Acetylation at Lysine 183 of Progesterone Receptor by p300 Accelerates DNA Binding Kinetics and Transactivation of Direct Target Genes

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Background: The identification of steroid receptor acetylation was based on the consensus motif (K/R)KK. This study reports the discovery by mass spectrometry of a novel progesterone receptor acetylation site at Lys-183 that is not in the consensus motif. In vivo acetylation and mutagenesis experiments revealed that Lys-183 is a primary site of progesterone receptor (PR) acetylation. Lys-183 acetylation is enhanced by p300 overexpression and abrogated by p300 gene silencing, suggesting that p300 is the major acetyltransferase for Lys-183 acetylation. Furthermore, p300-mediated Lys-183 acetylation is associated with heightened PR activity. Accordingly, the acetylation-mimicking mutant PRB-K183Q exhibited accelerated DNA binding kinetics and greater activity compared with the wild-type PR on genes containing progesterone response element. In contrast, Lys-183 acetylation had no influence on PR tethering effect on the nuclear factor κ-light chain enhancer of activated B cells (NFκB). Additionally, increases of Lys-183 acetylation by p300 overexpression or inhibition of deacetylation resulted in increases of Ser-294 phosphorylation levels. In conclusion, PR acetylation at Lys-183 by p300 potentiates PR activity through accelerated binding of its direct target genes without affecting PR tethering on other transcription factors. The effect may be mediated by enhancing Ser-294 phosphorylation.

The ovarian steroid hormone progesterone is essential for normal mammary and uterine development. It is also imperative for embryo implantation and maintenance of pregnancy. The progesterone receptor (PR) is the principal mediator of progesterone action. There are two major subtypes of human PR, PR-A and PR-B, that are translated from transcripts generated from two distinct promoters in the gene (1). PRA lacks the 165 amino acids found in the N terminus of PRB. PR isoforms are differentially expressed in a cell- or tissue-specific manner (2–4). PR belongs to the nuclear receptor superfamily that is characterized by a modular domain structure. The structure of PR begins with a poorly conserved N-terminal transactivation domain (NTD) that includes the activation function-1 (AF-1) originally defined to a core region of amino acids 456–546 (5). This is followed by the DNA binding domain responsible for receptor dimerization and DNA binding. The C-terminal ligand binding domain contains AF-2. AF-1 synergizes with AF-2 to bring about optimal receptor activity (6). In addition, an AF-3 is contained within the first 164 amino acids unique to PRB and synergizes with both AF-1 and AF-2 to maximize PRB activity. There is also a flexible hinge region, connecting the DNA binding domain and ligand binding domain (7). Hinge region contains the nuclear localization signal. It has been reported that PR hinge region also plays a role in regulating PR transactivation through its acetylation (8).

The PR function is regulated by a host of transcription coregulators. Agonist binding results in a conformational change in the ligand binding domain that allows the receptor to interact directly or indirectly with a diverse set of co-regulatory proteins (7). These include steroid receptor coactivator (SRC) family members (i.e., SRC-1, SRC-2, and SRC-3), which act primarily as anchoring surfaces to bridge the binding of other coregulators, such as protein-modifying enzymes acetyltransferases and methyltransferases (9–13). p300, its paralog CREB-binding protein (CBP), and p300/CBP-associated factor (PCAF) are the best characterized mammalian acetyltransferases. These proteins co-regulate the activities of a variety of transcription factors, including PRs and other nuclear receptors (14–21). They are recruited to PR in a ligand-dependent manner via interactions with SRC proteins (13) and acetylate nucleosomal histones and transcription coregulators to modulate transcriptional activity (22). For example, acetylation could facilitate recruitment of transcription factors to specific target DNA sequences by loosening nucleosomal structure and facilitate transcription factor binding to gene promoters. In addi-
PR Acetylation at Lysine 183 Promotes PR Transactivation

PR acetylation at Lys-183 enhances chemiluminescence (ECL) (GE Healthcare) or Immobilon Western Chemiluminescent HRP substrate (Millipore) according to the manufacturer’s protocol on x-ray films (Eastman Kodak Co.).

**PR Immunoprecipitation**—For MS identification of PR acetylation, ABC28 cell lysates were prepared in lysis buffer and incubated overnight at 4 °C with anti-PR antibody (H-190, Santa Cruz Biotechnology) precaptured by protein A/G-agarose beads (Santa Cruz Biotechnology). The immune complexes were subsequently washed two times with lysis buffer and two more times with high salt (500 mM NaCl) lysis buffer to remove nonspecific binding. Bound proteins were eluted by boiling in Laemmli sample buffer. The immunoprecipitated proteins were resolved by SDS-PAGE, fixed, and stained by Coomassie Blue G-250 (Sigma-Aldrich).

**In-gel Tryptic Digestion**—The PR gel bands were excised, chopped into small pieces (~1 × 1 mm), and transferred to Eppendorf tubes. They were washed with Milli-Q water; destained with 50% acetonitrile (ACN), 50% 25 mM NH₄HCO₃ via vigorous vortexing for 30 min three times; and dehydrated with 100% ACN until the gel particles became white. They were then reduced with 10 mM dithiothreitol (DTT) at 56 °C for 1 h and alkylated with 55 mM iodoacetamide for 45 min in the dark followed by successive washes with 25 mM NH₄HCO₃ and 50% ACN, 50% 25 mM NH₄HCO₃. Finally, they were dehydrated with 100% ACN and dried in vacuum. Trypsin (Trypsin Gold, Mass Spectrometry Grade, Promega, Madison, WI) was added in a weight ratio of 1:30. After the trypsin solution was completely absorbed by gel particles, 25 mM NH₄HCO₃ was added to completely cover the particles. They were then incubated at 37 °C overnight. Tryptic peptides were extracted from gel particles with 50% ACN containing 0.1% formic acid under sonication for 2 × 20 min. The combined extracts were dried in vacuum.

**LC-MS/MS and Data Analysis**—LC-MS/MS was carried out as described previously (26). Briefly, peptides were separated and analyzed on a liquid chromatograph ( Dionex UltiMate 3000 Nano-LC Systems) at a 300-nl/min flow rate coupled to an LTQ-FT Ultra system (Thermo Electron, Bremen, Germany). Mobile phase A (0.1% formic acid in H₂O) and mobile phase B (0.1% formic acid in ACN) were used to establish the 60-min gradient composed of 45 min of 5–35% B, 8 min of 35–50% B, and 2 min of 80% B followed by re-equilibration at 5% B for 5 min. Peptides were then analyzed on the LTQ-FT system with an ADVANCE™ CaptiveSpray™ source (Michrom BioResources) at an electrospray potential of 1.5 kV. A gas flow of 2, ion transfer tube temperature of 180 °C, and collision gas pressure of 0.85 millitorrs were used. A full MS scan (350–1600 m/z range) was acquired in the FT-ICR cell at a resolution of 100,000 and a maximum ion accumulation time of 1000 ms. The automatic gain control target for FT was set at 1e+06, and precursor ion charge state screening was activated. The linear ion trap was used to collect peptides and to measure peptide fragments generated by collision-induced dissociation. The default automatic gain control setting was used (full MS target at 3.0e+04, MS² at 1e+04) in the linear ion trap. The 10 most intense ions above a 500-count threshold were selected for fragmentation in collision-induced dissociation (MS³), which was
performed concurrently with a maximum ion accumulation time of 200 ms. For collision-induced dissociation, the activation Q was set at 0.25, isolation width (m/z) was 2.0, activation time was 30 ms, and normalized collision energy was 35% (27).

The MS/MS spectra in the raw data were first extracted into the dta format using the extract_msn (version 4.0) in Bioworks Browser (version 3.3, Thermo Fisher Scientific), and then the dta files were converted into the Mascot generic file format using an in-house program. Intensity values and fragment ion m/z ratios were not manipulated. The Uniprot human protein database (downloaded on April 5, 2013, with 132,655 sequences; 43,940,360 residues) was used for database searches. The database search was performed using an in-house Mascot server (version 2.4.0, Matrix Science, London, UK) with MS tolerance of 10 ppm, option 13C of 2, and MS/MS tolerance of 0.8 Da. Two missed cleavage sites of trypsin were allowed. Carbamidomethylation (Cys) was set as a fixed modification, and oxidation (Met), deamidation (Asn-Gln), acetylation (Lys), methylation (Lys-Arg), dimethylation (Lys-Arg), and trimethylation (Lys) were set as variable modifications. The obtained database search results were exported to Microsoft Excel using the export_dat_2.pl script of Mascot for further analysis. Only pepsearch results were exported to Microsoft Excel using the option13C of 2, and MS/MS tolerance of 0.8 Da. Two missed cleavage sites of trypsin were allowed. Carbamidomethylation (Cys) was set as a fixed modification, and oxidation (Met), deamidation (Asn-Gln), acetylation (Lys), methylation (Lys-Arg), dimethylation (Lys-Arg), and trimethylation (Lys) were set as variable modifications. The obtained database search results were exported to Microsoft Excel using the export_dat_2.pl script of Mascot for further analysis. Only peptides with E-values of less than 0.05 and Mowse scores greater than the identity or homology scores were further manually analyzed.

**PR Mutant Construction by Site-directed Mutagenesis—**

cDNA3.1 expression vectors encoding human PRB have been described previously (28). Point mutations of the Lys residue at site 183 of PR to glutamine and arginine were generated using the QuikChange® XL site-directed mutagenesis kit (Stratagene) according to the manufacturer’s protocol. The site-directed primers were designed using a Web-based program known as QuikChange® Primer Design. The mutations were verified by full-length cDNA sequencing. The sequences of mutagenic sense and antisense primers used were as follows: K183Q_sense, 5’-cgg cag ctc gcc atc agc tgt gcc ccc gg-3’; K183Q antisense, 5’-ggg cag cgg ctc attg gcc agc tgt cg-3’; K183R_sense, 5’-ggg cag cag ctc attg gcc agc tgt cg-3’; K183R antisense, 5’-ggg cag cgc ctc attg gcc agc tgt cg-3’.

**Small Interfering RNA (siRNA) Transfection—**

Predesigned control and p300 siRNAs (Ambion, Inc.) were transfected into ABC28 cells using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s instructions. ABC28 cells were plated in phenol red-free DMEM supplemented with 5% charcoal-stripped FBS and incubated at 37 °C for 48 h prior to transfection. For each well of a 6-well plate, 6 μl of Lipofectamine 2000 reagent and 40 nm siRNA were added into a final 2 ml of transfection medium and incubated for 8 h prior to a medium change. The sequences of p300 siRNA used were as follows: S1RNA1_sense, 5’-GCCUGGUAUAAUACCCGGAT (Ambion S2RNA ID S4697); S1RNA1 antisense, 5’-UCCGGUUAUAA-ACCAGGCat (Ambion S2RNA ID S4697); S1RNA2_sense, 5’-GGACUACCCUAUACCCAGA (Ambion S2RNA ID S4695); S1RNA2 antisense, 5’-UUACUGUAGGGGUGGUC (Ambion S2RNA ID S4695).

**Plasmid Transfection—** COS7 or HeLa cells were plated in 35-mm dishes at a density of 2.5 × 10^5 cells in antibiotic-free phenol red-free DMEM. The cells were then transfected with 0.5 μg of pc-DNA3.1, pc-DNA3.1wt-PRB, or the various pc-DNA3.1 Lys-183-PRB mutants using polyethyleneimine (PEI) (CELLnTEC, Switzerland) after 24 h of plating. The cells were then treated with 0.1% ethanol, 10 nm R5020 for 1 h at 24 h post-transfection. The cell lysate was then collected to test for the expression of total PR and phospho-PR.

**In Vitro Histone Acetyltransferase Assays of PR Peptides and PR Protein—** The in vitro acetylation assay was carried out as described (24) with minor modifications. PR peptide (KYGDS-SGTAAAHK183VLPRGLSPARQ (wild type) or KYGDSGTAAAHK183VLPRGLSPARQ (K→Q)), 1 μg of rh recombinant histone H3 protein (as positive control), or immunoprecipitated PR was incubated with 0.25 μCi of [3H]acetyl coenzyme A (CoA) (Amersham Biosciences) and 10 ng of recombinant p300 enzyme (Active Motif) in 30 μl of acetylation buffer containing 50 mM Tris (pH 8.0), 5% glycerol, 0.1 mM EDTA, 50 mM KC1, 1 mM DTT, 1 mM PMSF, and 10 mM sodium butyrate. Immunoprecipitated HA-CBP or FLAG-PCAF were also tested for their activity in acetylating PR in in vitro acetylation experiments. Reaction mixtures were incubated at 30 °C for 1 h, stopped by the addition of 5× Laemmli buffer, and resolved by SDS-PAGE. The reactions were analyzed first by Coomassie Blue staining to verify the amounts of proteins used in each reaction, and the same gel was subsequently subjected to autoradiography to evaluate acetylation.

**In Vivo Acetylation Assay—** The in vivo acetylation assay was carried out as reported (8). Briefly, PR-deficient COS7 cells were grown in DMEM containing 7.5% FBS and were transfected with pcDNA3.1-FLAG-PR in the presence or absence of pCI-FLAG-p300 using PEI. The transfected COS7 cells were pretreated with 1 μM TSA for 30 min followed by vehicle control or 10 nm R5020 treatment for another 1 h before cell lystate collection. Protein lystate was collected for PR immunoprecipitation by FLAG antibody, and captured FLAG-PR and FLAG-p300 were resolved by SDS-PAGE. Acetylated proteins were probed by a pan-anti-acetylated lysine antibody (catalog no. 9441, Cell Signaling).

**PR Luciferase Reporter Assay—** Luciferase assay procedures were adapted from the manufacturer’s protocol provided for the Dual-Luciferase Reporter System kit (Promega). Briefly, HeLa cells were seeded onto 60-mm dishes and were transfected with PEI with 5 ng of pcDNA3.1, pcDNA3.1wt-PRB, or pcDNA3.1-PRB-K183Q/R in addition to 1500 ng of reporter plasmids, such as PRE-Luc reporter or NFκB-Luc reporter. 1 ng of Renilla pRL-CMV vector was co-transfected as normalization control. 24 h post-transfection, the cells were treated with 0.1% EtOH or 10 nm R5020 for 12 h before the were lysed by 1× passive lysis buffer provided in the Dual-Luciferase Reporter System kit (Promega). 20 μl of lystate were analyzed using a computer-controlled microplate luminometer (Thermo Scientific Fluoroskan® Ascent FL). The activities of the Renilla and firefly luciferase were measured according to the manufacturer’s protocol. PRE/NFκB-dependent luciferase activity was normalized by Renilla reading and expressed as the average relative light units (RLU) of triplicate or four replicate measures ± S.E. Fold induction by ligand was calculated as the ratio of ligand-induced sample RLU to the corresponding vehicle control-treated sample RLU.
RNA Extraction and Quantitative RT-PCR—Total RNA was extracted with TRIzol reagent (Invitrogen) based on the manufacturer’s instructions. RNA was reverse transcribed with SuperScript II reverse transcriptase (Invitrogen) based on the manufacturer’s protocol. Quantitative RT-PCR was carried out with SYBR Green master mix (Bio-Rad) on an ABI Prism 7700 sequence detection system (Applied Biosystems) based on the manufacturer’s protocol. Primer sequences used in real-time PCR are as follows: FKBP5_sense, 5'-ccc cgg cgg cgg cgg cct tgc ttc ttc ctc taa tag agg gcc ggc cca tgc tga ct-3'; FKBP5_antisense, 5'-ccc atc atc ggc gtt tcc tca cca; 11ß-HSD2_sense, 5'-cca ggg gcc gca ttc tga ct-3'; 11ß-HSD2_antisense, 5'-ggc gca gct ctt tgg gct gga gct ggg t-3'; IL-6_sense, 5'-ggg aca tcc tgc agc gca ttc ttc tgc tgc tt-3'; IL-6_antisense, 5'-ggt ctc ctt tgg gcc ttc ttc ttc ttt ccc-3'; p300_sense, 5'-ggg aca gaa cag cca agc acc tc-3'; p300_antisense, 5'-ggg taa ggt tgc caa gtc ctg gct-3'; PGR_sense, 5'-ggg gat agc tct gag tcc gag ga-3'; PGR_antisense, 5'-ggg gag tgg gat gtc gtt ggc gtc ttc cat-3'; 36B4_sense, 5'-ggc atg ttc cca gca ggt tg-3'; 36B4_antisense, 5'-gcc tgt acct ttt tca caa ag-3'; MUC1_sense, 5'-aga cgt cag cgt gta tgc ctg-3'; MUC1_antisense, 5'-cag cag cgg gta ctt ttc ctc gc-3'; CSF2_sense, 5'-ggt acct aca agc agc agc agc-3'; CSF2_antisense, 5'-aaa cag cag ctg acc atc ctc gtc cc-3'; IER3_sense, 5'-tgc tgt aaa tgc agg tct ctt g-3'; IER3_antisense, 5'-aga cag cag gag tgg atg aga gca tgc t-3'. Real-time PCR for each targeted gene was performed in triplicates. Human acidic ribosomal phosphoprotein P0 (RPLP0), 36B4, was included as a RNA loading control for normalization of the quantity of the cDNA sample used in each experiment. The fold induction or repression for each gene expression between ethanol (vehicle control) and hormone treatment samples was calculated by normalizing Ct values with 36B4 Ct values based on the following formula:

Relative expression

\[ \frac{2^{\left(\text{Ct}_{\text{control}} - \text{Ct}_{\text{hormone}}\right)}}{2^{\left(\text{Ct}_{\text{control}} - \text{Ct}_{\text{hormone}}\right)}} \]  

(Eq. 1)

After the amplification process, a melting curve analysis using the LightCycler instrument was performed to verify the specificity of the PCR products.

Chromatin Immunoprecipitation—Chromatin immunoprecipitation (ChIP) analyses were performed with procedures modified from protocols described previously (29). The ABC28 cell line was grown in DMEM supplemented with 5% dextran charcoal-stripped FBS for 3 days. Cells were then treated with R520 (10^{-8} M) for various durations, as indicated in the figure legends, before collection for the ChIP assay. Cells were fixed with formaldehyde at a final concentration of 1% for 10 min at room temperature; cross-linking was stopped by the addition of glycerol to a final concentration of 0.125 M. Cells were then harvested, and the remaining steps followed standard protocols for ChIP experiments. Anti-PR antibody (H-190, Santa Cruz Biotechnology) was used for ChIP assays. PCR was performed to analyze immunoprecipitated chromatin in studies with ABC28 cells. The steps of PCR were as follows: 34 cycles with 1 min of denaturing at 94 °C, annealing at 60 °C, and extension at 68 °C. After DNA purification (Geneaid), immunoprecipitated DNA was assayed for the binding of the human FKBP5 (FK506-binding protein 5) gene promoter sequences. Primers for the human intronic PRE region were as follows: sense, 5'-taa tag agg gcc gag aag gca ga-3'; antisense, 5'-ggt aag tgt gtc tgc ctc ctc a-3'. PCR products were analyzed by agarose gel (2%) electrophoresis.

Plasmid Immunoprecipitation—Plasmid immunoprecipitation experiments were performed as described previously with minor modifications (30). HeLa cells were grown in DMEM supplemented with 5% charcoal-dextran-stripped serum for 1 day and transfected with 10 ng of WT PRB or PRB-K183Q/R expression vectors, in addition to 1.5 μg of PRE-TATA luciferase reporter plasmid and/or 100 ng of PCI-FLAG-p300 or PCI vector control. 24 h post-transfection, cells were treated with either R520 (10^{-8} M) or vehicle control (0.01% EtOH) for various durations. Cross-linking and termination of cross-linking of cells were as described for the ChIP assay. Cells were then harvested, and the remaining steps followed standard protocols for ChIP experiments. Anti-PR antibody (H-190, Santa Cruz Biotechnology) was used for PR-PRE-TATA-luciferase plasmid immunoprecipitation. The resulting immunoprecipitated and input DNA was analyzed by PCR or real-time PCRs with the following pair of primers for the detection of PRE luciferase sequences: sense, 5'-cta gca aaa tag gct gtc cc-3'; antisense, 5'-tat gtt ttt ggc gtc ttc cat-3'. PCR products were analyzed by 2% agarose gel electrophoresis.

Statistical Analysis—Data were analyzed using unpaired, two-tailed Student’s t test with a 95% confidence interval using the program GraphPad Prism version 5.

RESULTS

Identification of PR Acetylation at Lys-183 by LC-MS/MS—To further elucidate the mechanisms of progesterone action, sensitive LC-MS/MS tools were exploited to identify novel PR post-translational modifications using PR-transfected MDA-MB-231 cell line ABC28 that has been well characterized previously (25). Large scale immunoprecipitations of endogenously expressed PRA and PRB from ABC28 cells were carried out. The gel bands containing immunoprecipitated PR were excised and processed to tryptic peptides for LC-MS/MS analysis. Lys-183-modified peptides with a mass gain of 42.01 Da in lysine residue were consistently identified in almost all preparations. As is shown in Fig. 1, A and B, two tryptic peptides of 3+ charge state with monoisotopic masses at 522.620 and 536.623 Da, respectively, were detected. These two peptides have similar MS spectra but differ in mass by 42.01 Da (536.623 – 522.620) × 3, suggesting that the peptide with a mass of 536.623 is modified by acetylation or trimethylation. The MS/MS spectrum of a triply charged precursor ion at m/z 522.62022 (calculated molecular mass 1567.86066 Da) of the peptide VGDGGTAAAHKVLPR was detected at a retention time of 16.88 min (Fig. 1A). The MS/MS spectrum of a triply charged ion at m/z 536.623430 corresponding to the mass of the corresponding modified peptide (calculated molecular mass 1609.87029 Da) was identified at a later retention time, 22.17 min (Fig. 1B).

The precursor ions of the unmodified and modified peptides detected in the MS spectra were fragmented by MS/MS to identify the peptide and to assign the modification site. The MS/MS spectra and the fragment ion assignments were shown in Fig. 2. The fragment ions of the peptides were assigned to the b or y ion series by Mascot and confirmed by manual inspection of the
MS/MS spectra. The Lys-183 is sandwiched by a series of b and y ions in Fig. 2, B and C. From the manual inspection of the doubly charged y ions series, we observed mass shifts of 42 Da in the y2+/H11001 ion series of the modified peptide spectrum from y52+/H11001 to y152+/H11001 (Fig. 2B) when compared with the corresponding y2+/H11001 ion series of the unmodified peptide (Fig. 2A). For example, the increase in m/z value between y52+/H11001 of the modified peptide (m/z = 484.43) and y52+/H11001 of the unmodified peptide (m/z = 462.45) is 122 Da, corresponding to the mass of lysine (128.17) and an additional mass of 42 Da. The manual inspection of the b ion series flanking Lys-183 also indicates that the positive mass shift of 42 Da takes place at Lys-183. The detailed b and y ion profiles of this peptide from Mascot search is provided in Mascot Peptide View in supplemental data 3.

The peptide VGDSSGTAAAHK(Ac)VLPR was also detected in endogenously expressed PR in breast cancer cell line T47D. Manual inspection of the y ions series also confirmed the site of the 42-Da mass shift to be at Lys-183 (supplemental data 4 and 5). The detailed b and y ion profiles of the peptides from Mascot search are provided in Mascot Peptide View in supplemental data 6 (unmodified) and 7 (modified).

To further confirm Lys-183 acetylation, we analyzed FLAG-tagged PRB because a larger amount of PRB can be pulled down by FLAG antibody. MDA-MB-231 cells were stably transfected with FLAG-tagged PR, and the isolated FLAG-PRB was analyzed by LC-MS/MS. The modified peptides with the mass gain of 42 Da were consistently identified with very high confidence. Fig. 2C illustrates a representative MS/MS spectrum from a doubly charged precursor ion and its fragment ion assignment to VGDSSGTAAAHK(Ac)VLPR peptide. The peptide was identified by Mascot with an ion score of 115, and manual inspection of the b ion series and an almost complete y ion series that sandwiched the Lys-183 site indicates that the positive mass shifts of 42 Da started from y5 and extended to y13 but were not observed from y2 to y4, which confirms the presence of modification at Lys-183. For example, the mass difference between y4 (m/z = 484.43) and y5 (m/z = 526.45) of the modified peptide is 42 Da, which corresponds to the mass of lysine (128.17) and an additional mass of 42 Da. The manual inspection of the b ion series flanking Lys-183 also indicates that the positive mass shift of 42 Da takes place at Lys-183. The detailed b and y ion profiles of this peptide from Mascot search is provided in Mascot Peptide View in supplemental data 3.

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The observed mass shift of 42 Da in precursor and fragments ions could be attributable to Lys-183 acetylation (42.0106 Da) or trimethylation (42.0470 Da). Because the LTQ-FT Ultra instrument provides high resolution and mass accuracy with mass error of <10 ppm, the Lys-183 modification is positively identified as acetylation based on the accurate detection of the mass difference of 42.01 Da in modified and unmodified precursor ions.

The unambiguous identification of Lys-183 as acetylation but not trimethylation is further supported by the charge states detected in the non-acetylated and acetylated peptides. The
non-acetylated VGDSSGTAAAHKVLPR peptide was detected in 3+ and 4+ charge states, whereas the acetylated VGDSSG-TAAAHK(Ac)VLPR peptide was detected in 2+ and 3+ charge states. This is consistent with the fact that acetylation removes the protonation site at lysine, but trimethylation does not. Moreover, acetylation at lysine increases the hydrophobicity of the peptide due to loss of the protonation site at Lys-183, resulting in a significant increase in the peptide retention time from 16.88 min (unmodified) to 22.17 min (acetylated) in the reverse phase C18 column during LC-MS/MS analysis.

Verification of Lys-183 Acetylation by Acetyl-lysine Antibody—PR acetylation at Lys-183 was further verified with a pan-acetyl-lysine antibody (Fig. 3). WT PRB or mutant K183R was transiently transfected into COS7 cells. Immunoprecipitated PR was analyzed for acetylation by Western blotting analysis. PR acetylation is increased following treatment with progestin R5020 or trichostatin A (TSA), a histone deacetylase (HDAC) inhibitor. Combined treatment of TSA and R5020 resulted in greater
induction of PR acetylation than either treatment alone. More importantly, PR acetylation was not detectable in K183R mutant under all treatments, indicating that Lys-183 is the major site of acetylation on PR.

**PR Is Acetylated at Lys-183 by p300 and CBP by in Vitro Acetylation Assay**—Acetylation of steroid receptors has been shown to be catalyzed by histone acetyltransferases. Several acetyltransferases, such as p300, CBP, and PCAF, have been shown to acetylate nuclear receptors and modulate their activities (31). To identify the enzyme that acetylates PR at Lys-183, we conducted an *in vitro* acetylation assay with the potential acetyltransferases (recombinant FLAG-p300 or immunoprecipitated HA-CBP and FLAG-PCAF). It was found that all three enzymes could acetylate full-length PRB (Fig. 4, A–C). However, because PCAF is associated with p300/CBP, it is not clear if the activity comes from PCAF itself or the associated proteins.

We next asked if these enzymes could specifically acetylate Lys-183 of PR because Lys-183 is not contained in the typical acetylation consensus sequence (K/R)|X|KK. The peptide acetylation assay showed that both recombinant p300 and HA-CBP acetylated Lys-183, whereas the substitution of the residue Lys-183 with glutamine abolished acetylation (Fig. 4, D and E). On
the other hand, PCAF failed to acetylate Lys-183 of PR under the same assay conditions, although the enzyme strongly acetylated the positive control full-length recombinant histone H3 protein (Fig. 4F). This is consistent with our earlier notion that FLAG-PCAF-associated p300 or CBP could be involved in the acetylation of full-length PR, but the amount of co-immunoprecipitated p300 and CBP was insufficient to generate a detectable acetylation signal of the peptide. This is also supported by the observation that the peptide acetylation signals by p300 and CBP were also low, and hence a large amount of the enzymes would be needed to generate a detectable signal. Therefore, p300 and CBP but not PCAF can target PR for acetylation at Lys-183. However, the data cannot rule out the possibility that PCAF acetylates a previously reported acetylation motif, lysines 638–641 on PR (8).

**PR Is Acetylated at Lys-183 by p300 in Vivo—Because p300 and CBP are closely related transcriptional co-activators with extensive sequence and structural homology (32) and overlapping functions (33), we chose from here on in to focus on p300 for its role in PR acetylation and function. To confirm that Lys-183 as an authentic *in vivo* acetylation site by p300, we carried out an *in vivo* PR acetylation assay by co-expressing p300 and PRB in COS7 cells, and PR acetylation was detected by pan-acetyl-lysine antibody following co-immunoprecipitation. As is shown in Fig. 4G, PR acetylation was markedly increased by p300 overexpression, and PR acetylation by p300 was further increased in the presence of PR ligand R5020.

Having established that PR was acetylated *in vitro* and *in vivo* by p300, we next assessed whether p300 was the major acetyltransferase responsible for PR acetylation. p300 in ABC28 cells was knocked down using siRNA against p300, and its effect on PR acetylation level was assessed. After p300 knockdown, the PR protein was immunoprecipitated and visualized by Western blotting for acetylation (Fig. 4H). The transfection of p300 siRNAs resulted in undetectable levels of p300 by Western analysis while causing minimal change in PR protein levels (Fig. 4H). The silencing of p300 abrogated PR acetylation. Taken together, these data indicate that p300 is primarily responsible for PR acetylation at Lys-183.

**PR Acetylation at Lys-183 Enhances PR Activity Specifically at PRE Promoter—** We next tested the effect of Lys-183 acetylation on PR activity using Lys-183 mutants in a cell-based PR reporter gene assay. The luciferase reporter gene is driven by a PRE-containing Target Genes in Breast Cancer Cells—Data obtained with a reporter gene assay showed that PR acetylation at Lys-183 by p300 promotes PR-mediated transactivation on PRE-driven gene reporter but has no effect on the NFκB reporter gene. We then asked if the same mechanism of regulation occurs with endogenous genes in the PR-transfected MDA-MB-231 cell line ABC28. We did this by determining if p300 gene silencing could attenuate specifically PRE-containing gene expression. ABC28 cells were transiently transfected with negative control siRNAs (scrambled siRNA) or two different p300 siRNAs, which reduced p300 mRNA expression by 60–80% (Fig. 6A). Western blotting analysis showed that both p300 siRNAs also reduced the protein levels of p300 by at least 70% (Fig. 6C). We also verified that PR mRNA (Fig. 6B) and
protein levels (Fig. 6C) were not significantly reduced by p300 siRNA as compared with the control siRNA. Furthermore, ligand-induced PR protein band upshift as a result of PR phosphorylation occurs normally following p300 gene silencing.

Next, the effect of p300 siRNA on progestin-induced expression of three well characterized PRE-containing target genes and progestin-attenuated expression of three genes downstream of NFκB pathways were studied in ABC28 cells. The three direct PR target genes are FKBP5, 11β-HSD2 (11β-hydroxysteroid dehydrogenase type 2), and MUC1 (mucin 1), which contain PRE sequence in their enhancers (29, 36, 37). R5020 treatment induced a 2.8-, 3.3-, and 3.2-fold increase in the expression of FKBP5, 11β-HSD2, and MUC1, respectively (Fig. 6, D–F). p300 siRNAs significantly reduced R5020-induced expression of these genes. On the other hand, silencing of p300 did not cause any effect on the PR-mediated repression of NFκB target genes, CSF2 (colony-stimulating factor 2), IER3 (immediate early response 3), and IL-6 (interleukin-6) (38–40). Thus, it is likely that acetylation of Lys-183 exerts its effects specifically by enhancing PR interaction with its target gene promoter.

**PR Acetylation by p300 Accelerates PR-PRE Binding Kinetics**—Like other members of the nuclear receptor family, PR regulates gene transcription through binding to specific gene sequence and recruiting transcription co-regulators to the gene promoter (41). Upon ligand binding, PR recruits SRC-1, which in turn recruits p300/CBP to the gene promoter. To understand the mechanisms by which PR acetylation by p300 enhances specifically PR transcriptional activity on its direct target genes, we tested the hypothesis that Lys-183 acetylation enhances the rate or duration of PR-DNA binding. ChIP assays were carried out to monitor the influence of PR acetylation on the recruitment of PR to the well characterized intronic PRE on the promoter of FKBP5. Fig. 7A shows that p300 gene silencing resulted in a decrease of PR recruited to FKBP5 promoter (38–40). Thus, it is likely that acetylation of Lys-183 exerts its effects specifically by enhancing PR interaction with its target gene promoter.
PRE on the FKBP5 gene. As is shown in Fig. 7B, there is a time-dependent increase of PRE binding following R5020 treatment both in the absence and presence of TSA. At every time point, more PR was recruited to the PRE in the presence of TSA. Moreover, PRE binding with TSA treatment was detected at an earlier time point than that without TSA treatment. Clearly, TSA-treated sample after 10-min R5020 treatment shows more PRE binding than that without TSA at 30 min. These observations suggest that PR acetylation not only increases but also accelerates its DNA binding.

To evaluate the specific involvement of p300 acetylation of Lys-183 in the PR-PRE binding, we compared the PRE binding kinetics of WT PRB with that of acetylation-mimicking mutant PRB-K183Q. Plasmid immunoprecipitation assays coupled with quantitative real-time PCR were carried out to measure PR binding to the PRE-Luc vector. The assay allows us to compare the PRE binding kinetics between WT PRB and acetylation mimic mutant PRB-K183Q with multiple samples in transient transfection experiment. First, we confirmed using duplication samples that PRB-K183Q pulled down the canonical 2XPRE-Luc sequence more than WT PRB both in the absence and presence of R5020 (Fig. 7D). The magnitude of increase in DNA binding for PRB-K183Q as compared with WT PRB was ~30–40%, which is similar to its increase of transcriptional activity in PRE-luciferase assays and the increased recruitment to PRE with p300 overexpression. The ligand-independent binding is probably responsible for the ligand-independent activity detected in the PRE-Luc assays. Next, we compared their PRE-Luc binding kinetics in 5-min intervals over a 45-min period. It appears that K183Q mutant was more rapidly recruited to the PRE sequences following R5020 treatment (Fig. 7E). More interestingly, K183Q binding exhibited a cyclic fluctuation every 20 min on the synthetic PRE promoter. It reached peak promoter occupancy after 10 min of hormone treatment and returned to base line after 20 min.

**FIGURE 6.** p300 siRNA reduced R5020-induced expression of direct PR target genes, whereas it had minimal effect on PR trans-repression of NFκB target genes in ABC28 cells. ABC28 cells were transfected with NC or p300 siRNAs (40 nM) for 72 h before they were treated with or without 10 nM R5020 for 3 h. The cells were then harvested for RNA and protein analysis. A, p300 expression was reduced by p300 siRNA1 and siRNA2 by 60 and 80%, respectively, relative to scrambled siRNA control. B, PR mRNA levels were not affected by p300 siRNA transfection. C, Western blotting analyses of p300 and PR proteins following p300 siRNA treatment. β-Actin was probed as loading control. D–F, RNA expression of FKBP5 (D), 11β-HSD2 (E), and MUC1 (F) in p300 siRNA 1 and 2-transfected ABC28 cells were significantly reduced in response to R5020 treatment. G–I, R5020-induced suppression of CSF2 (G), IER3 (H), and IL-6 (I) in p300 siRNA-transfected ABC28 cells was not significantly affected. Statistical significance was determined using Student’s unpaired t test, and asterisks denote statistical significance (±S.E.; *, p < 0.05; ***, p < 0.001; ****, p < 0.0001), whereas n.s. denotes non-significant change as determined by unpaired Student’s t tests. Error bars, S.E.
before the next cycle. In contrast, WT PRB binding increased slowly over the period tested and had yet to reach peak promoter occupancy by 45 min. The cyclic fluctuations in the promoter binding of nuclear receptors and its co-regulators are well reported phenomena (29, 42). These data suggest that acetylation mimic K183Q mutant assembles onto the PRE promoter at a faster rate and renews the assembly more quickly. Thus, the acetylation of PR by p300 could enhance the kinetics of its occupancy on the promoter.

PR Acetylation at Lysine 183 Promotes PR Transactivation

Figure 8. Acetylation of PRB is associated with increased PR phosphorylation. A, the stabilization of acetylated PR by TSA enhances its phosphorylation at Ser-294. COS7 cells were transiently transfected with FLAG-tagged PRB. Cells were serum-starved for 24 h and treated with TSA as described in the legend to Fig. 3, followed by R5020 treatment for different time points (indicated in the figure) before collection for PR immunoprecipitation. Proteins resolved on Western blot were detected with pan-acetyl-lysine, PR, and phospho-Ser-294 antibodies. B, in vivo acetylation of PR by p300 increases PR phosphorylation. COS7 cells were transiently transfected with FLAG-tagged PRB in addition to vector control or FLAG-tagged p300 (4 μg). Cells then treated with 10 nM R5020 for different time points indicated in the figure. Proteins were detected using the respective antibodies. C, acetylation mimic mutant PRB-K183Q shows higher levels of Ser-294 phosphorylation than WT PRB or PRB-K183R in response to various R5020 dosages (10⁻⁹, 10⁻⁸, or 10⁻⁷ M). Western blotting analysis was carried out to detect PR and PR phosphorylation at Ser-294 using the respective antibodies. GADPH was probed as a loading control.
time point, sample without TSA treatment showed no detectable Ser-294 phosphorylation (Fig. 8A). Similarly, overexpression of p300, which elevated the levels of acetylated PR with or without R5020 treatment in a time-dependent manner, resulted in time-dependent increases of phosphorylation at Ser-294 as compared with cells with basal p300 expression (Fig. 8B).

To further characterize the specific effects of Lys-183 acetylation on PR phosphorylation, we compared Ser-294 phosphorylation among WT PRB, PRB-K183Q, and PRB-K183R following transient transfection in COS7 cells. As is shown in Fig. 8C, Ser-294 phosphorylation levels increases with increasing concentration of R5020 in all three forms of PRB. However, PRB-K183Q has higher levels of phosphorylation at Ser-294 at all R5020 dosages tested as compared with WT PRB and K183R. Taken together, these findings suggest that PR acetylation at Lys-183 facilitates PR phosphorylation at Ser-294, which, in turn, may lead to greater PR activity.

DISCUSSION

To date, the identification of acetylation of steroid hormone receptors has been based on the presence of acetylation consensus motif (K/R)-XKK (31). By LC-MS/MS analysis, this study reports the identification of a novel acetylation site of PR at Lys-183 that is not contained within such a consensus motif. The acetylation was detected in both the endogenously and exogenously expressed PR from breast cancer cell lines. Close inspection of the peptide sequence containing Lys-183, TAAAHX\textsuperscript{183}/VLPRG, shows that there is a positively charged Arg residue at the Lys-183 + 4 position, which fits the p300 substrate preference reported (45). Indeed, p300 directly acetylates PR at Lys-183 by both \textit{in vitro} and \textit{in vivo} acetylation assays. Lys-183 acetylation is associated with accelerated ligand-induced PR binding to its cognate PRE sequences and heightened PR activation kinetics on direct PR target genes, highlighting a critical involvement of Lys-183 of NTD in PR-DNA interaction.

Our study is consistent with an earlier study that PR acetylation is ligand-induced and can be increased with deacetylase inhibitor (8). However, the study has reported that PR is acetylated at two or more lysine residues at the acetylation consensus motif \textsuperscript{638}KXKK\textsuperscript{641} in the hinge region (8). Mutation of all three lysines is required to abolish the acetylation detection by antibody against acetylated lysines. This is in contrast to our finding that a single mutation, K183R, diminished PR acetylation \textit{in vivo} (Fig. 3), using the same pan-antibody against acetylated lysine. There may be several explanations for the discrepancies. First, although the Lys-183 mutant shows no detectable acetylation \textit{in vivo} under our experimental conditions, the detection of the remaining acetylation may be limited by the assay sensitivity. Second, the three lysines (\textsuperscript{638}KFKK\textsuperscript{641}) in the hinge region may be important for PR acetylation at Lys-183; the mutation of all three abolishes Lys-183 acetylation. It is also possible that the site and level of PR acetylation are cellular context- and experimental condition-dependent. Under our experimental conditions, Lys-183 of PRB is a primary acetylation site.

PR can regulate gene expression through ligand-induced recruitment to the cognate PR binding sites, known as PRE, of the target genes (46). PR also regulates the activity of other transcription factors, such as NFkB, through tethering mechanisms (35, 47, 48). Three lines of evidence support the notion that PR acetylation by p300 at Lys-183 specifically enhances PR activation of direct target genes with cognate PR binding sites. First, acetylation mimic mutation K183Q significantly increased progestin-induced PRE-Luc activity but had no effect on NFkB reporter gene activity. Second, p300 overexpression or TSA treatment that increased the levels of Lys-183 acetylation increased markedly WT PRB-mediated PRE-Luc activity. In contrast, K183Q and K183R mutants that can no longer be acetylated are significantly less responsive to the acetylation-enhancing agents. Consistently, p300 gene silencing significantly decreased progestin-induced expression of PRE-containing genes \textit{FKBP5}, 11\textbeta-HSD2, and \textit{MUC1} but had no influence on NFkB target genes. This specific effect of Lys-183 acetylation on PRE-containing target genes supports the notion that Lys-183 acetylation facilitates PR-DNA interaction.

Steroid receptors are known to exhibit dynamic association/dissociation/reassociation with their target gene promoters in a temporal manner depending on the promoter context and the presence of transcription co-regulators (29, 42, 49). It has been reported that p300 could potentiate PR activity by promoting the initiation and reinitiation process in PR transcription using cell-free chromatin transcription assays (13). Data presented here indicate that the effect of p300 on initiation/reinitiation process is mediated, at least in part, through Lys-183 acetylation. First, we observed that p300 gene silencing decreased PR-PRE binding (Fig. 7A), and the treatment with HDAC inhibitor TSA was associated with accelerated PR binding to the canonical PRE on the \textit{FKBP5} gene in the time point experiment (Fig. 7B). Significant binding was detected in sample treated with TSA after 5 min of progestin treatment. This is in contrast to the lack of detectable binding signal in sample without TSA treatment. Second, a PRE-Luc plasmid immunoprecipitation assay showed that acetylation mimic mutant K183Q displayed more rapid PR-PRE binding kinetics in response to progestin (Fig. 7E). When PRE binding was measured in 5-min intervals over a 45-min period, PRB-K183Q binding exhibited a cyclic fluctuation every 20 min on the synthetic PRE promoter. On the other hand, WT PRB binding to PRE has yet to reach peak promoter occupancy by 45 min, which is consistent with the average cycling time of 60–75 min reported for PR and estrogen receptor \(\alpha\) (29, 42). These data suggest that PRB-K183Q is recruited onto the PRE promoter at a faster rate (faster initiation) and renews the assembly more quickly (reinitiation), leading to greater transcriptional outcomes. It has been reported that CBP/p300 can recognize and bind acetylated lysine residues in histones and transcription factors through their bromodomains (50–53). It is plausible that p300 acetylates Lys-183 of PR to create a docking site for a better grip or more efficient recruitment of general transcription machinery. An increase of Lys-183 acetylation by p300 can thus serve to amplify PR activity in response to cellular cues.

Phosphorylation of PR has been shown to be a pivotal regulator of the transcriptional response to progestin (44, 54–56).
Ser-294 phosphorylation is one of the most important PR post-translational modifications. It is involved in regulating multiple aspects of PR activity, including nucleocytoplasmic shuttling, protein ubiquitination, and PR activation (57). For example, phosphomimic mutation by replacement of Ser-294 with aspartic acid (S294D) created hyperactive PR, whereas S294A mutant is hypactive (58). Interestingly, the heightened activity of acetylated PR at Lys-183 is associated with increased levels of PR phosphorylation at Ser-294 (Fig. 8). p300 overexpression and TSA treatment both elevated Lys-183 acetylation, resulting in evident increases of Ser-294 phosphorylation in a time-dependent manner. More specifically, the Lys-183 acetylation mimic, PRB-K183Q, exhibits higher levels of phosphorylation at Ser-294 than WT PRB and K183R. It is conceivable that increased Ser-294 phosphorylation in association with Lys-183 acetylation plays a part in acetylation-induced increase of PR activity.

Past studies have reported both negative and positive interplays between acetylation and phosphorylation. Acetylation of STAT1 negatively regulates interferon-induced STAT1 phosphorylation by recruiting tyrosine phosphatase TCP45 (59). On the other hand, p53 acetylation at Lys-373 leads to its hyperphosphorylation, and this allows better binding of p53 with promoters with which p53 normally interacts with low affinity (60). Note that in the present study, the detection of earlier and higher Ser-294 phosphorylation in association with Lys-183 acetylation also coincides with a faster DNA binding kinetics and greater amount of DNA binding. Because Ser-294 phosphorylation is a positive regulator of PR transcription, higher levels of phosphorylation at 294 in acetylated PR probably drive enhanced PR-DNA binding dynamics.

Combinations of different modifications have been proposed to serve as a “code” in regulating diverse function of proteins. In p53, different acetylation sites have differential effects on its target gene expression patterns via influencing p53 phosphorylation at different sites (60). Our study suggests that the combination of PR post-translational modifications, such as acetylation and phosphorylation, also presents an attractive regulatory mechanism for fine tuning the control of PR target genes. It is likely that interplays among the modifications in different combinations add dimension to the gene regulation strategy in nature that allow the same protein to exert diverse functions in different tissues (e.g. mammary gland and uterus) and under various physiological conditions.

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