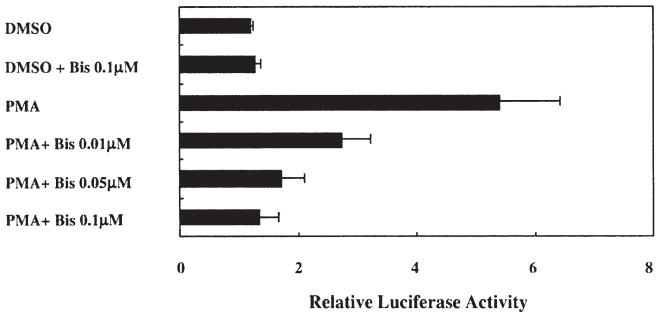

Figure 8. PKC inhibitor bisindolylmaleimide I (Bis I) inhibits PMA-mediated activation of MUC5AC promoter activity in SW1990 cells. SW1990 cells transfected with the -1982/+68 MUC5AC promoter-reporter construct were pretreated for 1 h with several concentrations of bisindolylmaleimide I (Bis) prior to treatment with PMA (0.5 μM) for 4 h.

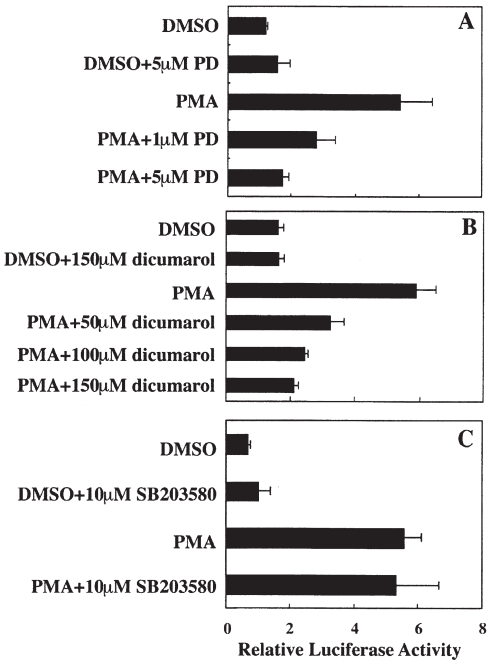

Figure 9. Effects of MAPK inhibitors on MUC5AC promoter activity in SW1990 cells treated with PMA. SW1990 cells transfected with the MUC5AC -1982/+68 promoter-reporter construct were treated with several concentrations of PD98059, dicumarol and SB208530 for 1 h and before addition of PMA (0.5 μM) for 4 h.

dimeric complex composed of Fos/Jun heterodimers, or Jun homodimers. Fos is a family comprised of cFos, FosB, Fra-1, and Fra-2, and Jun includes cJun, JunB, and JunD isoforms.

Different dimers exhibit differences in DNA binding and transactivation, so supershift assays were used to identify the subunits bound to the MUC5AC probe. The addition of either antibodies against cFos or cJun caused a reduction in the band intensity, suggesting that these specific Fos and Jun isoforms were involved in the up-regulation of MUC5AC transcription. A reduction in band intensity and the appearance of supershifted bands was produced by the addition of antibodies against pan-Fos and pan-Jun. The addition of a 100-fold excess of unlabeled AP-1 consensus oligonucleotide or unlabeled MUC5AC AP-1 nucleotide blocked the binding of nuclear proteins, indicating the specificity of the binding.

Western blotting showed activation of MAP kinases. Phosphorylation of MAP kinases in response to PMA stimulation was evaluated by Western blotting. Fig. 7 illustrates time-dependent changes in the phosphorylation of MAP kinases after PMA treatment. Both phosphorylated and non-phosphorylated ERK protein increased within 15 min of the addition of PMA, peaking at 1 h after treatment and decreasing thereafter. JNK and p38 proteins showed very similar transient phosphorylation, whereas levels of non-phosphorylated forms remained constant.

Inhibitor studies indicate that PKC, ERK and JNK are involved in PMA mediated up-regulation of MUC5AC. Pretreatment with PKC inhibitor bisindolylmaleimide I (Bis I) reduced the PMA-stimulated activity of the -1982/+68 MUC5AC promoter in a dose-dependent manner, indicating that PKC is involved in MUC5AC gene up-regulation by PMA (Fig. 8). Western blotting indicated that p38, JNK and ERK were phosphorylated in response to PMA; so, in order to identify which MAP kinase pathways were involved in MUC5AC gene transcription, promoter-reporter assays were performed using specific protein kinase inhibitors. PD98059 (MEK inhibitor) and dicumarol (JNK inhibitor) attenuated PMA-stimulated promoter activity in a dose-dependent manner (Fig. 9A and B). We also observed inhibition with JNK inhibitor SP600125 (data not shown) whereas p38 inhibitor SB203580 had no effect (Fig. 9C). These results suggest a pathway for PMA-stimulated MUC5AC expression involving PKC, MEK and JNK, but not p38.

Discussion

De novo expression of MUC5AC gastric foveolar mucin in invasive ductal adenocarcinoma of the pancreas and its precursor lesions prompted this study of its transcriptional regulation in pancreatic epithelial cancer cell lines (7). We analyzed the 3.8-kb 5'-flanking region of the MUC5AC gene, which contains numerous cognate AP-1 binding sites, Sp1/CACCC box elements and a TATA-like box upstream of its start site. Transfection assays in both SW1990 and HPAF cells indicated that promoter-reporter activity gradually decreased with the length of the included promoter region, down to base pair -1679. This activity appears to be attributable to the presence of multiple AP-1 binding sites, all of which lie upstream of -1679. A second significant reduction in reporter activity was observed when the proximal promoter region from -323 to -63 was deleted, which contains two Sp1 binding sites and a CACCC box element. These results suggest that both Sp1 and AP-1 transcription factors are involved in basal MUC5AC gene transcription. EMSA supershift analysis also demonstrated that Sp1 and AP-1 (Fos/Jun complex) transcription factors bind to oligonucleotides containing these putative Sp1 and AP-1 sites.

Sp1 is one of the family of Sp transcription factors that exists in a constitutive active from in the nucleus (19,20). Sp3 is another family member that represses Sp1 function (21). The Sp1 supershifted band was observed in our EMSA study (Fig. 3A). However, other bands were not supershifted by Sp1 antibody, suggesting that other Sp-family transcriptional factors, such as Sp3, might be present in these bands.

The large number of putative recognition sites complicated investigation into the role of AP-1 in MUC5AC transcription. Promoter-reporter assays with deletion constructs suggested that multiple sites may be involved, possibly in a co-operative fashion, precluding many of the conventional site-directed mutagenesis and EMSA approaches to promoter analysis. Because PMA is a potent activator of AP-1 (22-24) we decided to use this compound to analyze the MUC5AC promoter.

Our studies demonstrated that PMA increased MUC5AC message levels and increased the transcriptional activity of MUC5AC promoter/deletion constructs transiently transfected into the SW1990 cell line, indicating that PMA up-regulation occurs at the transcriptional level. However, the transcriptional activity of promoter/deletion constructs shorter than base -1679 were not increased by PMA. Since these constructs possess no AP-1 binding sites, these results indicate that AP-1 is important in the induction of MUC5AC by PMA. AP-1 binding was also confirmed by EMSA study. The intensity of the AP-1 EMSA band was increased by PMA, indicating that AP-1 nuclear protein binding was up-regulated by PMA. Supershift analysis showed that some Fos and Jun group transcription factors, especially cFos and cJun, are involved in this reaction. Thus, the present study clearly shows that AP-1 is involved in the transcription of the MUC5AC gene, not only in a basal condition but also in a PMA-stimulated condition.

In order to identify the signaling pathway involved in PMA-mediated MUC5AC up-regulation, the effects of various chemical inhibitors on PMA stimulated MUC5AC transcription were examined. Phorbol esters such as PMA function as tumor promoters and are known to modulate diverse cellular responses through a protein kinase C (PKC) signaling pathway. These include the regulation of gene transcription, cellular growth and differentiation, programmed cell death, immune response and receptor densitization. PMA can substitute for diacylglycerol, a natural transducer in activating protein kinase C (25-28). AP-1 is strongly induced by PKC activation. Since PKC inhibitor Bis I blocked MUC5AC transcriptional activity by PMA, PKC activation appears to be necessary for the PMA induction of MUC5AC promoter activity. The downstream effectors of the PKC pathway are mitogen-activated protein kinases (MAPKs). MAPKs are serine/threonine kinases that are activated by dual phosphorylation on threonine and tyrosine residues in response to a wide array of extracellular stimuli. Three distinct groups of MAPKs have been identified, including the extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 (29,30). Western blotting confirmed the expression of non-phosphorylated ERK, JNK and p38 in SW1990 cells under basal conditions. ERK, JNK and p38 were constitutively expressed in basal conditions. However, the level of ERK was increased 15 min after the stimulation of PMA, while those of JNK and p38 did not change. Phosphorylated ERK, JNK and p38 were also constitutively expressed under basal conditions and were up-regulated 15 min after PMA stimulation, reaching maximum levels after 1 h. These results indicate that PMA induced protein synthesis and phosphorylation of ERK while JNK and p38 were only phosphorylated by PMA, and that ERK inhibitor PD98059 and JNK inhibitors dicumarol and SP600125 inhibited the PMA-induced transcriptional activity of the MUC5AC promoter. p38 inhibitor

SB203580, on the other hand, had no such effect. This indicates that ERK and JNK, but not p38, are important mediators of PMA-induced MUC5AC transcription.

AP-1 subunit cFos has a serum response element (SRE) in its gene promoter. The AP-1 transcription factor complex is usually controlled by both phosphorylation and protein synthesis levels. Both ERK and JNK can activate SRE via phosphorylation of the ternary complex factor (TCF) and induce cFos protein synthesis (31,32). Another AP-1 subunit, cJun, has TRE in its promoter region. JNK can phosphorylate cJun. cJun itself can bind to TRE of cJun and induce protein synthesis of cJun. FRK, but not ERK, can phosphorylate cFos (33); however, another AP-1 subunit, Fra-1, has been reported to be phosphorylated by ERK (34). These AP-1 subunits might be involved in the induction of MUC5AC transcription. Putative NF-κB and AP-2 binding sites have been identified downstream of base -1366 in the MUC5AC promoter (16). NF-κB and AP-2 are also downstream effectors of the PKC pathway. However, PMA fails to up-regulate promoter activities of MUC5AC deletion constructs shorter than -1369/+68. Thus, our results suggest that these putative binding sites are probably non-functional.

In conclusion, the present study indicates that Sp1 is involved in MUC5AC promoter activity under basal conditions and that AP-1 is involved in both basal and PMA-induced MUC5AC promoter activity in pancreatic cancer cells. Thus, SP-1, PKC/ERK/AP-1 and PKC/JNK/AP-1 pathways appear to be important in regulating MUC5AC transcription.

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