The Progesterone Receptor Hinge Region Regulates the Kinetics of Transcriptional Responses Through Acetylation, Phosphorylation, and Nuclear Retention

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Progesterone receptors (PRs) are critical regulators of mammary gland development and contributors to breast cancer progression. Posttranslational modifications of PR have been shown to alter hormone responsiveness. Site-directed mutagenesis demonstrated that upon hormone binding, PR is acetylated at the consensus sequence, KXKK (amino acids 638–641), located within the hinge region. We created an acetylation-deficient (K-A) mutant as well as acetylation mimics (K-Q or K-T). Interestingly, similar to K-A PR, PR acetylation mimics (K-Q or K-T) displayed delayed phosphorylation and nuclear entry relative to wild-type (wt) PR-B, indicative of disruption of PR nuclear-cytoplasmic shuttling. Wt PR-B, but not K-mutant PRs, induced c-myc at 1 h of progestin treatment. However, at 6 h of treatment, c-myc induction was comparable with levels induced by wt PR-B, suggesting that the precise timing of PR phosphorylation and nuclear retention are critical for cells to rapidly initiate robust transcriptional programs. In contrast to c-myc, progestin-induced serum- and glucocorticoid-regulated kinase (SGK) expression displayed sensitivity to PR acetylation but not nuclear entry. Namely, in the presence of progestin, acetylation-deficient (K-A) mutant PR-B up-regulated SGK mRNA relative to wt PR; progesterone response element-luciferase assays confirmed this result. However, K-Q and K-T acetylation mimics only weakly induced SGK expression independently of nuclear retention. These data reveal the ability of PR acetylation to alter the magnitude of transcriptional response at selected (slow response) promoters (SGK), whereas the hinge region dictates the kinetics of the transcriptional response to hormone at other (rapid response) promoters (c-myc). In sum, the PR hinge region is multifunctional. Understanding the ability of this region to couple acetylation, phosphorylation, and nuclear entry may provide clues to mechanisms of altered hormone responsiveness. (***Molecular Endocrinology*** **24: 2126–2138, 2010)**

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Progesterone receptors (PRs) are ligand activated transcription factors and members of the nuclear receptor superfamily. PR-B isoforms are critical for proper mammary gland development, including the massive expansion of the gland that occurs during pregnancy in preparation for lactation (1, 2). The proliferative effects of these receptors have been implicated in the early progression of breast malignancy (3). Clinical data confirmed this by showing that the addition of progestins to hormone replacement therapy increased both the incidence and inva-

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Abbreviations: aa, Amino acid; AR, androgen receptor; CDK2-TY, constitutively active cell-cycle dependent protein kinase; DAPI, 4′,6-diamidino-2-phenylindole; DBD, DNA-binding domain; DCC, dextran-coated charcoal-stripped serum; HBD, hormone binding domain; MEK1R4F, mitogen-activated protein kinase kinase; NLS, nuclear localization signal; ΔNLS, NLS deficient; PR, progesterone receptor; PRE, progesterone response element; qPCR, quantitative real-time PCR; SGK, serum- and glucocorticoid-regulated kinase; TF, tissue factor; TSA, trichostatin A; WCL, Whole-cell lysates; wt, wild type.

siveness of breast cancers diagnosed in postmenopausal women (4, 5). In breast cancer cells, progestins have been shown to activate transcriptional programs that increase cell survival (6) and proliferation as well as prime cells for hyperresponsiveness to mitogenic growth factors (7–9). Thus, the molecular mechanisms that govern PR transcriptional activation in response to ligand are relevant to understanding early events in breast cancer progression.

PR are heavily posttranslationally modified proteins. They are phosphorylated on at least 14 sites, ubiquitinated, and sumoylated (10–12). These modifications have been shown to regulate PR function by changing hormone responsiveness, altering promoter selectivity, and modulating receptor turnover (13, 14). Varying combinations of these modifications may allow cells to respond to hormonal signals in a multitude of ways. Previous studies have reported that in addition to those listed above, steroid receptors, such as androgen receptors (ARs), estrogen receptors, and glucocorticoid receptors also undergo modification by acetylation on lysine (Lys; K) residues within conserved acetylation motifs (15). The functional effects of acetylation on steroid receptors include transcriptional activation, transcriptional repression, increased DNA binding, altered coregulator recruitment, and changes in hormone responsiveness (16–18).

The domain structure of PR includes an N-terminal domain, a DNA-binding domain (DBD), a hinge region, and a hormone binding domain (HBD). The hinge region contains consensus sites for posttranslational modifications, such as phosphorylation and acetylation, which have yet to be characterized (15). This domain has also been implicated in processes such as receptor dimerization, ligand-independent nuclear localization, and DNA and coregulator binding. Deletion mutants of PR that contain only the HBD are unable to dimerize with partner receptors; however, if the hinge region is added, dimerization capacity of PR is restored (19). A portion of the PR nuclear localization signal (NLS), located in the hinge region [amino acids (aa) 638–642], is homologous to the SV40 large T antigen NLS. Upon deletion or mutation of this region, PR is localized predominantly in the cytoplasm in the absence of progestin and exhibits delayed nuclear entry in the presence of hormone (20–22). Additionally, the PR hinge region or C-terminal extension has been shown to interact with the minor grove of DNA. Mutation of R637 and K638 of the conserved (*i.e.* in steroid receptors) motif, RKXKK, resulted in decreased binding of PR to progesterone response element (PRE) and PRE half-site sequences (23), and disruption of this motif in AR elicited similar results (24). In PR, this domain has also been shown to interact with coregulatory molecules such as high-mobility group protein B and Jun dimerization protein 2 to alter PR transcriptional activity (25, 26). These phenotypes indicate the importance of the hinge region in interrelated processes required for optimal PR function.

Here, we sought to understand the functional significance of PR acetylation in breast cancer cells. We demonstrate that ligand binding induces rapid PR acetylation at an acetylation motif (KXKK) located in the PR hinge region and conserved among steroid receptors. Disruption of these Lys by mutation to either alanine (Ala; A), glutamine (Gln; Q), or threonine (Thr; T) results in receptor hinge regions that are either uncharged (K-A) or carrying functional groups designed to mimic acetylated molecules (K-Q or K-T). These mutants were expressed in PR-null HeLa and T47D breast cancer cells to help define the consequences of PR acetylation on promoter selectivity and hormone responsiveness.

Results

Hormone-dependent regulation of PR acetylation

PR is known to contain an acetylation consensus sequence, KXKK (aa 638–641), in the hinge region (15). Initially, we investigated whether PR was acetylated in the presence of progestins. Wild-type (wt) PR-B was transiently transfected into Cos-1 cells to achieve high expression. The cells were then serum starved and exposed to trichostatin A (TSA), a histone deacetylase inhibitor, and the synthetic progestin, R5020, for increasing time. PR protein was immunoprecipitated and visualized by Western blotting using a pan acetylated-Lys antibody (Fig. 1A). Wt PR-B exhibited undetectable basal acetylation. However, R5020 induced acetylation as early as 30 min and persisted out to 4 h of treatment.

Phosphorylation events have been shown to alter posttranslational modifications of PR downstream of activated protein kinases (11, 13). To examine the possibility that kinases regulate PR acetylation, we performed acetylation assays in the presence of constitutively active kinases known to induce direct PR phosphorylation (12). Cos-1 cells were transiently transfected with wt PR-B and either a vector control, constitutively active cell-cycle dependent protein kinase (CDK2-TY) (27), or mitogen-activated protein kinase kinase (MEK1R4F) (28), serum starved, and treated (2 h) with R5020. PR was immunoprecipitated and blotted using antibodies against acetylated-Lys (Fig. 1B). Equal levels of PR were immunoprecipitated in each lane, and no significant difference in progestin-induced acetylation was visible between vector, CDK2-TY, and MEK1R4F expressing cells. Similar assays were performed using a Ser400 phosphorylation-deficient mutant and wt PR-B (Fig. 1C). Despite obtaining

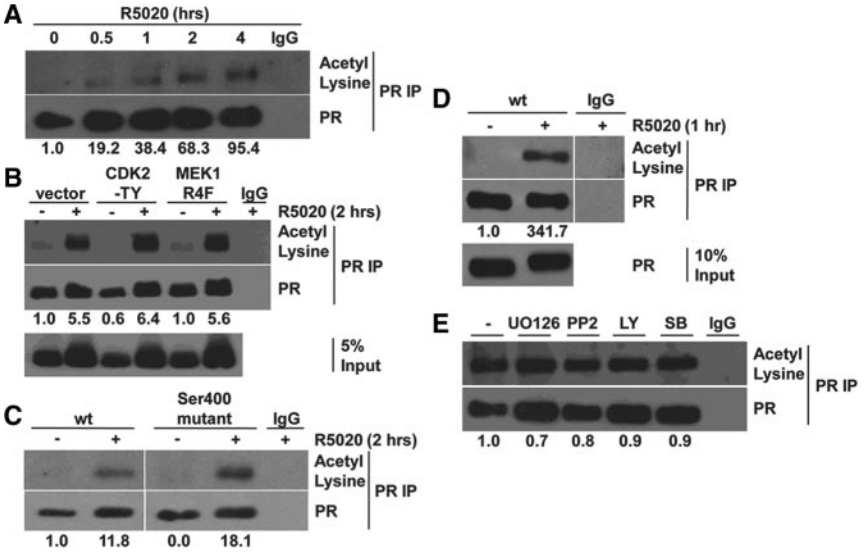

FIG. 1. Hormone-dependent regulation of PR acetylation. A, PR is acetylated upon hormone binding. Cos-1 cells were transfected with wt PR-B, starved for 24 h, then treated for 30 min with 10 μM TSA before R5020 (10^{-8} M) for 0, 0.5, 1, 2, and 4 h. Wt PR-B was immunoprecipitated and Western blotted for acetylated Lys or total PR. Densitometric values are shown for acetyl Lys-containing PR normalized to total PR. B, Constitutively active kinases do not alter PR acetylation. Wt PR-B and CDK2-TY, MEK1R4F, or vector control were transfected into Cos-1 cells, serum starved, and pretreated with 10 μM TSA before vehicle or R5020 (10^{-8} M) for 2 h. Lysates were immunoprecipitated using antibodies against total PR or normal IgG as a control. Immunoprecipitates were Western blotted for acetylated Lys and total PR. Values represent densitometry of acetyl Lys-containing PR as compared with total PR. C, Ser400 phosphorylation-deficient PR is acetylated. Cos-1 cells transfected with wt or Ser400-deficient PR-B were pretreated with 10 μM TSA before vehicle or R5020 (10^{-8} M) for 2 h and then were subjected to acetylation assays. Lysates were Western blotted as noted in B. *Each row* represents data acquired from the same SDS-PAGE and film exposure. Densitometry was performed, and values shown represent acetyl Lys-containing PR normalized to total PR. D, Stably expressed PR-B is acetylated upon ligand treatment. T47D cells stably expressing PR-B were serum starved overnight and treated for 1 h with R5020 (10^{-8} M) after pretreatment for 30 min with TSA (10 μM). Acetylation assays were completed, and immunoprecipitates were Western blotted for acetylated Lys and total PR. Densitometry-derived values for acetyl Lys-containing PR normalized to total PR are shown. E, PR is acetylated independently of blockade of multiple kinase pathways. Acetylation assays were performed in T47D cells stably expressing wt PR-B with 10 μM U0126, PP2, LY294002, or SB203580 before 10 μM TSA and R5020 (10^{-8} M) treatment for 1 h. Lysates were immunoprecipitated and Western blotted as described in D. After densitometric analysis, acetyl Lys-containing PR was normalized to total PR (values are shown); experiments were completed two to three times. IP, Immunoprecipitates; LY, LY294002; SB, SB203580.

equal levels of immunoprecipitated PR protein, the Ser400 mutant consistently displayed slightly increased acetylation relative to wt PR-B, perhaps related to the delayed nuclear localization of this mutant upon hormone binding (29).

To asses PR acetylation in cells continually expressing the receptor, T47D cells stably expressing wt PR-B were serum starved and pretreated with TSA followed by R5020 treatment (1 h). PR was immunoprecipitated and Western blotted with antibodies against total PR and acetyl-Lys (Fig. 1D). Similar to PR-B transiently expressed in Cos-1 cells, acetylation of PR-B stably expressed in T47D cells was induced to a significant degree by the presence of hormone. Progestin-induced PR acetylation in T47D cells was not further altered by blockade of selected kinases, including Mek1/2 (UO126), cellular-Src (PP2), phosphatidylinositol 3 kinase (LY294002), or p38 MAPK (SB203580) (Fig. 1E). Taken together, these data suggest that phosphorylation events do not significantly alter PR acetylation, a posttranslational modification that appears to be exclusive to hormonal regulation.

Acetylation maps to the hinge region of PR

To confirm that PR acetylation was indeed occurring at the putative consensus sequence (Fig. 2A) located in the hinge region (15), we created a series of mutations. Initially, we mutated the last two aa of the KXKK motif to Ala, dubbed KK6, because this uncharged residue is unable to accept acetyl group modification. As a control, this construct was compared with a C-terminal double Lys to Ala mutant PR (KK9; aa 932–933). Acetylation assays were performed demonstrating impaired acetylation of KK6 PR-B relative to wt and KK9, yet KK6 PR-B was still weakly acetylated (data not shown). This prompted us to mutate all three Lys residues in the hinge region motif to Ala (K-A) (Fig. 2A). Cos-1 cells were transiently transfected with wt, KK6, or K-A PR-B, serum starved, and treated with vehicle or R5020 (2 h) to promote acetylation (Fig. 2B). Equal levels of PR-B were immunoprecipitated from each sample (Fig. 2B, *middle panel*). Again, KK6 displayed decreased levels of acetylation, suggestive that some K638 is acetylated in this mutant receptor. However, K-A PR-B, containing all three Lys to Ala mutations, appeared unmodified relative to R5020-treated wt PR-B. These data indicate that PR-B is acetylated at two or more Lys residues within the KXKK motif in the hinge region and disruption of all three Lys residues is necessary to abolish PR acetylation.

PR acetylation mutants exhibit delayed phosphorylation

To study the effects of PR acetylation on PR functions in breast cancer cells, we created T47D breast cancer cells stably expressing acetylation deficient, K-A PR-B. Multiple clones expressing mutant K-A PR-B were generated

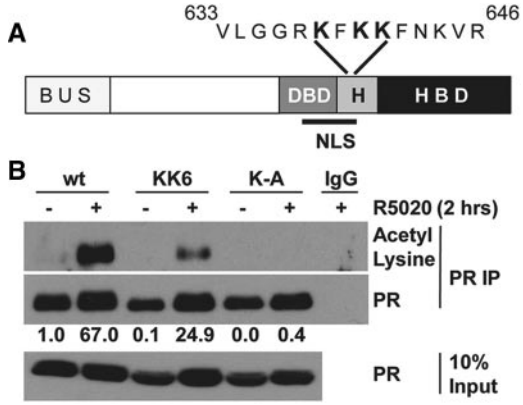

FIG. 2. Acetylation maps to the hinge region of PR. A, PR-B contains an acetylation consensus site, KXKK. The domains of PR-B include a B-upstream segment (BUS), an N-terminal domain, a DBD, a hinge region (H), and a HBD. The acetylation consensus sequence, KXKK, is located within the hinge region at aa 638–641. The NLS overlaps the acetylation consensus site and is located within both the hinge domain and the DBD. B, K-A PR-B is not acetylated. Cos-1 cells were transfected with wt, KK6, or K-A PR-B. Cells were serum starved for 24 h and then pretreated with TSA (10 μM) for 30 min before R5020 (10^{-8} M) treatment for 2 h. Acetylation assays were performed, and immune complexes were Western blotted with acetyl-Lys and total PR antibodies. Densitometry-derived values are shown for acetyl Lys-containing PR normalized to total PR. Experiments were carried out three times. IP, Immunoprecipitates.

and compared with wt PR-B expressing cells via PR Western blotting (Fig. 3A). Multiple independent clones were used throughout our studies to confirm results obtained in K-A mutant cells (data not shown).

Activation or inhibition of protein kinase inputs to PR regulation did not appreciably alter PR acetylation (Fig. 1). However, hormone-induced phosphorylation events may require PR acetylation, possibly for proper receptor folding or subcellular localization that may facilitate exposure of specific substrate residues to regulatory kinases. To characterize the K-A PR acetylation mutant with regard to phosphorylation events, T47D cells stably expressing wt and K-A PR-B were serum starved and treated with R5020 for 0, 10, or 60 min before Western blotting for PR (Fig. 3B). Cells expressing wt PR-B exhibited lower levels of PR protein relative to cells expressing K-A PR-B. After 60 min of progestin treatment, wt PR-B exhibited a slower migrating band or upshift in the polyacrylamide gel, whereas the K-A PR-B failed to upshift in the presence of progestin. Progestin-induced PR upshift has previously been attributed to increased global PR phosphorylation after ligand binding (30). However, this shift has not been credited to phosphorylation at specific sites because individual PR phosphomutants, including Ser190, Ser294, Ser345, and Ser400, all appear to shift equally to wt PR-B in response to ligand treatment (11, 29, 31, 32).

The observation that acetylation-deficient mutant PR-B does not shift in polyacrylamide gels in response to progestin (Fig. 3B) led us to suspect that this receptor may also have phosphorylation defects. We first tested K-A PR-B phosphorylation relative to wt using Western blotting and the available antibodies to CDK2 phosphorylation sites Ser190 and Ser400 and to the MAPK sites Ser294 and Ser345 (29, 31). Ser190 is a basal PR phosphorylation site, whereas Ser400 phosphorylation has been shown to regulate ligand-independent transcription and mediate rapid ligand-dependent nuclear entry (29). PR Ser294 phosphorylation prevents PR sumoylation at Lys388 (13), and thereby effectively links increased PR transcription to rapid ligand-dependent receptor down-regulation (33). Phosphorylation of PR Ser345 is induced upon progestin-activated rapid signaling to cellular-Src/MAPK and is critical for PR-SP1 tethering (31). T47D cells stably expressing either wt or K-A PR-B were serum starved and treated for 0, 30, or 180 min with R5020. Whole-cell lysates (WCL) were Western blotted using total or phospho-specific PR antibodies (Fig. 3C). Wt PR underwent a ligand-dependent upshift at 30 min of treatment. However, acetylation-deficient mutant PR exhibited a delay for at least 3 h of progestin exposure. Basally phosphorylated Ser190, however, is comparatively well phosphorylated between wt and K-A PR-B. Wt PR displayed heightened Ser294, Ser345, and Ser400 phosphorylation after 30 min of progestin, whereas K-A phosphorylation at these sites was not robust until at least 3 h. Interestingly, the peak of Ser294, Ser345, and Ser400 phosphorylation correlated with gel upshift of these receptors. To account for differences in PR expression, we quantified the relative levels of wt and K-A PR-B Ser400 phosphorylation using a two-color infrared Western blotting technique (13). Triplicate cultures of HeLa cells transiently expressing either wt or K-A PR-B were serum starved and treated with R5020 (1 h). Lysates were Western blotted using total and phospho-PR antibodies and secondary antibodies conjugated to red or green emitting flourophors. Bands were then quantified, and phospho-Ser400 was normalized to total PR protein within the same sample (Fig. 3D). Acetylation-deficient PR-B again displayed a significant decrease in Ser400 phosphorylation both in the absence and presence of progestin. Notably, wt PR-B exhibited basal Ser400 phosphorylation relative to weakly detectable phosphorylation of the K-A mutant. This difference is perhaps due to variations in basal nuclear localization (see below) and translates to comparable hormone-induced fold changes in Ser400 phosphorylation between wt and K-A PRs. These data indicate that disrupting specific Lys in the hinge region dramatically delay PR phosphorylation events known to be critical for tran-

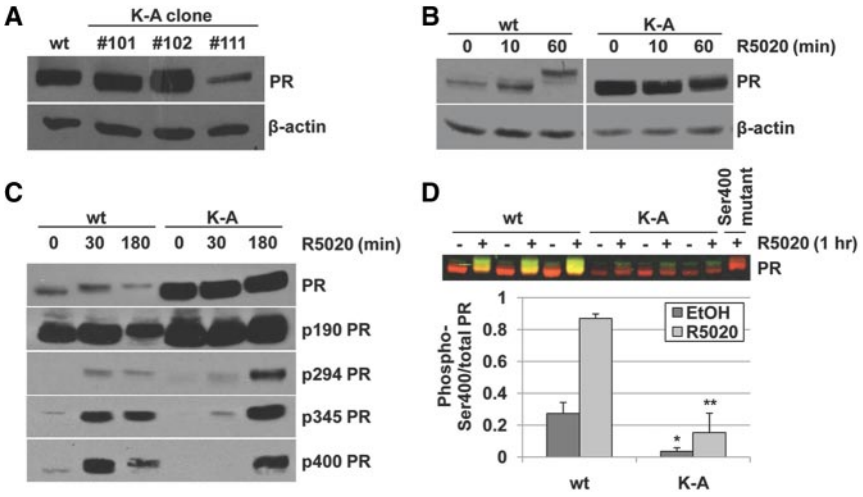

FIG. 3. Acetylation-deficient PR displays delayed phosphorylation. A, Stable expression of acetylation-deficient PR in breast cancer cells. T47D cells stably expressing wt or K-A PR-B (clone nos. 101, 102, and 111) were Western blotted for total PR and β-actin. B, K-A PR-B exhibits altered SDS-PAGE upshift. T47D cells stably expressing K-A or wt PR-B were serum starved for 24 h and treated for 0, 10, or 60 min with R5020 (10^{-8} M). Cell lysates were Western blotted for total PR and β-actin as a loading control. Data correspond to images from the same SDS-PAGE and film exposure. C, Acetylation-deficient PR displays a lag in phosphorylation on specific sites. T47D cells stably expressing wt or K-A PR-B were serum starved for 24 h and treated for 0, 30, or 180 min with R5020 (10^{-8} M). Cell lysates were Western blotted with phospho-specific antibodies against Ser190, Ser294, Ser345, and Ser400, as well as total PR. D, K-A PR-B exhibits less Ser400 phosphorylation compared with wt PR-B. Triplicate cultures of HeLa cells were transfected with wt or K-A PR-B, serum starved, and treated for 1 h with vehicle or R5020 (10^{-8} M). Lysates were Western blotted with antibodies against total PR and phospho-Ser400 PR. Total PR (*red*) and phospho-Ser400 PR (*green*) band intensities were quantified using an Odyssey Imaging System. Phosphorylation was normalized to total PR levels (±SD; *, $P < 0.005$; **, $P < 0.001$). Each experiment was performed three to four times. EtOH, Ethanol.

scriptional activation and nuclear accumulation/retention (29, 31, 33, 34).

Acetylation motif mutant PR displays defective nuclear retention

Previous reports identified an area in the PR hinge region, which includes the acetylation motif (aa 638–641), that is critical for ligand-independent nuclear retention (35), similar to the overlapping acetylation/NLS region in AR (36). Deletion of aa 638–642 (Δ638–642) created a predominantly cytoplasmic PR-B in the absence of ligand. Δ638–642 PR exhibited delayed nuclear entry upon progestin treatment by utilizing an alternative portion of the NLS within the DBD (20). Given this functional overlap, PR acetylation may mediate changes in PR nuclear retention. Likewise, altered PR localization may induce persistent changes in PR phosphorylation status; we have previously reported that Ser400 phosphorylation increases ligand-dependent PR nuclear retention (29). Additionally, the Lys in the acetylation motif may provide the positive charge necessary for the NLS in this region to function, similar to that of the well-characterized SV40 NLS, which contains both Lys and arginine (Arg; R) residues (37). Disruption of the positive charges within this region alone may thus ablate this portion of the NLS independent of PR acetylation. To test this possibility we transiently transfected HeLa cells with wt or K-A PR-B, serum starved, and then treated cells with R5020 (1 h) before subcellular fractionation and Western blotting (Fig. 4A). WCL were run next to cytoplasmic and nuclear fractions as controls for total PR levels among the cells. Wt PR-B was present in both the cytoplasm and the nucleus in the absence of progestin. Upon treatment, a robust band appeared in the nuclear fraction. Acetylation-deficient PR-B, however, was not detectable in the nucleus in both the absence and presence of ligand. Notably, wt PR-B exhibits a ligand-dependent upshift in the nuclear fraction that is not present in the cytoplasmic fraction, due to an increase in phosphorylation events.

To confirm these data, we performed confocal microscopy in HeLa cells. These cells possess large cytoplasmic spaces and well-defined nuclei, thus providing a highly sensitive assay of PR localization. Representative images of HeLa cells transiently transfected with wt or K-A PR-B after 0, 30, or 240 min of progestin treatment using antibodies against total PR are shown (Fig. 4B). In the absence of ligand, wt PR-B localizes to both the cytoplasm and the nucleus. After only 30 min of progestin treatment, wt PR appeared entirely nuclear. Acetylation motif mutant K-A PR localized largely to the cytoplasmic compartment in the absence of ligand and was retained in the nucleus to a similar extent as wt PR after 4 h of progestin exposure. The time course of delayed nuclear retention was then quantified using ImageJ to calculate the levels of total PR in the nucleus [as defined by 4′,6-diamidino-2-phenylindole (DAPI) staining] and the cytoplasm (described in *Materials and Methods*). HeLa cells transiently transfected with wt or K-A PR-B were serum starved and treated with R5020 for 0–4 h (Fig. 4C). Notably, K-A PR-B exhibited a defect in nuclear retention in the absence of ligand and delayed hormone-induced nuclear accumulation that approached that of wt PR-B after 4 h. Our observations suggest a link between nuclear retention, PR gel mobility upshift, and phosphorylation on specific serines. These events coincide whether they are early events (15 min to 1 h) for wt or late events (3–6 h) for the K-A PR-B.

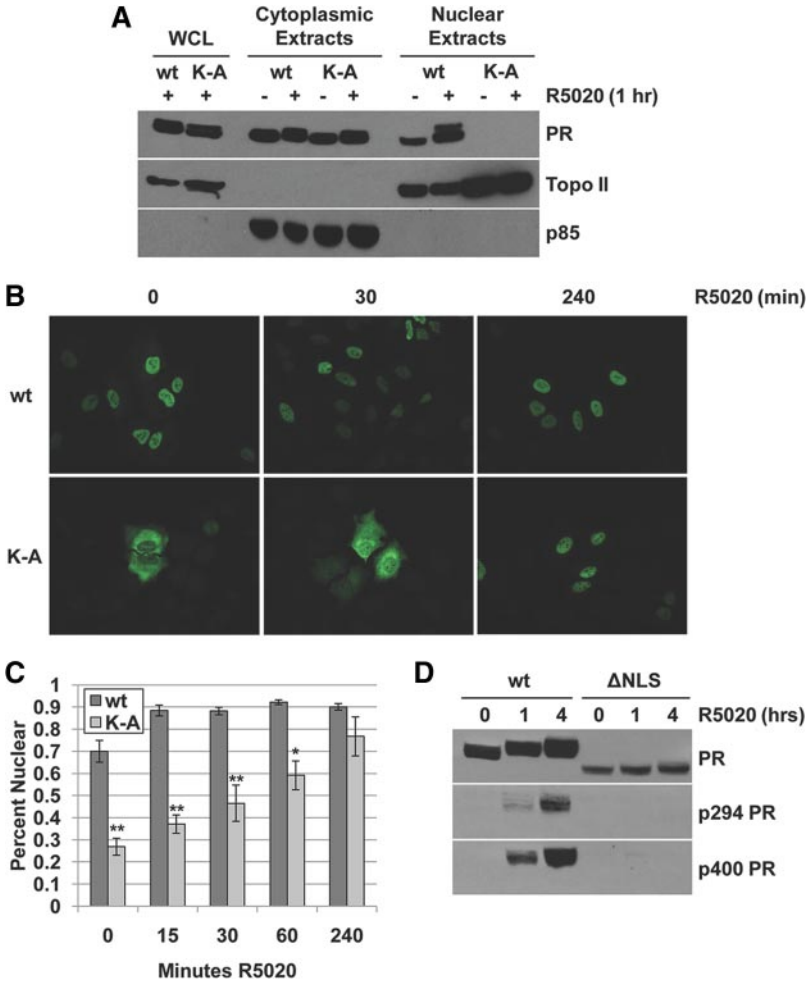

FIG. 4. Acetylation motif mutant exhibits a defect in nuclear retention. A, K-A PR-B displays a nuclear retention defect. HeLa cells were transfected with wt or K-A PR-B, serum starved for 24 h, and treated for 1 h with R5020 (10^{-8} M). Fractionation of lysates was completed, and fractions were Western blotted with total PR, topoisomerase II (nuclear control), or p85 (cytoplasmic control) antibodies. B, Acetylation-deficient PR-B displays a ligand-induced delay in nuclear accumulation. HeLa cells were transfected with wt or K-A PR-B, treated with 10^{-8} M R5020, and PR was visualized using PR-specific antibodies and confocal microscopy. C, Wt and K-A PR-B are largely nuclear by 4 h of R5020 treatment. HeLa cells transfected with wt and K-A PR-B were incubated with PR antibodies and DAPI and were subjected to confocal microscopy. PR fluorescence intensity of whole cells and nuclei was assessed, and percentages of nuclear occupancy were calculated. Values represent the average fluorescence intensity between triplicate cultures. Statistical significance between corresponding wt and K-A treatment groups was calculated using *t* tests (±SD; *, $P < 0.001$; **, $P < 0.0003$). D, HeLa cells were transiently transfected with wt or ΔNLS PR-B, serum starved, then treated for 0, 1, or 4 h with R5020 (10^{-8} M). Lysates were Western blotted with antibodies against total PR and antibodies specific for PR phosphorylation on Ser294 and Ser400. Experiments were completed three to four times.

To determine whether PR nuclear localization precedes select phosphorylation events, we examined the ability of an NLS-deficient (ΔNLS) PR-B to undergo phosphorylation on specific sites. ΔNLS PR-B is an exclusively cytoplasmic mutant with both the DBD and hinge region portions of the NLS deleted (38). HeLa cells were transiently transfected with wt or ΔNLS PR-B, serum starved, and treated for 0, 1, or 4 h with R5020 (Fig. 4D). Western blotting showed that wt PR-B was upshifted and phosphorylated on Ser294 and Ser400 upon 1 and 4 h of ligand exposure. ΔNLS PR-B, however, was unable to undergo upshift or phosphorylation on these sites. Ser190 was used as a control to demonstrate that ΔNLS PR-B receptors undergo phosphorylation (data not shown). Cotransfection of wt and ΔNLS PR-B results in heterodimers that are able to localize to the nucleus (38). We repeated these experiments in HeLa cells coexpressing both wt and ΔNLS PR-B. Upon nuclear entry, ΔNLS PR-B gel upshifted (data not shown), indicating that this mutant, when sequestered in the nucleus by wt PR, is capable of being phosphorylated. Taken together, these data indicate that rapid nuclear localization precedes efficient PR-B phosphorylation events; nuclear retention may facilitate persistent PR phosphorylation.

PR acetylation motif mutants exhibit delayed transcriptional activation

Given the delayed phosphorylation and nuclear retention of K-A PR-B, we speculated that this mutant might exhibit a lag in its transcriptional response to progestins. We sought to compare wt and K-A PR-B on early response PR target gene promoters. We tested T47D-CO cells (expressing endogenous PR) for a rapid transcriptional response on the c-myc promoter, a known immediate early gene for multiple mitogenic signaling pathways (39) and PR target gene (40). Cells were starved in 5% dextran-coated charcoal-stripped serum (DCC) and treated for 0, 30, 60, and 120 min with R5020 (Fig. 5A). To assess c-myc mRNA levels relative to β-actin, quantitative real-time PCR (qPCR) was performed; c-myc mRNA was rapidly induced and peaked after 60 min of progestin treatment.

In previous reports, we showed that PR Ser294 phosphorylation regulates progestin induction of c-myc protein expression (33). To examine the impact of delayed PR nuclear entry/retention on the early response PR target gene, c-myc, T47D cells stably expressing either wt or K-A PR-B were starved in 5% DCC and treated for either 1 h (Fig. 5B) or 6 h (Fig. 5C) with R5020 or vehicle control; c-myc mRNA expression was analyzed relative to β-actin using qPCR. Wt, but not K-A PR-B, induced c-myc mRNA at 1 h. The inability of

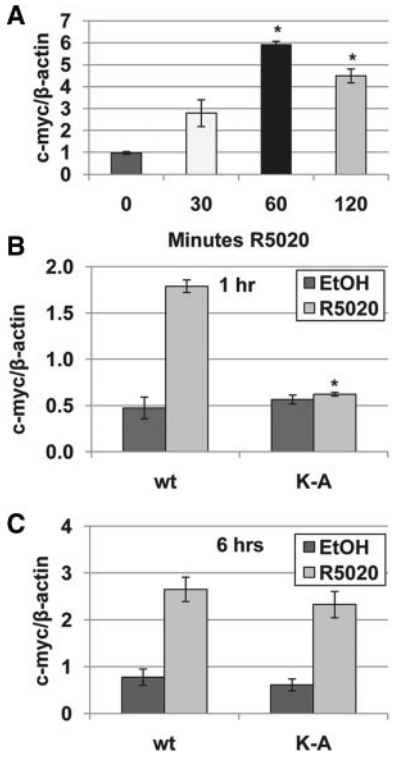

FIG. 5. Acetylation motif mutant displays delayed transcriptional activation. A, PR rapidly activates c-myc upon progestin exposure. T47D-CO cells expressing endogenous PR were plated in triplicate and starved in 5% DCC overnight before R5020 (10^{-8} M) treatment for 0, 0.5, 1, and 2 h; c-myc levels were assessed by qPCR and were normalized to β-actin. Significance between untreated and treated groups was determined using *t* tests (±SEM; *, $P < 0.005$). B, K-A PR-B fails to activate c-myc at early progestin time points. Triplicate cultures of T47D cells stably expressing wt or K-A PR-B were starved in 5% DCC overnight and treated with vehicle or R5020 (10^{-8} M) for 1 h. Evaluation of c-myc mRNA levels was performed as described in A. Progestin-induced c-myc expression was significantly different between wt and K-A PR-B (±SD; *, $P < 0.002$). C, Wt and K-A PR-B activate c-myc expression at delayed time points. T47D cells stably expressing wt or K-A PR-B were starved in 5% DCC followed by treatment with vehicle or R5020 (10^{-8} M) for 6 h; c-myc mRNA was assessed by qPCR and normalized to β-actin (±SD). All experiments were repeated three times. EtOH, Ethanol.

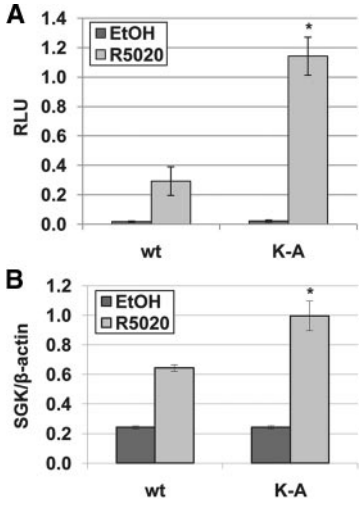

FIG. 6. Acetylation regulates transcriptional activity. A, K-A PR-B displays increased transcriptional activation. HeLa cells were transfected with wt or K-A PR-B as well as the 2XPRE-Luciferase plasmid and *Renilla* for a control. Cells were serum starved and treated for 18 h with vehicle or R5020 (10^{-8} M). Luciferase activity was measured in triplicate cultures, and statistical significance was determined using a *t* test (±SD; *, $P < 0.02$). B, Acetylation-dependent gene regulation. Triplicate cultures of T47D cells stably expressing wt or K-A PR-B were starved in 5% DCC and treated with vehicle or R5020 (10^{-8} M) for 18 h. Levels of SGK mRNA were measured by qPCR and were normalized to β-actin mRNA. Statistical significance was determined by comparing K-A treatment groups with wt treatment groups (±SD; *, $P = 0.04$). Experiments were performed three times. EtOH, Ethanol; RLU, relative light units.

K-A PR to induce c-myc is likely due to failed nuclear retention at this early time point (Fig. 4B). Interestingly, however, K-A PR-B exhibited robust activation of c-myc upon 6 h of progestin treatment, similar to wt PR-B (Fig. 5C). These data demonstrate that the precise timing of PR nuclear entry and phosphorylation events that function to retain PR in the nucleus (29, 34) are critical for the transcriptional activation of hormone "early response" genes. These data repeated in multiple clones of K-A PR expressing cells (data not shown).

PR acetylation negatively regulates transcriptional activity at selected promoters

To separate the effects of altered PR localization from other functional consequences of PR acetylation, we examined the effects of acetylation on PR transcriptional activity at late time points of progestin treatment, after both wt and K-A PR-B have accumulated in the nucleus (Fig. 4). HeLa cells were transiently transfected with wt or K-A PR-B in addition to 2XPRE-Luciferase reporter and *Renilla* control plasmids. Cells were then serum starved and treated with vehicle or R5020 (18 h), and luciferase activity was measured (Fig. 6A). Acetylation mutant K-A PR-B displayed a marked increase in progestin-induced transcriptional activity relative to wt PR-B. Because acetylation status clearly altered PR-B transcriptional response to progestins in reporter gene assays, we asked whether endogenous PR target genes, serum- and glucocorticoid-regulated kinase (SGK) and tissue factor (TF), were similarly affected (41, 42). T47D cells stably expressing mutant wt or K-A PR-B were starved in 5% DCC and treated with vehicle or R5020 (18 h). To determine target gene mRNA levels relative to β-actin, qPCR was performed. Similar to luciferase-reporter gene readouts, cells expressing acetylation-deficient mutant PR exhibited an increase in progestin-induced SGK expression relative to wt PR-B (Fig. 6B). Progestin regulation of TF, however, occurred independently of acetylation (data not shown). Taken together, these data suggest that acetylation, when coupled to nuclear retention (see below), can positively modulate the kinetics of gene expression at selected "rapid response" genes (c-myc).

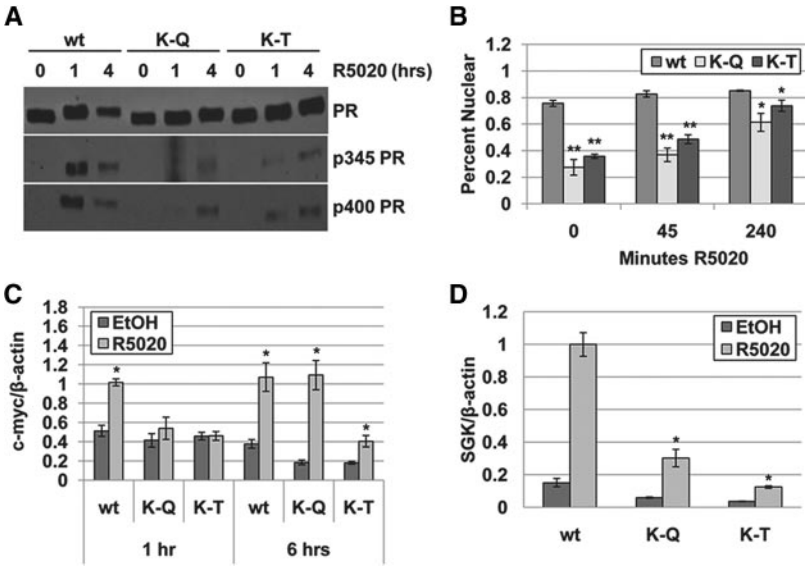

FIG. 7. PR acetylation mimics display altered transcriptional activation. A, Acetylation mimics exhibit delayed phosphorylation. T47D cells stably expressing wt, K-Q, or K-T PR-B were serum starved and treated with R5020 (10^{-8} M) for 0, 1, or 4 h. Cells were subjected to Western blotting for PR phosphorylation on Ser345 and Ser400 as well as total PR. B, Acetylation mimics display a lag in nuclear retention. Serum starved T47D cells stably expressing wt, K-Q, or K-T PR-B were treated with R5020 (10^{-8} M) for 0, 45, or 240 min. Coverslips incubated with PR antibody and stained with DAPI were subjected to confocal microscopy. Fluorescence intensity of whole cell and nuclear PR-B was calculated using ImageJ software. *P* values represent statistical significance between wt PR-B and K-Q or K-T PR-B (±SD; *, $P < 0.01$; **, $P < 0.002$). C, K-Q and K-T PR-B exhibit delayed c-myc induction. T47D cells stably expressing wt, K-Q, or K-T PR-B were starved in 5% DCC and treated with vehicle or R5020 (10^{-8} M) for 1 or 6 h. Triplicate cultures were subjected to qPCR, c-myc mRNA levels were normalized to β-actin, and statistical significance was evaluated by comparing vehicle and R5020-treated conditions within cell lines (±SEM; *, $P < 0.03$). D, PR acetylation mimics exhibited suppressed activation of SGK expression. T47D cells stably expressing wt, K-Q, or K-T PR-B were plated in triplicate cultures, serum starved, and treated for 18 h with vehicle or R5020 (10^{-8} M). SGK mRNA levels were normalized to β-actin and measured using qPCR. Statistical significance between groups was evaluated (±SD; *, $P < 0.002$). All experiments were completed three times. EtOH, Ethanol.

However, independent of nuclear retention, selected "late response" promoters (SGK) are negatively regulated by PR acetylation.

To further investigate how hinge region acetylation motif/NLS regulates PR phosphorylation status, nuclear retention, and transcriptional activity, we generated a series of new mutants. First, we mutated all the Lys in the acetylation motif to Arg (K-R), which are reportedly unable to accept acetyl groups while still retaining the positive charge of Lys residues (36). Second, we mutated the three Lys in the acetylation motif to Gln (K-Q) or Thr (K-T), which mimic acetylation of nuclear receptors (16). Notably, these aa changes (K to Q or T) remove the positive charge afforded by Lys and replace it with polar, more negative residues, mimicking that of an acetyl group.

We initially confirmed that each mutant was indeed acetylation deficient. Unexpectedly, we detected acetylation of the K-R mutant (data not shown). Notably, K-A mutant PRs are clearly acetylation null (Fig. 2), making it unlikely that other sites contribute to K-R PR acetylation. Additionally, numerous functional assays of PR action failed to distinguish the K-R mutant from wt PR-B. As predicted, K-Q and K-T PR were not acetylated (data not shown).

We next generated T47D cells stably expressing K-Q or K-T PR-B for comparison with wt PR-B expressing cells. Cells were then plated, serum starved, and treated with R5020 (1 or 4 h) before Western blotting for total and phospho-PR (Fig. 7A). As predicted, in the presence of progestin (1 h), wt PR-B exhibited a phosphorylation-induced gel upshift. Both PR acetylation-mimic constructs (K-Q and K-T) failed to upshift until 4 h of progestin treatment, similar to acetylation-deficient, K-A, PR. We also examined the ability of PR acetylation mimics to undergo Ser345 and Ser400 phosphorylation, the same sites deficient in the K-A mutant at early time points of progestin treatment (Fig. 3B). Similar to K-A PR-B, both K-Q and K-T PR-B appeared to have delayed phosphorylation at these residues relative to wt PR. These receptors were underphosphorylated on Ser345 and Ser400 at 1 h, but levels of phosphorylated receptors were comparable with wt by 4 h of progestin. These data confirm the strong correlation between the ability of PR to undergo gel upshift and multisite phosphorylation.

Given these data, we predicted that PR acetylation mimics also lag in progestin- induced nuclear retention due to partial disruption of the hinge-region NLS. We performed confocal experiments using T47D cells stably expressing wt PR-B, K-Q, or K-T PR-B (Fig. 7B). The receptors also displayed nuclear retention defects relative to wt PR-B that largely recovered after 4 h of progestin treatment. Together, these data suggest that charged Lys in the hinge region/acetylation motif are required for nuclear entry/retention. However, PR acetylation does not appear to play a dominant role in nuclear localization because both the acetylation-deficient and acetylation-mimic receptors are phenotypically identical in this regard.

Because K-Q and K-T PR exhibit comparable delays with K-A PR in gel upshift, phosphorylation, and nuclear retention, we assessed whether they also fail to rapidly activate c-myc. Again, T47D cells stably expressing K-Q,

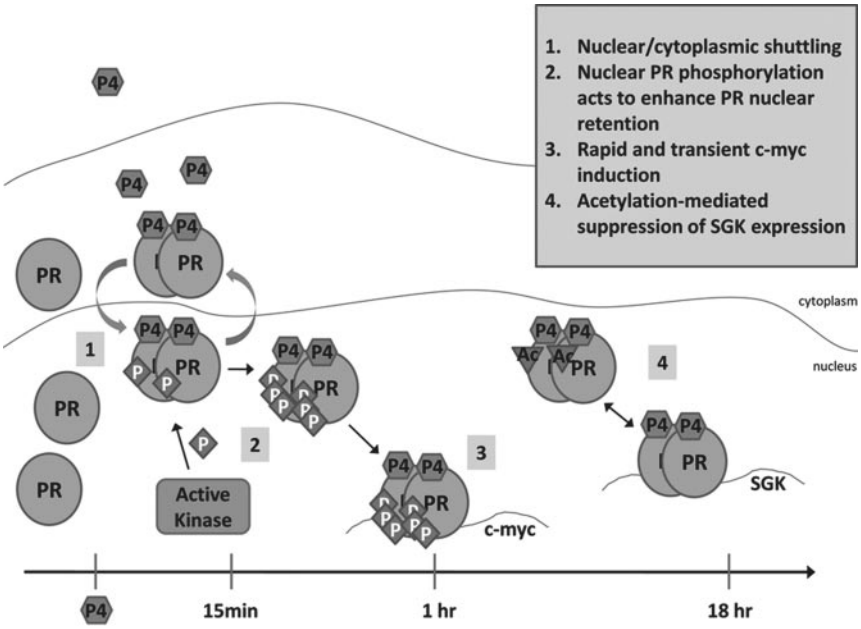

FIG. 8. PR hinge region mediates nuclear/cytoplasmic retention, acetylation, and transcriptional regulation. PR dynamically shuttles between the cytoplasm and the nucleus (1). Within 15 min of hormone binding, PR is localized to the nucleus via the NLS located in the hinge region. PR is rapidly phosphorylated and retained in the nucleus (2). The coordination of nuclear accumulation and phosphorylation leads to the rapid (1 h) and transient induction of genes, such as c-myc, by hypersensitive, phospho-PR (3). At late time points, ligand-induced PR acetylation in the hinge region leads to negative regulation of transcription on selected promoters (18 h), such as SGK, independently of rapid nuclear entry conferred by the hinge region (4).

K-T, or wt PR-B were starved in 5% DCC and subjected to progestin or vehicle treatment for 1 or 6 h. Similar to K-A PR-B, c-myc activation by K-Q or K-T PR occurred at 6 h but not 1 h of progestin treatment; wt PR-B induced c-myc expression at both time points (Fig. 7C). These findings suggest that kinetic regulation of PR gene programs relies primarily on the hinge region NLS, which controls the proper timing of hormone-induced PR nuclear accumulation and phosphorylation. Because this aspect of PR function is internally controlled among the K-mutants, the effect of PR acetylation may be revealed at late response genes using acetylation mimic receptors.

We therefore examined the ability of K-Q and K-T PR to induce SGK expression in response to progestin. Recall that acetylation-deficient mutant K-A PR displayed a heightened response to ligand on this endogenous promoter (Fig. 5B). T47D cells stably expressing wt, K-Q, or K-T PR-B were serum starved and treated with either vehicle or R5020 (18 h). SGK mRNA levels were evaluated using qPCR and normalized to β-actin (Fig. 7D). In response to progestin, wt PR-B robustly induced SGK expression, whereas both K-Q and K-T PR-B exhibited a weak transcriptional response to ligand. In similar experiments, TF gene regulation was assessed for wt, K-Q, and K-T PR-B at 18 h of progestin treatment. Again, regulation of TF mRNA in response to progestins was not altered by PR acetylation (data not shown). These data indicate that PR acetylation selectively inhibits hormone-dependent PR transcriptional responses on specific promoter contexts (SGK), whereas other promoters (c-myc and TF) remain insensitive to this modification (Fig. 8).

Discussion

PR-dependent transcriptional programs are largely proliferative in breast cancer cells (3). Our data demonstrate that the PR hinge region contains overlapping or embedded functional motifs (NLS and acetylation consensus sequence) that we experimentally separated here. Thus, we show that PR transcriptional activation is regulated by acetylation as well as the precise timing of phosphorylation events and nuclear retention of the receptor (Fig. 8) (29, 31). The disruption of any of these regulatory steps by mutation of PR has a profound impact on both the selection of gene targets (SGK) (Fig. 6) and the temporal management of gene expression (c-myc) (Fig. 5). The hinge region of PR is therefore a critical domain that is involved in controlling the kinetics and magnitude of hormone responsiveness at selected promoters in breast cancer cells.

PR acetylation, phosphorylation, and nuclear retention

Disruption of the Lys in the NLS/acetylation motif located in the hinge region of PR results in receptors that are retained in the cytoplasm in the absence of ligand and are unable to accumulate in the nucleus until at least 4 h of progestin treatment, in contrast to 15 min for wt PR (Figs. 4 and 7). Studies characterizing the homologous SV40 NLS have shown that the positive charge of the Lys residues is critical for its function; the basic property (positive charge) of the NLS enables binding to the acidic cleft on importin proteins for chaperoning through the nuclear pore (37). Likewise, our PR K-R mutant retained alkalinity and its ability to accumulate in the nucleus, whereas the neutral Ala mutation and the polar oxygen containing side-chain mimics, either carbonyl (Gln; Q) or a hydroxyl (Thr; T) groups, resulted in a nonfunctional NLS. Previous studies have shown that nonbasic posttranslational modifications within an NLS, such as phosphorylation,

can mask the positive charge of adjacent Lys and disrupt interactions with importin proteins rendering the NLS nonfunctional (43). Therefore, Lys in the hinge region of PR may be required for proper NLS function. Their modification by relatively negative acetyl groups counteracts their alkaline properties, thus decreasing the nuclear retention of PR when acetylated. Alternatively, acetylation has been shown to enhance nuclear/cytoplasmic shuttling of protein targets and in some cases is essential for this dynamic action. For example, POP-1 requires acetylation of certain Lys to gain entry to the nuclear compartment (44), whereas acetylation of proteins such as Max, c-Abl, and RECQL4 increases cytoplasmic localization (45–47). PR acetylation may function in a similar manner to mediate rapid or efficient nuclear/cytoplasmic shuttling. Phosphorylation of nuclear PRs may serve to block exit, perhaps by altering PR conformation or its binding partners and concealing the NLS, which is known to be required for PR export (38). Alternatively, acetylation may serve to enhance PR association with HSP90, a chaperone protein that has been implicated in PR cytoplasmic retention (22). This may help detain the transformed or active receptors in this compartment.

Indeed, phosphorylation of PR is a major regulator of the transcriptional response of cells to progestin (48). PR phosphorylation events are controlled by a variety of factors, including growth factor activation (9), expression level of kinases or kinase inhibitors (29), and ligand binding (12). Our data further indicate that PR phosphorylation is also linked to PR location within the cells. Phosphorylation on Ser294 and Ser400 is preceded by PR nuclear accumulation (Fig. 4D). Our previous reports suggest that PR Ser294 and Ser400 phosphorylation events may act to reduce the rate of PR nuclear export (29, 34), thus ensuring that progestin-induced transcriptional programs are carried out (discussed below).

The hinge region regulates PR transcriptional responses to hormone

Previous studies have illustrated that rapid transcriptional events in response to mitogens are critical for eliciting biological outcomes; immediate early response genes are typically transcription factors that are able to induce additional gene programs (49). Likewise, the strength and duration of protein kinase signaling can dictate the cells' ability to carry out these genetic programs via phosphorylation-dependent stabilization of early gene products (39). Our data suggest that the hinge region of PR is a critical regulator of the kinetics of the transcriptional response of cells to progestins for early events, such as the induction of c-myc. The execution of early PR genomic actions is dependent on the coordination of PR nuclear localization by the hinge region with proper PR phosphorylation, which occurs or is sustained exclusively in the nucleus. Thus, at early time points, when kinases are activated in part due to progestin-induced rapid signaling (50), it is likely that PR retention in the nucleus ensures that both PR and coregulators are simultaneously phosphorylated and activated. Just as PR Ser294 is required for progestin-induced c-myc (33), it is likely that these early transcriptional events are driven by kinase inputs to PR in PR-positive breast cancer cells.

The PR hinge region also functions to regulate "late" progestin-induced genomic events. Acetylation decreases PR hormone response on specific gene targets, such as SGK, a role separable from the NLS functions of the hinge region. Similarly, acetylation of estrogen receptor at Lys303 has been shown to decrease estrogen sensitivity (51). Additionally, acetylation of steroid receptors has also been shown to alter cofactor recruitment and DNA binding (16, 17). Residues within and adjacent to the PR acetylation motif participate in cofactor binding (23, 25, 26), as well as PRE interactions. Therefore, PR acetylation may hinder or interfere with binding to the noncanonical glucocorticoid response element (PRE) located within the SGK promoter (41). Furthermore, PR acetylation may act to shift the balance of coregulatory protein complexes, thus reducing PR's ability to stimulate late or sustained hormone-activated gene programs. The details of the mechanisms underlying the precise timing of promoter-specific gene regulation by steroid hormone receptors remain to be defined.

Materials and Methods

Cell culture and reagents

Cos-1, T47D-Y cells, T47D cells stably expressing PR-B, and HeLa cells were cultured as previously described (13, 29, 52). T47D-K-A, T47D-K-Q, T47D-K-T, and T47D-K-R stable cell lines were produced using T47D-Y cells transiently transfected with pSG5-hPR1 (K-A, K-Q, K-T, or K-R) and pSV-neo plasmids using FuGENE 6 (Roche Diagnostics, Indianapolis, IN) as described previously (13). Colonies formed from single cell clones were selected in 500 μg/ml and maintained in 200 μg/ml G418 (Calbiochem, La Jolla, CA).

Immunoblotting

Cell lysis and Western blotting procedures were performed as described previously (31). Ab-8 (NeoMarkers, Fremont, CA) and H-190 antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were used to blot for total PR, whereas antibodies recognizing Ser190 and Ser 294 phosphorylated PR were acquired from NeoMarkers. Ser345 and Ser400 phospho-PR antibodies were previously described (29, 31). Monoclonal β-actin antibody (Sigma-Aldrich, St. Louis, MO) was used for loading controls. For infrared Western blotting, the Odyssey

Infrared Imaging System (LI-COR Biosciences, Lincoln, NE) was used as previously described (13).

Acetylation assay

Cos, HeLa, or T47D-YB cells were plated at a density of 600,000 cells per 100-mm dish; 1 μg of plasmid DNA was transfected into Cos or HeLa cells using diethylaminoethyl-Dextran or FuGENE 6 (Roche Diagnostics) according to the manufacturer's instructions. Cells were starved for 24 h and then pretreated for 30 min with 10 μM TSA (Sigma-Aldrich) and/or 10 μM PP2 (Calbiochem), 10 μM UO126 (Calbiochem), 10 μM SB203580 (Calbiochem), and 10 μM LY294002 (Calbiochem) before treatment with 10^{-8} M R5020 (NEN, Boston, MA) or vehicle. RIPA Lite (150 mM NaCL, 0.01 M sodium phosphate, 0.01 M sodium dihydrogen phosphate, 2 mM EDTA, and 1% Triton X-100) was supplemented with inhibitors [0.1 M sodium fluoride, 0.2 M sodium vanadate, 0.1 M phenylmethylsulfonyl fluoride, 2 mg/ml aprotinin, 1 M β-glycerophosphate, a Complete Protease Cocktail Inhibitor Tablet (Roche Diagnostics), and a PhosSTOP tablet (Roche Diagnostics)] and used for cell lysis; 750–1000 μg of total protein was immunoprecipitated with Ab-8, total PR, antibody (NeoMarkers) or control normal mouse IgG (Santa Cruz Biotechnology, Inc.), and protein G agarose beads (Roche Diagnostics). Immune complexes were washed and subjected to SDS-PAGE for Western blotting. Membranes were immunoblotted with antiacetylated Lys antibodies (no. 9441; Cell Signaling, Beverly, MA) to detect acetylated proteins. Membranes were then stripped and blotted with H-190, total PR, antibodies (Santa Cruz Biotechnology, Inc.) to check efficacy of the immunoprecipitations.

Fractionation assay

HeLa cells were seeded in 100-mm dishes at a density of 800,000 cells/dish. FuGENE 6 (Roche Diagnostics) was used for transfection of 1 μg of plasmid DNA. Cells were starved for 24 h and then treated with 10^{-8} M R5020 or vehicle. Cells were collected in cold 1× PBS and spun at low speed for 5 min. To separate cytoplasmic fractions, cells were lysed in cytoplasmic extract buffer [0.25 M HEPES (pH 7.6), 0.15 mM potassium chloride, 4 μM EDTA, 2.5 μM dithiothreitol] containing 0.3% Nonidet P-40 plus inhibitors [5 nM sodium fluoride, 1 nM sodium vanadate, 1 nM phenylmethylsulfonyl fluoride, 0.02 mg/ml aprotinin, 5 nM β-glycerophosphate, and a Complete Mini Protease Cocktail Inhibitor Tablet (Roche Diagnostics)] and spun at low speed for 4 min. Supernatant was collected and spun at high speed for 5 min. Nuclear pellets were washed and resuspended in nuclear extract buffer [50 μM Tris solution (pH 8.0), 1.05 mM sodium chloride, 3.75 μM magnesium chloride, 0.5 μM EDTA, and 0.25% vol/vol glycerol] containing 0.155 μM NaCl plus inhibitors (as described above) and spun at high speed for 10 min. WCL were taken in supplemented RIPA buffer (53). WCL and nuclear and cytoplasmic fractions were subjected to Western blotting using total PR (Ab-8; NeoMarkers) antibodies. Topoisomerase II (TopoGEN, Port Orange, FL) and phosphatidylinositol 3 kinase p85 (Millipore, Billerica, MA) antibodies were used as markers of nuclear and cytoplasmic fractions, respectively.

Confocal microscopy

Confocal microscopy was performed as explained previously (34). Briefly, cells were plated on coverslips in six-well plates and starved overnight. After treatment, cells were fixed in 3.7% formaldehyde in PBS for 20 min at room temperature, washed three times, then permeabilized with 0.1% Triton X-100 in PBS for 5 min at room temperature. Coverslips were washed and blocked in 1% normal goat serum in PBS for 5 min. Primary antibody incubation was done with PR antibody, Ab-8 (NeoMarkers), diluted (1:200) in 0.5% normal goat serum in PBS for 1 h. After three washes, secondary antibody incubation was performed using Molecular Probes Alexa-Fluor 488 antimouse secondary antibody (Invitrogen, Carlsbad, CA) diluted (1:500) in 0.5% normal goat serum in PBS for 30 min. Washed coverslips were mounted on slides using ProLong Gold Anti-fade with DAPI (Invitrogen). Images were obtained with an Olympus FV-500 confocal microscope using 20 or 60× oil-immersion objectives (Olympus Corp., Center Valley, PA). Quantification of fluorescent images and determination of nuclear localization were performed by ImageJ version 1.42q software as described previously (54). Cell nuclei were defined using the DAPI signal. Division of the nuclear intensity of PR staining by the intensity of the total cellular PR staining was performed to determine the percent of nuclear localization.

Luciferase assay

PR transcriptional activity was quantified as described previously (55). Concisely, HeLa cells were plated in six-well plates at a density of 125,000 cells/well and were transfected using FuGENE HD (Roche Diagnostics) with 0.05 μg wt PR-B or K-A PR-B plasmid, 0.5 μg 2XPRE-Luciferase plasmid, and 0.03 μg pRL-TK, for *Renilla* control. Cells were serum starved overnight, treated for 18 h with 10^{-8} M R5020, lysed, and analyzed using the Dual-Luciferase Reporter Assay System from Promega according to the manufacturer's protocol (Promega, Madison, WI).

RT-PCR and qPCR

RNA isolation, synthesis of cDNA, and qPCR were executed as previously described (31). Briefly, T47D variant cell lines were starved in serum-free (*i.e.* for protein kinase-related studies) or 5% DCC-containing media (*i.e.* for experiments involving hormone addition), treated for various times with vehicle or R5020 (10^{-8} M), then harvested in Trizol for RNA isolation; no appreciable differences were observed between results obtained from cells routinely grown in DCC-containing media relative to serum-free conditions (within a given cell line).

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