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Keywords: histone acetylase; hormone receptor; nuclear receptor; post-translational modification (PTM); transcription
Acetylation at lysine 183 of progesterone receptor by p300 accelerates DNA binding kinetics and transactivation of direct target genes

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*Running Title: PR acetylation at lysine 183 promotes PR transactivation.

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Keywords: acetylation; progesterone receptor; transcriptional activity

Background: The identification of steroid receptors acetylation was based on the consensus motif K/RXKK.

Results: Mass spectrometry discovers PR acetylation at non-consensus lysine 183 by p300. K183 acetylation enhances PR-DNA binding kinetic and transcription activity.

Conclusions: PR acetylation at K183 promotes PR-gene enhancer interaction.

Significance: The study provides a novel insight into the regulation of PR function by acetylation.

ABSTRACT

The identification of lysine acetylation of steroid hormone receptors has previously been based on the presence of consensus motif K/RXKK. This study reports the discovery by mass spectrometry of a novel progesterone receptor acetylation site at K183 that is not in the consensus motif. In vivo acetylation and mutagenesis experiments revealed that K183 is a primary site of PR acetylation. K183 acetylation is enhanced by p300 over-expression and abrogated by p300 gene silencing, suggesting that p300 is the major acetyltransferase for K183 acetylation. Furthermore, p300 mediated K183 acetylation is associated with heightened PR activity. Accordingly, acetylation-mimicking mutant, PRB-K183Q exhibited accelerated DNA binding kinetics and greater activity compared to the wild type PRB on genes containing progesterone response element (PRE). In contrast, K183 acetylation had no influence on PR tethering effect on the nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB). Additionally, increases of K183 acetylation by p300 overexpression or inhibition of deacetylation resulted in increases of serine (S) 294 phosphorylation levels. In conclusion, PR acetylation at K183 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 S294 phosphorylation.

INTRODUCTION

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 cellular 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 amino terminal transactivation domain (NTD) that includes the activation function-1 (AF-1) originally defined to a core region of amino acid 456 - 546 (5).

This is followed by the DNA binding domain (DBD) responsible for receptor dimerization and DNA binding. The C-terminal ligand binding
domain (LBD) contains AF2. AF-1 synergizes with AF2 to bring about optimal receptor activity (6). In addition, an AF-3 is contained within the first 164 aa 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 DBD and LBD (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 LBD that allows the receptor to interact directly or indirectly with a diverse set of co-regulatory proteins (9). 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 (10-13) (14). p300, its paralog cAMP response element binding protein-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 (15-22). They are recruited to PR in a ligand-dependent manner via interactions with SRC proteins (14), and acylate nucleosomal histones and transcription coregulators to modulate transcriptional activity (23). For example, acetylation could facilitate transcription factors recruitment to specific target DNA sequences by loosening nucleosomal structure and facilitate transcription factor binding to gene promoters. In addition, p300 and CBP can also directly acetylate nuclear receptors to regulate their transcriptional activity (21,24,25). Although it has been reported that p300 is associated with PR during progesterone-induced transcriptional initiation (14), it is not known whether p300 directly acetylates PR to regulate its activity. Nonetheless, it has been reported that PR is acetylated at the consensus sequence, KXKK (amino acids 638–641) of hinge region (8). This PR acetylation was hormone-inducible and was able to regulate the kinetics of transcription of fast response genes and transcription magnitude of slow response genes (8).

Using LC-MS/MS analysis of immunoprecipitated PR from breast cancer cell lines T47D and ABC28, we have uncovered a novel acetylated site (K183) of high confidence located in the amino terminal domain of PR. Using various biochemical and cell-based assays, we have validated that K183 is the major target of PR acetylation by p300. In addition, we explored the mechanisms and functional consequences of K183 acetylation and demonstrated that K183 acetylation specifically enhances PR transactivation of genes containing PRE. Such regulation is likely to be important in modulating progesterone-dependent signaling in a variety of progesterone target tissues in both normal and pathological states.

**EXPERIMENTAL PROCEDURES**

### Cell Culture

MDA-MB-231, COS7 and HeLa cell lines were obtained from American Type Culture Collection (ATCC, Manassas, VA). ABC28 clone was derived by transfecting MDA-MB-231 cells with PR expression vectors hPR1 and hPR2 that contain human PR cDNA coding for PR isoform B and isoform A in pSG5 plasmid (26). Cells were routinely maintained in phenol red Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 7.5% fetal calf serum (FCS) (Sigma Aldrich) and 2 mM L-glutamine, at 37°C in a humidified atmosphere of 5% CO2–95% air. For experiments involving hormone treatment, cells were cultured in phenol red-free DMEM supplemented with 5% charcoal-stripped FBS and 2 mM L-glutamine for 2 days prior to treatment with progesterone, RU486 or R5020 (Sigma Aldrich).

### Protein lysate collection and Western blotting analysis

Cells were lysed with cold lysis buffer (50 mM HEPES, 150 mM NaCl, 1% Triton X-100, 5 μg/ml pepstatin A, 5 μg/ml leupeptin, 2 μg/ml aprotinin, 1 mM PMSF, 100 mM sodium fluoride, and 1 mM sodium vanadate (pH 7.5)) and proteins in the supernatant were collected after centrifugation at 12,000g for 12 min at 4°C. Protein concentration was determined using BCA™ Protein Assay Kit (PIERCE Biotechnology, Rockford, USA). Proteins were resolved by sodium dodecyl sulfate-
polyacrylamide gel electrophoresis (SDS-PAGE) before transferring onto a nitrocellulose membrane (Amersham, Buckinghamshire, UK). Anti-PR antibody, H-190 (Santa Cruz Biotechnology Inc., CA, USA) was used to probe for human PR isoforms, PR-A and PR-B. Phospho-S294 was probed by S294 antibody from Neomarkers (USA). Acetylated proteins were detected by a pan anti-acetylated lysine antibody (Cell Signaling). The secondary anti-mouse and anti-rabbit antibodies (GE Healthcare, UK) were used in dilutions 1:1000 and 1:2000 respectively, in reference to the primary antibody used. Anti-β-actin (Santa Cruz Biotechnology Inc., CA, USA) was used to probe for β-actin as loading control. Signal detection was carried out using enhanced chemiluminescence (ECL) (GE Healthcare, UK) or Immobilon Western Chemiluminescent HRP substrate (Millipore, Massachusetts, USA) according to manufacturer’s protocol on X-ray films (Eastman Kodak Co., New Haven, CT).

PR Immunoprecipitation (IP)

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) pre-captured 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 non-specific 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 (~1mm X 1mm) and transferred to Eppendorf tubes. They were washed with Milli-Q water, destained with 50% acetonitrile (ACN)/50% 25mM NH₄HCO₃ via vigorous vortexing for 30 min 3 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 (IAA) for 45 min in the dark followed by successive washes with 25 mM NH₄HCO₃ and 50% ACN/50% 25mM NH₄HCO₃. Finally, they were dehydrated with 100% ACN and dried in vacuum. Trypsin (Trypsin Gold, Mass Spectrometry Grade, Promega, Madison, WI) was added in the 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 (FA) under sonication for 2 x 20 minutes. The combined extracts were dried in vacuum.

LC-MS/MS and Data Analysis

LC-MS/MS was carried out as previously described (27). Briefly, peptides were separated and analyzed on a liquid chromatography (Dionex UltiMate 3000 Nano-LC Systems) at a 300 nl/min flow rate coupled to a LTQ-FT Ultra (Thermo Electron, Bremen, Germany). Mobile phase A (0.1% FA in H₂O) and mobile phase B (0.1% FA in ACN) were used to establish the 60 min gradient comprised 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 LTQ-FT 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 mTorr 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 setting 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 CID. The default automatic gain control setting was used (full MS target at 3.0e+04, MSⁿ at 1e+04) in the linear ion trap. The 10 most intense ions above a 500-count threshold were selected for fragmentation in CID (MS²), which was performed concurrently with a maximum ion accumulation time of 200 ms. For CID, 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%(28).

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, Inc.), and
then the dta files were converted into 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 5 April 2013 with 132655 sequences; 43940360 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, $^{13}$C of 2 and MS/MS tolerance of 0.8 Da. Two missed cleavage sites of trypsin were allowed. Carbamidomethylation (C) was set as a fixed modification, and Oxidation (M), Deamidated (NQ), Acetyl (K), Methylation (KR), Dimethylation (KR), and Trimethylation (K) were set as variable modifications. The obtained database search results were exported to Microsoft Excel using 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 identity or homology scores were further manually analyzed.

**PR Mutants construction by Site-directed mutagenesis**

pcDNA3.1 expression vectors encoding human PR-B have been described previously (29). Point mutations of the K residue at site 183 of PR to glutamine (Q) and arginine (R) 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 anti-sense primers used are: K183Q_sense- 5' cgg cag ctg ccc atc agg tgc tgc ccc gg3'; K183Q_antisense-5' cgg cag cac ctc atg ggc agc tgc cgt cc 3'; K183R_sense- 5' gga cgg cag ctg ccc ata gag tgc tgc cc3'; K183R_antisense-5’ ggg cag cac tct atg ggc agc tgc cgt cc 3’

**siRNA transfection**

Pre-designed control and p300 siRNAs (Ambion, Inc) were transfected into ABC28 cells using Lipofectamine 2000 reagent (Invitrogen, Inc.) according to 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 Lipofectamine 2000 reagent and 40 nM siRNA were added into a final 2 ml transfection medium and incubated for 8 h prior to a media change. The sequences of p300 siRNA used are: SiRNA1_sense-5' GCCUGGUAUAUAAACCGGAtt (Ambion SiRNA ID: S4697); SiRNA1_antisense-5' UCCGGUAUAUAAACCGGCat (Ambion SiRNA ID: S4697), SiRNA2_sense-5' GGACUAACCUAAGUAAtt (Ambion SiRNA ID: S4695), SiRNA2_antisense-5’ UUACUUAGGGGUAGUCCaa (Ambion SiRNA ID: S4695).

**Plasmid transfection**

COS7 or HeLa cells were plated in 35mm dishes at a density of $2.5 \times 10^5$ cells in antibiotic-free phenol-red-free DMEM. The cells were then transfected with 0.5 μg pc-DNA3.1, pc-DNA3.1 wt-PRB or the various pc-DNA3.1 K183-PRB mutants using Polyethylenimine (PEI) (CELLnTEC, Switzerland) after 24 h of plating. The cells were then treated with 0.1% ethanol/10nM 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 (HAT) assays of PR peptides and PR protein**

**In vitro** acetylation assay was carried out as described (25) with minor modifications. PR peptide (KVGDSSGTAAAHK$^{183}$ VLPRGLSPARQL(wildtype)) or KVGDSSGTAAHQ$^{183}$ VLPRGLSPARQL(K->Q), 1 μg recombinant Histone H3 protein (as positive control), or immunoprecipitated PR were incubated with 0.25 μCi [3H]acetyl coenzyme A (CoA) (Amersham) and 100 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 KCl, 1 mM DTT, 1 mM PMSF, and 10 mM sodium butyrate. Immunoprecipitated HA-CBP or Flag-PCAF were also tested for their activity in acetylation, 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**

In vivo acetylation assay was carried out as was 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 10 μM TSA for 30 min followed by vehicle control or 10 nM R5020 treatment for another 1 h before cell lysate collection. Protein lysate was collected for PR immunoprecipitation by Flag antibody and captured Flag-PR and Flag-p300 were resolved by SDS-PAGE. Acetylated proteins are probed by a pan anti-acetylated lysine antibody (Cat no. 9441, Cell Signaling).

**PR Luciferase Reporter Assay**

Luciferase assay procedures were adapted from manufacturer protocol provided by in Dual-Luciferase Reporter System kit (Promega). Briefly, HeLa cells were seeded onto 60mm dishes and were transfected with PEI with 5 ng of pcDNA3.1, pcDNA3.1-wt-PRB or pcDNA3.1-PRB-K183Q/R in addition to 1500ng 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 10nM R5020 for 12 h before lysed by 1X passive lysis buffer provided in Dual-Luciferase Reporter System kit (Promega). 20 μl of lysate 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 (±SEM). Fold induction by ligand was calculated as the ratio of ligand induced samples RLU to the corresponding vehicle control-treated samples RLU.

**RNA extraction and Quantitative Real Time Polymerase Chain Reaction (qRT-PCR)**

Total RNA was extracted with TRIzol reagent (Life Technologies) based on manufacturer instructions. RNA was reverse transcribed with SuperScript II reverse transcriptase (Invitrogen) based on manufacturer’s protocol. qRT-PCR was carried out with SYBR Green master mix (Biorad) on an ABI Prism 7700 Sequence Detection System (Applied Biosystems) based on manufacturer protocol. Primer sequences used in real-time PCR are as follows: FKBP5_sense: 5’ ccc cgc cgg cga cag gtt ctc tac, FKBP5_antisense: 5’ cca atc gtc tcc tca cca, 11β-HSD2_sense: 5’ caa ggg gcc gca tgc tga ct 3’, 11β-HSD2_antisense: 5’ gca gcc gct gtt gag gca gtt tg 3’, IL-6_sense: 5’ ggt acg ttc tgc aag gca tct 3’, IL-6_antisense: 5’ gcc agg gcc tgg agt acc tct 3’, p300_sense: 5’ gaa gaa gac cca age acc tc 3’, p300_antisense: 5’ cgg taa agt gcc tcc aat gt 3’, PGR_sense: 5’ ggg gaa gac tct tgc gag tgg ga 3’, PGR_antisense: 5’ ttt gcc ctt cag aag cgg 3’, 36B4_sense: 5’ gca atg ttg cca gtg tct 3’, 36B4_antisense: 5’ gcc tgg acc ttt tca gca ag 3’, MUC1_sense: 5’ aga cgt cag cgt gag tga tg 3’, MUC1_antisense: 5’ cag cgg ctc gta gtt tc 3’, CSF2_sense: 5’ gcc act acg age acc g 3’, CSF2_antisense: 5’ tcc aag atg acc atc ctc ag 3’, IER3_sense: 5’ tgc tgt aaa tgc agg tct ctt g 3’, IER3_antisense: 5’ aca cag acg gag tgc aga 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 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-treated samples was calculated by normalizing Ct values with 36B4 Ct values based on the following formula:

Relative expression = 2 ^ \left( \frac{Ct(\text{control}) \text{gene} X - Ct(\text{hormone}) \text{gene} X}{Ct(\text{control})36B4 - Ct(\text{hormone})36B4} \right)

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 previously described protocols (30). ABC28 cell line was grown in DMEM supplemented with 5% dextran charcoal-stripped FBS for 3 days. Cells were then treated with R5020 (10^{-8} M) for various duration as is indicated in the figure legends before collection for 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 glycine 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 was as followed: 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 gene promoter sequences. Primers for the human intronic PRE region were as follows: Sense: 5’- taa tag agg ggc gag aag gca ga-3’; and Antisense: 5’- ggt aag tgg gtg tgc tcg ctc a-3’. PCR products were analyzed by agarose gel (2%) electrophoresis.

Statistical Analysis

Data were analyzed using unpaired, two-tailed student t-test with 95% confidence interval using the program GraphPad Prism 5.

RESULTS

Identification of PR acetylation at K183 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 cells ABC28 that has been well-characterized previously (26). 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. K183 modified peptides with a mass gain of 42.01 Da in lysine residue were consistently identified in almost all preparations. As is shown in Figure 1A and Figure 1B, two tryptic peptides of 3+ charge state with monoisotopic masses at 522.620 Da 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)x3), 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 VGDSSGTAAAHKVLPR was detected at the retention time 16.88 min (Figure 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 (Figure 1B).

Plasmid immunoprecipitation

Plasmid immunoprecipitation experiments were performed as previously described with minor modifications (31). 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-Flagp300 or PCI vector control. 24 h post-transfection, cells were treated with either R5020 (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 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-PRE2-TATA-luciferase plasmid immunoprecipitation. The resulting immunoprecipitated and input DNA was analyzed by PCR or real-time PCR reactions with the following pair of primers for the detection of PRE luciferase sequences: Sense: 5’- cta gca aaa tag gtc cc-3’; Antisense: 5’- tgg tt ggc gtc ttc cat-3’. PCR products were analyzed by 2% agarose gel electrophoresis.
The MS/MS spectra and the fragment ions assignments were shown in Figure 2. The fragment ions of the peptides were assigned to the b- or y-ions series by Mascot and confirmed by manual inspection of the MS/MS spectra. The K183 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 y\(^2\) ions series of the modified peptide spectrum starting from \(y_5\) till \(y_{15}\) (Figure 2B) when compared to the corresponding y\(^2\) ion series of the un-modified peptide (Figure 2A). For example, the increase in m/z value between \(y_5\) of the modified peptide (m/z= 327.72) and \(y_5\) of the un-modified peptide (m/z=306.71) is 21 corresponding to 42 Da mass increase when multiply by its charge (+2). Therefore, it could be deduced that the modification took place at K183. The detailed b and y ions profiles of these two peptides from Mascot search is provided in Mascot Peptide View in Supplemental Data 1 (un-modified peptide) and 2 (modified peptide).

To further confirm K183 acetylation, we analyzed Flag-tagged PRB because a larger amount of PRB can be pull-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 42.01 were consistently identified with very high confidence. Figure 2C illustrates a representative MS/MS spectrum from a doubly charged precursor ion and its fragment ions 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 K183 site indicates that the positive mass shifts of 42 Da started from \(y_5\) to \(y_{13}\) but were not observed from \(y_2\) to \(y_4\) which confirms the presence of modification at Lys-183. For example, the mass difference between \(y_5\) (m/z= 484.43) and \(y_5\) (m/z=654.45) of the modified peptide is 170.02 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 K183 also indicates that the positive mass shift of 42.01 takes place at K183. The detailed b and y ions profiles of this peptide from Mascot search is provided in Mascot Peptide View in Supplemental Data 3.

The observed mass shift of 42 Da in precursor and fragments ions could attribute to K183 acetylation (42.0106 Da) or trimethylation (42.0470 Da). As the LTQ-FT Ultra instrument provides high resolution and mass accuracy with mass error < 10ppm, the K183 modification is positively identified as acetylation based on the accurate detection of the mass difference of 42.01 Da in modified and un-modified precursor ions.

The unambiguous identification of K183 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, while the acetylated VGDSSGTAAAHK(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 protonation site at K183, resulting in significantly increased in the peptide retention time from 16.88 min (unmodified) to 22.17 min (acylated) in reverse phase C18 column during LC-MS/MS analysis.

Verification of K183 acetylation by acetyl-lysine antibody

PR acetylation at K183 was further verified with a pan acetyl lysine antibody (Figure 3). Wild-type (wt) PR-B or mutant K183R was transiently transfected into COS7 cells. Immunoprecipitated PR was analyzed for acetylation by Western blotting analysis. Acetylation of PRB was undetectable in the absence of ligand. Consistent with the observations by Daniel et al (8), PR acetylation is increased following treatment with progesterin R5020 or trichostatin A (TSA), a histone deacetylases (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 K183 is the major site of acetylation on PR.

**PR is acetylated at K183 by p300 and CBP by *in vitro* acetylation assay**

Acetylation of steroid receptors (SRs) has been shown to be catalysed by histone acetyltransferases (HATs). Several acetyltransferases such as p300, CBP and PCAF have been shown to acetylate NRs and modulate their activities (32). To identify the enzyme that acetylates PR at K183, we conducted *in vitro* acetylation assay with the potent acetyltransferases (recombinant Flag-p300, or immunoprecipitated HA-CBP and Flag-PCAF). It was found that all three enzymes could acetylate full length PRB (Figure 4A, 4B and 4C). However, since 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 K183 of PR since K183 is not contain in the typical acetylation consensus sequence K(R)XKK. Peptide acetylation assay showed that both recombinant p300 and HA-CBP acetylated K183 while the substitution of the residue K183 with glutamine abolished acetylation (Figure 4D and 4E). On the other hand, PCAF failed to acetylate K183 of PR under the same assay conditions, although the enzyme strongly acetylated the positive control full-length recombinant Histone H3 protein (Figure 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 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 K183. However, the data cannot rule out the possibility that PCAF acetylates a previously reported acetylation motif K638–641 on PR (8).

**PR is acetylated at K183 by p300 *in vivo***

Since p300/CBP are closely related transcriptional co-activators with extensive sequence and structural homology (33) and overlapping functions (34), we chose from here on in to focus on p300 for its role in PR acetylation and function. To confirm K183 as an authentic *in vivo* acetylation site by p300, we carried out *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 Figure 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 small interfering RNA (siRNA) against p300 and its effects on PR acetylation level was assessed. After p300 knockdown, the PR protein was immunoprecipitated and visualized by western blotting for acetylation (Figure 4H). The transfection of p300 siRNAs resulted in undetectable levels of p300 by western analysis while causing minimal change in PR protein levels (Figure 4H). The silencing of p300 abrogated PR acetylation. Taken together, these data indicate that p300 is primarily responsible for PR acetylation at K183.

**PR acetylation at K183 enhances PR activity specifically at PRE promoter**

We next tested the effect of K183 acetylation on PR activity using K183 mutants in a cell-based PR reporter gene assay. The luciferase reporter gene is driven by a 2x canonical progesterone response element (PRE2-luc). Following treatment with 10 nM R5020 for 12 h, cells transfected with wt-PRB 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 K183. However, the data cannot rule out the possibility that PCAF acetylates a previously reported acetylation motif K638–641 on PR (8).
the other hand, the K183R mutant exhibited similar activity as the wt-PRB.

It is known that PR can be activated by the partial agonist property of PR antagonist RU486. We tested if PR acetylation had any effects on the partial agonist activity of RU486. Consistent with the results of R5020-induced PR activity, acetylation mimicking mutant PR-K183Q also exhibited heightened PRE transcriptional activity in response to RU486 treatment (wt-PRB - 5.07 fold, PRB-K183Q-7.12 fold and PRB-K183R - 5.19 fold) (Figure 5B). Together, these results indicate that K183 acetylation heightens the transcriptional activity of PRB in response to both progestin and agonist property of anti-progestin.

To demonstrate the role of p300 acetylation of PR on the transcriptional responses of PR on PRE activity, the effect of p300 overexpression on the transcriptional activity of wt-PRB, PRB-K183Q or PRB-K183R was investigated in PR reporter assay. p300 overexpression enhanced wt-PRB transcription activity on the PRE promoter by ~450% (Figure 5C). In contrast, the transcription enhancing effect of p300 on K183Q and K183R mutants was only 174% and 130%, respectively (Figure 5C). The data suggests that p300 enhanced PRB activity predominantly via K183 acetylation and K183 mutation markedly abolished the effect of p300. This notion is supported by the results that K183 is also important for histone deacetylase (HDAC) inhibitor TSA to enhance PR activation. TSA preserves protein acetylation through inhibiting of HDAC activity. As a result, TSA treatment led to an increase of 32% of wt PRB activity (Figure 5D). However, this effect on PRB-K183Q or PRB-K183R was only 18% and 12%, respectively, which were significantly lower (p<0.0001) than that on the wt-PRB. These results suggest that PR acetylation at K183 by p300 positively regulates PR transcriptional activity and the effect of p300 on K183 mutants is muted because the mutants were no longer acetylated by p300.

PR can regulate gene transcription through tethering with other transcription factors including Sp-1, AP-1 and NF-κB. For example, PR has been shown to attenuate Nuclear Factor Kappa-light-chain-enhancer of Activated B cells (NFκB) reporter activity in a ligand-dependent manner via the protein-protein interactions of PR with the p65 subunit of NFκB (35,36). We also addressed if K183 acetylation by p300 regulates the activity of PR on NFκB-mediated gene regulation using the well-characterized 5xRel-Luc reporter gene assay. The results showed that wt-PRB and K183 mutants attenuated the transcriptional activity of the NFκB to a similar extent (3-4 folds) (Figure 5E). Furthermore, p300 overexpression had no effect on PR activity in attenuating NFκB activity. Western blotting analysis showed similar protein expression of K183 mutants as compared to wt-PRB (Figure 5F). Thus, PR acetylation by p300 at K183 specifically regulates PR activity on its direct target genes while PR acetylation does not influence PR tethering with NFκB.

**K183 acetylation positively regulates the expression of PRE-containing target genes but has minimal effect on PR trans-repression on NFκB target genes in breast cancer cells.**

Data obtained with reporter gene assay showed that PR acetylation at K183 by p300 promotes PR-mediated transactivation on PRE-drive gene reporter but has no effect on NFκB reporter gene. We then asked if the same mechanism of regulation occurs with endogenous genes in PR-transfected MDA-MB-231 cells 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 (Scramble SiRNA) or two different p300 siRNA which reduced p300 mRNA expression by 60-80% (Figure 6A). Western blotting analysis showed that both p300 siRNAs also reduced the protein levels of p300 by at least 70% (Figure 6C). We also verified that PR mRNA (Figure 6B) and protein levels (Figure 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 NFκB pathways were studied in ABC28 cells. The three direct PR target genes are FK506 binding protein 5 (FKBP5), 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) and mucin 1 (MUC1) that contain PRE.
sequence in their enhancers (30,37-40). R5020 treatment induced 2.8-, 3.3- and 3.2-fold increase in the expression of FKBP5, 11β-HSD2 and MUC1, respectively (Figure 6D, E and F). p300 siRNA 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, colony stimulating factor 2 (CSF2), immediate early response 3 (IER3) and interleukin 6 (IL-6) (41-46) (Figure 6G, H and I). Thus, it is likely that acetylation of K183 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 (NR) family, PR regulates gene transcription through binding to specific gene sequence and recruiting transcription co-regulators to the gene promoter (47). 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 K183 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. Figure 7A shows that p300 gene silencing resulted in a decrease of PR recruited to FKBP5 PRE. Treatment of TSA (100 nM) increased ligand-induced PR recruitment to the FKBP5 promoter (Figure 7B). More importantly, TSA treatment is associated with accelerated PR binding to the canonical PRE on FKBP5 gene. As is shown in Figure 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 K183 in the PR-PRE binding, we compared the PRE binding between wt-PRB and PRB-K183R in the presence and absence of p300 overexpression in a time course experiment (Figure 7C). Similar to the effect of TSA treatment on PR-PRE recruitment, p300 overexpression is associated with increased and accelerated wt-PRB binding to the PRE sequences on the reporter plasmid. In contrast, p300 overexpression had no effect on PRB-K183R binding to PRE at all the time points tested. This suggests that the effect of p300 overexpression on increased DNA binding was mediated by K183 acetylation.

Since TSA treatment and p300 overexpression enabled PRB to associate with PRE at an earlier time, we compared the PRE binding kinetics of wt-PRB with that of acetylation mimicking mutant PRB-K183Q. Plasmid immuno precipitation assay coupled with quantitative real-time PCR were carried out to measure PR binding to 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 (Figure 7D). The magnitude of increase in DNA binding for PRB-K183Q as compared with wt-PRB was approximately 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 likely 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 (Figure 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 baseline after 20 min 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 (30,48). 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 is associated with increased PR phosphorylation at S294**

K183 is located in NTD, on which more than a dozen of phosphorylation sites are situated. It was reported that mutation of lysines at the KFKK (aa 638-641) acetylation motif delayed PR phosphorylation at S294, S345 and S400. Among these, S294 phosphorylation is the most studied phosphorylation site on NTD and is critical for PR activity (49,50). We asked the question if there is any functional interplay between K183 acetylation and S294 phosphorylation by testing if the increase of K183 acetylation level by HDAC inhibitor TSA could increase the levels of S294 phosphorylation. Expectedly, TSA treatment increased acetylated PRB levels compared to untreated controls (Figure 8A). This was associated with corresponding increases of S294 phosphorylation at both time points after R5020 treatment. It also appears that S294 phosphorylation occurred earlier in response to TSA treatment. Sample treated with TSA presents a prominent band at 15 min after R5020 treatment. At the same time point, sample without TSA treatment showed no detectable S294 phosphorylation (Figure 8A). This was associated with corresponding increases of S294 phosphorylation at both time points after R5020 treatment. 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 S294 as compared to cells with basal p300 expression (Figure 8B).

To further characterize the specific effects of K183 acetylation on PR phosphorylation, we compared S294 phosphorylation among wt-PRB, PRB-K183Q and PRB-K183R following transient transfection in COS7 cells. As is shown in Figure 8C, S294 phosphorylation levels increase with increasing concentration of R5020 in all three forms of PRB. However, PRB-K183Q has higher levels of phosphorylation at S294 at all R5020 dosages tested as compared with wt-PRB and K183R. Taken together, these findings suggest that PR acetylation at K183 facilitates PR phosphorylation at S294, 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/RXKK (51). By LC-MS/MS analysis this study reports the identification of a novel acetylation site of PR at K183 that is not contained within such 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 comprising K183, TAAAHK183VLPRG, shows that there is a positively-charged R residue at the K183 +4 position which fits the p300 substrates preference reported (52). Indeed, p300 directly acetylates PR at K183 by both in vitro and in vivo acetylation assays. K183 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 K183 of NTD in PR 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 638KXKK641 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 in vivo (Figure 3) using the same pan antibody against acetylated lysine. There may be several explanations for the discrepancies. First, although K183 mutant shows no detectable acetylation in vivo under our experimental conditions, the detection of the remaining acetylation may be limited by the assay sensitivity. Second, the three lysines (638KFKK641) in the hinge region may be important for PR acetylation at K183, the mutation of all three abolishes K183 acetylation. It is also possible that the site and level of PR acetylation is cellular context and experimental condition dependent. Under our experimental condition, K183 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 (53). PR also regulates the activity of other transcription factors such as NF-kB through tethering mechanisms (54-56). Three lines of evidence support the notion that PR acetylation by p300 at K183 specifically enhances PR activation of direct target genes with cognate PR binding sites. First, acetylation mimic mutation K183Q significantly increased progesterin-induced PRE-luc activity but had no effect on NFκB reporter gene activity. Second, p300 overexpression or TSA treatment that increased the levels of K183 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 progesterin-induced expression of PRE-containing genes FKBP5, 11β-HSD2 and MUC1 but had no influence on NFκB target genes. This specific effect of K183 acetylation on PRE-containing target gene supports the notion that K183 acetylation facilitates PR-DNA interaction.

Steroid receptors are known to exhibit dynamic association/dissociation/re-association with their target gene promoters in a temporal manner depending on the promoter context and the presence of transcription co-regulators (30,57,58). It has been reported that p300 could potentiate PR activity by promoting initiation and re-initiation process in PR transcription using cell-free chromatin transcription assays (14). Data presented here indicate that the effect of p300 on initiation/re-initiation process is mediated, at least partly, through K183 acetylation. First, we observed that p300 gene silencing decreased PR-PRE binding (Figure 7A) and the treatment with HDAC inhibitor TSA is associated with accelerated PR binding to the canonical PRE on FKBP5 gene in time point experiment (Figure 7B). Significant binding was detected in sample treated with TSA after 5 min of progesterin treatment. This is in contrast to the lack of detectable binding signal in sample without TSA treatment. Second, PR-Luc plasmid immunoprecipitation assay showed that acetylation mimic mutant K183Q displayed more rapid PR-PRE binding kinetics in response to progesterin (Figure 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 is 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 α (30,48). These data suggest that PRB-K183Q is recruited onto the PRE promoter at a faster rate (faster initiation) and renews the assembly more quickly (re-initiation), 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 bromo domains (59-61). It is plausible that p300 acetylates K183 of PR to create a docking site for a better grip or more efficient recruitment of general transcription machinery. Increase of K183 acetylation by p300 can thus serve to heighten PR activity in response to cellular cues.

Phosphorylation of PR has been shown to be a pivotal regulator of the transcriptional response to progestin (50,62-64). S294 phosphorylation is one of the most important PR phosphorylation. It is involved in regulating multiple aspects of PR activity including nucleocytoplasmic shuttling, protein ubiquitination and PR activation (65). For example, phospho-mimic mutation by replacement of S294 with aspartic acid (S294D) created hyperactive PR whereas S294A mutant is hypoactive (66). Interestingly, the heightened activity of acetylated PR at K183 is associated with increased levels of PR phosphorylation at S294 (Figure 8). p300 over-expression or TSA treatment, both elevated K183 acetylation, resulted in evident increases of S294 phosphorylation in a time-dependent manner. More specifically, K183 acetylation mimic, PRB-K183Q, exhibits higher levels of phosphorylation at S294 than wt-PRB and K183R. It is conceivable that increased S294 phosphorylation in association with K183 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 (67). On the other hand, p53 acetylation at K373 leads to its hyperphosphorylation and this allows better binding of p53 with promoters that p53 normally interacts with low affinity (68). Note that in the
present study, the detection of earlier and higher S294 phosphorylation in association with K183 acetylation also coincides with a faster DNA binding kinetics and greater amount of DNA binding. Since S294 phosphorylation is a positive regulator of PR transcription, higher levels of phosphorylation at 294 in acetylated PR likely 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 (69). 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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FOOTNOTES

The pCI-Flag-p300 expression vector was kindly provided by Dr. Lorenzo Puri (Dulbecco Telethon Institute, Rome). pCR3.1-SRC1, PRE2-Firefly Luciferase reporter and pRL-CMV (Renilla) plasmids were generously provided by Dr. Tsai M-J, Baylor (College of Medicine, Houston, Texas). HA-CBP and Flag-PCAF plasmid were generously given by Dr Mark Featherstone (School of Biological Sciences, Nanyang Technological University, Singapore). NFκB-luc reporter vector was kindly provided by Dr Peter Cheung (School of Biological Sciences, Nanyang Technological University, Singapore).

The abbreviations used are: AF-1, transcriptional activation function 1; AF-2, transcriptional activation function 2; AF-3, transcriptional activation 3; CBP, CREB-binding protein; ChIP, chromatin immunoprecipitation; CSF2, colony stimulating factor 2; DBD, DNA binding domain; DMEM, Dulbecco’s Modified Eagle Medium; DTT, dithiothreitol; ER, estrogen receptor; FA, formic acid; FKBP5, FK506 binding protein 5; 11β-HSD2, β-hydroxysteroid dehydrogenase type 2; HDAC, histone deacetylases; IAA, iodoacetamide; LBD, ligand binding domain; LC-MS/MS, liquid chromatography coupled to tandem mass spectrometry; LTQ, linear quadrupole ion trap; MS, mass spectrometry; NTD, amino terminal domain; PCAF, P300/CBP-associated factor; PEI, polyethylenimine; PGR, progesterone receptor; PI, phosphatase inhibitors; PR, progesterone receptor; PRE, progesterone receptor response element; PTM, post-translational modifications; wt-PRB, wild-type PRB; IER3, immediate early response 3; IL-6, interleukin 6; NFκB, nuclear factor kappa-light-chain-enhancer of activated B cells; MUC1, mucin 1; SiRNAs, small interfering RNA; SRC1, steroid receptor coactivator 1; TSA, Trichostatin A; XIC, extracted ion chromatogram.

FIGURE LEGENDS

Figure 1. Identification of PR acetylation/trimethylation by LC-MS/MS analysis. ABC28 cells were treated with 10 nM progesterone for 1 h before whole cell lysates were processed for LC-MS/MS analysis of PR modification. (A) Precursor ion mass spectrum of triply charged un-modified peptide VGDSSGTAAAHKVLPR. An isotopic distribution of the precursor peptide ion (monoisotopic mass = 522.620 Da) is observed. (B) Precursor ion mass spectrum of triply charged modified peptide (monoisotopic mass = 536.623 Da). Positive mass shifts of 42 Da are observed in the isotopic peaks distribution for the modified peptide in B as compared to un-modified peptide in A.

Figure 2. Assignment of acetylation to K183 (A) MS/MS spectrum of precursor ion m/z 522.6202201+ from the analysis shown in Figure 1A. The labeled peaks correspond to masses of y2+ ions of peptide fragments. (B) MS/MS spectrum of precursor ion m/z 536.6234303+ from the analysis shown in Figure 1B. The labeled peaks correspond to masses of y2+ ions of peptide fragments. By manual inspecting the y2+ ions series of this spectrum, the increment of m/z values by 21 (indicating 42 Da mass shifts) started from y5+ and continued to y15+ when compared to the corresponding y2+ ions series of the un-modified peptide from A, suggesting that the modification took place at K183. (C) MS/MS spectrum of precursor ion m/z 804.4309602+ from LC-MS/MS analysis of Flag-tagged PR expressed in MDA-MB-231 cells. The labeled peaks correspond to masses of band y ions of peptide fragments. Manual inspection of the b and y ions flanking K183 of this spectrum indicates that the positive mass increments of 42 Da started from y5 ion and continued till y13, which suggests modification to take place at K183. Mass shifts of 42 Da also started from b12 to b15 of the b ions series spanning the peptide in the opposite orientation, further validating the site of modification to be at K183.

Figure 3. Detection of acetylation at K183 by anti-acetyl-lysine antibody. COS7 cells were transiently transfected with vector control (pcDNA3.1), full length wt-PRB or PRB-K183R. Cells were serum starved for 24 h and pretreated with 10 µM TSA for 0.5 h, followed by 1 h of R5020 treatment before the whole cell lysates were collected for PRB immunoprecipitation. The protein resolved on the Western blot were detected with a pan acetyl lysine antibody, and re-probed for total.
PRB using PR antibody H190. AcK-PRB refers to acetylated PRB; PRB refers to total PRB. The figure shows that K183R mutation abrogated PRB acetylation.

**Figure 4. In vitro and in vivo acetylation of PRB at K183 by p300.** (A, B, C) PRB is acetylated *in vitro* by recombinant p300 (A), immunoprecipitated HA-tagged CBP (B) or immunoprecipitated Flag-tagged PCAF (C). Immunoprecipitated PRB was incubated with 500 nM acetyl coenzyme A and respective enzymes in 30 μl of acetylation buffer for 1 h at 30°C and final products were resolved by SDS-PAGE and then detected with anti-Flag, anti-HA, pan anti-acetyl lysine or anti-PR antibodies. 

**Figure 5. PRB-K183Q demonstrates heightened ligand-dependent activity.** (A, B) HeLa cells were transfected with 5 ng of wt-PRB, PRB- K183Q or PRB-K183R, together with PRE2-TATA-Luc (1.5 μg) and Renilla (1 ng) reporters. The PR-Luc activities were analysed under various conditions. (A) PR acetylation mimic PRB-K183Q induced higher PRE transactivation activity in response to R5020. (B) PR acetylation mimic PRB-K183Q induced higher PRE transactivation activity in response to Ru486. (C) p300 co-activation of PR is dependent on K183. HeLa cells were transfected with plasmids as described in A in addition to 50 ng of PCI-Flag p300 or PCI vector control. The increase of PRB-K183R or PRB-K183Q activity in response to p300 is only one third of that of wt-PRB. (E) The increases of PRB-K183R and PRB-K183Q activity in response to HDAC inhibitor TSA are 50% or less than that of PRB. Following transfection as in A, HeLa cells were treated with 100 nM TSA or vehicle control in the absence or presence of 10 nM R5020 for 1 h before analysis for PRE-Luc activity. (E) Relative protein expression of wt-PRB, PRB-K183Q and PRB-K183R transfected cells. β-actin was detected as loading control. 

**Figure 6. p300 siRNA reduced R5020-induced expression of direct PR target genes while had minimal effect on PR trans-repression of NFkB target genes in ABC28 cells.** ABC28 cells were transfected with NC or p300 siRNAs (40 nM) for 72 h before were treated with or without 10 nM R5020 for 3 h. The cells were then harvested for RNA and protein analysis. (A) p300 expression is reduced by p300 siRNA1 and siRNA2 by 60% and 80%, respectively relative to scramble siRNA control. (B) PR mRNA levels were not affected by of p300 siRNAs. (C) Western blotting analyses of p300 and PR proteins following p300 siRNA treatment. β-actin was probed as loading control. (D, E) mRNA expression of FKBP5 (D), 11β-HSD2 (E) and MUC1 (F) in p300 siRNAs 1 and 2 transfected ABC28 cells were significantly reduced in response to R5020 treatment. (G, H, I) R5020 induced suppression of CSF2 (G), IER3 (H) and IL-6 (I) in p300 SiRNAs transfected ABC28 cells were not significantly affected. Statistical significance was determined using unpaired t-test and asterisks denoted statistical significance (+SEM; *, p<0.05; ***, P<0.001; ****, P< 0.0001) while n.s denoted non-significant change as determined by unpaired Student’s t tests.
Figure 7. PR acetylation at 183 accelerates PR-PRE binding kinetics. (A) p300 silencing reduces PR recruitment to FKBP5 promoter. ABC28 cells were treated with vehicle or 10 nM R5020 for 15 min. Fixed DNA-protein complex was immunoprecipitated with PR antibody H190 or rabbit IgG control. Relative levels of PR-PRE promoter interaction were measured by PCR in duplicates and 1% DNA input was included as loading control. (B) HDAC inhibitor TSA accelerates the recruitment of PR to FKBP5 PRE. ABC28 cells were treated with R5020 according to the time points indicated in the figure and ChIP assays were performed as described in A. (C) Accelerated recruitment of PRB to PRE-luc by p300 is dependent on K183. HeLa cells were transfected with 10 ng of pcDNA3.1 vector, wt-PRB or PRB-K183R, in addition to 1.5 µg of PRE2-TATA-luciferase vector and 100 ng of PCI-Flag p300 or PCI vector control. Cells were then treated and analyzed for PR-PRE binding according to the time points indicated in the figure. Relative levels of PR-PRE promoter interaction were measured by PCR and 1% DNA input was included as loading control. (D) PRB-K183Q shows increased binding to PRE-Luc vector by plasmid immunoprecipitation assay as described in C. Relative levels of PR-PRE promoter interaction were measured as described in C in duplicates. (E) PRB-K183Q exhibits faster PRE binding kinetics. Plasmid immunoprecipitation assay was carried out as described in C according to the time points indicated in the figure. Immunoprecipitated plasmid DNA is quantified by real-time PCR. The results are expressed as relative binding of PR-PRE following normalization against 1% DNA input control (mean ± SEM).

Figure 8. Acetylation of PRB is associated with increased PR phosphorylation. (A) The stabilization of acetylated PR by TSA enhances its phosphorylation at S294. COS7 cells were transiently transfected with Flag-tagged PRB. Cells were serum starved for 24 h and treated with TSA as described in Figure 3, followed by R5020 treatment for different time points (indicated in figure) before collection for PR immunoprecipitation. Proteins resolved on Western blot were detected with pan anti-acetyl lysine acetylated-K, PR and phospho-S294 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 (4µg). 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 S294 phosphorylation than wt-PRB or PRB-K183R in response to various R5020 dosages (10^{-5}, 10^{-6} or 10^{-7} M). Western blotting analysis were carried out to detect PR and PR phosphorylation at S294 using the respective antibodies. GADPH was probed as a loading control.
Figure 1.

A

091106HwaHwa_ABC28_PRB_P #1616 RT: 16.88 AV: 1 NL: 4.68E3
T: FTMS + c ESI Full ms [350.00-1600.00]

B

091106HwaHwa_ABC28_PRB_P #2280 RT: 22.17 AV: 1 NL: 1.36E5
T: FTMS + c ESI Full ms [350.00-1600.00]
Figure 2.

Un-modified PRB K183 from ABC28 cells

Modified PRB K183 from ABC28 cells

Modified PRB K183 from exogenous Flag-PRB expressing MDA-MB-231 cells
Figure 3.
Figure 4.
Figure 5.
Figure 6.

A

B

C

PRE containing genes

D

E

F

NFκB target genes

G

H

I
Figure 7.

A  **Recruitment of PR to FKBP5 PRE**  

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B  **Kinetic Recruitment of PR to FKBP5 PRE**  

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C  **p300 enhanced wt-PRB but not PRB-K183R binding to PRE-luciferase plasmid**  

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D  **Recruitment of PRB to exogenously expressed PRE2-Luciferase reporter**  

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E  **Kinetic Recruitment of PRB to exogenously expressed PRE2-Luciferase reporter**  

[Graph showing Kinetic Recruitment of PRB to exogenously expressed PRE2-Luciferase reporter]
Figure 8.

A

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TSA

- pPRB(S294)
- AcK-PRB
- PRB

B

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Flag-p300

- pPRB(S294)
- AcK-PRB
- PRB
- Flag-p300

C

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- pPRB(S294)
- PRB
- GAPDH