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Lysine Methylation of Progesterone Receptor at Activation Function 1 Regulates both Ligand-independent Activity and Ligand Sensitivity of the Receptor*1

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Background: Activation function 1 (AF-1) is important for the activity of progesterone receptor (PR), but its functional motif is not known.
Results: AF-1 of progesterone receptor (PR) is monomethylated at Lys-464. Lys-464 mutation markedly alters PR properties and ligand sensitivity.
Conclusion: Lys-464 is critical for AF-1 function.
Significance: Lys-464 is a potential target for modulating AF-1 activity of PR.

Progesterone receptor (PR) exists in two isoforms, PRA and PRB, and both contain activation functions AF-1 and AF-2. It is believed that AF-1 is primarily responsible for the ligand-independent activity, whereas AF-2 mediates ligand-dependent PR activation. Although more than a dozen post-translational modifications of PR have been reported, no post-translational modification on AF-1 or AF-2 has been reported. Using LC-MS/MS-based proteomic analysis, this study revealed AF-1 monomethylation at Lys-464. Mutational analysis revealed the remarkable importance of Lys-464 in regulating PR activity. Single point mutation K464Q or K464A led to ligand-independent PR gel upshift similar to the ligand-induced gel upshift. This upshift was associated with increases in both ligand-dependent and ligand-independent PR phosphorylation and PR activity due to the hyperactivation of AF-1. In contrast, mutation of Lys-464 to the bulkier phenylalanine to mimic the effect of methylation caused a drastic decrease in PR activity. Importantly, PR-K464Q also showed heightened ligand sensitivity, and this was associated with increases in its functional interaction with transcription co-regulators NCoR1 and SRC-1. These results suggest that monomethylation of PR at Lys-464 probably has a repressive effect on AF-1 activity and ligand sensitivity.

Progestosterone is involved in the regulation of multiple physiological processes including reproduction, immunity, and neural function. It is also essential for the normal mammary development and has long been implicated in the development of breast cancers (1). The pro-cancer role of progesterone has been suggested by two clinical trials, the Women’s Health Initiative (2) and the Million Women study (3). A follow-up study has suggested by two clinical trials, the Women’s Health Initiative (2) and the Million Women study (3). A follow-up study concluded that combined use of estrogen plus progestin was associated with greater breast cancer incidence and more aggressive disease compared with estrogen therapy alone (4). Laboratory studies reported that progesterone can promote the expansion of the cancer stem cell-like population in the hormone-sensitive breast cancers and enabled these cells to escape endocrine therapy (5, 6). This is believed to be mediated by progesterone-induced down-regulation of miR-29 that normally targets Krüppel-like factor 4 (Klf4) that helps maintain the pool of cancer stem cells (7). However, the therapeutic benefit of anti-progestin for breast cancer is yet to be demonstrated. On the other hand, progestin megestrol acetate has been surveyed in several hormone therapy trials with clinical benefit similar to that of aromatase inhibitor letrozol in a phase III clinical trial (8). This discordance reflects complexity in the modes of progesterone action.

Progesterone receptor (PR) belongs to the superfamily of nuclear receptors that are transcription factors. It exists in two major isoforms, PRA and PRB. PRB lacks the first 164 amino acids that are present at the N terminus of PRB. PRA is a weaker transactivator than PRB but can repress the activity of PRB because of its active inhibitory domain at the N terminus (9). Variations in PRA/PRB ratios in breast tumors appear to affect clinical outcome; breast tumors with high PRA/PRB ratios are reportedly linked to a more aggressive disease and poorer disease-free survival (10, 11).

PR is characterized by a modular structure common to all nuclear receptors including a variable N-terminal domain (NTD), a highly conserved DNA-binding domain, a C-terminal ligand-binding domain (LBD), and a hinge region that connects the DNA-binding domain and LBD (12). PRs contain three transactivation domains. AF-1 and AF-2 are present in both PRA and PRB, whereas AF-3 is in the N-terminal domain.

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2 The abbreviations used are: PR, progesterone receptor; AF-1, -2, and -3, transcriptional activation function 1, 2, and 3, respectively; CIP, cAMP-dependent protein kinase; CRE, cAMP-responsive element; IGF, insulin-like growth factor; LBD, ligand-binding domain; LTP, transcriptional repression; MTF, mineralocorticoid receptor; NCoR1, nuclear receptor corepressor 1; NCoR2, nuclear receptor corepressor 2; PRA, progesterone receptor A; PRB, progesterone receptor B; PRD, progesterone receptor domain; PRG, progesterone receptor gene; PRM, progesterone receptor mRNA; PRP, progesterone receptor protein; PRS, progesterone receptor-specific; PRX, progesterone receptor behaving like X; RAR, retinoic acid receptor; RXR, retinoid X receptor; SRC-1, coactivator; TBP, transcriptional activation domain.
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unique to PRB. AF-1 mapped to the minimal region of amino acids 456–546 at the NTD, and AF-2 is within the LBD (13–15). AF-1 is responsible for the ligand-independent activity of PR (15), but it also synergizes with the ligand-dependent activation function of AF-2. This was demonstrated by agonist-dependent interaction between the AF-1-containing NTD and AF-2-containing LBD using mammalian two-hybrid assays and by protein–protein interaction studies (16). There has also been evidence that the AF-1 domain of the steroid hormone receptor is an important element in mediating promoter- and tissue-selective activity (17–19). For example, a recent study with mice reported that the AF-1 domain of estrogen receptor α (ERα) is specifically required for the development of trabecular bone and uterus but not cortical bone (18). The AF-1 domain also significantly contributes to the hormone-independent growth of breast cancer (20–24).

The activities of AF-1 and AF-2 can be regulated at multiple levels of the receptor activation pathways. These include the input from cytoplasmic signaling, the activities of transcription co-regulators, and promoter accessibility. Post-translational modifications can regulate each of these steps by modifying the protein’s property and altering its interaction with its ligand, other proteins, and gene promoters (25). So far, sumoylation, acetylation, and over a dozen phosphorylation sites on PR have been reported (12, 26, 27). Notably, all but two post-translational modification sites (Ser-676 phosphorylation and acetylation, and over a dozen phosphorylation sites on PR have been reported) (28). The activities of AF-1 and AF-2 significantly contribute to hormone-independent growth of breast cancer (20–24).

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—MCF-7, MDA-MB-231, COS7, and HeLa cancer cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA). T47D cells were kindly provided by Dr. Suet Fueng Ching (University of Cambridge). ABC28 clone was derived by transfecting MDA-MB-231 cells with PR expression vectors hPR1 and hPR2, which contain human PR cDNA coding for PR isoform B and isoform A in pSG5 plasmid (28). The 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. Cells were maintained 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).

**PR Immunoprecipitation (IP)**—For MS identification of PR methylation, T47D cell lysates with endogenously expressed PR were prepared in IP 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 incubated overnight at 4 °C with anti–PR antibody (H-190, Santa Cruz Biotechnology, Inc.) pre-immunization with protein A/G-agarose beads (Santa Cruz Biotechnology). For MS quantification of Lys-464 methylation, COS7 cell lysates exogenously expressed FLAG-PRB were prepared in IP buffer and incubated overnight at 4 °C with anti-FLAG M2 affinity gel (Sigma-Aldrich). The immune complexes were washed twice with IP buffer and twice with high salt (500 mM NaCl) IP buffer to remove nonspecific binding. Bound proteins were eluted by boiling in Laemmli sample buffer. Immunocomplexes were resolved by SDS-PAGE, fixed, and stained by Coomassie Blue G-250 (Sigma-Aldrich).

**In-gel Tryptic Digestion**—The gel strips of PR bands were excised, cut into small pieces, and transferred to Eppendorf tubes. They were washed with Milli-Q water, then destained three times with 50% ACN, 50% 25 mM NH₄HCO₃ via vigorous shaking for 30 min each time. The gel was then dehydrated with 100% ACN until the gel particles became white. They were then reduced with 10 mM 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 a 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 20 min twice. The combined extracts were dried in a vacuum dryer.

**LC-MS/MS and Data Analysis**—LC-MS/MS was carried out as described previously (29). Briefly, peptides were separated and analyzed on a liquid chromatograph (Dionex UltiMate 3000 Nano-LC Systems, Dionex, Singapore) at a 300 nl/min flow rate coupled to an LTQ-FT Ultra mass spectrometer (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 8–35% 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 millitorr were used. The LTQ-FT system was set to perform data acquisition in the positive ion mode as described previously except that the m/z range of 350–1600 was used in the full MS scan (30).

The MS/MS spectra in the raw data were first extracted into the dta format using extract_msn (version 4.0) in Bioworks Browser (version 3.3, Thermo Fisher Scientific, Inc.), 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 IPI human protein database (version 3.34, 67,758 sequences, 28,836,807 residues) was used for database searches. The database search was performed using an in-house Mascot server (version 2.2.07, Matrix Science, London, UK) with MS tolerance of 10 ppm, 13C of 2, and
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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), phosphorylation (Ser, Thr, and Tyr), acetylation (Lys), monomethylation (Lys and Arg), dimethylation (Lys and 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 ion scores greater than identity or homology scores were further manually analyzed.

Plasmid Construction—pcDNA3.1 expression vectors encoding human PRB, PRA, and PRBΔLBD have been described previously (31). Point mutations of the lysine residue at site 464 of PR to glutamine, arginine, alanine, or phenylalanine 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 are as follows: K464A_fwd, 5′-agt gca ttt gca ggc gcg ccg-3′; K464A_rev, 5′-gcc ctc cgc tct gta cag gat gca ctc cag-3′; K464Q_fwd, 5′-gag tgc acct gta agg atg cac tc-3′; K464Q_rev, 5′-gcg acg tgt ccg cct ccg gct atg ctc gct ac-3′; K464R_fwd, 5′-cac gcc aag gag tgt cag gaa gga ggg c-3′; K464R_rev, 5′-gcc ctc gcg tct gta cag gat gca ctc cag-3′; K464F_fwd, 5′-gag tgc acct gta cag gga ggg c-3′; K464F_rev, 5′-cg gct ctc gct gta cag gat gca ctc cag-3′; K464A0_fwd, 5′-ctg gag tgt cag gat gca ctc cag-3′; K464A0_rev, 5′-gcc ctc cgc tct gta cag gat gca ctc cag-3′; K464F0_fwd, 5′-gcc ctc cgc tct gta cag gat gca ctc cag-3′; K464F0_rev, 5′-cg gct ctc gct gta cag gat gca ctc cag-3′.

Transfection—COS7 or HeLa cells were plated in 35-mm dishes at a density of 2.5 × 10⁵ cells in antibiotic-free phenol red-free DMEM. The cells were then transfected with various plasmid expression vectors using polyethylenimine (CELLnTEC, Switzerland) as described in more detail in the legends of Figs. 4–7.

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)). Lysates were homogenized using chilled 29-gauge syringes. Protein supernatants were collected after centrifugation at 20,227 × g for 12 min at 4 °C. Protein concentration was determined using the Bio-Rad GS-800 scanning densitometer.

Dephosphorylation Assay—Dephosphorylation of PR was carried out to diminish PR gel upshift due to phosphorylation (32). Cells were harvested with cold lysis buffer as was described earlier without phosphatase inhibitors (PI) sodium fluoride and sodium vanadate. 30 μg of total protein lysate from each sample was then incubated with 0.5 units of calf intestinal alkaline phosphatase (CIP)/μg of protein in CIP buffer for 60 min at 37 °C before it was analyzed by Western blotting.

Luciferase Reporter Assay—Luciferase assay procedures were adapted from the manufacturer protocol provided by the Dual-Luciferase Reporter System kit (Promega). Briefly, HeLa cells were seeded onto 60-mm dishes and were transfected with polyethylenimine and 5 ng of pcDNA3.1, pcDNA3.1-WT PRB, or pcDNA3.1-PRB-K464Q/F/A in addition to reporter plasmids, such as 1.5 μg of PRE2-TATA-Firefly luciferase (PRE2-Luc) reporter plasmid and 5 ng of Renilla pRL-CMV vector (both generously provided by Dr. M.-J. Tsai, Baylor College of Medicine, Houston, TX). To study the functional interaction between PR or its mutants and the co-regulators of steroid receptors, 5 ng of PRB or mutant plasmids was transfected with various amounts of pCR3.1-SRC-1 (generously provided by M.-J. Tsai), pSG5-Myc-NCoR1, or pSG5-Myc-SMRT kindly provided by Martin L. Privalsky, University of California, Davis, CA), together with 1.5 μg of PRE2-Luc and 1 ng of Renilla plasmids as described in the legends of Figs. 5–7.

24 h post-transfection, the cells were treated with 0.1% ethanol (EtOH) or 10 nM R5020 for various durations before they were lysed by 1× passive lysis buffer provided in the Dual-Luciferase Reporter System kit (Promega). 20 μl of lysate was analyzed using a computer-controlled microplate luminometer (Thermo Scientific Fluoroskan® Ascent FL). Experiments were done in either triplicates or quadruplicates.

Mammalian Two-hybrid Assay—Mammalian two-hybrid assays were performed with minor modifications (33). Briefly, HeLa cells were transfected with 500 ng of VP16/WT PRB or VP16/PRB-K464Q/F in addition to 500 ng of GAL, GAL/ NCoR1-RID, or GAL/SMT-RID and 500 ng of 5× GAL4-RE- luc reporter plasmid. 24 h post-transfection, the cells were treated with 0.1% EtOH or 10 nM R5020 for 24 h before they were lysed by 1× reporter lysis buffer provided in the Luciferase Reporter System kit (Promega). 20 μl of lysate was analyzed using a computer-controlled microplate luminometer (Thermo Scientific Fluoroskan® Ascent FL). Experiments were done in triplicates. The relative light units (RLU) of each sample are normalized against its protein concentration, as determined by the BCA™ protein assay kit (Pierce). Fold induction by R5020 is obtained by expressing the ratio of the normalized value of R5020-treated samples against vehicle control samples.

RNA Extraction and Quantitative Real-time 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 real-time 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. Real-time PCR for each targeted gene was performed in triplicates. Human acidic ribosomal phosphoprotein P0 (RPLP0), 36B4, was included as an RNA loading control for normalization of the quantity of 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 formula, relative expression = 2^(-1\*\(\text{Ct(control)}\) - \(\text{Ct(hormone)}\)), where x = \(\text{Ct(control)}\) gene X - \(\text{Ct(hormone)}\) gene X - \(\text{Ct(control)}\) 36B4 - \(\text{Ct(hormone)}\) 36B4).

After the amplification process, melting curve analysis using the LightCycler instrument was performed to verify the quality of the PCR products. Primer sequences used in real-time PCR are as follows: FKBP5_fwd, 5'—tgt gtg cca gtc ttc cat-3'; FKBP5_rev, 5'—ccc cgg cgg cga gtt ctc tac-3'; F2HSD2_fwd, cag ggc ggc tca tga ctg gaa gtt ttc tca caa-3'; F2HSD2_rev, gca gaa ggt gta gaa g-3'; R5020 (10 nM) or vehicle control (0.1% EtOH) for 20 min. Cells were harvested, lysed by PCRs with the following pair of primers used for the detection of luciferase PREs: sense, 5'—gtg acc ata tga ccc agg aat g-3'; antisense, 5'—cgc aga ggt aat ctg ctc ttt-3'.

**RESULTS**

**Identification of PR Methylation by LC-MS/MS Analysis**—In this study, we exploited proteomics techniques to determine if PR is methylated at lysine or arginine residues. Large scale immunoprecipitations of endogenously expressed PRA and PRB from breast cancer T47D cells were carried out. The immunoprecipitated proteins were resolved by SDS-PAGE, and protein bands corresponding to PRA and PRB at 81 and 116 kDa were excised and then processed for LC-MS/MS analysis. Lys-464 monomethylation was consistently identified in both PRA and PRB isoforms in two unique peptides, ATPSRPGEAAVTAAPASVSSASSSGTLECYK-Me (Me) and ATPSRPGEAAVTAAPASVSSASSSGTLECYK-Me (Me) AEG-APPQQGPFPAPPCK, in eight replicate experiments. Fig. 1A shows the representative MS spectra from one experiment of the unmodified peptide ATPSRPGEAAVTAAPASVSSASSSGTLECYK (ion score of 102 and expect value of 4.4 e-3000) and modified peptide ATPSRPGEAAVTAAPASVSSASSSGTLECYK(Me) (ion score of 76 and expect value of 0.00015), both identified in the +3 charge state when the MS/MS spectra were searched with the IPI human database. The figure depicts a positive mass shift of 14 Da, indicating the incorporation of one methyl group to the Lys residue of the peptide. The monomethylation of peptide was further confirmed by observing the retention time shift in the XIC of the unmodified and modified peptides. Fig. 1B is the XIC of the unmethylated peptides (top) integrated within 5 ppm at the monoisotopic peaks showing the relative abundances of the peptides and a shift to longer retention time of the methylated peptide (middle). The methylation at lysine slightly increases the overall hydrophobicity of the peptide, which enhances the interaction of the peptides with the stationary phase of the C18 HPLC column, resulting in the increase in the retention time of the modified peptide. Another longer peptide which contains Lys-464 in the middle, ATPSRPGEAAVTA-
FIGURE 1. **Identification of Lys-464 as site of methylation by LC-MS/MS.** A, MS spectrum showing a pair of unmodified and Lys-464-monomethylated ATPSRPGEAAVTAAPASVSSASSGSSSTLECIKYK (Me) peptides from cells cultured in medium. B, extracted ion chromatogram of ATPSRPGEAAVTAAPASVSSASSGSSSTLECIKYK (top) and ATPSRPGEAAVTAAPASVSSASSGSSSTLECIKYK (Me) (middle) peptides integrated within 5 ppm at the monoisotopic peaks showing the relative abundances of the peptides and the shift in retention time of the methylated peptide due to the increase in hydrophobicity. Bottom, extracted ion chromatogram of ATPSRPGEAAVTAAPASVSSASSGSSSTLECIKYK (Me) AEAPPQQGPFAPPCK.
have the same demonstrated 14-Da positive mass shifts as compared with the Fig. 3 and supplemental Data 3, the Lys-464 methylation site is significantly increased when the methylation site is detected in the end of the tryptic peptide, there is no b ion, and y ion sandwiched Lys-464. Because the Lys-464 monomethylation is found at the C-terminal site of methylation to be at Lys-464. For example, the positive mass shifts of 14 Da do not occur until b36; the mass difference between b36 (m/z = 1586.78) and b36 (m/z = 1739.36) is 305.16 Da, which corresponds to the mass of tyrosine and lysine modified by monomethylation.

Taken together, the LC-MS/MS results showed that both PRA and PRB are endogenously monomethylated at Lys-464 in T47D cells. Lys-464 monomethylation was also abundantly detected in ectopically expressed PR from breast cancer MDA-MB-231 cells and monkey kidney fibroblast cell line COS7. To determine if the Lys-464 methylation is regulated by the ligand, COS7 cells transfected with FLAG-PRB were grown in media with and without progesterin R5020 for 1 h before the cell lysates were collected by IP for LC-MS/MS analysis. In addition to PRB, PRA was also expressed from the second ATG of PRB cDNA in detectable amounts on a Coomassie Blue-stained gel. Both PRA and PRB bands were excised and processed for LC-MS/MS analysis of Lys-464 methylation. The XICs of the unmodified and modified ATPSRPGEAAVTAPASAVSSASSGSGTLECIYK peptides were extracted within 5 ppm at the corresponding monoisotopic peaks. Both PRA and PRB were found to be methylated. The ratio of the area of the XIC of the unmodified peptide and the Lys-464 monomethylated peptide is about 1:1, and the ratio does not change in response to ligand treatment, indicating that the Lys-464 monomethylation probably is not regulated by the ligand (supplemental Data 4).

Lys-464 Mutants PR-K464Q and PR-K464A Exhibit Ligand-independent Gel Upshift—Lys-464 lies within the minimal region of AF-1 that was originally mapped to amino acids 456–546 (14, 15). AF-1 is known to mediate the ligand-independent PR activation and to synergize with the ligand-dependent activity of AF-2 (15, 16, 35, 36). In order to define the functional importance of Lys-464 methylation in regulating AF-1 activity, we first searched for the lysine methyltransferase that methylates Lys-464 but found no candidate. We then generated four PRB-Lys-464 mutants (PRB-K464A, PRB-K464Q, PRB-K464R, and PRB-K464F) that would enable us to deduce the functional role of Lys-464 and its methylation. The choice of amino acid was based on the understanding that lysine methylation increases both the local bulkiness and hydrophobicity of the distal R group (37). The hydrophilic lysine was replaced with the smaller, hydrophobic alanine to introduce maximal change of local structure so as to demonstrate if Lys-464 is important for PR function. Although glutamine is widely regarded as an amino acid mimic for lysine acetylation, it is in fact similar to unmodified lysine based on the size (both have a molecular mass of ~128 Da) and Kyte-Doolittle hydrophobicity scores (~3.5 versus ~3.9) (38). The difference is that Gln is zwitterionic, whereas Lys is positively charged at physiological pH. Gln has been suggested as a mimic for unmethylated lysine in the study of methylation of retinoic acid receptor (39). Arginine, on the other hand, resembles methylated lysine the most because of the bulky guanidinium group and of its positive charge. In fact, the steric structure of arginine is similar to the dimethylated lysine, so the K464R mutant was expected to mimic the function of wild type PR. We also tested the effect of replacing Lys-464 with a bulkier and hydrophobic phenylalanine, which was reported to mimic the effect of lysine trimethylation or arginine monomethylation (39, 40).

PR can be phosphorylated at more than a dozen serine residues within NTD, and the majority of the phosphorylation is ligand-induced (12). Progestin-induced phosphorylation causes PR “gel upshift” on denaturing SDS-PAGE due to a decrease in the electrophoretic mobility of the phosphorylated PR (12, 41). The band upshift on a Western gel is therefore a hallmark of PR activation. Interestingly, both PRB-K464Q and PRB-K464A displayed ligand-independent gel upshift akin to that of ligand-induced WT PR (Fig. 4A). The gel upshift was also clearly observed in PRA mutants, a minor product resulting from the second ATG of the PRB cDNA. Following R5020 (a synthetic progestin) treatment, K464Q and K464A mutants exhibited a further gel upshift that is higher than that of WT PR. In contrast, K464R and K464F mutants, which have increased local bulkiness and hydrophobicity, did not display PR band upshift (Fig. 4B). Because protein gel upshift is commonly associated with protein phosphorylation, it is plausible that K464Q or K464A replacement may have caused changes in the protein structure that is accessible for PR phosphorylation by kinases.

Because the ligand-independent gel upshift of PRB-K464Q and PRB-K464A resembles the ligand-induced PR gel upshift as a result of PR phosphorylation, we tested if there is an increase of ligand-independent PR phosphorylation at serine 294 and 400, the two most well studied PR phosphorylation sites. Phosphorylation at both sites can be ligand-induced, although PR phosphorylation at Ser-400 is also evident in the absence of ligand (42, 43). Indeed, both PRB-K464Q and PRB-K464A displayed higher basal levels of phosphorylation than PRB at Ser-294 and Ser-400 in the absence of ligand (Fig. 4C). Furthermore,
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A

ATPSRPGEAAVTAAPASVSSASSSGSTLECILYK

b ions:

b^2 ions:

ATPSRPGEAAVTAAPASVSSASSSGSTLECILYK
MS/MS of 1161.24 [M + 3H]^3

Relative Abundance

m/z

B

ATPSRPGEAAVTAAPASVSSASSSGSTLECILYK(Me)

b ions:

b^2 ions:

ATPSRPGEAAVTAAPASVSSASSSGSTLECILYK(Me)
MS/MS of 1165.92 [M + 3H]^3

Relative Abundance

m/z
FIGURE 2. MS/MS spectra and fragment ions assignments by Mascot of Lys-464-unmethylated peptide and Lys-464-monomethylated PR peptides. ATPSRPGEAAVTAAPASVSSASSSGSTLECILYK (A) and ATPSRPGEAAVTAAPASVSSASSSGSTLECILYK(Me) (B) were both identified in the 3+ charge state when the MS/MS spectra were searched with the IPI human database. The labeled peaks correspond to masses of b and y ions of peptide fragments. Positive mass shifts of +14 Da (marked in red) started from b2'. Manual inspection of fragmented b and y ion series spanning the peptide in opposite orientation confirmed the site of monomethylation to be at Lys-464.

FIGURE 3. MS/MS spectra and fragment ions assignments by Mascot of Lys-464-methylated long peptide ATPSRPGEAAVTAAPASVSSASSSGSTLECILYK(Me) AEGAPPQQGPFAPPCK. The detailed Mascot Peptide View is provided in supplemental Data 3. The labeled peaks correspond to masses of b and y ions of peptide fragments. Positive mass shifts of +14 Da (marked in red) started from b36'. Manual inspection of fragmented b and y ion series flanking Lys-464 confirmed the site of monomethylation to be at Lys-464.
both mutants also displayed higher levels of ligand-induced phosphorylation at both sites.

To further verify if the ligand-independent band upshift of the K464Q mutant is due to phosphorylation, we determined if a dephosphorylation reaction would abolish the band upshift. Protein dephosphorylation can be conveniently brought about by collecting cell lysates in buffer without PI that are routinely added to the lysis buffer for preserving protein phosphorylation and with the addition of CIP. As expected, the gel upshift of PRB-K464Q was visibly reduced in the dephosphorylation buffer (Fig. 4D). The gel upshift of the PRA-K464Q form (derived from PRB cDNA) was also evidently reduced in the dephosphorylation buffer. These results suggest that the ligand-independent gel upshift of PRB-K464Q and PRB-K464A mutants was to a large extent due to the increases in ligand-independent phosphorylations. This notion is further substantiated by the observation that the gel upshift of PRB-K464Q−/H9004LBD was also reduced under dephosphorylation conditions (Fig. 4E).

K464Q Mutant Exhibits Heightened Ligand-independent AF-1 Activity—Because PR band upshift and phosphorylation are associated with ligand-induced PR activation, we determined if PRB-K464Q is more active than WT PRB in the absence of ligand using a PRE-luciferase assay. Although there were no consistent increases in the ligand-independent activity of the full-length PRB-K464Q compared with WT PRB, the ligand-independent activity of PRB-K464QΔLBD was 3 times that of PRBΔLBD in HeLa cells (Fig. 5A), confirming that K464Q mutation led to higher ligand-independent AF-1 activity. The observed difference in activity was not due to unequal protein expression, as demonstrated by the corresponding PR protein immunoblot of the two plasmids (Fig. 5B).

PR-K464Q and PR-K464A Exhibit Heightened Ligand-dependent Activity—Lys-4644 mutations also have a significant effect on the ligand-induced activity of PR. Following treatment with

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**FIGURE 4.** K464Q and K464A mutants display slower gel electrophoresis mobility. A, unliganded PRB-K464Q and PRB-K464A mutants exhibit slower gel mobility that is similar to that of ligand-treated WT PRB. COS7 cells transfected with 100 ng of WT PRB, PRB-K464Q, or PRB-K464A plasmids were treated with 0.1% EtOH or 10 nM R5020 for 1 h before lysates were collected for Western blotting analysis using specific antibody against PRB. B, PRB-K464R and PRB-K464F mutants did not exhibit ligand-independent gel upshift. C, higher levels of Ser-294 and Ser-400 phosphorylation were detected in PRB-K464Q or PRB-K464A than in WT PRB treated with (+) or without (−) R5020. COS7 cells transfected with 100 ng of WT PRB and Lys-464 mutant plasmids were treated with either 0.1% EtOH or 10 nM R5020 for 1 h before lysates were collected for Western blotting analysis using specific antibodies against PRB, phospho-Ser-294, or phospho-Ser-400 on PRB. β-Actin was probed as a loading control. The immunoblots were analyzed by densitometry. The numbers below the blots depict the relative densitometry ratios of phospho-PR to total PR.

**FIGURE 5.** Ligand-independent transcriptional activity of K464QΔLBD is heightened. A, K464QΔLBD exhibits higher transcriptional activity. HeLa cells were transfected with 5 ng of either WT PRBΔLBD or PRB-K464QΔLBD vector, in addition to fixed amounts of PRE2-TATA-luciferase (1.5 μg) and Renilla (1 ng) expression vectors. All PRE-driven luciferase activity was normalized to Renilla and expressed as the average RLU of triplicate readings (± S.E. (error bars)) Relative PRE-luc activity was calculated as the ratio of the RLU of WT PRBΔLBD- or PRB-K464QΔLBD-transfected samples divided by the RLU of vector control (pcDNA3.1)-transfected samples. Asterisks denote statistical significance (****, p < 0.0001) determined by unpaired Student’s t tests. B, K464QΔLBD expressed in HeLa cells exhibits gel upshift in Western blotting analysis. β-Actin was probed as a loading control.
10 nM R5020 for 12 h, PRE-Luc activities in cells transfected with PRB-K464Q and PRB-K464A were 3–4 times that of the WT PRB (Fig. 6A). Hence, the ligand-induced increase of PR activity is similar to the ligand-independent increase of PRB-K464Q/H9004/LBD activity. Furthermore, this increase in ligand-independent activity of PRB-K464Q/H9004/LBD was due to the hyperactivity of AF-1 rather than AF-3 because the K464Q mutant of PRA, which does not contain AF-3, also exhibited a similar increase of activity compared with WT PRA (supplemental Data 5).

We further characterized the effect of the K464Q mutation on PRB activity under different experimental conditions. First, it was found that PRB-K464Q was consistently more active than WT PRB when transfected with various amounts (1, 2, and 5 ng)
of the expression vectors (Fig. 6B). Second, a short time course experiment showed that the ligand-induced activity of PRB-K464Q initiated earlier than WT PRB. After 1 h of R5020 treatment, PRB-K464Q activity was increased by 7.5-fold, whereas the activity of WT PRB remained at the basal level (Fig. 6C). This accelerated ligand-induced activity of PRB-K464Q is probably related to the ligand-independent phosphorylation, so the journey to activation may be shorter. Indeed, the plasmid immunoprecipitation assay showed that more PRB-K464Q was already bound to canonical 2XPRE sequences in the absence of ligand than WT PRB (Fig. 6D). There was also a marked increase in PRB-K464Q-PRE binding upon ligand treatment compared with WT PRB. This phenomenon is in concordance to its enhanced ligand-independent and ligand-dependent transcriptional activity, as observed in various luciferase reporter assays (Figs. 5A and 6A–C).

Although both arginine and lysine are positively charged, arginine is bulkier sterically due to the quanidinium group and therefore resembles the methylated lysine. Accordingly, the transcriptional activity of PRB-K464R was similar to that of WT PRB (Fig. 6E). On the other hand, replacement of Lys-464 with the bulkier and highly hydrophobic phenylalanine resulted in more than 50% reduction of PR activity.

Lys-464 Mutation Modifies Ligand Sensitivity—The results presented so far suggest that Lys-464 is critical for both the ligand-independent and ligand-dependent activity of AF-1. Lys-464 methylation may exert a repressive role. We next examined if Lys-464 mutation modifies PR sensitivity to PR ligand. Transcriptional activities of WT PRB, PRB-K464Q, and PRB-K464F in response to various concentrations of R5020 (0.01, 0.1, 1, and 10 nM) were measured by a PRE-Luc assay in PRB-K464F in response to various concentrations of R5020 (0.01, 0.1, 1, and 10 nM) were measured by a PRE-Luc assay in HeLa cells. Fig. 6F shows that PRB-K464Q was consistently more sensitive to progestin than WT PRB and PRB-K464F. For example, PRB-K464Q demonstrated similar activity in response to 0.1 nM R5020 as WT PRB to 1 nM R5020. In contrast, PRB-K464F required 10 times more R5020 (10 nM) than PRB (1 nM) to display similar activity. This suggests that Lys-464 methylation at AF-1 may regulate ligand sensitivity.

Lys-464 Affects the Recruitment of PR Co-activators—PR activity can be regulated through its ability to recruit transcription co-regulators, which in turn modify chromatin structure to facilitate the recruitment of basal transcription machinery. Nuclear receptor co-repressor 1 (NCoR1) and silencing mediator of retinoid and thyroid hormone receptor (SMRT) are well known NR co-repressors (44–46). We asked whether the heightened effect of K464Q mutation on PR activity resulted from an inhibition of recruitment of NCoR1 and SMRT. Surprisingly, co-transfection of 10 or 50 ng of NCoR1 expression vector with 5 ng of WT PRB stimulated the transcriptional activity of both WT PRB and the mutants in response to 0.1 nM R5020 (Fig. 7A). Furthermore, PRB-K464Q was more responsive, whereas PRB-K464F less responsive to the co-activating effect of NCoR1 than WT PRB. The absolute -fold increase of PRB activity due to 10 ng NCoR1 was 50-fold. In contrast, increased activity of PRB-K464Q was 180-fold, and that of PRB-K464F was merely 8-fold (Fig. 7A). It is also interesting that the increase of PR activity due to NCoR1 overexpression was proportional to the activities of PR and Lys-464 mutants in the absence of NCoR1 overexpression. We speculate that in the absence of overexpression, endogenous NCoR1 also played a role in the heightened or weakened activity of the Lys-464 mutants. Although SMRT was also a co-activator to PRB and Lys-464 mutants in this experimental setting, the absolute -fold increase of PR activity due to SMRT was not markedly different between PRB and the Lys-464 mutants (Fig. 7B). The data thus indicate that Lys-464 is critical for the functional interaction with NCoR1, which apparently functioned as a co-activator of PR under our experimental conditions. Other studies have also reported that NCoR1 could be recruited by either agonist- or antagonist-bound PR (47, 48) and acted like a co-activator in response to low doses of R5020 (49).

We also examined if Lys-464 mutation alters functional interaction with SRC-1 (steroid receptor coactivator), a bona fide nuclear receptor co-activator that is known to functionally interact with both AF-1 and AF-2 of PR (16, 50, 51). There was an about 600-fold increase of R5020-induced PRB-K464Q activity in cells co-transfected with 10 ng of SRC-1 compared with empty vector control. This is in contrast to a 300-fold increase of WT PRB activity and a 100-fold increase of PRB-K464F in response to SRC-1. With a larger amount of SRC-1 vector (50 ng), the difference among PRB and the mutants was reduced. The effect of a large amount of SRC-1 overexpression may have achieved a saturation effect such that the effect difference between PR and Lys-464 mutants was narrowed (Fig. 7C).

Co-repressors (e.g. NCoR1 and SMRT) are known to interact via their receptor interaction domains (RIDs) with the LBD in the C-terminal half of steroid/nuclear receptors (52). It is also reported that, other than LBD, the AF-1 domain of glucocorticoid receptors and PR is also important for mediating the interaction with the RID of co-repressors NCoR1 and SMRT (33). In particular, it was shown that PR N-terminal amino acids 468–508 are critical for PR interaction with the RID of NCoR1 or SMRT (33). Because Lys-464 is directly adjacent to the fragment and could participate in the interaction with the RIDs, we determined whether Lys-464 mutations (PRB-K464Q and PRB-K464F) alter PR interaction with RID of NCoR1 or SMRT. A modified mammalian two-hybrid analysis was conducted as was described in the above mentioned study. Chimeras of NCoR1-RID and SMRT-RID fused to the GAL4 DNA-binding domain were used in the assays to detect the association of co-repressors with either wild-type PRB or PRB-Lys-464 mutants fused with the VP16 activation domain. The interactions between the RIDs and PRB or the mutants were indicated from an inhibition of recruitment of NCoR1 and SMRT. Surprisingly, co-transfection of 10 or 50 ng of NCoR1 expression vector with 5 ng of WT PRB stimulated the transcriptional activity of both WT PRB and the mutants in response to 0.1 nM R5020 (Fig. 7A). Furthermore, PRB-K464Q was more responsive, whereas PRB-K464F less responsive to the co-activating effect of NCoR1 than WT PRB. The absolute -fold increase of PRB activity due to 10 ng NCoR1 was 50-fold. In contrast, increased activity of PRB-K464Q was 180-fold, and that of PRB-K464F was merely 8-fold (Fig. 7A). It is also interesting that the increase of PR activity due to NCoR1 overexpression was proportional to the activities of PR and Lys-464 mutants in the absence of NCoR1 overexpression. We speculate that in the absence of overexpression, endogenous NCoR1 also played a role in the heightened or weakened activity of the Lys-464 mutants. Although SMRT was also a co-activator to PRB and Lys-464 mutants in this experimental setting, the absolute -fold increase of PR activity due to SMRT was not markedly different between PRB and the Lys-464 mutants (Fig. 7B). The data thus indicate that Lys-464 is critical for the functional interaction with NCoR1, which apparently functioned as a co-activator of PR under our experimental conditions. Other studies have also reported that NCoR1 could be recruited by either agonist- or antagonist-bound PR (47, 48) and acted like a co-activator in response to low doses of R5020 (49).
with GAL4/SMRT-RID more than WT PRB did, whereas PRB-K464F interacted less (Fig. 7E). Western blotting analysis indicates that equal amounts of VP16/PRB, VP16/PRB-K464Q, and VP16/PRB-K464F were expressed in HeLa cells (Fig. 7F). Collectively, the data from the mammalian two-hybrid assay support the notion that Lys-464 of PR AF-1 plays a key role in PR physical and functional interaction with NCoR1 and SMRT. K464Q mutation facilitates, whereas K464F mutation hinders, the interaction. The altered interaction explains at least partly the mechanisms by which K464Q mutation heightens, whereas K464F mutation weakens, PR activity. Consistent with the effect on PR activity in the PRE-Luc assay, Lys-464 mutations alter markedly PR-mediated progestin response in breast cancer cells—The PR reporter gene assay measures the ability of PR to interact with PRE and recruit transcription machinery. This assay may not reflect the whole cellular effect of PR because many target genes of PR do not contain the typical palindromic PRE sequence, and the biological effects of PR are also significantly attributed to PR interac-

FIGURE 7. PRB-K464Q and PRB-K464F display differential sensitivity to the co-regulator’s effects of NCoR1. HeLa cells were transfected with 5 ng of WT PRB, PRB-K464Q, or PRB-K464F together with various amounts of NCoR1, SMRT, or SRC1 in addition to fixed amounts of PRE2-TATA-luciferase (1.5 μg) and Renilla (1 ng) expression vectors. Cells were then treated with either vehicle control (0.1% EtOH) or 10 nM R5020 for 12 h. PRE-driven luciferase activity was measured and calculated as was described in the legend to Fig. 5. Results are means ± S.E. (error bars). A, effects of NCoR1 co-transfection (0, 10, and 50 ng) on WT PRB, PRB-K464Q and PRB-K464F transcriptional activity. B, effects of SMRT co-transfection (0 and 10 ng) on WT PRB, PRB-K464Q, and PRB-K464F transcriptional activity. C, effects of SRC1 co-transfection (0, 10, and 50 ng) on WT PRB, PRB-K464Q, and PRB-K464F transcriptional activity. All results are shown to be significant with unpaired Student’s t test, two-tailed (p < 0.01). D, PRB-K464Q demonstrated enhanced NCoR1-RID interaction with R5020 induction. HeLa cells were transfected with 500 ng of VP16/WT PRB or VP16/PRB-K464Q/F, in addition to 500 ng of NCoR1-RID and 5× GAL4-RE-luc reporter plasmid. After transfection, the cells were incubated with either 0.1% ethanol or 100 nM R5020 for 24 h and subsequently harvested for luciferase and protein assays. The data are presented as -fold induction by expressing the normalized RLU (against the respective protein concentrations) of R5020-treated samples as a ratio of the normalized RLU of 0.1% ethanol control samples. The data from a representative experiment are shown. Error bars, S.E. F, Lys-464 mutants displayed similar SMRT-RID interaction as WT PRB. Cells were transfected with VP16/WT PRB or VP16/PRB-Lys-464 mutations in addition to 500 ng of NCoR1-RID and 5× GAL4-RE-luc, treated, and analyzed as in D. F, relative protein levels of VP16/WT PRB or VP16/PRB-Lys-464 mutations as detected by PR-specific antibody H190. β-Actin was probed as a loading control. Asterisks denote statistical significance (*, p < 0.05; **, p < 0.01; ***, p < 0.001; ****, p < 0.0001) determined by unpaired Student’s t tests. n.s., not significant.
tion with other transcription factors, such as activator protein 1 (NF-κB), through a tethering mechanism (53–56). To assess the whole cellular effect of K464Q mutation, we generated multiple clones of stably transfected pcDNA3.1, WT PRB, PRB-K464Q, and PRB-K464F MDA-MB-231 cells, and three clones of similar levels of PR protein (Fig. 8A) from each group were selected and pooled during the experiment to evaluate the effect of Lys-464 mutations on gene expression, cell adhesion, and growth. Fig. 8, B–D, shows the effect of Lys-464 mutation on progestin-induced mRNA expression of three PR target genes, mucin 1 (*MUC1*), FK506-binding protein 5 (*FKBP5*), and 11β-hydroxysteroid dehydrogenase (*11β-HSD2*) (Fig. 8D), all known to contain PRE in their promoters (57–59). In concordance with observations from PRE luciferase reporter assays, the PRB-K464Q mutant was significantly more potent than WT PRB in up-regulating these genes, whereas PRB-K464F was significantly less active.

It was reported previously that progestins were able to inhibit cell cycle progression in PR-transfected MDA-MB-231 cells (28, 60). We assessed the effects of Lys-464 mutations on the growth-inhibitory effects of R5020 using MDA-MB-231 cells expressing the different PRB-Lys-464 mutants. Consistent with previous studies, progestin inhibited the growth of WT PRB-transfected MDA-MB-231 cells by 38% after 72 h of treatment. PRB-K464Q mutation heightened PR-repressive activity on cell growth, and the total cell number was reduced by 65% (*p* < 0.001). On the other hand, PRB-K464F mutant exhibited similar activity in growth inhibition (36%) as WT PRB (Fig. 8E). We
also examined the effects of PRB-Lys-464 mutations on progesterone modulation of mRNA expression of two cell cycle-regulatory genes, kinesin-like protein 1 (KIF11) (Fig. 8f) and cyclin A2 (CCNA2) (Fig. 8g), in the MDA-MB-231 clones. The treatment of progesterone reduces the gene expression of CCNA2 and KIF11 in WT PRB and PRB-Lys-464 mutation clones. In concordance with the observations from the proliferation assay, PRB-K464Q exhibits the greatest reduction of mRNA levels of CCNA2 and KIF11, but PRB-K464F represses CCNA2 and KIF11 mRNA levels similarly to WT PRB. The data seem to suggest that, whereas the K464Q mutation heightens PR activity on growth inhibition, the K464F mutation does not alter PR activity on growth inhibition significantly.

It is known that progesterone induces remarkable focal adhesion and cell spreading in PRB-transfected MDA-MB-231 cells (62). We evaluated if there is any difference in progesterone-induced focal adhesion and cell spreading between PRB and PRB-Lys-464 mutants (K464Q and K464F). All clones exhibited similar cellular morphology and diameter under control conditions (Fig. 9, A and B). Upon treatment, PRB-K464Q clones exhibit a greater degree of spreading and occupied a greater surface area than WT PRB cells ($p < 0.05$), whereas PRB-K464F cells have a lesser degree of spreading (statistically insignificant because of high variation) with WT PRB (Fig. 9, A and B). The morphology of the K464Q cells and WT PRB was then further evaluated by visualizing using phalloidin-FITC conjugate while the focal adhesions were stained with anti-paxillin antibody. In response to R5020, PRB-K464Q-transfected cells showed a significantly greater degree of increase of stress fibers and focal adhesions than WT PRB-transfected cells (Fig. 9, A and C). In contrast, cells transfected with PRB-K464F had a significantly lesser number of focal adhesions ($p < 0.01$).

A genome-wide expression study has reported that progesterone modulates genes involved in cell adhesion and motility in PR-transfected MDA-MB-231 cells (63). Lumican (LUM) and PDZ and LIM domain 1 (PDLIM1) are among the cell adhesion genes up-regulated by progesterone in these cells. LUM and PDLIM1 (Fig. 9, D and E) were selected to test the effects of PRB-Lys-464 mutations on their regulation by progesterone because of their functional involvement in cell adhesion. LUM is an extracellular matrix protein and belongs to the family of small leucine-rich proteoglycans. In addition to regulating collagen fibrillogenesis, LUM has been reported to inhibit cell migration in melanoma cells and human mesenchymal stem cells via integrin $\beta 1$ (64, 65). PDLIM1/Elfin binds to filamentous actin-associated proteins and regulates organ development (66, 67). In agreement with its heightened activity on cell spreading, PRB-K464Q mediated greater induction of LUM and PDLIM1 by R5020 as compared with WT PRB (Fig. 9, D and E). In contrast, PRB-K464F mediated less induction of LUM and PDLIM1 (Fig. 9, D and E). These gene expression analyses provide the molecular basis for the hyper- and hypoactivity of K464Q and K464F mutants on cell adhesion.

**DISCUSSION**

It has been increasingly recognized that lysine and arginine methylation of nuclear receptors plays pivotal roles in regulating both the nuclear and cytoplasmic signaling (68–71). The first report was on trimethylation of retinoic acid receptor at Lys-347 in the ligand binding domain (39). Mutation of Lys-347 to alanine or glutamine disrupted the interaction of retinoic acid receptor with cofactors p300/CPB and with its heterodimer partner RXR. Subsequently, ER$\alpha$ was found to be methylated at lysine 302 and arginine 260 (69, 71). Lys-302 methylation by SET7 is necessary for efficient promoter binding, whereas arginine methylation at Arg-260 of the DNA-binding domain is critical for the cytoplasmic signaling of ER$\alpha$. The present study reports the first evidence of PR methylation at Lys-464 of AF-1 by LC-MS/MS analysis of both endogenous PR expressed in T47D cells and transfected PR in MDA-MB-231 and COS7 cells. The methylation site was sandwiched by a series of b and y ions from collisional activated dissociation of the longer peptide precursor ion, indicating the methylation site was identified with very high confidence. Assuming that the methylation is not lost during the sample preparation, about 50% of PR carries the monomethylation mark at Lys-464 based on the XIC area. This may represent a subclass of PR that is regulated by Lys-464 methylation.

Although the lysine methyltransferase responsible for Lys-464 methylation remains elusive, the functional characterization of four different Lys-464 mutants has shed light on the functional significance of Lys-464 methylation. The results argue for a repressive role of Lys-464 methylation on PR activity based on the understanding that lysine methylation increases the local bulkiness and hydrophobicity. Replacement of Lys-464 with bulkier arginine, which resembles methylated lysine because of the bulky quanidinium group and its positive charge, mimicked the activity of WT PRB. PRB-K464F mutation, which introduced a bulkier phenylalanine, significantly inhibited PR activity. On the other hand, mutation of Lys-464 to glutamine and alanine, which are either similar to or smaller than lysine, led to heightened PR activity. Nonetheless, there is no bona fide amino acid mimic of lysine monomethylation. Knowledge of specific lysine methyltransferase for Lys-464 will help to further verify the role of PR methylation.

It is remarkable that the single amino acid at position Lys-464 dominates the structural organization of PR such that PRB-K464Q or PRB-K464A, but not PRB-K464R or PRB-K464F, resulted in ligand-independent PR gel upshift (Fig. 4) that is commonly seen in ligand-activated PR (12, 41). Lys-464 resides within ER-interacting domain II, mapped to amino acids 456–546. ER-interacting domains I and II flank a proline-rich motif of amino acids 421–428 responsible for PR binding to the Src homology 3 domain of various cytoplasmic signaling proteins, including c-Src (72, 73), which is involved in progesterone-induced PR phosphorylation (54). It is plausible that Lys-464 is an important part of the interaction interface, and Lys-464 methylation hinders the interaction between the proline-rich motif and Src homology 3 domain-containing signaling molecules. K464Q or K464A mutations overcome this hindrance, leading to an increase in ligand-independent PR phosphorylation and AF-1 activation. On the other hand, there is probably other post-translational modification-associated change that contributes to the gel upshift of PRB-K464Q or PRB-K464A mutants because the dephosphorylation reaction reversed...
some but not all of the gel upshift of these mutants (Fig. 4, D and E).

The study of Lys-464 mutants has provided intriguing evidence that AF-1 of PR is also involved in regulating the ligand sensitivity. PRB-K464Q required nearly 10 times less R5020 than WT PRB and 100 times less R5020 than PRB-K464F to display similar levels of activities (Fig. 6F). This implies that gene polymorphism/mutation at Lys-464 could significantly
alter tissue response to progesterone. Progesterone hypersensitivity or resistance is known to cause serious clinical conditions. For example, autoimmune progesterone dermatitis, in which patients experience skin disorders such as eczema and angioedema during the luteal phase of the menstrual cycle, is associated with progesterone hypersensitivity (74, 75). On the other hand, progesterone resistance is common in cases of endometriosis and infertility (76–78). To our knowledge, there has been no report on gene polymorphism or mutation involving Lys-464, and this is an interesting area for future investigation.

One of the mechanisms by which Lys-464 mutations modify the activity of PR seems to be mediated by changes in the functional interaction between PR and transcription co-regulators. Both NCoR1 and SRC-1 are known to interact with AF-1 of PR to enhance the AF-1 activity (33, 50). In the present study, heightened activity of liganded PRB-K464Q was associated with increased co-activating effect by NCoR1 and SRC-1 and to a less extent by SMRT (Fig. 7). Conversely, decreased PRB-K464F activity was associated with weakened effect of NCoR1, SMRT, and SRC-1 (Fig. 1). More interestingly, a mammalian two-hybrid assay showed that Lys-464 is critical for physical interaction between PRB and NCoR1/SMRT. Consistent with their activities, PRB-K464Q interacted with NCoR1 more than WT PRB, whereas PRB-K464F interacted less with NCoR1. Accordingly, SMRT interacted more with PRB-K464Q but less with PRB-K464F than with PRB, which is also consistent with its co-activating potential on the PRB and mutants. It is likely therefore that Lys-464 methylation modulates PR interaction with NCoR1 and SMRT, which function as PR co-activators in HeLa Cells.

Altered functional interaction between SRC-1 and PR Lys-464 mutants may also be responsible for the changes in progesterin sensitivity of the mutants. SRC-1 is known to be important for receptor response to steroid hormones. SRC-1 knock-out mice displayed attenuated decidual response in response to progesterone treatment (79). It is known that agonist-induced PR activation involves direct intramolecular association between the N terminus of AF-1 and the C terminus of AF-2. SRC-1 can facilitate the interaction by binding simultaneously both the N- and C-terminal domains (16). Lys-464 may be critical for SRC-1 and AF-1 interaction that in turn facilitates the interaction between AF-1 and AF-2.

PR-transfected MDA-MB-231 cells demonstrate remarkable morphologic change (e.g. cells become more spread out and flattened) upon progesterin treatment. Such morphologic changes are associated with the decrease in cell migration and metastatic ability of breast cancer cells (80). Progesterone treatment is also found to induce the reversion of the mesenchymal phenotypes of basal phenotype breast cancer cell to epithelial phenotypes via PI3K/Akt pathway (81). Our data indicate that Lys-464 mutation of the AF-1 domain affects markedly the response to progesterin-induced focal adhesion. In accordance with their activity on gene expression and promoter binding, PRB-K464Q mutant exhibited greater activity in inducing focal adhesion and cell adhesion genes (LUIM and PDLIM1), whereas PRB-K464F mutant exhibited less activity (Fig. 9). Interestingly, although PRB-K464Q exhibited stronger growth-inhibitory activity in response to progesterin, the activities of PRB-K464F on growth and growth-related genes are similar to that of WT PRB. This raises an interesting possibility that AF-1 in general (and Lys-464 specifically) is important in the pathway-selective activity of PR. Although the K464F mutation impaired PR activity upon the induction of cell adhesion genes and PRE-containing genes (MUC1, FKBP5, and 11β-HSD2), the mutation did not exhibit significant effect on cell cycle-related genes.

In summary, the present study provides the first evidence of PR methylation at Lys-464 of AF-1. This is also the first identification of a critical functional motif in PR AF-1. We highlight three significant features of the findings. First, the evidence that a single mutation at Lys-464 (K464Q or K464A) led to a ligand-independent gel upshift of PR and ligand-independent PR phosphorylation supports the notion that Lys-464 is critical for NTD folding and interaction with other proteins under cellular conditions. Future identification of interacting proteins with the unliganded mutants will shed light on the mechanisms of the ligand-independent phosphorylation. Second, despite the increase of ligand-independent phosphorylation, the functional impact of Lys-464 mutation is largely on the ligand-dependent PR activity. We showed that the PRB-K464Q mutation heightened progesterin-induced PRB activity on promoter binding, gene expression, and cell adhesion characteristics, whereas the PRB-K464F mutation impaired it. This implies that AF-1 plays a key role in ligand-induced PR activity at the whole cellular level, including the regulation of cell adhesions and cytoskeletal structure formation of breast cancer cells. Last, the findings set the stage for further functional delineation of AF-1 in different tissues and in breast cancer. It is known that AF-1 of steroid receptors is important for their promoter and tissue-selective functions (18, 61, 82). The relative involvement of AF-1 of PR in its target tissues (e.g. mammary gland and uterus) and in breast cancer has not been clarified. The findings of this study provide an important basis for future studies of AF-1 in vivo. Conceivably, knowledge of cell/tissue-selective molecular function of AF-1 will shed light on how PR mediates both stimulatory and inhibitory effects of progesterin on breast cancer.

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Supplementary data 1.
Mascot Peptide view of triply charged unmethylated PR peptide ATPSRPGEAAVTAAPASASVSSASSSGSTLECILYK\textsuperscript{464} with an ion score 102 and expect value of 4.4 e\textsuperscript{-008}. PR was isolated from T47D cells treated with 100 nM Progesterone. The y and b ions of the peptide detected by LC-MS/MS are indicated in red. Comparison of the y and b ions profiles of the peptides in supplementary data 1 and 2 suggests that the positive mass shift of 14 Da takes place at K464 in the C-terminal end of the peptide.
Mascot Search Results

Peptide View

MS/MS Fragmentation of ATPSRGEEAVTAAPASVSSASSGSGTLEICLYK
Found in sple106401|PRGR_HUMAN in Xumprot_human, Progesterone receptor OS=Homo sapiens GN=PRG PE=1 SV=4

Match to Query 4481: 3480.711792 from(1161.244540,3+) intensity(40272.5508) rtinseconds(1812) scans(3324) index(2346)
Title: File3764 Spectrum2385 scans: 3324
Data file K:\Hwa\Hwa_ALL-Acetylation\111116_VaI.in\MGF\111116_VaI.in_PCPR_04.mgf

Click mouse within plot area to zoom in by factor of two about that point
Or, Plot from 300 to 2000 Da Full range
Label all possible matches Label matches used for scoring
Show Y-axis

Monoisotopic mass of neutral peptide Mr(calc): 3480.7042
Fixed modifications: Carbamidomethyl (C) (apply to specified residues or termini only)
Ions Score: 102 Expect: 4.6e-008
Matches : 99/400 fragment ions using 170 most intense peaks  [help]

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# Mascot Search Results: Peptide View

**NCBI BLAST** search of **ATPSRPGEAAVTAAPASASYVSSASSSSGILYK**  
(Parameters: blastp, nr protein database, expect=20000, no filter, PAM30)  
Other BLAST [web gateways](#)

### All matches to this query

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**Mascot** [http://www.matrixscience.com/](http://www.matrixscience.com/)
**Supplementary data 2.**

Mascot Peptide view of triply charged methylated peptide ATPSRPGEAAVTAAPASVSSASSSGSTLECILYK_{464}(Me) with an ion score 76 and expect value of 0.00015. PR was isolated from T47D cells treated with 100 nM Progesterone. The y and b ions of the peptide detected by LC-MS/MS are indicated in red. Comparison of the y and b ions profiles of the peptides in supplementary data 1 and 2 suggests that the positive mass shift of 14 Da takes place at K464 found in the C-terminal end of the peptide.
Mascot Search Results: Peptide View

Peptide View

MS/MS Fragmentation of ATPSRPGEAATAPAASSVSSASSGSTLCEILYK

Found in sp|P06401|PRGR_HUMAN, Progesterone receptor OS=Homo sapiens GN=PRG PE=1 SV=4

Match to Query 4494: 3494.729262 from(1165.917030.3+) intensity(16352.4150) scans(3353) runseconds(1823) index(2371)

Title: File1482 Spectrum2410 scans: 3353

Data file X:\HwaHwa\MascotResult\20111116_ValLin_PRdata\RAW2mgf6000\111116_ValLin_PCPR_04.mgf

Click mouse within plot area to zoom in by factor of two about that point

Or, Plot from 300 to 2000 Da Full range

Label all possible matches Label matches used for scoring

Monoisotopic mass of neutral peptide Mr(calc): 3494.7199

Fixed modifications: Carbamidomethyl (C) (apply to specified residues or termini only)

Variable modifications:
K36 : Methylkrr (KK)

Ions Score: 76 Expect: 0.00015

Matches : 68/400 fragment ions using 136 most intense peaks (help)
Mascot Search Results: Peptide View

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NCBI BLAST search of ATPSRPGEAAVPTAPASVSSASSSSTLECLYK
(Parameters: blastp, nr protein database, expect=20000, no filter, PAM30)
Other BLAST web gateways

Mascot: http://www.matrixscience.com/
Supplementary data 3
Mascot Peptide view of methylated peptide
ATPSRPGEAAVTAAPASAVSSASSSGSTLECILYK_{464} (Me)AEGAPPQGPFAPPCK identified in its 4+ charge state with an ion score of 68 and expect value of 0.00074. PR was isolated from T47D cells treated with 100 nM Progesterone. The y and b ions of the peptide detected by LC-MS/MS are indicated in red. Manual inspection of b² ions flanking K464 of the peptide confirms that the site of methylation is at K464.
Mascot Search Results

Peptide View

MS/MS Fragmentation of **ATPSRPGEAAVTAAPASASVSSASSGTLCELYKAEGAPPQGPFAPP**

Found in sp|P06401|PRGR_HUMAN, Progesterone receptor OS=Homo sapiens GN=PGR PE=1 SV=4

Match to Query 5083: 5224.538336 from(1307.141860.4+) intensity(8810.8643) scans(4079) rinseconds(2097) index(2987)

Title: File1482 Spectrum3026 scans: 4079

Data file X:\Hwa\Hwa\MascotResult\20111116_VaLin_PRdata\RAW2mgf6000\111116_VaLin_PCPR_04.mgf

Click mouse within plot area to zoom in by factor of two about that point
Or, Plot from 300 to 2000 Da Full range

Label all possible matches □ Label matches used for scoring □

Monoisotopic mass of neutral peptide Mr(calc): 5224.5445

**Fixed modifications:** Carbamidomethyl (C) (apply to specified residues or termini only)

**Variable modifications:**

K36 : Methyl1kr (KR)

**Ions Score:** 68 **Expect:** 0.00074

**Matches:** 84/584 fragment ions using 133 most intense peaks (help)

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NCBI BLAST search of ATPSRPGEAAVTAAPASVSSASSGHTLUCYKAEAPPQQGPFAPPCPK
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Other BLAST web gateways

All matches to this query
| Mascot | http://www.matrixscience.com/ |
Supplemental Data 4. Ligand does not regulate the level of K464 methylation.
Quantitation of K464 methylation by LC-MS/MS analysis of 3 independent experiments. Each 100 mm dish of COS7 cells was transfected with 1 µg of pcDNA3.1 Flag-PR and was treated with either vehicle (0.1% EtOH) or 10 nM R5020 for 1 hr before harvesting for immunoprecipitation. Equal amounts of protein from the vehicle control or R5020 treated samples were utilized for Flag-PR immunoprecipitation by anti-Flag M2 affinity gel. Protein bands corresponding to PRB and PRA were excised and analyzed by LC-MS/MS. The XIC of the unmodified and modified ATPSRPGEAAVTAAPASVSSASSSGSTLECILYK peptides were extracted within 5ppm at the corresponding monoisotopic peaks. Ratio of the area of XIC of the unmodified peptide and the K464 monomethylated peptide was calculated for each data set. Effect of ligand on K464 methylation is indicated by the fraction of methylated K464 of the R5020 treated samples over the fraction of methylated K464 of untreated samples. Data shown is the average of 3 independent experiments (mean ± SEM)
Supplemental Data 4. Ligand does not regulate the level of K464 methylation. Quantitation of K464 methylation by LC-MS/MS analysis of 3 independent experiments. Each 100 mm dish of COS7 cells was transfected with 1 µg of pcDNA3.1 Flag-PR and was treated with either vehicle (0.1% EtOH) or 10 nM R5020 for 1 hr before harvesting for immunoprecipitation. Equal amounts of protein from the vehicle control or R5020 treated samples were utilized for Flag-PR immunoprecipitation by anti-Flag M2 affinity gel. Protein bands corresponding to PRB and PRA were excised and analyzed by LC-MS/MS. The XIC of the unmodified and modified ATPSRPGEAAVTAAPASASVSSASSSGSTLECILYK peptides were extracted within 5ppm at the corresponding monoisotopic peaks. Ratio of the area of XIC of the unmodified peptide and the K464 monomethylated peptide was calculated for each data set. Effect of ligand on K464 methylation is indicated by the fraction of methylated K464 of the R5020 treated samples over the fraction of methylated K464 of untreated samples. Data shown is the average of 3 independent experiments (mean ± SEM).

Supplemental Data 5. K464Q mutation heightens PRA activity. (A) Protein expression of wt-PRA and PRA-K464Q mutants are similar with β-actin as loading control. (B) PRA-K464Q mutant displays higher ligand-dependent transcriptional activity. HeLa cells were transfected with either 5 ng of wt-PRA and PRA-K464Q expression vector vector, in addition to the fixed amounts of PRE2-TATA-luciferase (1.5 µg) and Renilla (1 ng) reporter plasmids. Cells were then treated with either vehicle control (0.1% EtOH) or 10 nM R5020 for 12 hr. PRE-driven luciferase activity was normalized to Renilla and expressed as the average relative light units (RLU) of triplicate readings (±SEM). Fold induction by R5020 was calculated as the ratio of the relative luciferase activity (RLU) of R5020-treated samples divided by the RLU of corresponding EtOH-treated samples. Asterisks denote statistical significance (****, P<0.0001) determined by unpaired Student’s t test.