Dynamic Regulation of Cyclooxygenase-2 Promoter Activity by Isoforms of CCAAT/Enhancer-binding Proteins*

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To elucidate the mechanism by which isoforms of CCAAT/enhancer-binding proteins regulate cyclooxygenase-2 expression, we determined by a novel technique binding of six isoforms of this transactivator to two sequence-specific CCAAT/enhancer-binding protein (−132/−125) and cyclic AMP (−59/−53) regulatory elements in human foreskin fibroblasts treated with phorbol 12-myristate 13-acetate for 4 h. The δ isoform bound to these two elements at basal state, which was displaced by full-length as well as two truncated β isoforms, a 41-kDa liver-enriched activating protein and a 16-kDa liver-enriched inhibitory protein, after phorbol ester stimulation. Kinetic analysis shows time-dependent changes in β and δ binding that were concordant with time-dependent increase in cyclooxygenase-2 induction. Overexpression of the 16-kDa δ isoform blocked the promoter activity and protein level induced by phorbol ester. Paradoxically, it increased binding of β isoforms to the sequence-specific promoter DNA but suppressed cyclooxygenase-2 promoter activation by p300 cotransfection. These findings provide new insight into the regulation of cyclooxygenase-2 promoter by an interplay between two opposite β isoforms and p300 co-activator.

Cyclooxygenase-2 (COX-2) plays diverse pathophysiological roles notably in inflammation, tissue damage, and tumorigenesis (1–3). COX-2 is induced by myriad mitogenic and inflammatory mediators (4). Transcriptional regulation of COX-2 by various stimuli has been extensively investigated, and a large body of data has been reported. However, the mechanisms by which COX-2 transcription is activated and regulated are not entirely clear. NF-kB activation and binding to its cognate site on the COX-2 promoter region has been reported to mediate COX-2 transcription induced by tumor necrosis factor α and hypoxia (5, 6), while binding of CCAAT/enhancer binding protein (C/EBP) to its cognate site on COX-2 promoter has been reported to be crucial for promoter activation by stimuli such as phorbol 12-myristate 13-acetate (PMA), interleukin-1 and growth factors (7–9). Results from a recent report have shown that PMA increased C/EBPβ binding to the C/EBP regulatory element of human COX-2 promoter region by a process depending on C/EBPβ phosphorylation (10). C/EBPβ belongs to the basic leucine zipper C/EBP family that comprises six members, and C/EBPβ is closely related to C/EBPα and C/EBPβ but is distantly related to C/EBPγ, C/EBPε, and C/EBPδ (11, 12). Several truncated forms of C/EBPβ have been noted. These truncated forms originate from the use of alternative translation start sites (13). A major truncated form with molecular weight close to the full-length C/EBPβ was originally shown in liver cells to activate transcription and was named liver-enriched transcription activating protein (LAP), while a small molecular weight form was shown to repress transcription in liver cells and was named liver-enriched transcription inhibitory protein (LIP) (14). LAP and LIP have been shown to express in other types of cells, and LIP is considered as a dominant negative mutant of C/EBPβ (15). Transient transfection of LIP has been shown to suppress COX-2 promoter activity (8). However, the physiological role of C/EBPβ-LIP and C/EBPβ-LAP in regulating COX-2 expression has not been reported. The roles of other C/EBP isoforms in regulating COX-2 expression are also unknown. In this study, we tested the hypothesis that PMA caused changes in C/EBP isoform binding to COX-2 promoter regulatory elements, which result in a dynamic control of COX-2 expression. Our results show that PMA increased binding of full-length C/EBPβ, C/EBPβ-LF, C/EBPβ-LAP, and C/EBPβ-LIP to C/EBP and CRE sites while it reduced C/EBPε level and its binding to the C/EBP site. Overexpression of C/EBPβ-LIP suppressed COX-2 promoter activity and protein expression by interfering with the activity of a transcription co-activator p300.

EXPERIMENTAL PROCEDURES

Cell Culture—Human foreskin fibroblasts (HFF) were purchased from ATCC and cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100 μg/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a 5% CO2 incubator. In all experiments, 85–90% confluent HFF were serum-starved for 24 h prior to treatment with PMA.

Plasmid DNA—Luciferase expression vector pGL3 basic containing a COX-2 promoter region (~891/+9) was constructed as previously described (16). Site-directed mutation of C/EBP and CRE sites was previously described (10). Plasmids expressing C/EBPβ were generously provided by Dr. Steven McKind. Plasmid expressing C/EBPβ was kindly provided by Dr. Philip Auron and was used as the template to generate C/EBPβ-LIP by PCR. For LIP synthesis, forward primer 5′-GACAACGTATGGCGGCCTCCGGTAC-3′ and reverse primer 5′-GACCTCGAGCTAGATGCGCGAGGAGGC-3′ were obtained from Integrated DNA Technologies. The PCR product was purified after double digestion with HindIII/XhoI and was cloned into the HindIII/XhoI sites of an expression vector pCMV-Tag2 (Stratagene). Plasmid expressing p300 was a gift from Dr. Joan Boyes.

Western Blot Analysis—Whole cell lysates were prepared by lysing...
HFF with RIPA buffer (1× phosphate-buffered saline, 1% igepl CA-630, 0.5% sodium deoxycholate, and 0.1% SDS) containing a protease inhibitor mixture (Roche) and phosphatase inhibitors (10 mM NaF, 25 mM β-glycerophosphate, and 1 mM sodium orthovanadate). The lysates were centrifuged at 12,000 rpm for 10 min after sonication. The supernatants were mixed with 2× SDS loading buffer followed by boiling for 3 min. 20–50 μg of solubilized lysate proteins were separated on a 4–15% gradient SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane. After blocking with 5% non-fat dried milk in phosphate-buffered saline/Tween buffer, the blots were incubated with polyclonal antibodies against isoforms of C/EBPs (Santa Cruz Biotechnology) and COX-2 protein (Cayman). Horseradish peroxidase-conjugated secondary antibody was added, and the bands were visualized by enhanced chemiluminescence.

DNA-Protein Binding Assay—As the conventional electrophoretic mobility shift assay is difficult to identify and quantify multiple C/EBP isoforms bound to a sequence-specific DNA probe, we developed a novel method by using streptavidin-coated beads to bind biotinated DNA probe, which was used to interact with nuclear extract proteins. After centrifugation, the pelleted beads were collected and washed, and proteins were eluted by loading buffer and separated by 4–15% polyacrylamide gel electrophoresis. The separated proteins were analyzed by Western blots. In the experiments here, we incubated 600 μg of nuclear extract proteins with 6 μg of biotinated COX-2-specific C/EBP sequence or CRE sequence (Integrated DNA Technologies) and 60 μl of 4% beaded agarose (Sigma) mixed with 70% slurry at room temperature for 1 h with shaking. The bead concentration was in excess to lessen binding of nuclear extract proteins to free biotinated probes. Beads were pelleted and washed with cold phosphate-buffered saline for three times. Proteins bound to the beads were eluted and separated by SDS-PAGE. Western blot analysis was done as described above. 5′-biotinylated wild-type and mutated C/EBP sequences were: C/EBP wild-type, 5′-biotin/ACCGGCTTACGCAATTTTTTAAG-3′ and mutant, 5′-
biotin/ACCGGCGCGATAGCTTTTTTTAAG-3'/H11032
/biotin/H11032 -biotinated wild-type and mutant CRE sequences were CRE wild-type, 5'-/H11032 -/biotin/CAGTCATCGAGT-CACATGGG-3'/H11032, and mutant, 5'-/H11032 -/biotin/CAGT-CATTTCGTCACATGGG-3'/H11032. Nuclear extracts were prepared from HFF by a method previously described (10). This novel DNA-protein binding assay had much less nonspecific binding than electrophoretic mobility shift assay.

**Transient Transfections**—Transfection of HFF with a pGL3 luciferase expression vector containing COX-2 5'-flanking DNA fragment 891 to 9 was carried out as previously described (10). The expressed luciferase activity was determined in a luminometer. Cotransfection of pGL3-COX-2 promoter with C/EBP-containing plasmids was done by mixing 1 µg of pGL3-COX-2 promoter and 0.5 µg of C/EBP isoform plasmid DNA with 6 µl of FuGENE 6 transfection reagent (Roche Diagnostics, Indianapolis, IN), and the mixture was added to each well of HFF in a 6-well plate. After incubation for 8 h, the cells were washed twice with fresh medium and incubated in fresh medium containing 10% fetal bovine serum for 18 h. Cells were washed and incubated in serum-free medium for 24 h. The quiescent cells were treated with PMA (100 nM) or control vehicle for 4 h, and luciferase activity was measured.

**RESULTS**

**PMA Selectively Reduced C/EBPβ Protein Levels**—Consistent with previously reported results (10), PMA induced a significant amount of COX-2 protein in HFF after a 4-h treatment accompanied by a significant induction of the promoter activity (Fig. 1A). Since the C/EBP isoform protein levels in HFF have not been previously reported, we determined all six isoforms of C/EBP by Western blot analysis. A basal level of C/EBPα, C/EBPβ, and C/EBPδ were detected in unstimulated HFF (Fig. 1B) but C/EBPγ, C/EBPε, and C/EBPζ were undetectable (data not shown). Two isoforms of C/EBPα, the 42-kDa full-length and a 30-kDa truncated form, and three isoforms of C/EBPβ, the 46-kDa full-length (C/EBPβ-FL), a 41-kDa C/EBPβ-LAP, and a 16-kDa C/EBPβ-LIP, were expressed in unstimulated...
cells (Fig. 1B). C/EBP\(\delta\) was detected as a single 36-kDa protein (Fig. 1B). After PMA treatment for 4 h, there was a significant reduction of C/EBP\(\beta\) protein levels, while neither C/EBP\(\alpha\) nor C/EBP\(\beta\) isoforms were altered (Fig. 1B).

**PMA Altered Binding of C/EBP Isoforms to C/EBP and CRE Sites of COX-2 Promoter**—The CRE site (–59/–53) and C/EBP site (–123/–125) at the 5′-untranslated region of COX-2 genes are essential for PMA-induced promoter activity. To evaluate the involvement of C/EBP isoforms in COX-2 promoter function, we determined binding of a COX-2-specific C/EBP or CRE sequence to C/EBP isoforms in nuclear extracts prepared from HFF treated with or without PMA for 4 h. In unstimulated cells, C/EBP\(\delta\) was the predominant isoform bound to C/EBP probe (Fig. 2A). C/EBP\(\beta\)-LIP binding was also detected. PMA treatment resulted in a reduction in C/EBP\(\delta\) and an increase in C/EBP\(\beta\)-LIP, C/EBP\(\beta\)-FL (46 kDa), and C/EBP\(\beta\)-LAP binding. Binding of all three isoforms of C/EBP\(\delta\) was abrogated when a C/EBP mutant replaced the wild-type C/EBP as the probe (Fig. 2A). PMA did not induce C/EBP\(\alpha\) binding to the C/EBP site. There was basal binding of C/EBP\(\beta\) isoforms and C/EBP\(\delta\) to COX-2-specific CRE probes in unstimulated cells (Fig. 2B). PMA increased C/EBP\(\beta\)-LAP and C/EBP\(\beta\)-LIP binding to the CRE site without changing the binding of C/EBP\(\delta\) (Fig. 2B). There was no detectable C/EBP\(\alpha\) binding to the CRE site (data not shown).

**Kinetics of PMA-induced C/EBP Binding and COX-2 Protein Expression**—These results suggest that COX-2 expression is regulated by a dynamic change in binding of C/EBP isoforms to the C/EBP and CRE sites of the COX-2 promoter. To discern the relationship of C/EBP isoform binding and COX-2 expression, we measured the kinetics of COX-2 protein levels, C/EBP isoform levels, and C/EBP binding to C/EBP and CRE probes in cells treated with PMA. COX-2 proteins were detectable at 2 h and increased with time after PMA treatment (Fig. 3). C/EBP\(\beta\) isoforms were not significantly changed over time, while the C/EBP\(\delta\) level was reduced with time (Fig. 3). The COX-2 protein increase was concordant with a time-dependent reduction in C/EBP\(\alpha\) levels, a reduction in C/EBP\(\delta\) binding to the C/EBP probe (Fig. 4A), and an increase in C/EBP\(\beta\)-LAP, -FL, and -LIP binding (Fig. 4B) despite a lack of change in C/EBP\(\beta\) isoform levels (Fig. 5). These results are consistent with the interpretation that PMA increased C/EBP\(\beta\) binding through post-translational modification of C/EBP\(\beta\) (10). Results from this study further revealed a reduction in C/EBP\(\alpha\) binding to the C/EBP site because of suppression of C/EBP\(\delta\) protein expression by PMA. There was also a time-dependent increase in binding of C/EBP\(\beta\) isoforms to the CRE (Fig. 5A) while there was no significant time-dependent change in C/EBP\(\delta\) binding to the CRE site (Fig. 5B).

**Effect of Transient Transfection of C/EBP Isoforms on COX-2 Promoter and C/EBP Binding Activities**—Transient overexpression of C/EBP\(\beta\)-LIP (Fig. 6A) reduced COX-2 promoter stimulation by PMA to the basal promoter activity (Fig. 6B). C/EBP\(\delta\) overexpression (Fig. 6A) increased basal level
without suppressing PMA-induced COX-2 promoter activity (Fig. 6B). Interestingly, C/EBPβ-FL overexpression had no significant effect on basal or PMA-induced COX-2 promoter activity (Fig. 6B).

We suspected that inhibition of COX-2 promoter by C/EBPβ-LIP overexpression may be attributed to its interfering with binding of C/EBPβ-LAP to C/EBP or the CRE site. To our surprise, C/EBPβ-LIP overexpression did not inhibit binding of C/EBPβ-LAP but instead increased basal and PMA-induced C/EBPβ-LAP binding to the C/EBP site (Fig. 7A) and the CRE site (Fig. 7B). Overexpression of C/EBPβ increased the basal binding of C/EBPβ-LAP and C/EBPβ-LIP to the C/EBP site (Fig. 8A) and the CRE site (Fig. 8B) and basal binding of C/EBPβ to the C/EBP site (Fig. 8A). Interestingly, its binding to the C/EBP site was significantly reduced in PMA-treated cells despite its overexpression (Fig. 8A). C/EBPβ overexpression did not influence δ binding to the CRE site (Fig. 8B).

Overexpression of C/EBPβ-LIP Inhibited COX-2 Promoter Activation by p300—Since C/EBPβ-LIP did not block binding of C/EBPβ-LAP and yet suppressed COX-2 promoter activity induced by PMA, we suspected that C/EBPβ-LIP overexpression may act at the level of interaction between DNA-bound C/EBPβ-LAP and co-activator p300. Transfection of cells with p300 plasmids increased basal and PMA-induced COX-2 promoter activity (Fig. 9). This increase was completely abrogated by overexpression of C/EBPβ-LIP (Fig. 9).

C/EBPβ-LIP Inhibited COX-2 Protein Levels—In agreement with the COX-2 promoter results, overexpression of C/EBPβ-LIP by transient transfection reduced COX-2 protein levels induced by PMA (Fig. 10), while neither C/EBPβ-FL nor C/EBPβ overexpression altered the basal or PMA-stimulated COX-2 protein levels (data not shown). However, unlike the promoter data, p300 overexpression did not increase basal COX-2 protein level and increased PMA-induced COX-2 protein expression to a lesser extent than the promoter activity (Fig. 10). We suspect that the difference between promoter and protein stimulation by p300 is
due to an artificially high luciferase activity expressed by the transfected naked DNA. Nevertheless, COX-2 protein levels induced by PMA plus p300 overexpression were suppressed by cotransfection with C/EBPβ-LIP (Fig. 10).

**DISCUSSION**

Results from this study indicate that PMA induces a dynamic switch of C/EBP isoform binding to C/EBP and CRE sites on the 5'-untranslated region of COX-2 promoter, thereby stimulating COX-2 promoter activity. PMA causes three key switches: 1) increased binding of C/EBPβ-LIP to C/EBP and CRE sites; 2) reduced binding of C/EBPβ to the CEBP site; and 3) increased C/EBPβ-LIP binding to C/EBP and CRE sites. Increased binding of C/EBPβ-LIP to these two regulatory elements plays a key role in transactivation of COX-2 promoter, while increased C/EBPβ-LIP binding provides a positive regulation of COX-2 promoter activity. The role of C/EBPβ in this chain of events is less clear. Our data suggest that it is involved in the control of basal COX-2 promoter activity. Its binding to the C/EBP site is correlated with the protein level. When its protein expression is aberrantly augmented, such as in skin carcinogenesis, it causes an autonomous COX-2 protein expression that contributes to cancer growth (17). PMA reduced C/EBPβ protein levels, and this reduction resulted in a reduced binding of C/EBPβ to the CEBP site. It is unclear whether the effect of PMA is at the transcriptional or post-transcriptional level of C/EBPβ expression. That PMA also reduced C/EBPβ binding in overexpression driven by a viral promoter suggests that PMA might accelerate C/EBPβ degradation. Reduced C/EBPβ binding due to a lower C/EBPβ protein level coupled with an increased C/EBPβ-LAP binding is likely to be pivotal for COX-2 induction by PMA and other stimuli signaling via the protein kinase C pathway. It is interesting to note that C/EBPβ binding to the CRE site was not reduced despite a reduced C/EBPβ level and an increased C/EBPβ-LAP binding to this site. This result suggests that following stimulation by PMA, the affinity of C/EBPβ-LAP binding to the CEBP site is greatly enhanced, which leads to displacement of C/EBPβ from the CEBP site.

PMA increased the binding of all three isoforms of C/EBPβ to CREB and CRE sites without increasing their protein expression. A previous study showed that PMA induced phosphorylation of C/EBPβ, and the phosphorylated C/EBPβ exhibited an increased DNA binding activity (18). Our recent study showed that dephosphorylation of C/EBPβ resulted in a marked reduction of C/EBPβ binding to CREB site on COX-2 promoter (10). Protein kinase C does not phosphorylate C/EBPβ directly (18) but mitogen-activated protein kinase, calcium-dependent calmodulin kinase II, and ribosomal S6 kinase can directly phosphorylate C/EBPβ at different serine/threonine residues (19–21). It is unclear as to which kinase phosphorylates C/EBPβ, thereby increasing its binding activity in response to exogenous stimulation by inflammatory or mitogenic factors. It is also unknown whether C/EBPβ-LIP binding depends on phosphorylation. C/EBPβ-LIP retains the C-terminal DNA binding domain and the leucine zipper region but loses the N-terminal transactivation domain of C/EBPβ-FL or C/EBPβ-LAP (14). It can form a homodimer or heterodimer with C/EBPβ-FL or C/EBPβ-LAP and bind to CREB or CRE site. Our results suggest that it can also form a heterodimer with C/EBPβ and bind to the CRE site. Results from our transient transfection experiment are consistent with previous reports that C/EBPβ-LIP suppresses COX-2 promoter activity induced by exogenous stimuli (8). In this study, we provide evidence that C/EBPβ-LIP blocks PMA-induced promoter activity and protein expression. C/EBPβ-FL/LAP has been reported to recruit p300 family co-activators to the promoter region through its direct interaction with the co-activator (22). Since C/EBPβ-LIP did not block the binding of C/EBPβ-FL/LAP, we reasoned that it may interfere with the interaction of p300 with C/EBPβ-FL/LAP. Our results show for the first time that overexpression of C/EBPβ-LIP abrogated the stimulatory effect of p300 on COX-2 promoter activity and protein expression. We speculate that the inhibitory effect of C/EBPβ-LIP is attributable to binding of an increasing amount of LIP homodimers and LIP/LAP heterodimer to the cognate site, which no longer interact with p300, and thus interfere with recruitment of transcription machinery to the TATA region for RNA polymerase II to initiate transcription.

In summary, we have provided several novel aspects of COX-2 promoter stimulation by PMA. 1) Increased C/EBPβ-LAP binding to CEBP and CRE sites plays a pivotal role in COX-2 transcriptional activation. 2) Sequence-specific binding of C/EBPβ-LAP recruits p300 for initiation of COX-2 transcription. 3) Concurrent increase in C/EBPβ-LIP binding to these two sites interferes with CEBP-LAP interaction with p300 and provides a dynamic control of COX-2 promoter function.

**ACKNOWLEDGMENTS**—We thank Dr. Marcus Kuo, University of Texas M. D. Anderson Cancer Center for providing laboratory space to perform the p300 transfection experiments during the flood disaster and Susan Mittinger for editorial assistance.

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J. Biol. Chem. 2002, 277:6923-6928.
doi: 10.1074/jbc.M108075200 originally published online December 10, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M108075200

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