Nucleic Acids Research, 2006, Vol. 34, No. 1 ***217–231***
doi:10.1093/nar/gkj422

Functional role of NF-IL6β and its sumoylation and acetylation modifications in promoter activation of *cyclooxygenase 2* gene

Ju-Ming Wang[1], Chiung-Yuan Ko[1], Lei-Chin Chen[1,2], Wen-Lin Wang[1] and Wen-Chang Chang[1,*]

[1]Department of Pharmacology, College of Medicine, National Cheng Kung University, Tainan, Taiwan and [2]Department of Medical Nutrition, I-Shou University, Dashu Township, Kaohsiung County, Taiwan

Received September 13, 2005; Revised and Accepted December 14, 2005

ABSTRACT

NF-IL6β regulates gene expression and plays function roles in many tissues. The EGF-regulated cyclooxygenase-2 (*cox-2*) expression is mediated through p38^{MAPK} signaling pathway and positively correlates with NF-IL6β expression in A431 cells. NF-IL6β coordinated with c-Jun on *cox-2* transcriptional activation by reporter and small interfering RNA assays. NF-IL6β could directly bind to CCAAT/enhancer-binding protein (C/EBP) and cyclic AMP-response element (CRE) sites of the *cox-2* promoter by *in vitro*-DNA binding assay. The C/EBP site was important for basal and, to a lesser extent, for EGF-regulated *cox-2* transcription, while the CRE site was a more specific response to EGF inducibility of *cox-2* gene. SUMO1 expression attenuated EGF- and NF-IL6β-induced *cox-2* promoter activities. NF-IL6β was found to be sumoylated by *in vivo*- and *in vitro*-sumoylation assays, and the SUMO1-NF-IL6β (suNF-IL6β) lost its ability to interact with p300 in *in vitro*-binding assay. NF-IL6β was also acetylated by p300, and acetylation of NF-IL6β enhanced the *cox-2* promoter activity stimulated by NF-IL6β itself. *In vivo*-DNA binding assay demonstrated that EGF stimulated the recruitment of p300 and NF-IL6β to the *cox-2* promoter, yet promoted the dissociation of SUMO1-modificated proteins from the promoter. These results indicated that NF-IL6β plays a pivotal role in the regulation of basal and EGF-induced *cox-2* transcription.

INTRODUCTION

Prostaglandins play important roles in many biological processes, including cell division, immune responses, blood pressure regulation, ovulation, bone development and wound healing. The cyclooxygenase (COX, prostaglandin endoperoxide synthase) is a key enzyme in prostaglandin, prostacyclin and thromboxane biosynthesis from arachidonic acid. Two COX isoforms were described (1). COX-1 is constitutively expressed in most tissues and cells in animal species. COX-2 is induced by a wide-range of stimulators, such as IL-1β (2,3), TNF-α (4), IL-18 (2), epidermal growth factor (EGF) (5) or LPS (6), in many distinct cell types (7–9) and is regulated mainly at the level of transcription. Human *cox-2* promoter region contains a twin arginine translocation A and multiple regulatory elements, including two putative nuclear factor-κB (NF-κB) binding sites, one nuclear factor interleukin-6 (NF-IL6)/CCAAT/enhancer-binding protein (C/EBP) binding site and one cyclic AMP-response element (CRE) (10). Recent studies on human *cox-2* promoter have shown that *cox-2* transcription is regulated by different transcription factors, including NF-κB (11), NF-IL6/C/EBP (11–14), C/EBPδ (12), CREB (12,13,15) and activation protein 1 complex (AP-1) (5,11), supporting that regulation of *cox-2* gene expression could involve complex interactions among diverse transcription factors. Thus, transcriptional mechanism of *cox-2* induction relies on cell type-specific as well as combined interactions of several *cis*-acting regulatory elements, transcription factors and signal transduction pathways.

The C/EBP family contains three main activating members, C/EBPα, C/EBPβ and C/EBPδ, that recognize the same DNA sequence. These three members have a common structure: an N-terminal domain bearing the transaction domain, a basic DNA-binding domain and a C-terminal leucine zipper domain that allows the homo- or hetero-dimerization of these factors.

*To whom correspondence should be addressed. Tel: +886 6 235 3535 Ext. 5496; Fax: +886 6 274 9296; Email: wcchang@mail.ncku.edu.tw

C/EBPδ is involved in the ligand-stimulated transcriptional regulation of *cox-2* gene (12). However, the detail molecular mechanism of human C/EBPδ, NF-IL6β, in the regulation of *cox-2* gene transcription is unclear.

Post-translational modification of proteins by sumoylation is an important regulatory mechanism and has been found to be utilized in many cellular processes (16–18). SUMO modification of several transcription factors has been reported, including the androgen receptor (19), LEF1 (20), c-Myb (21), TEL (22), Sp3 (23,24), p53 (25), c-Jun (26) and C/EBPs (26). SUMO conjugation has been shown to regulate several different protein functions including protein stability, subcellular localization and transcriptional activation regulation (18,27,28). The consensus sequence, (I/L)KXE, for sumoylation has been defined (29). The C/EBP family belongs to the large family of basic leucine zipper (bZIP) transcription factors. The repression domain I of C/EBPϵ was demonstrated to be modified by SUMO1 (26), and this modification was proposed to be important for the inhibitory function of this domain. Kim *et al*. (25) also reported that conserved SUMO target sequences are present in C/EBPα, C/EBPβ and C/EBPδ, and that these isoforms can be conjugated to SUMO1 (26). However, the function of sumoylated C/EBPs is largely unknown, especially in the case of NF-IL6β.

Coactivator p300 and CREB-binding protein (CBP) serve as an integrator for gene transcription. Several reports have suggested involvement of p300 coactivator in *cox-2* transcriptional regulation (4,5,30). p300 contains histone acetyltransferase (HAT) activity that modulates the acetylation of histones or transcription factors, thus affecting the DNA binding and transcriptional activation. p300 and CBP have been shown to participate in C/EBPs-mediated gene transcription (31–33). C/EBP family members trigger the phosphorylation of p300 and consequently increase p300-mediated transcriptional activation (34).

Several reports have shown C/EBPβ and C/EBPδ's involvement in *cox-2* gene expression (35,36). However, the effects of C/EBPs on *cox-2* transcription are dependent on cell type and stage of differentiation. Gain or loss of function of C/EBPδ and C/EBPβ regulate *cox-2* promoter activity in various cell types (35,37,38). In our previous study, we found that induction of c-Jun is involved in EGF-induced *cox-2* expression (5). In addition, we found that the level of NF-IL6β is also elevated by EGF treatment in human epidermoid carcinoma A431 cells (37). In this study, we extended our work to investigate the functional role of NF-IL6β in regulating *cox-2* promoter activity in the basal and EGF-induced transcriptional state, and the effects that sumoylation and acetylation of NF-IL6β play function roles on the promoter. Our results indicated that NF-IL6β mediates the basal and EGF-induced *cox-2* promoter state, and sumoylation of NF-IL6β attenuates the activation of *cox-2* promoter, while p300 can acetylate NF-IL6β and participate in the NF-IL6β-enhanced *cox-2* promoter regulation.

MATERIALS AND METHODS

Materials

Human EGF was purchased from Peprotech (Rocky Hill, NJ). SB203580 was obtained from Calbiochem (San Diego, CA). Antibodies against COX-2, NF-IL6β, SUMO1 and α-p300-conjugated agarose were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against acetyl-lysine were purchased from Upstate (Charlottesville, VA). Monoclonal α-HA antibody was purchased from BM (Boehringer, Mannheim, Germany). Lipofectamine 2000, Dulbeco's modified Eagle's medium (DMEM), SuperScriptTM III and Opti-MEM medium were obtained from Invitrogen (Carlsbad, CA). All oligonucleotides were synthesized by MDBio Inc. (Taipei, Taiwan). Fetal bovine serum (FBS) was from HyClone Laboratories (Logan, UT). Streptavidin–Sepharose beads were purchased from Amersham Biosciences (Buck, UK). *In vitro* transcription/translation kit was purchased from Promega (Madison, WI). Expression plasmid pcDNA3/HA was a gift of Dr. Hsin-Fang Yang-Yen (Institute of Molecular Biology, Academia Sinica, Tapei, Taiwan). p*Sliencer*™ 3.0 vector was purchased from Ambion (Austin, TX). The sumoylation kit was purchased from LAE Biotechnology Co. (Taichung, Taiwan). The recombinant p300 protein was purchased from Active Motif (Carlsbad, CA). The cloning vector, yTA vector, was purchased from Yeastern Biotech. Co. (Taipei, Taiwan). DNA polymerase kit and BD Advantage GC™ PCR kit for PCR-cloning were purchased from BD Biosciences (Palo Alto, CA). Protein concentration column, Amicon® Centriprep® Filter Devices, was purchased from MILLIPORE (Billerica, MA). All other reagents used were of the highest purity obtainable. The expression vector TAM-67 encoding the truncated human c-Jun was the generous gift of Dr M. Birrer (NCI, National Institutes of Health, Rockville, MD). Small interfering RNA (siRNA) pool for c-Jun and a non-specific control siRNA were purchased from Darmacon (Lafayette, CO). pSUPERc-Jun siRNA was designed and constructed by KRII International Co. (Taipei, Taiwan).

Plasmid transfection and reporter gene assay

A431 and HeLa cells were maintained in DMEM supplemented with 10% FBS, 100 μg/ml streptomycin, and 100 U/ml penicillin. All EGF treatments of A431 cells were in the concentration of 50 ng/ml. Cells were transfected with plasmids by lipofection using Lipofectamine 2000 according to the manufacturer's instruction. Cells were replated 24 h before transfection at an optional density in 3 ml of fresh culture medium in a 3.5 cm plastic dish. For usage in transfection, 5 μl of Lipofectamine 2000 were incubated with reporter plasmid and the expression plasmids as indicated in each experiment, in 2 ml of Opti-MEM medium for 30 min at room temperature. Total DNA concentration for each experiment was matched with empty vector. Cells were transfected by changing the medium with 2 ml of Opti-MEM medium containing the plasmids and Lipofectamine 2000, unless otherwise stated. Cells were stimulated with EGF when necessary and incubated for 16 h. The luciferase activities in cell lysates were measured by the luciferase assay system and determined as described (37). Luciferase activity was normalized per microgram of extract protein.

Small interfering RNAs assay

Two oligonucleotides were synthesized according to the oligonucleotide design procedure described in the Ambion's

manual. They were as follows: 5′-gATCCgCCAggAgATg-CAgCAgAAgTTCAAgAgACTTCTgCTgCATCTCCTggTT-TTTTggAAA-3′ and 5′-AgCTTTTCCAAAAAACCAggA-gATgCAgCAgAAgTCTCTTgAACTTCTgCTgCATCTCCT-ggCg-3′. The 5′ ends of the two oligonucleotides were non-complementary and formed the BamHI and HindIII restriction site overhangs that facilitated efficient directional cloning into the p*Sliencer*™ 3.0 vector. Cell transfection separately with NF-IL6β siRNA expression vectors (pSi-1) and p*Sliencer*™ 3.0 negative control vectors (pSi-C), which encode a hairpin siRNA whose sequence is not found in the human genome databases was purchased from Ambion, were carried out by Lipofectamine 2000 or lipofectin according to the manufacturer's instruction. After 24 h recovery in complete medium, RT–PCR or western blotting assay was performed from the transfectants, which had been previously starved for 6 h and re-stimulated with EGF for 2 h.

DNA affinity precipitation assay

Nuclear extracts from A431 cells with or without EGF treatment were prepared, and DNA affinity precipitation assay was performed according to the method described previously (38,39). The 200 μg of lysates extracted from each group were incubated with 1 μg of biotinylated C/EBP or CRE oligonucleotides in the presence of DNA binding buffer containing 10 mM Tris–HCl pH 7.5, 50 mM NaCl, 1 mM DTT, 1 mM EDTA, 10% (v/v) glycerol, 10 mM NaF, 1 mM PMSF, 1 μg aprotinin/ml, 1 μg leupeptin/ml, 1 mM Na_3VO_4 and 2 μg poly(dI–dC). After 1 h of incubation at 4°C, 40 μl of streptavidin–Sepharose were added to the reaction mixture and the incubation was continued for 1 h. The complexes were then precipitated by centrifugation and washed three times with DNA binding buffer before they were resolved by SDS–PAGE and subsequently analysed by immunoblotting with α-C/EBPδ antibodies.

Chromatin immunoprecipitation assay

The chromatin immunoprecipitation (ChIP) assay was carried out essentially as described by Saccani *et al*. (40). Briefly, A431 cells with or without prior stimulation with EGF were treated with 1% formaldehyde for 15 min. The cross-linked chromatin was then prepared and sonicated to an average size of 300–500 bp. The DNA fragments were immunoprecipitated with antibodies specific to p300, SUMO1 and NF-IL6β or control rabbit IgG at 4°C, overnight. After reversal of cross-linking, the immunoprecipitated chromatin was amplified by PCR amplification of specific regions of the *cox-2* genomic locus. The primers were as follows: COX-2/F-186: 5′-CTGGGTTTCCGATTTTCTCA-3′, COX-2/R+49; 5′-GAGTTCCTGGACGTGCTCCT-3′, COX-2/F+800; 5′-CTAAGGCAGGTTAAAAAATTGTATTTCC-3′ and COX-2/R+1200: 5′-TCCCTTGAAGTGGGTAAGTAT-GTAGTG-3′. The amplified DNA products were resolved by agarose gel electrophoresis and confirmed by sequencing.

In vitro expression of NF-IL6β proteins

In vitro transcription/translation of NF-IL6β was performed using 1 μg of pcDNA3-HA/NF-IL6β and a wheat germ coupled transcription/translation system according to the instructions provided by the manufacturer. Recombinant His-tagged NF-IL6β (His/NF-IL6β) and NF-IL6βK120A (His/NF-IL6βK120A) were generated from the pET-28a (+) vector. The recombinant plasmids were transformed into BL21 (DE3) cells. Isopropyl-β-D-thiogalactopyranoside (IPTG) was used to induce recombinant protein expression in the transformants. His/NF-IL6β and His/NF-IL6βK120A were purified according to the instructions provided by the manufacturer and dialysed with dialysis buffer (50 mM HEPES pH7.4, 100 mM NaCl and 1 mM DTT). The dialysed proteins were concentrated by Amicon® Centriprep® Filter Devices.

In vitro- and *in vivo*-SUMO modification assays

In vitro-SUMO modification of NF-IL6β was performed using sumoylation kit. Briefly, assays were performed with 2 μl of SAEI and SAEII (7.5 μg/ml), 2 μl of UBC9 (50 μg/ml), 2 μl of 10× sumoylation reaction buffer (200 mM HEPES, pH 7.5, 50 mM $MgCl_2$ and 20 mM ATP), 2 μl of SUMO1 (50 μg/ml) and 2 μl of *in vitro*-translated HA/NF-IL6β. The reaction mixture was incubated at 37°C for 30 min and then quenched with SDS–PAGE sample buffer. The samples were subsequently analysed by SDS–PAGE and immunoblotting analysis with α-HA antibodies. *In vivo*-sumoylation assay was carried out in A431 cells. Cells were transfected with pcDNA3-HA/NF-IL6β expression vectors in the presence or absence of SUMO1-GG. Cell extracts were prepared in sample buffer (5% SDS, 0.15 M Tris–HCl pH 6.7 and 30% glycerol) and then diluted 1:3 with RIPA buffer (25 mM Tris–HCl pH 8.2, 50 mM NaCl, 0.5% NP-40, 0.5% deoxycholate and 0.1% SDS), containing 20 mM *N*-ethylmaleimide and 1 mM PMSF, 1 μg aprotinin/ml and 1 μg leupeptin/ml for subsequent immunoprecipitation assay.

In vitro- and *in vivo*-acetylation assay

Purified p300 protein (50 ng) and 0.5 μg of the indicated His/NF-IL6β protein or His/NF-IL6βK120 were incubated in a reaction mixture containing 50 mM Tris–HCl, pH 8.0, 10% glycerol, 0.1 mM EDTA, 1 mM DTT and 40 μM acetyl-coenzyme A for 1 h at 30°C. The reaction mixture was subjected to SDS–PAGE and analysed by western blotting using anti-α-acetyl-lysine antibodies. *In vivo*-acetylation assay was performed by transfecting cells with the pcDNA3-HA/NF-IL6β expression vectors in the presence or absence of p300 expression plasmid. Cells were lysed in sample buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 1 mM EDTA and 1% Triton X-100) and then diluted 1:4 with TE buffer (10 mM Tris–HCl, pH 7.5 and 0.1 mM EDTA), containing 100 μM sodium butyrate, 1 mM PMSF, 1 μg aprotinin/ml and 1 μg of leupeptin/ml for immunoprecipitation assay.

Construction of reporter plasmids and expression vectors

The *cox-2* promoter fragment from −207 to +49 bp (−207/+49wt) was obtained by PCR from the pXC918 reporter (5). The primers used were as follows: COX-2/KpnI-207: 5′-GGGGTACCTGCTCCCAAATTGGGGCAGC-3′, COX-2/HindIII+49: 5′-GGAAGCTTGAGTTCCTGGACGTGCTC-C-3′. The PCR fragments were cloned into yTA vector and verified by sequencing. A KpnI/HindIII fragment was subcloned into the multi-cloning sites of the promoter-less

vector pGL2-basic. Mutant reporter plasmids were derived from −207/+49wt by site-directed mutagenesis of each individual region as indicated. Plasmid pGL2-promoter/COX-2/1XCEBP (pGL2-promoter 1XC/EBP) was derived by inserting one copy of the DNA fragment containing the C/EBP motif sequence (5′-GGGCTTACGCAATTTTTTTAA-3′) into the SmaI site of the pGL2-promoter vector. NF-IL6β was generated from human liver cDNA library by PCR using BD Advantage GC^{TM} PCR kit and using the following oligonucleotides: 5′-CGGGATCCAGCGCCGCGCTCTTCAGCCTG-3′ and 5′-GGCCTCGAGGCCGCGCGTTACCGGCAGTC-3′. The amplified fragment was digested with BamHI and XhoI and inserted into BamHI- and XhoI-digested pcDNA3-HA to produce HA-tagged NF-IL6β (HA/NF-IL6β). The *cox-2* promoter plasmids pXC80 and pXC918 have been described previously (5).

RT–PCR

Total RNA was isolated from A431 cells using the TRIzol RNA extraction kit. Of the isolated RNA 1 μg was subjected to reverse transcription with SuperScriptTM III. Specific primers for COX-2: 5′-CCCACTTCAAGGGATTTT-3′ and 5′-CCAGACCAAAGACCTCCT-3′, and for NF-IL6β: 5′-AGCGCAACAACATCGCCGTG-3′ and 5′-GTCGGGTCTGAGGTATGGGTC-3′, were used for analyses. The PCR products were separated by electrophoresis in 1% agarose gel and visualized with ethidium bromide staining.

p300 pull-down assay

In vitro-translated HA/NF-IL6β (2 μl) were incubated with purified p300 protein in a buffer composed of 50 mM Tris–HCl, pH 8.0, 10% glycerol, 0.1 mM EDTA and 1 mM DTT. After pulling down by α-p300-conjugated agarose (p300-AC), the immunoprecipitation pellets were washed and separated by SDS–PAGE for subsequent detection by immunoblotting with α-HA antibodies.

RESULTS

p38^{MAPK} inhibitor attenuates EGF-induced *cox-2* transcription

Recent studies have shown that the known C/EBP family proteins can modulate *cox-2* gene expression through interactions with each other or other transcription factors. Hence prior to this study, we have verified the expression of C/EBPs in the presence of EGF (Figure 1A). According to these data, the expression pattern of C/EBPα is not affected by EGF treatment, whereas C/EBPβ expression can be greatly increased by EGF treatment, but only after a 6 h delay. Otherwise, NF-IL6β pre-existed before EGF treatment and enhanced by EGF treatment. The induction pattern of NF-IL6β is similar with c-Jun and COX-2 expression patterns after EGF treatment.

We previously found that the p38^{MAPK} signaling pathway mediates the EGF-induced *NF-IL6β* transcription in A431 cells (37). To examine the possibility that NF-IL6β regulates *cox-2* gene expression in A431 cells, we first studied the correlation between p38^{MAPK} activation and *NF-IL6β*/*cox-2* gene transcription. RT–PCR assay was carried out for this study.

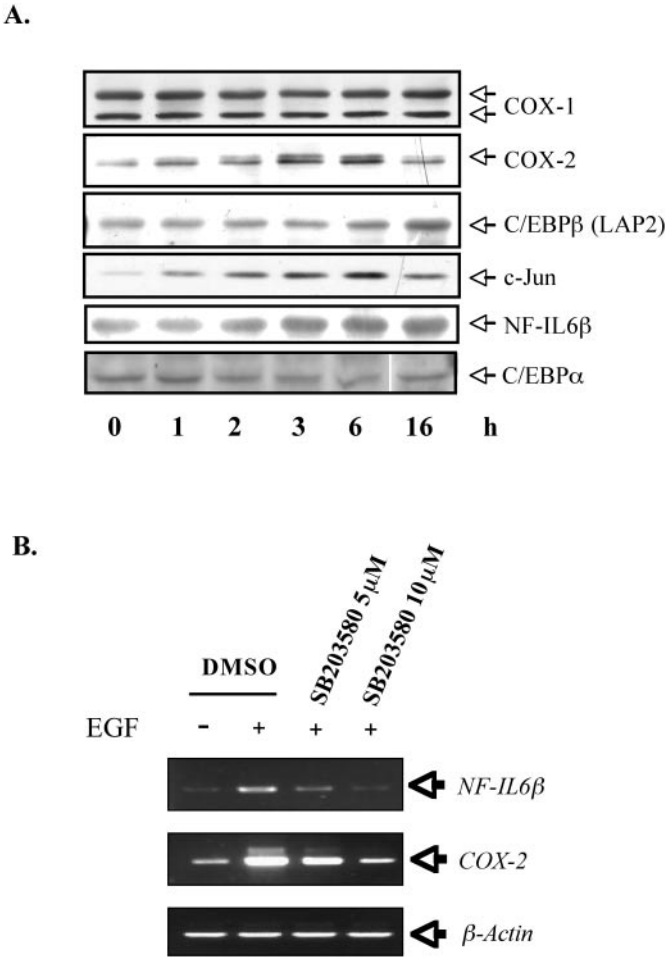

Figure 1. (**A**) EGF-induced COX-2 expression and C/EBP protein levels in A431 cells. Cell lysates were harvested after EGF treatment. Antibodies to recognize the COX-1, COX-2, c-Jun and C/EBPs protein levels performed by western blot analysis. (**B**) SB203580, p38 inhibitor, attenuates EGF-induced *cox-2* transcription in a dose-dependent manner. A431 cells deprived of serum were pretreated with p38 inhibitor SB203580 for 30 min prior to stimulation with EGF for 90 min. After stimulation, cells were lysed, and total RNA was prepared for RT–PCR analysis of *NF-IL6β* and *cox-2* mRNA levels.

The levels of EGF-induced *NF-IL6β* mRNA and *cox-2* mRNA were attenuated by pretreatment with p38^{MAPK} inhibitor, SB203580, in a dose-dependent manner (Figure 1B). The results suggest a close relationship between EGF-induced NF-IL6β expression and *cox-2* transcription through p38^{MAPK} signaling activation.

NF-IL6β plays a functional role in *cox-2* promoter activity

To address and connect whether NF-IL6β would activate the *cox-2* promoter, cells were cotransfected with an NF-IL6β expression vector and a reporter construct controlled by the *cox-2* promoter. As shown in Figure 2A, overexpression of NF-IL6β enhanced *cox-2* promoter activity in a dose-dependent manner. The effect could be enhanced by EGF treatment. The results suggest that NF-IL6β might play a functional role in the basal and EGF-induced expression of COX-2.

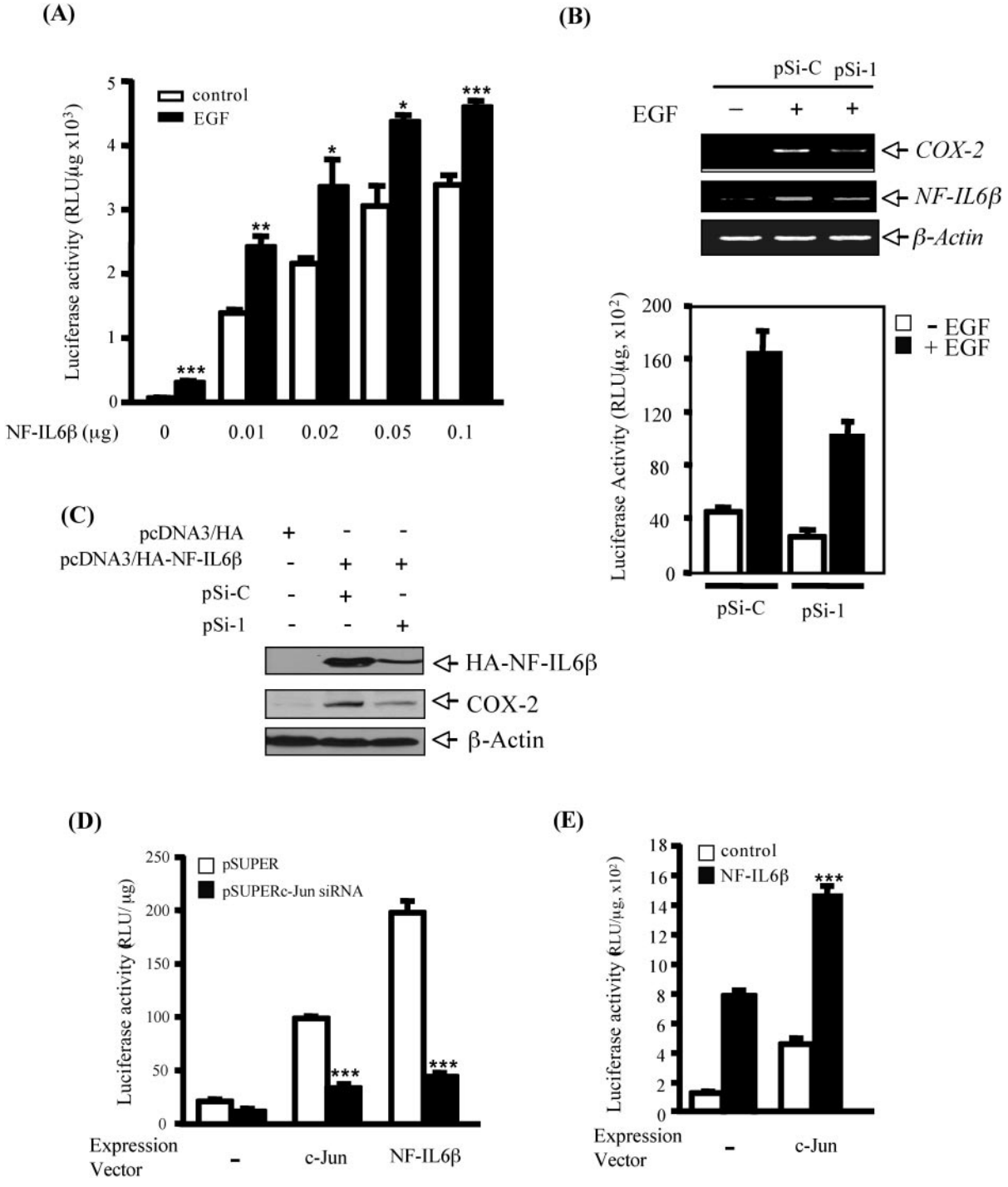

Figure 2. NF-IL6β plays a role in transcription of *cox-2* gene. (**A**) Overexpression of NF-IL6β enhances the *cox-2* reporter activity. A431 cells were transfected with 0.2 μg of reporter vector pXC80 carrying *cox-2* promoter (−80/+49 bp) together with expression vector of NF-IL6β in 1 ml of Opti-MEM medium. After medium change, cells were treated with or without EGF for 13 h. Cell lysates were then prepared, and luciferase activity was assayed. (**B**) Silencing of NF-IL6β expression attenuates EGF-induced *cox-2* reporter activity. Cells were transfected with 1 μg of pXC918 carrying *cox-2* promoter (−918/+49 bp) together with 1 μg of pSi-C or pSi-I. The upper panel shows that the transfection of NF-IL6*β* expression vectors attenuated the pXC918 reporter activity. The lower panel shows that the transcriptional products of *cox-2* and *NF-IL6β* genes which were examined by RT–PCR analysis. The pSi-C represents the p*Silencer*™ negative control plasmid. The pSi-I indicates the specific NF-IL6β knockdown expression vector. (**C**) Reduction of NF-IL6β expression decrease COX-2 expression. The transfection of pcDNA3/HA-NF-IL6β with pSi-C or pSi-I was performed in HeLa cells. Cellular lysates were harvested and analysed by western blotting probed with α-HA, α-COX-2 and α-β-actin antibodies. β-Actin was used as an internal loading control. (**D**) Silencing of c-Jun expression attenuates NF-IL6β-induced *cox-2* reporter activity. A431 cells were transfected with 0.2 μg of pXC80, together with 0.5 μg of expression vector pSUPERc-Jun siRNA and 0.2 μg of each expression vector of c-Jun and NF-IL6β in 1 ml of Opti-MEM medium. Statistical significance between pSUPERc-Jun siRNA-transfected and untransfected cells was analysed by Student's *t*-test. (**E**) Cooperation of NF-IL6β with c-Jun in promoter activation of *cox-2* gene. A431 cells were transfected with 0.2 μg of pXC80 reporter vector together with 0.05 μg of expression vector of NF/IL6β and 0.02 μg of expression vector of c-Jun in 1 ml of Opti-MEM medium. Cell lysates were then prepared, and luciferase activity was assayed. Statistical significance between c-Jun-transfected and untransfected cells was analysed by Student's *t*-test.

To investigate the effect of endogenous NF-IL6β on transcriptional activation of *cox-2* gene, complementary specific oligonucleotides aimed to inhibit NF-IL6β gene expression by RNA interference were designed. Transfection of A431 cells with the NF-IL6β siRNA expression vector resulted in reduction of EGF-induced NF-IL6β mRNA and a concomitant decrease of *cox-2* mRNA (Figure 2B, upper panel). Consistent with the RT–PCR results, cells cotransfected with a luciferase reporter construct controlled by *cox-2* promoter, pXC918 (5), and an NF-IL6β siRNA expression vectors, pSi-1, expressed significantly less luciferase activity than that cotransfection with negative control, pSi-C (Figure 2B, lower panel). Cotransfection of HA/NF-IL6β expression vectors with pSi-C increased COX-2 expression, but the transfectant with pSi-1 decreased the COX-2 expression (Figure 2C). These results suggest that NF-IL6β plays a role in regulating COX-2 expression. Since c-Jun is involved in EGF-induced *cox-2* transcriptional activation through the CRE binding site (5,41), we examined whether activation of *cox-2* promoter, which bears a CRE binding site, by NF-IL6β requires the cooperation with c-Jun. As shown in Figure 2C, cells cotransfected with c-Jun siRNA expression vector reduced NF-IL6β-induced *cox-2* promoter activity, indicating that the activation of *cox-2* promoter by NF-IL6β is, at least in part, dependent on c-Jun. Furthermore, cotransfection of cells with c-Jun and NF-IL6β expression vectors resulted in a synergistic activation of the *cox-2* promoter (Figure 2D). Taken together, these data indicate that NF-IL6β and c-Jun likely co-regulate EGF-dependent *cox-2* expression.

C/EBP and CRE motifs are important for *cox-2* gene activation in A431 cells

To further study whether C/EBP and CRE motifs are involved in the basal and EGF-induced *cox-2* gene regulation, reporter expression controlled by the wild-type or mutant *cox-2* gene promoter was assessed in transient transfection studies. The results are summarized in Figure 3B. A point mutation at the C/EBP motif resulted in 60–70% loss of the basal promoter activity, but only a 20–30% decrease in EGF-inducible increase of the promoter activity (compare −207/+49wt with −207/+49mCEBP). In contrast, a point mutation at the CRE motif lost ∼40% of the basal promoter activity and ∼45–55% of EGF-inducible activity (compare −207/+49wt with −207/+49mCRE). The stimulatory effect of EGF was abolished when both CRE and C/EBP sites were mutated (−207/+49mCE/C), suggesting that C/EBP and CRE motifs are essential for *cox-2* promoter activity. These results demonstrate that CRE motif is more important in EGF response, while C/EBP site is more important in regulating the basal *cox-2* expression. To evaluate the effect of NF-IL6β on C/EBP and CRE motifs, the NF-IL6β expression vector was cotransfected with various mutant *cox-2* promoters in A431 cells. The results are summarized in Figure 3C. The mutation at C/EBP and CRE sites diminished 75 and 40%, respectively, of the stimulatory effect of NF-IL6β on the *cox-2* promoter (lanes 2 and 3). While the NF-IL6β effect seems to be more prominent at the C/EBP motif, double mutation at both sites almost completely abolished the NF-IL6β response (lane 4). Since NF-IL6β played a functional role in the transcriptional activity of *cox-2* promoter, we examined whether NF-IL6β binds to the C/EBP or CRE motifs of *cox-2* promoter. To test the binding activity between CRE and C/EBP motifs, DNA affinity precipitation assay was performed with nuclear extracts prepared from control and EGF-treated A431 cells. As seen in Figure 3D (lanes 5 and 8), EGF apparently increased the amount of NF-IL6β bound to the C/EBP and CRE motifs of *cox-2* promoter.

Sumoylation plays a negative regulatory role in *cox-2* promoter activation

Base on these results, we have verified that NF-IL6β play a functional role in *cox-2* transcription. We then tried to elucidate the mechanism in which NF-IL6β modulates the basal and EGF-induced transcriptional regulation of *cox-2* gene. C/EBPδ was reported to be a SUMO1 substrate *in vitro* and *in vivo*, and a K120A mutant of the Gal4-C/EBPδ(1–142) fusion protein lost ∼60% of its repressive activity, as compared with the wild-type Gal4-C/EBPδ(1–142) protein (26). To investigate whether sumoylation acts on the transcription factors for the *cox-2* promoter, transient reporter assay was performed. Cotransfection of the SUMO1 expression vector significantly repressed the NF-IL6β-enhanced pXC918 reporter activity (Figure 4A, compare lanes 3 and 4 with lanes 5 and 6). To specifically examine whether the C/EBP-binding complex participated in the SUMO-mediated repressive effect, a heterologous reporter pGL2 promoter-C/EBP was used, and the NF-IL6β-enhanced pGL2 promoter-C/EBP reporter activity could be attenuated by exogenously expressed SUMO1 (compare lanes 9 and 10 with lanes 11 and 12). These results suggest that sumoylation might suppress *cox-2* promoter activity under the EGF-deprived condition, and that the C/EBP motif might be the site of SUMO-mediated effect.

NF-IL6β is a SUMO substrate

Although the mouse C/EBPβ has been shown to be a target for SUMO1, sumoylation of human C/EBPδ (NF-IL6β) and its biological function have not been elucidated. By sequence comparison between mouse C/EBPδ and NF-IL6β, the consensus sequence for SUMO attachment, LKREP, in the regulatory domain motif (RDM) was conserved. To examine whether NF-IL6β is a SUMO substrate, *in vitro*-sumoylation assay was carried out with purified E1 (SAEI and SAEII), E2 (Ubc9) and *in vitro*-transcribed/translated HA/NF-IL6β. SUMO1 or SUMO3 could be covalently conjugated to HA/NF-IL6β *in vitro* (Figure 5A, lanes 3 and 4). To map the site of sumoylation, we first examined the sequence and found a region between amino acid 110 and 151 in HA/NF-IL6β contained a potential sumoylation site (data not shown). The lysine 120 in this region has been reported to be a potential site of sumoylation (26). A K120A mutation was then generated by mutagenesis in HA/NF-IL6β to see whether sumoylation of the protein was affected. As shown in Figure 5B (lane 4), the K120A mutant protein could not be sumoylated by *in vitro*-sumoylation assay, suggesting that lysine 120 is the site of sumoylation. Since post-translational modifications of transcription factor often affect their DNA binding, the DNA-binding activity of NF-IL6β and suNF-IL6β was examined by gel-shift assay using *in vitro*-translated HA/NF-IL6β protein and labeled CRE or C/EBP probes. NF-IL6β bound to DNA as a homodimer (our observation, data not shown).

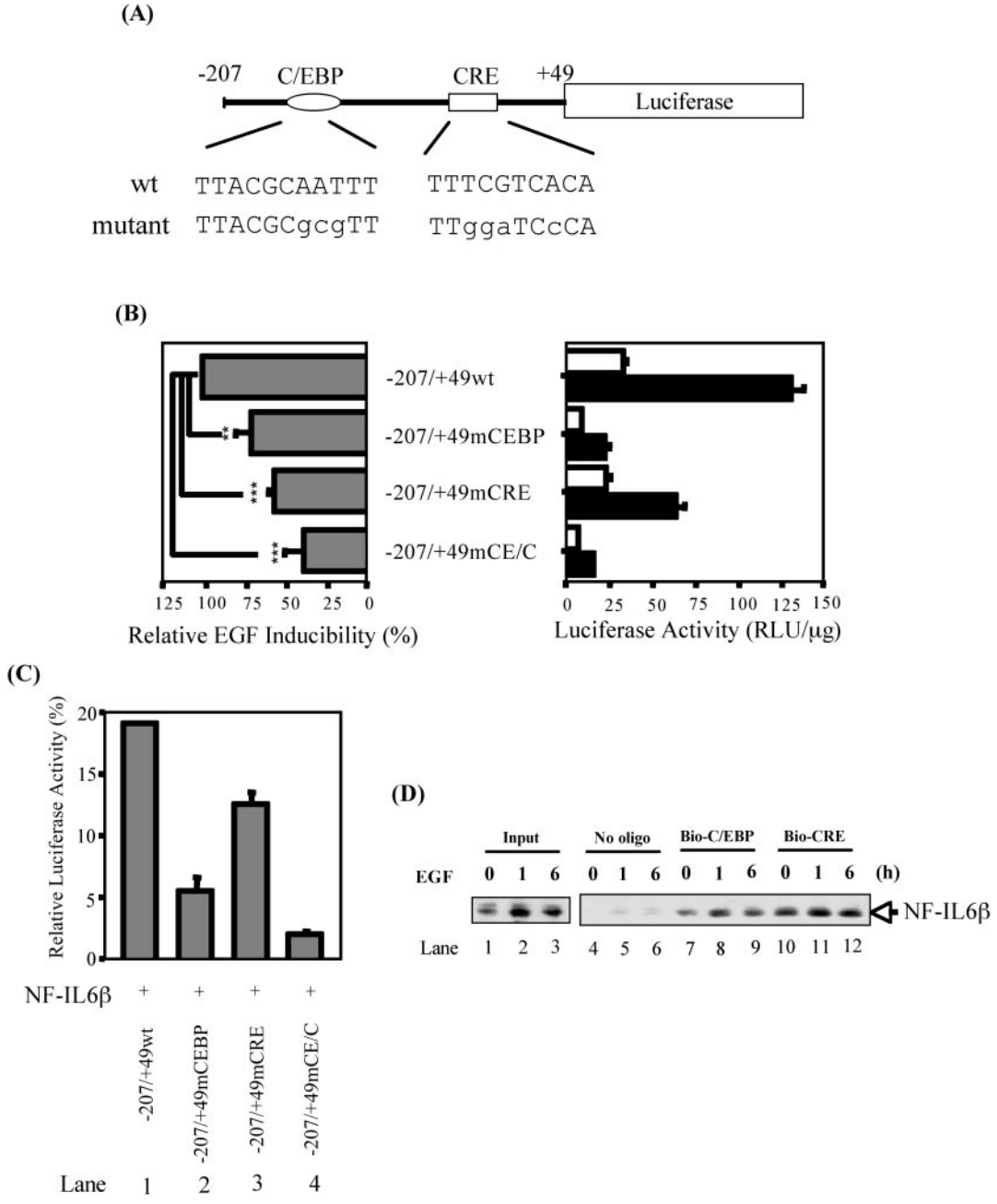

Figure 3. C/EBP and CRE motifs play functional role in EGF-induced *cox-2* gene activation in A431 cells. (**A**) Schematic representation of constructs used in this study. The mutated sequences in each individual construct are shown in lowercase. (**B**) The C/EBP and CRE motifs are important for *cox-2* gene activation. A431 cells transfected with 2 μg of indicated reporters containing site-directed mutagenesis of *cox-2* promoter were treated with (black column) or without (white column) EGF for 16 h (right panel). Cell lysates were then prepared, and luciferase activity was assayed. Each group was performed with triplicate assays. In the left panel, three independent assays were performed, and the relative EGF inducibility to wild and mutant promoters was compared. Statistical analysis was performed by Student's *t*-test. (**C**) Both CRE and C/EBP motifs are important on *cox-2* promoter activity. A431 cells, transfected with 1 μg of indicated reporters together with an expression vector encoding NF-IL6β or an empty vector, were left in medium without EGF treatment for 16 h. Cell lysates were prepared, and luciferase activity was assayed. The graph shown is a relative result. Ratio between the luciferase activity of each reporter cotransfected with NF-IL6β expression vector and that cotransfected with empty vector was calculated, and then normalized by assigning the relative luciferase ratio of −207/+49wt to a value of 100%. (**D**) NF-IL6β binds to the C/EBP and CRE elements in DNA affinity precipitation assay. Nuclear extracts were harvested from cells treated with EGF and incubated with biotin-labeled oligonucleotides. The precipitated products pulled down by streptavidin–Sepharose were analysed by western blotting with α-C/EBPδ antibodies.

The homodimerized HA/NF-IL6β could bind to the C/EBP motif at the *cox-2* promoter (Figure 5C, lane 2). Interestingly, it also bound to the CRE motif (Figure 5C, lane 5), a result subsequently confirmed with purified His/NF-IL6β protein (data not shown). Using equal amount of the proteins, we found no appreciable differences between HA/NF-IL6β and sumoylated HA/NF-IL6β, in term of DNA-binding and homodimerization activities (Figure 5C, compare lane 2 with lane 3, or lane 5 with lane 6). We next examined whether the turnover rates (half-life) of NF-IL6β and NF-IL6βK120A are

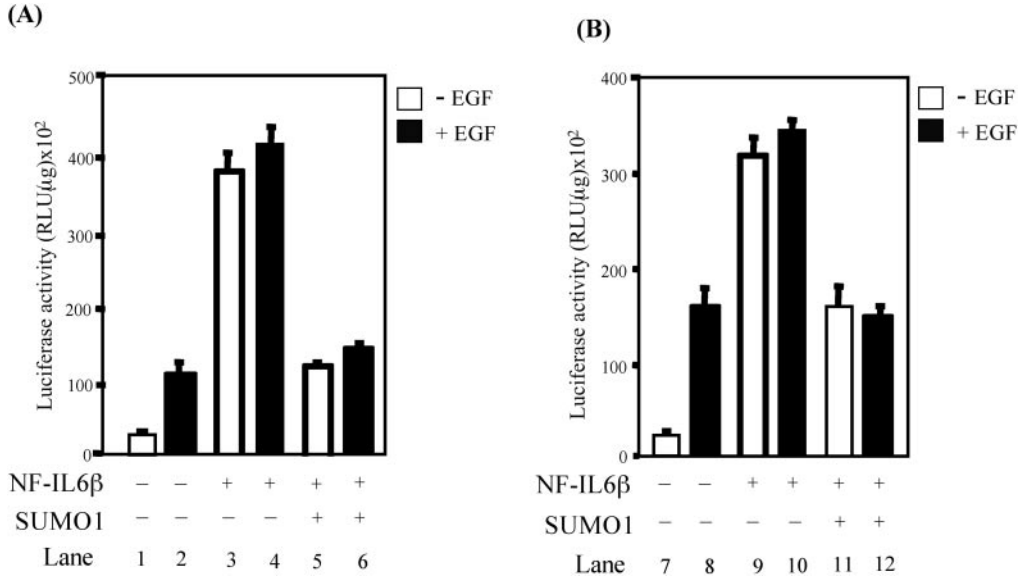

Figure 4. Sumoylation plays a negative regulation role in *cox-2* promoter. Cells transfected with 1 μg of pXC918 luciferase reporter vector (**A**) or C/EBP-heterologous reporter vector (**B**) together with 0.5 μg of indicated expression vector were treated with or without EGF for 16 h. Cell lysates were performed, and luciferase activity was assayed. Each group was performed with triplicate assays.

different. Transfected cells were harvested at different time points post-cycloheximide treatment, and exogenously expressed HA/NF-IL6βwt or HA/NF-IL6βK120A in cell lysates were analysed by western blotting. We measured the different time points of remained HA/NF-IL6β proteins and separately normalize with the '0' time point. As shown in Figure 5D, the turnover half-life of HA/NF-IL6βwt was ~3.5–4 h, similar to that of HA/NF-IL6βK120A. There was also no appreciable difference in the patterns of nuclear localization between the wild-type and K120A mutant NF-IL6β proteins (data not shown). These results suggest that NF-IL6βK120A has the same DNA-binding activity, protein half-life and nuclear localization as NF-IL6βwt. We then moved on to address the issue of whether NF-IL6βK120A K120A is involved in the transactivation activity of *cox-2* transcription.

The regulatory domain motif of C/EBPs is critical for inhibitory domain function (26). To test whether the SUMO-conjugated NF-IL6β is important for silencing *cox-2* transcription, we first examined whether NF-IL6β is a sumoylated protein in A431 cells. The *in vivo*-sumoylation assay was performed by transfecting cells with expression vectors of SUMO1 active form and HA/NF-IL6β or HA/NF-IL6βK120A. western blotting analysis indicated that the suHA/NF-IL6β was detectable in A431 cells. HA/NF-IL6β was endogenously sumoylated in cells (Figure 6A, compare lane 1 with lane 3), and the sumoylated HA/NF-IL6β was increased when cells were cotransfected with SUMO1 (Figure 6A, compare lane 3 with lane 4). However, the no SUMO-conjugated patterns were seen when HA/NF-IL6βK120A was cotransfected with the SUMO1-GG expression vector (Figure 6A, compare lanes 3 and 4 with lanes 5 and 6). Sumoylation of proteins is agent-dependent. For example PMA can induce Elk-1, but not IκBα, sumoylation in COS7 cells (42). The constitutive sumoylation is also observed for STAT1 at the lysine703 residue (43). We therefore investigated whether EGF regulates SUMO1 conjugation to NF-IL6β in A431 cells. Using immunoprecipitation and western analysis, we found EGF attenuated the content of suNF-IL6β in A431 cells (Figure 6B, compare lane 1 with lane 2, and lane 3 with lane 4). Finally, the transactivation activity of NF-IL6βwt and NF-IL6βK120A mutant was evaluated in co-transfection studies. The results indicate that the K120A mutant contributes higher reporter activities than the wild-type NF-IL6β on the −207/+49wt reporter (Figure 6C, compare lanes 1 and 2 with lanes 5 and 6) suggesting that an intact lysine 120 of NF-IL6β is necessary for the repression in *cox-2* transcription.

NF-IL6β is a HAT (p300) substrate

A previous study reported that PU.1 could enhance p300 mediated-C/EBPβ acetylation (44). Although C/EBPs were reported to interact with p300 (31,33,45), no evidence to date suggests that NF-IL6β is an acetylated protein including C/EBPδ. To address this issue and verify that EGF regulates *cox-2* transcription through p300 (41), we investigated whether acetylated NF-IL6β (acNF-IL6β) is detectable in A431 cells. Using α-acetyl-lysine antibodies, we were able to analyse immunoprecipitated products, from the cell lysates containing endogenous p300. We found that EGF enhanced the acetylation of HA/NF-IL6β (Figure 7A, compare lanes 3 with 4). Furthermore, exogenously expressed p300-enhanced NF-IL6β acetylation (Figure 7B, compare lanes 3 and 4 with lanes 5 and 6). To confirm our *in vivo* data, an *in vitro*-acetylation assay was performed using purified p300 with His/NF-IL6β or His/NF-IL6βK120A. Despite removing the acetylation site at lysine 120, p300 is still able to acetylate NF-IL6βK120A at other unknown site (Figure 7B, compare lanes 3 with 5). To examine whether NF-IL6β was involved in an increase in p300 transactivation activity, we perform a reporter assay by cotransfection of p300

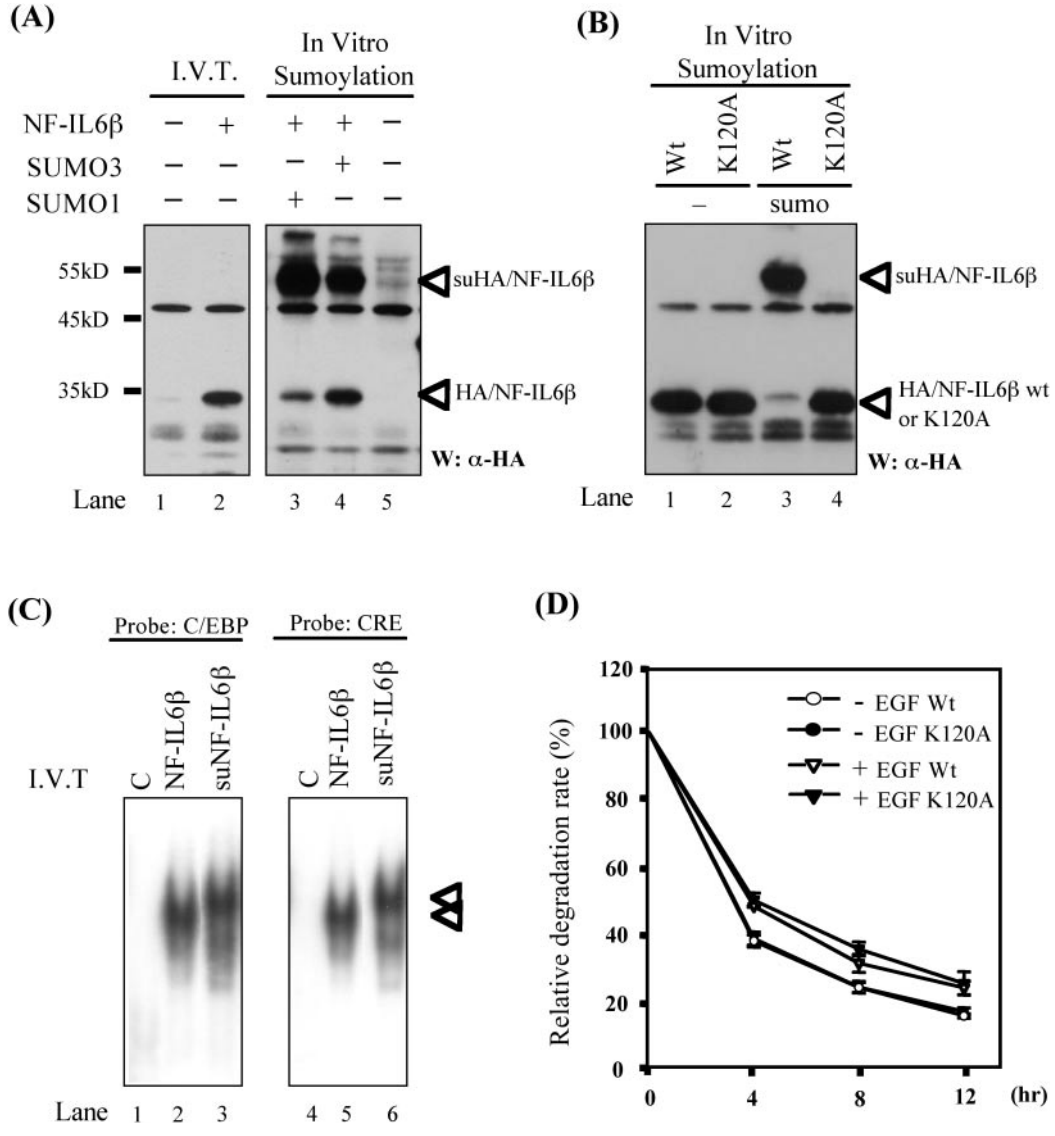

Figure 5. NF-IL6β is a target for attachment of SUMO. (**A**) NF-IL6β is a SUMO1 and SUMO3 substrate *in vitro*. *In vitro*-sumoylation assay using *in vitro*-translated HA/NF-IL6β and SUMO1 or SUMO3 protein were performed as described in Materials and Methods. The reaction mixtures were separated by SDS–PAGE and blotted with α-HA antibodies. (**B**) Lysine120 (K120) of NF-IL6β is a SUMO1-conjugated site. *In vitro*-sumoylation assay was performed as in A. The reaction mixtures were separated by SDS–PAGE and blotted with α-HA antibodies. (**C**) suNF-IL6β does not affect DNA-binding activity. Gel-shift assay with ^{32}P-labeled C/EBP or CRE probes and equal mass of *in vitro*-translated NF-IL6β (lanes 2 and 6), or suNF-IL6β (lanes 3 and 6) was performed. (**D**) K120A mutant has the same protein turnover rate with wild-type NF-IL6β. Cells transfected with 0.5 μg of expression vector of HA/NF-IL6βwt or HA/NF-IL6βK120A were treated with or without EGF in the presence of 10 μg/ml cycloheximide. Cell lysates were then prepared, and the immunoblotting was performed using α-HA antibodies. The graph was plotted by normalizing the level of HA/NF-IL6β or HA/NF-IL6βK120A at each time point, and each protein level at time zero point was assigned to a value of 100%.

and NF-IL6β expression vectors with or without knockdown expression vectors of NF-IL6β. The knockdown of exogenous expression of NF-IL6β inhibited p300/NF-IL6β-involved *cox-2* reporter activity (Figure 7C). Additionally, to dissect whether K120A mutant participated in the p300 transaction activity, *cox-2* reporter assay was carried out by combination of p300 with NF-IL6β or K120A expression vectors. Exogenous p300 proteins exhibited higher *cox-2* promoter/reporter activity regardless whether wild-type or K120A mutant of NF-IL6β was used (Figure 7D, compare lanes 1 with 3 and lanes 2 with 4). These results suggest that NF-IL6β is a HAT (p300) substrate and the position of lysine120 on NF-IL6β is not absolutely critical for p300 action.

SUMO1 represses p300-enhanced *cox-2* promoter activity

Our results proposed that NF-IL6β acts as a bifunctional transcription factor on *cox-2* gene suppression and activation by post-translational modification. To examine whether post-translated NF-IL6β could bind to the *cox-2* promoter *in vivo,* a ChIP assay was performed for NF-IL6β, SUMO1 and p300. EGF treatment increased the level of binding of NF-IL6β to the *cox-2* promoter. (Figure 8A, compare lanes 3 with 4). The binding activity of SUMO1-modified proteins was abundant on *cox-2* promoter but decreased after EGF treatment (Figure 8A, compare lanes 5 with 6). Lastly, without

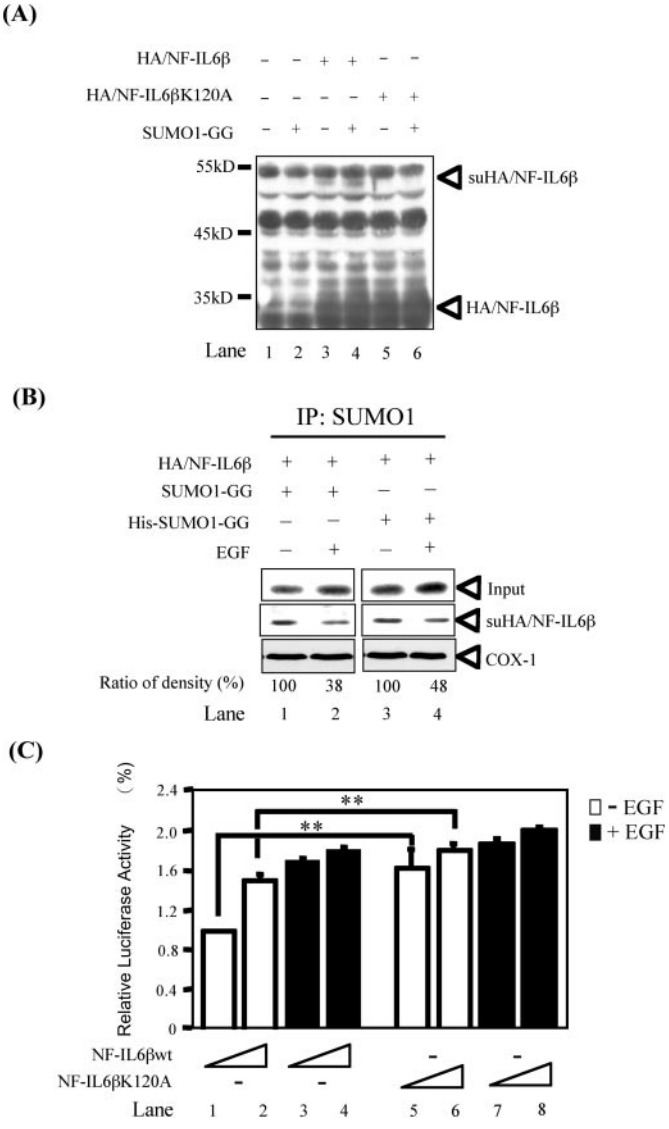

Figure 6. EGF attenuates NF-IL6β sumoylation. (**A**) NF-IL6β sumoylation *in vivo*. Cell lysates were prepared from A431 cells transfected with 1 μg of each indicated expression vector, and the western blotting was performed with α-HA antibodies. Arrow sign represents the position of suHA/NF-IL6β (lanes 3 or 4). (**B**) EGF attenuates HA/NF-IL6β sumoylation in A431 cells. Cells transfected with expression vectors as indicated were treated with or without EGF. Cell lysates were then prepared, and immunoprecipitated with specific SUMO1 antibodies. The immunoprecipitated products were analysed by α-HA antibodies and quantified with densitometer. The 'Input' represents the lysates containing exogenous HA/NF-IL6β before immunoprecipitation step. (**C**) NF-IL6βK120A increased *cox-2* reporter activity. Cells transfected with plasmid 0.5 μg of −207/+49wt together with 0.1 or 0.5 μg of each expression vectors as indicated were treated with or without EGF. Cell lysates were then prepared, and luciferase activity was assayed.

EGF treatment, p300 is unable to associate with the *cox-2* promoter (Figure 8A, compare lanes 7 with 8). The results indicated that EGF increased the binding of p300 and NF-IL6β but reduced the binding of SUMO1-modified proteins on the A region of *cox-2* promoter.

Since EGF decreased sumoylation and increased acetylation of NF-IL6β in A431 cells, we then studied whether suNF-IL6β lost its ability to interact with p300. Using western blotting assay, we demonstrated that p300 could not bind to the suNF-IL6β (Figure 8B, compare lanes 3 and 4 with lanes 7 and 8). This result suggested that non-sumoylated or de-sumoylated NF-IL6β is available for the recruitment of p300 and support our hypothesis that suNF-IL6β mediates transcriptional inactivation, while p300 and NF-IL6β/acNF-IL6β could mediates transcriptional activation of *cox-2* gene.

DISCUSSION

A number of recent reports described a role of MAPKs signaling in the induction of *cox-2* gene in several cell types (46–49). In A431 cells, we demonstrated that U0126, a MEK1 inhibitor, and SP600125, a JNK inhibitor, repressed EGF-induced *cox-2* transcription (5). Multiple cell signaling pathways including Src− focal adhesion kinase (FAK), PI3-K, p70S6 kinase, and MAPKs (p38 and ERK1/2) are involved in type I collagen-induced activation of C/EBP and CREB in serum-stimulated macrophages (12). Pharmacological inhibition of PI3-K, ERK1/2 activation, and p38 MAPK activity suppressed *cox-2* induction by EGF in CaSki human cervical cancer cell line (50). Our previous study also indicated that PI3-K/$p38^{MAPK}$ pathways mediate EGF-regulated NF-IL6β transcriptional activation (37). Thus, $p38^{MAPK}$ contributes to the *cox-2* transcriptional activation was verified, and we also provide a possible linkage of $p38^{MAPK}$/NF-IL6β pathway regulated *cox-2* transcription in A431 cells.

CREB and AP-1 (c-Jun/c-Fos) have been reported to bind to the CRE *cis*-acting element in human *cox-2* promoter (5,50–53). We previously demonstrated that c-Jun is involved in EGF-induced *cox-2* transcription through transactivation of CRE binding site in A431 cells (5,41). AP-1 transcription factor mediates bombesin-stimulated *cox-2* expression in intestinal epithelial cells (46), but has not been linked to the modulation of endogenous expression in epithelial cancer cells. In this study, we provided several lines of evidence suggesting that NF-IL6β is involved in the regulation of *cox-2* by transactivating the CRE site on promoter. The C/EBPs binding consensus sequence is T (T/G)NNGNAA(T/G). Several reports (11–14) have shown that C/EBPβ or C/EBPδ could bind to the C/EBP motif, CTTACGCAATG, of human *cox-2* promoter using *in vitro*-binding assay. However, the CRE motif, ATTTCGTCACATG, is also a putative C/EBPs-like motif (the underlined sequence). By gel-shift assay, we found that purified or *in vitro*-translated NF-IL6β could directly bind to the C/EBP and CRE motif on *cox-2* promoter (Figure 5C) and EGF treatment enhanced the binding of NF-IL6β to the CRE site (Figure 3D). The same phenomenon of C/EBPδ binding to the C/EBP and CRE sites was also observed in the LPS- and TPA-regulated *cox-2* gene (54). Moreover, overexpression of NF-IL6β transactivated not only C/EBP motif but also CRE motif on *cox-2* promoter (Figure 3C) and cotransfection of cells with c-Jun and NF-IL6β expression vectors resulted in a synergistic activation of the *cox-2* promoter bearing CRE site (Figure 2E). These results support the notion that NF-IL6β-bound C/EBP motif coordinates with the CRE motif transactivated by NF-IL6β and c-Jun in the promoter activation of *cox-2* gene upon by EGF treatment. EGF activated heterologous pGL2 promoter-C/EBP reporter activity about 4- to 5-folds (Figure 4B), suggesting that C/EBP motif is responsive to EGF stimulation.

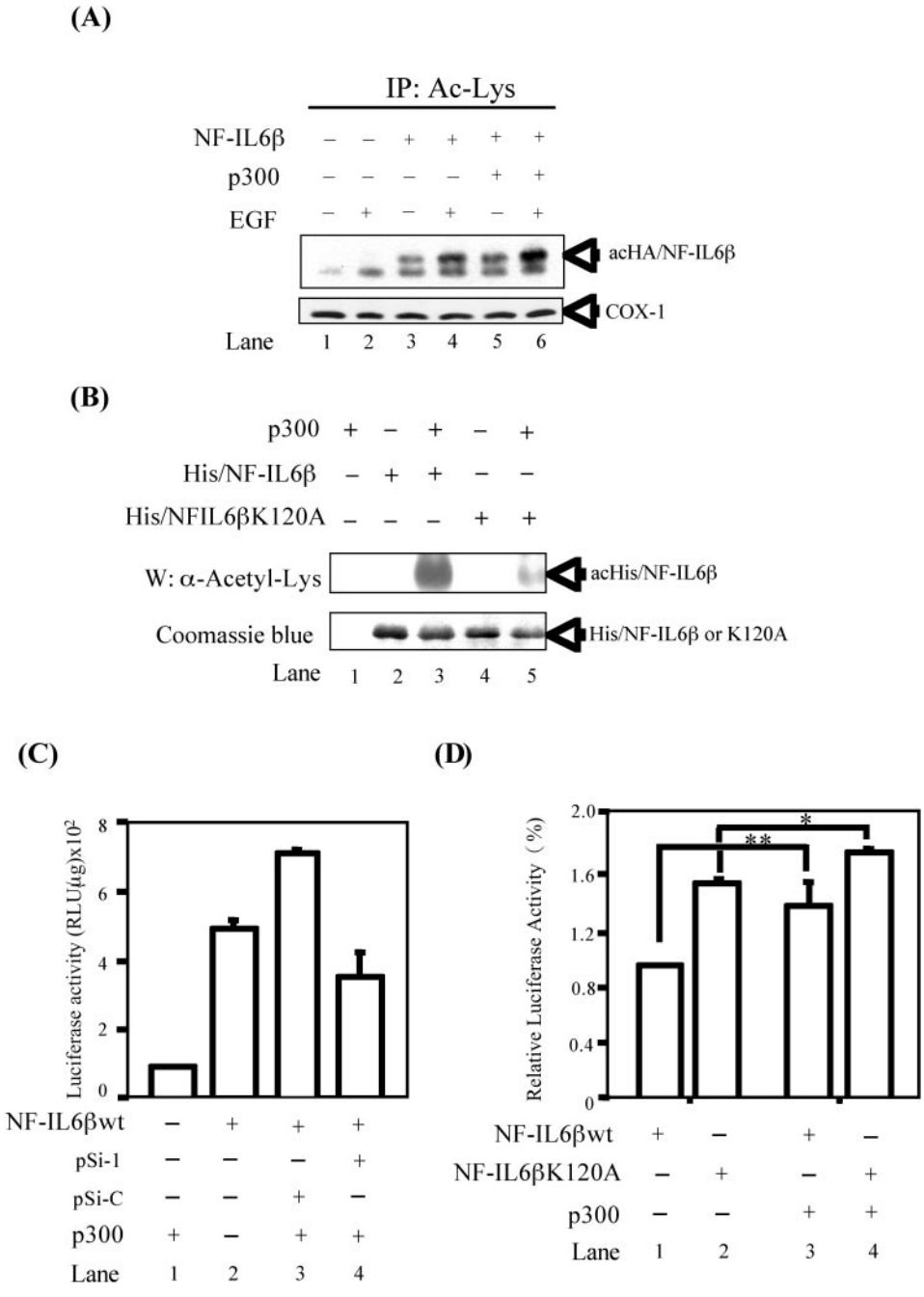

Figure 7. NF-IL6β is a HAT (p300) substrate. (**A**) EGF stimulation increases NF-IL6β acetylation *in vivo*. The lysates, prepared from cells transfected with 1 μg of each indicated expression vector with or without EGF treatment, were immunoprecipitated with specific anti-acetyl-lysine antibodies. The immunoprecipitation products were analysed by α-HA antibodies. (**B**) p300 acetylates NF-IL6β and NF-IL6βK120A *in vitro*. *In vitro*-acetylation assay using purified p300 protein and His/NF-IL6β or His/NF-IL6β was performed as described in Materials and Methods. (**C**) Silencing of NF-IL6β expression attenuates p300/NF-IL6β-enhanced *cox-2* promoter activity. Cells transfected with 0.5 μg of reporter plasmid −207/+49wt together with 0.5 μg of each expression vector as indicated were left in medium without EGF treatment for 16 h. Cell lysates were prepared, and luciferase activity was assayed. (**D**) Overexpression of p300 increases NF-IL6β-induced −207/+49wt reporter activity. Cells transfected with 0.5 μg of reporter plasmid −207/+49wt together with 0.5 μg of each expression vector as indicated were left in medium without EGF treatment for 16 h. Three independent experiments were performed, and the statistic analysis was performed by Student's *t*-test.

Activation of the basal transcription activity by overexpression of NF-IL6β was observed using pXC918 (−918/+49)(Figure 4A, lane 3), −207/+49wt (JM Wang *et al*., unpublished data) and C/EBP-heterologous reporters (Figure 4B, lane 9). However, EGF could not obviously enhance these *cox-2* reporter activities, suggesting that the overexpressed NF-IL6β may have enough ability to directly recruit the cofactors and create a more intact transcription initiation complex, such as CBP or p300 (31), to mimic EGF stimulation. These results suggested that NF-IL6β plays a role at the switch control in *cox-2* transcriptional regulation.

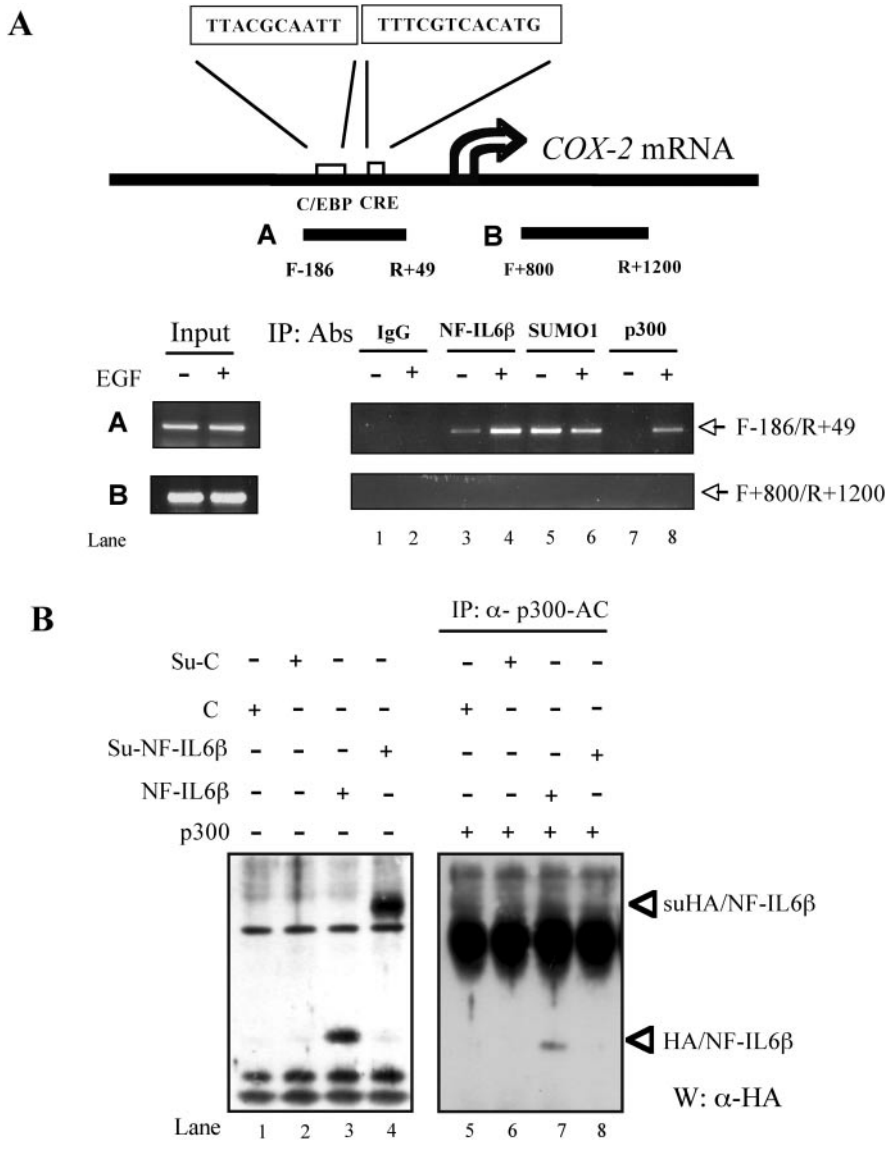

Figure 8. NF-IL6β binds to the *cox-2* gene promoter *in vivo*. (**A**) ChIP analysis was performed as described in Materials and Methods. The upper panel indicates the scheme of 5′-flanking region of *cox-2* gene, and the location of designed primers for PCR. Chromatin from A431 cells with or without EGF treatment was immunoprecipitated with specific antibodies as indicated and the *cox-2* promoter region was amplified by PCR. (**B**) Purified p300 proteins cannot interact with suNF-IL6β. The pull-down assay was performed using the mixtures of purified p300 protein incubated with *in vitro*-translated HA/NF-IL6β with or without sumoylation enzymes as described in Materials and Methods. The 'C' represents the control products of *in vitro* transcription/translation reaction with pCDNA3/HA vector; the 'Su-C' represents the products were performed by *in vitro*-translated control products in *in vitro*-sumoylation reaction. Western blots of reaction mixture (left panel) and α-p300-immunoprecipitated pellet (right panel), using α-HA antibodies, are indicated.

In resting cells, a low-level of mRNA expression and SUMO-mediated suppression of the promoter have been reported (27,28,55). Sumoylation plays a role in *cox-2* gene expression (Figure 4). Several possible regulating proteins have been proposed for the transcription regulation of *cox-2* gene, such as histone H4 (56), C/EBPs (5) and p300 (5,38). However, nobody has yet examined the transient change between basal level and ligand-stimulation of *cox-2* gene expression. Our study focuses on a novel NF-IL6β-regulated pathway, involving post-translational modification of the protein to regulate *cox-2* transcription. Recently, Kim *et al*. (25) reported the inhibitory function of C/EBPs by Gal4 fusion protein assay system and showed that lysine 120 was a potential SUMO-conjugated site on C/EBPδ. Therefore, we demonstrated NF-IL6β is a SUMO1 and HAT substrate and can regulate the *cox-2* transcriptional regulation. We introduced the same point mutation into NF-IL6β and confirmed that lysine 120 was likely a SUMO-conjugated site (Figure 5B and 6A). The K120A mutant of NF-IL6β retained the same DNA binding activity, homodimerization activity, protein stability and nuclear localization as the wild-type NF-IL6β (Figure 5C and D). However, comparing with NF-IL6βwt, it enhanced the transcriptional activation (Figure 6C) suggesting that the post-translational modification might be involved in the modulation of critical protein–protein interaction. Although our reporter assay suggested that suNF-IL6β plays a repressive function role on *cox-2* promoter (Figure 6C), we did not rule out the possibility that other

SUMO-modified proteins might be involved in the maintenance of the state of repression. Several SUMO-regulated transcription factors could interact with various HDACs resulting in gene repression. For example, sumoylation of Elk-1 results in the recruitment of HDAC-2 and hence transcriptional repression at Elk-1 target genes (4). HDAC1 decreases LPS-induced *cox-2* gene expression had been reported, although the mechanism of recruitment is still not clear (4). Our preliminary results from the DNA affinity precipitation assay demonstrated that suNF-IL6β could increase the interaction with some HDACs including HDAC1 (JM Wang *et al*., unpublished data.) These results suggest that suNF-IL6β has the ability to recruit HDACs to regulate gene expression.

p300 acts as a coactivator for many transcription activators to modulate basal- and enhancer-regulated transcriptional activation (57). C/EBPβ binding and p300 recruitment are required for phorbol 12-myristate 13-acetate (PMA)-induced *cox-2* transcription (38). We previously reported that p300 plays a functional role in EGF-induced *cox-2* promoter activity (5). Transcriptional activation by C/EBPα and C/EBPβ coordinated the coactivators, CBP and p300, which promote transcription by acetylating histones and recruiting basal transcription factors (58,59). p300/CBP acetylates the histone tails of nucleosomes, thus favoring chromatin remodeling and activation of transcription (60). In this study, exogenously expressed p300 could increase the NF-IL6β-mediated *cox-2* promoter activity (Figure 7D). We also demonstrated that p300 could directly acetylate NF-IL6β *in vitro* (Figure 7B) and EGF enhances acetylation of NF-IL6β *in vivo* (Figure 7A). The most interesting thing is lysine 120 of NF-IL6β can be a sumoylation or acetylation site. The same phenomenon occurs in Sp3 (61). The *in vivo* studies of something site capable of being both sumoylated and acetylated are difficult to carry out. Nevertheless, it can explain why the repression and activation effect of NF-IL6βK120A is not obvious. In addition, our experiments in this paper suggest that p300 and NF-IL6β/AcNF-IL6β were involved in the EGF-induced *cox-2* transcription. This covalent modification might further enhance the architectural stability of the whole general basal transcription factors on *cox-2* promoter. By ChIP assays, we showed that EGF enhanced *cox-2* promoter binding activities of both p300 and NF-IL6β. (Figure 8A). The inability of suNF-IL6β to interact with p300 was also demonstrated by *in vitro*-binding assay (Figure 8B). Taken together, these results indicate that suNF-IL6β and acNF-IL6β are involved, respectively, in the silencing and activation of *cox-2* transcription. The dynamic pattern of histone H4 acetylation has been demonstrated to associate with *cox-2* transcription by bradykinin and IL-1β (62). Thus, different acetylation patterns of histones, in conjunction with acNF-IL6β or other modulators, may result in conformational changes of chromatin and selective association of transcription factor to the *cox-2* promoter. We provided evidence to support that NF-IL6β is a sumoylated protein and also acetylated by EGF treatment in A431 cells. It brings us to deliberate the interplay between the post-translational modification and ligands-induction of NF-IL6β in downstream target genes. C/EBP proteins could be modified by SUMO-1 attachment within their RDM sequences (25). *In vitro*-acetylation assay shown NF-IL6β at least have two acetylation sites (Figure 7B). The RDM sequences of C/EBP proteins exist in the related region. Moreover, C/EBPβ was identified to be an acetylated protein (63). The similar phenomenon of acetylation site of C/EBPβ occurs nearby to its DNA binding domain. Align and compare the lysine residues in NF-IL6β−lysine 184, mouse C/EBPδ-lysine 184, human C/EBPβ-lysine 264 and mouse C/EBPβ-lysine 215, we found these lysine residues close to DNA binding domain are conserved. However, the exact acetylation site on NF-IL6β and whether the reciprocal action of suNF-IL6β and acNF-IL6β is involved in chromatin remodeling needs to be examined.

In addition to stabilization and *de novo* synthesis of transcription activators, a gene can also be regulated by post-translational modifications of pre-existing transcription factors. Here, we use the NF-IL6β-regulated *cox-2* system to establish a model that links pre-existing NF-IL6β and its post-translationally modified form to regulate *cox-2* transcription. The present study clearly indicated that NF-IL6β and its sumoylation and acetylation modifications play a functional role in the regulation of *cox-2* promoter. NF-IL6β regulated the basal and EGF-induced *cox-2* gene expression (Figure 9). In resting cells, sumoylation of NF-IL6β attenuated the activation of *cox-2* gene promoter. Upon EGF treatment, the recruitment of p300 and NF-IL6β to the *cox-2* gene promoter is enhanced, while the sumoylated form of NF-IL6β on gene promoter is attenuated. It suggests that EGF treatment could result in a decrease in SUMO1-modified proteins or suNF-IL6β, an increase of NF-IL6β protein bound to the *cox-2* promoter and the recruitment of p300 and involvement of NF-IL6β acetylation activate the promoter activity of *cox-2*

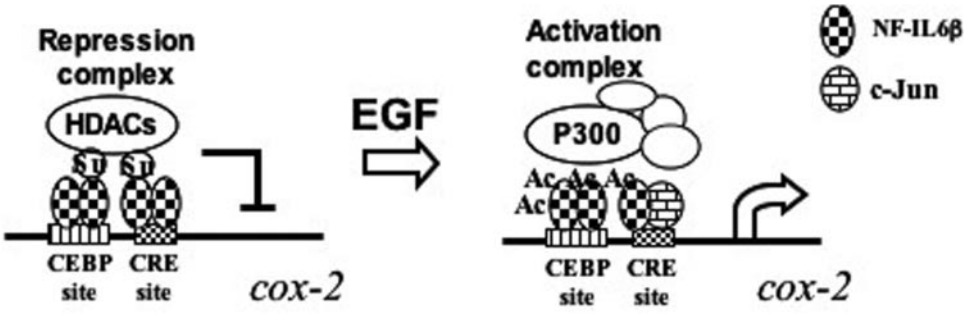

Figure 9. NF-IL6β is a bifunctional protein in *cox-2* transcription. In the resting cell stage, suNF-IL6β recruits HDACs to play a repressor role and result in inactivation of *cox-2* transcription. Presence of EGF, p300 could bind to NF-IL6β. Once recruited, p300 can acetylate NF-IL6β, cooperate with NF-IL6β/ c-Jun complex and result in conformational changes of chromatin to form a more stable transcription initiation complex.

gene. These results demonstrated a possible interaction between p300 and the post-translational modification of NF-IL6β in controlling the *cox-2* gene expression.

ACKNOWLEDGEMENTS

Thanks are due to Drs Wai-Ming Kan and Rong-Fong Shen for critical review of this manuscript. This work was supported by the Ministry of Education Program for Promoting Academic Excellent of University under the grant number 91-B-FA09-1-4 of Taiwan, Republic of China. Funding to pay the Open Access publication charges for this article was provided by the Ministry of Education.

Conflict of interest statement. None declared.

REFERENCES

1. Herschman,H.R. (1996) Prostaglandin synthase 2. *Biochim. Biophys. Acta*., **1299**, 125–140.
2. Lee,J.K., Kim,S.H., Lewis,E.C., Azam,T., Reznikov,L.L. and Dinarello,C.A. (2004) Differences in signaling pathways by IL-1beta and IL-18. *Proc. Natl Acad. Sci. USA*, **101**, 8815–8820.
3. Jiang,B., Xu,S., Hou,X., Pimentel,D.R., Brecher,P. and Cohen,R.A. (2004) Temporal control of NF-kappaB activation by ERK differentially regulates interleukin-1beta-induced gene expression. *J. Biol. Chem*., **279**, 1323–1329.
4. Deng,W.G., Zhu,Y. and Wu,K.K. (2003) Up-regulation of p300 binding and p50 acetylation in tumor necrosis factor-alpha-induced cyclooxygenase-2 promoter activation. *J. Biol. Chem*., **278**, 4770–4777.
5. Chen,L.C., Chen,B.K., Chang,J.M. and Chang,W.C. (2004) Essential role of c-Jun induction and coactivator p300 in epidermal growth factor-induced gene expression of cyclooxygenase-2 in human epidermoid carcinoma A431 cells. *Biochim. Biophys. Acta*., **1683**, 38–48.
6. Cho,Y.H., Lee,C.H. and Kim,S.G. (2003) Potentiation of lipopolysaccharide-inducible cyclooxygenase 2 expression by C2-ceramide via c-Jun N-terminal kinase-mediated activation of CCAAT/enhancer binding protein beta in macrophages. *Mol. Pharmacol*., **63**, 512–523.
7. Kujubu,D.A., Reddy,S.T., Fletcher,B.S. and Herschman,H.R. (1993) Expression of the protein product of the prostaglandin synthase-2/TIS10 gene in mitogen-stimulated Swiss 3T3 cells. *J. Biol. Chem*., **268**, 5425–5430.
8. Reddy,S.T. and Herschman,H.R. (1994) Ligand-induced prostaglandin synthesis requires expression of the TIS10/PGS-2 prostaglandin synthase gene in murine fibroblasts and macrophages. *J. Chem. Biol*., **269**, 15473–15480.
9. Kamata,R., Reddy,S.T., Wolner,B. and Herschman,H.R. (1995) Prostaglandin synthase 1 and prostaglandin synthase 2 both participate in activation-induced prostaglandin D2 production in mast cells. *J. Immunol*., **155**, 818–825.
10. Tazawa,R., Xu,X.M., Wu,K.K. and Wang,L.H. (1994) Characterization of the genomic structure, chromosomal location and promoter of human prostaglandin H synthase-2 gene. *Biochem. Biophys. Res. Commun*., **203**, 190–199.
11. Lee,A.K., Sung,S.H., Kim,Y.C. and Kim,S.G. (2003) Inhibition of lipopolysaccharide-inducible nitric oxide synthase, TNF-alpha and COX-2 expression by sauchinone effects on I-kappaBalpha phosphorylation, C/EBP and AP-1 activation. *Br. J. Pharmacol*., **139**, 11–20.
12. Cho,M.K., Cho,Y.H., Lee,G.H. and Kim,S.G. (2004) Induction of cyclooxygenase-2 by bovine type I collagen in macrophages via C/EBP and CREB activation by multiple cell signaling pathways. *Biochem. Pharmacol*., **67**, 2239–2250.
13. Tamura,M., Sebastian,S., Yand,S., Gurates,B., Fang,Z., Okamura,K. and Bulun,S.E. (2003) Induction of cyclooxygenase-2 in human endometrial stromal cells by malignant endometrial epithelial cells: evidence for the involvement of extracellularly regulated kinases and CCAAT/enhancer binding proteins. *J. Mol. Endocrinol*., **31**, 95–104.
14. Cieslik,K., Zhu,Y. and Wu,K.K. (2002) Salicylate suppresses macrophage nitric-oxide synthase-2 and cyclo-oxygenase-2 expression by inhibiting CCAAT/enhancer-binding protein-beta binding via a common signaling pathway. *J. Biol. Chem*., **277**, 49304–49310.
15. Bradbury,D.A., Newton,R., Zhu,Y.M., El-Haroun,H., Corbett,L. and Knox,A.J. (2003) Cyclooxygenase-2 induction by bradykinin in human pulmonary artery smooth muscle cells is mediated by the cyclic AMP response element through a novel autocrine loop involving endogenous prostaglandin E2, E-prostanoid 2 (EP2), and EP4 receptors. *J. Biol. Chem*., **278**, 49954–49964.
16. Hay,R.T. (2001) Protein modification by SUMO. *Trends Biochem. Sci*., **26**, 332–333.
17. Gill,G. (2004) SUMO and ubiquitin in the nucleus: different functions, similar mechanisms? *Genes Dev*., **18**, 2046–2059.
18. Muller,S., Hoege,C., Pyrowolakis,G. and Jentsch,S. (2001) SUMO, ubiquitin's mysterious cousin. *Nat. Rev. Mol. Cell Biol*., **2**, 202–210.
19. Poukka,H., Karvonen,U., Janne,O.A. and Palvimo,J.J. (2000) Covalent modification of the androgen receptor by small ubiquitin-like modifier 1 (SUMO-1). *Proc. Natl Acad. Sci. USA*, **97**, 14145–14150.
20. Sachdev,S., Bruhn,L., Sieber,H., Pichler,A., Melchior,F. and Grosschedl,R. (2001) PIASy, a nuclear matrix-associated SUMO E3 ligase, represses LEF1 activity by sequestration into nuclear bodies. *Genes Dev*., **15**, 3088–3103.
21. Bies,J., Markus,J. and Wolff,L. (2002) Covalent attachment of the SUMO-1 protein to the negative regulatory domain of the c-Myb transcription factor modifies its stability and transactivation capacity. *J. Biol. Chem*., **277**, 8999–9009.
22. Chakrabarti,S.R., Sood,R., Nandi,S. and Nucifora,G. (2000) Posttranslational modification of TEL and TEL/AML1 by SUMO-1 and cell-cycle-dependent assembly into nuclear bodies. *Proc. Natl Acad. Sci. USA*, **97**, 13281–13285.
23. Ross,S., Best,J.L., Zon,L.I. and Gill,G. (2002) SUMO-1 modification represses Sp3 transcriptional activation and modulates its subnuclear localization. *Mol. Cell*, **10**, 831–842.
24. Muller,S., Berger,M., Lehembre,F., Seeler,J.S., Haupt,Y. and Dejean,A. (2000) c-Jun and p53 activity is modulated by SUMO-1 modification. *J. Biol. Chem*., **275**, 13321–13329.
25. Kim,J., Cantwell,C.A., Johnson,P.F., Pfarr,C.M. and Williams,S.C. (2002) Transcriptional activity of CCAAT/enhancer-binding proteins is controlled by a conserved inhibitory domain that is a target for sumoylation. *J. Biol. Chem*., **277**, 38037–38044.
26. Gill,G. (2003) Post-translational modification by the small ubiquitin-related modifier SUMO has big effects on transcription factor activity. *Curr. Opin. Genet. Dev*., **13**, 108–113.
27. Verger,A., Perdomo,J. and Crossley,M. (2003) Modification with SUMO. *EMBO Rep*., **4**, 137–142.
28. Eaton,E.M. and Sealy,L. (2003) Modification of CCAAT/enhancer-binding protein-beta by the small ubiquitin-like modifier (SUMO) family members, SUMO-2 and SUMO-3. *J. Biol. Chem*., **278**, 33416–33421.
29. Subbaramaiah,K., Cole,P.A. and Dannenberg,A.J. (2002) Retinoids and carnosol suppress cyclooxygenase-2 transcription by CREB-binding protein/p300-dependent and -independent mechanisms. *Cancer Res*., **62**, 2522–2530.
30. Mink,S., Haenig,B. and Klempnauer,K.H. (1997) Interaction and functional collaboration of p300 and C/EBPbeta. *Mol. Cell. Biol*., **17**, 6609–6617.
31. Kovács,K.A., Steinmann,M., Magistretti,P.J., Halfon,O. and Cardinaux,J.R. (2003) CCAAT/enhancer-binding protein family members recruit the coactivator CREB-binding protein and trigger its phosphorylation. *J. Biol. Chem*., **278**, 36959–36965.
32. Guo,S., Cichy,S.B., He,X., Yang,Q., Ragland,M., Ghosh,A.K., Johnson,P.F. and Unterman,T.G. (2001) Insulin suppresses transactivation by CAAT/enhancer-binding proteins beta (C/EBPbeta). Signaling to p300/CREB-binding protein by protein kinase B disrupts interaction with the major activation domain of C/EBPbeta. *J. Biol. Chem*., **276**, 8516–8523.
33. Schwartz,C., Beck,K., Mink,S., Schmolke,M., Budde,B., Wenning,D. and Klempnauer,K.H. (2003) Recruitment of p300 by C/EBPbeta triggers phosphorylation of p300 and modulates coactivator activity. *EMBO J*., **22**, 882–892.
34. Thomas,B., Berenbaum,F., Humbert,L., Bian,H., Bereziat,G., Crofford,L. and Olivier,J.L. (2000) Critical role of C/EBPdelta and C/EBPbeta factors in the stimulation of the cyclooxygenase-2 gene transcription by interleukin-1beta in articular chondrocytes. *Eur. J. Biochem*., **267**, 6798–6809.

35. Wadleigh,D.J., Reddy,S.T., Kopp,E., Ghosh,S. and Herschman,H.R. (2000) Transcriptional activation of the cyclooxygenase-2 gene in endotoxin-treated RAW 264.7 macrophages. *J. Biol. Chem.*, **275**, 6259–6266.
36. Caivano,M., Gorgoni,B., Cohen,P. and Poli,V. (2001) The induction of cyclooxygenase-2 mRNA in macrophages is biphasic and requires both CCAAT enhancer-binding protein beta (C/EBP beta) and C/EBP delta transcription factors. *J. Biol. Chem.*, **276**, 48693–48701.
37. Wang,J.M., Tseng,J.T. and Chang,W.C. (2005) Induction of human NF-IL6β by epidermal growth factor is mediated through the p38 signaling pathway and CREB activation in A431 cells. *Mol. Bio. Cell*, **16**, 3365–3376.
38. Zhu,Y., Saunders,M.A., Yeh,H., Deng,W.G. and Wu,K.K. (2002) Dynamic regulation of cyclooxygenase-2 promoter activity by isoforms of CCAAT/enhancer-binding proteins. *J. Biol. Chem.*, **277**, 6923–6928.
39. Dignam,J., Lebovitz,R. and Roeder,R. (1983) Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Res.*, **11**, 1475–1489.
40. Saccani,S., Pantano,S. and Natoli,G. (2001) Two waves of nuclear factor kappaB recruitment to target promoters. *J. Exp. Med.*, **193**, 1351–1359.
41. Chen,L.C., Chen,B.K. and Chang,W.C. (2005) Activating protein 1-mediated cyclooxygenase-2 expression is independent of N-terminal phosphorylation of c-Jun. *Mol. Pharmacol.*, **67**, 2057–2069.
42. Yang,S.H., Jaffray,E., Hay,R.T. and Sharrocks,A.D. (2003) Dynamic Interplay of the SUMO and ERK Pathways in Regulating Elk-1 Transcriptional Activity. *Mol. Cell*, **12**, 63–74.
43. Rogers,R.S., Horvath,C.M. and Matunis,M.J. (2003) SUMO modification of STAT1 and its role in PIAS-mediated inhibition of gene activation. *J. Biol. Chem.*, **278**, 30091–30097.
44. Joo,M., Park,G.Y., Wright,J.G., Blackwell,T.S., Atchison,M.L. and Christman,J.W. (2004) Transcriptional regulation of the cyclooxygenase-2 gene in macrophages by PU.1. *J. Biol. Chem.*, **279**, 6658–6665.
45. Erickson,R.L., Hemati,N., Ross,S.E. and MacDougald,O.A. (2001) p300 coactivates the adipogenic transcription factor CCAAT/enhancer-binding protein alpha. *J. Biol. Chem.*, **276**, 16348–16355.
46. Guo,Y.S., Hellmich,M.R., Wen,X.D. and Townsend,C.M.,Jr (2001) Activator protein-1 transcription factor mediates bombesin-stimulated cyclooxygenase-2 expression in intestinal epithelial cells. *J. Biol. Chem.*, **276**, 22941–22947.
47. Scherle,P.A., Ma,W.G., Lim,H., Dey,S.K. and Trzaskos,J.M. (2000) Regulation of cyclooxygenase-2 induction in the mouse uterus during decidualization. An event of early pregnancy. *J. Biol. Chem.*, **275**, 37086–37092.
48. Chen,W., Tang,Q., Gonzales,M.S. and Bowden,G.T. (2001) Role of p38 MAP kinases and ERK in mediating ultraviolet-B induced cyclooxygenase-2 gene expression in human keratinocytes. *Oncogene*, **20**, 3921–3926.
49. Subbaramaiah,K., Hart,J.C., Norton,L. and Dannenberg,A.J. (2000) Microtubule-interfering agents stimulate the transcription of cyclooxygenase-2. Evidence for involvement of ERK1/2 AND p38 mitogen-activated protein kinase pathways. *J. Biol. Chem.*, **275**, 14838–14845.
50. Kulkarni,S., Rader,J.S., Zhang,F., Liapis,H., Koki,A.T., Masferrer,J.L., Subbaramaiah,K. and Dannenberg,A.J. (2001) Cyclooxygenase-2 is overexpressed in human cervical cancer. *Clin. Cancer Res.*, **7**, 429–434.
51. Han,S., Inoue,H., Flowers,L.C. and Sidell,N. (2003) Control of COX-2 gene expression through peroxisome proliferator-activated receptor gamma in human cervical cancer cells. *Clin. Cancer Res.*, **9**, 4627–4635.
52. Subbaramaiah,K., Lin,D.T., Hart,J.C. and Dannenberg,A.J. (2001) Peroxisome proliferator-activated receptor gamma ligands suppress the transcriptional activation of cyclooxygenase-2. Evidence for involvement of activator protein-1 and CREB-binding protein/p300. *J. Biol. Chem.*, **276**, 12440–12448.
53. Subbaramaiah,K., Marmo,T.P., Dixon,D.A. and Dannenberg,A.J. (2003) Regulation of cyclooxgenase-2 mRNA stability by taxanes: evidence for involvement of p38, MAPKAPK-2, and HuR. *J. Biol. Chem.*, **278**, 37637–37647.
54. Inoue,H., Yokoyama,C., Hara,S., Tone,Y. and Tanabe,T. (1995) Transcriptional regulation of human prostaglandin-endoperoxide synthase-2 gene by lipopolysaccharide and phorbol ester in vascular endothelial cells. Involvement of both nuclear factor for interleukin-6 expression site and cAMP response element. *J. Biol. Chem.*, **270**, 24965–24971.
55. Yang,S.H. and Sharrocks,A.D. (2004) SUMO promotes HDAC-mediated transcriptional repression. *Mol. Cell*, **13**, 611–617.
56. Shiio,Y. and Eisenman,R.N. (2003) Histone sumoylation is associated with transcriptional repression. *Proc. Natl Acad. Sci. USA*, **100**, 13225–13230.
57. Vo,N. and Goodman,R.H. (2003) CREB-binding protein and p300 in transcriptional regulation. *J. Biol. Chem.*, **276**, 13505–13508.
58. Nerlov,C. and Ziff,E.B. (1995) CCAAT/enhancer binding protein-alpha amino acid motifs with dual TBP and TFIIB binding ability co-operate to activate transcription in both yeast and mammalian cells. *EMBO J.*, **14**, 4318–4328.
59. Kowenz-Leutz,E. and Leutz,A. (1999) A C/EBP beta isoform recruits the SWI/SNF complex to activate myeloid genes. *Mol. Cell*, **4**, 735–743.
60. Huang,Z.Q., Li,J., Sachs,L.M., Cole,P.A. and Wong,J. (2003) A role for cofactor–cofactor and cofactor–histone interactions in targeting p300, SWI/SNF and Mediator for transcription. *EMBO J.*, **22**, 2146–2155.
61. Sapetschnig,A., Rischitor,G., Braun,H., Doll,A., Schergaut,M., Melchior,F. and Suske,G.. (2002) Transcription factor Sp3 is silenced through SUMO modification by PIAS1. *EMBO J.*, **21**, 5206–5215.
62. Nie,M., Pang,L., Inoue,H. and Knox,A.J. (2003) Transcriptional regulation of cyclooxygenase 2 by bradykinin and interleukin-1beta in human airway smooth muscle cells: involvement of different promoter elements, transcription factors, and histone h4 acetylation. *Mol. Cell. Biol.*, **23**, 9233–9244.
63. Xu,M., Nie,L., Kim,S.H. and Sun,X.H. (2003) STAT5-induced Id-1 transcription involves recruitment of HDAC1 and deacetylation of C/EBPβ. *EMBO J.*, **22**, 893–904.