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Phosphorylation of p300 at Serine 89 by Protein Kinase C*

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CREB-binding protein (CBP)/p300 plays an important role in the connection of many different signal transduction pathways and the promotion of certain differentiation and proliferation processes. This role depends upon the ability of CBP/p300 to serve as coactivator for transcription factors. It has been suggested that CBP/p300 is regulated by phosphorylation, but the nature of the phosphorylation, the responsible kinase *in vivo*, and its physiological significance are still unclear. Here, we demonstrate the first identification of an *in vivo* phosphorylation site, conserved serine 89, in p300. Signal-dependent protein kinase C is able to phosphorylate serine 89 and mediates this phosphorylation event *in vivo*. Different from other phosphorylation observed so far in CBP/p300, this serine 89-specific phosphorylation represses the transcriptional activity of p300. This phosphorylation-mediated regulation of p300 function represents a previously unrecognized signal transduction pathway for protein kinase C to regulate cell growth and differentiation.

Adenoviral E1A-binding protein p300 and CREB-binding protein (CBP)[1] are highly conserved in their primary sequences and closely related in their functions (1–5). Homologues of CBP/p300 have been found in *Drosophila* (6), *Xenopus* (7), and *Caenorhabditis elegans* (8). Many studies have suggested that CBP/p300 is critically involved in the control of development, proliferation, differentiation, and apoptosis (9, 10).

Most studies of CBP/p300 have focused on their functions as transcriptional coactivator (1, 11). Consequently, the activities of CBP/p300 have been proposed as essential and integral for many fundamental biological events (9, 12). Precisely how CBP/p300 activates transcription remains uncertain, however. CBP/p300 have been shown to interact with RNA polymerase II and several other general transcription factors required for gene activation (11, 13–15), suggesting a role as a transcriptional integrator or adaptor. CBP/p300 have also been found to contain histone/factor acetyltransferase (HAT/FAT) activities (16–18). Through the activities of intrinsic or associated HATs, CBP/p300 may activate transcription through acetylating histones or other transcription factors (19–23).

Compared with the extensive analysis of the CBP/p300 transcriptional and HAT activities, little effort has been devoted to understanding the regulation of CBP/p300 function. Binding of E1A to CBP/p300 inhibits its HAT and transcriptional activities (4, 5, 24, 25), although the precise mechanism of this inhibition remains unclear. E1A has been proposed to interact directly with the CBP/p300 HAT domains, as well as to disrupt the interactions of associated HATs, such as P/CAF (26). Interactions between CBP/p300 and components of the RNA polymerase II holoenzyme complex may also be disrupted by E1A (14); SV40 T antigen is also able to interact with p300 and repress p300-mediated transcription activation (27). It has also been proposed that activation of the Ras-MAPK pathway allows $pp90^{rsk}$ to associate with CBP and inhibit cAMP-mediated gene transcription in a manner similar to E1A (28).

Surprisingly few studies have addressed the role of phosphorylation in regulating CBP/p300 function. Phosphorylated p300 has been found in both quiescent and proliferating cells, and the level of p300 phosphorylation changes along with cell cycle progression (29). Several recent reports have suggested that CBP/p300 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 CBP/p300 activities (12, 30–33). However, it is still unclear for the most part which kinases are responsible for CBP/p300 phosphorylation *in vivo* and where the phosphorylation sites occur. More importantly, the functional links between specific phosphorylation events and CBP/p300 activities remain largely unknown. Elucidating the nature of CBP/p300 phosphorylation is essential for understanding the regulation of CBP/p300 function.

To address these issues, we examined the phosphorylation of p300 *in vivo*. We first identified a major p300 phosphorylation site and then determined that protein kinase C (PKC) was responsible for phosphorylation of this site *in vivo*. By utilizing a p300 construct bearing a point mutation at the phosphorylation site, we examined the effect of the phosphorylation on p300 function. Our data suggest that phosphorylation of p300 represses the transcriptional activity of p300.

EXPERIMENTAL PROCEDURES

Phosphorylation Assay—p300 fragments fused to GST on beads were incubated for 30 min at 30 °C in kinase buffer (20 mM Hepes, pH 7.4, 10 mM magnesium acetate, 1 mM dithiothreitol, 100 μM ATP) plus HeLa nuclear extracts (10–50 μg) (34). The beads were washed with BC400 (20 mM Tris-Cl, pH 7.9, 400 mM KCl, 0.2 mM EDTA) containing 1% Triton X-100. The proteins were eluted in Laemmli's buffer and separated on an SDS-polyacrylamide gel. PKC kinase assays (35) were carried out in the kinase buffer supplemented with 0.5 mM $CaCl_2$, 100 ng/ml phorbol 12-myristate 13-acetate, 100 μg/ml phosphatidylserine, using the p300 fragment (amino acids 74–163) fused to GST (rp300n) on beads as substrate. Either purified PKC βII (100 ng) or other PKC isotypes immunoprecipitated from HeLa whole cell lysate or nuclear

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[1] The abbreviations used are: CBP, CREB (cAMP response element-binding protein)-binding protein; HAT, histone acetyltransferase; PKC, protein kinase C; GST, glutathione *S*-transferase; CAT, chloramphenicol acetyltransferase; ER, estrogen receptor; HPLC, high pressure liquid chromatography; LC/ESI/MS/MS, liquid chromatography electrospray ionization tandem mass spectrometry; CNBr, cyanogen bromide; AP-1, activator protein-1; Rb, retinoblastoma; *m/z*, ratio of mass to charge; Mes, 4-morpholineethanesulfonic acid.

extracts (200–500 μg) were used in the assays (36).

Protein Digestion—HeLa nuclear extracts adjusted to 350 mM KCl were immunoprecipitated with anti-p300-specific monoclonal antibodies (29) and digested directly for MS analysis. Alternatively, proteins were separated first on an SDS-polyacrylamide gel, electroblotted onto a nitrocellulose membrane, and stained with Ponceau S (Sigma). The desired bands were excised and minced into 1–2-mm pieces. Trypsin digestion and cyanogen bromide (CNBr, Aldrich) cleavage were carried out as described (37, 38). CNBr-cleaved peptides were further digested in 50 mM phosphate buffer (pH 7.8) with endoproteinase Glu-C (Sigma) overnight at 37 °C.

Manual Edman Degradation—Manual Edman sequencing of labeled peptides was carried out precisely as described (39). Briefly, peptides were immobilized covalently on an acrylamide-Sequelon disc (Millipore) using 10 mg/ml 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide in 0.1 M Mes (pH 5.0). The unbound peptides were removed by washing the disc with trifluoroacetic acid and methanol. The discs were then transferred into an Eppendorf tube and subjected to Edman degradation.

Metabolic Labeling—HeLa cells grown in Dulbecco's modified Eagle's medium containing 5% fetal calf serum in 10-cm dishes at 80% confluency were used for metabolic labeling. Cells were washed with phosphate-buffered saline and fed with 3 ml of either phosphate-free or methionine-free Dulbecco's modified Eagle's medium containing 10% dialyzed fetal bovine serum. ^{32}P-labeled orthophosphate or [^{35}S]methionine was added to the corresponding dishes (1 mCi/dish), which were then incubated at 37 °C for 4 h. The medium was aspirated, and cells were washed with phosphate-buffered saline before harvesting.

Peptide Mapping—The CNBr-cleaved peptides were dissolved in pH 1.9 buffer containing 88% formic acid, acetic acid, and deionized H_2O (25:78:897) and loaded onto a cellulose plate (20 × 20 cm). First-dimension electrophoresis was carried out at 1 kV for 30 min in pH 1.9 buffer. Second-dimension chromatography was developed in buffer containing 1-butanol, pyridine, acetic acid, and deionized H_2O (15:10:3:12) for 6–8 h.

LC/ESI/MS/MS Analysis—The protein digest from immunoprecipitated p300 was applied to a Targa 0.5 × 150-mm C18 column (Higgins Analytical) using a 10 μl/min flow rate. Mobile phases contained 0.1% acetic acid and peptides were eluted with a 0–32% acetonitrile gradient over 80 min followed by a 32–65% acetonitrile gradient over 10 min. Peptides were analyzed on-line by electrospray ionization mass spectrometry (ESIMS) using an LCQ ion trap mass spectrometer (Finnigan) to measure both peptide whole masses (MS) and masses of peptide fragments produced by collision-induced dissociation (MS/MS). Initial experiments detected potential phosphopeptides whose abundance was too low to trigger data dependent MS/MS scans. Therefore, the instrument was programmed to specifically select these ions for MS/MS analysis. Relative collision energy of 40% was used, with a maximum ion time of 500 ms. A total of 8 microscans was summed for each MS/MS spectra.

Immunodetection of Serine 89-phosphorylated p300—A phosphoserine 89-specific antiserum was prepared by immunizing rabbits with a p300-derived oligopeptide containing phosphoserine 89 (RSGSpSPNLNMGV). The specificity of the antiserum was examined using enzyme-linked immunosorbent assay with phosphorylated and nonphosphorylated oligopeptides at various concentrations. Subsequently, the antiserum was tested by Western analysis using various amounts of nonphosphorylated rp300n and rp300n phosphorylated by HeLa nuclear extracts. In both the enzyme-linked immunosorbent assay and Western analyses, the antiserum recognized the serine 89-phosphorylated protein with a high degree of specificity. The optimal dilution of the antiserum was used in Western analysis of immunoprecipitated p300 to measure the level of serine 89 phosphorylation *in vivo*.

Transfections and CAT Assays—HeLa cells at 50% confluency in 10-cm plates were transfected with LipofectAMINE (16 μl, Life Technologies, Inc.) as described by the manufacturer. The cells for transfection with the ER construct were grown in phenol red-free Dulbecco's modified Eagle's medium containing 5% dextran-coated charcoal-treated fetal calf serum (40). Ethanol (as a control) or estradiol (E2, 10 nM) dissolved in ethanol was added 1 h post-transfection. One or two days after transfection, the cells were lysed in 100 μl of buffer (15) followed by a CAT assay using the organic phase extraction procedure (11). CAT activity was normalized against cotransfected β-galactosidase activity (15).

RESULTS

p300 Is Phosphorylated at Serine 89 in Vitro by HeLa Nuclear Extracts—To identify the sites of p300 phosphorylation, we examined a series of bacterially expressed recombinant fragments representing the entire length of p300. Only the N-terminal 243-amino acid fragment (rp300N) was phosphorylated after incubation with HeLa nuclear extracts (Fig. 1*A*). This region contains many potential phosphorylation sites. To identify these sites, we first digested the phosphorylated rp300N protein with trypsin. Tryptic peptides were separated by HPLC using a reverse phase C18 column (VYDAC, 218TP52) (Fig. 1*B*), and each fraction was monitored by Cerenkov counting in a scintillation counter. Two major radiolabeled fractions (*peaks A* and *B* at retention times of 61 and 70 min, respectively) were detected, and these were subjected to automated Edman sequencing. Both fractions contained peptides with N-terminal residues corresponding to position 87 in p300. The two radiolabeled fractions were further digested with CNBr, separated by HPLC, and again subjected to automated sequencing. Residue 87 was again identified as the N terminus of the radiolabeled peptide. The labeled peptide ^{87}SGSSPNLNh^{95} (where h is either homoserine or homoserine lactone derived from methionine by CNBr cleavage) contains serines at positions 87, 89, and 90. Manual Edman degradation (39) was used to further characterize the radiolabeled tryptic peptide collected from *peak A*. At each cycle, both the eluted solution and the filter bearing the remaining peptide were subjected to Cerenkov counting. Only serine 89 at cycle 3 was labeled (Fig. 1*C*), indicating the site of phosphorylation. Mutating residue 89 from serine to alanine in the recombinant p300 N-terminal construct (rp300NS89A) markedly decreased the level of phosphorylation (Fig. 1*D*) and completely eliminated labeling of the CNBr peptide as measured by two-dimensional peptide map-

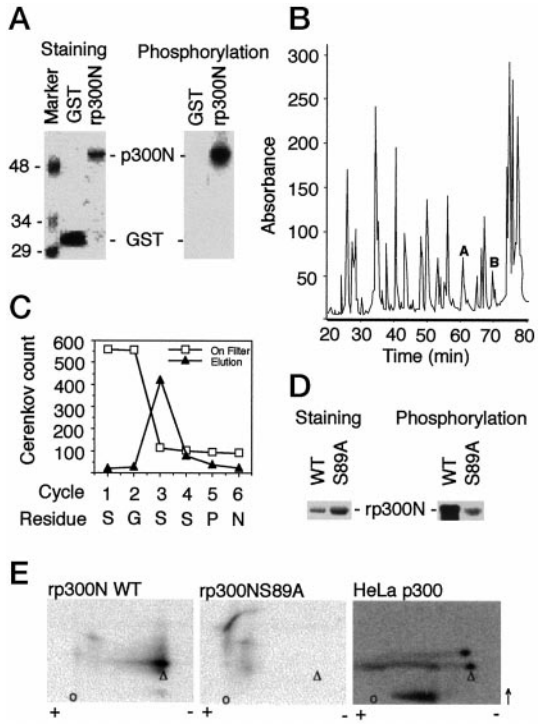

FIG. 1. **Serine 89 is a major phosphorylation site in p300.** *A*, phosphorylation of rp300N (GST-p300, amino acids 1–243) by HeLa nuclear extracts (*right*). Coomassie-stained input proteins (*left*). *B*, HPLC chromatogram of tryptic fragments of ^{32}P-labeled rp300N; *peaks A* and *B* fractions contained 60% of the radioactive label. *C*, manual sequencing of peptides in *peak A*. *D*, phosphorylation of rp300N (*WT*) and the point mutant (*S89A*) (*right*). Coomassie-stained input proteins (*left*). *E*, two-dimensional mapping of CNBr fragments of recombinant rp300N and endogenous p300 proteins. *o*, origin of sample application; + and −, polarity of the first-dimension electrophoresis; *arrow* indicates direction of second-dimension chromatography; *triangle* designates the position of the phosphoserine 89-containing peptide.

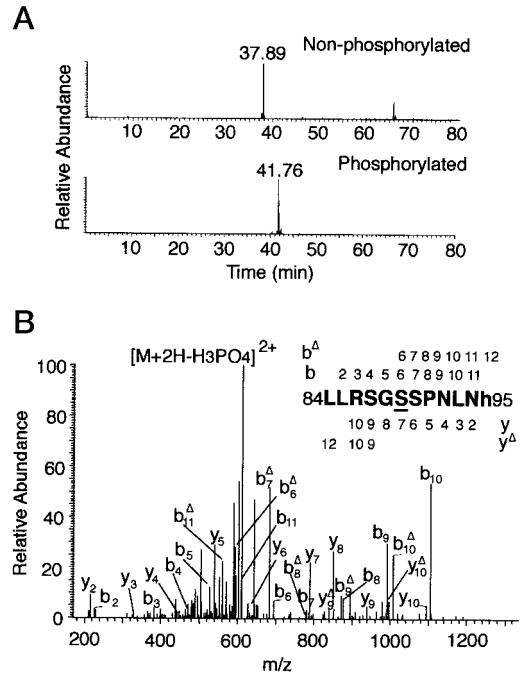

FIG. 2. **Phosphoserine 89 in endogenous p300 as determined by LC/ESI/MS/MS analysis.** p300 was immunoprecipitated from HeLa cells, digested with CNBr and then with Glu-C before analysis by LC/ESI/MS/MS. *A*, chromatogram displaying currents for ions with calculated *m/z* at 620.8 (nonphosphorylated) and 660.8 (phosphorylated) for the doubly charged ion ^{84}LLRSGSSPNLNh^{95}. *B*, MS/MS spectrum of the phosphorylated ion with *m/z* at 660.7. △, denotes loss of phosphate from the fragment ions. The recovered *b* and *y* fragment ions are listed *above* and *below* the sequence, respectively. The *underline* in the sequence indicates the phosphorylated residue.

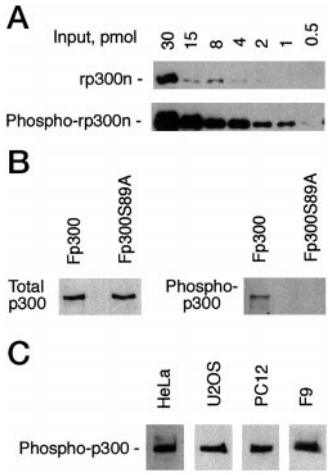

FIG. 3. **p300 is phosphorylated at serine 89 *in vivo*.** *A*, Western analysis using an antiserum generated against a phosphoserine 89-containing peptide antigen. *B*, the Flag-tagged p300 (*Fp300*) and point mutant (serine 89 to alanine, *Fp300S89A*) were transfected and expressed in HeLa cells, immunoprecipitated with anti-Flag antibodies from whole cell lysates, and analyzed by Western blot using anti-p300 and phosphoserine 89-specific antiserum. *C*, the p300 protein was immunoprecipitated from whole cell lysates prepared from different cell lines and analyzed by Western blot using the phosphoserine 89-specific antiserum.

ping (Fig. 1*E*). These observations suggest that serine 89 is a major phosphorylation site of the rp300N fragment of p300 *in vitro*.

Serine 89 Is Also Phosphorylated in Endogenous p300—To determine whether serine 89 is phosphorylated *in vivo*, we metabolically labeled the endogenous p300 protein by growing HeLa cells in ^{32}P. Two-dimensional peptide mapping of the labeled p300 after digestion with CNBr revealed a labeled phosphopeptide that migrated at the same position as the radiolabeled peptide from rp300N (Fig. 1*E*). Thus, we presume that serine 89 is also phosphorylated in endogenous p300. To test this hypothesis further, we used mass spectrometry to identify the phosphorylation site in the endogenous p300. p300 immunoprecipitated from nuclear extracts was digested with CNBr and endoproteinase Glu-C. The digested peptides were subjected to online capillary liquid chromatography electrospray tandem mass spectrometry (LC/ESI/MS/MS). During ionization, the peptide bearing serine 89 (84LLRSGSSPNLNh95) could be either singly or doubly charged. The calculated ratios of mass to charge (*m/z*) are 1240.7 and 620.8 for the unphosphorylated species, and 1320.7 and 660.8 for the species phosphorylated at one residue. Ions with either the expected *m/z* value for the unphosphorylated or phosphorylated peptides eluted at 37.9 and 41.8 min, respectively (Fig. 2*A*). The *m/z* values 1240.9 and 620.9 measured in the MS spectra were assigned to the unphosphorylated peptide, and the values 1320.8 and 660.7 were assigned to the phosphorylated peptide. These peptide ions were further isolated and subjected to collision-induced dissociation to produce MS/MS spectra. This caused fragmentation of the parent ions along peptide bonds to produce both *y* fragment ions (loss of successive residues from the N terminus) and *b* fragment ions (loss of successive residues from the C terminus). The identity of the unphosphorylated peptide was confirmed by the presence of 15 of 22 possible *b* and *y* series ions in the MS/MS spectra. The MS/MS spectra for the phosphorylated parent ions determined the identity of the phosphopeptide and the phosphorylation site at serine 89. Fig. 2*B* shows a MS/MS spectrum for the doubly charged phosphopeptide ion. The dominant peak with an *m/z* value of 611.9 in the spectrum is consistent with the loss of one phosphate from the parent ion. Sixteen *b* ions and 11 *y* ions were also assigned. Six of these *b* ions and two of these *y* ions resulted from the additional loss of phosphate (*b*Δ and *y*Δ). Based on the *m/z* values, none of the *b2–b5* and *y2–y6*, which contained serine 87 and 90, respectively, but not serine 89, were phosphorylated. On the other hand, the *b6–b11* and *y7–y10*, which contained serine 89, were phosphorylated and were able to lose phosphoric acid. This sequence information obtained from the MS/MS spectrum provides unambiguous evidence that serine 89 is an *in vivo* site of p300 phosphorylation.

To detect serine 89-phosphorylated endogenous p300 easily, we made a phosphoserine 89-specific antiserum. The specificity of the antiserum was examined by enzyme-linked immunosorbent assay using phosphorylated and nonphosphorylated oligopeptides (data not shown) and then by Western analysis using various amounts of nonphosphorylated and phosphorylated recombinant p300 fragment rp300n (amino acids 74–163), which contains serine 89 (Fig. 3*A*). In both cases, the antiserum recognized the serine 89-phosphorylated protein with a high degree of specificity. In addition, serine 89 phosphorylation of p300 could be detected by the antiserum when a Flag-tagged p300 was transfected and expressed in HeLa cells but not when a Flag-tagged p300 bearing a point mutation at serine 89 to alanine was expressed (Fig. 3*B*). We subsequently used the antiserum to detect endogenous p300 phosphorylated at serine 89 in different cell lines (Fig. 3*C*). The serine 89-specific phosphorylation of p300 in these cells suggests that this phosphorylation is a common phenomenon.

PKC Mediates the Phosphorylation of p300 at Serine 89—Each of the N-terminal sequences of human and mouse p300 and CBP contains a consensus (R*XX*S) phosphorylation site similar to that used by PKC (45, 46) (Table I). This observation prompted us to test whether PKC was able to phosphorylate

TABLE I
Alignment of human and mouse CBP/p300 N-terminal sequences

p300 human	84 LL**R**SG**S**SPNLNMG 96
p300 mouse	84 LL**R**SG**S**SPNLNMG 96
CBP human	73 LL**R**GG**S**GSSINPG 85
CBP mouse	72 LL**R**GGSGSSINPG 84
Kinase consensus[a]	. . **R** . . **S** . . . . .

[a] R*XX*S is similar to phosphorylation consensus used by cAMP-dependent kinases (PKA), cGMP-dependent kinases, calcium/calmodulin-dependent kinases, and protein kinase C.

rp300n. Purified PKC βII, a classical PKC isotype, phosphorylated rp300n but not its serine 89 to alanine point mutant (rp300nS89A) (Fig. 4*A*). In contrast, PKA (cAMP-dependent kinase) and CaM kinase IV (calcium/calmodulin-dependent kinase), which also recognize R*XX*S phosphorylation sites (45) failed to phosphorylate serine 89 of p300 (data not shown). However, PKC βII was not detected in HeLa nuclear extracts (Fig. 4*B*), and therefore it was not the kinase we were looking for. By Western analysis, we found that several isotypes of PKC exist in HeLa nuclei. Of them, only a classical type α and a novel type δ were shown to exist in both HeLa nuclei and phosphorylate serine 89 in rp300n specifically (Fig. 4, *A* and *B*).

To test whether PKC mediates phosphorylation of p300 at serine 89 *in vivo*, we treated HeLa and U2OS cells with calphostin C (47). Calphostin C inhibits PKC specifically by interacting with its regulatory C1 domain (48), which is common in all PKC isotypes and binds to its key activator, diacylglycerol (49). Levels of serine 89-phosphorylated p300 were measured using the phosphoserine 89-specific antiserum. The level of the serine 89-phosphorylated p300 was reduced markedly in calphostin C-treated cells, whereas the level of total p300 protein did not change (Fig. 4*C*). Thus, we suggest that PKC mediates phosphorylation of p300 at serine 89 in different cells.

Phosphorylation of p300 at Serine 89 Represses Its Function as a Transcriptional Coactivator—We next investigated whether phosphorylation at serine 89 affected p300 function. Expression constructs containing a Gal4 DNA-binding domain fused to either wild type human p300 (Galp300) or a cDNA containing a point mutation at serine 89 to alanine (Galp300S89A) were used in this study. When transfected into HeLa cells, the wild type and point mutant fusion proteins were expressed at the same level (Fig. 5*A*). The point mutant was significantly more active than the wild type p300, however, when tested using a CAT reporter driven by Gal4 binding sites (Fig. 5*B*). These observations suggest that phosphorylation at serine 89 repress the transcriptional ability of p300. We further tested whether the p300 point mutation affected transcription of specific promoters. For these studies, we utilized promoters containing an ER-binding site or activator protein-1 (AP-1) site derived from the collagenase gene (40, 50). The serine 89 to alanine point mutant was also more active than the wild type p300 in potentiating both promoters (Fig. 5, *C* and *D*). These observations indicate that phosphorylation of p300 at serine 89 represses its ability as a transcriptional coactivator and thus suppresses the ER and AP-1 activities.

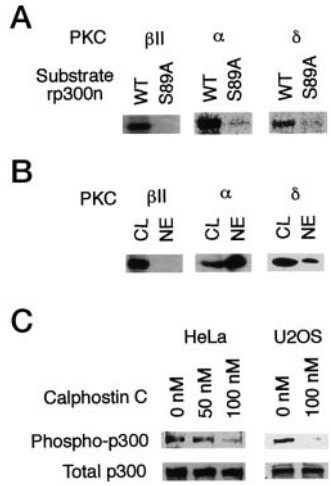

FIG. 4. **Protein kinase C mediates phosphorylation of p300 at serine 89.** *A*, phosphorylation of wild type (*WT*) or point mutant (*S89A*) rp300n (GST-p300 amino acids 74–163) by purified PKC βII and other PKC isotypes immunoprecipitated from HeLa cells. *B*, Western analysis of the PKC proteins in whole cell lysate (*CL*) and nuclear extracts (*NE*), each prepared from an approximately equal number of cells. *C*, Western analysis of p300 phosphorylated at serine 89 after immunoprecipitated with anti-p300 antibodies from HeLa and U2OS cells treated with calphostin C.

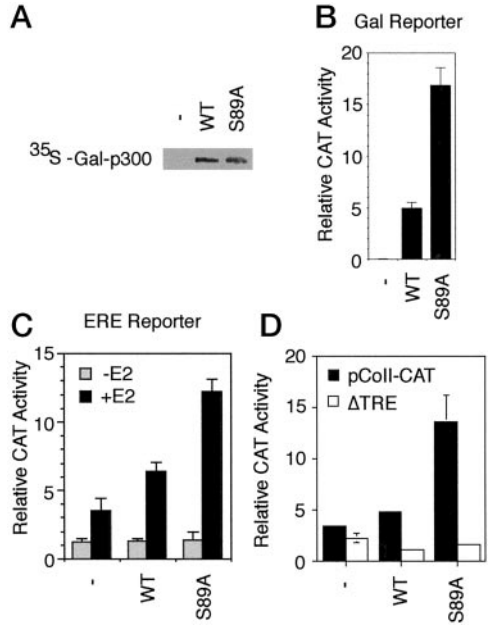

FIG. 5. **p300 transcriptional activity is inhibited by phosphorylation at serine 89.** *A*, cells were labeled with [^{35}S]methionine after transfection with Gal4 1–147 (−), Galp300 (*WT*), or Galp300S89A (*S89A*). Proteins were immunoprecipitated with anti-Gal4 antibodies. *B–D*, transfection with either Gal4 1–147 (−), Galp300 (*WT*), or Galp300S89A (*S89A*) (0.5 μg) and different reporters (1 μg each): a Gal4 reporter pG5e1bCAT (*B*), pERE-TATA-CAT with ER construct pHEO (0.4 μg) in the presence or absence of estradiol (*E2*, 10 nM) (*C*), and the collagenase reporter pCoII (−517/+63)-CAT (pCoII-CAT) or pCoII (−517/+63 ΔTRE or AP-1)-CAT (ΔTRE) (*D*). CAT activities were normalized to cotransfected β-galactosidase activity. *Error bars* represent the standard deviation (n = 3–5). ERE, ER-binding site.

DISCUSSION

Phosphorylation is known to be an important mode of transcription factor regulation. Components of the basal transcriptional machinery and elements of chromatin are also regulated by phosphorylation. Surprisingly, despite the essential roles of CBP/p300 in transcriptional signaling, characterization of their regulation by phosphorylation remains relatively unexplored. Several studies have suggested that CBP and p300 are phosphorylated (12, 30–33), but in most of these cases, it remains unclear which kinases are responsible for CBP/p300 phosphorylation *in vivo* and which specific residues are phosphorylated. Here, we present the first identification of an *in vivo* phosphorylation site in CBP/p300, characterize the responsible kinase, and demonstrate the repression of p300 function by this phosphorylation. These observations indicate that p300 and presumably CBP are indeed regulated by phosphorylation. Serine 89 of p300 and its surrounding PKC consensus sequence (R*XX*S) are conserved among human and mouse p300

and CBP. Similar to p300, an N-terminal fragment of CBP (amino acids 1–270) containing the corresponding serine was phosphorylated by PKC.[2] This highly conserved phosphorylation, plus its inhibitory role in the regulation of p300 function, implies its importance in cellular control.

p300 was identified originally through its ability to interact with the adenoviral E1A oncoprotein. To achieve a full level of cellular transformation, E1A must bind to both Rb and p300, which implies important regulatory roles for both of these cellular factors in cell growth (3, 51). Rb is well known as a tumor suppressor and a G_1 checkpoint regulator. Inactivation of Rb via phosphorylation by G_1 cyclin-dependent kinases is crucial to cell cycle progression. Recent studies show that CBP and p300 are also tumor suppressors (1, 52, 53). The requirement for CBP/p300 in cell proliferation has been shown in studies using p300-deficient mice (54) and after ribozyme ablation of CBP/p300 in tissue culture (55). Both studies suggest that the level of CBP/p300 is critical to its role in the regulation of proliferation. Little is known about how CBP/p300 subserves this function, however, or whether the growth/tumor suppressor actions of CBP/p300 are similarly regulated by phosphorylation. Our observations that phosphorylation at serine 89 by PKC represses p300 function suggest a mechanism by which p300 could down-regulate cell growth. Conceivably, the loss of the serine 89-phosphorylation sites in the fusions of MOZ and MLL to CBP or p300 could contribute to the mechanisms that link these translocations to the development of hematological malignancies (10).

The PKC family of proteins are signal-activated kinases of importance in major cellular functions. Release of diacylglycerol and calcium stimulate PKC activity within a short period of time, whereas tumor-promoting phorbol esters (phorbol 12-myristate 13-acetate) may regulate PKC activity within a relative long term (56). The PKC isotypes play distinct roles in the regulation of growth and differentiation depending on which cell types are being examined (57). Our studies show that PKCα and PKCδ have the ability to phosphorylate p300 at serine 89 and repress its function in HeLa cells. This regulation thus represents a previously unsuspected signal transduction pathway for PKC to participate in cellular controls. Consistent with this hypothesis, p300 is expected to participate in certain differentiation pathways (4) and overexpression of PKCα and PKCδ upon phorbol ester stimulation-induced differentiation in a mouse myeloid progenitor cell line (58). This induction of differentiation by PKCα and PKCδ may be mediated in part by phosphorylation of p300. In addition, CBP/p300 as well as PKCα and PKCδ express universally in many different tissues (51, 56), indicating a possible global event of phosphorylation of p300 at serine 89 by PKCα and PKCδ. Indeed, we detected phosphorylation of p300 at serine 89 in all of the cell lines examined.

Previous studies showed that the C-terminal fragments of CBP could be phosporylated *in vitro* by cyclin E-Cdk2 kinase (32). This phosphorylation caused an increase in the HAT activity of CBP. This observation, combined with our study here, suggests that there are two types of regulation by phosphorylation in CBP/p300: a positive effect by phosphorylation, causing a short term stimulation of function, and a repressive effect by phosphorylation at serine 89, possibly serving a checkpoint to CBP/p300 function.

In summary, our findings suggest that p300 is phosphorylated at serine 89 by PKC and that this phosphorylation represses its function. It will be particularly interesting to investigate whether this phosphorylation and repression event of p300 is regulated in the cell cycle and how this event contributes to specific cellular decisions, such as withdrawal from the cell cycle and differentiation.

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