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) 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 (19–23). 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 the responsible 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 Hapes, 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 a SDS-polyacrylamide gel. PKC kinase assays (35) were carried out in the kinase buffer supplemented with 0.5 mM CaCl₂, 100 ng/ml phosphor 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
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, electrophoetized 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) containing 1-butanol, pyridine, acetic acid, and deionized H2O (15:10:3:12) overnight at 37 °C. Manual Edman sequencing of labeled peptides was dissolved in pH 1.9 buffer containing 88% formic acid, acetic acid, and deionized H2O (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 H2O (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 μM/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 experiment 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 used by immunizing rabbits with a p300-derived oligopeptide containing phosphoserine 89 (RSGSpSPN-LNMGV). The specificity of the antiserum was examined using enzyme-linked immunosorbent assay with phosphorylated and nonphosphorylated oligopeptides at various concentrations. The antibody 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% dextan-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. 1A). 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. 1B), and each fraction was monitored by Cerenkov counting in a scintillation counter. Two major radioiodinated 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 radioiodinated 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 radioiodinated peptide. The labeled peptide [35S]rp300N Kg (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 radioiodinated tryptic peptide collected from peak A. At each cycle, the in situ solution A filter bearing the remaining peptide was subjected to Cerenkov counting. Only serine 89 at cycle 3 was labeled (Fig. 1C), 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. 1D) and completely eliminated labeling of the CNBr peptide as measured by two-dimensional peptide map.
Phosphorylation of p300 by PKC

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 32P. Two-dimensional peptide mapping of the phosphorylated parent ions determined the identity of the phosphorylated peptide. The MS/MS spectra of the phosphorylated peptide were consistent with the loss of residue from the parent ion. Sixteen b and y series ions were assigned. Six of these b ions and two of these y ions resulted from the additional loss of phosphate (b5 and y5). Based on the m/z value of 611.9 in the spectrum, 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 was immunoprecipitated from nuclear extracts was digested with CNBr and endoproteinase Glu-C. The digested peptides were analyzed by Western blotting using antibodies specific for phosphorylated serine 89. 

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, a chromatogram displaying currents for ions with calculated m/z at 620.8 (nonphosphorylated) and 660.8 (phosphorylated) for the doubly charged ion 84LLRSGSSPNLNh95. 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.

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 (RXXS) phosphorylation site similar to that used by PKC (45, 46) (Table I). This observation prompted us to test whether PKC was able to phosphorylate...
were expressed at the same level (Fig. 5). In HeLa cells, the wild type and point mutant fusion proteins (Galp300S89A) were used in this study. When transfected into cells fused to either wild type human p300 (Galp300) or a cDNA containing a Gal4 DNA-binding domain, whether phosphorylation at serine 89 affected p300 function.

We next investigated using the phosphoserine 89-specific antiserum. The level of the p300 phosphoprotein was measured by immunoblot analysis of p300 phosphorylated (12, 30–33), but in most of these cases, it remains unclear which kinases are responsible for CBP/p300 phosphorylation. Here, we present the first identification of a novel kinase, and demonstrate the repression of p300 function by this phosphorylation. These observations indicate that phosphorylation at serine 89 represses 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 which is similar to phosphorylation consensus used by cAMP-dependent kinases (PKA), cGMP-dependent kinases, calcium/calmodulin-dependent kinases, and protein kinase C.

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 isoforms and binds to its key activator, diacylglycerol (49). Levels of serine 89-phosphorylated p300 were measured in vivo 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. 5C). 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 cells, the wild type and point mutant fusion proteins were expressed at the same level (Fig. 5A). 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. 5B). These observations suggest that phosphorylation at serine 89 represses 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 which is similar to phosphorylation consensus used by cAMP-dependent kinases (PKA), cGMP-dependent kinases, calcium/calmodulin-dependent kinases, and protein kinase C.

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 phosphorylation at serine 89 represses the transcriptional ability of p300.

**TABLE I**

| Alignment of human and mouse CBP/p300 N-terminal sequences |
|-----------------------------------------------------------|
| p300 human | 84 LLRSGGSSPNLNGM 96 |
| p300 mouse | 84 LLRSGGSSPNLNGM 96 |
| CBP human | 84 LLRSGGSSPNLNGM 96 |
| CBP mouse | 84 LLRSGGSSPNLNGM 96 |

**Fig. 4.** Protein kinase C mediates phosphorylation of p300 at serine 89. A, phosphorylation of wild type (WT) or point mutant (S89A) p300n (GST-p300 amino acids 74–163) by purified PKC βII and other PKC isoforms 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 [35S]methionine after transfection with Gal4 1–147 (–), Gal300 (WT), or Gal300S89A (S89A). Proteins were immunoprecipitated with anti-Gal4 antibodies. B–D, transfection with either Gal4 1–147 (–), Gal300 (WT), or Gal300S89A (S89A) (0.5 μg) and different reporters (0.5 μg each): a Gal4 reporter p45E1bCAT (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 II (–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.
and CBP. Similar to p300, an N-terminal fragment of CBP (amino acids 1–270) containing the corresponding serine was phosphorylated by PKC. 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 adenosinergic 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 G1 checkpoint regulator. Inactivation of Rb via phosphorylation by G1 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 ribosome 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 PKC might regulate PKC activity within a relative long term (56). The PKC isoforms play distinct roles in the regulation of growth and differentiation depending on which cell type 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 by PKCδ. Indeed, we detected phosphorylation of p300 at serine 89 by PKCδ. This regulation 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.

Acknowledgments—We acknowledge assistance from P. W. Howard in two-dimensional mapping, from L. L. David in MS analysis, from P. W. Howard in making the phosphoserine 89-specific antiserum, and from F. Caldwell during manuscript preparation. We thank P. Chambon for pHEO and pERE-TATA-CAT plasmids, Yang Shi for pc0II-CAT and ΔTRE constructs, A. Newton for PKCβII protein, and M. L. Dell’Acqua and A. Edwards for anti-PKC antibodies and helpful discussions.

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