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

Ju-Ming Wang, Joseph T. Tseng, and Wen-Chang Chang

Department of Pharmacology, College of Medicine, National Cheng Kung University, Tainan 701, Taiwan

Submitted February 22, 2005; Revised May 3, 2005; Accepted May 5, 2005
Monitoring Editor: Carl-Henrik Heldin

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 p38MAPK 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 p38MAPK. Our results suggested that PI3-kinase/p38MAPK/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 Guillemin, 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 (Wkedel and Ziegler-Heitbrock, 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 G0 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
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 G0 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-IL6b).

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 cyclcalAMP-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 isofoms 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 p90RSK may be responsible for CREB phosphorylation at Ser133, both in vitro and in vivo, in response to growth factor stimulation (Bohm 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 p38MAPK, 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 CREB mitogenic properties (Wiley et al., 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 CREB mitogenic properties (Wiley et al., 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 CREB mitogenic properties (Wiley et al., 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 CREB mitogenic properties (Wiley et al., 1995; Treisman, 1996).

Molecular Biology of the Cell
Nuclear Extract and Gel Shift Assays

Gel shift assays were carried out essentially as described previously (Kawamoto et al., 1984). Briefly, the 32P-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(dIdC). After 20 min of incubation at room temperature, the reaction mixtures were resolved in a 5% native polyacrylamide gel (acylamide/bisacylamide ratio, 3:1) at 4°C, and the specific protein complexes were visualized by autoradiography. The CRE binding huamnoid contained 10 M 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 MgCl2, 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 a-CREB, a-Sp1, and a-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-IL6CRE (hCRE), 5′-GGGCGCTGACGTACGGCCGGG-3′; NF-IL6mCRE (murCRE), 5′-GGGCGCTGATCCACCCGGGG-3′; NF-IL6Sp1-1, 5′-AAGGCTCGGG-GGCGTCCGCAGGG-3′; NF-IL6Sp1-2, 5′-CCGGACTGCGGGCGGCGGTGCG-3′; mouse C/EBP-6CRE (mCRE), 5′-GGGCGCTGACGTACGGCCGGG-3′; and consensus Sp1 oligonucleotide (sp1), 5′-ATTCCGATCCGGGCGGCGGGAAGG-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 being immunoprecipitated with antibodies 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-IL6 genomic locus, the NF-IL6 genomic fragment ranging from 9 to 1717 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-IL6 gene ranging from −1717 to +9 base pairs and the C/EBPβ 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/EBPβ promoter were performed. Both 5′-serial deletion of promoter regions of mouse C/EBPβ, −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/EBPβ transcription is through the APRE and Sp1 motifs (Okazaki et al., 2002). To clarify the function of Sp1/APRE reporter in human NF-IL6β promoter, DNA gel-shift assay using the predicted APRE-like oligonucleotide 5′-GGCGCGCGCTCCACGCCCAGGGG-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.

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

By comparing with the proximal region sequences of human, mouse, and rat C/EBPβ 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/EBPβ 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/EBPβ gene range from −1680 to +14

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 analysis of NF-IL6β and C/EBPβ protein expression was correlated with that of β-actin mRNA level (Figure 1A). It was previously reported that IL-6-induced mouse C/EBPβ transcription is through the APRE and Sp1 motifs (Okazaki et al., 2002). To clarify the function of Sp1/APRE reporter in human NF-IL6β promoter, DNA gel-shift assay using the predicted APRE-like oligonucleotide 5′-GGCGCGCGCTCCACGCCCAGGGG-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-IL6βSp1-1 and NF-IL6βSp1-2 with the individually mutated Sp1 site resulted in a more significant attenuation of
the basal promoter activity than NF-IL6mCRE 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-IL6/H9252-347/mSp1-1, NF-IL6/H9252-347/mSp1-2, or NF–IL6/H9252-347/mCRE) diminished the EGF induction of the promoter activity by 30–40% respectively, whereas the triple mutant, NF-IL6/H9252-347/9mC/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-IL6β promoter.

**Binding of CREB to NF-IL6β Promoter**

To further identify the transcription factors bound to the CRE site of NF-IL6β 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/EBPβ 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-IL6 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-IL6β 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
petition 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 p38MAPK and PI3-Kinase Signal Transduction Pathway in NF-IL6β Regulation**

EGF interacts with EGFRI 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
p38*MAPKα- 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
p38MAPK played a functional role in CREB activation in EGF-stimulated A431 cells.

To further determine the relationship between PI3-kinase, P38MAPK, 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 p38MAPK 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 p38MAPK and CREB, and p38MAPK was a major CREB Ser133 activator in A431 cells.

To examine whether the \(-347/+9\) fragment of NF-IL6 promoter is required for PI3-kinase and p38MAPK activation, the NF-IL6-347/ +9 was cotransfected with different expression vectors, including wild-type expression vectors of CREB; p38α; two constitutive activation forms of p38MAPK upstream activators, MKK3Ac and MKK6Ac; and p110α, 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-IL6-347/+9 (lanes 4 and 5), and p110α also contributed the same effect (lane 6). Furthermore, a dominant negative mutant of p38α (DN-p38α) attenuated the transcriptional activity enhanced by p110α (Figure 8B). These results strongly suggested that PI3-kinase/p38MAPK signaling was involved in the transcriptional activation of human NF-IL6β promoter.

Mediation of the p38MAPK Signal through CREB in Activation of Human NF-IL6β Promoter Activity

To further confirm whether CREB was a downstream target of p38MAPK and p38MAPK-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-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 p38αMAPK 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

Vol. 16, July 2005 3371

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.

Figure 6. Wortmannin and SB203580 attenuated EGF-inducted NF-IL6β mRNA and protein expression. (A and B) Cells were pre-treated 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 lysates 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.
containing CRE motif, pGL2NF-IL6/1xCRE, 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 p38 was mediated through phosphorylation of CREB Ser133 to regulate the CRE motif. Cells were cotransfected with p38 expression vectors enhanced the heterologous reporter activity of pGL2NF-IL6/1xCRE 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-IL6/1xCRE could be attenuated by dominant negative forms of p38 or CREB. The results shown in Figure 9C indicated that coexpression of DN-p38 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 p38 played a functional role in EGF-induced NF-IL6 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/EBPβ, which are binging in the vicinity of the transcriptional initiation site. The APRE site is important for IL-6-regulated C/EBPβ 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-kB (NF-
The promoter activity of pGL-2C/EBP or CRE-heterologous reporter genes combined with the control (pCDNA3) or the pCDNA3/CREBS133A (DN-CREB) expression vectors. After transfection, cells were cultivated in medium without or with 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-IL6βxCRE reporter in A431 cells. For the normalization, the pGL2NF-IL6βxCRE reporter activity cotransfected with control vector, pCDNA3, was assigned a value of 100. The reporter activities of pGL2NF-IL6βxCRE combined with DN-p38α or DN-CREB were normalized to the control. The data shown are means ± standard deviations of two independent experiments.

Figure 9. Functional role of p38MAPK 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 without or with 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-IL6βxCRE reporter in A431 cells. For the normalization, the pGL2NF-IL6βxCRE reporter activity cotransfected with control vector, pCDNA3, was assigned a value of 100. The reporter activities of pGL2NF-IL6βxCRE combined with DN-p38α or DN-CREB were normalized to the control. The data shown are means ± standard deviations of two independent experiments.
p38MAPK, mTOR, and p70S6 kinase (Barros et al., 1997; Sidhu and Omiecinski, 1998; Khaledhpour 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 p38MAPK 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 p38MAPK. p38MAPK 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 p38MAPK 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 p38MAPK 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 (Soltot et al., 1994). Using a number of different approaches, several pieces of evidence indicated that p38MAPK contributed to the EGF induction of NF-IL6β. SB203580, which selectively inhibits p38α and p38β isoforms but has no effect on JNK and ERK (Cuenda 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 wortmannin partially inhibited p38 MAPK and CREB activation to the phosphorylation of NF-IL6β gene in A431 cells.

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 p38MAPK. The phosphorylated p38MAPK 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/ p38MAPK/CREB pathway plays a functional role in EGF-induced transcription of NF-IL6β gene in A431 cells.

ACKNOWLEDGMENTS

We thank Drs. Wai-Ming Kan, Hsin-Fang Yang-Yen, and Shen K. Yang for critical reviewing and editing of the manuscript. We also thank for Drs. Jiahuai Han for the plasmid expressing DN-p38α, Ming-Zong Lai for the plasmids expressing MKK3Ac and MKK6Ac, Hsin-Fang Yang-Yen for the plasmid p1102, and Jeffrey Y. Yen for the plasmids expressing DN-CREB and CREB. This work was supported in part by grant the Ministry of Education Program for Promoting Academic Excellent of University under the grant number 91-B-FA09-1 of the Republic of China.

REFERENCES


De Cesare, D., Fimia, G. M., and Sassone-Corsi, P. (1999). Signaling routes to CREB. This work was supported in part by grant the Ministry of Education Program for Promoting Academic Excellent of University under the grant number 91-B-FA09-1 of the Republic of China.

Molecular Biology of the Cell
J.-M. Wang et al.


