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Activation of Extracellular Signal-Regulated Kinase Signaling by Epidermal Growth Factor Mediates c-Jun Activation and p300 Recruitment in Keratin 16 Gene Expression

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ABSTRACT

In studies of gene regulation of keratin 16, we reported previously that simian virus 40 promoter factor 1 shows a functional cooperation with c-Jun and coactivators p300/CBP in driving the transcriptional regulation of epidermal growth factor (EGF)-induced keratin 16 gene expression. In the present study, we found that the stimulated expression of keratin 16 by EGF was mediated mainly through the mitogen-activated protein kinase kinase–extracellular signal-regulated kinases 1 and 2 (ERK1/2) signaling pathway. Ser63 and Ser73 on the c-Jun NH_2-terminal transactivation domain could be phosphorylated in cells treated with EGF; nevertheless, we found that the c-Jun COOH terminus played a pivotal role in EGF-induced expression of keratin 16. The activation of keratin 16 by EGF treatment could not be enhanced by the overexpression of myc-c-JunK3R, in which three putative acetylation lysine residues on the c-Jun COOH terminus were all mutated into arginines, suggesting that c-Jun acetylation on the COOH terminus might partially play a functional role in this system. In addition, by using a chromatin immunoprecipitation assay and a DNA affinity precipitation assay, EGF treatment up-regulated the p300 recruitment through ERK signaling to the promoter region in regulating keratin 16 transcriptional activity. Furthermore, the enhancement of acetyl-histone H3 to the keratin 16 chromatin promoter induced by EGF was also mediated via ERK activation. In conclusion, these results strongly suggest that both c-Jun induction and p300 recruitment to gene promoter, mediated through ERK activation, played an essential role in regulating keratin 16 gene expression by EGF. p300 mediated and regulated EGF-induced keratin 16 gene expression, at least in part, through multiple mechanisms, including a selective acetylation of c-Jun and histone H3.

Keratinocytes are the most abundant of the cell types in the epidermis, which forms the external surface of the skin. The maintenance of homeostatic balance in epidermal keratinocytes is dependent on the coordinate regulation of differentiation and activation (Paramio et al., 1999). The most prominent cytoskeletal proteins in keratinocytes are keratins, a large family of proteins that form the intermediate filament network in all epithelial cells (Fuchs and Weber, 1994). The programmed expression of keratins, which is determined by the location and function of the keratinocytes within the epidermis, is commonly used as a phenotypic marker of epithelial development and differentiation (Fuchs, 1990). In the skin diseases characterized by hyperproliferation such as psoriasis, keratin 16 has been reported to be the marker in vivo and in vitro (Leigh et al., 1995; Komine et al., 2000). Therefore, keratin 16 is usually referred to as an activation-, hyperproliferation-, and disease-associated keratin.

The earliest event in the EGF signaling pathway involves coupling of EGF to receptor tyrosine kinase, and then Ras activation subsequently leads to the activation of Raf-1. Raf-1 phosphorylates and activates mitogen-activated protein kinase kinase (MEK), which in turn phosphorylates and activates p44/p42 mitogen-activated protein kinase (MAPK), also named as extracellular signal-regulated kinases 1 and 2 (ERK1/2). ERK1/2 are serine/threonine kinases that regulate gene expression by phosphorylating nuclear substrates. Activation of the Ras-MEK-ERK cascade is a common event in

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ABBREVIATIONS: EGF, epidermal growth factor; Sp1, simian virus 40 promoter factor 1; AP1, activating protein 1; ERK, extracellular signal-regulated kinase; JNK, c-Jun NH_2-terminal kinase; HAT, histone acetyltransferase; MEK, mitogen-activated protein kinase kinase; MAPK, mitogen-activated protein kinase; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; bp, base pair; HA, hemagglutinin; Ac, acetyl histone; C_T, threshold cycle; SP600125, anthra(1,9-*cd*)pyrazol-6(2*H*)-one 1,9-pyrazoloanthrone; U0126, 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophynyltio)butadiene.

cellular proliferation and transformation that usually lead to tumors (Mansour et al., 1994). Ras can also activate Rac, which in turn leads to the phosphorylation and activation of c-Jun amino-terminal kinase (JNK).

One of the early and crucial events caused by mitogenic stimulation of mammalian cells is the expression of the proto-oncogene c-Jun (Shaulian and Karin, 2002). The regulation of c-Jun activity by mitogens is mediated by two mechanisms at the transcriptional and the post-translational levels (Morton et al., 2003). First, mitogens induce the transcription of c-Jun through promoter activation (Gupta and Prywes, 2002), and second, phosphorylation of the NH_2-terminal transactivation domain of c-Jun, mostly through JNK signaling, activates its transcriptional activation function (Davis, 2000). The transactivation activity of c-Jun can be regulated through the phosphorylation of clusters of serine/threonine residues in the NH_2 terminus (Karin et al., 1997). Moreover, it has been reported that several agonists induce the phosphorylation of two major serine phosphoacceptor sites on c-Jun at Ser63 and Ser73 (May et al., 1998) and two threonine residues at Thr91 and Thr93 (Papavassiliou et al., 1995). However, the identity of the protein kinase that phosphorylates Thr91 and Thr93 in vivo is still unknown.

It is now widely accepted that the versatile coactivator p300 promotes gene transcription by three mechanisms (Chan and Thangue, 2001). First, it provides a bridge between DNA binding transcription factors and the basal transcription machinery. Second, it acts as a scaffold for the assembly of multiprotein complexes. p300 cooperates with multiple nonhistone proteins, notably transcriptional factors such as p53 (Somasundaram and El-Deiry, 1997), cAMP response element-binding protein (Smith et al., 1996), c-Jun (Bannister et al., 1995), and Sp1 (Suzuki et al., 2000). Third, p300 possesses intrinsic histone acetyltransferase (HAT) activity that acetylates transcription factors and chromatin to facilitate transactivation (Ogryzko et al., 1996). Several experiments have suggested the importance of histone acetylation in the transcription of a variety of genes (Shikama et al., 1997). In addition, acetylation of transcriptional factors by p300 leads to changes in protein-protein interaction and protein-DNA interaction, which subsequently can result in increased or decreased transcription (Sterner and Berger, 2000). It has been reported that Lys271 on the COOH terminus basic region of c-Jun is acetylated by p300 in vitro and in vivo (Vries et al., 2001). Moreover, p300-mediated acetylation turns Sp3 from a transcriptional repressor to an activator (Ammanamanchi et al., 2003).

We have provided a proposed model for the transcriptional regulation of the keratin 16 gene, indicating that Sp1 recruits c-Jun to the promoter through the binding of Sp1 to the Sp1 site and coactivators p300/CBP interact with Sp1 and AP1 proteins and participate in the transcriptional regulation for EGF induction of keratin 16 gene expression in keratinocytes (Wang and Chang, 2003). The aim of the present work is to further investigate the signaling pathway and the regulatory mechanism of these nuclear factors involved in the transcriptional control of keratin 16 upon EGF treatment. We found that EGF stimulated c-Jun biosynthesis through ERK and JNK cascades and up-regulated p300 recruitment through ERK signaling to the promoter in regulating keratin 16 transcriptional activity in human keratinocytes, which involves multiple mechanisms, including a selective acetylation of c-Jun and histone H3.

Materials and Methods

Materials. Human EGF (natural, culture grade) was purchased from Pepro Technology (Rocky Hill, NJ). Monoclonal antibody against keratin 16 was from NEOMARKERS (Fremont, CA). Monoclonal antibodies against c-Jun, ERK2, and p300 were obtained from Transduction Laboratories (Lexington, KY). Protein A-agarose beads, agarose conjugated to c-Jun antibodies, and polyclonal antibodies against Sp1, c-Jun, p300, JNK1, and normal rabbit IgG were from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal antibodies directed against acetylated-lysine and the phosphorylated form of Thr202/Tyr204 ERK1/2, Thr183/Tyr185 JNK, Ser63 c-Jun, and Ser73 c-Jun were purchased from Cell Signaling Technology (Beverly, MA). Antibodies against ERK1/2, acetyl-H3, acetyl-H4, and expression vector of activated MEK1 and HA-p300 were obtained from Upstate Biotechnology (Lake Placid, NY). Antibody against HA was from Roche Diagnostics (Penzberg, Germany). The inhibitor of MEK1, U0126, was from Promega (Madison, WI). Inhibitor of JNK, SP600125, was from Tocris Cookson Inc. (Ballwin, MO). Salmon sperm DNA and streptavidin agarose were from Sigma-Aldrich (St. Louis, MO). Protein A Sepharose CL-4B was from GE Healthcare (Little Chalfont, Buckinghamshire, UK). Biotinylated oligonucleotides were obtained from MDBio, Inc. (Taipei, Taiwan). The luciferase assay system was from Promega. Luciferase plasmid pXP-1 was a gift from Dr. T. Sakai of Kyoto Prefecture University of Medicine. TRIzol RNA extraction kit, SuperScript III, Lipofectamine, Dulbecco's modified Eagle's medium, and Opti-MEM medium were obtained from Invitrogen (Carlsbad, CA). Expression vector of K52R ERK2 was provided by Dr. M. Z. Lai (Academia Sinica, Taipei, Taiwan). Vector TAM-67 was kindly provided by Michael J. Birrer of the National Institutes of Health (Bethesda, MD). Expression vectors of pMT-108 and pMT-161 were kindly provided by Dr. B. C. Chung (Academia Sinica). Expression vectors of myc-c-Jun and myc-c-JunK3R were kindly provided by Dr. A. Zantema (Leiden University Medical Center, Leiden, the Netherlands). Expression vector of E1A was a kind gift of Dr. H. M. Shih (National Health Research Institutes, Taipei, Taiwan). Expression vectors of p300 and p300ΔHAT were provided by Dr. K. K. Wu (University of Texas Health Science Center, Houston, TX). Fetal bovine serum was from Hyclone Laboratories (Logan, UT). All other reagents used were of the highest purity obtainable.

Cell Culture and EGF Treatment. HaCaT cells, a spontaneously immortalized human epidermal keratinocyte cell line, were grown at 37°C under 5% CO_2 in 10-cm plastic dishes containing 8 ml of Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum, 100 μg/ml streptomycin, and 100 IU/ml penicillin. In this series of experiments, cells were treated with 30 ng/ml EGF in optimal serum-free conditions, unless stated otherwise.

Reverse Transcription-PCR. Cells maintained for 2 days in serum-free medium were incubated with EGF for the indicated time period as described and harvested. Total RNA was isolated using the TRIzol RNA extraction kit, and 5 μg of RNA was subjected to reverse transcription-PCR with SuperScript III. Keratin 16-specific primers and glyceraldehyde-3-phosphate dehydrogenase primers were used as controls. The PCR products were separated by 1% agarose-gel electrophoresis and were visualized with ethidium bromide staining.

Preparation of Cell Lysates. Cells from 10-cm plastic dishes were washed twice with PBS and lysed with radioimmunoprecipitation assay buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% IGEPAL CA-630, and 0.5% sodium deoxycholate) containing 1 mM EDTA, 1 mM sodium orthovanadate, 2 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin. The lysates were centrifuged at 7500*g* for 5 min. The supernatants were collected and stored at −70°C until used.

Preparation of Nuclear Extracts. Cells from 10-cm plastic dishes were washed twice with PBS and scraped in 500 μl of PBS. Cells were collected by centrifuging at 7500*g* for 20 to 30 s, resuspended in 400 μl of buffer A (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, and 10 mM KCl) and stood on ice for 10 min. Nuclei were pelleted by centrifugation at 7500*g* for 20 to 30 s. Pellets were resuspended in 100 μl of buffer C [20 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 420 mM NaCl, and 25% (v/v) glycerol] and stood on ice for 20 min. The suspension was centrifuged at 7500*g* for 2 min. The supernatants were collected and stored at −70°C until used. Buffers A and C contained 0.5 mM dithiothreitol, 2 μg/ml leupeptin, 1 mM orthovanadate, 2 μg/ml pepstatin A, and 0.5 mM phenylmethylsulfonyl fluoride.

Western Blots. Analytical 10% SDS-polyacrylamide slab gel electrophoresis was performed. The cell nuclear extracts or lysates (30–200 μg of protein of each) prepared from control, and EGF-treated cells were analyzed. The supernatants were mixed with 5× SDS loading buffer followed by boiling for 5 min. For immunoblotting, proteins in the SDS gels were transferred to a polyvinylidene difluoride membrane by an Electroblot apparatus (Bio-Rad, Hercules, CA). After blocking with 5% nonfat dried milk in 1× PBS, the blots were incubated with antibodies against specific proteins (as the primary antibodies) individually. Horseradish peroxidase-conjugated secondary antibodies were added, and the bands were detected by the SuperSignal enhanced chemiluminescence kit (Pierce Biotechnology, Rockford, IL). The density of the immunoblots was determined by an image analysis system installed with a software BIO-ID (Vilber Lourmat, Mame-Ia-Valee, France).

Immunoprecipitation Assay. One milligram of nuclear extracts was immunoprecipitated with 30 μl of anti-c-Jun antibodies-agarose conjugated in immunoprecipitation buffer [20 mM HEPES, pH 7.9, 2 mM $MgCl_2$, 0.2 mM EDTA, 0.1 mM KCl, 10% (v/v) glycerol, and 1 mM dithiothreitol] under gentle shaking at 4°C overnight. Immunoprecipitated beads were pelleted and washed three times with washing buffer (1× PBS containing 0.5% IGEPAL CA-630). Protein was removed from the beads by boiling in 1× SDS loading buffer for 5 min and separated by SDS-PAGE, followed by Western blot analysis probed with antiacetylated lysine and c-Jun antibodies.

Transfection with Lipofectamine and Reporter Gene Assay. Cells were transfected with plasmids by lipofection using Lipofectamine according to the manufacturer's instruction with a slight modification. Cells were replated 24 h before transfection at an optimal cell density in 2 ml of fresh culture medium in a 3.5-cm plastic dish. For use in transfection, 12.5 μl of Lipofectamine was incubated with 0.5 μg of pXK-5-1 luciferase plasmid that contains the EGF response region of keratin 16 gene (Wang and Chang, 2003), or the indicated plasmids as described in each experiment, in 1 ml of Opti-MEM medium for 30 min at room temperature. Total DNA concentration for each transfection was matched with pcDNA3.1. Cells were transfected by changing the medium with 1 ml of Opti-MEM medium containing the plasmids and Lipofectamine, followed by incubation at 37°C in a humidified atmosphere of 5% CO_2 for 24 h. After a change of Opti-MEM medium to 2 ml of fresh culture medium, cells were stimulated with EGF if necessary and then incubated for an additional 24 h. The luciferase activities in cell lysates were measured by the luciferase assay system and determined as described previously (Liu et al., 1997). Luciferase activity was normalized per microgram of extract protein.

Chromatin Immunoprecipitation Assay and Real-Time PCR. HaCaT cells were treated with or without inhibitors for 30 min followed by EGF treatment for 24 h. Cells were cross-linked with 1% formaldehyde at 37°C for 15 min, washed twice with PBS, lysed with L1 buffer [50 mM Tris, pH 8.0, 2 mM EDTA, 0.1% (v/v) IGEPAL CA-630, and 10% (v/v) glycerol], and then resuspended with L2 buffer (50 mM Tris, pH 8.0, 5 mM EDTA, and 1% SDS). The lysates were sonicated to shear the size of DNA to 500 to 1000 bp. Two hundred micrograms of sonicated extracts were diluted 10-fold with dilution buffer [50 mM Tris, pH 8.0, 0.5 mM EDTA, 0.5% (v/v) IGEPAL CA-630, and 0.2 M NaCl] followed by incubation with 20 μl of salmon sperm DNA-saturated 50% protein A-Sepharose at 4°C for 30 min for precleaning. Immunoprecipitation was performed with specific antibodies with rotating at 4°C overnight, followed by adding 40 μl of salmon sperm DNA-saturated 50% protein A-Sepharose at 4°C for 2 h. Immunoprecipitated beads were pelleted and washed with high-salt and low-salt washing buffer each three times. DNA-protein complex was eluted in an elution buffer (1× Tris-EDTA buffer containing 1% SDS) with rotating at room temperature for 15 min, and the immune complex cross-link was reversed by heating at 65°C overnight, followed by treatment with 100 μg/ml proteinase K at 50°C for 1 h. DNA was extracted twice with phenol/chloroform, precipitated with ethanol, and dissolved in 50 μl of H_2O. L1 buffer, L2 buffer, and dilution buffer contained 0.5 mM phenylmethylsulfonyl fluoride, 1 mM orthovanadate, 2 μg/ml pepstatin A, 2 μg/ml leupeptin, 5 mM sodium fluoride, and 1 μg/ml aprotinin. Specific sequences in the immunoprecipitates were detected by PCR amplification or quantitative real-time PCR. The primer pair 5′-AAAGCTGGGTGGGAACTCTGAGCC-3′ and 5′-GGTGCCAAGGAGGGAGGTGAGC-3′ was used to amplify a 256-bp fragment in the human keratin 16 promoter. The PCR product was separated by 1% agarose-gel electrophoresis and visualized with ethidium bromide staining.

Quantitative real-time PCR was performed by LC FastStart DNA Master SYBR Green I and LightCycler instrument from Roche Diagnostics using the following conditions: 10 min at 95°C, and then 35 cycles of denaturation (95°C, 10 s), annealing (60°C, 5 s), and extension (72°C, 20 s). The DNA from target gene and input fractions were diluted (1/4 for target gene fraction and 1/10 for input fraction). After SYBR Green PCR amplification, data acquisition and subsequent data analyses were performed using the LightCycler Software (version 3.5; Roche Applied Science). The number of PCR cycles required to reach a threshold set arbitrarily is called the threshold cycle (C_T). Target DNA copy number and C_T values are inversely related. The absolute levels of keratin 16 in the experimental samples were determined by the C_T values from the standard curve.

DNA Affinity Precipitation Assay. The binding assay was performed by mixing 200 μg of nuclear extract proteins, 2 μg of biotinylated keratin 16-specific wild-type oligonucleotide, and 20 μl of streptavidin-agarose beads (4%) with a 50% slurry. The mixture was incubated at room temperature for 1 h with rotating. Beads were pelleted and washed three times with PBS (4°C). The binding proteins were eluted by loading buffer and separated by SDS-PAGE, followed by Western blot analysis probed with specific antibodies. 5′-Biotinylated wild-type sequence was SpAP, 5′-biotin-GTTAGGAGGGCCCCGCCTTCCCCAGG-3′.

Results

Effect of ERK and JNK Inhibitors on EGF-Induced Expression of Keratin 16. EGF has been shown to activate the MAPK signaling pathways in several cell lines. Treatment of HaCaT cells with EGF resulted in a rapid phosphorylation of ERK1/2 (Fig. 1A) and JNK1/2 (Fig. 1B), but not p38 MAPK (data not shown). There were no changes in the levels of total ERK2 and JNK1. To determine whether EGF-induced expression of keratin 16 was mediated by ERK and JNK activation, U0126 and SP600125, which are inhibitors of MEK1 and JNK, respectively, were used. EGF-induced expression of keratin 16 mRNA (Fig. 1C) and protein (Fig. 1D) were inhibited by the treatment of cells with U0126 in a dose-dependent manner. Treatment of cells with U0126 and transfection of cells with ERK2 dominant-negative mutant K52R ERK2 both dose-dependently inhibited the EGF-induced promoter activation of keratin 16 (Fig. 1E). More than 70% of inhibition on EGF-induced keratin 16 expression and promoter activity was observed when cells were treated with

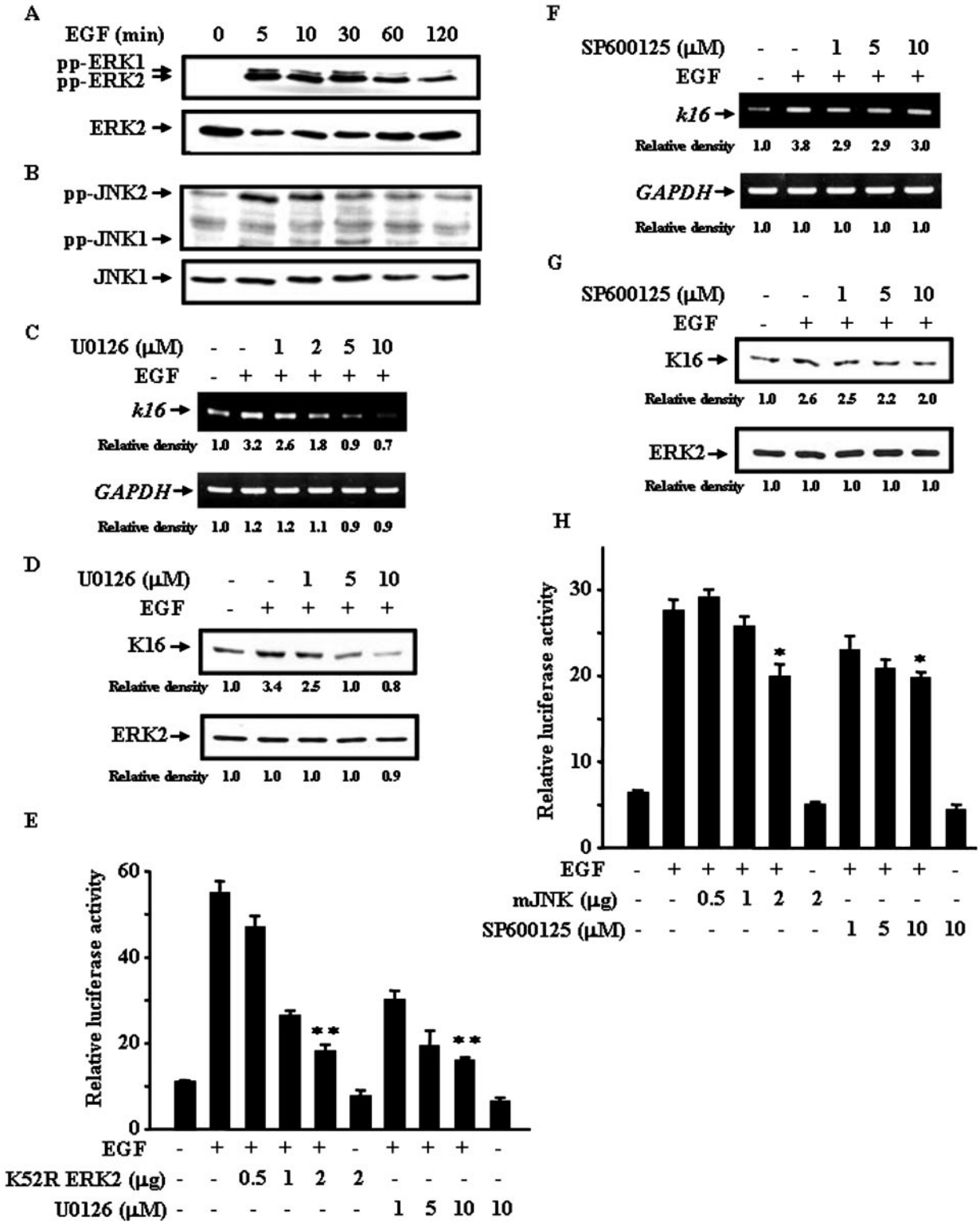

Fig. 1. Effect of U0126 and SP600125 on EGF-induced expression of keratin 16 gene. HaCaT cells maintained in serum-free medium for 24 h were treated with 30 ng/ml EGF for 5 to 120 min. Whole-cell lysates were prepared and subjected to Western blot analysis using antibodies specific for phosphorylated form of ERK (A) and JNK (B) and those against ERK2 and JNK1. Cells maintained in serum-free medium for 2 days were treated with a different amount of U0126 (C) or SP600125 (F) for 30 min followed by EGF treatment for up to 24 h. Reverse-transcription PCR was performed as described under *Materials and Methods*. Cells maintained in serum-free medium for 5 days were treated with a different amount of U0126 (D) or SP600125 (G) for 30 min followed by EGF treatment for up to 48 h. Western blot analysis of keratin 16 protein expression was performed, and ERK2 protein was used as control. The relative density of blots was quantified as indicated. Cells transfected with pXK-5-1 luciferase plasmid by the lipofection method as described under *Materials and Methods* were treated with a different amount of U0126 for 30 min or cotransfected with a different amount of K52R ERK2 (E) and SP600125 for 30 min or mJNK (H) followed by EGF treatment for up to 24 h. The luciferase activities and protein concentrations were then determined and normalized. Values of luciferase activity are means ± S.E. of three determinations. Statistical analyses by comparing with EGF treatment alone were performed by Student's *t* test.

10 μM U0126 and transfected with 2 μg of K52R ERK2. On the other hand, inhibition of the JNK signaling cascade with SP600125 induced a slight decrease in keratin 16 expression after EGF treatment, in the levels of mRNA (Fig. 1F), protein (Fig. 1G) , and promoter activation (Fig. 1H). Less than 30% of inhibition was observed when cells were treated with 10 μM SP600125, and only a minor inhibitory effect of transfection with 2 μg of JNK dominant-negative mutant mJNK on EGF-induced promoter activation was also observed (Fig. 1H). These results indicated that ERK activation by EGF was mainly required for the regulation of keratin 16 gene expression.

Effect of ERK and JNK Inhibitors on EGF-Induced Expression and Phosphorylation of c-Jun. We found previously that Ras-ERK and Ras-Rac-JNK signaling, followed by the activation of c-Jun induction, was essential for mediating EGF-induced gene expression of 12(*S*)-lipoxygenase in human epidermoid carcinoma A431 cells (Chen et al., 2000). Furthermore, we have reported that c-Jun is essential for EGF-induced keratin 16 gene expression in HaCaT cells (Wang and Chang, 2003). We then studied the effect of U0126 or SP600125 treatment on EGF-induced c-Jun expression in HaCaT cells. As shown in Fig. 2A, the expression of c-Jun induced by EGF for 1 h was partly inhibited (approximately 50%) by either 10 μM U0126 or 10 μM SP600125 individually, but it was completely eliminated by a combination of these two inhibitors. These results supported the notion that ERK and JNK activation, during MAPK signaling, were both required for c-Jun induction by EGF. The transactivation activity of c-Jun can be regulated mostly through phosphorylation of two serine residues at Ser63 and Ser73 in the NH_2 terminus (May et al., 1998). Phosphorylation of Ser63 and Ser73 on the c-Jun NH_2-terminal transactivation domain was indeed detected in HaCaT cells treated with EGF (Fig. 2B). To further study the effect of U0126 and SP600125 on c-Jun phosphorylation at Ser63 and Ser73 induced by EGF, we established a stable transfectant of the NR-22 cell line with a truncated form of c-Jun, to rule out the interference of inhibition by these inhibitors in endogenous c-Jun expression induced by EGF in cells. N1-220 represents the expression vector of mutant c-Jun, which lacks the DNA binding domain and leucine zipper domain but contains the transactivation domain of wild-type c-Jun (Fig. 2C). As expected, c-Jun (N1-220) was stably expressed, and no change in it was observed in NR-22 cells treated with EGF or inhibitors. A dose-dependent inhibition with SP600125 on c-Jun phosphorylation at Ser63 and Ser73 induced by EGF was observed, but no apparent effect on the state of phosphorylation of c-Jun could be found in cells treated with U0126 (Fig. 2D). These results suggested that phosphorylation at serine residues Ser63 and Ser73 on the c-Jun NH_2 terminus was mainly through JNK activation, and induction of transcription through c-Jun pro-

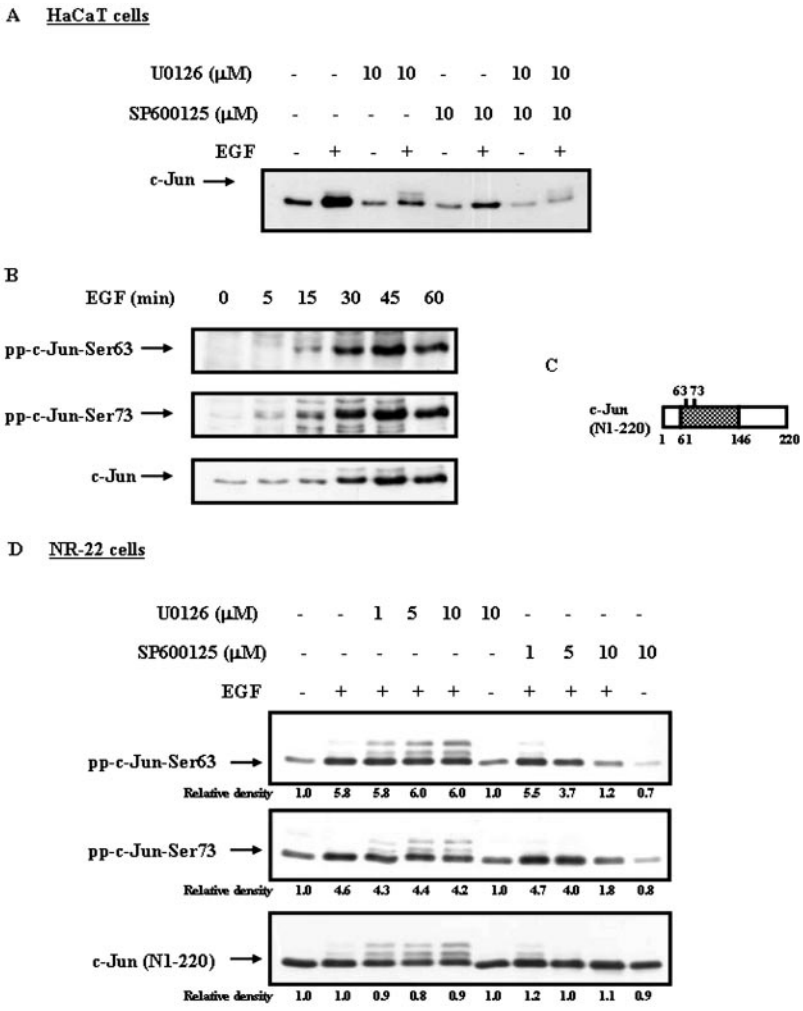

Fig. 2. Effect of U0126 and SP600125 on EGF-induced expression and phosphorylation of c-Jun in HaCaT cells and NR-22 cells. A, HaCaT cells maintained in serum-free medium for 24 h were treated with 10 μM U0126 or SP600125 for 30 min, followed by EGF treatment for 1 h. Western blot analysis of c-Jun expression in nuclear extracts was performed. B, HaCaT cells were starved for 24 h in serum-free medium and then treated with 30 ng/ml EGF for a different time period as indicated. Western blot analysis of c-Jun phosphorylation and expression in nuclear extracts was performed. C, schematic representation of truncated c-Jun, which lacked DNA binding domain and leucine zipper domain of wild-type c-Jun. D, NR-22 cells maintained in serum-free medium for 24 h were treated with a different amount of U0126 or SP600125 for 30 min, followed by EGF treatment for 15 min. Western blot analysis of c-Jun (N1-220) phosphorylation and expression in nuclear extracts was performed. The relative density of blots was quantified as indicated.

moter activation was mainly through ERK pathway, which was consistent with the finding of Gupta et al. (2002).

Essential Role of COOH-Terminal Domain of c-Jun for EGF-Induced Expression of Keratin 16. To investigate the requirement of NH_2-terminal transactivation domain of c-Jun on EGF-induced expression of keratin 16, various expression vectors were used, as shown in Fig. 3A. First, to determine whether the phosphorylation sites on c-Jun at Ser63 and Ser73 were critical for EGF-induced keratin 16 expression, an expression vector, c-Jun-S63/73A, in which Ser63 and Ser73 were both replaced by alanines, was used (Fig. 3A). Transient transfection assays revealed that overexpression of c-Jun mutant c-Jun-S63/73A (Fig. 3B, lanes 7–10) and its respective wild-type c-Jun (Fig. 3B, lanes 3–6) had the same stimulatory effect on keratin 16 promoter activity, both for basal response and for EGF response. Second, to further study whether any other phosphorylation sites besides Ser63 and Ser73 on the c-Jun NH_2-terminal transactivation domain were required for EGF-induced keratin 16 expression, we used the expression vector pMT161, in which the putative phosphorylation sites of c-Jun NH_2 terminus, including Ser58, Thr62, Ser63, Ser73, Thr89, Thr91, and Thr93, were all replaced by alanines (Fig. 3A). Overexpression of c-Jun mutant pMT161 (Fig. 3B, lanes 15–18) had a stimulatory effect on keratin 16 promoter activity, both for basal response and for EGF response, which was also similar to the effect of its respective wild-type c-Jun pMT108 (Fig. 3B, lanes 11–14). These results suggested that NH_2-terminal phosphorylation of c-Jun was not involved in EGF-induced expression of keratin 16. Furthermore, to examine whether

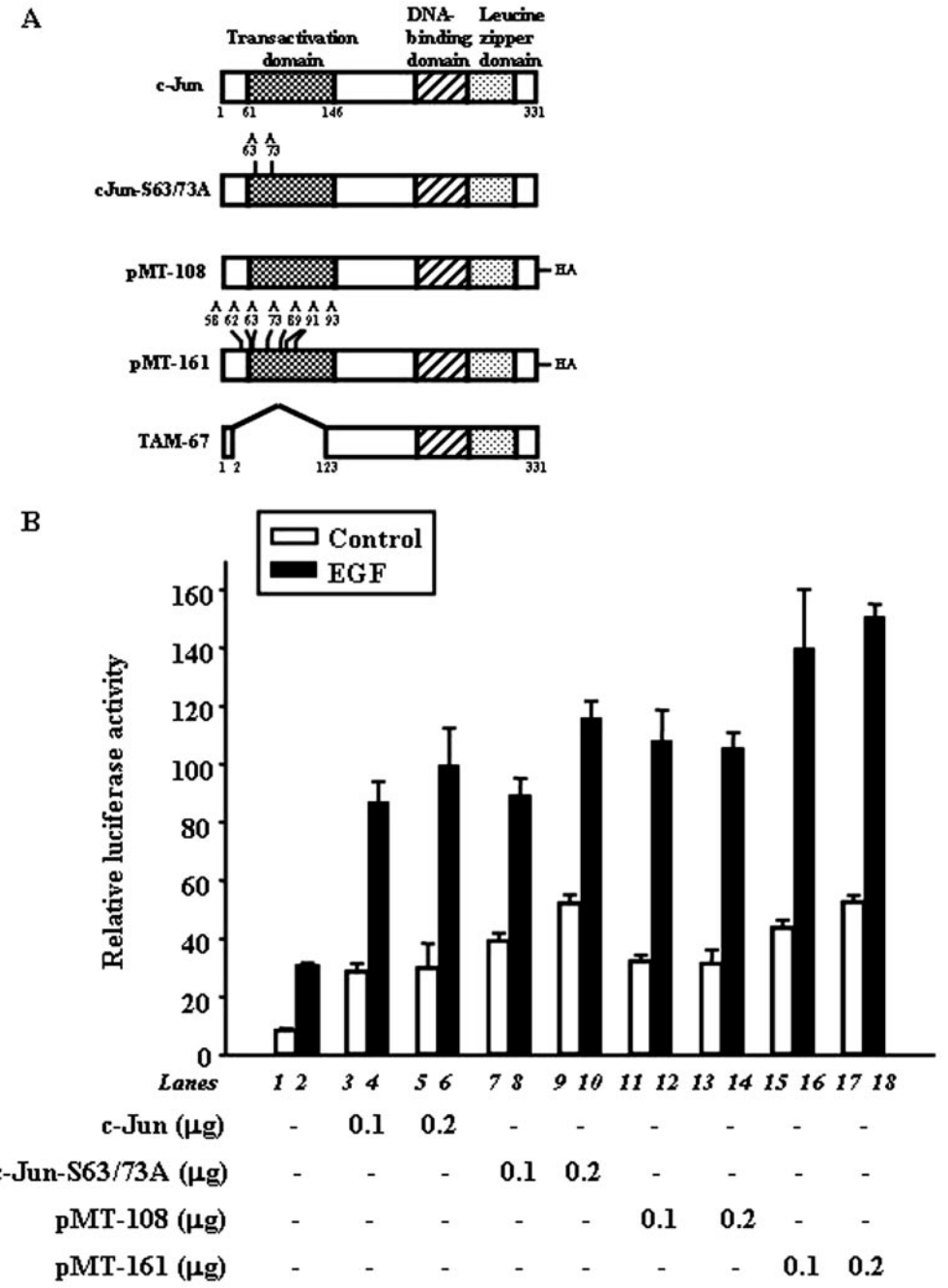

Fig. 3. Analysis of NH_2 and COOH terminus of c-Jun on EGF-induced expression of keratin 16. A, schematic representations of c-Jun and its various mutants. Cells were transfected with pXK-5-1 luciferase plasmid and a different amount of expression vectors encoding wild-type c-Jun (c-Jun and pMT-108), c-Jun mutant (c-Jun-S63/73A and pMT-161; B and E), or truncated c-Jun (TAM-67; D and E) and were treated with EGF for 24 h as indicated. Cells were transfected with pXK-5-1 luciferase plasmid, along with c-Jun (0.5 μg), and a different amount of expression vector TAM-67 as indicated (C). Plasmid transfection was performed as described under *Materials and Methods*. The luciferase activities and protein concentrations were then determined and normalized. Values of luciferase activity are the means ± S.E. of three determinations. Fifty micrograms of transfected cell lysates was subjected to Western blot analysis using antibodies against c-Jun (of D and E). Cells were transfected with either the empty vector pcDNA3.1 (lane 1) or the expression vector TAM-67 (lane 2) and were treated with EGF for 24 h. Nuclear extracts of transfected cells were collected and analyzed by DNA affinity precipitation assay performed as described under *Materials and Methods*. Binding of Sp1, c-Jun, and TAM-67 proteins to the wild-type probe (SpAP) was analyzed by Western blot (F).

the COOH terminus of c-Jun was required for EGF-induced expression of keratin 16, we used the expression vector TAM-67, a truncated form of c-Jun that lacked the NH_2-terminal transactivation domain but contained the COOH-terminal DNA binding domain and leucine zipper domain (Fig. 3A). The promoter activation of keratin 16 induced by c-Jun overexpression was inhibited by TAM-67 in a dose-dependent manner, confirming that TAM-67 indeed played its functional role of dominant-negative mutant of wild-type c-Jun (Fig. 3C). However, transfection of TAM-67 in cells dose-dependently increased EGF-induced promoter activation of keratin 16 (Fig. 3D), which was similar to our previous but unusual results shown in Fig. 3B. We further compared the transactivation activity of full-length vectors (c-Jun and c-Jun-S63/73A) and deletion mutant (TAM-67) of c-Jun upon EGF treatment as shown in Fig. 3E. Transient transfection assays showed that overexpression of TAM-67 had less stimulatory effect than that of full-length c-Jun in unstimulated cells (lanes 9, 11, and 13 were compared with lanes 3, 5, and 7 or lanes 15, 17, and 19), suggesting that c-Jun NH_2 terminus was essential for keratin 16 expression upon basal condition, which was consistent with the inhibitory effects of TAM-67 in Fig. 3C. The EGF response by TAM-67 (5.1- to 6-fold) was even slightly higher than those by the two full-length c-Jun (3- to 5.3-fold). These results strongly suggested that the c-Jun COOH terminus, including the DNA binding domain and leucine zipper domain, was essential and sufficient for EGF induction of keratin 16 gene expression. In addition, the transfected protein expression level of c-Jun (middle, lanes 4, 6, and 8), c-Jun-S63/73A (middle, lanes 16, 18, and 20), and TAM-67 (bottom) was dose-dependently increased from 0.02 to 0.1 μg. To confirm whether TAM-67 could bind to the EGF-responsive region from −162 to −114 bp, an oligonucleotide covering both the Sp1 site (−127 to

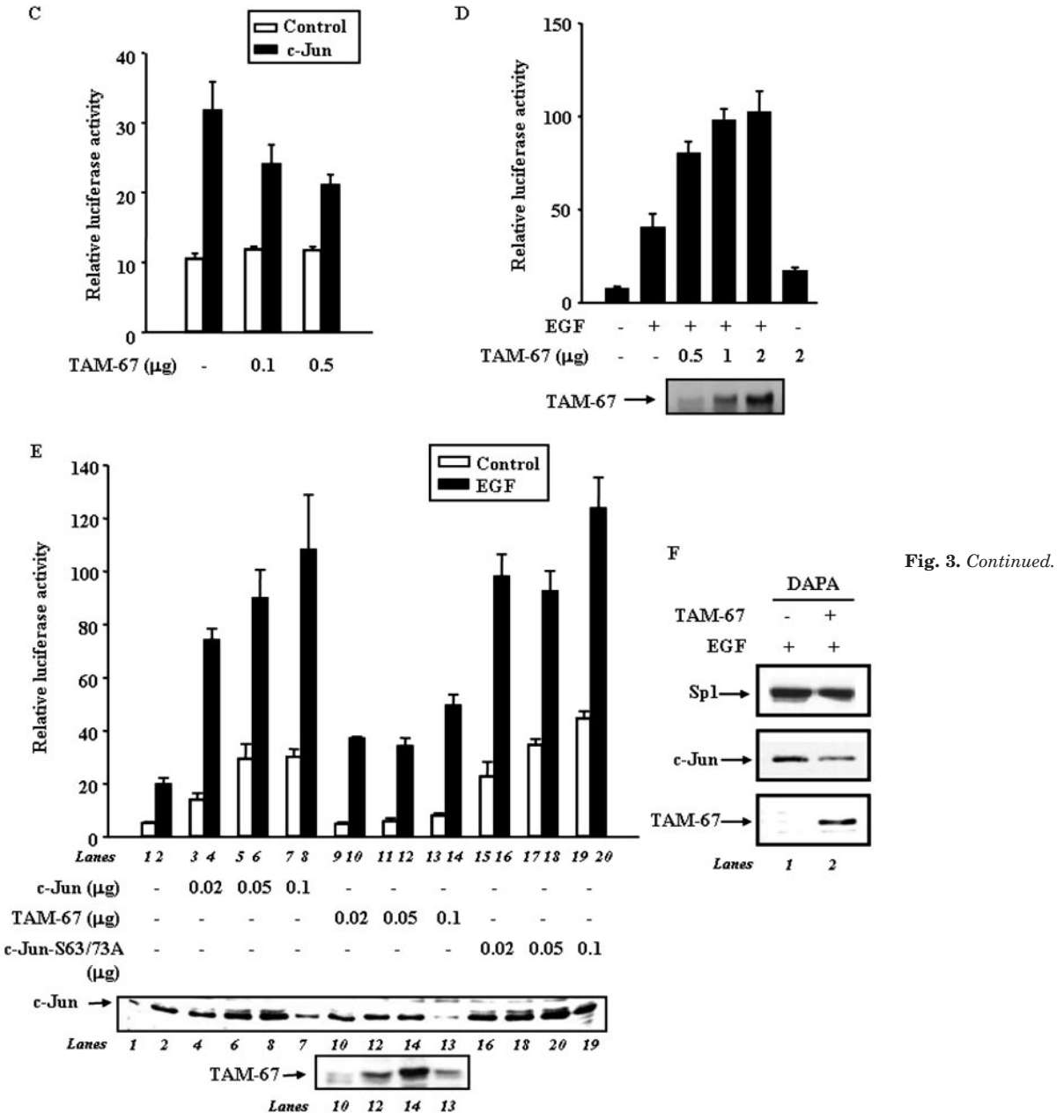

Fig. 3. *Continued.*

−122 bp) and AP1 site (−148 to −142 bp) (SpAP) were synthesized, labeled with biotin, and used as a probe. By using a DNA affinity precipitation assay, which provides quantitative information of transactivator binding, we found that TAM-67 indeed bound to this essential EGF-responsive region and competed the binding of endogenous c-Jun (Fig. 3F, lane 2).

Involvement of Acetylation of the c-Jun COOH Terminus in Regulating Keratin 16 Expression Induced by EGF. Because there was no requirement of c-Jun NH_2-terminal phosphorylation in the keratin 16 regulatory system, we then studied the functional role of the c-Jun COOH terminus. Vries et al. (2001) reported previously that the DNA binding domain of the COOH terminus of c-Jun is acetylated by p300. In this study, we first investigated whether c-Jun is also acetylated in cells upon EGF treatment by using immunoprecipitation assay with c-Jun antibodies. EGF increased the expression of endogenous c-Jun protein in a time-dependent manner as expected (Fig. 4A). As shown in Fig. 4B, a significant enhancement of acetylated lysine of immunoprecipitated c-Jun was detected in cells treated with EGF, and the induction was sustained for at least up to 2 h. To ascertain whether acetylation of c-Jun was involved in this regulatory system, we used the expression vector myc-c-JunK3R, in which three lysine residues, Lys268, Lys271, and Lys273, on the c-Jun DNA binding domain were all mutated into nonacetylatable arginines to maintain the positive charge while preventing acetylation (Fig. 4C). As shown in Fig. 4D, HaCaT cells were cotransfected with c-Jun mutant myc-c-JunK3R and its respective wild-type c-Jun myc-c-Jun, individually. The activation of the keratin 16 promoter by EGF treatment could not be enhanced by overexpression with a different amount of myc-c-JunK3R, distinct from the stimulatory effect of overexpression of wild-type myc-c-Jun. Meanwhile, the transfected protein expression level of myc-c-JunK3R was dose-dependently increased from 0.5 to 1 μg (bottom, lanes 5–8) and was similar to that transfected with 0.5 μg of wild-type myc-c-Jun (lower, lanes 3–6). In addition

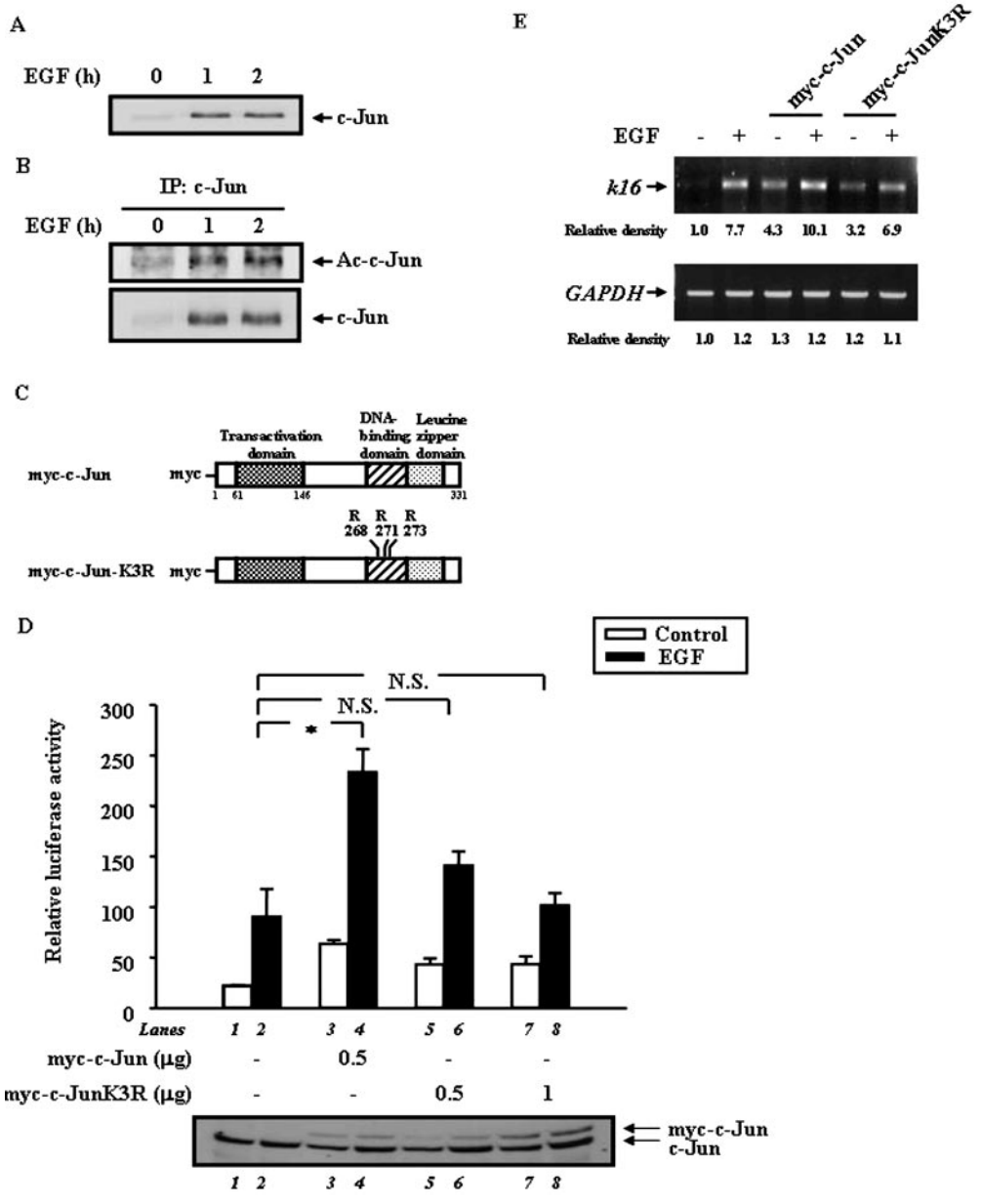

Fig. 4. Effect of c-Jun acetylation in regulating keratin 16 expression. A, cells were starved for 24 h in serum-free medium and then treated with 30 ng/ml EGF for a different time period as indicated. Nuclear extracts (20 μg) were prepared and subjected to Western blot by using antibodies specific for c-Jun. B, nuclear extracts (500 μg) from EGF-treated cells were immunoprecipitated with antibodies against c-Jun bound to protein A-agarose. Immunoprecipitates were subjected to 10% SDS-PAGE followed by Western blot with antibodies against acetyl-lysine and c-Jun, respectively. C, schematic representation of c-Jun mutant that all three lysines in the wild-type c-Jun COOH terminus mutated into arginines. D, cells were transfected with pXK-5-1 luciferase plasmid, along with 0.5 μg of myc-c-Jun or a different amount of myc-c-JunK3R, and were treated with EGF for 24 h as indicated. Plasmid transfection was performed as described under *Materials and Methods*. The luciferase activities and protein concentrations were then determined and normalized. Values of luciferase activity are the means ± S.E. of three determinations. Statistical analysis was performed by Student's *t* test. Values of lane 4, lane 6, and lane 8 were compared with that of lane 2 (top); N.S., not significant. Fifty micrograms of transfected cell lysates was subjected to Western blot analysis using antibodies against c-Jun (bottom). E, cells were transfected with 4 μg of myc-c-Jun or myc-c-JunK3R and were treated with EGF for 24 h as indicated. Reverse-transcription PCR was performed as described under *Materials and Methods*. The relative density of blots was quantified as indicated.

to the reporter gene analysis, reverse-transcription PCR was performed to confirm the effect of myc-c-JunK3R at endogenous keratin 16 mRNA level (Fig. 4E). We found that overexpression of wild-type myc-c-Jun increased the keratin 16 mRNA expression in the absence or presence of EGF treatment. However, the induction of the keratin 16 mRNA by EGF treatment could not be enhanced by the overexpression of myc-c-JunK3R, which was similar to the results in Fig. 4D. These results suggested that acetylation of the c-Jun COOH terminus by EGF treatment might be a part of multiple mechanisms in regulating keratin 16 gene transactivation.

Up-Regulation of p300 Recruitment and Histone H3 Acetylation to the Keratin 16 Promoter through MEK-ERK Activation upon EGF Treatment. In addition to the essential role of c-Jun, the associated proteins and cofactors bound to the keratin 16 promoter with EGF treatment might be another potential activating mechanism. To investigate the dynamics of the chromatin keratin 16 promoter-associated proteins for EGF response, a chromatin immunoprecipitation assay was performed. Chromatin was immunoprecipitated with specific antibodies, and the keratin 16 promoter region, containing the essential Sp1 and AP1 binding sites for promoter activation induced by EGF, was amplified by PCR with the specific primers (Fig. 5A). As shown in Fig. 5B, chromatin immunoprecipitation assays using specific antibodies detected recruitment of p300, acetyl-histone H3 (Ac-H3) and acetyl-histone H4 (Ac-H4) to the chromatin keratin 16 promoter region in unstimulated cells, and EGF treatment resulted in a time-dependent enhancement only in p300 and acetyl-H3 binding. Immunoprecipitation performed with rabbit IgG, which did not show detectable keratin 16 promoter fragment, was used for negative control. To further clarify whether the recruitment of p300 and acetyl-H3 to the keratin 16 promoter was regulated through MAPK signaling pathway in vivo, U0126 and SP600125 were used. We found that an up-regulation of p300 recruitment to chromatin induced by EGF treatment was blocked by U0126, but no apparent inhibitory effect was observed with SP600125 (Fig. 5C). Moreover, the increased binding of acetyl-H3 to the core keratin 16 promoter by EGF stimulation was further analyzed by quantitative real-time PCR. EGF treatment resulted in an increase in the level of acetyl-H3 on the keratin 16 chromatin, reducing the C_T value from 25.6 to 23.6, which corresponded to a 3.5-fold increase in the amount of

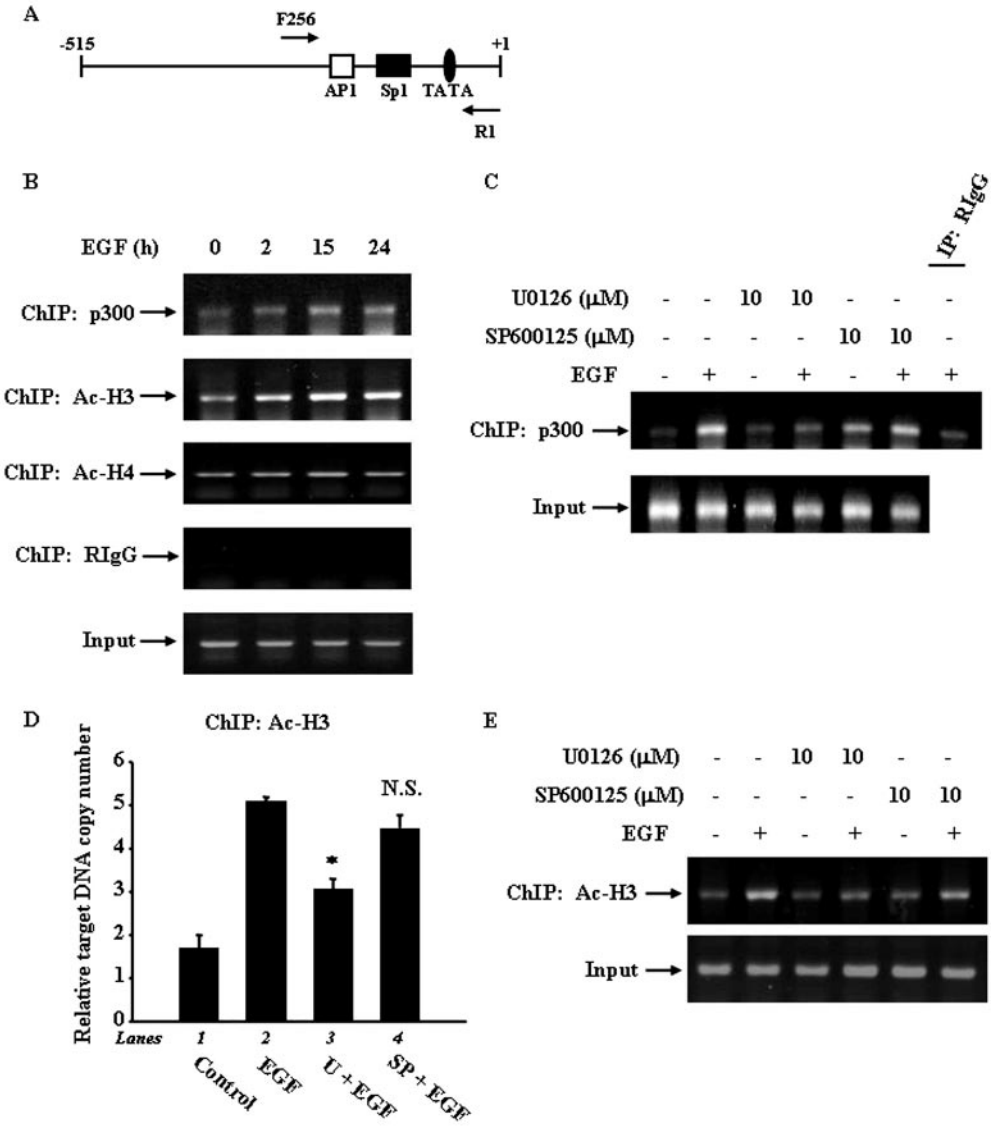

Fig. 5. Chromatin immunoprecipitation assay of p300 recruitment and histone H3 acetylation to the chromatin keratin 16 promoter by EGF treatment. The dynamics of the chromatin keratin 16 promoter-associated proteins for EGF response were analyzed by chromatin immunoprecipitation assay as described under *Materials and Methods*. The primers used were marked by the solid arrows in A. Cells maintained in serum-free medium for 24 h were treated with 30 ng/ml EGF for a different time period as indicated. Chromatin DNA with associated proteins was cross-linked with formaldehyde, sheared, and precipitated by a variety of antibodies. The DNA fragments were then released and amplified by PCR to target specifically the promoter region (B). Cells maintained in serum-free medium for 24 h were treated with U0126 or SP600125 for 30 min followed by EGF treatment for 24 h. Recruitment of p300 protein (C) and acetylation of histone H3 (D and E) to the keratin 16 promoter in vivo were performed using anti-p300 and anti-acetyl-H3 antibodies, respectively. The levels of acetyl-H3 on the keratin 16 chromatin were analyzed by real-time PCR after chromatin immunoprecipitation as described under *Materials and Methods*. The histograms represent the relative target DNA copy number compared with control. Results are expressed as the means ± S.E. in triplicate. Statistical analysis was performed by Student's *t* test. Values of lanes 3 and 4 were compared with that of lane 2; N.S., not significant (D). Final PCR products were separated on 1% agarose-gel electrophoresis and visualized with ethidium bromide (E). Input, 1% of total chromatin used to verify equal loading of chromatin components before precipitation. Immunoprecipitation performed with rabbit IgG (RIgG) was used to serve as negative controls.

acetyl-H3 (Fig. 5D, lane 2). U0126 apparently inhibited this EGF effect at the keratin 16 locus (Fig. 5D, lane 3), whereas SP600125 had no such effect (Fig. 5D, lane 4). Final PCR products were separated by 1% agarose-gel electrophoresis and visualized with ethidium bromide staining as shown in Fig. 5E.

We next used the DNA affinity precipitation assay to evaluate the effect of EGF on p300 and other abovementioned nuclear factors involved in keratin 16 gene expression such as Sp1 and c-Jun binding to a biotinylated keratin 16 promoter sequence. Transcription factors present in the complex were analyzed by Western blot. As shown in Fig. 6A, a significant recruitment of p300 to the probe SpAP was detected in cells after EGF treatment. However, U0126, but not SP600125, blocked the recruitment of p300 to SpAP by EGF (Fig. 6A), whereas no change in endogenous p300 protein expression was found in response to EGF or in combination with the inhibitors (Fig. 6B). Moreover, the binding (Fig. 6A) and protein expression (Fig. 6B) of c-Jun induced by EGF were both significantly inhibited by either U0126 or SP600125 individually, and no changes in Sp1 binding (Fig. 6A) and protein expression (Fig. 6B) were found in the same situation. These results indicated that the effects of U0126 or SP600125 treatment on Sp1 and c-Jun binding by DNA affinity precipitation assay were consistent with those on endogenous Sp1 and c-Jun protein expression. To further confirm the role of MEK-ERK activation on p300 recruitment, cells were transfected with either the expression vector of HA-tagged activated MEK1 (Fig. 6C, lanes 2 and 4) or the empty vector (Fig. 6C, lanes 1 and 3) as control. In the HA-MEK1-transfected cells, no change of endogenous p300 and Sp1 were observed (Fig. 6C, lane 4). However, a significant increase of precipitation of p300 with the SpAP probe was detected (Fig. 6C, lane 2). Taken together, these results strongly suggested that up-regulation of p300 recruitment and histone H3 acetylation to the keratin 16 promoter by EGF was mediated through the MEK-ERK signaling pathway. ERK1/2 could not be recruited to the keratin 16 gene promoter upon EGF stimulation, although an increased recruitment of p300 to the probe SpAP has been observed in cells treated with EGF for 1 h (Fig. 6D, lanes 1–4). It might be that the physical association of substrate (p300) with kinase enzyme (ERK) is quite transient because of the relatively high rates of catalysis and substrate turnover. Thus, little ERK would be expected to be precipitated with DNA.

Functional Role of p300 in the Keratin 16 Gene Expression for EGF Response. To confirm the specificity of the recruitment of p300 to the keratin 16 promoter induced by EGF, an adenoviral oncoprotein E1A, which targets and inactivates p300 (Hu et al., 1999), was used. As shown in Fig. 7A, the recruitment of p300 to SpAP induced by EGF was inhibited by E1A overexpression by using a DNA affinity precipitation assay (lane 2 compared with lane 4), whereas no change in endogenous p300 protein expression was found under the same conditions (lanes 5–8). Meanwhile, the binding of Sp1 and c-Jun to SpAP induced by EGF was not

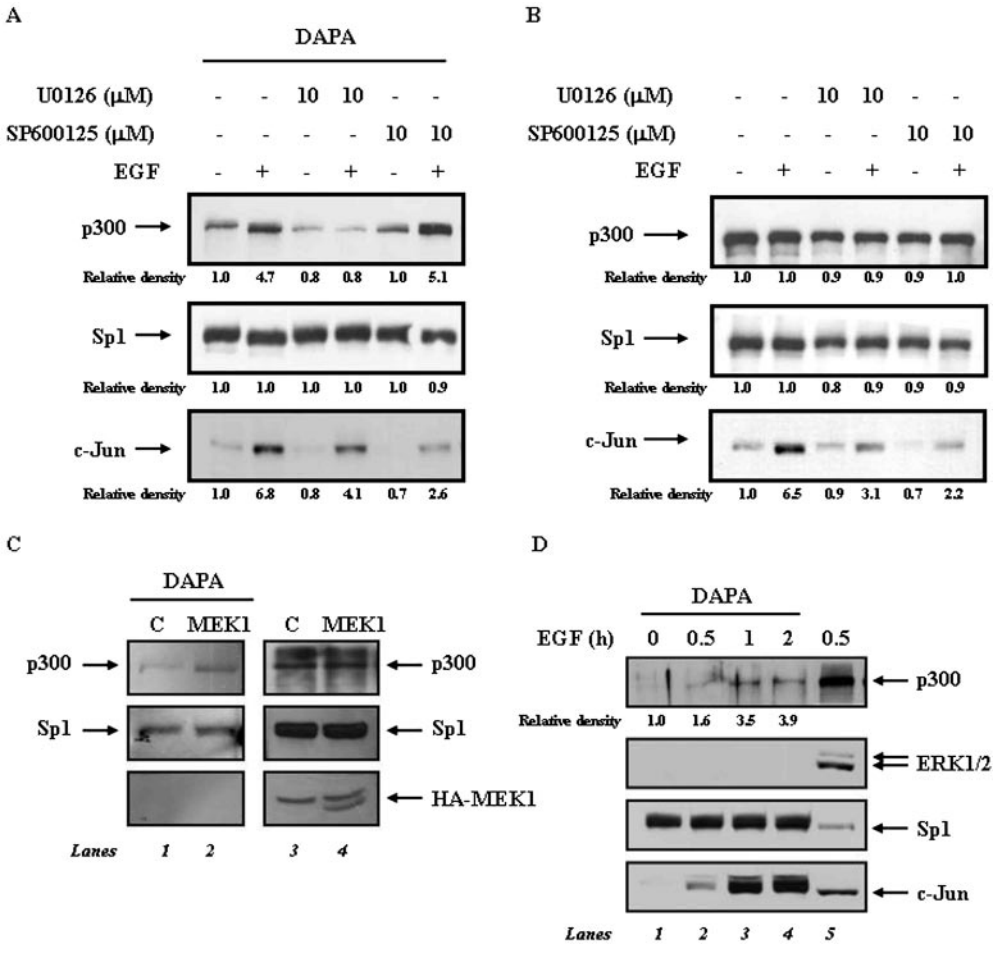

Fig. 6. Effect of MEK-ERK activation on p300 recruitment to the keratin 16 essential promoter region by EGF treatment. A, cells maintained in serum-free medium for 24 h were treated with 10 μM U0126 or SP600125 for 30 min followed by EGF treatment for 24 h. Binding of p300, Sp1, and c-Jun proteins to the wild-type probe (SpAP) was performed by DNA affinity precipitation assay and analyzed by Western blot as described under *Materials and Methods*. B, Western blot analysis of protein expression in nuclear extracts was performed. C, cells were transfected with either the expression vector of HA-tagged MEK1 (lanes 2 and 4) or the empty vector (lanes 1 and 3). Total cell lysates of transfected cells were collected and analyzed with specific antibodies by Western blot (lanes 3 and 4). Binding of p300, Sp1, and HA proteins to the SpAP probe was performed by DNA affinity precipitation assay and analyzed by Western blot (lanes 1 and 2). D, cells maintained in serum-free medium for 24 h were treated with 30 ng/ml EGF for a different time period as indicated. Binding of p300, ERK1/2, Sp1, and c-Jun proteins to the wild-type probe (SpAP) was performed by DNA affinity precipitation assay and analyzed by Western blot as described under *Materials and Methods*. Lane 5 denotes 10% of nuclear extract proteins without assay. The relative density of blots was quantified as indicated.

influenced by E1A overexpression, which presented a similar pattern of endogenous Sp1 and c-Jun protein expression. To further study the functional role of p300 in modulating keratin 16 gene expression for EGF response, the expression vector encoding wild-type p300 (HA-p300) was used. As shown in Fig. 7B, the induction of keratin 16 promoter activity by HA-p300 overexpression (lane 3) was enhanced upon EGF treatment (lane 4); however, the stimulatory effect was dose-dependently blocked by E1A overexpression (lanes 5–7). Moreover, in the cells expressing E1A, the endogenous keratin 16 mRNA induced by EGF was also obviously suppressed (Fig. 7C, compare lane 2 with lane 4). Taking these results together, the inhibitory effect of E1A on EGF-mediated transcription of keratin 16 correlated with the ability of E1A to block the specific recruitment of p300 to the promoter. To explore whether the intrinsic HAT activity of p300 was involved in this EGF regulatory system, we used the expression vector p300ΔHAT, a truncated form of p300 that lacks a HAT domain. As shown in Fig. 7D, overexpression of wild-type p300 augmented keratin 16 promoter activity by EGF stimulation (compare lane 2 with lane 5). Nevertheless, this augmenting effect was greatly attenuated when p300 HAT domain was deleted (compare lane 3 with lane 6). These results suggested that the HAT activity of p300 was crucial for the regulation of keratin 16 transactivation for EGF response.

Discussion

Our previous results indicated that the induction of c-Jun biosynthesis through the Ras signaling pathway in cells is an essential step in EGF-induced expression of keratin 16. In this present work, we further demonstrated that the enhanced recruitment of p300 through ERK activation was the key event on triggering transcriptional activation of keratin 16. We found that ERK activation was the major signaling pathway (more than 70%) in keratin 16 gene expression upon EGF stimulation (Fig. 1). However, the expression of c-Jun induced by EGF treatment was only partly inhibited (approximately 50%) by either U0126 or SP600125 individually (Fig. 2A). Taken together, these results clearly indicated that, in addition to the essential role of c-Jun, there might be another potential activating mechanism in regulating keratin 16 through ERK activation.

By using a chromatin immunoprecipitation assay in vivo (Fig. 5, B and C) and a DNA affinity precipitation assay in

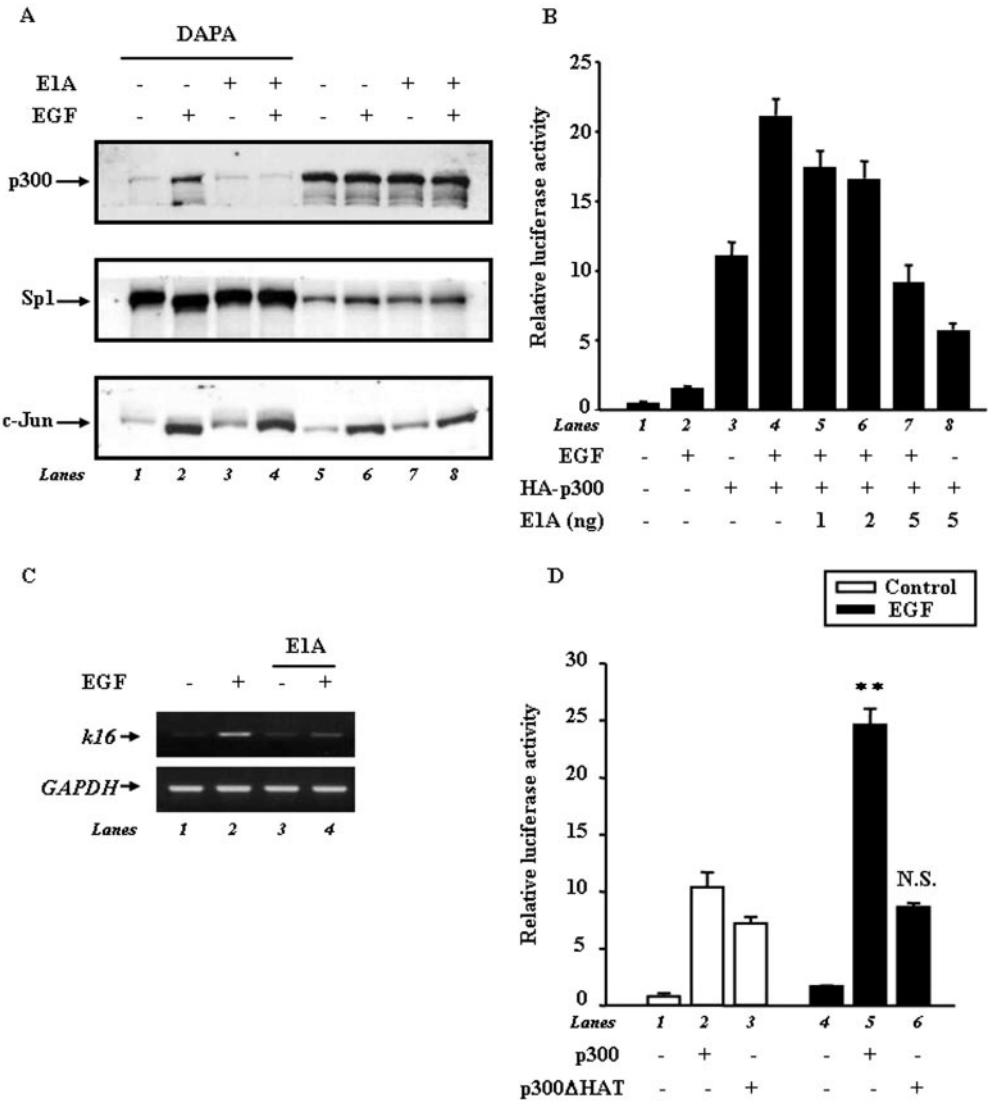

Fig. 7. Functional role of p300 in the keratin 16 gene expression for EGF response. Cells were transfected with the expression vector E1A (4 μg) and were treated with EGF for 24 h as indicated. A, binding of p300, Sp1, and c-Jun proteins to the SpAP probe was performed by DNA affinity precipitation assay and analyzed by Western blot (lanes 1–4). Western blot analysis of proteins expression in nuclear extracts was performed (lanes 5–8). C, reverse-transcription PCR was performed as described under *Materials and Methods*. Cells were transfected with pXK-5-1 luciferase plasmid, along with 1 μg of HA-p300 or a different amount of E1A (B), 1 μg of p300, or p300ΔHAT (D) and were treated with EGF for 24 h as indicated. Plasmid transfection was performed as described under *Materials and Methods*. The luciferase activities and protein concentrations were then determined and normalized. Values of luciferase activity are means ± S.E. of three determinations. Statistical analysis was performed by Student's *t* test. The value of lane 2 was compared with that of lane 5, and the value of lane 3 was compared with that of lane 6; N.S., not significant (D).

vitro (Fig. 6A), p300 recruitment was up-regulated in cells treated with EGF, which correlated with the promoter activation of keratin 16 induced by EGF. On the contrary, EGF-induced p300 recruitment was blocked by the viral oncoprotein E1A (Fig. 7A), which correlated with the repressive effect of E1A on EGF-mediated transcription of keratin 16 (Fig. 7, B and C). Moreover, differential effects of ERK and JNK inhibitors on EGF-mediated p300 recruitment to the keratin 16 promoter were found. U0126 but not SP600125 inhibited the recruitment of p300 to promoter by EGF treatment (Fig. 5, A and C). Taken together, we concluded that up-regulation of p300 recruitment to promoter induced by EGF through the Ras-MEK-ERK signaling cascade was the key event in regulating transcriptional activation of keratin 16.

According to the document reported previously, growth factor-dependent phosphorylation of the coactivator CBP controls its recruitment to the transcription complex and subsequent transcriptional activation (Zanger et al., 2001). Further studies in our system are necessary to elucidate whether the recruitment of p300 to the keratin 16 promoter by ERK activation was also dependent on its phosphorylation level. Several recent reports have suggested that p300/CBP may be phosphorylated by various kinases that are important in either cell-cycle regulation or different signal transduction pathways, and these phosphorylation events have been speculated to affect various p300/CBP activities (Janknecht and Hunter, 1996). Therefore, elucidating the nature of p300/CBP phosphorylation is essential for understanding the regulation of p300/CBP function and its target gene transcription. It has been reported previously that serine 89 with the NH_2 terminus of p300 has been shown to be phosphorylated by protein kinase C in vivo (Yuan and Gambee, 2000). The transcriptional activity of p300 is stimulated by phenylephrine through p42/p44 MAPK cascade (Gusterson et al., 2002), and the COOH-terminal transactivation domain (amino acids 1572–2370) of p300 is phosphorylated by MAPK in vitro (Sang et al., 2003). However, it is still unclear for the most part which kinases are responsible for p300 phosphorylation in vivo upon growth-factor treatment. More importantly, the functional links between the specific phosphorylation events and the downstream gene regulations remain largely unknown.

Another interesting finding from this study was that phosphorylation of serine/threonine residues in the NH_2-terminal transactivation domain of c-Jun, which is well known to be a critical role for c-Jun transcriptional activation (Karin et al., 1997), was not required in regulating keratin 16 gene expression by EGF. With the transient transfection assays in HaCaT cells, overexpression of the putative NH_2-terminal phosphorylation site-mutated c-Jun mutants such as c-Jun-S63/73A (Fig. 3B, lanes 7–10) and pMT161 (Fig. 3B, lanes 15–18) had significant effects on keratin 16 promoter activity for basal response and for EGF response, which were also similar to the effects of its individual wild-type c-Jun (Fig. 3B, lanes 3–6) and pMT108 (Fig. 3B, lanes 11–14). Furthermore, transfection of the NH_2 terminus truncated-c-Jun TAM-67 in cells also increased EGF-induced promoter activation of keratin 16 in a dose-dependent manner (Fig. 3, D and E). Evidence obtained from these results supported the notion that the COOH-terminal domain of c-Jun played an essential role for EGF-induced expression of keratin 16. In our system, the increased expression of c-Jun might be more important than the direct phosphorylation of the protein, which was similar to the study of cyclooxygenase-2 (Chen et al., 2005) and vimentin gene expression (Wu et al., 2003). In addition, our study indicated that acetylation of the c-Jun COOH terminus by EGF treatment might be involved in regulating keratin 16

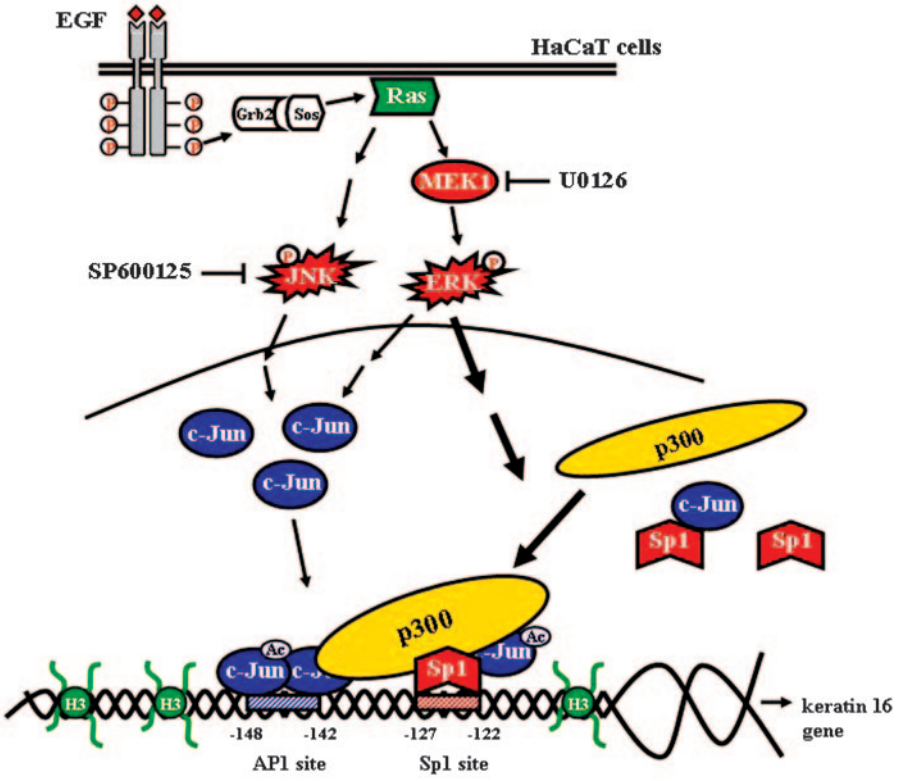

Fig. 8. Possible model for c-Jun activation and p300 recruitment through the MAPK pathway in EGF-induced keratin 16 gene expression. A diagram of transcription factor activation and coactivator recruitment by EGF treatment of the keratin 16 promoter described in this text is shown. EGF stimulated ERK and JNK activation, followed by the biosynthesis of c-Jun. ERK activation-dependent recruitment of the coactivator p300 to gene promoter might sequentially lead to acetylation of c-Jun and histone H3 and played a pivotal role in EGF-induced expression of keratin 16 gene.

transactivation (Fig. 4). The acetylation of transcription factors and factors related to transcription has been shown to be an important regulatory mechanism of transcription (Shikama et al., 1997). Levy et al. (2004) reported previously that acetylation of β-catenin by p300 regulates nuclear β-catenin/Tcf4 complex interaction. Therefore, it might be likely that acetylation of the c-Jun COOH terminus remained more important to recruit the transcription apparatus in driving keratin 16 gene expression upon EGF treatment.

In the studies of gene regulation of keratin 16 in cells upon EGF treatment, the functional role of nuclear protein Sp1, in addition to p300 and c-Jun, could not be ignored. It has been reported that p42/p44 MAPK directly phosphorylates Sp1 on threonines 453 and 739 both in vitro and in vivo and then increases Sp1 binding activity and transcriptional efficiency of the vascular endothelial growth factor promoter in a derivative of fibroblasts stably expressing an estrodiol-inducible Raf-1 (Milanini et al., 2002). Moreover, EGF stimulates gastrin promoter through the activation of Sp1 kinase activity (Chupreta et al., 2000). EGF-stimulated a*poA-I* gene expression is mediated solely through the Ras-MAPK cascade, and enhanced activity of this pathway requires Sp1 with an intact phosphorylation site at Thr266 in human hepatoma (Zheng et al., 2001). Whether any post-translational modification of Sp1 by EGF treatment occurs and then promotes keratin 16 gene activation by protein-protein interaction with other nuclear factors such as p300 remains to be elucidated.

Although the regulation of Sp1, c-Jun, and c-Fos by signaling pathways initiated by EGF has been defined in several culture cell systems, including our previous report on gene regulation of 12(*S*)-lipoxygenase in human epidermoid carcinoma A431 cells (Chen and Chang, 2000), the molecular interaction mechanism among the transcription factors might be different depending on the specificity of genes and cells studied. We found previously that the direct interaction between Sp1 and c-Jun induced by EGF (Chen and Chang, 2000) and phorbol 12-myristate 13-acetate (Chen et al., 2002) cooperatively activated expression of the 12(*S*)-lipoxygenase gene and that Sp1 may function as an anchor protein to recruit c-Jun to the promoter, thus activating the transcriptional activity of the 12(*S*)-lipoxygenase gene. In the present study, we compared the effect of the c-Jun dominant-negative mutant TAM-67 on the gene regulation of 12(*S*)-lipoxygenase in A431 cells and keratin 16 in HaCaT cells. We found that overexpression of TAM-67 in cells attenuates the gene expression of 12(*S*)-lipoxygenase but stimulates that of keratin 16 after EGF treatment (Fig. 3D). The model of 12(*S*)-lipoxygenase gene seems to be different from that of keratin 16 because neither the known AP1-binding sequence in the promoter region responsive to EGF nor the apparent binding between transcription factor AP1 and EGF-responsive promoter DNA was observed in the human 12(*S*)-lipoxygenase gene (Liu et al., 1997).

Taken together with our previous findings (Wang and Chang, 2003), the signal transduction and gene regulation of EGF-induced gene expression of keratin 16 in HaCaT cells can be summarized in the proposed model shown in Fig. 8. EGF stimulated ERK and JNK activation, followed by the induction of c-Jun biosynthesis. The endogenous nuclear Sp1 acted as an anchor protein to recruit c-Jun to the gene promoter of keratin 16. EGF also stimulated the p300 recruitment to the gene promoter of keratin 16 through ERK signal activation. Because overexpression of cells with E1A completely blocked the EGF-induced p300 recruitment and keratin 16 gene activation, our results indicated that the ERK-mediated p300 recruitment played a pivotal role in EGF-induced keratin 16 gene expression. p300 then interacted with the transcription factor complex of c-Jun and Sp1 and activated the transcription machine at least in part through the acetylation of c-Jun and histone H3. The three transcription factors, c-Jun, Sp1, and p300, cooperated with each other in regulating keratin 16 gene expression in keratinocytes.

Acknowledgments

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