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2019

Woo, A. R. E., Sze, S. K., Chung, H. H., & Lin, V. C. L. (2019). Delineation of critical amino acids in activation function 1 of progesterone receptor for recruitment of transcription coregulators. *Biochimica et Biophysica Acta (BBA) - Gene Regulatory Mechanisms*, 1862(4), 522-533. doi:10.1016/j.bbagr.2019.01.004

<https://hdl.handle.net/10356/143287>

<https://doi.org/10.1016/j.bbagr.2019.01.004>

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Delineation of critical amino acids in activation function 1 of progesterone receptor for recruitment of transcription coregulators

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Running Title: Positively charged amino acids in activation function 1 are critical for the activity of PR

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Keywords: Positively charged residues; progesterone receptor; activation function 1, transcriptional activity, coregulators

Abstract

The activation functions AF1 and AF2 of nuclear receptors mediate the recruitment of coregulators in gene regulation. AF1 is mapped to the highly variable and intrinsically unstructured N terminal domain and AF2 lies in the conserved ligand binding domain. The unstructured nature of AF1 offers structural plasticity and hence functional versatility in gene regulation. However, little is known about the key functional residues of AF1 that mediates its interaction with coregulators. This study focuses on the progesterone receptor (PR) and reports the identification of K464, K481 and R492 (KKR) as the key functional residues of PR AF1. The KKR are monomethylated and function cooperatively. The combined mutations of KKR to QQQ render PR isoform B (PRB) hyperactive, whereas KKR to FFF mutations abolishes as much as 80% of PR activity. Furthermore, the hyperactive QQQ mutation rescues the loss of PR activity due to E911A mutation in AF2. The study also finds that the magnitudes of the mutational effect differ in different cell types as a result of differential effects on the functional interaction with coregulators. Furthermore, KKR provides the interface for AF1 to physically interact with p300 and SRC-1, and with AF2 at E911. Intriguingly, the inactive FFF mutant interacts strikingly stronger with both SRC-1 and AF2 than wt PRB. We propose a tripartite model to describe the dynamic interactions between AF1, AF2 and SRC-1 with KKR of AF1 and E911 of AF2 as the interface. An overly stable interaction would hamper the dynamics of disassembly of the receptor complex.

Abbreviations: PR, Progesterone Receptor; Estrogen receptor α (ER α); AF, Activation Function, SRC, Steroid Receptor Coactivator; NTD, Amino terminal domain; LBD, Ligand Binding Domain; CBP, CREB binding protein; NCoR1, Nuclear receptor corepressor; SMRT, silencing mediator of retinoic acid and thyroid hormone receptor; wt, wild-type, hLBD, hinge-LBD; EtOH, Ethanol; R5020, promegestone; co-IP, co-immunoprecipitation; Progesterone Receptor Response element, PRE; Luciferase, Luc.

1 Introduction

The progesterone receptor (PR) is a member of the nuclear receptor (NR) superfamily. It is critically involved in various reproductive processes including ovulation, implantation and maintenance of pregnancy [1, 2]. It is also essential for mammary side branching and alveolar development [3, 4]. PR exists in two isoforms that are transcribed from alternative promoters with PRB being 164 aa longer than PRA at the N-terminal domain (NTD) [5]. Like other NRs, PR activity is primarily mediated by Activation Functions AF1 and AF2. The core AF1 is mapped to aa 456 – 555 in the highly variable and intrinsically unstructured NTD, whereas the AF2 lies in the highly structured ligand binding domain (LBD). In addition, PRB contains a third activation function (AF3) at its N terminus [6]. AF1 was originally reported for its hormone-independent activity when the deletion of the LBD resulted in a constitutively active protein in reporter gene assay [7, 8]. It is now well established that AF1 also synergizes with AF-2 for the ligand-dependent activity of steroid receptors. Agonist-induced interaction between the AF1-containing NTD and AF2-containing LBD is involved in the functional synergy between the two activation functions [9].

Transcription coregulators are general mediators between NRs and the general transcription machinery [10]. Since the discovery of steroid receptor coactivator-1 (SRC-1) in a yeast two-hybrid screening [11], more than 400 coregulators have been reported. They can function as coactivators that are generally recruited by agonist-bound NRs or as corepressors that can be recruited by antagonist-bound NRs [12] [13]. The p160 family of steroid receptor coactivators (SRC-1, SRC-2, and SRC-3) is the most studied. The ligand-induced recruitment of SRCs would in turn recruit chromatin remodeling enzymes such as p300, CREB binding protein (CBP) and coactivator-associated arginine methyltransferase (CARM1) that facilitate chromatin accessibility by the general transcription machinery [14]. Nuclear receptor corepressor (NCoR1) and silencing mediator of retinoic acid and thyroid hormone receptor (SMRT) are among the best-studied NR corepressors. They were initially identified based on their suppressive effect on target genes of the retinoic acid receptor and thyroid receptor [15, 16]. They are also recruited to NRs upon antagonist binding and repress gene transcription through their histone deacetylase activity or by recruiting histone deacetylases such as HDAC3 which induces a closed chromatin conformation [17]. NCoR1/SMRT can also physically interact with components of the general transcriptional machinery, such as transcription factor IIB (TFIIB) to block them in a non-functional conformation [18, 19]. Both coactivators and corepressors play key roles in gene regulation by NRs.

It is well established that coregulators are recruited by AF2 of NRs in response to ligand binding. This is putatively mediated through the signature LXXLL motif known as NR box on coregulators [20, 21]. The coactivator interaction surface of LBD is believed to form a hydrophobic pocket consisting of residues from helix 3 and 12 [22, 23]. Coregulators can also interact directly with AF1. SRC-1 has been reported to interact with the AF1 regions of the androgen receptor (AR) via the glutamine-rich region to promote gene activation [24]. CBP/p300 has also been reported to interact with AF1 of AR, glucocorticoid receptor (GR) and estrogen receptor α (ER α) [25-28]. Studies of the cryo-EM structure of the ER/SRC-3/p300 complex have indicated that AF1 of ER α is positioned adjacent to AF2 [29]. It was suggested that AF1 is involved in the ligand-induced recruitment of SRC3 which in turn binds to p300. However, there is generally a lack of knowledge about the contact points of AF1 with transcription coregulators.

The intrinsically unstructured nature of AF1 can conceivably render it the plasticity to form a variety of interfaces depending on the context, which in turn offers functional versatility in response to ligand [30, 31]. This is believed to be the molecular basis for the gene and cell-specific activity of steroid receptors [32-34]. Studies of mice with AF1 domain deletion (aa 2 - 148) in (ER α) have led to the understanding of tissue-specific involvement of AF1 in several physiological processes. For example, AF1 is required for the development of trabecular bone and uterus but not cortical bone and

the growth plate closure [35, 36]. The activity of ER α AF1 is also ligand-selective. Although the protective effect of 17 β -estradiol against obesity and insulin resistance is AF1-independent, the protective effect conferred by tamoxifen is mediated by the selective activation of ER α -AF1 [37]. It is unclear how AF1 exerts these tissue-selective effects. Genetic models with functional point mutations would thus be more useful for gaining insight into the molecular mechanism of AF1 activity.

PR is also known to exert cell context-dependent effects. It promotes the proliferation of mammary luminal cells through paracrine signaling but suppresses estrogen-induced endometrial cell proliferation via stromal-epithelial crosstalk [38, 39]. The effects of progesterone and progestins on breast cancer are also known to be dependent on the clinical and cellular context [40-52]. For example, clinical trials of hormone replacement therapy in post-menopausal women concluded that the use of progestin increases the risk of breast cancer [47, 48], but progestins have been used in the past as a second-line endocrine therapy in advanced breast cancer with appreciable clinical benefit [50-52]. The role of AF1 in these context-dependent activities of PR is not clear. It has been reported that PR AF1 and AF2 interact with multiple regions in SRC-1 [53]. It has also been shown that the aa 468 – 508 of PRB AF1 were necessary for its interaction with the receptor interaction domain of NCoR1/SMRT [54]. However, the specific residues involved in the interaction with coregulators in general are not known. The objective of this study was to identify the critical functional residues in PR AF1 so as to establish functional mutants as genetic tools for a clear elucidation of AF1 function. The study has identified K464, K481, and R492 as the critical AF1 residues that function cooperatively. These residues form the interaction interface with SRC-1, and AF2 and p300. These functional mutants provide excellent genetic tools for the characterization of the promoter- and tissue-specific function of AF1 *in vivo*.

2 Materials and Methods

2.1 Cell culture and hormone treatment

HeLa, MDA-MB-231, MCF7, T47DC42 (from Dr Carol A. Lange, University of Minnesota, USA), and COS7 were routinely maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing phenol red (Nacalai Tesque, Japan), supplemented with 7.5% fetal bovine serum (FBS) (Hyclone, South Logan, Utah, USA) and 2mM L-glutamine (Hyclone). The cells were cultured at 37°C in a humidified 5% CO₂-95% air. For hormone treatment, the cells were plated in phenol-red free DMEM supplemented with 5% dextran-charcoal-treated FBS (DCC-FBS) and 2mM L-glutamine for 48h prior to treatment with progesterone (P8783, Sigma-Aldrich) or promegestone (R5020) (PerkinElmer) in 0.01% ethanol. Control cells received 0.01% ethanol only.

2.2 Plasmids and site-directed mutagenesis

pCR3.1-SRC-1, PRE2-TATA-Firefly Luciferase reporter and Renilla pRL-CMV plasmids were kindly provided by Dr. Ming-Jer Tsai (Baylor College of Medicine, Houston, Texas, USA). pCR3.1-Flag-JDP2, VP16-vector, VP16-BN (1-550 aa of PRB)(annotated as VP16-NTD), GAL4-RE-LUC, GAL4-DBD, GAL4-hLBD and GAL4-SRC-1 were kindly provided by Dr Dean Edwards (Baylor College of Medicine, Houston, Texas, USA) [9]. The plasmids pSG5-Myc-NCoR1 and pSG5-Myc-SMRT were kindly provided by Dr. Martin L. Privalsky [55]. Flag-p300 plasmid was kindly provided by Dr Lorenzo Puri (Dulbecco Telethon Institute, Rome) [56]. HA-CBP vector was kindly provided by Dr Mark Featherstone (Nanyang Technological University, Singapore).

Point mutations of lysine (K), arginine (R) or glutamic acid (E) residues in PRB – K464, K481, R492, K531, R546 and E911, to glutamine (Q), phenylalanine (F) or alanine (A) were generated via site-directed mutagenesis polymerase chain reaction (PCR) using Pfu Turbo Polymerase (Thermo Scientific, as per manufacturer's protocol). Generation of multiple mutations, sequential site-directed mutagenesis was performed. The site-directed primers were designed using the web-based program,

QuikChange® Primer Design. The sequences of the sense and antisense primers for K464Q and K464F are as described [57]. The sense and antisense primers for other mutants are the following:

K481Q_sense 5'-gccgccctgccaggcgccggg-3';
K481Q_antisense 5'-cccggcgctggcagggcggc-3';
K481F_sense 5'-cgccgccctgcttcgcccggcg-3';
K481F_antisense 5'-gcgcccggcggaagcagggcggc-3';
R492Q_sense 5'-cctgtcccgcaggacggcctgc-3';
R492Q_antisense 5'-gcaggccgtcctgcgggagcagg-3';
R492F_sense 5'-gctgcctgctcccgttcgacggcctgcct-3';
R492F_antisense 5'-gagggcaggccgtcgaacgggagcaggcagc-3';
K531Q_sense 5'-ccgccgtgctccaggagggcctg-3';
K531Q_antisense 5'-cagggcctcctggagcacggcgg-3';
R546Q_sense 5'-ccctatctcaactacctgcagccggattcagaagccag-3';
R546Q_antisense 5'-ctggcttctgaatccggatgcaggtagttgagataggg-3';
E911A_sense 5'-gaattccagaaatgatgtctgcagttattgctgcacaattacc-3';
E911A_antisense 5'-ggtaattgtgcagcaataactgcagacatcatttctggaaattc-3'.

Site-directed mutagenesis were conducted using pcDNA3.1 expression vector containing human PRB cDNA (wild-type, K464Q and K464F), flag-tagged PRB cDNA, VP16-BN (1-550 residues of PRB, annotated as VP16-NTD) and GAL4-hLBD [9]. Flag-NTD (aa 1-550 + nuclear localization signal (NLS)) was cloned from pcDNA3.1-Flag-PRB (wild-type and triple AF1 mutants) expression vectors using cloning primers:

PRB_Flag_Kpn1_sense 5'-ccgggtaccgccaccatggactacaaggacgacgatgataagactgagctgaaggcaaagggt-3';
PR_AF1-NLS_Xbal-antisense 5'-ccgtctagatcagactttattgaacttttaatttcgtgggctctggctggct-3'.

To generate AF1 mutants, site-directed mutagenesis was performed on the derived cloned product. All derived mutations were verified by full-length cDNA sequencing.

2.3 PR immunoprecipitation from T47D breast cancer cells

To screen for post translational modifications of PR by mass spectrometry, cell lysates from T47D breast cancer cells were prepared using ice-cold lysis buffer (described in detail under *Protein Lysate Collection and Western blotting*). Endogenous PR in the cell lysate was immunoprecipitated using anti-PR antibody (sc-7208, Santa Cruz Biotechnology Inc.) that was pre-conjugated to AG Agarose beads (sc-2003, Santa Cruz Biotechnology Inc.). The bound PR were resolved via SDS-PAGE, fixed and stained by Coomassie Blue G-250 (Bio-Rad).

2.4 In-gel Tryptic Digestion

Gel strips of PR bands were excised after Coomassie Blue staining. The strips were diced into small pieces and washed with Milli-Q water. The gel pieces were then de-stained with 25mM NH_4HCO_3 in 50% ACN (Acetonitrile) until colorless. The de-stained gel pieces were dehydrated with 100% ACN until they appeared white. They were then reduced with 10mM DTT for 1h at 56°C, and alkylated in the dark for 45min with 55mM Iodoacetamide (IAA) before being washed with 25mM NH_4HCO_3 , followed by washes with 25mM NH_4HCO_3 in 50% ACN. The gel pieces were then dehydrated with 100% ACN and dried completely using speed vacuum concentrator. The dehydrated gel pieces were treated with Trypsin (Trypsin Gold, Mass Spectrometry Grade, Promega, Madison, WI) in a weight ratio of 1:30. Upon complete absorption of trypsin by the gel particles, 25mM NH_4HCO_3 was added to completely submerge the particles and incubated at 37°C overnight. The digested peptides were then eluted from the gel pieces by sonicating the pieces twice for 20min in 50% ACN containing 0.1% formic acid. The eluted peptides from each elution process were combined, and dried in speed vacuum concentrator.

2.5 LC-MS/MS and Data analysis

To detect PR methylation, the 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) as described in detail [57]. The LTQ-FT system was used to perform data acquisition in the positive ion mode using an m/z range of 350-1600 in the full MS scan. The MS/MS spectra in the raw data was extracted into the dta format using `extract_msn` (version 4.0) in Bioworks Browser (version 3.3, Thermo Fisher Scientific, Inc.), and converted into the Mascot generic file formatting using an in-house program. The intensity values and fragment ion m/z ratios were not manipulated. Database searches using an in-house Mascot server (version 2.2.07, Matrix Science, London, UK) were conducted with reference to the IPI human protein databased (version 3.34, 67,758 sequences, 28,836,807 residues) with MS tolerance of 10ppm, and MS/MS tolerance of 0.8 Da [57].

2.6 Protein lysate collection and western blotting

Cells were lysed with ice-cold lysis buffer (50mM HEPES, 150mM NaCl, 1% Triton X-100, 5 μ g/mL pepstatin A, 5 μ g/mL leupeptin, 2 μ g/mL aprotinin, 1mM PMSF, 100mM sodium fluoride and 1mM sodium vanadate) (Sigma-Aldrich). The lysate was subsequently homogenized using 29-Gauge syringes and centrifuged down at 13,800 rpm (20,227 rcf) for 12min at 4 $^{\circ}$ C. The concentrations of the supernatants were quantitated using BCATM Protein Assay Kit (Pierce, Rockford, IL, USA), as per manufacturer's protocol. The protein samples were boiled at 100 $^{\circ}$ C for 8min in Laemmli sample buffer (50mM Tris-Cl pH 6.8, 2% sodium dodecyl sulphate (SDS), 10% glycerol, 1% 2-mercaptoethanol, 0.002% bromophenol blue). Equal amounts of proteins were loaded into 5% or 8% SDS-polyacrylamide gel. The protein samples were resolved via SDS-polyacrylamide gel electrophoresis (SDS-PAGE) before transferring onto a nitrocellulose membrane (Bio-Rad Laboratories). The membranes were blocked in 5% non-fat skimmed milk in TBST before incubation with the primary antibodies overnight in 4 $^{\circ}$ C with gentle agitation. Total PR expression was detected using anti-PR (sc-7208, Santa Cruz Biotechnology Inc.). Anti GAPDH (Ambion), anti- β -actin (sc-47778, Santa Cruz Biotechnology Inc.) and anti- β -tubulin (M2008, SABio) antibodies were used to probe GAPDH, β -actin and β -tubulin respectively for loading control. The secondary anti-mouse and anti-rabbit antibodies (GE Healthcare) were used in dilutions 1:2000 and 1:3000 respectively. Detection of the signals was conducted using Immobilon Western HRP Substrate (Merck Millipore) as per manufacturer's protocol. The chemiluminescence signals were then exposed to X-ray films (Eastman Kodak Co., New Haven, CT). Densitometry readings were quantified using Quantity One (Bio-Rad).

2.7 Luciferase Reporter Assay

HeLa, MDA-MB-231, MCF7 or T47DC42 cells were seeded onto 60 mm dishes in phenol-red free DMEM supplemented with 5% DCC-FBS and 2mM L-glutamine. The cells were transfected with 5ng pcDNA3.1/pcDNA3.1-PRB (wild-type or AF1 and/or AF2 mutants), 1 μ g PRE-2X-TATA-LUC and 0.5ng Renilla vector pRL-CMV, with or without 20ng of coregulators, using Polyethylenimine (PEI) transfection reagent (Polysciences). After 24h, the cells were treated with progesterone or R5020 in 0.01% ethanol for 16h. The cells were collected with 1X passive lysis buffer provided in Dual-Luciferase Reporter System (E1960, Promega) and the lysate was analyzed using a computer-controlled microplate luminometer, GloMax [®]-Multi+ Microplate Multimode Reader with Instinct[®] (Promega). The activities of the Renilla and Firefly luciferase were measured as per manufacturer's protocol. Data were generally plotted as R5020-induced luciferase units, which are obtained as the ratio of the RLUs of the samples treated with R5020 divided by those obtained in the ethanol-treated samples; unless indicated otherwise in figure legends.

2.8 Co-immunoprecipitation (Co-IP)

HeLa cells were transfected with 1 μ g pcDNA3.1-PRB (wild-type, AF1 mutants or PR NTD and the mutants), 4 μ g pCI-Flag-p300, or 2 μ g pCR3.1-SRC-1 in different experiment. The transfected cells were treated with 10 nM R5020 in 0.01% ethanol prior to the collection of cell lysate. The supernatant after centrifugation was subjected to immunoprecipitation. Anti-Flag[®] M2 Affinity Gel (A2220, Sigma-

Aldrich) were used to pull-down Flag-p300 with PR, Flag-PR with SRC-1, or Flag-NTD with SRC-1. The immunoprecipitated proteins were resolved by SDS-PAGE and detected using anti-PR antibody (sc-7208, Santa Cruz Biotechnology Inc.), anti-p300 antibody (sc-585, Santa Cruz Biotechnology Inc.), or anti-SRC-1 antibody (#2191, Cell Signaling Technologies). Data presented is a representative IP immunoblot of 3 independent experiments. Densitometry readings of detected signals were quantified and analyzed using Quantity One® 1-D Analysis Software (Bio-Rad).

2.9 In Vivo Acetylation Assay

The *in vivo* acetylation assay was carried out as described in [58]. COS7 cells were transfected with 1 µg pcDNA3.1-PRB (wild-type or AF1 mutants) together with 4 µg pCI-Flag-p300. The transfected cells were exposed to 10 µM Trichostatin A (TSA) for 30 min before the treatment with 10 nM R5020 in 0.1% Ethanol for 1 h. The cell lysate was collected for PR immunoprecipitation by anti-PR antibody (sc-7208, Santa Cruz Biotechnology Inc.) that was conjugated to AG Agarose beads (sc-2003, Santa Cruz Biotechnology Inc.). The captured PR was resolved by SDS-PAGE. Acetylated PR was detected using a pan-anti-acetylated lysine antibody (#9441, Cell Signaling Technologies). Levels of p300 and PR were validated using anti-p300 and anti-PR antibodies (sc-585 and sc-7208, Santa Cruz Biotechnology Inc.). Data presented is a representative IP immunoblot of 3 independent experiments.

2.10 Mammalian-Two-Hybrid Interaction Assay

Mammalian two-hybrid assays were performed according to the published protocol with minor modifications [9]. COS7 cells were transfected with 500 ng VP16-vector/NTD (wt/QQQ/FFF) and 500 ng GAL4-vector/hLBD/hLBD-E911A/SRC-1, together with 500 ng reporter plasmid GAL4-RE-LUC and 0.5 ng Renilla. The transfected cells were treated with 10 nM R5020 in 0.01% ethanol for 48 h before lysis with 1X Passive lysis buffer provided by Dual Luciferase Reporter System (Promega) as described above (*Luciferase reporter assay*). The activities of the Renilla and GAL4-activities were measured as per manufacturer's protocol. Experiments were done in either duplicates or triplicates with at least 3 independent experiments. Unless specified otherwise, the data were generally plotted as the ratio of the RLUs of the samples treated with R5020 divided by those obtained in the ethanol-treated samples.

2.11 Statistical Analysis

Data obtained were expressed as means ± SEM (standard error mean). Statistical analyses of the data were determined using one-way or two-way ANOVA, followed by post-hoc Tukey test with 95% confidence interval using the program GraphPad Prism 6. For all tests, difference was considered significant if the p-value < 0.05. Degree of statistical significance is indicated with asterisks *, p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001. Data not statistically significant is indicated as *n.s.* unless indicated otherwise.

3 Results and Discussion

3.1 AF1 is monomethylated at K481 and R492

The core AF1 of PR is localized to aa 456 - 555 and contains five positively charged residues, K464, K481, K531, R492 and R546. We have previously reported that AF1 is monomethylated at K464 [59]. The K464Q mutation enhanced PR activity by 2 - 3 times, whereas the K464F mutation, which increases local hydrophobicity and bulkiness [60, 61], impaired it by 50%. To establish functional mutants with a greater gain or loss of function for functional and mechanistic studies of AF1, additional screening of post-translational modifications in AF1 was conducted. The tandem mass spectrometry (MS/MS) data was collected from liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) analysis of endogenously expressed PRA and PRB from breast cancer cells T47D. The MS/MS spectra were matched to proteins in the Uniprot human protein database using an in-house Mascot database search engine. Two putative monomethylation sites at K481 and R492 were identified in PR. The Mascot annotated MS/MS spectra of the methylated peptides are shown in [Supplementary Data 1](#). The methylation sites at K481 and R492 of PR were consistently identified with high confidence (peptide expect value < 0.05 and peptide score > identity score). Monomethylation at K481 of PR was identified in multiple tryptic peptides: AEGAPPQQGPFAPPPCK^{Me} (Mascot pep score: 79.2, expect value: 4.8E-6), AEGAPPQQGPFAPPPCK^{Me}APGASGCLLP (Mascot pep score: 53.2, expect value: 0.0022) and AEGAPPQQGPFAPPPCK^{Me}APGASGCLLP^{Me} (Mascot pep score: 60.1, expect value: 4.8E-4). Similarly, R492 monomethylation was also identified in two PR peptides, AEGAPPQQGPFAPPPCKmeAPGASGCLLP^{Me} (Mascot pep score: 60.1, expect value: 4.8E-4) and APGASGCLLP^{Me} (Mascot pep score: 38.0, expect value: 0.049). Figure 1 shows the three pairs of annotated MS/MS spectra of unmodified and monomethylated peptides. Each precursor ion was isolated in LTQ-FTMS and fragmented by collisionally activated dissociation (CAD) to generate the product b- and y-ions. The fragmentation patterns of the unmodified and monomethylated peptides are similar except that the b- and y-ions containing the modification sites were shifted by +14Da. In addition, both K481^{Me} and R492^{Me} were identified in the peptide AEGAPPQQGPFAPPPCKMeAPGASGCLLP (Figure 1C and 1D). In both spectra, K481^{Me} was sandwiched by a series of b- and y-ions. Together with the annotated spectrum shown in Figure 1B, the K481^{Me} has been unambiguously determined. R492^{Me} was identified at the C-terminal R residue in the spectra shown in Figure 1D and 1F. All y-ion fragments were shifted by +14Da. Moreover, the y2-ion in Figure 1D has a 286.19 m/z, which indicates a methylation modification in the PR fragment. As P cannot be modified by methylation, the methylation site is positively assigned to the terminal residue R492.

To confirm the methylation sites, we performed an analysis to determine the peptide retention time in C18 reverse phase liquid chromatography (RPLC). The extracted ion chromatogram (XIC) of the unmodified peptide AEGAPPQQGPFAPPPCK with double charges at 874.925 m/z was integrated within +/-5ppm at the monoisotopic peak. The corresponding modified peptide AEGAPPQQGPFAPPPCK^{Me} at 881.933 m/z was also integrated. The two isotopic clusters corresponding to the two peptides are identified at 20.14 and 21.21 mins in the C18 reverse phase chromatography ([Supplementary Figure 1](#)). As the monomethylated peptide is slightly more hydrophobic than the unmodified peptide, this retention time shift of the modified peptides further confirms the monomethylation at K481. Similarly, the XICs of another pair of R492 containing peptides APGASGCLLP (549.790 m/z) and APGASGCLLP^{me} (556.798 m/z) were analyzed by the same method. We consistently observed that the unmodified peptide was eluted at 17.65 min and the methylated peptide at 18.59 min ([Supplementary Figure 1](#)), indicating longer retention time for the more hydrophobic methylated peptides in the C18 RPLC. Together, the MS data confirm the methylation modification at the K481 and R492 residues in the AF1 domain.

Figure 1. Three pairs of annotated MS/MS spectra of unmodified and monomethylated peptides. Each precursor ion was isolated in a LTQ-FTMS and fragmented by collisionally activated dissociation (CAD) to generate the product b- and y-ions. The fragmentation patterns of the unmodified and monomethylated peptides are similar except that the b- and y-ions containing the modification sites were shifted by +14Da. (A) The annotated MS/MS spectrum of unmodified peptide AEGAPPQQGPFAPPPCK. (B) The annotated MS/MS spectrum of the methylated peptide AEGAPPQQGPFAPPPCK^{Me}, in which the K481^{Me} was detected at the C-terminal. The m/z of the whole series of y-ions was shifted by +14Da, corresponding to the mass shift of monomethylation. (C) The unmodified peptide AEGAPPQQGPFAPPPCKAPGASGCLLP was positively identified by this annotated MS/MS spectrum. (D) Both K481^{Me} and R492^{Me} are identified in this spectrum. Together with the annotated spectrum showed in B, the K481^{Me} can be confirmed with high confidence. (E) The annotated MS/MS spectrum shows the identification of the unmodified peptide APGASGCLLP. (F) The corresponding monomethylated APGASGCLLP^{Me} peptide was identified at the C-terminal of the peptide. The R492^{Me} was confirmed by the detection of the same modification site in both D and F.

3.2 Positively charged amino acids of AF1 are involved in the ligand-induced PR activity in PRE-luciferase assay

The evidence of mono-methylation in 3 out of the 5 positive residues suggests that the positively charged residues are potentially important for AF1 activity. To verify this, each of the 5 positive residues was mutated to the neutral polar glutamine (Q) in PRB. The side-chain conformation

entropy of Q is similar to that of lysine [62]. As such, the substitution would allow us to evaluate the relevance of these positive amino acids in AF1 activity. Interestingly, while the expression levels of the mutants were similar in transient transfection assays (Figure 2A), all mutants except for K531 showed twice as much activity as the wt PRB in response to 10 nM progestin R5020 in PRE-luciferase (Luc) assay (Figure 2B). To address whether these positively charged amino acids function cooperatively to modulate AF1 activity, various combinations of mutations were tested. The 3 monomethylated residues (464, K481 and R492) were found to display the most cooperative activity. Notably, the combinations of K464Q/K481Q and K464Q/R492Q mutations had a significant additive effect on ligand-induced PRB activity compared to each of the single mutation, whereas the activity of the K481Q/R492Q mutant was similar to the single mutant's activity (Figure 2C). However, the triple mutation K464Q/K481Q/R492Q enhanced PRB activity by a further 30% compared to the double mutation K464Q/K481Q. The fact that the QQQ mutation enhances PR activity suggests that the positive charge is not favorable for transcription efficiency.

Since the monomethylation of lysine or arginine increases local bulkiness and hydrophobicity, phenylalanine (F) has been used as a methylation mimic [59-61]. Although the single mutation K481F, R492F or in combination had no effect, double mutant K464F/K481F showed a greater repressive effect on PR activity by ~30% compared to K464F (Figure 2D). The activity of the triple mutant K464F/K481F/R492F (FFF) was further reduced such that it has only 20% of the activity of the wt PRB. It can be concluded that K464, K481 and R492 function cooperatively. The overall increase in the hydrophobicity and bulkiness in PR AF1 due to methylation likely disrupted its gene regulatory activity but the removal of positive charges by KKR-QQQ mutation enhanced the activity of PRB.

The effect of the AF1 mutations described above was tested in response to 10 nM synthetic progestin R5020. We next analyzed the responses to different concentrations of the natural hormone, progesterone. It is evident from the dose response curve that the activity of QQQ mutant is on average 4 times more than that of the wt PRB at each concentration tested (Figure 2E). In contrast, the ligand response of the FFF mutant was significantly attenuated, with its activity being 4 times lower than wt PRB at each of the concentrations tested. In addition, the R5020-induced QQQ activity followed an evidently faster kinetics than the wt PR and FFF mutant; the activity of QQQ mutant reached 20 RLU, 3-times of wt PR activity in the first hour of R5020 treatment and its activity increased much more steeply in the next 15 hours than that of the wt PRB and FFF (Figure 2F).

Figure 2. The cooperative action of the positively charged residues K464, K481 and R492 are important for AF1 function. The transcriptional activity of wt PRB or various mutants were measured by PRE-Luc assay in HeLa cells with or without treatment of 10 nM R5020. (A) *The expression levels of PRB, K464Q, K481Q, R492Q, K531Q and R546Q were similar.* (B) *Mutation of lysine or arginine residues, except K531, to glutamine AF1 increased the transcriptional activity of PRB.* (C) *Combined mutation of K464, K481, and R492 to glutamine increased the transcriptional activity of PRB cooperatively.* (D) *Combined mutation of K464, K481, and R492 to phenylalanine attenuated the transcriptional activity of PRB cooperatively.* Data in B, C and D were plotted as percentage R5020-induced activity against PRB (mean $\pm$ SEM, 2 independent experiments, triplicates). (E) *Transcriptional activity of PRB and AF1 mutants in response to various doses of progesterone (Prog). QQQ exhibits increased ligand sensitivity.* Data were plotted as relative luciferase units (RLUs) against empty vector (pcDNA3.1) control at 0 nM progesterone (mean $\pm$ SEM, representative of 2 independent experiments, triplicates). Data obtained is statistically significant ($p < 0.001$) by two-way ANOVA. (F) *QQQ responds faster to R5020.* Time point PRE-transactivation assay of PRB and AF1 mutants. Data were plotted as relative luciferase units against pcDNA3.1 at 0min (mean $\pm$ SEM, 2 independent experiments, triplicates). Data obtained is statistically significant ($p < 0.0001$) by two-way ANOVA.

3.3 KKR of AF1 is not involved in PRB regulation of transcriptional activity of NFκB and AP1

In addition to regulating gene expression through its interaction with cognate gene enhancer sequences known as the progesterone response element (PRE), PR also regulates gene transcription through an indirect effect by tethering to other transcriptional factors such as activating protein 1 (AP1), nuclear factor kappa-B (NFκB) and specificity protein 1 (SP1) [63-69]. The outcome is usually the repression of gene activation by these transcription factors and this mode of action has been implicated in the anti-inflammatory function of progesterone [64, 65]. Interestingly, neither QQQ nor FFF mutation had any effect on the ligand-induced transrepressive activity of PRB on NFκB or AP1 promoter (**Supplementary Figure 2**). This confirms that the AF1 exerts its influence specifically on the direct target genes of PR.

3.4 The magnitude of gain and loss of PR activity in AF1 mutants are cell type-dependent

Since AF1 action is known to be tissue context-dependent, we asked if these AF1 mutations influence PR activity differently in different cell lines. In addition to HeLa cells, we tested 3 breast cancer cell lines. MDA-MB-231 is a basal breast cancer cell line and is triple-negative for ER, PR and HER2; T47DC42 cells are a PR-negative subline of T47D cell line [70, 71]; MCF7 cells are ER- and PR-positive but the endogenous PR expression is dependent on estrogen stimulation and hence very low in steroid-deprived culture medium [72]. Consistently, the QQQ mutant was hyperactive and the FFF was hypoactive in all the cell lines tested. However, the mutants exhibited differences in the magnitude of gain and loss of the activities. In particular, the AF1 mutants showed the highest magnitude of gain and loss of activity in HeLa cells and the lowest in MDA-MB-231 cells (Figure 3A). These observations are consistent with the understanding that the AF1 activity is cell type-dependent.

The AF1 was originally discovered for its ligand-independent and cell type-dependent activity in the LBD-deleted receptor [8, 33]. Interestingly, the effect of these AF1 mutations on LBD-deleted PRB (PRBΔLBD) is very different from that of the full-length protein. In HeLa and MDA-MB-231 cells, FFFΔLBD is approximately 2 - 3 times more active than the activity of the wt PRBΔLBD, but QQQ activity is similar to or slightly higher than the wt PRBΔLBD (Figure 3C). In MCF-7 and T47D-C42 cells, however, the activity of FFFΔLBD is similar to the wt PRBΔLBD, and QQQΔLBD mutant exhibits 2.5 times the activity of wt PRBΔLBD. Collectively, the QQQ and FFF mutants behave differently in full length and in LBD deleted PRB, and in different cell types.

Figure 3. AF1 mutants display cellular specificity in PR activation. (A) QQQ exhibits variable transcriptional activity in different cell lines. PRE-transactivation assay of full-length PRB and AF1 mutants in HeLa, MDA-MB-231, T47DC42 and MCF7 in response to 10 nM R5020. Data were plotted as R5020-induced luciferase units (mean $\pm$ SEM, 3 independent experiments, triplicates). Data obtained are statistically significant ($p < 0.001$), unless indicated as *n.s.* ($p > 0.05$) by two-way ANOVA. (B) PR protein levels of PRBΔLBD, QQQLΔLBD and FFFΔLBD in HeLa cells was analyzed by western blotting using antibodies against PR and β -tubulin (loading control). (C) AF1 mutants exhibit distinctively different transactivation capacities as compared to full-length PR in different cell lines. PRE-transactivation assay of DLBD truncated PRB and AF1 mutants in (i) HeLa cells, (ii) MDA-MB-231, (iii) T47DC42 and (iv) MCF7 cells. Data were plotted as a relative PRE-luciferase (PRE-LUC) units against vector control (mean $\pm$ SEM, 2 independent experiments, triplicates).

3.5 The hyperactivity of AF1 mutant QQQ rescues the loss of AF2 function

The above data indicate that K464, K481 and R492 of PR AF1 cooperate to play a pivotal role in ligand-induced activity of PR. Despite of its reputation of being a ligand-independent transactivation domain, the QQQ and FFF mutations of the full-length PR had no detectable effect on the ligand-independent activity of PRB in all the cell lines tested (Supplementary Figure 3). Since AF1 and AF2 have been reported to function in synergy [73], we investigated whether a functional AF2 is required for the hyperactivity of the QQQ mutant. E911 is conserved among the steroid hormone receptors in the AF2 core domain [74]. It has been reported that the E911A mutation diminished PR activity without affecting ligand and DNA binding and coactivator recruitment [75, 76]. Whilst the PRB-E911A mutant lost ~80% of the activity in HeLa cells, QQQ mutation in E911A (QQQA) restored the activity of PRB to that of the wt PRB (Figure 4A). Likewise, QQQA exhibited similar levels of activity as the wt PRB in MDA-MB-231 cells (Figure 4B). On the other hand, there is an expected further loss of PR activity in E911A mutant with additional FFF mutations in both cell lines.

Transcription coregulators are important for ligand-induced NR activity through chromatin remodeling and assembly of the pre-initiation complex for gene transcription [77-80]. Since QQQ mutation rescued the loss of AF2 activity resulting from E911A mutation, we thought of using this model to identify AF1-specific coregulators involved in facilitating the hyperactive AF1. Surprisingly, SRC-1, SRC-2, CBP, and p300 were all able to increase the activity of E911A mutant and the effect of these coregulators on QQQ-E911A are all significantly and proportionally higher than that on E911A (Figure 4C). For example, the activity of E911A was increased by SRC-1 from ~50 to ~400 RLU (absolute increase of ~350 RLU), whereas the activity of E911A-QQQ was increased by SRC-1 from ~400 to ~1300 (an absolute increase of 900 RLU). Similarly, E911A activity was increased by SRC-2 from ~50 to ~1000 RLU (absolute increase of ~950 RLU). This is in contrast with an absolute increase of 1900 RLU in E911A-QQQ by SRC-2. Taken together, the results suggest that the hyperactivity of AF1 in QQQA is not due to an enhanced interaction with a specific AF1 coactivator. Instead, the hyperactive AF1 is achieved through greater functional cooperation with a range of coactivators (Figure 4C).

Figure 4. The hyperactive AF1 mutant QQQ can rescue the loss of AF2 activity due to E911A mutation in both HeLa and MDA-MB-231 cells. The transcriptional activity of wt PRB or various mutants were measured by PRE-Luc assay in response to 10 nM R5020. (A) QQQ mutant rescued the loss of PRB activity in the E911A mutant in (A) HeLa and (B) MDA-MB-231 cells. Data were plotted as percentage of R5020-induced activity as described in Figure 1A (mean $\pm$ SEM, representative of 3 independent experiments, each in triplicates). (C) The hyperactive AF1 recovered the loss of AF2 response to transcription coactivators SRC-1, SRC-2, p300 and CBP in HeLa cells (mean $\pm$ SEM, 3 independent experiments, duplicates). (D) QQQA mutant exhibited similar activation kinetics as the wt PRB in HeLa cells. Data were plotted as described in 1E (mean $\pm$ SEM, representative of 3 independent experiments, triplicates). Data presented (A-D) is statistically significant by one-way or two-way ANOVA, unless indicated as *n.s.* ($p > 0.05$),

The mechanism behind the impaired activity in the E911A mutant may shed light on how QQQ function to rescue of loss of AF2 activity. It has been shown that the E911A mutant is capable of ligand and DNA binding, and recruitment of coactivators such as SRC-1 and SRC-3 to the steroid

hormone responsive MMTV promoter [75, 76]. This is also supported by our data that the activity of E911A mutant increases in response to coactivator overexpression (Figure 4C). However, chromatin immunoprecipitation (ChIP) assay shows that the E911A mutant has impaired ability in driving a progressive RNA polymerase II on its target gene [76]. It is plausible that the hyperactive AF1 can rescue this defect of E911A. This is supported by the evidence that the activation kinetics of the QQQA is almost identical to wt PR. This is in contrast to the much slower rate of transactivation by E911A mutant (Figure 3D). We propose that PR AF1 facilitates the assembly of a functionally progressive RNA polymerase complex for efficient gene transcription.

3.6 AF1 mutants exhibit different levels of gain and loss of functional interaction with transcription coregulators in different cell types

Data in Figure 2A shows that AF1 mutants exhibit the least gain and loss of activity in MDA-MB-231 cells and the most in HeLa cells. Since the functional role of AF1 and AF2 is to mediate the recruitment of transcription coregulators [77-80], we hypothesized that the gain or loss of PR activity resulting from the AF1 mutation is due to the changes in its functional cooperation with transcription coregulators. Hence, the effect of AF1 mutation on its functional interaction with a panel of 4 coactivators (SRC-1, SRC-2, CBP, and p300) and 2 corepressors (NCoR1 and SMRT) were examined in HeLa and MDA-MB-231 cells. In HeLa cells, SRC-1, CBP and p300 enhanced the activity of QQQ more than that of wt PRB in terms of the absolute increase of RLU, and their co-activating effect on FFF mutant is very limited (Figure 5A). For example, SRC-1 enhanced the activity of PRB by ~500 RLU, of QQQ mutant by 1000 RLU, and of FFF mutant by ~100 RLU in HeLa cells (Figure 5A). Similarly, CBP and p300 also enhanced the activity of PRB and the AF1 mutants proportionally according to their original activities. In MDA-MB-231 cells, however, only CBP enhanced the activity of QQQ mutant (by ~540 RLUs) as compared to that without CBP. In contrast, CBP did not have any detectable effect on the activity of PRB and FFF mutant (Figure 5B). As for SRC-1, SRC-2 and p300, they all increased the activity of wt PRB, QQQ and FFF mutants similarly, suggesting that the AF1 mutations did not affect the function interaction with these coactivators in MDA-MB231 cells. These differences in the functional interaction of the mutants with coactivators likely contributed to the greater gain of activity of QQQ in HeLa cells as compared to that in MDA-MB-231 cells.

K464, K481 and R492 are adjacent to or within the reported corepressor binding motif of AF1 (residues 468 – 508) [81]. In HeLa cells, we and others have reported that NCoR1 enhances PR activity in response to R5020 [59, 82]. Consistently, this study also shows in HeLa cells that NCoR1 enhances the activity of both wt PRB and QQQ mutant, whereas it has no effect on the activity of FFF mutant. On the other hand, SMRT suppressed the activity of PRB, QQQ and FFF mutant to a similar extent (Figure 5A). What is also more striking is that, in MDA-MB-231 cells, NCoR1 and SMRT suppressed the activity of QQQ by 30% and 40%, respectively, but they had no repressive effect on FFF activity (Figure 5B). These strong repressive effects of NCoR1 and SMRT on QQQ would conceivably contribute to a smaller gain of QQQ activity in MDA-MB-231 cells. Similarly, the lack of effect on FFF activity would lead to a smaller loss of FFF activity. Collectively, the gain and loss of activity of the AF1 mutants in different cell type are likely due to the combined changes of their functional interaction with coregulators.

Considering that PR transcriptional activity is dynamically regulated by its interaction with its coregulators, the differential expression of coregulators in different cell lines could be involved in the cell-specific PR transactivity. Although real-time PCR analysis of various coregulators in our study showed differences in their expression of SRC-1 and SRC-2 in these cell lines (Supplementary Figure 4), the expression levels of the coregulators in different cell lines cannot explain the differences in the cell type-dependent activity of the AF1 mutants or their functional cooperation with the coregulators.

JDP2 has been reported to be a ligand-dependent AF-1 coactivator for PR although the effect is mediated by the DBD [83]. Consistent with the report, JDP2 was found to increase the ligand-independent activity of wt PR and the AF1 mutants in both HeLa cells and MDA-MB-231 cells (Supplementary Figure 5). However, it enhanced the R5020-induced activity of PRB and the AF1 mutants similarly in both HeLa and MDA-MB-231 cells, suggesting that these AF1 mutations had no effect on how JDP2 interacts with PRB.

Figure 5. The QQQ and FFF mutations lead to differential response to coregulators in a cell-dependent manner. R5020-induced transcriptional activities of PRB and AF1 mutants in (A) HeLa and (B) MDA-MB-231 cells in the presence of control vector, SRC-1, SRC-2, CBP, and p300, NCoR1 and SMRT. Data were plotted as relative R5020-induced PRE-luciferase units (mean $\pm$ SEM, 3 independent experiments, triplicates). Data are statistically significant between vector and coregulators by two-way ANOVA ($p < 0.05$), unless indicated as *n.s.* ($p > 0.05$).

3.7 K464/K482/R492 form interface for PR interaction with p300 and SRC-1

Since QQQ and FFF mutants exhibited greater and smaller functional interaction with transcription coactivators in HeLa cells, respectively, we tested whether this is due to corresponding changes in their physical interaction. p300 is crucial for chromatin remodeling and enhancer formation during hormone-induced gene transactivation. It also acetylates PR directly [84, 85]. Intriguingly, regardless of the loss of positive charges or the gain in bulkiness and hydrophobicity, both QQQ and FFF mutants displayed impaired interaction with p300 as compared to the wt PRB (Figure 6A). However, this loss of interaction had no effect on R5020-induced PR acetylation (Figure 6B). This suggests that the KKR residues forms part of the interaction interface with p300, but p300-mediated PR acetylation does not require stable PR binding with p300.

Unexpectedly, FFF mutant showed the most interaction with SRC-1, which is approximately 6 times that of wt PRB at both 20 and 45 min after R5020 treatment. On the other hand, QQQ interaction with SRC-1 is only slightly more than the wt PRB (Figure 6C). Serine 294 (S294) phosphorylation of PR is the hallmark of PR activation in response to progestin. It is important for transcriptional activity of PR and subsequent PR ubiquitination [86, 87]. Interestingly, FFF was also

equally phosphorylated at S294 compared to PRB and QQQ despite of its lack of activity (Figure 6C). This partly explains why FFF is still able to interact fully well (and much better) with SRC-1.

Figure 6. K464/K481/R492 are critical for PR interaction with transcription coregulators. (A) Mutations in the AF1 attenuated the interaction between PRB and p300 in response to R5020 treatment. (B) Acetylation of PRB by p300 is not affected by the AF1 mutation. (C) FFF mutations enhances the interaction between PRB and SRC-1 in response to R5020 treatment in co-IP assay. The relative band intensity (normalized against pcDNA-EtOH) of the detected SRC-1 interaction at each time point is indicated beneath the SRC-1 blot. The PR band was first probed with phospho-S294 antibody before probing with antibody against PR. Note that the immunoprecipitated PR band in R5020-treated samples appear hollow in the middle due to the effect of prior probing with phospho-S294 antibody. (D) Interaction between SRC-1 and NTD (aa 1-550) is enhanced by FFF mutation. The interaction was evaluated by Mammalian Two-hybrid assay between VP16-NTD (wt or AF1 mutants) and GAL4-SRC-1 in COS7 cells. Data was plotted as fold-induction against vector+GAL4-SRC-1 (mean $\pm$ SEM, 2 independent experiments, triplicates). Values obtained are statistically significant against wt samples ($p < 0.05$) by one-way ANOVA, unless indicated as *n.s.* ($p > 0.05$). (E) FFF mutation promoted the physical interaction between PR-NTD and SRC-1 in co-IP assay.

Mammalian Two-hybrid assay and co-IP assay showed that the enhanced interaction of FFF with SRC-1 does not require AF2. In mammalian two-hybrid assay, the interaction of VP16-NTD with GAL4-SRC-1 is measured by GAL4-driven luciferase activity. Consistently, VP16-NTD with FFF mutation interacted with GAL4-SRC-1 the most, whereas wt NTD and QQQ-NTD interacted with SRC-1 similarly (Figure 6D). The co-IP experiment also confirmed that NTD-FFF interacts with SRC-1 more strongly than the wt NTD and NTD-QQQ, whose interactions were not detectable under the experimental conditions (Figure 6E). These observations indicate that enhanced interaction between FFF and SRC-1 is mediated specifically at AF1 through FFF residues as the interface.

3.8 K464/K482/R492 of AF1 and E911 of AF2 are required for the interaction between AF1 and AF2

Ligand-induced interactions between the NTD and LBD have been reported for several nuclear receptors including PR [9, 53, 88]. We asked whether KKR of AF1 is involved in the interaction between NTD and LBD using the mammalian two-hybrid assay using VP16-NTD and GAL4-DBD (Figure 7A). As expected, R5020 induced interaction between the wt NTD and LBD. Interestingly, E911A mutation abolished this interaction, suggesting that E911 is critical for NTD-LBD interaction. QQQ mutation in NTD had no effect on R5020-induced interaction with the LBD (GAL4-hLBD). In contrast, FFF mutation enhanced the ligand-dependent interaction between the AF1/NTD and AF2/hLBD by ~10 folds and E911A mutation completely abolished this interaction. This is the first demonstration that E911 in AF2 is essential for AF1- AF2 interaction. Furthermore, KKR of AF1 provides the interface for AF1-AF2 interaction. Mutations of KKR to the bulky and hydrophobic phenylalanine remarkably enhanced the interaction.

Figure 7. KKR of AF1 and E911 of AF2 are required for the interaction between AF1 and AF2. (A) The interaction between AF1/NTD and AF2/LBD was analyzed by the Mammalian Two-hybrid assay between VP16-NTD (wild-type (wt) or AF1 mutants) and GAL4-hLBD (wt or E911A) in COS7 cells. Data was plotted as relative GAL4-RE-luciferase units against vector+GAL4-hLBD (EtOH) (mean $\pm$ SEM, 3 independent experiments, duplicates). (B) The tripartite model to describe the interactions among PR AF1, AF2 and SRC-1. K464/K481/R492 of AF1 are involved in the interaction with both SRC-1 and AF2. E911 (in yellow triangle) in AF2 is required for AF1-AF2 interaction. The interactions are believed to be transient and dynamic to allow the recycling of PR transcription complex on the gene enhancer. The strong and stable interaction of FFF mutant with both SRC-1 and AF2 puts a constraint on the dynamics of the tripartite interaction such that it hampers the dynamic recycling of the transcription complex. In the absence of AF2, PRB Δ LBD-FFF would be freed from this tripartite constraint and its stronger interaction with SRC-1 promotes transcription.

It is intriguing that the loss of functional cooperation (in PRE-luc assay) of the full length FFF mutant with SRC-1 was associated with a markedly stronger physical interaction with SRC-1. One consideration for the apparent contradiction is the involvement of these interactions in the dynamics of the receptor-coregulator assembly and disassembly. Hormone-induced coactivator recruitment and transcription complex assembly is a cyclic process that involves rapid association and dissociation of coregulators and basal transcription factors [89, 90]. The formation of the receptor-coregulator complex is said to be transient to facilitate the dynamic process [91]. The rigid and stable interaction of FFF-AF1 with SRC-1 may hamper the disassembly and reassembly of PR transcription complex, and delay the recycling of SRC-1 for the formation of productive enhancers. This is also supported by the evidence that progesterin-induced degradation of SRC-1 is critical for the transcriptional activity of PR [92]. On the other hand, the moderate increase of interaction between QQQ and SRC-1 could be optimal for the formation of a dynamic productive enhancer and perhaps enhancer-promoter looping [93].

If the loss of activity of FFF is due to the rigid and stable interaction between full length FFF and SRC-1, why FFF Δ LBD is hyperactive despite of its equally strong interaction with SRC-1? We believe that this has to do with AF2 in LBD. FFF Δ LBD interacts with both SRC-1 and AF2 more strongly than the wt protein, suggesting that PR AF1 interacts with SRC-1 and AF2 through the same region and maybe adjacent residues. Since SRC-1 also interacts with AF2 [53], AF1, AF2 and SRC-1 effectively form a tripartite structural relationship. A stronger interaction of AF1 with AF2 and SRC-1 could make the tripartite structure more stable, which may hamper the dynamics of the receptor transcription complex. In the absence of AF2, PRB Δ LBD-FFF is freed from this tripartite constraint, and its greater ability to recruit SRC-1 results in enhanced transcriptional activity. We therefore propose a tripartite model to describe the functional and structural relationship among AF1, AF2 and SRC-1 (Figure 7B). The strength and duration of the interaction of the three components may be intricately regulated by processes such as post-translational modification to meet the demand of the dynamic process. This model is supported by the cryo-EM structure of full length ER α complex with SRC-3 and p300 [29]. The spatial organization of the structure has clearly indicated that AF1 and AF2 are right next to each other and that both are involved in the recruitment of SRC-3.

Previous studies of AF1 have generally reported the importance of hydrophobic and acidic amino acids in AF1 activity. Some of these mutagenesis analysis (AR and HNF-4) were carried out using only a sub-region of NTD or AF1 fused to Gal4-DBD or LexA-DBD in the absence of LBD/AF2 [94, 95], but studies on GR and retinoic acid receptor β 2 were performed in both isolated AF1 core domain and full length receptors [96, 97]. In addition, the study of GR also showed importance of non-hydrophobic amino acids. However, none of these studies scrutinized the involvement of positively charged residues. As amino acid composition of AF1 among NR is highly variable, the usefulness of specific properties of the amino acids in AF1 function may vary among nuclear receptors. Different nuclear receptors may also use different strategies to coordinate AF1, AF2, and coregulator activity depending on the primary sequence of AF1.

This is the first report on the involvement of positive residues in AF1 activity of NR. The data suggest that KKR residues are the interface for AF1 interaction with AF2, SRC-1 and p300. It appears that the positive charges are required for PR interaction with p300 as both QQQ and FFF mutants were unable to interact with p300 well. On the other hand, the positive charges is not necessary for the transient and dynamic interaction with SRC-1 because the removal of the positive charge by QQQ mutation did not have marked effect on the interaction. It is conceivable that monomethylation of KKR would modulate PR interaction with SRC-1. Although the replacement with phenylalanine increases the local bulkiness and hydrophobicity that mimic methylation to some degree, the structure of phenylalanine is evidently different from a methylated lysine or arginine. Future studies will examine the effect of chemical methylation on AF1-SRC-1 interaction using recombinant proteins.

In summary, the present study reports 3 novel features regarding the mechanism of AF1 action. Firstly, the study provides strong evidence that the positively charged residues K464, K481 and R492 are functional residues for AF1. The post-translational modification such as methylation of these residues would conceivably modulate PR activity in a cell and promoter-dependent manner. This is supported by the evidence that the mutation to amino acids with different properties had different effects on the activity of full length PRB and PR Δ LBD in different cell lines (Figure 3). The study also shows that PR AF1 can function independent of AF2 because the hyperactive QQQ mutation rescued the loss of PR activity caused by the E911A mutation (Figure 4). Since the AF2 mutant at E911A is defective in supporting a progressive RNA polymerase II activity for gene transcription [76], we propose that an important aspect of AF1 function is to ensure the formation of productive RNA polymerase II complex. Secondly, AF1 interacts with a broad range of coregulators since AF1 mutations altered the coregulatory activities of both coactivators and corepressors in a cell context-dependent manner. The magnitudes of gain and loss of function of QQQ and FFF in different cell lines are the results of combined changes of the functional cooperation with the coactivators and corepressors. Thirdly, we discovered that K464/K481/R492 forms the interaction interface with coregulators such as p300 and SRC-1 (Figure 6), as well as AF2. Importantly E911 of AF2 critical for AF1-AF2 interaction at KKR because E911A mutation abolished LBD interaction of both wt and mutant NTD (Figure 7). True to its unstructured nature, intricately versatile and calibrated interactions of AF1 with AF2 and coregulators are important for a productive enhancer and dynamic cycling of the transcription complex. An abnormally strong and rigid interaction of FFF-AF1 with AF2 and SRC-1 diminished PR activity. It is also intriguing that AF1 uses the same interface/region to interaction with both AF2 and SRC-1. The interactions may occur sequentially, or different residues of KKR are involved in the interaction with AF2 and SRC-1, respectively. The classical model of steroid receptor activation states that ligand-induced folding of AF2 forms the interaction surface with coactivators. However, sequence of events leading to AF1 binding to AF2 and coregulators is not clear. Future studies will address these questions using genetic, biochemical and structural tools. With the understanding of cell- and gene-selective effect of AF1, there have been efforts to screen for AF1-targeting compounds for clinical applications. For example, small molecules EPI-001 and Sintokamide A targeting AF1 have been reported to be effective in inhibiting the activity of androgen receptor in prostate cancer cells [98, 99]. Since the change of properties (size, charge) in PR AF1 residues leads to opposite outcomes, these residues are also potential targets for the development of PR antagonist.

Acknowledgements

This work is supported by Singapore Ministry of Education Academic Research Fund Tier 2, MOE2014-T2-2-125. We thank Dr Or Yu Zuan and Mr Lim Chew Leng for their technical assistance (Nanyang Technological University, Singapore).

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