ERK2-mediated C-terminal Serine Phosphorylation of p300 Is Vital to the Regulation of Epidermal Growth Factor-induced Keratin 16 Gene Expression*1

Received for publication, January 10, 2007 and in revised form, July 3, 2007 Published, JBC Papers in Press, July 9, 2007, DOI 10.1074/jbc.M700264200

Yun-Ju Chen‡§, Ying-Nai Wang¶, and Wen-Chang Chang†‡¶

From the ‡Department of Pharmacology, ¶Institute of Basic Medical Sciences, College of Medicine, †Center for Gene Regulation and Signal Transduction Research, National Cheng Kung University, Tainan 701, Taiwan and the §Department of Molecular and Cellular Oncology, University of Texas MD Anderson Cancer Center, Houston, Texas 77030

We previously reported that the epidermal growth factor (EGF) regulates the gene expression of keratin 16 by activating the extracellular signal-regulated kinase 1 and 2 (ERK1/2) signaling which in turn enhances the recruitment of p300 to the keratin 16 promoter. The recruited p300 functionally cooperates with Sp1 and c-Jun to regulate the gene expression of keratin 16. This study investigated in detail the molecular events incurred upon p300 whereby EGF caused an enhanced interaction between p300 and Sp1. EGF apparently induced time- and dose-dependent phosphorylation of p300, both in vitro and in vivo, through the activation of ERK2. The six potential ERK2 phosphorylation sites, including three threonine and three serine residues as revealed by sequential analysis, were first identified in vitro. Confirmation of these six sites in vivo indicated that these three serine residues (Ser-2279, Ser-2315, and Ser-2366) on the C terminus of p300 were the major signaling targets of EGF. Furthermore, the C-terminal serine phosphorylation of p300 stimulated its histone acetyltransferase activity and enhanced its interaction with Sp1. These serine phosphorylation sites on p300 controlled the p300 recruitment to the keratin 16 promoter. When all three serine residues on p300 were replaced by alanine, EGF could no longer induce the gene expression of keratin 16. Taken together, these results strongly suggested that the ERK2-mediated C-terminal serine phosphorylation of p300 was a key event in the regulation of EGF-induced keratin 16 expression. These results also constitute the first report identifying the unique p300 phosphorylation sites induced by ERK2 in vivo.

Keratins, which are the most prominent cytoskeletal proteins in keratinocytes, belong to a large family of ~30 epithelially specific intermediate filament proteins that form the cytoskeleton (1). Among these, keratin 16 is usually referred to as an activation- and hyperproliferation-associated keratin because it is reported to be the marker in the hyperproliferated skin diseases (2, 3). Furthermore, whereas 10% of the invasive breast carcinomas are positive, in either a diffuse or focal pattern, with the keratin 16 antibodies, normal breast tissue and noninvasive breast carcinomas are nearly negative with the keratin 16 antibodies (4). A recent study indicates that keratin 16 is induced in human papilloma virus-infected tissues at the transcriptional level, and more importantly, the induction leads to the proliferation of keratinocytes, a known characteristic of human papilloma virus infection (5). Therefore, a full understanding of the gene regulation of keratin 16 might contribute to therapies beneficial to those hyperproliferated diseases.

It is reported that p44/p42 mitogen-activated protein kinases (MAPKs), 2 also known as extracellular signal-regulated kinase 1 and 2 (ERK1/2), phosphorylate specific substrates, including transcription factors like Elk-1 (6, 7) and coactivators such as p300/CBP (8, 9). Once activated, MAPKs directly phosphorylate proteins containing the minimal consensus phosphoacceptor motif Ser/Thr-Pro (10). In addition to the MAPK phosphoacceptor consensus sites, it is becoming increasingly clear that in many cases, additional determinants, known as docking domains, are required to specify for the MAPK substrates (11). MAPKs bind directly to the docking domain on the substrate or bind indirectly to the substrate through an adaptor possessing the docking domain (10).

p300, as well as its related protein CBP, are well known transcriptional coactivators. Both play pivotal roles in coordinating and integrating multiple signal-dependent events with the transcription machinery and participate in diverse physiological processes, including development, proliferation, differentiation, and apoptosis (12, 13). Several functional domains of p300/CBP, such as CH1 and CH3 domains, are important for protein-protein interactions (12). To date, the versatile coactivators p300/CBP enhance gene transcription mainly via three mechanisms (12). First, they create a bridge between the transcription factors and basal transcription machinery. Second,

* This work was supported in part by Grant NSC 95-2320-B-006-025 from the National Science Council of Republic of China and by the National Cheng Kung University Program for Promoting Academic Excellence and Developing World Class Research Centers. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S4.

§ To whom correspondence should be addressed. Tel: 886-6-235-3535, ext. 5496; Fax: 886-6-274-9296; E-mail: wcchang@mail.ncku.edu.tw.

2 The abbreviations used are: MAPK, mitogen-activated protein kinase; EGF, epidermal growth factor; WT, wild type; ERK, extracellular signal-regulated kinase; PKC, protein kinase C; CBP, CAMP-response element-binding protein-binding protein; aa, amino acid; PBS, phosphate-buffered saline; HAT, histone acetyltransferase; HA, hemagglutinin; siRNA, short interfering RNA; GST, glutathione S-transferase; DAPA, DNA affinity precipitation assay.
they act as a scaffold for the assembly of multiprotein complexes, including transcription factors and cofactors through their CH1, CH3, and KIX domains, etc. The simultaneous interaction between p300/CBP and multiple transcription factors is thought to contribute to the transcriptional synergy. For example, p300 cooperates with Sp1 to regulate p21<sup>wafl/cip1</sup> promoter activation (14). In addition, it is reported that the acetyltransferase region of p300 interacts with the DNA-binding domain of Sp1 to regulate the DNA binding activity of Sp1 (15). Third, p300/CBP possesses intrinsic histone acetyltransferase (HAT) activity, which acetylates transcription factors and histones and thus activates transcription (16).

Recently, many studies have reported that p300 and CBP are direct targets of cellular signaling, resulting in their post-translational modifications (17). Of these modifications, phosphorylation is extensively studied especially in vitro. For example, activated ERK2 phosphorylates GST-p300 (aa 1572–2370) in vitro, and as a result, the transcriptional activity of p300 is enhanced (9). The C-terminal phosphorylation of CBP by ERK1 stimulates its HAT activity in vitro (18, 19). Nonetheless, it is still not entirely clear how phosphorylation may regulate the p300 or CBP activity in vivo. Basically, it is not known which kinases are responsible for the p300/CBP phosphorylation in vivo, nor is it known exactly where the phosphorylation sites may take place. Most important of all, the functional links between the specific phosphorylation events and the downstream gene regulation remain largely unknown. Up to this point, very few phosphorylation sites on p300/CBP have been identified in vivo. These identified phosphorylation sites from in vivo studies are described as follows. The PKC or AMP-activated protein kinase-mediated Ser-89 phosphorylation of p300 attenuates its HAT activity or its interaction with individual nuclear receptors (20–22). The growth factor-induced Ser-436 phosphorylation of CBP through phosphatidylinositol 3-kinase–PKC pathway controls the recruitment of CBP to the transcription complex (23). The Ser-301 phosphorylation of CBP by calmodulin kinase IV mediates the CBP-dependent transcriptional activation (24). Moreover, the Akt-mediated Ser-1834 phosphorylation of p300 increases the p300 recruitment to the ICAM-1 promoter and enhances its associated HAT activity, resulting in the transcriptional activation of ICAM-1 gene (25). We have previously proposed a model for the transcriptional regulation of keratin 16 (26, 27). We suggest that the epidermal growth factor (EGF) up-regulates the recruitment of coactivator p300 to the promoter of keratin 16 through the activation of ERK, the last being of critical importance for the EGF regulation of keratin 16. We also show that the recruited p300 functionally cooperates with Sp1 and c-Jun to enhance the gene expression of keratin 16. Furthermore, the HAT domain of p300 is absolutely required for the keratin 16 promoter activation incurred upon EGF treatment. Therefore, the aim of this work was to study how the ERK activation regulated the recruitment of p300, thereby activating the keratin 16 gene expression. We found that the ERK2-mediated C-terminal serine (Ser-2279, Ser-2315, and Ser-2366) phosphorylations of p300 were the major targets by EGF, resulting in the stimulation of its HAT activity and its interaction with Sp1. These ERK2-mediated serine phosphorylation sites on p300 apparently controlled the p300 recruitment to the keratin 16 promoter, thus explaining the activation of the transcriptional activity of keratin 16 caused by EGF. Furthermore, this study was the first to identify the exact ERK2 phosphorylation sites on p300 in vivo.

**EXPERIMENTAL PROCEDURES**

**Materials**—Human EGF (natural, culture grade) was purchased from PePro Technology (Rocky Hill, NJ). U0126, an inhibitor of MEK1, and the luciferase assay system were from Promega (Madison, WI). TRizol RNA extraction kit, SuperScript<sup>™</sup> III, Dulbecco’s modified Eagle’s medium, Opti-MEM medium, Sp1-specific siRNA, and control siRNA were obtained from Invitrogen. Recombinant p300 was purchased from Active Motif (Carlsbad, CA). Polyclonal antibodies against ERK1/2, monoclonal antibody against phosphoserine/threonine-nine-proline (phospho-Ser/Thr-Pro), recombinant histone H3, HA-p300 expression vector, protein A-agarose/salmon sperm DNA, and HAT assay kit were from Upstate Biotechnology, Inc. (Lake Placid, NY). Recombinant activated ERK2 and inactivated ERK2 were obtained from Biomol (Plymouth Meeting, PA). Biotinylated oligonucleotides were purchased from MDBio, Inc. (Taipei, Taiwan). Arrest-In reagent was from Open Technology (Taipei, Taiwan). Pfu-turbo polymerase was obtained from Stratagene (La Jolla, CA). Streptavidin-agarose was purchased from Sigma. Monoclonal antibodies against ERK2, c-Jun, and p300 were obtained from BD Transduction Laboratories. Agarose conjugated to p300 and Sp1, polyclonal antibodies against Sp1, monoclonal antibody against GST, normal mouse IgG, and normal rabbit IgG were from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal antibodies directed against acetyl-lysine, phospho-ERK1/2, and agarse conjugated to phospho-ERK1/2 were purchased from Cell Signaling Technology (Beverly, MA). Antibodies against HA and affinity matrix-conjugated to HA were from Roche Applied Science. 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<sub>2</sub> 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. A431 cells, a human epidermoid carcinoma cell line, were grown in the same condition as HaCaT cells. In this series of experiments, both cells were treated with 30 ng/ml EGF in optimal serum-free conditions, unless stated otherwise.

**Immunoprecipitation Assay**—Nuclear extracts and cell lysates were prepared as described before (27). Nuclear extracts (80–1000 μg of protein of each) or lysates (1000–1800 μg of protein of each) were immunoprecipitated with 20 μl of p300 antibodies agarose-conjugated, phospho-ERK1/2 antibodies agarose-conjugated, Sp1 antibodies agarose-conjugated, or HA antibodies affinity matrix-conjugated individually in immunoprecipitation buffer (20 mM Hepes (pH 7.9), 2 mM MgCl<sub>2</sub>, 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 500 μl of washing buffer (1× PBS containing 0.5% Nonidet P-40).
Protein was removed from the beads by boiling in 2× SDS loading buffer for 5 min and separated by SDS-PAGE, followed by Western blot analysis, as described before (27), probed with phospho-Ser/Thr-Pro antibodies, or as indicated.

**In Vitro Kinase Assay**—The commercially obtained full length of recombinant p300 protein was incubated at 30 °C for 30 min with recombinant activated or inactivated ERK2 in the presence of 23.3 μM cold ATP in MAPK buffer (25 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 5 mM glycerol phosphate, 2 mM dithiothreitol, and 0.1 mM Na₃VO₄). The reaction was stopped by adding an equal volume of 5× SDS loading buffer and by heating at 95 °C for 5 min. After separation through 10% SDS-PAGE, the gel was transferred and followed by Western blot analysis probed with phospho-Ser/Thr-Pro antibodies.

**In Vitro Acetylation Assay and Nonradioactive Histone Acetyltransferase Activity Assay**—The commercially obtained full length of recombinant p300 protein was subjected to an in vitro kinase assay, as described previously, and then mixed with purified 0.5 μg of histone H3 and 2 μg of acetyl-CoA in the presence of acetylation assay buffer (50 mM Tris–HCl (pH 8.0), 10% glycerol, 0.1 mM EDTA, and 1 mM dithiothreitol) and incubated at 30 °C for 30 min. In addition, the immunoprecipitates of HA-p300-WT or HA-p300-S3A with or without EGF treatment were subjected to this in vitro acetylation assay. The reaction was stopped by adding an equal volume of 5× SDS loading buffer and by heating at 95 °C for 5 min. After separation through a 12% SDS-PAGE, the gel was followed by Western blot analysis probed with acetyl-lysine antibodies. Nonradioactive HAT activity assay was performed by using a HAT assay kit according to the manufacturer’s instruction. The commercially obtained full length of recombinant p300 protein was subjected to an in vitro kinase assay as described previously, and nuclear extracts with HA-p300-WT (wild type) or HA-p300-S3A (with Ser-2279, Ser-2315, and Ser-2366 all replaced by alanine) were transfected with plasmids by lipofection using Arrest-In (DNA:Arrest-In = 1:5) was incubated with 0.2 μg of pXK-5-1 luciferase plasmid that contained the EGF-responsive region of the keratin 16 gene (26) or the indicated plasmids as described in each experiment in 60 μl of Opti-MEM medium for 10 min at room temperature. Total DNA concentration for each transfection was matched with HA-pcDNA3.0. Cells were transfected by changing the medium with 1 ml of Opti-MEM medium containing the plasmids and Arrest-In, followed by incubation at 37 °C in a humidified atmosphere of 5% CO₂ for 5 h. A change of Opti-MEM medium to 2 ml of fresh culture medium and overnight incubation, cells were stimulated with EGF if necessary and then incubated for an additional 24 h. For cells transfected in a 6-cm plastic dish, 2 μg of indicated plasmids as described in each experiment were transfected in 2 ml of Opti-MEM medium, and the residual transfection procedure was similar to that as described above. The luciferase activities in cell lysates were measured by the luciferase assay system and determined as described previously (28). Luciferase activity was normalized per μg of extract protein.

**DNA Affinity Precipitation Assay (DAPA)**—The binding assay was performed by mixing nuclear extract proteins, 2 μg of biotinylated keratin 16-specific wild type oligonucleotide, and 20 μl of streptavidin-agrose 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 cold 1× PBS. The binding proteins were eluted by 2× SDS loading buffer and separated by SDS-PAGE, followed by Western blot analysis probed with specific antibodies. 5′-Biotinylated wild type sequence was SpAp (where SpAp is a wild type sequence containing Sp1- and Ap1-binding sites), 5′-biotin-GTATAGAGGCGCCCCCTTCCTCCAGG-3′ (27).

**Chromatin Immunoprecipitation Assay**—Stable transfectants of HaCaT cells with HA-p300-WT or HA-p300-S3A expression were treated with or without inhibitors for 30 min, followed by EGF treatment for 24 h. Cells were cross-linked with 1% formaldehyde at room temperature for 15 min, washed once with 1× PBS, lysed with L1 buffer (50 mM Tris (pH 8.0), 2 mM EDTA, 0.1% (v/v) Nonidet P-40, 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 500–1000 bp. One μg of sonicated extracts was diluted 10-fold with dilution buffer (50 mM Tris (pH 8.0), 0.5 mM EDTA, 0.5% (v/v) Nonidet P-40, and 0.2 mM NaCl), followed by incubation with 40 μl of protein A–agarose/salmon sperm DNA at 4 °C for 30 min for pre-cleaning. Immunoprecipitation was performed with specific antibodies and rotating at 4 °C overnight, followed by adding 40 μl of protein A–agarose/salmon sperm DNA at 4 °C for 1.5 h. Immunoprecipitated beads were pelleted and washed with high salt and low salt washing buffer each four 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/reaction proteinase K at 50 °C for 1 h. DNA was extracted once with phenol/chloroform, precipitated with ethanol, and dissolved in 45 μl of H₂O. 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. The PCR product was separated by 1.5% agarose gel electrophoresis and visualized with ethidium bromide staining (27).

**Construction and Purification of GST-p300 Fusion Proteins**—To create various GST-p300 fragments, PCR-generated fragments encoding amino acids 2–331, 302–670, 565–966, 952–1145, 1140–1570, 1570–1968, 1964–2197, and 2188–2375 of human p300 were inserted into the Sall/NotI sites of...
pGEX4T-1 individually. In addition, these constructs were subjected to QuikChange® XL site-directed mutagenesis kit for obtaining single point or triple points mutations of GST-p300 fragments. *Escherichia coli* containing expression vectors of respective GST-p300 fragment were cultured in LB medium and induced with 1 mm isopropyl β-D-thiogalactopyranoside for 3–6 h at 18–37 °C as indicated. Cells were harvested, and incubated in 1× PBS buffer containing 2 μg/ml lysozyme, 1 mm EDTA, 5 mm dithiothreitol, 1 mm phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, and 0.5 μg/ml pepstatin A at 37 °C for 30 min. After sonication, samples were centrifuged at 7,500 × g for 5 min, and the supernatant was then incubated with glutathione-Sepharose™ 4B at 4 °C for 2 h to overnight as indicated, followed by elution with elution buffer (50 mm Tris (pH 9.6), 20 mm glutathione, and 1 mm EDTA) at 4 °C for 30 min twice. These elutions were stored at −70 °C until used.

**GST Pulldown Assay**—Equal molar concentrations of GST or GST-Sp1 fusion protein were incubated with 250 ng of recombinant p300 protein at 4 °C overnight. The p300 protein that interacted with GST-Sp1 was precipitated with glutathione-Sepharose™ 4B. The reaction was stopped by boiling for 5 min in 2× SDS loading buffer and analyzed by Western blot probed with p300 and GST antibodies.

**Reverse Transcription-PCR**—HaCaT cells with stable expression of HA-p300-WT or HA-p300-S3A maintained for 2 days in serum-free medium were incubated with EGF for 24 h and then harvested. Total RNA was isolated by using the TRizol RNA extraction kit, and 2 μg of RNA were subjected to reverse transcription-PCR with SuperScript™III. Specific primers for keratin 6, 16, 17 and glyceraldehyde-3-phosphate dehydrogenase were used. The PCR products were separated by 1% agarose-gel electrophoresis and visualized with ethidium bromide staining.

**RESULTS**

**ERK2-dependent Phosphorylation of p300 by EGF**—Several studies indicate that direct phosphorylation of p300 or CBP controls their recruitment to the transcription complex (23, 25). In addition, our previous results indicate that the ERK activation regulates the recruitment of p300 to the keratin 16 promoter (27). Therefore, we studied here whether ERK caused the phosphorylation of p300. At first, we studied the interaction between p300 and ERK by coimmunoprecipitation with p300 antibodies. As shown in Fig. 1A, significant bind-
ings of phospho-ERK2 and ERK2 to p300 in the nucleus were observed in HaCaT cells treated with EGF (IP, compare lane 2 with lane 1). Furthermore, these interactions induced by EGF were blocked by U0126, a specific MEK1 inhibitor (Fig. 1A, IP, compare lane 4 with lane 2), which presented a similar pattern of endogenous protein expressions (Fig. 1A, Nuclear extracts).

Next, to confirm whether p300 was a direct substrate of ERK2, an in vitro kinase assay was performed by using phospho-Ser/Thr-Pro antibodies. The antibodies were chosen because the minimal ERK consensus phosphoacceptor motif is Ser/Thr-Pro (10). As shown in Fig. 1B, purified full-length p300 protein was phosphorylated by recombinant activated ERK2-A in vitro (lane 2). Inactivated ERK2-I was here used as a negative control (Fig. 1B, lane 1). The inputs of p300 (Fig. 1B, lanes 3 and 4) and ERK2 (lanes 5 and 6) were shown as equal. To further confirm the phosphorylation of p300 by activated ERK2-A in vitro was not because of the different binding affinities between purified p300 protein and these two recombinant ERK2 proteins, immunoprecipitation assay with p300 antibodies was then performed. The results shown in Fig. 1C indicated that both activated ERK2-A and inactivated ERK2-I had similar binding affinities to p300 protein in vitro (middle panel, lanes 3 and 4).

We then assessed whether EGF induced phosphorylation of p300 in vivo. The p300 protein was immunoprecipitated from the nuclear extracts of HaCaT cells with p300 antibodies and then immunoblotted with phospho-Ser/Thr-Pro antibodies. The results are shown in Fig. 1D. EGf induced the phosphorylation of p300 in a time-dependent manner. The induction of p300 phosphorylation by EGF was maximized at 30 min (Fig. 1D, lane 3) and sustained at least up to 90 min (lane 6) after EGF treatment. It was also observed that this EGF-induced phosphorylation of p300 was dose-dependent (see supplemental Fig. S1A, middle panel). These results showed that the phosphorylation of immunoprecipitated p300 was maximized when HaCaT cells were treated with EGF for 30 ng/ml. Similar results were observed in A431 cells (Fig. 1E). When A431 cells were transiently overexpressed with HA-p300-WT (wild type) and then the lysates were immunoprecipitated with HA antibodies, EGF was found to induce the phosphorylation of HA-p300-WT after cells were treated with EGF for 30–45 min. Furthermore, to confirm the specificity of EGF on the effect of p300 phosphorylation, a selective EGF receptor kinase inhibitor, AG1478, was used. As shown in Fig. 1F, the EGF-induced phosphorylation of p300 was inhibited by AG1478 (compare lane 4 with lane 2), whereas the p300 proteins were immunoprecipitated equally (lanes 5–8).

To clarify whether the EGF-induced phosphorylation of p300 was regulated through the ERK activation in vivo, U0126 was used. The results shown in Fig. 1G indicated that whereas U0126 showed no effect on its own (lane 6), it blocked the phosphorylation of p300 caused by EGF, specifically at the 30-min time point (compare lane 5 with lane 4). Furthermore, we confirmed the effect of U0126 on the EGF-induced phosphorylation of p300 by using the stable transfectants of HaCaT cells with HA-p300-WT or empty expression vector. Nuclear extracts from this stable cell line were immunoprecipitated with HA antibodies and then probed with phospho-Ser/Thr-Pro antibodies. It was found that EGF induced the phosphorylation of p300 (see supplemental Fig. S2A, compare lane 8 with lane 7), and this induction was blocked by U0126 (lane 10), whereas U0126 showed no effect on its own (lane 9). To further confirm that the ERK activation specifically mediated the EGF-induced phosphorylation of p300, an in vitro kinase assay was performed. In this assay, the substrates were from the immunoprecipitates of endogenous p300 using p300 antibodies in HaCaT cells without EGF treatment, and the kinases were from the immunoprecipitates of endogenous p300 using phospho-Ser/Thr-Pro antibodies in HaCaT cells treated with or without EGF. The results are shown in Fig. 1H. We found that indeed endogenous phospho-ERK in EGF-treated HaCaT cells enhanced the phosphorylation level of endogenous p300 in vitro (Fig. 1H, upper panel, compare lane 2 with lane 1), and the inputs of p300 and phospho-ERK were shown in middle and lower panels. Taken together, these results strongly suggested that EGF mediated the phosphorylation of p300 through the ERK2 activation both in vitro and in vivo.

Identification of ERK2 Phosphorylation Sites on p300 in Vitro—We next attempted to identify the ERK2 phosphorylation sites on p300. Previous reports indicate that the ERK-specific phosphorylation motif features a Ser-Pro or Thr-Pro specific phosphorylation motif (29). Nevertheless, initial sequence screening
revealed that there was no DEF in the vicinity of numerous Ser-Pro or Thr-Pro sequences on p300.

To dissect the potential ERK2 phosphorylation sites on p300, several GST-p300 truncated proteins were constructed to study which fragments were involved in ERK2-stimulated phosphorylation. These purified proteins, including GST-p300 (aa 2–331), GST-p300 (aa 302–670), GST-p300 (aa 565–966), GST-p300 (aa 952–1145), GST-p300 (aa 1140–1570), GST-p300 (aa 1570–1968), GST-p300 (aa 1964–2197), and GST-p300 (aa 2188–2375), were expressed in vitro (Fig. 2A). Then, every purified fragment of GST-p300 was subjected to an in vitro kinase assay using phospho-Ser/Thr-Pro antibodies. Among these fragments, GST-p300 (aa 952–1145), GST-p300 (aa 1140–1570), and GST-p300 (aa 1964–2197) were not phosphorylated by activated ERK2-A (data not shown). However, five fragments, including GST-p300 (aa 2–331), GST-p300 (aa 302–670), GST-p300 (aa 565–966), GST-p300 (aa 1570–1968), and GST-p300 (aa 2188–2375), were phosphorylated by activated ERK2-A. To identify the exact ERK2 phosphorylation sites within these five fragments, we mutated every potential ERK2 phosphorylation site with the consensus sequence Ser-Pro or Thr-Pro.

As shown in Fig. 2B, the activated ERK2-A phosphorylated GST-p300 (aa 2–331)-WT (wild type) (lane 2), whereas inactivated ERK2-I could not (lane 1). When we further mutated Thr-317 within the fragment (aa 2–331) to alanine, the activated ERK2-A-mediated phosphorylation was completely inhibited (Fig. 2B, lane 3). The results indicated that within this fragment (aa 2–331), Thr-317 was a potential ERK2 phosphorylation site. In Fig. 2C, we observed that activated ERK2-A (lane 3), but not inactivated ERK2-I (lane 2), caused the phosphorylation of GST-p300 (aa 565–966). Similarly, when Thr-317 within this fragment was mutated to alanine, the activated ERK2-A-mediated phosphorylation was completely inhibited (Fig. 2C, lane 4). The results shown in Fig. 2, B and C, indicated that Thr-317 was the only ERK2 phosphorylation site on p300 between amino acids 2 and 670 in vitro. In addition to Thr-317, two other residues, Thr-938 (Fig. 2D, compare lane 5 with lane 3) and Thr-1960 (Fig. 2E, compare lane 3 with lane 1), were identified in fragments aa 565–966.
and aa 1570–1968 respectively. Meanwhile, mutation of Ser-499 (Fig. 2C, lane 5), Thr-594 (Fig. 2D, lane 4), or Thr-1909 (Fig. 2E, lane 2) to alanine was used as a negative control for the three fragments, respectively.

In fragment aa 2188–2375 (Fig. 2F), when Ser-2279 (lane 4), Ser-2315 (lane 5), or Ser-2366 (lane 6) was replaced to alanine individually, only a slight decrease in the phosphorylation was seen when compared with the activated ERK2-A-mediated phosphorylation of wild type fragment (lane 3). However, a complete inhibition was observed when all these three serines were mutated (Fig. 2F, lane 7). In this fragment (aa 2188–2375), there were three ERK2 phosphorylation sites, Ser-2279, Ser-2315, and Ser-2366. Thus, six potential ERK2 phosphorylation sites, including three threonine residues (Thr-317, Thr-938, and Thr-1960) and three serine residues (Ser-499, Ser-594, and Ser-1909), were identified in p300.

**FIGURE 3.** Confirmation of ERK2 phosphorylation sites on p300 by EGF treatment in vivo. A, six in vitro identified ERK2 phosphorylation sites on p300 are shown in the diagram. B, A431 cells with 60–80% confluence were transiently overexpressed with HA-p300-WT or HA-p300-6A (with those six in vitro identified ERK2 phosphorylation sites mutated to alanine) expression vectors, allowed to recover in complete medium, and then treated with or without EGF for 30 min. Nuclear extracts were immunoprecipitated (IP) with HA antibodies and subjected to Western blot with phospho-Ser/Thr-Pro antibodies to determine in vivo ERK2 phosphorylation sites on p300. C, A431 cells were transiently overexpressed with expression vectors of HA-p300-WT, HA-p300-S3A (with those three in vitro identified serine residues on p300 mutated to alanine), or HA-p300-T3A (with those three in vitro identified threonine residues on p300 mutated to alanine). Then similar procedures were performed as described in B. D, A431 cells were transiently overexpressed with expression vectors of HA-p300-WT or HA-p300-S3A, allowed to recover in complete medium, and then pretreated with or without 10 μM U0126 for 30 min, followed by EGF treatment for 30 min. Then similar procedures were performed as described in B. E, A431 cells were transiently overexpressed with expression vectors of individual HA-p300-S2279A, HA-p300-S2315A, or HA-p300-S2366A as well as HA-p300-WT. Similar procedures were then performed as described in B. F, A431 cells were transiently overexpressed with expression vectors of individual HA-p300-S2279A, HA-p300-S2315A, or HA-p300-S2366A as well as HA-p300-WT, HA-p300-S3A, and HA-p300-T3A, allowed to recover in complete medium, and then treated with EGF for 15 min. Nuclear extracts were immunoprecipitated with HA antibodies and subjected to Western blot with phospho-ERK1/2 or ERK2 antibodies to analyze the interaction between these mutations of HA-p300 and ERK2. Similar results were obtained in two or three independent experiments.
p300 Phosphorylation by ERK2 Regulates Keratin 16 Expression

serine residues (Ser-2279, Ser-2315, and Ser-2366), were identified in these in vitro kinase assays (Fig. 3A).

Confirmation of ERK2 Phosphorylation Sites on p300 by EGF Treatment in Vivo—Fig. 2 indicated that we have successfully identified six potential ERK2 phosphorylation sites, including three threonine residues and three serine residues, from in vitro experiments. To further confirm whether these six residues were phosphorylated by EGF in vivo, several mutations of HA-tagged p300 expression vectors were constructed. As shown in Fig. 3B, A431 cells were transiently overexpressed with HA-p300-WT and HA-p300-6A (with these six residues all replaced by alanine), and immunoprecipitation assay was performed by using HA antibodies. The results indicated that the EGF-induced phosphorylation in cells overexpressing HA-p300-6A was almost completely inhibited (Fig. 3B, lane 5) when compared with that seen in cells overexpressing HA-p300-WT (lane 3). Furthermore, the EGF-induced phosphorylation in cells overexpressing HA-p300-S3A (with three serine residues all replaced by alanine) was thoroughly inhibited (Fig. 3C, compare lane 5 with lane 3), whereas that seen in cells overexpressing HA-p300-T3A (with three threonine residues all replaced by alanine) was only slightly inhibited (Fig. 3C, compare lane 7 with lane 3). Moreover, the EGF-induced phosphorylation of HA-p300-WT was completely blocked by U0126 (Fig. 3D, compare lane 5 with lane 3). The results indicated that these three serine residues on p300 were the major ERK2 phosphorylation sites by EGF in vivo.

We further analyzed whether all these three serine residues were individually phosphorylated by EGF. Individual serine mutations of HA-tagged p300 expression vector were overexpressed in cells and immunoprecipitated with HA antibodies. As shown in Fig. 3E, a significant decrease in the EGF-induced phosphorylation was observed in cells overexpressing individual serine mutations (lanes 5, 7, and 9) as compared with that seen in cells overexpressing HA-p300-WT (lane 3). To rule out the possibility that different abilities of protein interactions resulted in variations of p300 phosphorylation levels, we also assessed the interactions between these HA-tagged expression vectors of p300 and activated ERK2. The results shown in Fig. 3F indicated that all these mutations of HA-tagged p300 expression vectors interacted with phospho-ERK2 to similar levels (lanes 2–7). Taken together, these findings suggested that these three C-terminal serine phosphorylation sites on p300 were the major phosphorylation targets by ERK2 when cells were treated with EGF in vivo.

Effect of C-terminal Serine Phosphorylation on p300 HAT Activity in Vitro—Several recent reports suggest that p300/CBP is phosphorylated by various kinases that are important in either cell cycle regulation or divergent signal transduction pathways. These phosphorylation events have been speculated to affect various p300/CBP activities such as the HAT activity (13). We have demonstrated in the past that the HAT domain of p300 is absolutely required for the keratin 16 promoter activity when cells were treated with EGF (27). We were interested next in whether the C-terminal serine phosphorylation by the ERK2 activation increased the HAT activity of p300, thus leading to the regulation of keratin 16 gene expression. Therefore, an in vitro acetylation assay was performed. The purified p300 was subjected to an in vitro kinase assay with activated ERK2-A or inactivated ERK2-I, and then proceeded to an in vitro acetylation assay using purified histone H3 as a substrate that could be detected by using acetyl-lysine antibodies. As shown in Fig. 4A, the results indicated that the activated ERK2-A-induced phosphorylation of p300 caused an increase of p300 HAT activity in vitro (upper panel, compare lane 4 with lane 3). There was no acetylation of histone H3 when histone H3 was omitted from the sample (Fig. 4A, upper panel, lanes 1 and 2). The inputs of histone H3 and p300 were shown in Fig. 4A, middle and lower panels. Furthermore, nuclear extracts from A431 cells overexpressed with HA-p300-WT or HA-p300-S3A were immunoprecipitated with HA antibodies as acetylases and subjected to an in vitro acetylation assay using purified histone H3 as a substrate. Fig. 4B indicated that a stimulated HAT activity was observed in EGF-treated HA-p300-WT (upper panel, compare lane 3 with lane 2), whereas the stimulation was attenuated in EGF-treated HA-p300-S3A (upper panel, compare lane 5 with lane 4). The inputs of histone H3, HA-p300-WT and HA-p300-S3A are shown in Fig. 4B, middle and lower panels.

Moreover, similar results were observed by using the nonradioactive histone acetyltransferase assay. Detection of the acetylated histone H3 was performed by enzyme-linked immunosorbent assay. The full length of recombinant p300 protein was subjected to an in vitro kinase assay and then proceeded to this assay. As shown in Fig. 4C, these results indicated that the activated ERK2-A-induced phosphorylation of p300 caused a stimulation of p300 HAT activity in vitro (Fig. 4C, compare lane 6 with lane 5), whereas neither inactivated ERK2-I nor activated ERK2-A had effect per se (lanes 3 and 4). When nuclear extracts of stable transfectants of HaCaT cells with HA-p300-WT or HA-p300-S3A expression were used as the sources of HAT enzymes, then subjected to this nonradioactive HAT activity assay, a stimulation of HAT activity was found in EGF-treated HA-p300-WT (Fig. 4D, compare lane 3 with lane 2), whereas the stimulation was completely inhibited in EGF-treated HA-p300-S3A (Fig. 4D, compare lane 5 with lane 4). All these results strongly indicated that the ERK2-dependent C-terminal serine phosphorylation of p300 regulated the HAT activity of p300 in the presence of EGF.

Effect of C-terminal Serine Phosphorylation of p300 on Protein-Protein Interactions—It is now widely accepted that p300 acts as a scaffold for multiple non-histone proteins, notably transcriptional factors such as Sp1 (14). The interaction between the acetyltransferase region of p300 and the DNA-binding domain of Sp1 has been demonstrated in the yeast two-hybrid system (15). We reported here that the purified full-length p300 directly interacted with full-length GST-Sp1 in vitro as demonstrated by the GST pulldown assay (Fig. 5A, lane 3). Furthermore, by performing the immunoprecipitation assay as shown in Fig. 5B, a significant binding of p300 to immunoprecipitated Sp1 was observed in EGF-treated HaCaT cells with Sp1 antibodies (compare lane 2 with lane 1). No nonspecific binding of p300 to Sp1 was detected with IgG antibodies (Fig. 5B, lanes 3 and 4) whereas Sp1 proteins were immunoprecipitated in equal amount (lanes 6 and 7). We then studied whether the ERK2-mediated phosphorylation of p300 increased the p300 interaction with Sp1. A431 cells were transiently overex-
Effect of C-terminal serine phosphorylation of p300 on its HAT activity

Figure 4. Effect of C-terminal serine phosphorylation of p300 on its HAT activity in vitro. A, recombinant p300 protein was subjected to an in vitro acetylation assay, and then purified histone H3 and acetyl-CoA were added to the mixture followed by an in vitro acetylation assay as described under “Experimental Procedures” in the acetylation reaction buffer. After separation through a 12% SDS-PAGE, the gel was followed by Western blot analysis probed with acetyl-lysine antibodies. B, A431 cells with 60–80% confluence were transiently overexpressed with HA-p300-WT or HA-p300-S3A expression vectors, allowed to recover in complete medium, and then treated with EGF for 30 min. Nuclear extracts immunoprecipitated (IP) with HA antibodies as acetylases mixed with purified histone H3 and acetyl-CoA and then were subjected to an in vitro acetylation assay. Western blot was used for detecting the acetylation of histone H3 by using acetyl-lysine antibodies. Immunoprecipitated HA-p300-WT and HA-p300-S3A proteins and histone H3 were analyzed with HA and histone H3 antibodies, respectively. C, recombinant p300 protein was subjected to an in vitro kinase assay, and acetyl-CoA was then added to the mixture with the acetylation reaction buffer, followed by a nonradioactive histone acetyltransferase activity assay as described under “Experimental Procedures.” D, nuclear extracts from HaCaT cells stably expressing HA-p300-WT or HA-p300-S3A with or without EGF treatment for 1 h was mixed with acetyl-CoA in the acetylation buffer and then subjected to a nonradioactive histone acetyltransferase activity assay as described under “Experimental Procedures.” Values of O.D. were means ± S.E. of two determinations. Statistical analysis was performed by Student’s t test. *p < 0.05; **p < 0.01. N.S., not significant; A, activated; I, inactivated. Similar results were obtained in two or three independent experiments.

Effects of C-terminal serine phosphorylation of p300 on its recruitment to the keratin 16 promoter—The results above indicated that the C-terminal serine phosphorylation of p300 by ERK2 activation was important for p300 HAT activity and its interaction with Sp1. Therefore, we next studied the effect of C-terminal serine phosphorylation of p300 on the regulation of EGF-induced keratin 16 expression. We previously observed that EGF up-regulates the recruitment of p300 to the keratin 16 promoter through ERK activation (27). Here we investigated whether the C-terminal serine phosphorylation of p300 affected its recruitment to the keratin 16 promoter by using DAPA. As shown in Figure 6A, EGF up-regulated the recruitment of HA-p300-WT to the keratin 16 promoter (upper panel of DAPA, compare lane 3 with lane 2), whereas this up-regulation was completely blocked in the stable transfectants of HaCaT cells with HA-p300-S3A expression (upper panel of DAPA, compare lane 7 with lane 6). Moreover, it was found that this up-regulated recruitment of HA-p300-WT was inhibited by the pretreatment of U0126 (Fig. 6A, upper panel of DAPA, compare lane 5 with lane 3). Sp1 proteins were constitutively bound to the keratin 16 promoter (Fig. 6A, lower panel of DAPA).
p300 Phosphorylation by ERK2 Regulates Keratin 16 Expression

To further confirm the results observed in Fig. 6A, the chromatin immunoprecipitation assay was performed by using extracts from HaCaT cells stably expressing HA-p300-WT or HA-p300-S3A with or without EGF treatment. The results are shown in Fig. 6B. HA-p300-WT was found to slightly bind to the chromatin keratin 16 promoter region in unstimulated cells (Fig. 6B, compare lane 2 with lane 1). This binding was apparently enhanced in the presence of EGF (Fig. 6B, compare lane 3 with lane 2), whereas the EGF effect on the recruitment to the chromatin keratin 16 promoter region was not seen in cells with stable expression of HA-p300-S3A (compare lane 6 with lane 5). Furthermore, this up-regulated recruitment of HA-p300-WT by EGF was blocked by pretreatment of U0126 (Fig. 6B, compare lane 4 with lane 3), which was not seen in cells with stable expression of HA-p300-S3A (compare lane 7 with lane 6). Sp1 proteins were constitutively bound to the chromatin keratin 16 promoter region (Fig. 6B, lanes 8–14). Immunoprecipitation performed with mouse IgG, which did not show detectable keratin 16 promoter fragment, was used for negative controls (Fig. 6B, lanes 15–21). As shown in Fig. 6B, input, 5% of total chromatin was used to verify equal loading of chromatin components before precipitation (bottom panel). These findings were of decisive importance because we reported previously that the EGF-induced keratin 16 gene expression is completely inhibited when the recruitment of p300 is blocked (27). These results also implicated the ERK2-dependent C-terminal serine phosphorylation of p300 as a critical step in the recruitment of p300 to the promoter of keratin 16 in response to EGF.

Effect of ERK2 Activation-dependent Phosphorylation of p300 on EGF-induced Transcriptional Activity of Keratin 16—We confirmed further whether these phosphorylation sites on p300 affected the EGF control of keratin 16 promoter activity. Individual mutation of the six phosphorylation sites on p300 was thus undertaken. At first, we confirmed the effect of threonine phosphorylation of p300 on the EGF-induced keratin 16 promoter activity. As shown in Fig. 7A, HaCaT cells overexpressing either HA-p300-T3A (lanes 9), HA-p300-T317A (lane 10), HA-p300-T1960A (lane 11), or HA-p300-T3A (lane 12)
FIGURE 7. Effect of ERK2 activation-dependent phosphorylation of p300 on EGF-induced keratin 16 transcriptional activity. A and B, QuikChange®XL site-directed mutagenesis kit was used to introduce single point or triple point mutations of full-length HA-tagged p300 expression vectors. HaCaT cells with 60–80% confluence were transiently transfected with pXK-5-1 luciferase plasmid, along with various HA-p300 expression vectors and then treated with EGF for 24 h as indicated. Detailed procedures were described under "Experimental Procedures." The luciferase activities and protein concentrations were determined and normalized. Values of luciferase activity were means ± S.E. of three determinations. Statistical analysis was performed by Student’s t test. The value of each HA-p300 expression vector mutant was compared with that of HA-p300-WT upon EGF treatment. *, p < 0.05; **, p < 0.01. Protein expressions in the presence of EGF are shown in the lower panels. C, HaCaT cells with stable expression of HA-p300-WT or HA-p300-S3A maintained for 2 days in serum-free medium were incubated with EGF for 24 h and then harvested. Total RNA was isolated, and reverse transcription PCR was performed as described under "Experimental Procedures." Similar results were obtained in two or three independent experiments.
showed no effect on the keratin 16 promoter activity upon EGF treatment, when compared with that seen in HA-p300-WT-overexpressing cells treated with EGF (lane 8). However, as shown in Fig. 7B, we observed a significant inhibition in cells overexpressing either HA-p300-S2279A (lane 10), HA-p300-S2315A (lane 11), or HA-p300-S2366A (lane 12) as compared with that seen in HA-p300-WT-overexpressing cells (lane 9). More importantly, an almost complete inhibition in the EGF-induced keratin 16 promoter activity was observed when the three serine residues on C terminus of p300 were all mutated (Fig. 7B, compare lane 13 with lane 9). These indicated that the C-terminal serine phosphorylation was important for the regulation of EGF-induced transcriptional activity of keratin 16. It has been reported that Ser-1834 of p300 is phosphorylated by Akt (25). To clarify whether the Ser-1834 phosphorylation of p300 was involved in the regulation of EGF-induced keratin 16 promoter activity, the expression vector HA-p300-S1834A (with Ser-1834 replaced by alanine) was constructed. The results showed that no significant difference was found in cells overexpressing HA-p300-S1834A upon EGF treatment (Fig. 7B, compare lane 14 with lane 9). This suggested that the Akt-mediated Ser-1834 phosphorylation of p300 was not important for the regulation of EGF-induced keratin 16 transcriptional activity.

Furthermore, we investigated the effect of ERK2-dependent C-terminal serine phosphorylation of p300 on the EGF-induced mRNA expression of endogenous keratin 16. In HaCaT cells stably expressing HA-p300-S3A, no apparent EGF effect was observed on the keratin 16 mRNA (Fig. 7C, compare lane 6 with lane 5) as compared with that seen in cells stably expressing HA-p300-WT (Fig. 7C, compare lane 4 with lane 3). Meanwhile, we estimated the effect of C-terminal serine phosphorylation of p300 on the mRNA expression of other hyperproliferation-associated keratins, K6 and K17 (30). The results are shown in Fig. 7C. It was found that the C-terminal serine phosphorylation of HA-p300 had no significant effect on the mRNA expression of keratin 6 (Fig. 7C, lanes 9–12) and keratin 17 (lanes 15–18) as compared with that seen in keratin 16 (lanes 3–6), suggesting that the expression of keratin 6 and keratin 17 was not mediated through p300 regulation in these stable HaCaT cell lines. The expressions of HA-p300 in these stable HaCaT cell lines were evaluated as equal (Fig. 7C, lanes 27–30). These results suggested that these three keratins were regulated through different mechanisms. Taken together, it implicated that the ERK2-dependent C-terminal serine phosphorylation of p300 was a regulatory mechanism pivotal for the gene expression of keratin 16 by EGF.

**DISCUSSION**

Our previous studies have indicated that in addition to c-Jun and Sp1, p300 is another pivotal protein in the gene regulation of keratin 16 by EGF. We previously found that EGF increases the recruitment of p300 to the keratin 16 promoter and that EGF does so by activating ERK protein. In this study, we further investigated the mechanisms whereby ERK might cause an increased recruitment of p300 to the keratin 16 promoter. Several important findings from this study are thus summarized in Fig. 8. Briefly, 1) the phosphorylation of C-terminal serine residues (Ser-2279, Ser-2315, and Ser-2366) on p300 was the major action caused by EGF in vivo (Fig. 3). 2) The phosphorylation was mediated by the activation of ERK2 (Fig. 1). 3) The C-terminal serine phosphorylation of p300 stimulated the HAT activity of p300 (Fig. 4) and also enhanced its interaction with Sp1 (Fig. 5). 4) This C-terminal serine phosphorylation controlled the recruitment of p300 to the keratin 16 promoter (Fig. 6). 5) Once recruited, the phosphorylated p300 acetylated histone H3 on the keratin 16 promoter via its increased HAT activity. As a consequence, p300 enhanced EGF-induced gene expression of keratin 16.
importance of the phosphorylation of p300 on its functions both in vitro and in vivo. Previous studies (9, 18, 19) link phosphorylation of p300 to its ensued activities in vitro. Furthermore, Gusterson et al. (31) report that the phenylephrine-stimulated transcriptional activity of N terminus of p300 (aa 1–743) is p42/44 MAPK-dependent. They also indicate that phenylephrine enhances the Ser-89 phosphorylation on p300 in vitro (32). However, Ser-89 on p300 is the target of PKC (20). Furthermore, Ser-89 is not a consensus site for ERK. Thus, how the ERK activation regulates p300 is not totally clear. Our study provided a model on how EGF regulated p300 through the ERK2 activation and constituted the first report identifying ERK2 phosphorylation sites on p300 in vivo.

Several studies indicate that p44 MAPK (ERK1) phosphorylates the C terminus of CBP, causing stimulation of CBP HAT activity in vitro. In addition, the C terminus of CBP is required for the ERK1-stimulated HAT activity of CBP (18, 19). These results suggest that the C-terminal phosphorylated CBP causes a conformational change that exposes its HAT domain, leading to the stimulation of its HAT activity. It is known that p300 interacts with Sp1 through its HAT domain (15). In this study, we also observed that ERK2 mediated C-terminal serine phosphorylation of p300, resulting in the increase of p300 HAT activity. As these ERK2-dependent phosphorylation sites on p300 were not within the HAT domain of p300 (aa 1195–1673), it was thus likely that the EGF-induced C-terminal serine phosphorylation of p300 caused the exposure of the p300 HAT domain, leading to not only the stimulation of its HAT activity but also its increased affinity with Sp1. Whether this conformational change creates as yet a new interaction interface with Sp1 remains to be explored. Li et al. (33) indicate that MAPK-phosphorylated Elk-1 creates a new interaction domain with p300. In addition, we also observed that the Sp1/p300 interaction increased gradually and peaked at 24 h (see supplemental Fig. S1B, upper panel) and EGF induced this interaction in a dose-dependent manner (see supplemental Fig. S1A, upper panel). It is also reported that the HAT region of p300 interacts with the DNA-binding domain of Sp1 to regulate the DNA binding activity of Sp1. Once Sp1 binds to DNA, the p300/Sp1 interaction is inhibited (15). However, our current system indicated that Sp1 protein bound to DNA constitutively. Therefore, fully understanding how phosphorylated p300 affects its interaction with Sp1 awaits further studies.

Many studies indicate that most protein kinases, such as MAPK (9, 18), Akt (25), and cyclin E-Cdk2 (34), phosphorylate the C terminus of p300, resulting in the stimulation of p300 HAT activity or its transactivation potential. In this study, we found that the C-terminal serine phosphorylation of p300 played a pivotal role in the EGF-induced keratin 16 gene expression. As p300 is a common coactivator, its C-terminal phosphorylation might be one of the common mechanisms underlying its gene regulation activity in general, vis à vis its specific effect on transcription factors. We tested three genes in our system, including cyclooxygenase-2, 12(S)-lipoxigenase, and p21 waf1/cip1. Our previous results indicate that p300, by cooperating with c-Jun, is required for EGF-induced gene expression of cyclooxygenase-2 (35). Moreover, it is known that both EGF and phorbol 12-myristate 13-acetate induce 12(S)-lipoxigenase expression (36) and that p300 is required for the phorbol 12-myristate 13-acetate-induced 12(S)-lipoxigenase expression (37). A study also indicates that p300 collaborates with Sp1 to regulate the butyrate-induced p21 waf1/cip1 expression (14). In fact, we found that the C-terminal serine phosphorylation of p300 had a slight but significant effect (20–35%) on the EGF-induced gene expression of cyclooxygenase-2, 12(S)-lipoxigenase, and p21 waf1/cip1 (see supplemental Fig. S4). However, as seen from this study, the C-terminal serine phosphorylation of p300 had a much more dramatic effect on the EGF-induced keratin 16 gene expression. The difference might arise from these facts. (a) Keratin 16 is usually a marker for the hyperproliferation-associated diseases, and proliferation-associated ERK signaling is the major regulatory pathway (more than 70%) for the EGF-induced gene activation of keratin 16 (27); (b) EGF induces gene activation of keratin 16 by activating ERK protein to recruit p300 to the keratin 16 promoter (27). A note in passing, however, other EGF-induced downstream signaling, such as c-Jun N-terminal kinase (JNK) or PKC activation, might also cooperate to regulate the expression of the three genes. Understanding the regulation of p300 activity will shed light on how cells use this common coactivator to mediate specific cellular genetic responses to diverse stimuli. It is for certain that understanding the post-translational modifications of p300, such as phosphorylation, will remain a major topic for future studies. Whether the ERK2-mediated phosphorylation of p300 caused by EGF plays a role on other cellular functions such as cell cycle regulation, differentiation, and apoptosis deserves to be investigated extensively.

In conclusion, we reported here that the ERK2-mediated C-terminal serine (Ser-2279, Ser-2315, and Ser-2366) phosphorylation of p300 was the major signaling target triggered by EGF, particularly in vivo, which resulted in the stimulation of the HAT activity of p300. This regulation at least played a pivotal role in the EGF regulation of keratin 16 gene expression. These findings also provided a better understanding between the activity of p300 and disease-associated keratin 16 overexpression. As mentioned above, keratin 16 is usually referred to as an activation- and hyperproliferation-associated keratin specifically reported to be the marker in the hyperproliferated skin diseases, such as psoriasis (2, 3). Psoriasis is a common inflammatory skin disorder that affects about 0.1–3% of the world’s population (38). Although psoriasis does not ordinarily affect the general health of a patient, the unsightliness can be detrimental, although sometimes underestimated, to the social and psychological welfare of a patient. However, the understanding of its pathogenesis is still limited, and there has been less-than-optimal care until now. Analysis of the transcriptional regulation of the psoriasis-associated keratin 16 gene will provide important insights into the pathogenesis of psoriasis and may thus help to improve treatments by providing novel therapies in the future.

Acknowledgment—We thank Dr. Tsung-Ping Su for critical reviewing and editing the manuscript.
REFERENCES

1. Fuchs, E., and Weber, K. (1994) *Annu. Rev. Biochem.* 63, 345–382
2. Komine, M., Rao, L. S., Kaneko, T., Tomic-Canic, M., Tamaki, K., Freedberg, I. M., and Blumenberg, M. (2000) *J. Biol. Chem.* 275, 32077–32088
3. Leigh, I. M., Navsaria, H., Purkis, P. E., Bowden, P. E., and Riddle, P. N. (1995) *Br. J. Dermatol.* 133, 501–511
4. Wetzel, R. H., Kuipers, H. J., Lane, E. B., Leigh, I. M., Troyanovsky, S. M., Holland, R., van Haelst, U. J., and Ramaekers, F. C. (1991) *Am. J. Pathol.* 138, 751–763
5. McClowry, T. L., Shors, T., and Brown, D. R. (2002) *J. Med. Virol.* 66, 96–101
6. Janknecht, R., Ernst, W. H., Pingoud, V., and Nordheim, A. (1993) *EMBO J.* 12, 5097–5104
7. Gille, H., Kortenjann, M., Thomae, O., Moomaw, C., Slaughter, C., Cobb, M. H., and Shaw, P. E. (1995) *EMBO J.* 14, 951–962
8. Liu, Y. Z., Thomas, N. S., and Latchman, D. S. (1999) *Neuroreport* 10, 1239–1243
9. Sang, N., Stiehl, D. P., Bohensky, J., Leshchinsky, I., Srinivas, V., and Caro, J. (2003) *J. Biol. Chem.* 278, 14013–14019
10. Yang, S. H., Sharrocks, A. D., and Whitmarsh, A. J. (2002) *Biochim. Biophys. Acta* 1592, 205–211
11. Yuan, L. W., and Gambee, J. E. (2000) *J. Biol. Chem.* 275, 40946–40951
12. Yuan, L. W., Soh, J. W., and Weinstein, I. B. (2002) *Biochem. Biophys. Acta* 1592, 205–211