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Induction of Human NF-IL6β by Epidermal Growth Factor Is Mediated through the p38 Signaling Pathway and cAMP Response Element-binding Protein Activation in A431 Cells

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The CCAAT/enhancer binding protein δ (C/EBPδ, CRP3, CELF, *NF-IL6β*) regulates gene expression and plays functional roles in many tissues, such as in acute phase response to inflammatory stimuli, adipocyte differentiation, and mammary epithelial cell growth control. In this study, we examined the expression of human C/EBPδ (*NF-IL6β*) gene by epidermal growth factor (EGF) stimulation in human epidermoid carcinoma A431 cells. *NF-IL6β* was an immediate-early gene activated by the EGF-induced signaling pathways in cells. By using 5′-serial deletion reporter analysis, we showed that the region comprising the −347 to +9 base pairs was required for EGF response of the *NF-IL6β* promoter. This region contains putative consensus binding sequences of Sp1 and cAMP response element-binding protein (CREB). The *NF-IL6β* promoter activity induced by EGF was abolished by mutating the sequence of cAMP response element or Sp1 sites in the −347/+9 base pairs region. Both in vitro and in vivo DNA binding assay revealed that the CREB binding activity was low in EGF-starved cells, whereas it was induced within 30 min after EGF treatment of A431 cells. However, no change in Sp1 binding activity was found by EGF treatment. Moreover, the phosphatidylinositol 3 (PI3)-kinase inhibitor (wortmannin) and $p38^{MAPK}$ inhibitor (SB203580) inhibited the EGF-induced CREB phosphorylation and the expression of *NF-IL6β* gene in cells. We also demonstrated that CREB was involved in regulating the *NF-IL6β* gene transcriptional activity mediated by $p38^{MAPK}$. Our results suggested that PI3-kinase/$p38^{MAPK}$/CREB pathway contributed to the EGF activation of *NF-IL6β* gene expression.

INTRODUCTION

C/EBPδ belongs to the CCAAT/enhancer binding protein family that is involved in tissue differentiation, liver regeneration, metabolism, healing, and immune response (Ramji and Foka, 2002). All of the members of the C/EBP family have a C-terminal leucine zipper domain for dimerization and a basic domain for DNA binding. Recently, six distinct C/EBP isoforms have been identified: C/EBPα, C/EBPβ (also known as NF-IL6, LAP, AGP/EBP, IL-6DBP, or NF-M), C/EBPγ (immunoglobulin [Ig]/EBP or GPE1BP), C/EBPδ, C/EBPε (CRP1), and CHOP (gadd153) (Lekstrom-Mines and Xanthopoulos, 1998). The majority of the family members recognize similar DNA sequences in their target genes, where they bind either as homodimers or heterodimers with other C/EBP family members or with other leucine zipper factors (Hsu *et al*., 1994).

C/EBPδ has been implicated in the control of adipogenesis and in mediating the acute phase response to inflammatory stimuli (Wedel and Ziegler-Heibrock, 1995; Mandrup and Lane, 1997; Tanaka *et al*., 1997). Studies of the expression of mouse C/EBPδ show that it is typically undetectable in most cell types and tissue but that it is rapidly induced by stimulators, such as interleukin (IL)-1 (Okazaki *et al*., 2002), lipopolysaccharide (LPS) (Kravchenko *et al*., 2003; Liu *et al*., 2003), interferon (IFN)-α, IFN-γ (Tengku-Muhammad *et al*., 2000), IL-6 (Kamaraju *et al*., 2004), prostacyclin (Belmonte *et al*., 2001), and tumor necrosis factor-α (Cardinaux *et al*., 2000). Moreover, it has been reported that C/EBPδ expression is involved in cell cycle control. C/EBPδ mRNA and protein levels are markedly induced in cultured mouse mammary epithelial cells during G_0 growth arrest and apoptosis initiated by serum and growth factor withdrawal (O′Rourke *et al*., 1997). It also plays an important role in inducing growth arrest of mammary epithelium cells by oncostatin M and in promoting prostate epithelial cell growth arrest and/or apoptosis after androgen withdrawal (Yang *et al*., 2001; Hutt and DeWille, 2002). In mouse embryonic fibroblasts, the lacking of C/EBPδ results in genomic instability and centrosome amplification in vitro. These results suggest that C/EBPδ may play a substantial role in tumor suppression in vivo (Hung *et al*., 2004).

Studies on the signaling pathways that regulate transcription of C/EBPδ are still limited. Species-specific autoregulation has been proposed for the regulation mechanism of the C/EBPδ gene. For example, the autoregulation of the rat C/EBPδ is through two downstream binding sites at +3350 and +3700 of the C/EBPδ gene (Yamada *et al*., 1998). In contrast, the 5′ ends of the mouse and the ovine C/EBPδ

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Abbreviations used: ChIP, chromatin immunoprecipitation; CRE, cAMP response element; CREB, cAMP response element-binding protein; EGF, epidermal growth factor; MAPK, mitogen-activated protein kinase.

gene are sufficient for autoactivation (O'Rourke *et al.*, 1999; Davies *et al.*, 2000). STAT3 and Sp1 mediated the IL-6–induced mouse C/EBPδ gene expression in hepatoma cells (Cantwell *et al.*, 1998). STAT3 also is involved in the regulation of C/EBPδ gene expression in G_0 growth-arrested mouse mammary epithelial cells (Hutt *et al.*, 2000). In preadipocytes, activation of extracellular signal-activated kinase (ERK) and CREB was shown to increase the expression of mouse C/EBPδ (Belmonte *et al.*, 2001). However, there is no report about the transcription regulation of human *C/EBPδ* (*NF-IL6β*).

CREB is a member of the leucine zipper class of cAMP-responsive element binding proteins/activation transcription factor (CREB/ATF). It responds to a variety of external signals and plays important roles in cell proliferation and differentiation (De Cesare *et al.*, 1999; Shaywitz and Greenberg, 1999). CREB requires phosphorylation at Ser133 to become active that is induced by cyclical AMP-elevating agents, mitogens, or exposure to cellular stresses (De Cesare *et al.*, 1999; Shaywitz and Greenberg, 1999). When activated by mitogenic stimuli, such as the isoforms of mitogen-activated protein kinase (MAPK)-activated protein kinase 1 (MAPKAP-K1, also called RSK) and stress-activated protein kinase phosphorylate CREB at Ser133 in vitro (Deak *et al.*, 1998; Caivano and Cohen, 2000; Wiggin *et al.*, 2002). Recent evidence indicates that $p90^{rsk}$ may be responsible for CREB phosphorylation at Ser133, both in vitro and in vivo, in response to growth factor stimulation (Böhm *et al.*, 1995; Xing *et al.*, 1996; Monaco and Sassone-Corsi, 1997). Also, the stress-induced phosphorylation of CREB is prevented by SB203580, an inhibitor of another MAPK family member including stress-activated protein kinase 2 (SAPK2) and $p38^{MAPK}$, which is a component of a distinct signal transduction pathway (Deak *et al.*, 1998).

The epidermal growth factor (EGF) receptor (EGFR) is a 170-kDa transmembrane glycoprotein with intrinsic tyrosine kinase activity (Carpenter, 1987; Hunter and Cooper, 1985). On ligand binding, the EGFR undergoes autophosphorylation and initiates multiple intracellular signaling cascades, leading to the induction of cell growth (Hill and Treisman, 1995; Treisman, 1996). EGFR activation also induces other signaling pathways that turn off EGFR signaling through endocytosis. Attenuation of the signaling is important for the control of EGFR mitogenic properties (Wiley *et al.*, 1991; Sorkin and Waters, 1993). Many effector molecules were reported to be involved in the EGF signal cascades, including phospholipase C-γ1, Ras-mitogen-activated protein kinase kinase (MEK)-MAPK, phosphatidylinositol 3 (PI3)-kinase, Akt, Src, and STATs (Olayioye *et al.*, 2000; Yarden and Sliwkowski, 2001). Whereas EGF is a potent mitogen, it paradoxically induces apoptosis in cells that overexpress EGFR such as the human epidermoid carcinoma A431 cells (Haigler *et al.*, 1978). Its growth in monolayer culture had been shown to exhibit a biphasic response to EGF. A431 cells are weakly stimulated by picomolar concentrations of ligand but exhibit a marked inhibition of proliferation in the presence of nanomolar concentration of EGF (Barnes, 1982; Kawamoto *et al.*, 1984).

In this study, we demonstrated that EGF up-regulated the transcriptional activity of *NF-IL6β* gene under growth arrest condition in A431 cells. We further showed that CREB and Sp1 bound to the cAMP response element (CRE) and Sp1 sites of *NF-IL6β* gene promoter region, respectively. EGF induced the phosphorylation of $p38^{MAPK}$ in A431 cells. The activation of $p38^{MAPK}$ and CREB was involved in EGF-activated PI3-kinase signaling pathway. Pretreatment with p38 inhibitor SB203580 reduced both CREB phosphorylation in Ser133 and the transcriptional product of *NF-IL6β* gene after EGF stimulation. These results suggested that the EGF-induced *NF-IL6β* gene expression was mediated through the $p38^{MAPK}$ signaling pathway and CREB activation.

MATERIALS AND METHODS

Materials

Human EGF was purchased from Peprotech (Rocky Hill, NJ). All the chemical inhibitors, wortmannin, SB203580, PD98059, SP200126, anisomycin, and cycloheximide were obtained from Calbiochem (San Diego, CA). Antibodies against CREB, Sp1, Sp3, COX-1, NF-IL6β, and p38 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Phosphorylated CREB and p38 antibodies were purchased from Cell Signaling Technology (Beverly, MA). Lipofectamine 2000, Dulbecco's modified Eagle's medium, and Opti-MEM medium were obtained from Invitrogen (Carlsbad, CA). Fetal bovine serum (FBS) was purchased from Hyclone Laboratories (Logan, UT). All other reagents used were of the highest purity obtainable.

Methods: Cell Culture and Transfection

Human epidermoid carcinoma cell line, A431 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% FBS, 100 μg/ml streptomycin, and 100 units/ml penicillin. Transient transfection into A431 cells was carried out using Lipofectamine 2000 transfection reagent. For experiments with chemical inhibitors, the following concentrations and compounds were used: 0.1 μM wortmannin, 10 μM SB203580, 20 μM PD98059, 50 nM SP200126, 50 ng/ml anisomycin, and 10 μg/ml cycloheximide. All inhibitors were added 30 min before the 50 ng/ml EGF treatment of cells.

Western Blotting

An analytical 10% SDS-polyacrylamide slab gel electrophoresis was performed. The cell lysates were prepared from control and EGF-treated cells, and 70 μg of protein of each was analyzed. For immunoblotting, proteins in the SDS gels were transferred to a polyvinylidene difluoride membrane by an electroblot apparatus. Immunoblot analysis was carried out with goat or rabbit IgG antibody coupled to horseradish peroxidase. An enhanced chemiluminescence kit (Amersham Biosciences, Piscataway, NJ) was used for detection.

Reporter Plasmids and Luciferase Assay

Cells were transfected with plasmids by Lipofectamine 2000 according to the manufacturer's instruction. The longest promoter fragment was cloned by PCR of genomic DNA from strain 129 mice or A431 cells, and inserted into the TA cloning vector. The primers used are as follows: C/EBPδ-2015, 5′-CCAAGTCTGACAGTGCTCTCTG; C/EBPδ+14; 5′-GGGAAGCTTCCTG-GCGTCCA AGTTGGCTG-3′; NF-IL6β-1717; 5′-GGGGTACCTTGGTGTTC-CGACGCAGATC-3′; and NF-IL6β+9; 5′-GGGAAGCTTGGCTGTCAC-CTCGCTGGGCC-3′. The luciferase reporter plasmids containing various fragments of mouse *C/EBPδ* promoter (C/EBPδ-1680/+14, C/EBPδ-1268/+14, C/EBPδ-1065/+14, and C/EBPδ-275/+14) were generated from the C/EBPδ-2015 by digestion with various restriction enzymes, *Stu*I, *Pml*I, *Ssp*I, or *Sma*I. The shorter fragment of *NF-IL6β* promoter, NF-IL6β-347/+9 was generated by PCR with primers NF-IL6β+9 and NF-IL6β-347, 5′-GGGGTAC-CGAGGAGGTTCCAAGCCCAC-3′, and digested with *Kpn*I and *Hind*III. These treated fragments were subcloned into the multicloning sites of the promoterless vector pGL2-Basic (Promega, Madison, WI). The numbers in parentheses indicate the nucleotide position with respect to the transcriptional initiation site. Other site-directed mutagenesis reporters of the NF-IL6β promoter (NF-IL6β-347/+9mC, NF-IL6β-347/+9mSp1-1 NF-IL6β-347/+9mSp1-2, NF-IL6β-347/+9mCmSp1-1, NF-IL6β-347/+9mdSp1, NF-IL6β-347/+9mCmSp1-2, NF-IL6β-347/+9mCmdSp1) were derived from NF-IL6β-347/+9. Plasmid pGL2-NF-IL6β1xCRE was derived by inserting one copy of the DNA fragment containing CRE site (sequence as below NF-IL6βCRE) into the *Sma*I site of the pGL2-Promoter vector (Promega). Transfected cells were seeded in growth medium with or without EGF. Sixteen hours after transfection, cells were harvested and assayed for luciferase activity. Luciferase activities were normalized with the amount of protein in the cell lysate.

Reverse Transcription (RT)-PCR

A431 cells were maintained for 6 h in serum-free medium and restimulated with EGF under various time courses and chemical compounds treatment. Total RNA was isolated using the TRIzol RNA extraction kit, and 1 μg of RNA was subjected to RT-PCR with SuperScriptII. The NF-IL6β–specific primers 5′-AGCGCAACAACATCGCCGTG-3′ and 5′-GTCGGGTCTGAGG-TATGGGTC-3′ were used for analysis. The β-actin primers sense strain, 5′-CCCAAGGCCAACCGCGAGAAG-3′ and antisense strain, 5′-TCTTCAT-TGTGCTGGGTGCCA-3′ were used as controls. The PCR products were separated by 1% agarose-gel electrophoresis and visualized with ethidium bromide staining.

Nuclear Extract and Gel Shift Assays

Gel shift assays were carried out essentially as described previously (Kawamoto *et al*., 1984). Briefly, the ^{32}P-labeled oligonucleotide probes (−0.2 to 0.5 ng) containing the CRE, Sp1-1, or Sp1-2 site were incubated with 8 μg of nuclear extracts or 1 μl of in vitro-translated CREB in the specific binding buffer, as described below, containing 1 μg of poly(dI-dC). After 20 min of incubation at room temperature, the reaction mixtures were resolved in a 5% native polyacrylamide gel (acrylamide/bisacrylamide ratio, 30:1) at 4°C, and the specific protein complexes were visualized by autoradiography. The CRE binding buffer contained 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM dithiothreitol (DTT), 1 mM EDTA, and 10% (vol/vol) glycerol. The Sp1 binding buffer contained 20 mM HEPES, pH 7.9, 0.1 mM KCl, 2 mM $MgCl_2$, 15 mM NaCl, 0.2 mM EDTA, 5 mM DTT, 10% (vol/vol) glycerol, and 2% (wt/vol) polyvinyl alcohol. For antibody supershifting experiments, 1 μg of various indicated antibodies, such as α-CREB, α-Sp1, and α-Sp3 or control rabbit IgG was included in the binding reaction mixture. For competition experiments, a 100-fold molar excess of unlabeled wild-type or mutant oligonucleotides was included in the binding reaction mixture. The sense strand sequences of various oligonucleotides used are as follows: NF-IL6βCRE (hCRE), 5′-GGGGCGTGCACGTCAGCCGGG-3′; NF-IL6βmCRE (muthCRE), 5′-GGGGCGTGGATCCCAGCCGGG-3′; NF-IL6βSp1-1, 5′-AAGGCTCGGG-GCGGCTCCGGGG-3′; NF-IL6βSp1-2, 5′-CCGGAGTCGGGGCGGGGCGT-GC-3′; mouse C/EBPδCRE (mCRE), 5′-GGGGCGTGCGCGTCAGCTGGG-3′; and consensus Sp1 oligonucleotide (cSp1), 5′-ATTC GATCGGGGCGGGGC-GAGC-3′.

Chromatin Immunoprecipitation Assay

The chromatin immunoprecipitation (ChIP) assay was carried out essentially as described previously (Wang *et al*., 2003) with a minor modification. 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 300–400 base pairs before be immunoprecipitated with antibody specific to CREB, pCREB, and Sp1 or control rabbit IgG at 4°C for overnight. After reversal of cross-linking, the immunoprecipitated chromatin was amplified by PCR with various sets of primers as indicated. The amplified DNA products were resolved by agarose gel electrophoresis and confirmed by sequencing. For PCR amplification of specific regions of the *NF-IL6β* genomic locus, the NF-IL6β-347 and NF-IL6β+9 were used.

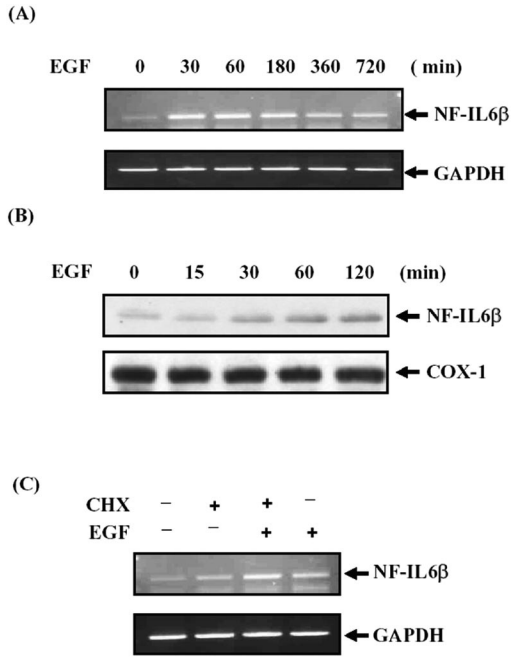

Figure 1. NF-IL6β production was increased by EGF in A431 cells. (A and B) A431 cells were starved for 6 h and then restimulated with 50 ng/ml EGF. Total RNA or cell lysates were harvested for different time points as indicated for examining by RT-PCR and Western blotting analysis of *NF-IL6β* mRNA and protein level, respectively. (C) Effect of cycloheximide treatment on the transcription of *NF-IL6β* mRNAs. The A431 cells were starved for 6 h and then pretreated with cycloheximide for 20 min before addition of EGF. Total RNA was isolated after 1 h of EGF treatment for RT-PCR assay.

RESULTS

Induction of NF-IL6β Expression by EGF Treatment

Treatment of A431 cells with EGF resulted in a rapid increase in the steady-state level of *NF-IL6β* mRNA without effect on β-actin mRNA level (Figure 1A). *NF-IL6β* mRNA induction was detectable in 30 min after EGF treatment and was maximal after 2 h with an increase of four- to fivefold. To assess whether NF-IL6β protein expression also was correlated with transcriptional regulation by EGF, Western blotting with NF-IL6β–specific antibodies was performed. The EGF-induced NF-IL6β protein expression was elevated in 30 min and sustained up to 2 h (Figure 1B). The induction of NF-IL6β protein expression was correlated with that of NF-IL6β mRNA increasing as shown in Figure 1A. From Figure 1C, treatment with cycloheximide did not affect the induction of *NF-IL6β* mRNA by EGF. It indicated that de novo protein synthesis was not required for the EGF response.

Identification of the EGF Responsive Element in NF-IL6β Promoter Region

By comparing with the proximal region sequences of human, mouse, and rat *C/EBPδ* promoters, several conserved transcription factor binding sites, including Sp1 and CRE motifs were found. However, no acute phase response element (APRE) exists in the human *NF-IL6β* promoter (Figure 2A). As shown in Figure 2B, transient transfection with full-length mouse *C/EBPδ* promoter, −1680/+14 base pairs, or human *NF-IL6β* promoter, −1717/+9 base pairs, resulted in an average of 4.5-fold increase in luciferase activity upon EGF treatment. These results demonstrated that the 5′-flanking regions of the *NF-IL6β* gene ranging from −1717 to +9 base pairs and the C/EBPδ gene range from −1680 to +14 base pairs provided inducibility for EGF response. To further determine the EGF responsive element in them, the reporters containing 5′-serial deletion of *NF-IL6β* and *C/EBPδ* promoter were performed. Both 5′-serial deletion of promoter regions of mouse *C/EBPδ*, −275/+14 base pairs, and human *NF-IL6β*, −347/+9 base pairs showed the same EGF inducibility as the individual full-length reporter. It suggested that the proximal regions near the transcriptional initiation sites were important for EGF activity in A431 cells.

It was previously reported that IL-6-induced mouse *C/EBPδ* transcription is through the APRE and Sp1 motifs (Okazaki *et al*., 2002). To clarify the function of Sp1/APRE region in human *NF-IL6β* promoter, DNA gel-shift assay using the predicted APRE-like oligonucleotide 5′-GGCTC-CGGGGGGCTCCCAGGGCG-3′ of human *NF-IL6β* promoter was performed. However, no slow mobility shifting pattern was observed (our unpublished data). An APRE-like oligonucleotide was inserted into the pGL2 Promoter vector containing a simian virus 40 (SV40) promoter. The luciferase reporter results indicated that the human APRE-like site and mouse APRE site did not confer EGF activity on the SV40 promoter in A431 cells (Figure 2C). However, EGF could induce the promoter activity of the heterologous reporter containing CRE site (Figure 2D). These results suggested that the CRE site, but not the APRE site, contributed to the EGF function in A431 cells.

To identify the responsive motifs involved in EGF activation of the *NF-IL6β* promoter, a series of reporters with mutations as illustrated in Figure 2E were constructed. NF-IL6βmSp1-1 and NF-IL6βmSp1-2 with the individually mutated Sp1 site resulted in a more significant attenuation of

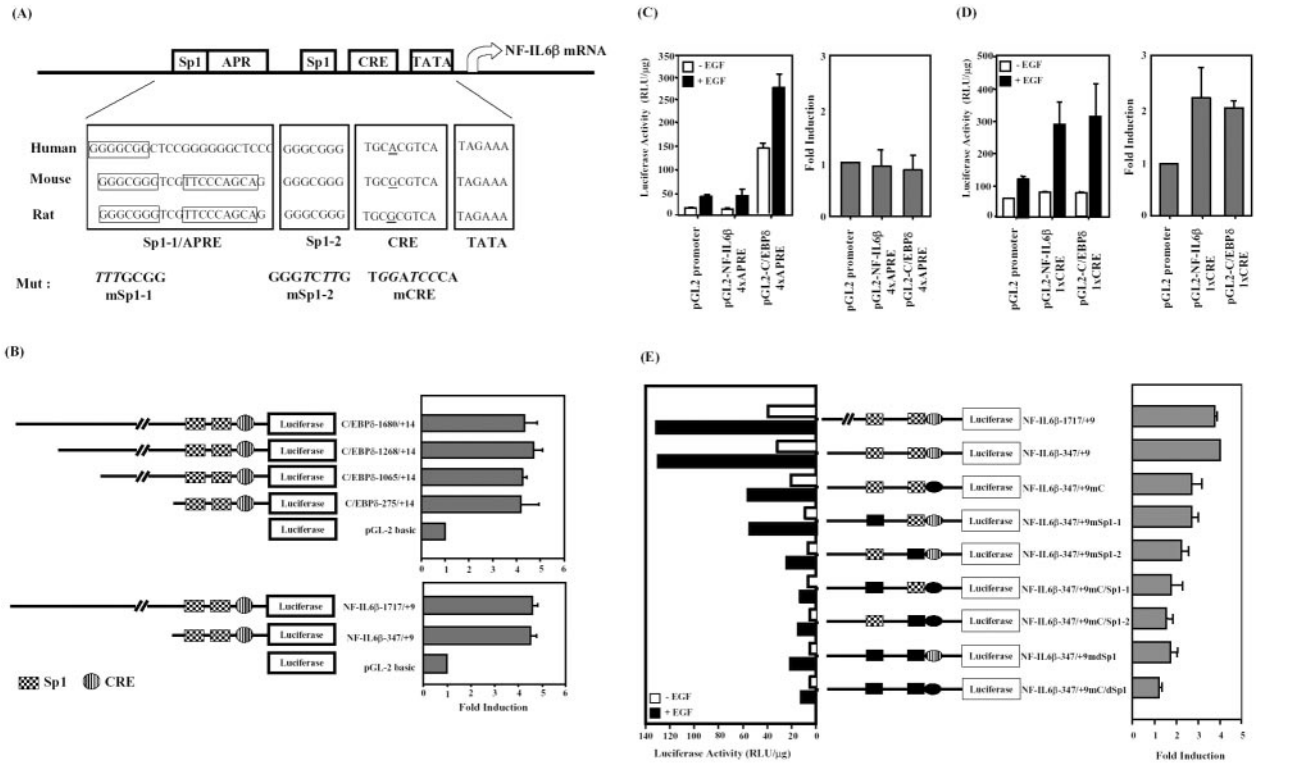

Figure 2. Mapping of promoter elements required for EGF activation of the NF-IL6β reporter gene. (A) Putative conserved sites alignment of promoter sequences of human, mouse and rat *C/EBP*δ genes. The consensus sequences of the Sp1/APRE, CRE, and TATA sites are blocked. The mutated sequence in each individual construct is shown in italics and bold. (B) Activation of the *C/EBP*δ and *NF-IL6*β promoter by EGF in A431 cells. A431 cells transfected with various luciferase reporters as indicated were stimulated with or without EGF. The average fold inductions by EGF for each construct are shown. The minimal EGF responsive regions of *C/EBP*δ and *NF-IL6*β gene were represented by bold. (C and D) Characterization of heterologous reporters containing APRE or CRE elements contributed to EGF response. The heterologous reporters were transiently transfected to A431 cells and treated with or without EGF for 15 h. (E) Functional roles of binding sites for Sp1- or CREB-related transcriptional factors in the *NF-IL6*β promoter activity. A431 cells transfected with various luciferase reporters as indicated were stimulated with or without EGF, and the lysates were analyzed for luciferase activity (left). Similar results were obtained from three independent experiments, and the data shown here were from one representative assay. The average fold induction (mean $\pm$ SD, n = 3) by EGF for each construct was analyzed (right).

the basal promoter activity than NF-IL6βmCRE with the mutated CRE site. It suggested that the Sp1 sites were critical for the basal promoter activity. Furthermore, the single site mutants of Sp1-1, Sp1-2, or CRE site (NF-IL6β-347/+9mSp1-1, NF-IL6β-347/+9mSp1-2, or NF–IL6β-347/+9mCRE) diminished the EGF induction of the promoter activity by 30–40% respectively, whereas the triple mutant, NF-IL6β-347/+9mC/dSp1 with two Sp1 sites and a CRE site, resulted in a complete elimination of EGF response. These results indicated that the Sp1-1, the Sp1-2, and the CRE motifs indeed played important roles in the EGF induction and the basal activity of *NF-IL6*β promoter.

Binding of CREB to NF-IL6β *Promoter*

To further identify the transcription factors bound to the CRE site of *NF-IL6*β promoter, gel shift assays with nuclear extracts prepared from EGF-treated A431 cells were performed. As shown in Figure 3A, CRE binding activity was low in cells deprived of EGF (lane 1) and was rapidly induced within 30 min after EGF stimulation (lanes 2 and 3) and decreased in 60 min (lane 4). The CRE motif of mouse *C/EBP*δ promoter (mCRE), specific for CREB/ATF-1 binding (Belmonte *et al*., 2001), was used as a competitor. The retarded band was competed out by 100-fold mCRE (lane 5), suggesting CREB/ATF-1 could be the binding protein of CRE motif. To determine the possible CRE-binding protein, the EGF-induced CRE-binding complex was examined by antibodies recognized CREB or ATF-2 in gel-shift assay. The α-CREB antibodies completely shifted and blocked the EGF-induced CRE-binding complex (lane 8), but α-ATF-2 antibodies did not (lane 9). Additionally, the CREB protein, synthesized in vitro by the TNT-coupled reticulocyte lysate system (Promega), bound specifically to the CRE probe (Figure 3B, lane 2). Moreover, addition of the CREB-specific antibodies, but not the control rabbit IgG shifted the specific CREB/CRE-binding complex to a higher molecular weight region (Figure 3B, compare lane 3 with lane 4). Excess human NF-IL6β CRE (hCRE, Figure 3B, lane 5) and mCRE (Figure 3B, lane 6) oligonucleotides competed with CREB for the formation of CREB/CRE binding complex, but mutant hCRE (muthCRE) oligonucleotide did not (Figure 3B, lane 7). These results indicated that the CREB protein bound to the CRE binding element in the human *NF-IL6*β promoter.

Involvement of Sp1 in EGF Stimulation of NF-IL6β *Gene Transcription through the Sp1 Motifs*

To determine whether Sp1 proteins bound to both of the Sp1 sites, gel-shift assay was carried out with probes containing individual Sp1 site and nuclear extracts from cells treated with EGF as indicated (Figure 4A). The binding pattern of

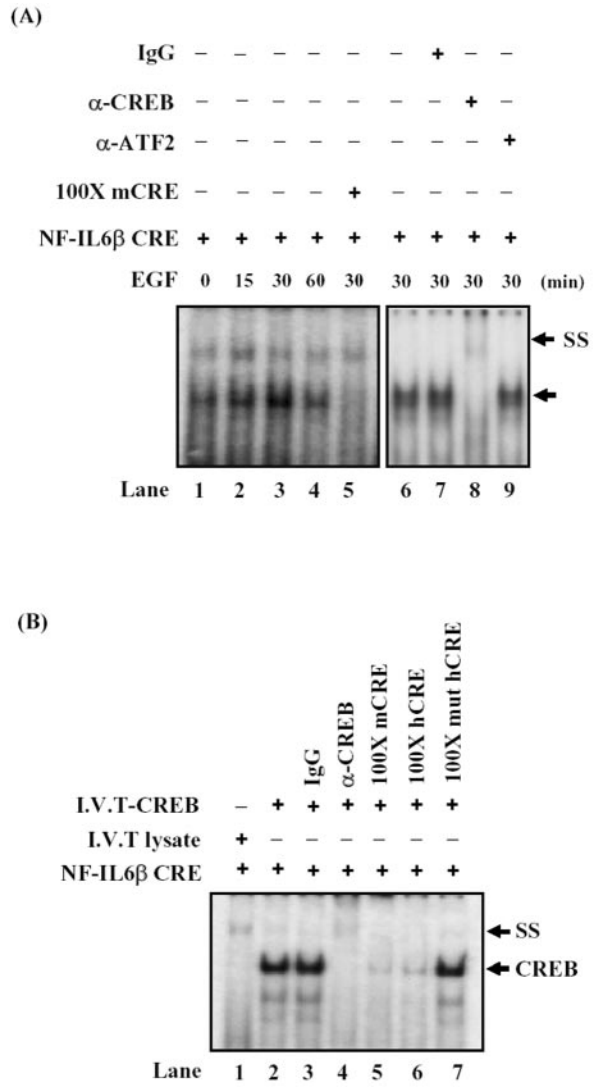

Figure 3. CREB was one component of the CRE-binding complex and directly bound to *NF-IL6β* promoter in vitro. (A) In lanes 1–4, nuclear extracts from A431 cells deprived of serum and restimulated with EGF for various time courses were incubated with the ^{32}P-labeled CRE element of *NF-IL6β* gene. In lane 5, competition analysis was carried out by the addition of an 100-fold molar excess of unlabeled mCRE oligonucleotides. In lane 6, no antibody was present in the binding reaction. In lanes 7, 8 and 9, binding assays were performed with control, CREB, or ATF2 specific antibody, respectively. The solid arrow indicates the specific DNA-protein complex, and the "SS" arrow indicates the band supershifted by the antibody. (B) The CREB protein synthesized in vitro by the TNT-coupled reticulocyte lysate system was allowed to bind to the CRE probe (lane 2). Incubation with a control rabbit IgG (lane 3), purified rabbit anti-CREB antibodies (lane 4), excess unlabeled mCRE (lane 5), hCRE (wt, lane 6) or hCRE-mutant (muthCRE, lane 7), were performed respectively, and then resolved on the nondenaturing gel. "CREB" represents the CREB retard shifting, and "SS" indicates the supershifted band by antibodies.

the Sp1 sites was different from that of the CRE sites. Both of the Sp1-1 and Sp1-2 binding activities were already near maximum in serum starvation cells and were not further induced by EGF treatment, as shown in Figure 4A (lanes 1–4 and lanes 9–12). To examine the binding specificity of Sp1 on Sp1-1 and Sp1-2 motifs, the excess oligonucleotides competition assays were performed using commercially available consensus Sp1 (cSp1) and wild-type (Wt) oligonucleotides. Both Sp1-1– and Sp1-2–binding complexes were completely abolished by the cSp1 oligonucleotides (Figure 4A, lanes 5 and 13) or the Wt oligonucleotides (Figure 4A, lanes 6 and 14). A supershift pattern also was observed when antibodies against human Sp1 were added to the mixture of Sp1 probes and nuclear extracts (Figure 4A, lanes 7 and 15); however, the α-Sp3 antibodies did not (Figure 4A, lanes 8 and 16). These results suggested that the Sp1 sites were specifically for Sp1 binding but not for Sp3. To study the functional role of Sp1 with or without EGF treatment, reporter gene assay was performed. Cells were cotransfected with *NF-IL6β* reporter genes together with either a control vector, pCDNA3, or a vector expressing Sp1 as shown in Figure 4B. Overexpression of Sp1 could transactivate the reporter activity of NF-IL6β-347/+9 but not NF-IL6β-347/+9mdSp1 without EGF stimulation (lanes 3 and 7). Although the reporter activities of both constructs were enhanced under EGF stimulation (compare lanes 1 and 2 with lanes 5 and 6, respectively). A further increase in reporter activity due to the overexpression of Sp1 was observed only in cells transfected with NF-IL6β-347/+9 reporter (compare lane 2 with lane 4), but not in cells transfected with NF-IL6β-347/+9mdSp1 reporter (compare lane 6 with lane 8). These results suggested that the two Sp1 motifs on the essential promoter region played important roles in regulating EGF-induced gene expression of NF-IL6β.

Binding of CREB and Sp1 to NF-IL6β Promoter In Vivo

To confirm the results of the in vitro-DNA binding assay, binding of CREB and Sp1 to the *NF-IL6β* gene promoter in vivo was examined by using the ChIP assay. The primers NF-IL6β-347 and NF-IL6β+9 were used to specifically amplify the promoter region containing CRE, Sp1-1 and Sp1-2 motifs of the *NF-IL6β* gene locus as illustrated in Figure 5A. The predicted size of PCR fragment was confirmed by agarose gel electrophoresis, which was further characterized by DNA sequencing. As shown in Figure 5B, CREB and Sp1 bound to the promoter region of *NF-IL6β* gene in control cells (lanes 10 and 13). On EGF treatment, antibodies recognizing the active form of the CREB (pCREB) or the CREB protein specifically coprecipitated with the fragment of *NF-IL6β* promoter in an EGF inducible manner (lanes 8 and 11). This inducible binding pattern of CREB was also observed in the IL-3–stimulated transcriptional regulation of *mcl-1* gene (Wang *et al*., 2003). Moreover, Sp1 constitutively bound to the promoter region of *NF-IL6β* gene. These results were consistent with the above-mentioned observation of gel-shift assay (Figure 4A).

Role of p38^{MAPK} and PI3-Kinase Signal Transduction Pathway in NF-IL6β Regulation

EGF interacts with EGFR to result in receptor autophosphorylation and initiates multiple intracellular signaling cascades, leading to the induction of cell growth (Hill and Treisman, 1995; Treisman, 1996). PI3-kinase and MAPKs pathways have been reported to play an important role in EGF signaling in A431 cells (Soltoff *et al*., 1994; Matthew and Jan, 2001; Chen *et al*., 2002). To determine the possible involvement of signaling pathways in EGF induction of *NF-IL6β* mRNA, the pharmacological inhibitors of signal transduction components were used to study the EGF-induced *NF-IL6β* regulation. The effect of various specific MAPK inhibitors, SP600125 (JNK inhibitor), SB203580 (p38 inhibitor), and PD98059 (MEK1 inhibitor), on *NF-IL6β* mRNA expression was studied. SB203580, a specific inhibitor of

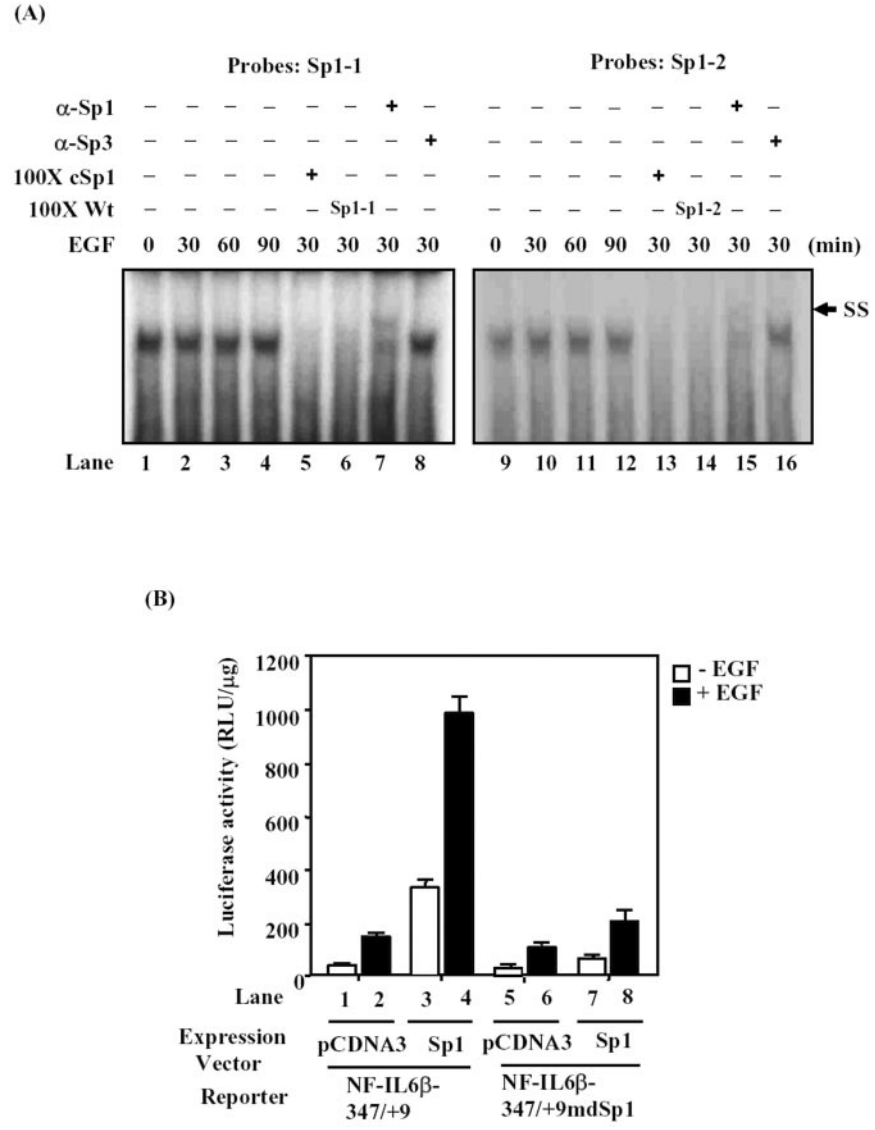

Figure 4. Sp1 was one component of the Sp1 binding complexes and played a role in the EGF stimulation of the NF-IL6β reporter. (A) Left and right, respectively, show that DNA binding pattern of Sp1-1 and Sp1-2 oligonucleotides. Lanes 1–4 and lanes 9–10 indicate the time course lysates extracted from EGF-restimulated A431 cells. The commercialized consensus Sp1 (cSp1, lanes 5 and 13) and wild-type oligonucleotides (Sp1-1 or Sp1-2, lanes 6 or 14) were, respectively, preincubated with nuclear extract for competition assay. The retard probe signals were determined with specific α-Sp1 (lanes 7 and 15) and α-Sp-3 (lanes 8 and 16) antibodies, and "SS" represents the supershifting band. (B) A431 cells were transfected with NF-IL6β-347/+9 (lanes 1–4) or containing Sp1 sites mutagenic reporter, NF-IL6β-347/+9mdSp1 (lanes 5–8), with a control or Sp1 expression vector, as indicated. The transfectants were stimulated with or without EGF for 12 h before the cell lysates were prepared and analyzed for luciferase activity.

p38$^{MAPK}\alpha$- and β-isoforms, apparently inhibited EGF-induced *NF-IL6β* transcriptional activity (Figure 6A, compare lane 2 with lane 4); however, no effects were found upon SP600125 (lane 3) or PD98059 (lane 5) treatment.

Overexpression of p110CAAX, a constitutively activated PI3-kinase, as well as insulin induces mRNA expression and nuclear expression of C/EBPβ and C/EBPδ in vascular smooth muscle cells (Sekine *et al*., 2002). To clarify the possible contribution of PI3-kinase to the human *NF-IL6β* gene regulation in A431 cells, the effect of PI3-kinase inhibitor wortmannin was studied. Pretreatment of cells with 100 nM wortmannin attenuated the effect on EGF induction of *NF-IL6β* mRNA (Figure 6B, compare lane 2 with lane 3). These results indicated that PI3-kinase and p38^{MAPK} were involved in EGF-induced NF-IL6β transcription. For investigating the signal pathway transduced by these two kinases on NF-IL6β transcription, combined treatment with wortmannin and SB203580 were performed, and it did not result in any synergistic inhibition of EGF-induced transcription of *NF-IL6β* mRNA (Figure 6B, compare lane 4 and lane 5). A similar inhibition phenomenon was observed at the NF-IL6β protein level (Figure 6C). These results strongly suggested that PI3-kinase and p38^{MAPK} activation was on the same signaling pathway to regulate NF-IL6β transcription under EGF-stimulation in A431 cells.

In rat osteosarcoma UMR cells, EGF activates members of the MAPK family, including p38^{MAPK} and ERKs. Treatment of cells with either SB203580 or PD98059 prevents phosphorylation of CREB at Ser133 induced by EGF (Swarthout *et al*., 2002). Because CREB bound to *NF-IL6β* promoter was demonstrated in our system, whether the p38^{MAPK} phosphorylated the CREB was conducted. By detecting with antibodies against phosphorylated Ser133 CREB, a rapid induction of the phosphorylation of Ser133 on CREB in A431 cells was found (Figure 7A, lanes 1–4). Under similar experimental condition, the EGF-induced phosphorylation of CREB was prevented by pretreatment of SB203580 (Figure 7A, lanes 5 and 6). It suggested that p38^{MAPK} was an activator mediating the EGF-induced CREB activation. To further confirm this observation, treatment with anisomycin, p38 ^{MAPK} activator, in A431 cells was carried out. Induction of Ser-133 phosphorylation of CREB by anisomycin was observed (our unpublished data). Together, these results suggested that

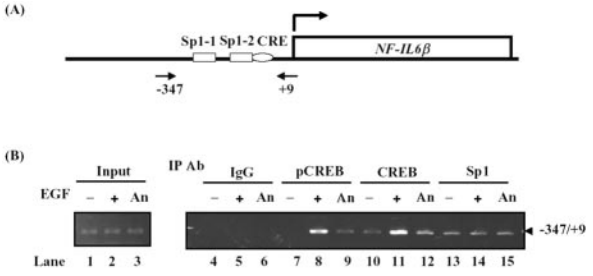

Figure 5. CREB and Sp1 bound to the *NF-IL6β* gene promoter in vivo. (A) Schematic representation of the *NF-IL6β* genomic locus spanning the promoter region. Primer NF-IL6β+9 and primer NF-IL6β-347 were generated for PCR reaction. (B) ChIP analysis of CREB or Sp1 binding to the *NF-IL6β* gene locus. Sheared formaldehyde cross-linked chromatin was immunoprecipitated with antibodies as indicated and processed for PCR amplification. As a positive control, PCR amplification also was carried out with input DNA from chromatin before the immunoprecipitation step (lanes 1, 2, and 3). The chromatin was isolated from cells with (lanes 5, 8, 11, and 14) and without (lanes 4, 7, 10, and 13) EGF treatments, or anisomycin treatments (lanes 6, 9, 12, and 15) and the immunoprecipitation (IP) step was performed with various antibodies (IP antibody), as indicated. The "−347/+9" indicates the PCR products after specific primers amplification with purified templates from specific antibody-IP step.

p38^{MAPK} played a functional role in CREB activation in EGF-stimulated A431 cells.

To further determine the relationship between PI3-kinase, P38^{MAPK}, and CREB, the chemical inhibition assay was performed by Western blotting assay. A431 cells were preincubated with or without wortmannin and then stimulated with EGF for various time courses as indicated in Figure 7B. Phosphorylation of p38^{MAPK} and CREB Ser133 was partially inhibited by wortmannin in the same time courses (compare lanes 2 with 5, lanes 3 with 6 and lanes 4 with 7). These results suggested that PI3-kinase was an upstream, but just partial, activator in the EGF-induced activation of p38^{MAPK} and CREB, and p38^{MAPK} was a major CREB Ser133 activator in A431 cells.

To examine whether the −347/+9 fragment of *NF-IL6β* promoter is required for PI3-kinase and p38^{MAPK} activation, the NF-IL6β-347/+9 was cotransfected with different expression vectors, including wild-type expression vectors of CREB; p38α; two constitutive activation forms of p38^{MAPK} upstream activators, MKK3Ac and MKK6Ac; and p110*, constitutive activation form of PI3-kinase, under the condition without EGF treatment. As shown in Figure 8A, both of the p38-mimic activators MKK3Ac and MKK6Ac increased the transcriptional activity of NF-IL6β-347/+9 (lanes 4 and 5), and p110* also contributed the same effect (lane 6). Furthermore, a dominant negative mutant of p38α (DN-p38α) attenuated the transcriptional activity enhanced by p110* (Figure 8B). These results strongly suggested that PI3-kinase/p38^{MAPK} signaling was involved in the transcriptional activation of human *NF-IL6β* promoter.

Mediation of the p38^{MAPK} Signal through CREB in Activation of Human NF-IL6β Promoter Activity

To further confirm whether CREB was a downstream target of p38^{MAPK} and p38^{MAPK}-regulated phosphorylation of CREB Ser133 contributes to NF-IL6β transcription, a dominant negative mutant of CREB, in which serine 133 was replaced by alanine (DN-CREB), was transfected to A431 cells to address this issue. A431 cells transfected with p38α-

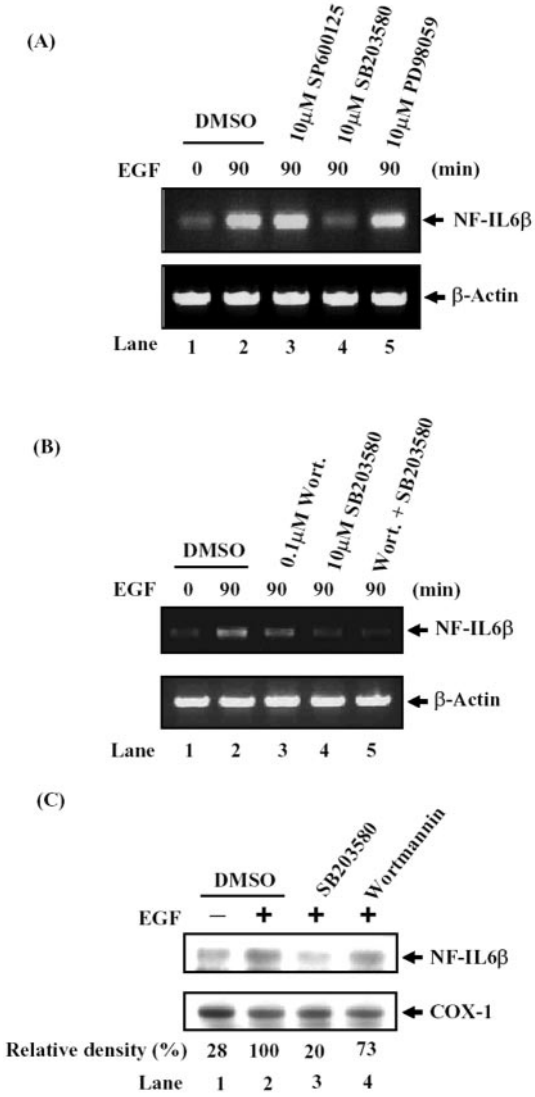

Figure 6. Wortmannin and SB203580 attenuated EGF-inducted *NF-IL6β* mRNA and protein expression. (A and B) Cells were pretreated with various MAPK inhibitors as indicated for 30 min before restimulation with EGF for 90 min. After stimulation, total RNA was prepared from these cells and analyzed by RT-PCR with specific NF-IL6β primers for detection of the *NF-IL6β* mRNA. (C) Cell were treated with compounds as indicated for 30 min before restimulation with EGF for 4 h. Lysates from various treatment were analyzed by Western blot with α-NF-IL6β or α-cyclooxygenase (COX)-1 antibodies. The relative density of NF-IL6β protein expression was normalized with COX-1 protein in densitometer. The pharmacological inhibitors were dissolved in dimethyl sulfoxide (DMSO), and the final 0.5% of DMSO was used in incubation.

expressing vector enhanced NF-IL6β-347/+9 reporter activity without EGF treatment (Figure 9A, compare lane 1 with lane 4), and the presence of EGF significantly enhanced reporter activity of NF-IL6β-347/+9 in the presence of p38α expression vector (Figure 9A, compare lane 2 with lane 5). Moreover, overexpressed DN-CREB repressed the reporter activity of NF-IL6β-347/+9 with or without p38α^{MAPK} expression under EGF stimulation (Figure 9A, compare lane 2 with lane 3, and lane 5 with lane 6). For specifically determining whether the CRE motif of the *NF-IL6β* promoter was a p38α-regulated CREB target site, a heterologous promoter

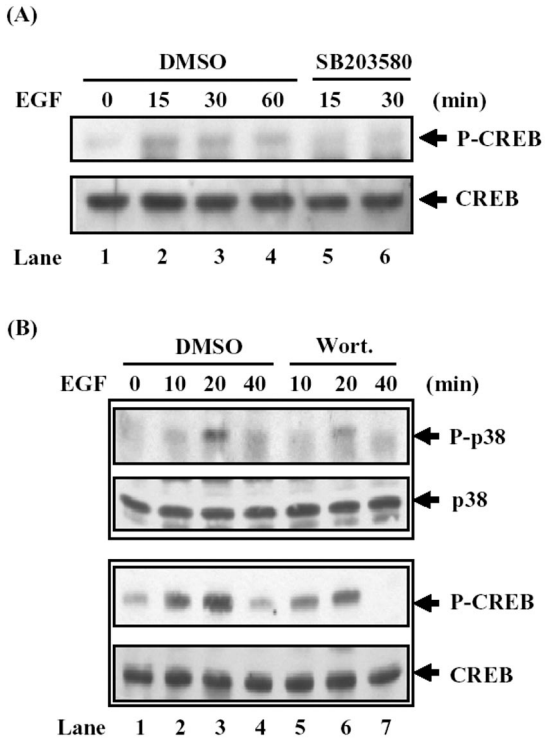

Figure 7. EGF activated the PI3-kinase/p38^{MAPK}/CREB signaling pathway in A431 cells. (A) Inhibition of EGF-induced CREB phosphorylation by SB203580. The A431 cells were pretreated with or without 10 μM SB203580 for 30 min and then stimulated with EGF as indicated. Western blot analysis with α-P-CREB and α-CREB antibodies was performed. (B) Wortmannin attenuated the activation of p38^{MAPK} and CREB after EGF treatment. Cell lysates from A431 cells stimulated with EGF for various times were analyzed by Western blotting with antibodies recognizing the active form p38 (P-p38), CREB (P-CREB), p38, and CREB. The pharmacological inhibitors were dissolved in dimethyl sulfoxide (DMSO), and the final 0.5% of DMSO was used in incubation.

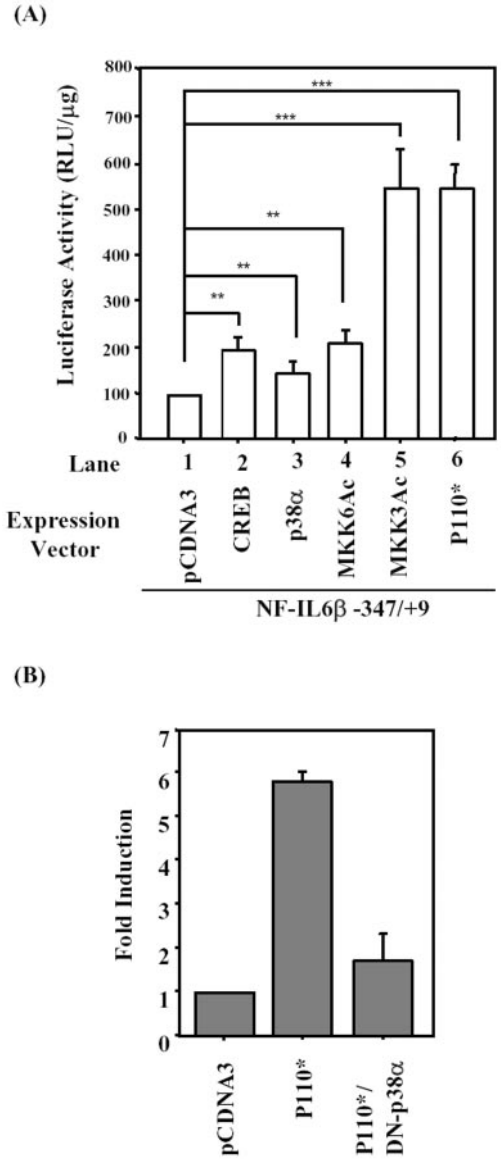

Figure 8. Overexpression of constitutively activated MKK3, MKK6, or p110* up-regulated the promoter activity of *NF-IL6β* gene. (A) A431 cells were cotransfected with reporter NF-IL6β-347/+9 and various expression vectors as indicated. (B) A431 cells were cotransfected with reporter NF-IL6β-347/+9 and p110* alone or DN-p38α combined with p110*. After growing in serum-free medium for 15 h, the luciferase assay was performed with lysates of those transient transfectants. Statistic analysis was performed by Student's *t* test.

containing CRE motif, pGL2NF-IL6β1xCRE, was constructed and analyzed. Insertion of a CRE site alone was sufficient to confer EGF activity on the SV40 promoter (Figure 9B, compare lane 2 with lane 7). We then examined whether p38α was mediated through phosphorylation of CREB Ser133 to regulate the CRE motif. Cells were cotransfected with p38α expression vectors enhanced the heterologous reporter activity of pGL2NF-IL6β1xCRE under EGF treatment (Figure 9A, compare lane 5 with lane 8). Moreover, similar pattern was observed in the experiment with cotransfection of CREB expression vector (Figure 9B, compare lane 6 with lane 9). We further examined whether the EGF inducibility on the pGL2NF-IL6β/1xCRE could be attenuated by dominant negative forms of p38α or CREB. The results shown in Figure 9C indicated that coexpression of DN-p38α or DN-CREB attenuated EGF inducibility effect on CRE reporter activity (compare lane 1 with lanes 2 or 3). These results suggested that the induction of phosphorylated CREB through activated p38α played a functional role in EGF-induced *NF-IL6β* transcription.

DISCUSSION

In this study, we provided several pieces of evidence suggesting that the Sp1 and the CRE sites in the essential promoter region were required for EGF-induced transcription of the human *NF-IL6β* gene. Several transcription factors, for example, STAT3 (Hutt *et al*., 2000; Alonzi *et al*., 2001), CREB/ATF-2 (Belmonte *et al*., 2001), RunX (McCarthy *et al*., 2000), and Sp1 (Alonzi *et al*., 2001), are involved in the transcriptional regulation of *C/EBPδ*, which are binging in the vicinity of the transcriptional initiation site. The APRE site is important for IL-6–regulated *C/EBPδ* promoter activity in hepatoma cells (Cantwell *et al*., 1998). However, the APRE-like sequence does not exist in human *NF-IL6β* promoter. By comparing the APRE regions on mouse and human promoter sequences, an Sp1, a nuclear factor-κB (NF-

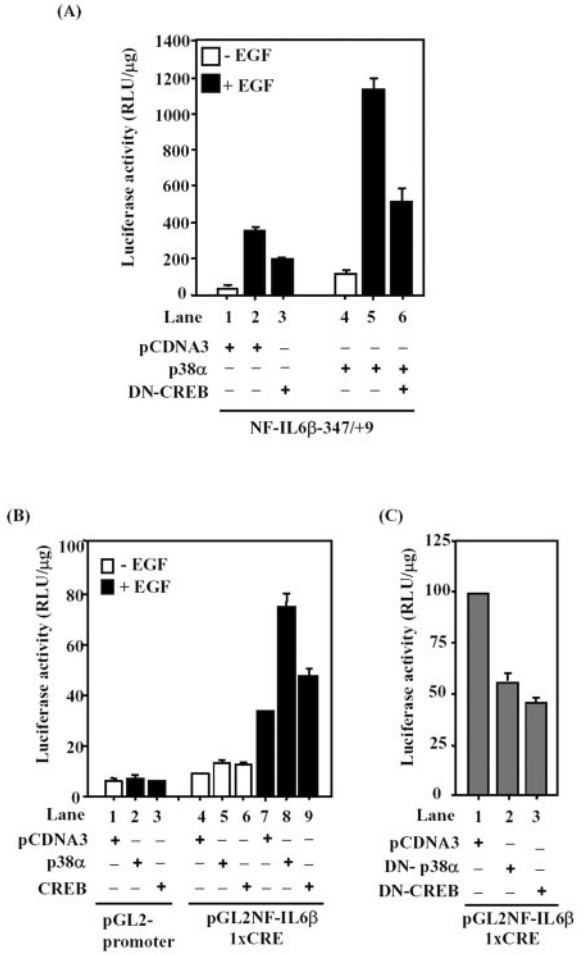

Figure 9. Functional role of $p38^{MAPK}$ and CREB in EGF stimulated *NF-IL6β* promoter activity in A431 cells. (A) A431 cells were transiently transfected with reporter genes as indicated along with a control (pCDNA3) or the pCDNA3/CREBS133A (DN-CREB) expression vectors. After transfection, cells were cultivated in medium with or without EGF. Cell lysates were then prepared and analyzed for luciferase activity. (B) A431 cells transfected with pGL2-promoter or CRE-heterologous reporter genes combined with the control, p38α, or CREB expression vectors, as indicated, were stimulated with or without EGF for 15 h. Cell lysates were then prepared and analyzed for luciferase activity. (C) Dominant negative forms of p38α or CREB were cotransfected with pGL2NF-IL6β1xCRE reporter in A431 cells. For the normalization, the pGL2NF-IL6β1xCRE reporter activity cotransfected with control vector, pCDNA3, was assigned a value of 100. The reporter activities of pGL2NF-IL6β1xCRE combined with DN-p38α or DN-CREB were normalized to the control. The data shown are means ± standard deviations of two independent experiments.

κB)–like, and STAT3 binding sites in mouse APRE region were observed (Figure 2A). The mouse Sp1 site corresponds to the human *NF-IL6β* Sp1-1 site, whereas no homologous sites of NF-κB and STAT3 exist in *NF-IL6β*. Transient transfection with the heterologous reporter containing the APRE sequence, C/EBPδ 5′-AGCGAGGGCGGGTCGTTCCCAGC-3′ (Sp1 site, underlined; NF-κB site, italics) and *NF-IL6β* 5′-GGCTCCGGGGGGCTCCCAGGGCG-3′ to A431 cells under EGF treatment was conducted in this study. By comparing with the control reporter construct, there was no induction observed in pGL2-NF-IL6β 4xAPRE (Figure 2C, left). However, the increased promoter activity of pGL-2C/EBPδ 4xAPRE might be due to the Sp1 binding element existed in the mouse APRE region (Figure 2C, right). On the other hand, both heterologous reporters containing CRE sequence showed twofold induction under EGF treatment (Figure 2D). These results indicated that *NF-IL6β* had different promoter usage in A431 cells from mouse *C/EBPδ* in other systems. The two Sp1 sites and CRE site, but not APRE region, were more important in the basal or in the EGF-induced transcriptional activities of *NF-IL6β* gene (Figure 2E).

Expression of dominant negative form of CREB dramatically reduces the leukemia inhibitory factor- and prostacyclin-stimulated *C/EBPδ* expression (Belmonte *et al*., 2001). In RAW264 cells, *C/EBPβ* induction seems not to require CREB, because it is not affected by the treatment that abolishes CREB activation (Caivano and Cohen, 2000). Nevertheless, in our study, the same treatments inhibited CREB phosphorylation and also abolished *NF-IL6β* induction (Figure 9, A and C). The *NF-IL6β* CRE site, GCACGTCA, has homologous sequence to the ATF/CRE sequence motifs (TGACGTCA) and like as the asymmetric and weak binding sites (Nichols *et al*., 1992). Transfection of the CRE heterologous reporters of *C/EBPδ* or *NF-IL6β* promoter enhanced the EGF induction of transcription activity in A431 cells (Figure 2D). From the results of gel shift and ChIP assays (Figures 3A and 5), it clearly indicated that CREB bound to the *NF-IL6β* promoter, whereas EGF enhanced the DNA binding activity of CREB through phosphorylation mechanism.

Sp1 is a ubiquitous nuclear factor that plays a key role in maintaining basal transcription of house-keeping genes. Many reports show that the posttranslational modification of Sp1, such as phosphorylation (Banchio *et al*., 2004) and acetylation (Ryu *et al*., 2003), is important for its regulation of target genes expression. Mutants of Sp1 sites on *NF-IL6β* promoter resulted in not only lose of the basal transcription activity but also lose of the EGF-induced activity (Figure 2). Constitutive binding of Sp1 to the *NF-IL6β* promoter was not affected by the EGF treatment as shown in the gel shift and ChIP assays (Figures 4 and 5). Overexpression of Sp1 also enhanced the basal transcription activity (Figure 4B, lanes 1 and 3), indicating that Sp1 played a major role in the basal transcriptional complex of *NF-IL6β* promoter. On EGF treatment, the *NF-IL6β* promoter did not recruit more Sp1 proteins to increase the transcription activity. Instead, the EGF-induced posttranslational modification of Sp1 might account for the elevation of transcription activity.

Several previous reports show that NF-κB pathway is important for transcriptional regulation of mouse *C/EBPδ* (Tengku-Muhammad *et al*., 2000; Okazaki *et al*., 2002), but there was no evidence to show that the direct binding of NF-κB to the promoter region of *C/EBPδ* gene is crucial. Induction of de novo production biosynthesis through NF-κB might be a possible mechanism to account for the LPS-regulation of mouse C/EBPδ (Caivano *et al*., 2001). In our hands, *NF-IL6β* was an immediate-early gene regulated by EGF treatment, and NF-κB could not bind to the APRE-like motif by gel-shifting assay (Wang and Chang, unpublished result). It indicated that NF-κB might not be involved in EGF-induced transcriptional regulation of *NF-IL6β* gene.

Cycloheximide failed to inhibit the induction of NF-IL6β by EGF, indicating that NF-IL6β is an immediate-early gene (Figure 1C). This result was the same as the immediate-early gene encoding transcription factor c-fos treated with cycloheximide (Morgan and Curran, 1991). NF-IL6β mRNA also is superinduced by cycloheximide (Figure 1C). An unappreciated property of protein translation inhibitors is their ability to activate numerous kinases, such as c-Jun NH_2-terminal kinase (JNK),

p38^{MAPK}, mTOR, and p70S6 kinase (Barros *et al*., 1997; Sidhu and Omiecinski, 1998; Khaleghpour *et al*., 1999). Cycloheximide superinduces glucocorticoid-mediated transcription of a gene encoding the α-epithelial sodium channel protein via a mechanism that can be suppressed by a p38 MAPK inhibitor (Itani *et al*., 2003). Our results demonstrated that p38^{MAPK} signaling pathway was involved in the EGF-induced NF-IL6β transcription that also might be involved in cycloheximide-induced manner.

Another interesting finding from this study is the demonstration that PI3-kinase was an upstream regulator of p38^{MAPK}. p38^{MAPK} is a JNK-related MAPK that is activated in response to a variety of stimuli, including growth factors, phorbol esters, cytokines, and environmental stress (Minden and Karin, 1997); and the different upstream activators of p38^{MAPK} were reported, such as MKK3/6 (Shuto *et al*., 2001), Rac (Xu *et al*., 2003), Src (Daly *et al*., 1999; Frey *et al*., 2004), or PI3-kinase (Gibbs *et al*., 2002; Xu *et al*., 2003; Gonzalez *et al*., 2004). IL-4 stimulates Rac and Cdc42, which seem to regulate a protein kinase cascade initiated at the level of PAK and lead to activation of p38^{MAPK} in A431 cells, and are finally able to produce IL-6 (Wery-Zennaro *et al*., 2000). PI3-kinase coprecipitates with the ErbB3 protein in response to EGF in A431 cells (Soltoff *et al*., 1994). Using a number of different approaches, several pieces of evidence indicated that p38^{MAPK} contributed to the EGF induction of *NF-IL6β*. SB203580, which selectively inhibits p38α and p38β2 isoforms but has no effect on JNK and ERK (Cuenda *et al*., 1997; Kumar *et al*., 1997), inhibited the EGF-induced *NF-IL6β* expression (Figure 6). In addition, *NF-IL6β* promoter activity was inhibited by expression of a dominant-negative p38α mutant and was activated by the overexpression of wild-type p38α (Figure 9). p38^{MAPK} can be phosphorylated and activated by the dual-specific protein kinases MKK3 and MKK6 (Derijard *et al*., 1995). Overexpression of the constitutively activated p38^{MAPK}-specific kinase MKK6 or MKK3 directly stimulated *NF-IL6β* promoter activity (Figure 8). Treatment of anisomycin that mimics the p38^{MAPK} activation increased the in vivo-CREB binding activity (Figure 5B) and CREB phosphorylation in A431 cells (Wang and Chang, unpublished result). The evidence concluded that p38 was involved in the signal pathway of EGF-induced *NF-IL6β* expression. We also investigated the signal transduction cascades that connect EGFR activation to phosphorylation of p38^{MAPK} in A431 cells. Several possible signaling transduction pathways that could be implicated in PI3-kinase–regulated p38^{MAPK} activation have been reported, such as the PI3-kinase/Rca/p38^{MAPK} pathway in Signet-ring cell carcinoma (Xu *et al*., 2003), the TGFβ1-induced PI3-kinase/p38^{MAPK}/Akt pathway in mesenchymal cells (Horowitz *et al*., 2004), or the involvement of PI3-kinase/p38^{MAPK}/Akt2 pathway in myogenesis (Gonzalez *et al*., 2004). The treatment of wortmannin partially inhibited p38^{MAPK} and CREB activation (Figure 7), suggesting that PI3-kinase might not be the only upstream activator.

Based on these observations, a tentative model for regulation mechanism of *NF-IL6β* gene in A431 cells under EGF treatment was proposed. In human epidermoid carcinoma A431 cells, EGF signal, at least in part, activated the PI3-kinase pathway that led to the phosphorylation of p38^{MAPK}. The phosphorylated p38^{MAPK} in turn induced the CREB phosphorylation and increased its binding to *NF-IL6β* promoter. Then, the Sp1 cooperated with the phosphorylated CREB to activate the transcription of *NF-IL6β* gene. In conclusion, these results indicated that induction of PI3-kinase/p38^{MAPK}/CREB pathway plays a functional role in EGF-induced transcription of *NF-IL6β* gene in A431 cells.

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