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Title: AP1-mediated cyclooxygenase-2 expression is independent of N-terminal phosphorylation of c-Jun

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Running title: Functional role of c-Jun in COX-2 expression

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Abbreviations: COX-2, cyclooxygenase-2; EGF, epidermal growth factor; PDGF, platelet-derived growth factor ; CRE, cyclic AMP response element; AP1, activating protein 1; MAPK, mitogen-activated protein kinase; JNK, c-Jun N-terminal kinase; bZip, basic region-leucine zipper; CBP, CREB-binding protein (where CREB is cAMP-response element-binding protein).

ABSTRACT

Transcriptional activation of the cyclooxygenase-2 (COX-2) gene is responsible for high level of prostaglandin production during inflammation and carcinogenesis. Previously, we found that c-Jun induction plays a crucial role in epidermal growth factor (EGF)-induced gene expression of COX-2. In this study, the functional role of c-Jun in EGF-induced transcriptional activation of COX-2 in A431 cells was investigated. We found that over-expression of c-Jun N-terminal phosphorylation site mutants had similar stimulatory effects on COX-2 promoter activity and protein expression as c-Jun wild-type. TAM-67, a mutant of c-Jun that lacks the N-terminal transactivation domain of c-Jun, also enhanced COX-2 promoter activity and protein expression in cells treated with EGF. *In vitro* DNA affinity precipitation and reporter assays revealed that regulation of c-Jun C-terminus by EGF enhanced c-Jun binding to COX-2 promoter and induced COX-2 expression. Furthermore, we also demonstrated that c-Fos, which provides transactivation function in Jun/Fos heterodimer, was required for EGF-induced expression of COX-2. These results indicated that c-Jun N-terminal phosphorylation was not required for EGF-induced expression of COX-2. c-Jun, which could recruit other transcription factors like c-Fos, was required for EGF-induced expression of COX-2 in A431 cells.

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INTRODUCTION

Cyclooxygenase (COX), also known as prostaglandin G/H synthase (PGHS), is the rate-limiting enzyme in the biosynthesis of prostaglandins from arachidonic acid. Two isoforms derived from distinct genes located on separate chromosomes have been characterized and are referred to as COX-1 and COX-2 (Herschman, 1996). A third COX isoform produced as an alternate splice variant of COX-1 gene is recently identified as COX-3 (Chandrasekharan et al., 2002). COX-1 is constitutively expressed in most tissues and mediates physiological responses such as the regulation of renal and vascular homeostasis and cytoprotection of the stomach. In contrast, COX-2 is an inducible enzyme and its pathophysiological role has been linked to inflammation (Hinz and Brune, 2002). Recently, a large body of genetic and biochemical evidence supports that COX-2 plays an important role in tumorigenesis (Evans and Kargman, 2004). Other studies also suggested that the expression of COX-2 is regulated largely at the transcription level by cytokines (Kuitert et al., 1997), growth factors (Xie and Herschman, 1996) and tumor promoters (Kujubu et al., 1991). The human COX-2 promoter contains a TATA box and a number of putative transcription factors binding sites including cyclic AMP response element (CRE), E-box, NF-IL6, NFκB, Sp1 and AP2 (Tohnai, 2002). Binding of transcription factors CREB, NFκB or C/EBPβ to the COX-2 promoter is important to the expression of the COX-2 gene. Previously, we reported that the EGF-induced expression of COX-2 in A431 cells was mediated through the Ras-mitogen-activated protein kinase (MAPK) signaling pathway and subsequent induction of c-Jun following MAPK activation (Chen et al., 2004). Xie and Herschman (Xie and Herschman, 1996) also reported that c-Jun could be activated by platelet-derived growth factor (PDGF) and binds to the CRE element of murine COX-2 promoter region which regulates COX-2 gene transcription. Although the requirement of c-Jun induction for

growth factor-induced transcription of COX-2 has been clearly demonstrated, little is known about the transactivation mechanism that modulates the effect of c-Jun in the regulation of the COX-2 promoter activity.

c-Jun, a component of the activating protein 1 (AP1) family of leucine zipper transcription factors, forms a variety of dimeric complexes with other basic region-leucine zipper (bZip) factors such as Jun-Jun or Jun-Fos dimers, as well as Jun-ATF dimers (Vogt, 2001). Many reports suggested that phosphorylation of c-Jun by c-Jun N-terminal kinase (JNK) at Ser-63 and Ser-73 is required for c-Jun transactivation activity (Dunn et al., 2002). In this study, we demonstrated, however, that N-terminal phosphorylation sites on the transactivation domain of c-Jun were not required for EGF-induced expression of COX-2. c-Jun, which could recruit other transcription factors like c-Fos, was required for EGF-induced expression of COX-2 gene in A431 cells.

Materials and Methods

Materials. Human EGF was purchased from Pepro Tech. (Rocky Hill, NJ). Monoclonal antibodies against c-Jun N-terminus and ERK2 were obtained from BD (Franklin Lakes, NJ). SuperSignal West Pico Chemiluminescent Substrate was purchased from Pierce Biotechnology (Rockford, IL). Polyclonal antibodies against c-Fos and streptavidin-agarose beads were purchased from Upstate (Lake Placid, NY). Polyclonal antibodies against COX-2, COX-1, and c-Jun C-terminus were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal antibodies directed against the phosphorylated form of Thr-183/Tyr-185 JNK were purchased from New England Biolabs (Beverly, MA). The luciferase assay kit was from Promega (Madison, WI). SP600125 was obtained from Tocris Cookson (Avonmouth, UK). Nucleobond plasmid purification system was from Macherey-Nagel (Mannheim, Germany). *pfu* DNA polymerase was purchased from Stratagene (La Jolla, CA). The expression vectors pcDNA3.1jun and TAM-67 encoding the wild or truncated human c-Jun proteins were the generous gifts of Dr M Birrer (NCI, Institutes of Health, Rockville, MD). The expression vectors encoding the human HA-tagged human wild-type and mutant c-Jun proteins (pMT108 and pMT161) were the gifts from Dr Bon C Chung (Institute of Molecular Biology, Academia Sinica, Taipei, Taiwan). Luciferase plasmid pXP-1 was a gift of Dr T Sakai (Kyoto Prefecture University of Medicine, Kyoto, Japan). Lipofectamine 2000, Dulbecco's modified Eagle's medium and Opti-MEM medium were obtained from Invitrogen (Carlsbad, CA). Fetal bovine serum was from HyClone Laboratories (Logan, UT). Biotinated oligonucleotides were synthesized by MDBio Inc. (Taipei, Taiwan). A small interfering RNA (siRNA) pool including four siRNAs targeting human c-Jun and a non-specific control siRNA pool were purchased from Dharmacon (Lafayette, CO). pSUPERc-Jun siRNA targeting human c-Jun (bases 105-123) was

designed and constructed by KRII International Co. (Taipei, Taiwan). All other reagents used were of the highest purity obtainable.

Cell culture. Human epidermoid carcinoma A431 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin, and 100 U/ml penicillin. In this series of experiments, cells were treated with 25 ng/ml EGF in culture medium supplemented with 10% fetal bovine serum.

Microsomes preparation. The general procedure for microsomes preparation was carried out as described previously (Kulmacz and Wu, 1989). The transfected A431 cells were scraped from the plates into ice-cold 0.1 M Tris-HCl, pH 7.4. Cells were sonicated briefly, and centrifuged at 10000 × g for 20 min. The supernatant liquid was centrifuged at 100000 × g for 1 h to pellet the microsomal fraction, which was resuspended in NP-40 lysis buffer. Microsomal proteins were subjected to Western blotting as described above.

Plasmids construction. The COX-2 promoter plasmids pXC80 and pXC44 have been described previously (Chen et al., 2004) and the mutants at the sites of CRE and E-box were constructed by site-directed mutagenesis method as previously described (Higuchi et al., 1988). The CRE site of -80/+49 fragment was mutated from -59 TTCGTCA -53 to TTatTCA, and the E-box site was mutated from -54 CACATG +49 to CACAct. Single or dual mutants were constructed into luciferase expression vector pXP1. The expression vector of c-Jun deletion mutant N1-220 was generated in pcDNA3.1 vector by PCR. The expression vectors of c-Jun mutant (pcDNA3.1junS63/73A and pcDNA3.1junM3A) were generated in pcDNA3.1 vector by

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PCR using the site-directed mutagenesis method (Higuchi et al., 1988). All constructs were verified by sequencing.

Transfection and reporter gene assay. Cells were transfected with plasmids or siRNA for c-Jun by lipofection using Lipofectamine 2000 according to the manufacturer's instruction with a slight modification as described previously (Chen et al., 2004). Each transfection was normalized with appropriate empty vector plasmids or control siRNA. After incubating the transfected cells at 37℃ for 44 h, the cell lysate was collected and subjected to luciferase activity or Western blot analysis. Luciferase activity was quantitated by using a luciferase assay kit and normalized to the protein concentration. Unless specially described, values expressed as relative luciferase activity, are average of three determinations. To establish the stable N1-220 and TAM-67-expressing clones, cells were transfected with equal amounts of N1-220, TAM-67 or pcDNA3.1 (neomycin-resistant gene expression plasmid) using Lipofectamine 2000, followed by selection with 0.3 mg/ml G418 for 1 month. The resistant clones were pooled, and the early passages of these cells were used for experiments.

Western blotting. An analytical 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was performed. For immunoblotting, proteins in the SDS gels were transferred to a polyvinylidene difluoride membrane by an Electroblot apparatus. Antibodies against human COX-2, COX-1, phospho-c-Jun (Ser-63), phospho-c-Jun (Ser-73), c-Jun, phospho-JNK, JNK-1, c-Fos, or β-actin were employed as the primary antibodies. Immunoblot analysis was carried out with secondary antibody coupled to horseradish peroxidase. SuperSignal West Pico

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Chemiluminescent Substrate was used for detection. The density of the immunoblots was determined by an image analysis system installed with a software BIO-ID.

Preparation of nuclear extracts. A431 cell nuclear extracts were isolated by a micropreparation technique as previously described (Andrews and Faller, 1991). Briefly, cell pellets were resuspended in buffer A containing 10 mM HEPES-KOH (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.2 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml leupeptin, and 1 μg/ml pepstatin on ice for 10 min. Cell nuclei were pelleted and resuspended in buffer C containing 20 mM HEPES-KOH (pH 7.9), 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF, 1 μg/ml leupeptin, and 1 μg/ml pepstatin on ice for 20 min. Cellular debris was removed by centrifugation at 12,000 × g for 2 min, and the supernatant fraction was stored in aliquot at −70°C.

DNA affinity precipitation assay. The DNA affinity precipitation assay was carried out as previously described (Zhu et al., 2002). In brief, oligonucleotides biotinated at 5'-termini and corresponding to the sense −67 to −42 bp and antisense strands or CRE/E-box element mutated oligonucleotides of the COX-2 promoter were annealed. The DNA affinity precipitation assay was performed by incubating 2 μg of biotinated DNA probe with 200 μg of nuclear extract and 20 μl of streptavidin-agarose beads in phosphate-buffered saline at room temperature for 1 h with rotation. Beads were collected and washed with cold phosphate-buffered saline for three times. Proteins bound to the beads were eluted and separated by 10% SDS-PAGE. Western blot analysis was carried out as described above.

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Chromatin immunoprecipitation (ChIP) assay. Chromatin immunoprecipitation assay was carried out as previously described (Saccani et al., 2001) with minor modification. Briefly, A431 cells were treated with 1% formaldehyde for 15 min. The cross-linked chromatin was then prepared and sonicated to an average size of 300 to 400 bp prior to being immunoprecipitated with antibodies specific to c-Jun, c-Fos or control rabbit IgG at 4 ℃ overnight. After reversal of cross-linking, the immunoprecipitated chromatin was PCR amplified with the following primers specific for the COX-2 promoter: F186-1, CTGGGTTTCCGATTTTCTCA and R49, GAGTTCCTGGACGTGCTCCT. The resulting PCR products were separated by agarose gel electrophoresis.

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Results

Essential role of c-Jun in EGF-induced expression of COX-2. We reported earlier that the 5'-flanking region of COX-2 gene ranging from -80 to -44 bp was required for EGF response (Chen et al., 2004). Sequence analysis of COX-2 gene promoter region ranging from -80 to -44 bp indicated the presence of a CRE (-57 to -53 bp) and an E-box elements (-54 to -49 bp). In order to further identify the EGF-response element in the promoter region from -80 to -44 bp, plasmids with mutated CRE and E-box elements were constructed by site-directed mutagenesis. Cells were transiently transfected with wild-type (pXC80) or mutant COX-2 promoter constructs and the effect of EGF on the reporter activity of these constructs is summarized in Fig. 1A. An 85% decrease was observed in pXC80CREmu with a mutation at CRE element. Plasmid with an E-box mutation (pXC80E-boxmu) attenuated the EGF response by 34%. Plasmid with a double mutation with CRE and E-box elements attenuated the EGF response by 81%, which was similar to the effect induced by pXC44. These results indicated that the CRE element in human COX-2 promoter region ranging from -57 to -53 bp played a critical role in EGF-induced transcription of COX-2 gene, while the E-box site (-54 to -49 bp) played only a minor role.

Our earlier studies also showed that c-Jun induction is important in EGF-induced expression of COX-2 (Chen et al., 2004). To further examine the role of c-Jun in COX-2 expression by EGF, the response element on COX-2 promoter region required by c-Jun over-expression was studied. Cells were co-transfected with c-Jun expression vectors and luciferase bearing vector pXC80 or plasmids with the CRE/E-box mutation. The effect of over-expression of c-Jun on these plasmids is summarized in Fig. 1B. A substantial decrease in the stimulatory response of c-Jun-transfection was observed in vectors bearing the promoter sequence with a deletion from -80 (pXC80) to -44 bp (pXC44), indicating that the DNA sequence ranging from -80 to -44 bp was

important for the c-Jun response of COX-2 promoter activation. Mutation at the CRE or E-box element (pXC80CREmu or pXC80E-boxmu) exhibited a 71% decrease in c-Jun response of COX-2 promoter activation. Double mutation at CRE and E-box (pXC80CRE/E-boxmu) dramatically reduced the promoter activity induced by c-Jun over-expression, which was similar to the effect of pXC44. These results suggested that the CRE/E-box element in the promoter region of human COX-2 played an important role in EGF and c-Jun response of COX-2 promoter activation.

To examine whether binding of c-Jun to COX-2 promoter was required for EGF-induced expression of COX-2, *in vitro* DNA affinity precipitation assay was used to quantify the DNA binding of c-Jun. The oligonucleotides corresponding to the sense −67 to −42 bp and antisense strands covering the CRE/E-box element were used as probes for DNA affinity precipitation assay. As shown in Fig. 2A, there was a significant amount of c-Jun bound to COX-2 promoter region ranging from -67 to -42 bp within the first 30 min of EGF treatment. The maximum binding of c-Jun to COX-2 promoter element was observed at 2 h after EGF treatment (Fig. 2A, upper panel) which paralleled c-Jun protein level in nucleus (Fig. 2A, lower panel). The *in vivo* binding of c-Jun to COX-2 promoter region was then evaluated by chromatin immunoprecipitation assay. Sonicated chromatin was precipitated with antibodies against c-Jun. Nonimmune rabbit IgG was used as control. The region of COX-2 promoter pulled down by immunoprecipitation was identified by PCR by using two COX-2-specific primers. The same sonicated lysates on chromatin immunoprecipitation was also checked by PCR. Binding of c-Jun to the CRE/E-box containing COX-2 promoter was enhanced in cells treated with EGF for 1 h, whereas nonimmune rabbit IgG failed to precipitate this COX-2 promoter region (Fig. 2B). These results suggested that the binding of c-Jun to COX-2 promoter region was required for

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EGF-induced expression of COX-2 *in vivo*. To further directly assess the essential role of c-Jun in EGF-induced expression of COX-2, we used an siRNA approach. As shown in Fig. 3A, c-Jun siRNA transient transfection led to a significant decrease in the expression of c-Jun (Fig. 3A, middle panel). Knockdown of c-Jun reduced the COX-2 protein expression induced by EGF (Fig. 3A, upper panel). Reporter assay also confirmed that 44 h after c-Jun siRNA transfection, the promoter activity induced by EGF was significantly attenuated in siRNA-transfected cells compared to that with empty vector (Fig. 3B). Altogether, these results strongly supported the essential role of c-Jun in EGF-induced expression of COX-2.

No requirement of c-Jun N-terminus phosphorylation for EGF-induced expression of COX-2. Phosphorylation of Ser-63 and Ser-73 on transactivation domain of c-Jun by JNK (Derijard et al., 1994) is necessary for transactivation of c-Jun on gene transcription (Binetruy et al., 1991; Smeal et al., 1991). To investigate whether phosphorylation of Ser-63 and Ser-73 of c-Jun was required for EGF-induced expression of COX-2, a selective pharmacological inhibitor of JNK, SP600125 (Bennett et al., 2001), was used to examine the effect of COX-2 expression induced by EGF. No effect of 5 μM SP600125 on JNK phosphorylation was observed, while 10 and 30 μM SP600125 completely inhibited it (Fig. 4B). These results indicated that SP600125 at concentrations of 10 and 30 μM might non-specifically inhibit the activities of MKK4 and MKK7 as previously reported (Bennett et al., 2001), and 5 μM SP600125 was a concentration for studying the specific function of JNK. As shown in Fig. 4C, treatment of cells with 5 μM SP600125 significantly inhibited the N-terminal phosphorylation of c-Jun, but no inhibitory effect on COX-2 protein expression was observed (Fig. 4A). These results suggested that EGF-

induced phosphorylation of Ser-63 and Ser-73 of c-Jun was not required for the gene expression of COX-2.

In order to further confirm that Ser-63 and Ser-73 phosphorylation of c-Jun was not required in EGF-induced expression of COX-2, an expression vector pcDNA3.1junS63/73A, a full length of c-Jun in which Ser-63 and Ser-73 were replaced by alanines, was constructed (Fig. 4D). Cells were transiently transfected with wild-type (pcDNA3.1jun) or mutant c-Jun (pcDNA3.1junS63/73A) and then treated with EGF for 15 min. Cell lysates were analyzed by Western blot to confirm whether the phosphorylation of Ser-63 and Ser-73 of c-Jun was abolished in cells transfected with pcDNA3.1junS63/73A. As shown in Fig. 4E, Ser-63 and Ser-73 on wild-type c-Jun could be phosphorylated in cells transfected with wild-type c-Jun. The phosphorylation level of wild-type c-Jun was increased by EGF treatment while the phosphorylation of Ser-63 and Ser-73 of c-Jun was absent in cells transfected with pcDNA3.1junS63/73A (Fig. 4E). To examine the effect of mutant plasmid (pcDNA3.1junS63/73A) of c-Jun on COX-2 promoter activity, cells were cotransfected with either expression vectors of wild-type or mutant c-Jun (pcDNA3.1jun or pcDNA3.1junS63/73A) together with the -80 to +49 bp COX-2 promoter reporter construct (pXC80). Over-expression of mutant c-Jun (pcDNA3.1junS63/73A) was able to activate the COX-2 promoter activity in a dose-dependent manner, which was similar to the effect of wild-type c-Jun on the promoter activity of COX-2 under the cell culture condition that the expression level of the c-Jun and c-JunS63/73A was almost the same (Fig. 5A, left panel). Under similar experimental condition, EGF stimulated the COX-2 promoter activity in a similar fashion as the cells over-expressing c-JunS63/73A and wild-type c-Jun (Fig. 5A, right panel).

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It has been reported that, in addition to Ser-63 and Ser-73 on c-Jun transactivation domain, Thr-91 and Thr-93 could also be phosphorylated upon growth factor stimulation (Morton et al., 2003). To further examine whether these two phosphorylation sites on transactivation domain of c-Jun were required for EGF-induced expression of COX-2, we used an expression vector pMT161 in which the putative phosphorylation sites Ser-58, Thr-62, Ser-63, Ser-73, Thr-89, Thr-91 and Thr-93 on c-Jun transactivation domain were replaced by alanines (Fig. 5B). Co-transfection of mutant c-Jun (pMT161) with COX-2 promoter construct also resulted in a 6-fold stimulation of the COX-2 promoter activity that was comparable to the induction by wild-type c-Jun (pMT108) and the protein expression level of c-Jun was the same (Fig. 5C). Moreover, a higher stimulation (13.1-folds) of COX-2 promoter activation was also observed in cells co-transfected with pMT161 and treated with EGF (Fig. 5C).

To examine if the enhancement of the COX-2 promoter activities stimulated by c-Jun mutants was correlated with the protein expression of COX-2 in A431 cells, cells were transfected with c-Jun N-terminus mutants and the microsomal fraction of transfected cells was analyzed by Western blot using antibodies against COX-2. Over-expression of c-JunS63/73A resulted in a 2.7-fold increase in COX-2 protein expression, which was similar to that by wild-type c-Jun (Fig. 5D). These results suggested that EGF-induced gene expression of COX-2 was independent on N-terminal phosphorylation of c-Jun.

Essential role of C-terminus domain of c-Jun for EGF-induced expression of COX-2. Since N-terminal phosphorylation of c-Jun was not involved in EGF-induced expression of COX-2, we next examined whether C-terminus of c-Jun was required for EGF-induced expression of COX-2. Deletion mutants of c-Jun (Fig. 6A) were used to identify the region of c-

Jun responsible for the activation of the COX-2 promoter. Cells were co-transfected with TAM-67 which lacked the N-terminal amino acids 3-122 but contained both of the basic region and leucine zipper domain, or with N1-220 which lacked the C-terminal amino acids 221-331 but contained the transactivation domain, together with the COX-2 promoter construct (pXC80), and the activity of the COX-2 promoter in the absence or presence of c-Jun mutants upon EGF stimulation was determined. A control experiment demonstrated that TAM-67 was able to act as a dominant negative mutant to inhibit c-Jun activity on pAP1-luc, an Ap1 responsive reporter plasmid (data not shown). Over-expression of TAM-67 resulted in a dose-dependent manner with the amount of transfected TAM-67 plasmid. Treatment of the TAM-67-over-expressed cells with EGF enhanced the promoter activity, which was also dependent on the dosage of TAM-67 added (Fig. 6B). However, no such activation was observed in cells transfected with expression vector N1-220 (Fig. 6B). These results suggested that C-terminus domain including basic region and leucine zipper domain of c-Jun was essential for EGF-induced expression of COX-2.

To further examine whether the protein expression of COX-2 stimulated by EGF was mediated through the regulation of c-Jun C-terminus, cells were stably transfected with TAM-67 and the COX-2 protein expression levels in cells treated with EGF were determined. Although the protein expression of TAM-67 in cells was slightly enhanced by EGF treatment, a 15-fold induction of the protein expression of COX-2 was observed upon EGF treatment (Fig. 6C). These results suggested that C-terminal domain of c-Jun played a pivotal role in EGF-induced expression of COX-2 in A431 cells. Regulation of c-Jun C-terminus by EGF might be involved in COX-2 gene expression.

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Enhancement of c-Jun and its C-terminus binding to COX-2 promoter region by EGF treatment. c-Jun has been reported to be constitutively phosphorylated at Thr-231, Ser-243, and Ser-249 which are located proximal to DNA-binding domain in resting human epithelial cells (Lin et al., 1992). Activation of protein kinase C results in the dephosphorylation of c-Jun at one or more of these sites which coincides with increased AP1 binding activity (Boyle et al., 1991). To examine whether the binding of c-Jun to COX-2 promoter could be enhanced by EGF treatment, cells were transfected with the expression vector of HA-tagged c-Jun and treated with EGF. Nuclear extracts of transfected cells were collected and subjected to DNA affinity precipitation assay. As shown in Fig. 7A, a significant binding (2.3-folds) of c-Jun to COX-2 promoter was observed in pMT108-transfected cells treated with EGF for 1 h, while a 5.5-fold c-Jun binding was observed in cells treated with EGF for 2 h. In order to further confirm if the regulation of c-Jun binding to COX-2 promoter by EGF was limited to the C-terminal region of c-Jun, EGF-treated cells were then determined for its expression of TAM-67. Under this experimental condition, cells treated with EGF for 10 min, the protein level of c-Jun in nucleus was not yet changed by EGF treatment (Fig. 7B, lower panel) while the protein level of TAM-67 in nucleus was equally in control and EGF-treated cells (middle panel). However, a 6.5-fold increase of TAM-67 binding to COX-2 promoter was observed in cells treated with EGF for 10 min compared with that in control cells (upper panel). These results suggested that DNA binding activity of c-Jun stimulated by EGF was mediated through the C-terminal regulation of c-Jun.

To test whether the dephosphorylation form of c-Jun might increase its DNA binding activity, an expression vector c-JunM3A, a full length of c-Jun in which Ser-231, Thr-243 and Thr-249 were replaced by alanines, was constructed (Fig. 8A). Cells were transfected with the expression vectors of either c-Jun or c-JunM3A and the nuclear extracts of transfected cells were

collected respectively which were subjected to DNA affinity precipitation assay. A higher binding activity (1.6-folds) of c-JunM3A to COX-2 promoter than that of wild-type c-Jun was observed in this *in vitro* DNA binding assay (Fig. 8B). In order to investigate whether the increased DNA binding activity of c-JunM3A correlated to its transactivation activity, cells were co-transfected with the COX-2 promoter construct. As shown in Fig. 8C and D, over-expression of cells with c-JunM3A had higher induction effect on gene promoter activation and COX-2 protein expression than that with wild-type c-Jun. Moreover, EGF also enhanced the effect of c-JunM3A or wild type c-Jun on COX-2 protein expression (Fig. 8D). These results indicated that the effect of EGF on COX-2 expression in transfected cells might be due to the enhancement of the expression of endogenous c-Jun. Taken together, these results clearly indicated that the DNA binding activity of c-Jun was essential for the transcriptional activation of COX-2 gene upon EGF treatment.

Cooperation of c-Jun with c-Fos to activate COX-2 gene. Since our results indicated that N-terminal phosphorylation of c-Jun by JNK was not required for the transcriptional activation of COX-2, we then studied how the transactivation function of c-Jun was driven. It has been reported that c-Jun forms homodimers or heterodimerizes with other Jun family members or with other bZip protein including members of the c-Fos and ATF/CREB families. Different complexes may then modulate the expression of target genes (Chinenov and Kerppola, 2001; van Dam and Castellazzi, 2001). It was recently reported that PDGF regulates AP1 by stimulating the expression of c-Fos and phosphorylation of c-Fos by ERK, and its transcriptional activity is thus enhanced (Monje et al., 2003). We therefore proposed that c-Fos of AP1 heterodimer might provide its transactivation function for the gene transcription of COX-2. To investigate whether

c-Fos was involved in EGF-induced expression of COX-2, the gene expression of c-Fos and its binding to COX-2 promoter region were assessed. As shown in Fig. 9A, (upper panel), exposure of A431 cells to EGF potently increased the protein level of c-Fos in nucleus. c-Fos was detectable as early as 15 min after stimulation and the maximum response was observed in cells treated with EGF for 1 h. In the analysis of the binding of c-Fos to COX-2 promoter by *in vitro* binding assay, we found that binding of c-Fos to COX-2 promoter region was consistent with the protein level of c-Fos in nucleus enhanced by EGF treatment (Fig. 9A, lower panel). No c-Fos binding to the CRE/E-box element mutated oligonucleotides compared with the wild-type oligonucleotides (Fig. 9B) was observed. The *in vivo* binding of c-Fos to COX-2 promoter region was further evaluated by chromatin immunoprecipitation assay. Binding of c-Fos to CRE/E-box containing COX-2 promoter was enhanced in cells treated with EGF for 1 h, whereas nonimmune rabbit IgG failed to precipitate this COX-2 promoter region (Fig. 9C). These results suggested that the binding of c-Fos to COX-2 promoter region was required for EGF-induced expression of COX-2 *in vivo*.

To further address whether c-Fos would activate the COX-2 promoter activity, cells were co-transfected with the c-Fos expression vector and the COX-2 promoter construct. As shown in Fig. 10, 0.4 μg of the c-Fos expression resulted in a significant induction of COX-2 promoter activity and this effect could be enhanced by EGF treatment. Increasing the concentration of pSV-fos up to 0.8 μg resulted in a greater activation (5.4-folds), while a higher EGF response (8.5-folds) was also observed. These results suggested that c-Fos might play a functional role in EGF-induced expression of COX-2.

We then examined whether c-Fos cooperated with c-Jun to activate COX-2 promoter activity. Cells were co-transfected with 0.2 μg of expression vector of c-Jun and various amount

of c-Fos plasmid. The effect on COX-2 promoter activity was determined. As shown in Fig. 11A, co-transfection of c-Jun and c-Fos expression vector resulted in a dose-dependent activation of the COX-2 promoter. Up to 20-fold increase in the activation of COX-2 promoter activity was observed in cells co-transfected with 0.75 μg of c-Fos expression vector and 0.2 μg of c-Jun expression vector. The cooperative effect of c-Fos and c-Jun on COX-2 promoter activity was enhanced by EGF-treatment up to 28-folds. Since N-terminal phosphorylation of c-Jun was not required for EGF-induced expression of COX-2, we further investigated whether N-terminal phosphorylation sites-mutated c-Jun could cooperate with c-Fos to activate the COX-2 promoter. Co-transfection of c-JunS63/73A (Fig. 11B) or pMT161 (Fig. 11D) expression vectors with c-Fos expression vector resulted in a similar induction as wild-type c-Jun (Fig. 11, A and C). Likewise, the COX-2 promoter activity was also enhanced by EGF stimulation. These results suggested that c-Fos cooperating with c-Jun to activate COX-2 promoter was independent of N-terminal phosphorylation of c-Jun. To further examine whether C-terminus of c-Jun without N-terminal transactivation domain could cooperate with c-Fos to activate COX-2 promoter, cells were co-transfected with TAM-67 and c-Fos expression vectors. As shown in Fig. 11E, a similar activation pattern as those of pMT108 and pMT161 was observed. Taken together, these results suggested that c-Fos induction and its cooperation with c-Jun under EGF treatment was required for the expression of COX-2, while the cooperation of c-Jun with c-Fos on COX-2 promoter activation was not due to the transactivation domain on c-Jun but due to the transactivation activity from c-Fos.

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Discussion

Previously, we reported that EGF-induced expression of COX-2 in A431 cells was mediated through the Ras-MAPK signaling pathway, and subsequent induction of c-Jun following MAPK activation was required for the EGF response (Chen et al., 2004). In the present study, we firstly used the chromatin immunoprecipitation assay (Fig. 2B) and c-Jun siRNA experiment (Fig. 3) to directly demonstrate the important role of c-Jun in the EGF-induced expression of COX-2 *in vivo*. The present study further provided additional evidence to support the conclusion that N-terminal phosphorylation of transactivation domain on c-Jun was not required for c-Jun transactivation activity in the EGF-induced COX-2 expression. It was reported that the transcriptional activity of c-Jun is increased following phosphorylation on Ser-63 and Ser-73 by JNK (Smeal et al., 1991). In contrast, we found no difference in the ability of wild-type c-Jun and the c-JunS63/73A mutant to enhance the promoter activity of COX-2 gene (Fig. 5A). Moreover, SP600125, a pharmacological inhibitor of JNK, efficiently abolished the Ser-63 and Ser-73 phosphorylation of c-Jun and JNK phosphorylation induced by EGF, while it had no effect on COX-2 protein expression (Fig. 4). Although it has been reported that c-Jun also could be phosphorylated by JNK on Thr-91 and Thr-93 (Morton et al., 2003), but these two phosphorylation sites on transactivation domain of c-Jun were not required for EGF-induced expression of COX-2 (Fig. 5C). Furthermore, over-expression of c-JunS63/73A resulted in an increase in the COX-2 protein expression in A431 cells (Fig. 5D). Our results indicated that the transactivation function of c-Jun enhanced COX-2 expression induced by EGF treatment was independent of JNK activation and N-terminal phosphorylation of c-Jun. Although this finding was in contrast to numerous reports indicating that N-terminal phosphorylation of c-Jun plays a pivotal role on its transactivation activity (Dunn et al., 2002), many other reports have also demonstrated that N-

terminal phosphorylation of c-Jun may not be required for gene expression. For example, calcium-activated c-Fos transcription mediated by c-Jun (Cruzalegui et al., 1999) and light–induced apoptosis of photoreceptor cells mediated by c-Jun (Grimm et al., 2001) are independent of N-terminal phosphorylation of c-Jun. The mechanism of c-Jun stimulated gene expression without N-terminus phosphorylation has been reported. Behre et al. (Behre et al., 1999) reported that c-Jun N-terminal phosphorylation sites mutant interact with ETS domain transcription factor PU.1 for M-CSF receptor promoter activation. Moreover, the formation of complex between p65 and TAM-67 on the κB site activates the IL-6 promoter activity (Faggioli et al., 2004), and C/EBPβ and TAM-67 on TNFα gene promoter synergistically activate the target gene expression (Zagariya et al., 1998). In this study, we also provided several pieces of evidence to indicate that c-Fos provided its transcription activity via a c-Jun/c-Fos heterodimer in EGF-induced expression of COX-2. First, exposure of A431 cells to EGF potently stimulated c-Fos protein expression and the binding of c-Fos to COX-2 promoter region *in vivo* (Fig. 9C). The binding of c-Fos to human COX-2 promoter region ranging from –67 to –42 bp covering CRE/E-box site upon EGF treatment was demonstrated by *in vitro* DNA binding assay (Fig. 9A and B). Second, over-expression of c-Fos resulted in a significant induction of COX-2 promoter activity, which was enhanced by EGF treatment (Fig. 10). Third, co-transfection of c-Fos with either wild-type or N-terminal phosphorylation sites-mutated c-Jun mutants resulted in a similar induction on promoter activity of COX-2. Moreover, co-transfection of c-Fos with N-terminal deletion mutant of c-Jun expression vector TAM-67 also resulted in an increase in promoter activity than that of c-Fos over-expression alone, and a synergistic activation of COX-2 promoter activity was observed following stimulation of EGF (Fig. 11). However, we could not rule out

the possibility that other members of AP1 or ATF/CREB families may interact with c-Jun to regulate EGF-induced expression of COX-2.

It has been reported that Fos/Jun dimer interacts with its cognate binding site and regulates a wide array of genes (Angel and Karin, 1991). The activity of AP1 regulated by growth factors has been the subject of intense investigation. Although N-terminal phosphorylation of c-Jun by JNK is important for the AP1 activity (Binetruy et al., 1991; Smeal et al., 1991), it is not required for COX-2 gene expression based on the results of this study. c-Fos contains several transcriptionally active regions including several autonomous transactivation domains (Jooss et al., 1994; McBride and Nemer, 1998). The modulation of transcription by c-Fos is via the increase in the DNA binding affinity of Fos/Jun dimer. However, the results of our present study suggested a transactivation functional role of c-Fos in COX-2 gene regulation. This finding was consistent with another report indicating that phosphorylation of the C-terminal transactivation domain of c-Fos by extracellular signal-regulated kinase modulates the transcription activity of AP1 (Monje et al., 2003). In our present study, we provided direct evidence to prove that c-Fos of Fos/Jun dimer acts as an activator for gene transcription in the absence of N-terminal phosphorylation of c-Jun.

Previously, several reports suggested that the general co-activator CREB-binding protein (CBP)/p300 stimulates c-Jun-dependent transcription which is mediated through c-Jun residue Ser-63 and Ser-73. However, the interaction of c-Jun N-terminal transactivation domain with CBP appears to be independent of c-Jun phosphorylation on Ser-63 and Ser-73 (Bannister et al., 1995). Moreover, it has been reported that c-Fos could interact with CBP/p300 and regulate gene expression (Chan and La Thangue, 2001). We previously reported that c-Jun induction and cooperation with p300 were essential for EGF-induced expression (Chen et al., 2004). In this

study, we further demonstrated that c-Fos may provide its transactivation activity for transactivation domain-truncated c-Jun. Taken together, the formation of multiprotein transcription complex including CBP/p300, c-Fos and c-Jun was required for EGF-induced expression of COX-2.

The activity of c-Jun is regulated at both transcriptional and post-translational levels. As indicated above, changing in N-terminal phosphorylation state of c-Jun was not required for its transactivation potential in our system. c-Jun has been reported to be phosphorylated at Thr-231, Ser-243, and Ser-249 (Boyle et al., 1991) located proximal to the DNA-binding domain when the binding of c-Jun to DNA is inhibited. The phosphorylated form of c-Jun is activated by dephosphorylation of these sites in response to protein kinase C activation and the DNA binding activity of c-Jun is increased. Several pieces of evidence were provided in this study to indicate that C-terminal dephosphorylation of c-Jun following the increase the DNA binding activity of c-Jun was required for EGF-induced expression of COX-2 in A431 cells. First, activation of TAM-67 by EGF treatment resulted in a significant activation of COX-2 promoter activity (Fig. 6B). This dominant-negative mutant of c-Jun has been shown to inhibit the function of endogenous AP1 protein through a “quenching” mechanism and inhibit gene expression (Brown et al., 1994). However, in COX-2 gene regulation, it acted as an enhancer in response to EGF (Fig. 6B). Moreover, a synergistic effect of COX-2 protein expression was observed in stable cell line over-expressing TAM-67 and treated with EGF (Fig. 6C). Second, the binding of ectopic expressed c-Jun to COX-2 promoter region was enhanced by EGF treatment (Fig. 7). These results further confirmed that binding of c-Jun to COX-2 promoter region was mediated through the C-terminal regulation of c-Jun by EGF. The possible functional role of c-Jun C-terminus phosphorylation state in promoter binding was furthermore studied by the site-directed mutagenesis approach.

The c-JunM3A expression vector having Thr-231, Ser-243, and Ser-249 replaced by alanines to mimic the dephosphorylated state of c-Jun, had higher DNA binding activity (Fig. 8B) and transcriptional activity (Fig. 8C and D) than wild-type c-Jun. These results strongly suggested that the phosphorylation/dephosphorylation state of c-Jun C-terminus might play a functional role in EGF-induced expression of COX-2 gene in A431 cells.

Acetylation is another important type of post-translational modification of transcription factors (Struhl, 1998). Recently, p300 was shown to acetylate c-Jun on C-terminus Lys268, Lys271, and Lys273 and to regulate the transcriptional activity of c-Jun (Vries et al., 2001). However, c-JunK3R, an expression vector of mutant c-Jun in which Lys268, Lys271, and Lys273 were replaced by non-acetylable arginines, had no significant effect on EGF-induced promoter activity of COX-2 (data not shown). Hence the possibility of c-Jun acetylation involved in EGF-induced expression of COX-2 could be ruled out.

In summary, we demonstrated in this study that the induction of COX-2 in A431 cells by EGF required c-Jun protein synthesis. However, N-terminal phosphorylation of c-Jun was not required for EGF-induced expression of COX-2. The regulation of c-Jun C-terminal by EGF was required for COX-2 gene transcription. c-Fos may provide the transactivation function for c-Jun-driven transcriptional activity on the gene expression of COX-2. Our results suggest a model in which c-Jun expression, induced by EGF, could recruit either c-Fos or other transcription factors to the promoter and regulate gene expression of COX-2 in A431 cells.

Acknowledgements

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Footnotes

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Legends for Figures

Fig. 1. Effect of EGF and c-Jun over-expression on COX-2 promoter activation. A, The promoter activity of site-directed mutants made in the COX-2 promoter region was analyzed by transient transfection into A431 cells treated with 25 ng/ml EGF for 3 h. Values are means ± S.E.M. of three to seven independent experiments. B, Cells were cotransfected with various mutants of pXC80 and pcDNA3.1jun by the lipofection method. The expression of luciferase and the concentration of total cell lysates were determined and normalized. Values for luciferase activity are means ± S.E.M. of five independent experiments. Statistical significance (* $P < 0.05$ and *** $P < 0.001$) between wild-type and mutant COX-2 promoter activities was analyzed by Student's t-test.

Fig. 2. Binding of c-Jun to COX-2 promoter region. A, Cells were starved for 24 h in serum free culture medium before treatment with 25 ng/ml EGF for different time as indicated. The nuclear extracts were prepared and subjected to DNA affinity precipitation assay (DAPA) as described under "Materials and Methods". Proteins bound to the beads were eluted and resolved by Western blotting using anti-c-Jun antibodies (upper panel). The protein level of c-Jun in nucleus was also analyzed by Western blotting (lower panel). The relative density of blots was quantified as indicated. B, Cells were treated with 25 ng/ml EGF for 1 h and subjected to ChIP assay as described under "Materials and Methods".

Fig. 3. Effect of c-Jun siRNA on EGF-induced gene expression of COX-2. A, After transfection with control or c-Jun siRNA at a final concentration of 100 nM, cells were treated with 10 ng/ml EGF for 90 min. The expression of COX-2 (upper panel), c-Jun (middle panel) and β-actin

(lower panel) in total cell lysates were analyzed by Western blotting. B, A431 cells were cotransfected with the -80/+49 human COX-2 promoter construct (pXC80) and various concentrations of expression vector of c-Jun siRNA. After transfection for 42 h, cells were treated with 25 ng/ml EGF for 3 h. Statistical significance (*** $P < 0.001$) between c-Jun siRNA and empty vector-transfected cells was analyzed by Student's t-test.

Fig. 4. Effect of EGF on phosphorylation of c-Jun N-terminus. A, Confluent A431 cell were treated with various concentrations of SP600125 for 30 min followed by 25 ng/ml treatment for 3 h. The whole-cell lysates were analyzed by Western blotting using anti-COX-2 (upper panel) and COX-1 (lower panel) antibodies. B, Cells were treated with various concentrations of SP600125 for 30 min followed by 25 ng/ml treatment for 5 min. The whole-cell lysates were analyzed by Western blotting using anti-phospho-JNK (upper panel) or JNK-1 (lower panel) antibodies. C, c-Jun N1-220 stable clone of A431 cells were treated with various concentrations of SP600125 for 30 min followed by 25 ng/ml treatment for 15 min. The whole-cell lysates were analyzed by Western blotting using anti-phospho-c-Jun (Ser-63) (upper panel), phospho-c-Jun (Ser-73) (middle panel) and c-Jun (lower panel) antibodies. D, Schematic representation of the mutated c-Jun form (c-JunS63/73A). The BR indicates basic region, and LZ indicates leucine zipper domain. E, After transfection of wild-type (c-Jun) or mutant c-Jun (c-JunS63/73A), cells were treated with EGF for 15 min. The whole-cell lysates were analyzed by Western blotting. The relative density of blots was quantified as indicated.

Fig. 5. Effect of over-expression of wild-type c-Jun and its N-terminus mutants on COX-2 gene expression. A, A431 cells were cotransfected with the -80/+49 human COX-2 promoter

construct (pXC80) and four different concentrations of expression vectors of wild-type (0.06, 0.1, 0.2, and 0.35 μg) or mutant c-Jun (0.02, 0.05, 0.1, and 0.3 μg) in order to ensure the equal protein expression in cells. After transfection for 42 h, cells were treated with 25 ng/ml EGF for 3 h. Luciferase activity was quantitated and normalized to the protein concentration. Ten μg of total cell lysates were analyzed by Western blotting by using anti-c-Jun and anti-COX-1 antibodies. B, Schematic representation of pMT108 and the mutated c-Jun form (pMT161). C, Cells were cotransfected with the -80/+49 human COX-2 promoter construct (pXC80) and expression vectors of wild-type or mutant c-Jun. Statistical significance (* $P < 0.05$ and *** $P < 0.001$) between EGF-treated and control cells was analyzed by Student's t-test (upper panel). Twenty μg of total cell lysates were analyzed by Western blotting by using anti-HA antibodies (lower panel). D, A431 cells were transfected with wild-type (c-Jun) or mutant c-Jun (c-JunS63/73A). The expression of COX-2 (upper panel) and COX-1 (middle panel) in microsomal fraction, and that of c-Jun (lower panel) in total cell lysates were analyzed by Western blotting.

Fig. 6. Effect of over-expression of c-Jun deletion mutants on COX-2 gene expression. A, Schematic representation of the mutated c-Jun forms. B, A431 cells were cotransfected with the -80/+49 human COX-2 promoter construct (pXC80) and various concentrations of expression vectors of N-terminus or C-terminus of c-Jun. Statistical significance (** $P < 0.01$ and *** $P < 0.001$) between TAM-67-transfected and untransfected cells was analyzed by Student's t-test (upper panel). Twenty μg of total cell lysates were analyzed by Western blotting by using anti-c-Jun antibodies (lower panel). C, A431 cell lines expressing the neomycin resistant gene alone (pcDNA3.1) or together with TAM-67 were treated with 25 ng/ml EGF for 3 h. The total cell

lysates were analyzed by Western blotting using anti-COX-2 (upper panel), c-Jun (middle panel) and β-actin (lower panel) antibodies. The relative density of blots was quantified as indicated.

Fig. 7. Enhancement of binding of c-Jun and its C-terminus to COX-2 promoter by EGF treatment. A, After transfection with empty vector or HA-tagged c-Jun expression vector (pMT108), cells were treated with 25 ng/ml EGF for different time as indicated. The nuclear extracts were prepared and subjected to DNA affinity precipitation assay as described under "Materials and Methods". Proteins bound to the beads were eluted and resolved by Western blotting using anti-HA antibodies (upper panel). The protein level of c-Jun in nucleus was also analyzed by Western blotting (lower panel). B, A431 cells expressing TAM-67 were treated with 25 ng/ml EGF for different time as indicated. The nuclear extracts were prepared and subjected to DNA affinity precipitation assay as described under "Materials and Methods". Proteins bound to the beads were eluted and resolved by Western blotting using anti-c-Jun antibodies (upper panel). The protein levels of TAM-67 (middle panel) and c-Jun (lower panel) in nucleus were also analyzed by Western blotting. The relative density of blots was quantified as indicated.

Fig. 8. Enhancement of c-Jun binding to COX-2 promoter and COX-2 promoter activity by over-expression of c-Jun with mutation of C-terminus phosphorylation sites. A, Schematic representation of the mutated c-Jun form c-JunM3A. B, Cells were transfected with expression vector of wild-type or mutant c-Jun. The nuclear extracts were prepared and subjected to DNA affinity precipitation assay as described under "Materials and Methods". Proteins bound to the beads were eluted and resolved by Western blotting using anti-c-Jun antibodies (upper panel). c-Jun expression of nuclear extract was also analyzed by Western blotting (lower panel). C, A431

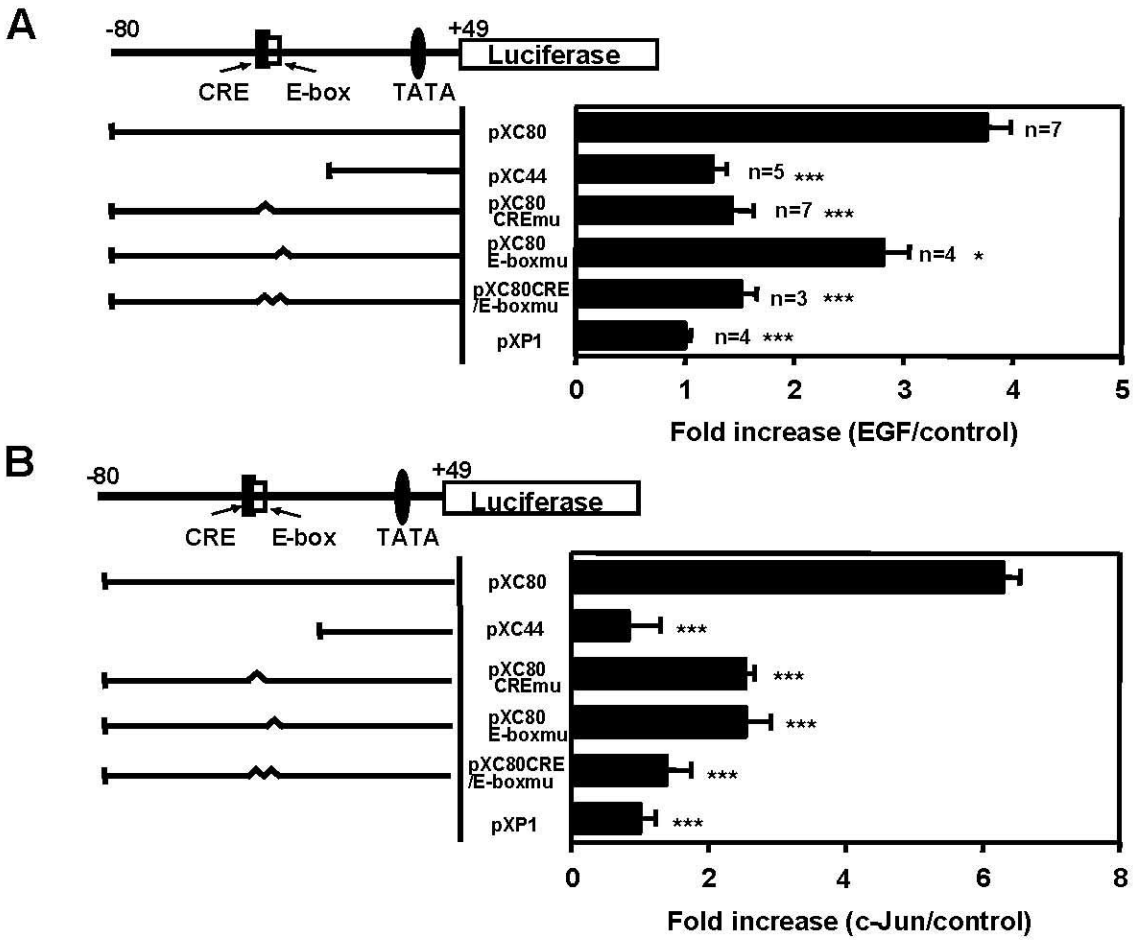
cells were cotransfected with the -80/+49 human COX-2 promoter construct (pXC80) and various concentrations of expression vectors (0.01, 0.02, and 0.03 μg) of wild-type or mutant c-Jun. Statistical significance (** $P < 0.01$) between wild-type and mutant c-Jun-transfected cells was analyzed by Student's t-test (upper panel). Ten μg of total cell lysates were analyzed by Western blotting by using anti-c-Jun and COX-1 antibodies (lower panel). D, A431 cells were transfected with wild-type (c-Jun) or mutant c-Jun (c-JunM3A). After transfection for 24 h, cells were treated with 10 ng/ml EGF for 90 min. The expression of COX-2 (upper panel), c-Jun (middle panel), and β-actin (lower panel) in total cell lysates were analyzed by Western blotting. The relative density of blots was quantified as indicated.

Fig. 9. Induction of c-Fos and its binding to COX-2 promoter by EGF treatment. A, Cells were starved for 24 h in serum free culture medium before treated with 25 ng/ml EGF for different time as indicated. The protein level of c-Fos in nucleus was analyzed by Western blotting (upper panel). The nuclear extracts were then subjected to DNA affinity precipitation assay as described under "Materials and Methods". Proteins bound to the beads were eluted and resolved by Western blotting using anti-c-Fos antibodies (lower panel). B, Cells were starved for 24 h in serum free culture medium before treated with 25 ng/ml EGF for 1 h. The nuclear extracts were then subjected to DNA affinity precipitation assay. Wild-type (WT) 5'-biotinated oligonucleotides or CRE/E-box element mutated oligonucleotides (CRE/E-box mu) corresponding to COX-2 promoter region ranging from –67 to –42 bp were used as probes for DNA affinity precipitation assay. Proteins bound to the beads were eluted and resolved by Western blotting using anti-c-Fos antibodies. C, Cells were treated with 25 ng/ml EGF for 1 h and subjected to ChIP assay as described under "Materials and Methods".

Fig. 10. Enhancement of COX-2 promoter activity by c-Fos over-expression. A431 cells were cotransfected with the -80/+49 human COX-2 promoter construct (pXC80) and various concentrations of expression vectors of c-Fos. Statistical significance (* $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$) between c-Fos-transfected and untransfected cells was analyzed by Student's t-test.

Fig. 11. Cooperation of c-Fos with c-Jun in promoter activation of COX-2 gene. A431 cells were cotransfected with the -80/+49 human COX-2 promoter construct (pXC80), expression vector of wild-type c-Jun (A and C) or mutant c-Jun (B, D and E) and various concentrations of expression vectors of c-Fos. After transfection for 42 h, cells were treated with 25 ng/ml EGF for 3 h. Statistical significance (* $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$) between c-Fos-transfected and untransfected cells was analyzed by Student's t-test.

Figure 1


A
-80
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Luciferase
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pXC44
pXC80 CREmu
pXC80 E-boxmu
pXC80CRE /E-boxmu
pXP1
n=7
n=5 ***
n=7 ***
n=4 *
n=3 ***
n=4 ***
0
1
2
3
4
5
Fold increase (EGF/control)
B
-80
+49
Luciferase
CRE
E-box
TATA
pXC80
pXC44
pXC80 CREmu
pXC80 E-boxmu
pXC80CRE /E-boxmu
pXP1
***
***
***
***
0
2
4
6
8
Fold increase (c-Jun/control)

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Figure 2

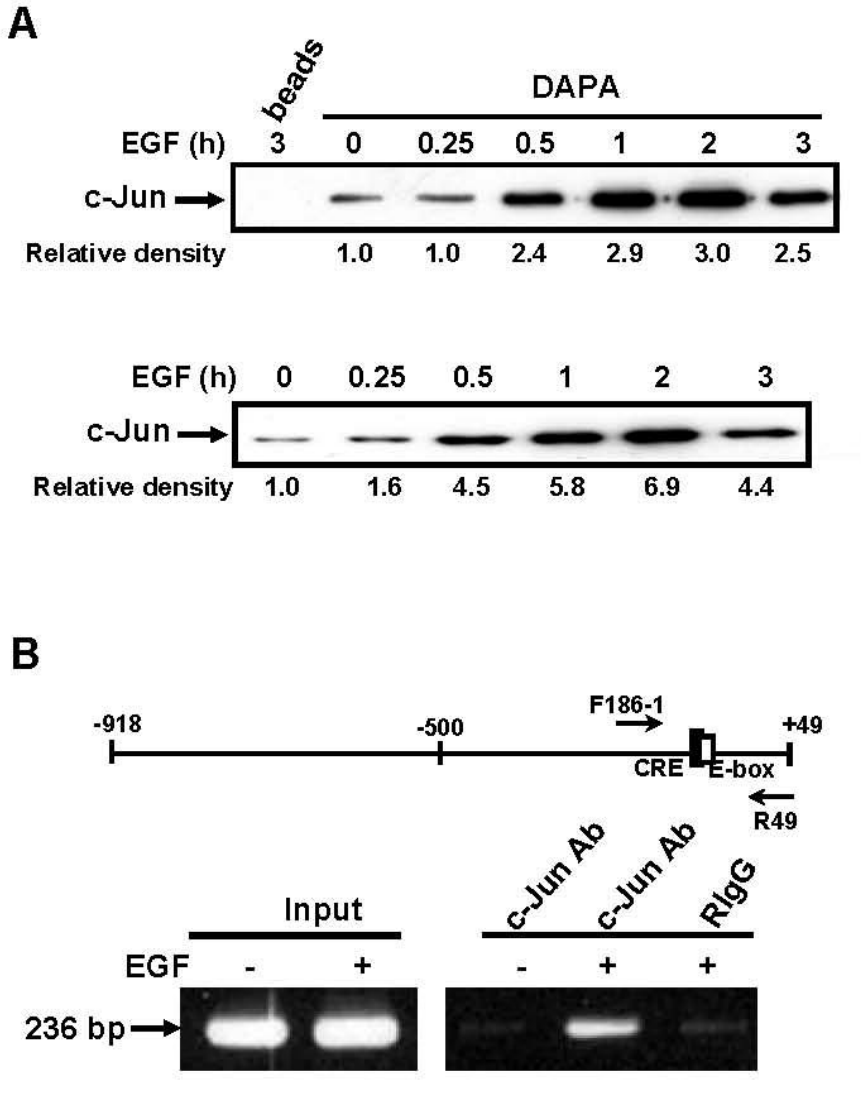

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Figure 3

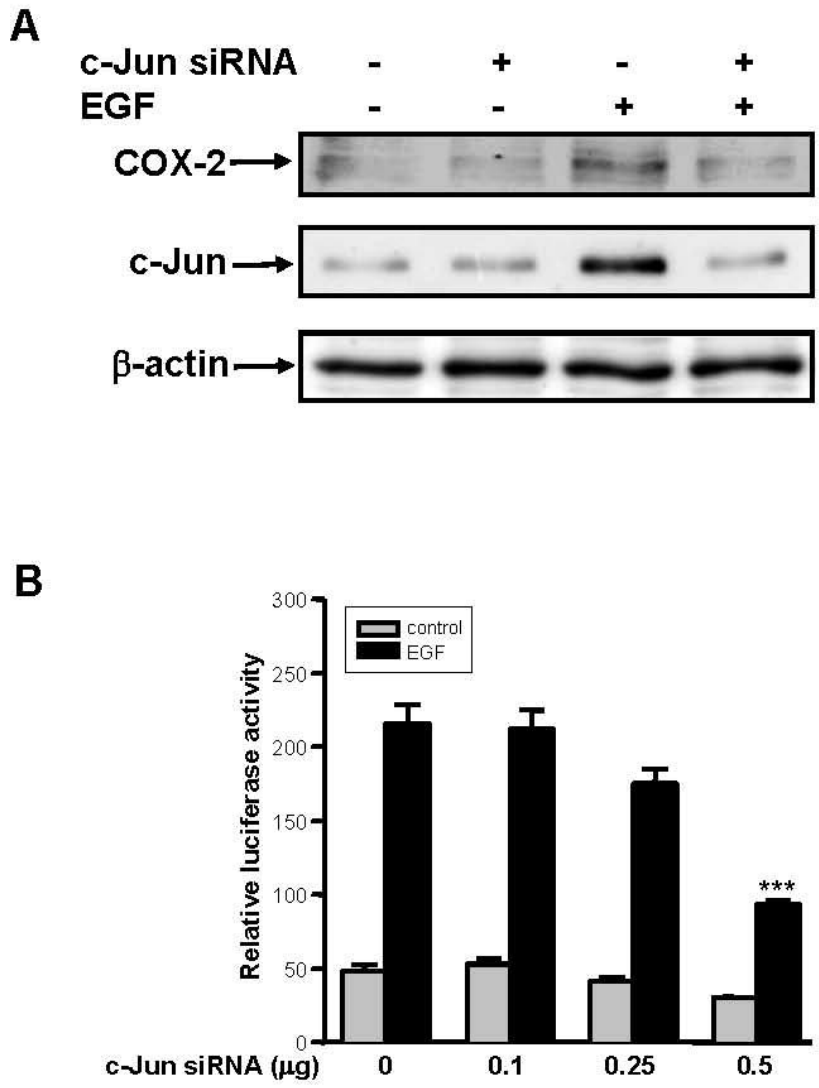

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Figure 4

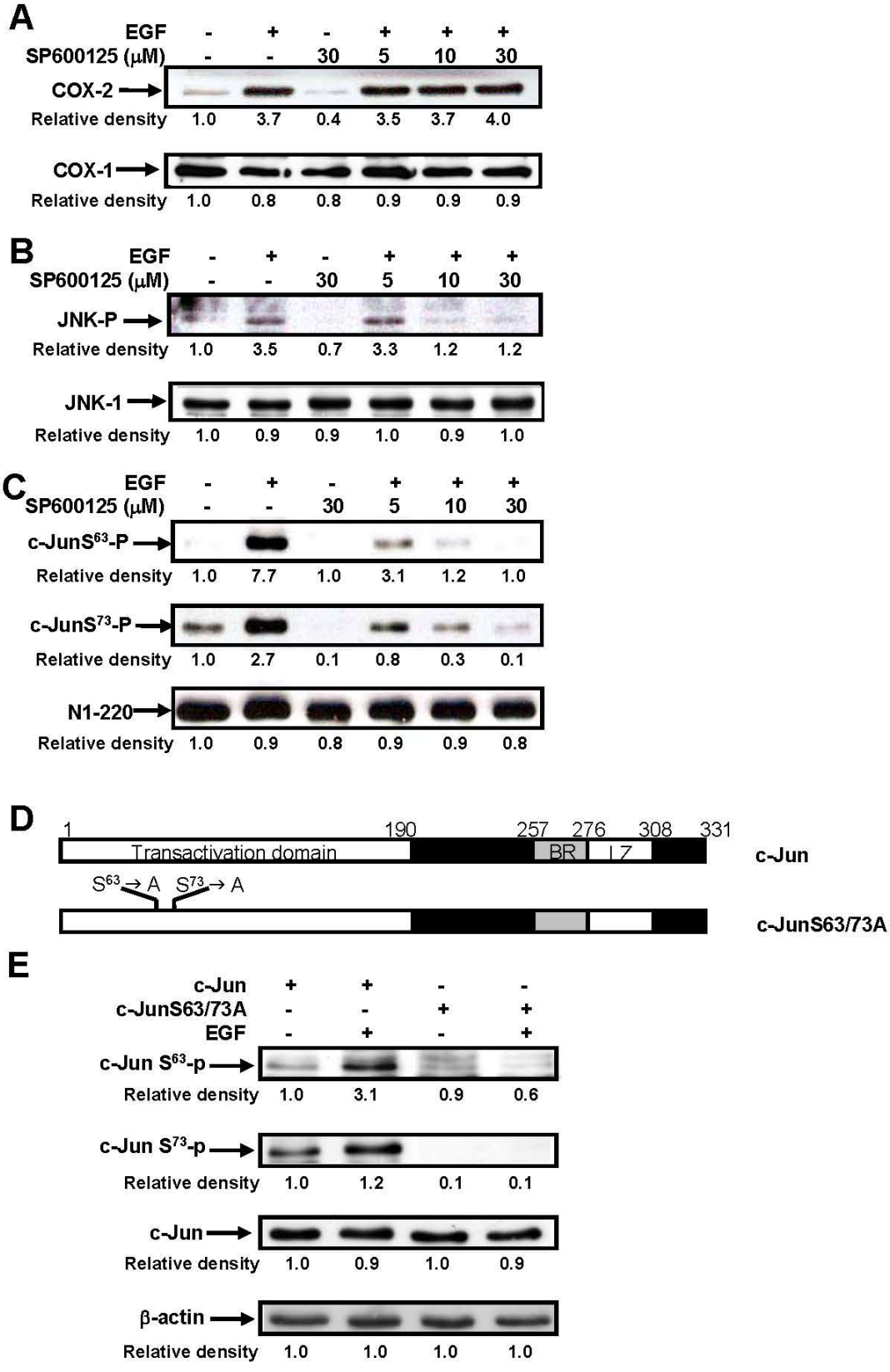

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Figure 5

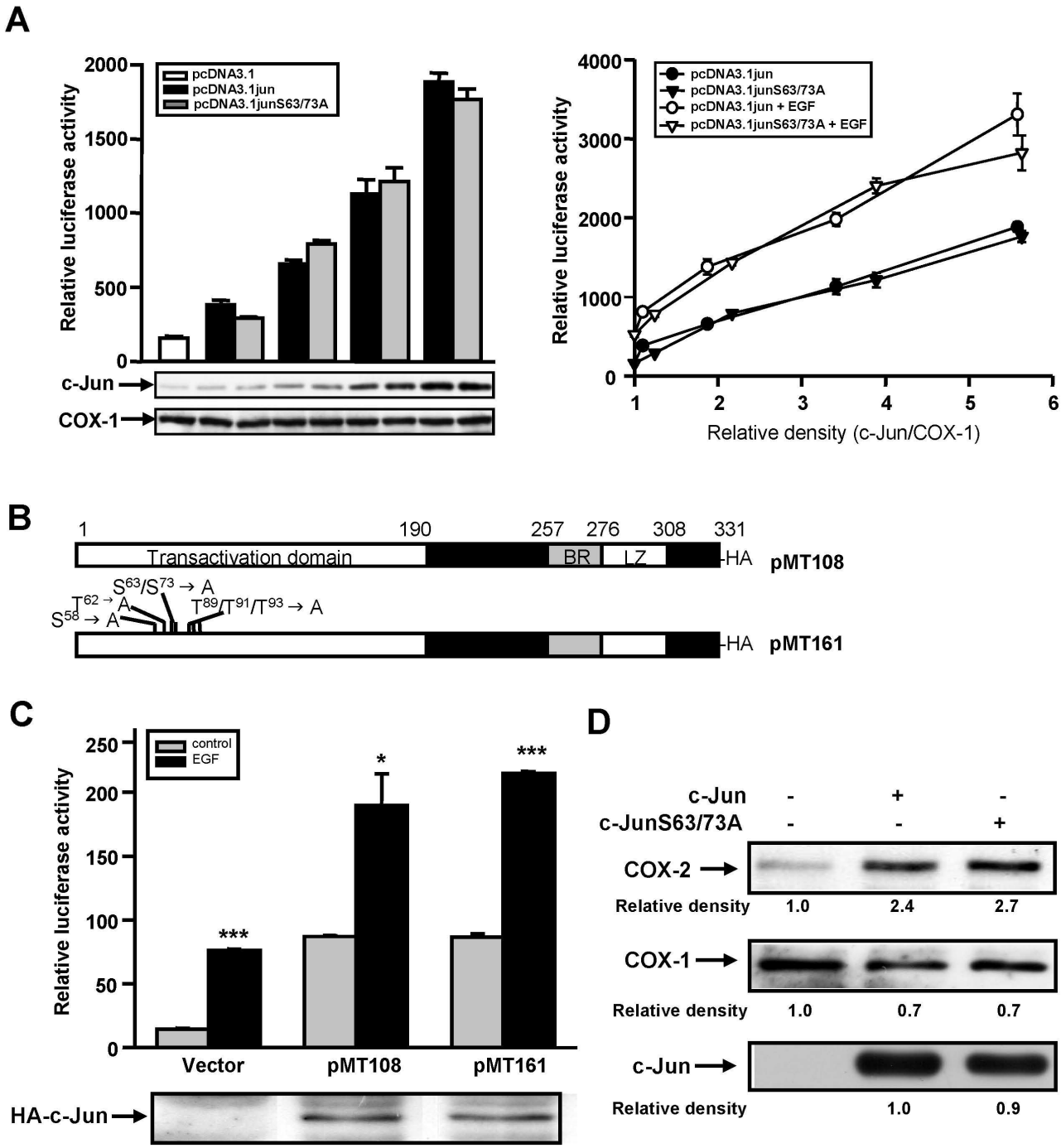

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Figure 6

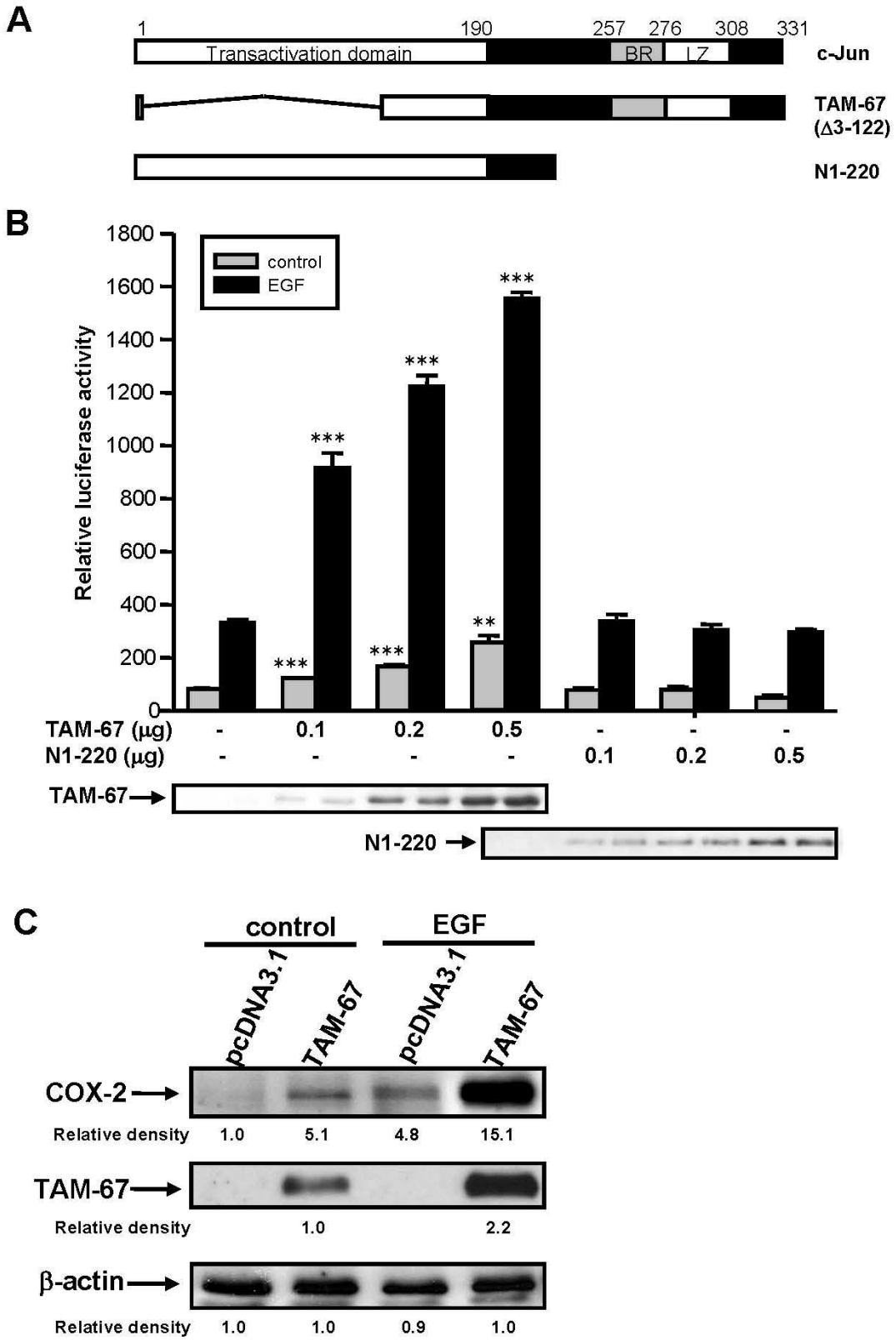

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Figure 7

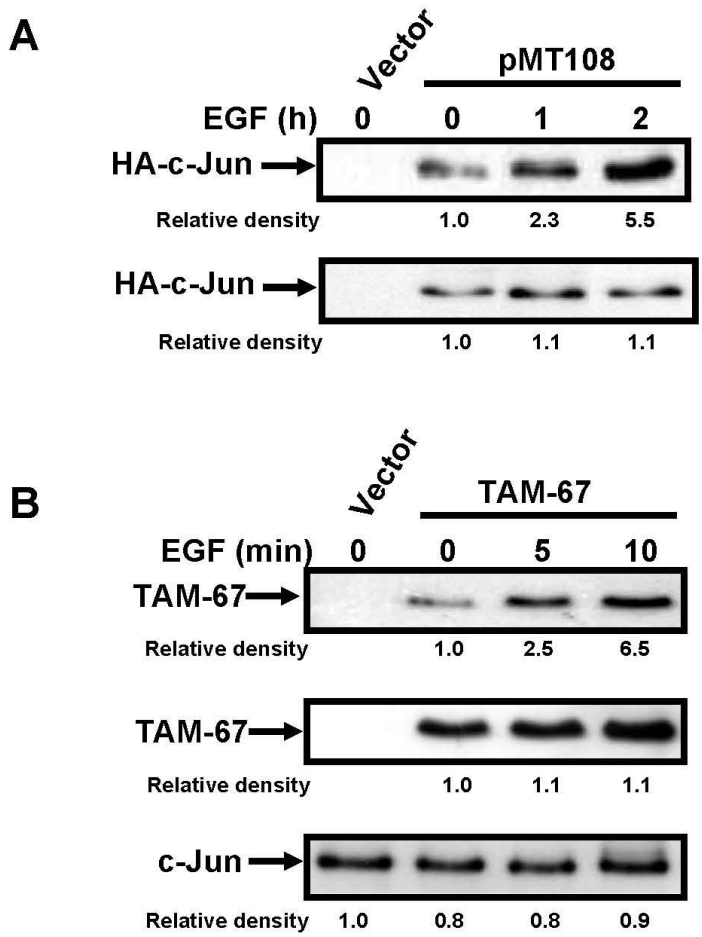

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Figure 8

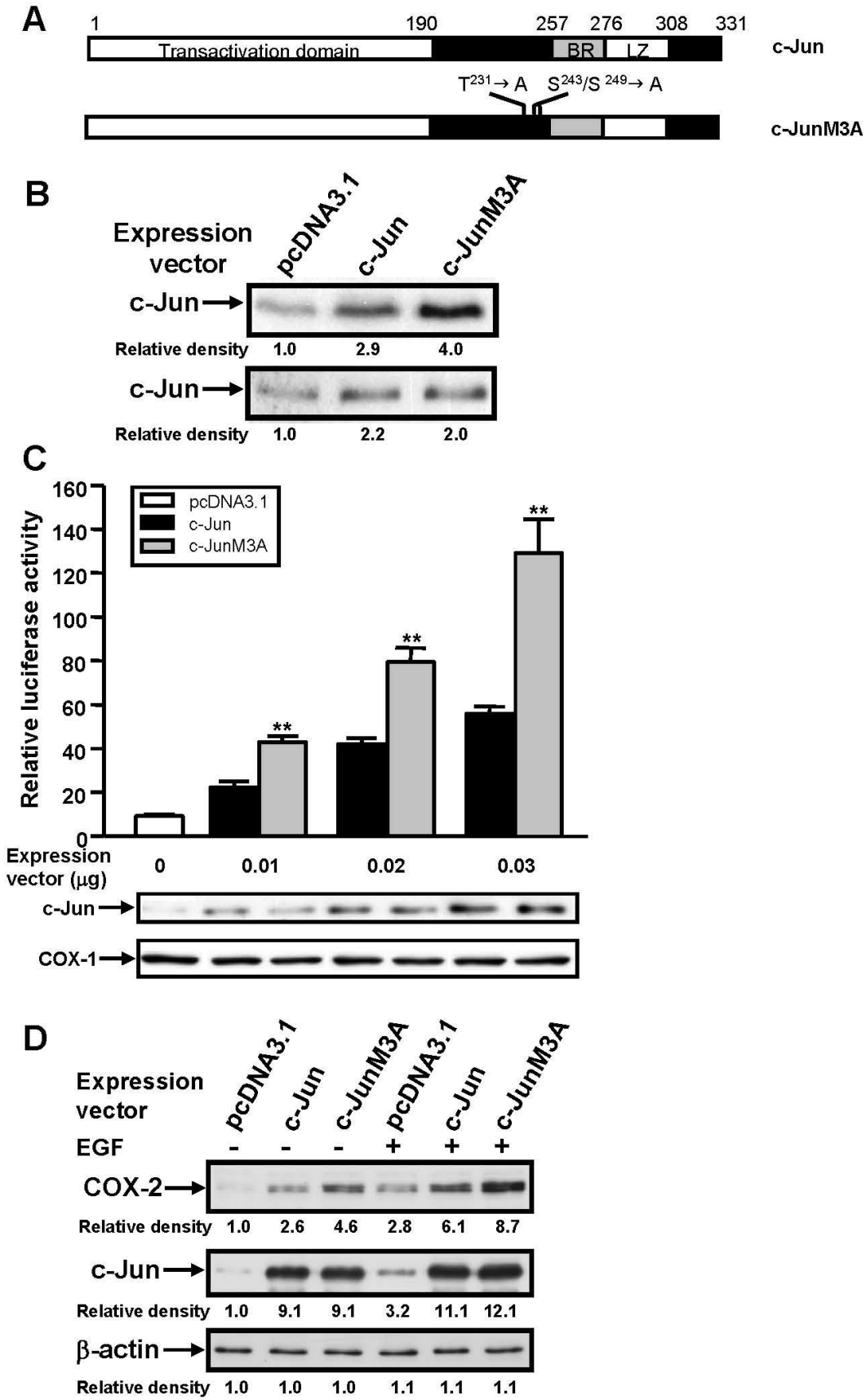

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Figure 9

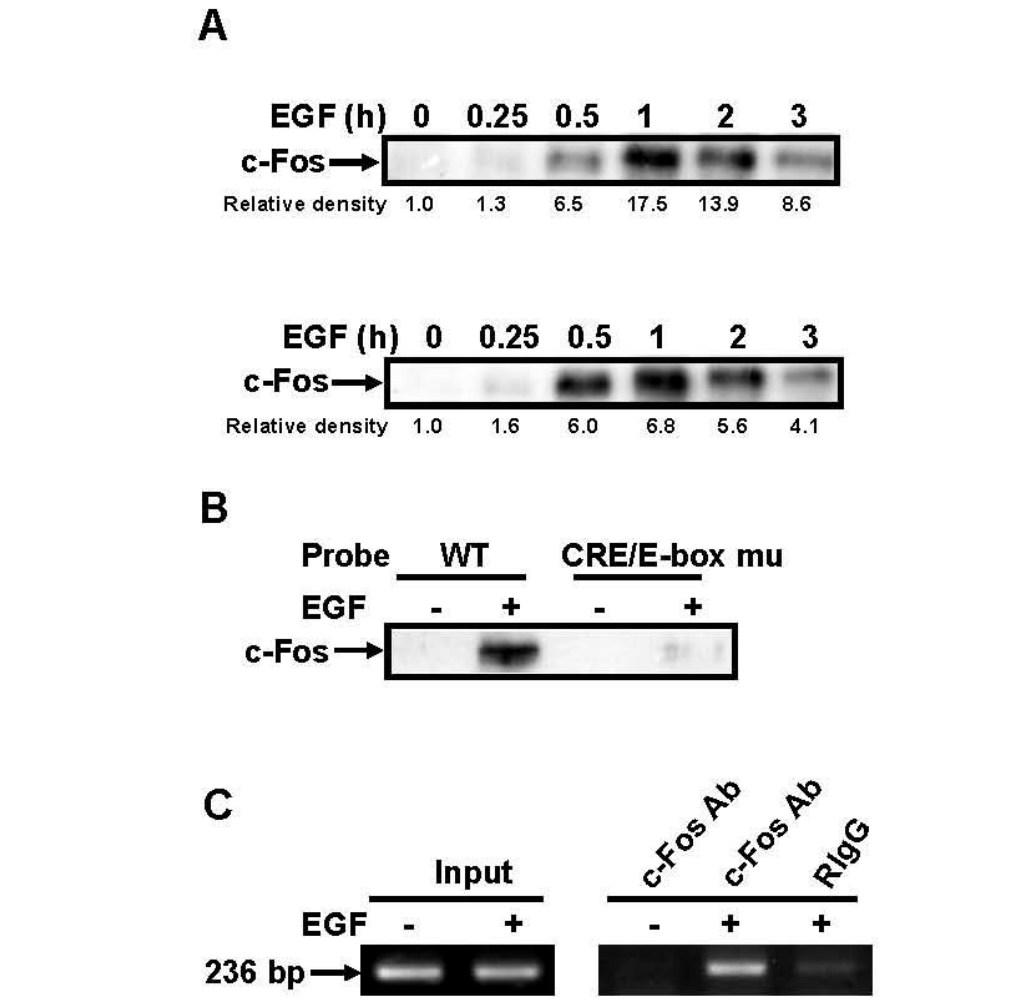

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Figure 10

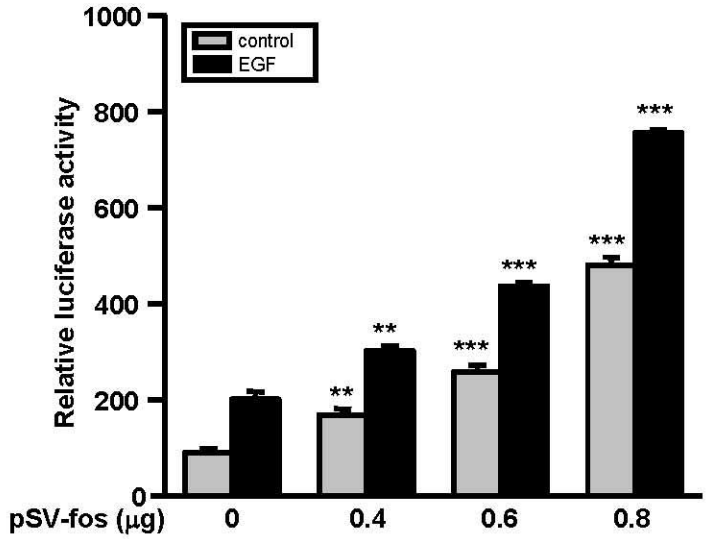

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Figure 11