UPREGULATION OF p300 BINDING AND p50 ACETYLATION IN TUMOR NECROSIS FACTOR-α INDUCED CYCLOOXYGENASE-2 PROMOTER ACTIVATION

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Running title: p300 binding and p50 acetylation
It is well established that p300 plays an important role in mediating gene expressions. However, it is less clear how its binding is influenced by physiological stimuli and how its altered binding affects transactivator acetylation and binding. In this study, we determined p300 binding to a core cyclooxygenase-2 (COX-2) promoter region by chromatin immunoprecipitation and streptavidin-agarose pulldown assays in basal and tumor necrosis factor-α (TNFα) treated human foreskin fibroblasts. We found basal binding of p300, p50/p65 NF-κB, cyclic AMP regulatory element binding protein-2, CCAAT/enhancer binding protein β and C-Jun. p50/p65 and p300 binding was selectively increased by TNFα. Immunoprecipitation confirmed direct interaction of p300 with NF-κB and the other involved transactivators. p50 acetylation was detected in resting cells which was increased by TNFα or lipopolysaccharide. Overexpression of p300 augmented p50 acetylation which was attenuated by deletion of its histone acetyltransferase domain. Enhanced p50 acetylation correlated with increased p50 binding to COX-2 promoter and transcriptional activation. Co-transfection of E1A with p300 abrogated p50 acetylation and p50 binding. These findings suggest that upregulation of p300 binding and its acetylation of NF-κB occupies a central position in COX-2 promoter activation.
INTRODUCTION

Cyclooxygenase-2 (COX-2) catalyzes the synthesis of robust prostaglandins and thromboxane. It is highly inducible in many cell types by cytokines, oncogenic and mitogenic factors. COX-2 has been shown to play an important role in inflammation, angiogenesis, and tumorigenesis. Its involvement in these pathophysiological processes depends largely on its transcriptional activation by diverse stimuli. Its promoter activation by pro-inflammatory mediators has been extensively investigated. Several regulatory elements located at the 5'-flanking untranslated region including a cyclic AMP response element (CRE) at -53 to -59, a CCCAAT/enhancer binding protein (C/EBP) element at -124 to -132 and two NF-κB sites at -438 to -447 and -213 to -222 are involved in human COX-2 transactivation. We have recently demonstrated binding of CRE binding protein (CREB), C/EBPβ and NF-κB to their respective binding sites in this region. Our results indicate that COX-2 induction in human fibroblasts and endothelial cells require binding of multiple transactivators. P300/CREB binding protein overexpression has been shown to upregulate COX-2 transcription suggesting an important role of p300 in bridging the multiple DNA-bound transactivators with general transcription factors to initiate COX-2 transcription. P300 belongs to a large class of transcription co-activators, which serve as an adaptor for transcriptional activation of diverse genes. It is a 2414-amino acid protein containing several domains for binding to transactivators, adenoviral E1A and general transcription factors. It is a histone acetyltransferase (HAT), which acetylates histone and induces chromatin remodeling to facilitate tranactivation. P300 has been shown to acetylate transactivators and enhance their binding to DNA.
p300 binding and p50 acetylation

(19, 20). Since p300 is capable of binding to CREB (21), C/EBPβ (22) and NF-κB (23), it is likely to be a major co-activator for COX-2 transcriptional activation. However, there was little reported data about p300 binding to COX-2 promoter nor was there information about p300 acetylation of COX-2 bound transactivators. In this study, we determined p300 interaction with transactivators bound to the core COX-2 promoter region and assessed the role of p300 HAT in regulating COX-2 transcriptional activity in human fibroblasts stimulated with tumor necrosis factor-α (TNFα). Our results show that TNFα upregulated binding of p50/p65 NF-κB which correlated with enhanced p300 recruitment to the promoter complex. Deletion mutation of p300 HAT reduced p300 recruitment and severely attenuated its ability to enhance basal and TNFα-induced COX-2 promoter activity. Our results further show that p300 acetylated p50 NF-κB but not p65, C/EBPβ or CREB-2. These findings indicate that p300 mediates and regulates COX-2 transactivation by multiple mechanisms including a selective acetylation of p50 NF-κB.
EXPERIMENTAL PROCEDURES

Plasmids - A promoter region of human COX-2 gene (-891 to +9 from the transcription start site) was constructed into the luciferase reporter vector pGL3 as previously described (8). The expression vectors containing full-length p300 (pCL.p300) and its HAT deletion mutant (CL.p300ΔHAT, Δ1, 472-1, 522) (19) were provided by Dr. Joan Boyes. The E1A expression vector (24) was provided by Dr. Pardip Raychaudhuri.

Cell culture and treatment - Human foreskin fibroblasts (HFb) were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 1:100 dilution of an antibiotic-antimycotic solution. For all experiments, 80-90% confluent cells were cultured in serum free medium for 24 h, washed with PBS and incubated in fresh medium in the presence or absence of 10 ng/ml TNFα (Sigma), 100 nM of phorbol 12-myristate 13-acetate (Sigma) or 2 μg/ml of lipopolysaccharide (E.coli 026:B6, Sigma) at 37°C for 4 h. After washing with chilled PBS three times, the cells were harvested and processed to prepare cell lysates or nuclear extracts. All the tissue culture reagents were obtained from Life Technologies, Inc.

Transient transfection - The transfection procedure was performed as previously described (10). In brief, 10 μl of Lipofectamine 2000 reagent (Invitrogen) and 4 μg of luciferase expression constructs were mixed, and the mixture was slowly added to each well of HFb grown in a 6-well plate and incubated for 24 h. The cells were washed, incubated in serum free medium and treated with TNFα (10 ng/ml). The expressed luciferase activity was measured in a luminometer (TD-20/20). To evaluate the effect of p300 or p300 ΔHAT on COX-2 promoter activation, 2 μg of pCL.p300, pCL.p300 ΔHAT or pCL.vector plus 4 μg of luciferase expression constructs were mixed with 15 μl of
**p300 binding and p50 acetylation**

Lipofetamine 2000 reagent and the mixture was added slowly to cells cultured in a 6-well plate. To evaluate the effect of p300 overexpression on p50 acetylation or binding, 10 µg pCL constructs were mixed with 25 µl of Lipofetamine 2000 reagent and the mixture was added to cells cultured in a 10 cm dish. Co-transfection of E1A and p300 was performed by mixing 4 µg E1A plasmid construct with 6 µg p300 construct and 25 µl of lipofetamine and the mixture was slowly added to ~90% confluent cells in serum-free medium in a 10-cm dish and incubated for 3 h. 10% FBS was added and incubated for an additional 21 h. Cells were washed and incubated in serum free medium for 24 h prior to addition of TNFα or vehicle control.

**Western blot analysis** - Western blot analysis was performed as previously described with minor modifications (25). In brief, cell pellets were lysed with lysis buffer containing 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 µg/ml leupeptin, 5 µg/ml aprotinin, 1% Nonidet P450, 0.5% sodium deoxycholate and 0.1% sodium dodecyl sulfate. The lysate was centrifuged and the supernatant was collected and boiled for 5 min. Protein concentration was determined. Lysate proteins were separated by electrophoresis in a 4-15% sodium dodecyl sulphate-polyacrylamide minigels (Bio-Rad) (SDS-PAGE) and then electrophoretically transferred to a nitrocellulose membrane (Amersham Pharmacia Biotech). Western blots were probed with a specific rabbit polyclonal anti-p300 antibody (Santa Cruz Biotechnology). The protein bands were detected by enhanced chemiluminescence (Pierce).

**Immunoprecipitation** - Nuclear extracts were prepared from HFb by a method previously described (26). 800 µg nuclear extracts were incubated with a specific rabbit polyclonal antibody against p300, C-Jun, CREB-2, C/EBPβ, p50 or p65 (all from Santa
p300 binding and p50 acetylation

Cruz Biotechnology) at a final concentration of 4 µg/ml each overnight at 4°C. Protein A/G plus agarose (Santa Cruz) was then added for 2 h at 4°C. Beads were washed four times in RIPA buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 2 mM NaF, 1 mM EDTA, 1 mM EGTA, 1 mM NaVO4, 1% Triton X-100) containing protease inhibitors (Roche), and the immunoprecipitated proteins were separated by SDS-PAGE and analyzed by Western blotting. Control immunoprecipitation was performed with a non-immune rabbit normal immunoglobulin (Santa Cruz).

DNA-protein binding assay - Binding of p300 to COX-2 promoter DNA sequence was assayed by a technique recently described (12). 80-90% confluent HFb were incubated in serum-free medium with 10 ng/ml TNFα for 4 h before nuclear extracts were prepared. The biotin labeled double-stranded oligonucleotides were synthesized by Integrated DNA Technologies based on human COX-2 promoter sequence -30 to -453 (8). A non-relevant biotinylated sequence 5'-AGAGTGGTCACTACCCCCTG-3' was included as control. The binding assay was performed by mixing 600 µg HFb nuclear extract proteins, 6 µg biotin-labeled DNA oligonucleotides and 60 µl streptavidin agarose beads (4%) with 70% slurry. The mixture was incubated at room temperature for 1 h with shaking. Beads were pelleted and washed with cold PBS for three times. The binding proteins were separated by SDS-PAGE followed by Western blot analysis probed with specific antibodies.

Chromatin Immunoprecipitation (ChIP) - The assay was done as described with minor modifications (27). 80-90% confluent HFb were serum starved for 24 h and treated with or without TNFα (10 ng/ml) at 37°C for 4 h. 1% formaldehyde was added to the culture medium and after incubation for 20 min at 37°C, cells were washed twice in
p300 binding and p50 acetylation

PBS, scraped and lysed in lysis buffer (1% SDS, 10 mM Tris-HCl pH 8.0, with 1 mM PMSF, pepstatin A and aprotinin) for 10 min at 4°C. Lysates were sonicated 5 times for 10 s each and the debris was removed by centrifugation. One third of the lysate was used as DNA input control. The remaining two thirds of the lysate were diluted 10-fold with a dilution buffer (0.01% SDS, 1% Triton X100, 1 mM EDTA, 10 mM Tris-HCl pH 8.0, and 150 mM NaCl) followed by incubation with antibodies against p300, C-Jun, C/EBPβ, CREB-2, p50, p65 NF-κB or a non-immune rabbit IgG (Santa Cruz) overnight at 4°C. Immunoprecipitated complexes were collected by using protein A/G-plus agarose beads. The precipitates were extensively washed and incubated in an elution buffer (1% SDS and 0.1 M NaHCO₃) at room temperature for 20 min. Cross-linking of protein-DNA complexes was reversed at 65°C for 5 h, followed by treatment with 100 µg/ml proteinase K for 3 h at 50°C. DNA was extracted 3 times with phenol/chloroform and precipitated with ethanol. Pellets were resuspended in TE buffer and subjected to PCR amplification using specific COX-2 promoter primers: 5’ primer, -709 CTGTGTAAAGCAACTTAGCT -690, and 3’ PRIMER -32 AGACTGAAAACCAAGCCCAT -51. The resulting product of 678 bp in length was separated by agarose gel electrophoresis.

Acetylation of transactivators - p50, p65, C/EBPβ, or CREB-2 in nuclear extracts was immunoprecipitated with a specific antibody and the immunoprecipitates were collected by using protein A/G-plus agarose beads. After extensive washing proteins were separated by SDS-PAGE and acetylated transactivators were detected on Western blots using a monoclonal antibody against acetylated lysine (1:1000 dilution, Cell Signaling Technology).
p300 binding and p50 acetylation

RESULTS

Upregulation of p300 binding to COX-2 promoter by TNFα - To determine whether p300 recruitment to COX-2 promoter-transactivator complex was altered by TNFα stimulation, we evaluated p300 binding in unstimulated as well as TNFα-stimulated HFb by ChIP. Chromatin was immunoprecipitated with a p300 antibody and a COX-2 promoter-enhancer region (-32 to -709) containing the essential binding sites for promoter activation was amplified by PCR. Vector-transfected HFb, like native HFb, shows trace p300 binding at basal state which was increased by TNFα treatment (Fig. 1). ChIP assays using specific transactivator antibodies also detected binding of C-Jun, CREB-2, C/EBPβ, p50 and p65 NF-κB to the core COX-2 promoter region in chromatin structure in unstimulated cells and TNFα treatment resulted in a significant increase only in p50 and p65 NF-κB binding (Fig. 1). Binding of p300 and transactivators to COX-2 promoter was specific as immunoprecipitation with a normal rabbit IgG did not show detectable COX-2 promoter fragment (Fig. 1).

We next used the streptavidin agarose pulldown assay, which provides quantitative information of transactivator binding, to evaluate the effect of TNFα on transactivator and p300 binding to a biotinylated COX-2 promoter sequence (-453 to -30). Nuclear extracts from HFb treated with and without TNFα were incubated with the biotinylated probe and streptavidin-agarose beads. Transactivators and p300 present in the complex were analyzed by Western blots. Consistent with the ChIP assays, C-Jun, CREB-2, C/EBPβ and p50/p65 NF-κB as well as p300 were detected in resting cells and only p300 and p50/p65 NF-κB binding was increased by TNFα stimulation (Fig. 2).
**p300 binding and p50 acetylation**

Interaction of p300 with COX-2 promoter-bound transactivators was determined by immunoprecipitation of nuclear extract proteins with antibodies against C-Jun, CREB-2, C/EBPβ, p50, p65 or nonimmune IgG and analysis of p300 in the complex by Western blots. P300 was co-precipitated with each of the transactivators in resting and TNFα-treated cells (Fig. 3a). p300 complexion with each transactivator tended to be increased by TNFα. However, quantitation of binding is difficult because of large p300 molecular mass and slow mobility in gel electrophoresis. Interaction of p300 with these transactivators was confirmed by immunoprecipitation of nuclear extract proteins with anti-p300 antibodies and identification of transactivators by Western blot analysis (Fig. 3b). Together, these results provide direct evidence for recruitment and binding of p300 to COX-2 promoter in resting cells and an upregulation of p300 binding as a result of increased p50/p65 binding to the promoter following TNFα stimulation.

**Attenuation of COX-2 promoter activation by p300 HAT deletion mutation** - HFb expressed a low basal level of p300 (Fig. 4). Transient transfection of p300 increased its level which was accompanied by a large increase in basal COX-2 promoter activity (Fig. 5), a result consistent with the reported data (12-14). Overexpression of a p300 HAT deletion mutant by transient transfection to a similar level as the overexpressed wild-type (WT) protein also increased the basal COX-2 promoter activity but the increase was only about 30% of that induced by the WT p300 (Fig. 5). TNFα did not significantly increase native or transduced p300 levels (Fig. 4). However, p300 overexpression augmented COX-2 promoter activity stimulated with TNFα and this augmenting effect was greatly attenuated when p300 HAT domain was deleted (Fig. 5). As the HAT domain of p300 is situated close to transactivator binding domains, its deletion (Δ1472-
p300 binding and p50 acetylation

1522) may influence p300 binding. We therefore compared recruitment of WT and ΔHAT p300 to the biotinylated probe by the streptavidin-bead pulldown assay. The ΔHAT mutant did not bind as well as the WT p300 and its binding was close to the basal p300 binding (Fig. 6). Together, these results suggest that p300 plays a major role in regulating COX-2 promoter activity and its HAT activity is crucial for the regulation of COX-2 transactivation and p300 recruitment.

Selective p50 acetylation by p300 - It is well established that p300 HAT contributes to promoter activation by acetylating core histones in chromatin structure. In this study, we investigated whether it acetylates COX-2 promoter-bound transactivators. p50, p65, CREB-2 and C/EBP transactivators were immunoprecipitated with their respective antibodies and the acetylated proteins were detected by Western blots using an antibody specific for acetylated lysine. A low level of acetylated p50 was detected at basal state which was increased by TNFα stimulation (Fig. 7a). Neither p65 nor other transactivators were acetylated. Acetylated p50 was increased slightly by stimulation with phorbol 12-myristate 13-acetate (100 nM) for 4 h and was significantly increased by stimulation with lipopolysaccharide (2 µg/ml) for 4 h (Fig. 7b). To determine whether p50 acetylation was mediated by p300, we overexpressed WT or ΔHAT p300 and assayed p50 acetylation. At the basal state, p300 transfection increased p50 acetylation whereas ΔHAT caused a much less increase (Fig. 8a). P300 overexpression augmented TNFα-induced p50 acetylation, which was attenuated by HAT deletion mutation (Fig. 8). As expected, p50 protein levels at basal state were low and were increased by TNFα stimulation. Neither p300 nor p300 ΔHAT transfection altered p50 protein levels (Fig. 8b).
To determine whether p50 acetylation was correlated with an increased p50 binding to the COX-2 promoter we measured p50 binding to the biotinylated COX-2 promoter in HFb transduced by WT, ΔHAT or its control vector. At basal state, p300 overexpression increased p50 binding by more than 2-fold while ΔHAT overexpression exerted a lesser increase (Fig. 9). TNFα increased p50 binding which was augmented by WT but not ΔHAT p300 overexpression (Fig. 9). ChIP assays were performed to evaluate the effect of p300 overexpression on p50 binding to chromatin COX-2 promoter region. Corresponding to the results of in vitro binding experiments shown in Fig. 9, overexpression of wild-type p300 augmented p50 binding to COX-2 promoter region in the chromatin structure of resting and TNFα-stimulated cells (Fig. 10). ΔHAT overexpression attenuated the increase in p50 binding (Fig. 10).

Inhibition of p50 acetylation by E1A - As adenoviral E1A is a potent inhibitor of p300 coactivator activities (28), we evaluated its effect on p50 acetylation and binding. Its overexpression suppressed COX-2 promoter activities stimulated by TNFα and p300 overexpression (data not shown). Overexpression of E1A by transient transfection abrogated p50 acetylation induced by p300 overexpression in the presence or absence of TNFα without altering the p50 level (Fig. 11). Inhibition of p50 acetylation by E1A was correlated with reduction of p50 binding to a COX-2 promoter probe (Fig. 12a) and to the chromatin COX-2 promoter region (Fig. 12b).
DISCUSSION

Results from this study show a low level of p300 recruited to the COX-2 promoter-bound transactivators in resting human fibroblasts. p300 recruitment was enhanced in cells treated with TNFα and further augmented by p300 overexpression. Enhanced p300 binding was correlated with an upregulation of COX-2 promoter activities. These enhancing activities of p300 were abrogated by E1A, an inhibitor of p300. These results indicate that p300 plays a crucial role in regulating COX-2 transcription. Our findings further indicate that p300-mediated COX-2 transcriptional activation depends on HAT. Deletion of the HAT domain resulted in a more than 60% reduction in COX-2 promoter activity induced by p300. p300 HAT is capable of acetylating the N-terminal lysine residues of core histones, thereby modifying the chromatin structure (15-17). This property of p300 is likely contributing to accessibility of COX-2 promoter regulatory elements for transactivator binding. In this study, our results shed light on another important property of p300 that enhances NF-κB dependent COX-2 transcription. Our results indicate that p300 HAT is capable of acetylating p50, thereby increasing p50/p65 NF-κB binding to COX-2 promoter. P300 has previously been shown to acetylate p53 (19) and GATA (20). To our knowledge, this is the first report of p300-mediated p50 acetylation under physiological conditions. Interestingly, p50 acetylation by p300 was demonstrated as a mechanism by which human immunodeficiency virus-1 replicates in host lymphocytes (29). Binding of host p50/p65 NF-κB to viral long terminal repeat region has been shown to play a key role in viral genome transcription. In the presence of a viral protein Tat, p300 HAT acetylates p50 at several lysine residues which results in enhanced p50 binding to its cognate sites. Our
results show p50 acetylation in TNFα-treated cells in the absence of the viral Tat. It is unclear whether under physiological conditions, p50 acetylation by p300 also depends on a Tat-like cofactor. That p300 HAT acetylates p50 without a concurrent acetylation of C/EBPβ, CREB-2, C-Jun or p65 suggests a stringent requirement of an appropriate lysine structural environment in p50 and possibly also p53 and GATA-1 for the action of p300 HAT.

NF-κB is a heterodimer typically comprising a p50 subunit that binds to promoter and a p65 subunit that interacts with p300. NF-κB is sequestered in cytosol and upon stimulation, it translocates to nucleus where it binds to its cognate sites. NF-κB has been shown to play a key role in COX-2 transcriptional activation stimulated by TNFα, lipopolysaccharide and other pathophysiological stresses. There are two NF-κB sites at the core promoter region of human COX-2 and mutation of either site results in loss of response to TNFα stimulation (10). In this study, our results show that TNFα selectively increased NF-κB binding to these sites which was accompanied by enhanced p300 recruitment and binding to the COX-2 promoter complex. In view of augmented p50/p65 binding and p300 recruitment by p300 overexpression and the requirement of HAT for the p300-induced binding activities, we propose that p300 binding to DNA-bound NF-κB is limited by a low level of p300 in cells and TNFα is capable of augmenting p300 binding by a positive feedback loop driven by p50 acetylation. p50 acetylation by HAT of p300 bound to the complex leads to an increased p50/p65 binding which in turn recruits additional p300 to the complex. This autoregulatory loop ensures upregulation of NF-κB mediated gene expression. As NF-κB plays a key role in transcriptional activation of
myriad proinflammatory genes, this regulatory mechanism has important implications in inflammation, tissue injury and tumorigenesis.

There is a large body of data which supports the notion that p300 recruitment to DNA-bound transactivators is essential for transcription of many genes. However, there is little information about the role of increased p300 binding in gene transcription under physiological condition. In this study, our results demonstrate increased p300 binding to chromatin as well as naked COX-2 promoter sequence by TNFα stimulation. Increased p300 binding correlated closely with an enhanced p50/p65 binding. Our results provide further evidence for complex formation between p300 and each involved transactivator and an upregulation of p300 and p50/p65 in the complex by TNFα. Together with our previously reported results (10-12), these findings indicate that at basal state, p300 is recruited to COX-2 promoter by interacting with constitutively bound CREB and C-Jun at the CRE site, C/EBPβ at CRE and C/EBP sites and p50/p65 NF-κB at both NF-κB sites. TNFα stimulation increases p50/p65 binding via p50 acetylation and the increased NF-κB bound to its specific sequence recruits additional p300 leading to amplified transcriptional activation. TNFα stimulation could therefore serve as a model for understanding COX-2 transcriptional stimulation by diverse cytokines, growth factors, angiogenic factors and environmental stress. These agonists induce COX-2 transcription by upregulating the binding of distinct groups of transactivators which in turn recruit p300. p300 binding to transactivators drives the transcriptional initiation complex via interaction with general transcriptional factors. Furthermore, p300 acetylates core histone, notably H3 and H4 lysines to modify chromatin structure and increase transactivator binding. In addition, p300 acetylates selective classes of
transactivators, such as p50, thereby further increasing transactivator binding and amplifying transactivation of genes. Increased transactivator binding results in recruiting additional p300, thus creating a positive regulatory loop for COX-2 transactivation. p300 thus occupies a central position in regulating COX-2 promoter activation through its pleiotropic actions.

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Abbreviations used in this paper: COX-2, cyclooxygenase-2; TNFα, tumor necrosis factor α; CREB, CRE binding protein; HAT, histone acetyltransferase; ΔHAT, HAT deletion mutant; WT, wild-type p300; HFb, human foreskin fibroblasts; C/EBP, CCAAT/enhancer binding protein; ChIP, Chromatin immunoprecipitation.
**FIGURE LEGENDS**

**Fig. 1.** TNFα increased p300 and p50/p65 NF-κB binding to chromatin COX-2 promoter region. Chromatin fragments prepared from HFb treated without or with TNFα were immunoprecipitated with specific antibodies against p300, C-Jun, CREB-2, C/EBPβ, p50 or p65 and the COX-2 promoter region (-32 to -709) in the chromatin precipitate was amplified by PCR under identical conditions. Rabbit non-immune IgG was included as a negative control. **a.** A representative set of results. **b.** Densitometric analysis of basal vs. TNFα treated samples from three experiments. Each bar denotes mean ± S.D. Only the difference in p300, p50 and p65 binding was statistically significant (p<0.05 for each).

**Fig. 2.** TNFα increased p300, p50 and p65 binding to a biotinylated COX-2 promoter probe. Nuclear extracts from HFb were incubated with streptavidin-agarose beads and a biotinylated COX-2 promoter sequence -30 to -453. Proteins in the complex were analyzed by Western blots. **a.** Results from a representative experiment. **b.** Comparison of the densitometry of basal vs. TNFα treated samples in three experiments. Each bar is mean ± S.D. Only the difference in p300, p50 and p65 binding levels was statistically significant (p<0.05).

**Fig. 3.** Co-immunoprecipitation of p300 with transactivators. **a.** Nuclear extract proteins were immunoprecipitated with the indicated specific antibody and proteins in the immuno-precipitates were detected by Western blot analysis using a p300 antibody. This figure is from one of two experiments with similar results. **b.** Nuclear extracts were immunoprecipitated with a p300 antibody and tranactivators in the complex were
p300 binding and p50 acetylation
detected by their respective antibodies. This figure is from one of two experiments with
similar results. IP control denotes IP with a normal rabbit IgG.

**Fig. 4.** TNFα did not influence the protein levels of p300 in HFb. p300 proteins were
determined by Western blot analysis. Upper panel shows a representative Western blot.
The lower panel shows densitometric analysis of Western blots from three experiments.
Each bar denotes mean ± S.D.

**Fig. 5.** p300 HAT deletion mutation attenuated COX-2 promoter activity. HFb were
transfected with wild-type (WT) p300, HAT deletion mutant (∆HAT) or vector followed by
treatment with or without TNFα. COX-2 promoter activity was expressed as relative light
unit (RLU) per µg of cell lysate proteins. Each bar denotes mean ± S.D. of three
experiments.

**Fig. 6.** Deletion of HAT reduced p300 binding. Nuclear extracts from HFb treated with or
without TNFα stimulation were incubated with biotinylated COX-2 promoter probe and
streptavidin-agarose beads. p300 in the complex was detected by Western blots.
Control denotes the use of a non-related biotinylated sequence as the probe. a.
Western blot of a representative experiment. b. Densitometric analysis of three
experiments. Each bar is mean ± S.D.

**Fig. 7.** TNFα and lipopolysaccharide increased p50 acetylation. a. Nuclear extracts from
HFb treated with or without TNFα for 4 h were immunoprecipitated with CREB-2,
C/EBPβ, p50 or p65 antibodies or a non-immune rabbit IgG and the acetylated
transactivator in the precipitate was detected by Western blot analysis. IP control
denotes IP of nuclear extracts from TNFα treated cells with non-immune IgG. Only
acetylated p50 was detected in unstimulated cells which was increased by TNFα.
Densitometric analysis of acetylated p50 (Ac-p50) shows a significant increase in Ac-p50 in TNFα treated cells (n=3, p < 0.05). a. Nuclear extracts from cells treated with or without phorbol 12-myristate 13-acetate (PMA, 100 nM) or lipopolysaccharide (LPS, 2 µg/ml) for 4 h were immunoprecipitated with a normal rabbit IgG (IP control) or a specific p50 antibody. Ac-p50 was detected by Western blots using an acetylated lysine antibody. LPS significantly increased Ac-p50 over the basal level (n=3, p < 0.05) whereas PMA did not significantly increase Ac-p50.

**Fig. 8.** p300 overexpression increased p50 acetylation. Nuclear extracts were immunoprecipitated with an anti-p50 antibody. a. Acetylated p50 (Ac-p50) and b. Total p50 were analyzed by Western blots using an anti-acetylated lysine antibody and an anti-p50 antibody, respectively. The upper panel shows a representative Western blot and the lower panel, densitometric analysis of three experiments.

**Fig. 9.** p300 overexpression increased p50 binding. Binding was carried out by streptavidin-agarose pulldown assay using a biotinylated COX-2 promoter sequence (-30 to -453) as the probe. a. A representative Western blot. b. Densitometric analysis of three experiments. Each bar is mean ± S.D. Control denotes the use of a non-relevant probe in the assay.

**Fig. 10.** p300 overexpression increased p50 binding to chromatin COX-2 promoter. Chromatin was immunoprecipitated with a specific p50 antibody or a non-immune rabbit IgG and the COX-2 promoter region was amplified by PCR. a. A representative gel showing COX-2 promoter fragments. Non-immune IgG column denotes immunoprecipitation with a normal rabbit IgG of chromatin from p300-transfected cells treated with TNFα for 4 h. b. Densitometric analysis of COX-2 promoter fragments in
*p300 binding and p50 acetylation*

chromatin complexes immunoprecipitated with a p50 antibody. Each bar represents mean ± S.D. of three experiments.

**Fig. 11.** E1A transfection abrogated Ac-p50 stimulated by p300. HFb co-transfected with E1A and p300 were treated with or without TNFα. Nuclear extracts were immunoprecipitated with a p50 antibody or a control IgG. **a.** Ac-p50 and **b.** p50 levels were detected by Western blot analysis using antibodies specific for acetylated lysine (a) or p50 (b). Densitometric analysis shows mean ± S.D. from three experiments.

**Fig. 12.** Inhibition of p50 binding to naked COX-2 promoter (a) and chromatin COX-2 promoter (b). Binding assays are described in Experimental Procedures. Control probe is a 22-bp biotinylated nonrelevant sequence. Each bar represents mean ± S.D. of three experiments.
Fig 1

a.

| IP antibody | DNA input | PCR control | Nonimmune IgG | Basal | + TNFα | Specific antibody |
|-------------|-----------|-------------|---------------|-------|--------|-------------------|
| p300        |           |             |               |       |        |                   |
| c-Jun       |           |             |               |       |        |                   |
| CREB-2      |           |             |               |       |        |                   |
| C/EBPβ      |           |             |               |       |        |                   |
| p50         |           |             |               |       |        |                   |
| p65         |           |             |               |       |        |                   |

IP antibody: p300, c-Jun, CREB-2, C/EBPβ, p50, p65

COX-2 promoter: 678 bp

b.

Densitometry

- Basal
- + TNFα

p300, c-Jun, CREB-2, C/EBPβ, P50, P65
Fig 2

a.

![Image of gel blots showing protein expression levels for p300, c-Jun, CREB-2, C/EBPβ, p50, and p65 under control, basal, and + TNFα conditions.]

b.

![Graph showing densitometry for p300, c-Jun, CREB-2, C/EBPβ, p50, and p65 under basal and + TNFα conditions. The bars represent the average densitometry values with error bars indicating standard deviation.]
Fig 3a

a. IP: anti-transactivators  WB: anti-p300

|        | c-Jun | CREB-2 | CEBPβ | p50 | p65 | IP control |
|--------|-------|--------|-------|-----|-----|------------|
| TNFα   | -     | +      | -     | +   | -   | +          |

← p300
b. IP: anti-p300    WB: anti-transactivors

Fig 3b
Fig 4

Vector                  +          - - +         - - +
p300-WT              - +         - - +          -
p300-ΔHAT          - - +               - - +
TNFα          - - - +         +         +

Densitometry

Vector          + - - + - -
p300-WT       - + - + - -
p300-ΔHAT     - - + - - +
TNFα          - - - + + +
Fig 6

a. Fig 6

|            | Basal | + TNFα |
|------------|-------|--------|
| Control    |       |        |
| Vector     |       |        |
| p300-WT    |       |        |
| p300-ΔHAT  |       |        |

b. Fig 6

|            |        |        |
|------------|--------|--------|
| Vector     | +      | -      | -      |
| p300-WT    | -      | +      | -      |
| p300-ΔHAT  | -      | -      | +      |
| TNFα       | -      | -      | +      |

Densitometry

Vector  p300-WT  p300-ΔHAT  TNFα

| + | - | - |
|---|---|---|
|   | + | + |
|   |   | + |
Fig 7

a.

| IP control | Basal | TNFα |
|------------|-------|------|
| Ac-CREB-2 |       |      |
| Ac-C/EBPβ |       |      |
| Ac-p50    |       |      |
| Ac-p65    |       |      |

Densitometry

b.

| IP control | Basal | PMA | LPS |
|------------|-------|-----|-----|
| Ac-p50     |       |     |     |

Densitometry
Fig 8

a.  

|          | Basal | + TNFα |
|----------|-------|--------|
| IP control |      |        |
| Vector |      |        |
| p300-WT |      |        |
| p300-ΔHAT |  |        |

Densitometry

- Ac-p50

b.  

|          | Basal | + TNFα |
|----------|-------|--------|
| IP control |      |        |
| Vector |      |        |
| p300-WT |      |        |
| p300-ΔHAT |  |        |

Densitometry

- p50
Fig 9

a. 

|          | Basal | + TNFα |
|----------|-------|--------|
| Control  |       |        |
| Vector   |       |        |
| p300-WT  |       |        |
| p300-ΔHAT|       |        |
| Vector   |       |        |

b. 

|          | Densitometry |
|----------|--------------|
| Vector   | +, -, -, +, -|
| p300-WT  | - +, -, - |
| p300-ΔHAT| - - +, - + |
| TNFα     | - - - + + + |
Fig 10

a. [Image of gel showing bands with annotations]

b. [Graph showing densitometry results]

DNA input  PCR control  Nonimmune IgG  Vector  p300-WT  p300-DHAT  Vector  p300-WT  p300-DHAT

Basal  + TNFα

Densitometry

Vector            +         - - +         - - 
p300-WT        - +         - - +         - 
p300-DHAT    - - +                  - - +

TNFα: - - - +        +        +
**Fig 11**

(a) 

IP: IgG  

p50 antibody  

[Image of Western blot showing Ac-p50]  

Densitometry  

---  

(b) 

[Image of Western blot showing p50]  

Densitometry  

| p300 | TNFα | E1A |
|------|------|-----|
| +    | +    | +   |
| +    | +    |     |
| +    | +    | +   |

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Fig 12

a. Probe: Control → COX-2 promoter →

![Image](image1)

Densitometry

|        | p300 | + | + | + | + | + |
|--------|------|---|---|---|---|---|
| TNF α  | -    | - | - | - | + | + |
| E1A    | -    | - | + | - | + | + |

b. IP: IgG → p50 antibody →

![Image](image2)

Densitometry

|        | p300 | + | + | + | + | + |
|--------|------|---|---|---|---|---|
| TNF α  | -    | - | - | - | + | + |
| E1A    | -    | - | + | - | + | + |
Upregulation of p300 binding and p50 acetylation in tumor necrosis factor-α induced cyclooxygenase-2 promoter activation
Wu-Guo Deng, Ying Zhu and Kenneth K. Wu

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