

Protein kinases mediate ligand-independent derepression of sumoylated progesterone receptors in breast cancer cells

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In advanced breast tumors, protein kinases are upregulated and steroid hormone receptors often function independently of ligand. Herein, we explored mechanisms of ligand-independent progesterone receptor (PR) activity. We showed previously that growth factor-induced phosphorylation of PR Ser-294 blocks PR Lys-388 sumoylation. SUMO-deficient mutant PR-B (K388R) thus provides a model receptor for the study of PR function in the context of high kinase activities. T47D cells stably expressing K388R PR-B exhibited increased ligand-independent proliferation and growth in soft agar relative to cells expressing wt PR-B or phospho-mutant (sumoylated) S294A PR-B. Expression of selected PR target genes (HB-EGF, IRS-1, and STC1) was significantly elevated in cells containing desumoylated (K388R) PR-B. Basal PR transcriptional activity occurred independently of progestins, was increased by activated CDK2, and attenuated by RU486. Notably, ChIP assays demonstrated that K388R PR-B and SRC1 were constitutively recruited to the STC1 promoter in the absence of progestin; PR Lys-388 sumoylation was required for HDAC3 recruitment. Knock-down of STC1 inhibited proliferation of cells expressing K388R PR-B. These data suggest a mechanism whereby phosphorylated, and thus desumoylated, PRs mediate increased expression of growth promoting genes. Our data explain why breast cancer models often remain insensitive to progestins, but are growth-inhibited by antiprogestins, and underscore the need to target PR-B and associated kinase activities as part of breast cancer therapy.

HDAC | phosphorylation | sumoylation

Progesterone receptors (PR) mediate lobulo-alveolar proliferation during breast development and contribute to breast cancer progression, in part by synergizing with peptide growth factors (1–3). Regulation of PR activity occurs via the integration of ligand activated rapid signaling events and transcriptional activation (4). Peptide growth factors diminish the requirement for steroid hormone ligands by activation of protein kinases that directly target PR and its co-activators (i.e., a function of rapid signaling events). For example, direct phosphorylation of PR by MAPK or CDK2 alters transcriptional activity in part by modulating other posttranslational modifications including ubiquitination and sumoylation (3, 5). Crosstalk between growth factor pathways and steroid receptors provides an exquisite mechanism for mammary epithelial cells to sense a dynamic range of hormone concentrations and selectively regulate specific promoters.

Sumoylation of steroid hormone receptors represses transcriptional activity (6). We showed that EGF and protein kinase-dependent (MAPK, CDK2) phosphorylation of PR-B on Ser-294 blocks sumoylation of PR Lys-388 (5). Undersumoylated receptors respond to low concentrations of ligand and are transcriptionally hyperactive at a subset of PRE (progesterone response element) containing target genes (HB-EGF). Sumoylation of PR is thus a key modulator of both the progesterone/PR dose–response curve and PR target gene selection. Breast cancer cells containing phosphorylated, undersumoylated PR display increased cell growth in response to progestins, suggesting that modified receptors target growth promoting genes (5).

Accumulating evidence suggests that ligand-independent actions of PR are biologically relevant (7, 8). Herein, we hypothesized that PR sumoylation/desumoylation contributes to the regulation of hormone-independent PR activity. We focused on SUMO-deficient mutant (K388R) PR-B as a model for PR action in breast cancer cells with high kinase activity, where phosphorylation events block PR Lys-388 sumoylation (5).

Results

Sumoylation of PR Represses Basal Proliferation in Breast Cancer Cells.

Sumoylation of PR-B is rapidly induced by both agonist (R5020) and antagonist (RU486) (5). We measured the levels of wt, K388R (SUMO-deficient mutant), or S294A (phospho-mutant) PR-B sumoylation in HeLa cells using the SUMO assay previously described for PR (5). Following 24 h treatment with either progestin (R5020) or vehicle (EtOH), unmodified and upshifted, sumoylated PRs were visualized by Western blotting (Fig. 1A). Consistent with previous reports (5, 9), wt PR-B, but not K388R PR-B, was sumoylated in the presence of exogenous SUMO-1, and PR sumoylation was increased in response to progestin (lane 4). Notably, we detected partial sumoylation of wt PR-B, but not K388R, in the absence of ligand (lane 3) indicating that this modification occurs in unstimulated (serum starved) cells. In a similar experiment, we measured sumoylation of wt PR-B relative to phospho-mutant S294A PR-B (Fig. 1B). Wt PR-B was partially sumoylated within 1 h of R5020 treatment. S294A mutant PR-B cannot undergo Ser-294 phosphorylation, an event that blocks K388 sumoylation (5). Consistent with negative regulation of PR sumoylation via phosphorylation at this site, S294A PR-B displayed increased sumoylation compared to wt PR-B in the absence and presence of R5020.

The detection of modest levels of basally sumoylated PRs (Fig. 1A) prompted us to examine the contribution of PR sumoylation to PR-induced breast cancer cell growth, particularly in the absence of ligand. For these experiments, we used T47D breast cancer cells stably expressing either wt, K388R, or S294A PR-B and selected clones for expression of equal receptor levels (Fig. 1C, inset) (5). Cells were cultured in steroid-free media supplemented with either vehicle or R5020 for 6 days and viable cells were quantified by MTT assay as a measure of their basal and progestin-stimulated growth (Fig. 1C). Progestin had no significant effect on the growth of T47D cells. However, surprisingly, multiple clones of T47D cells stably expressing the SUMO-deficient PR-B mutant (K388R) consistently displayed increased basal proliferation relative to wt PR-B expressing cells (Fig. 1C clone 7; [supporting information (SI) Fig. S1A clone 28]). In contrast, cells expressing heavily sumoylated

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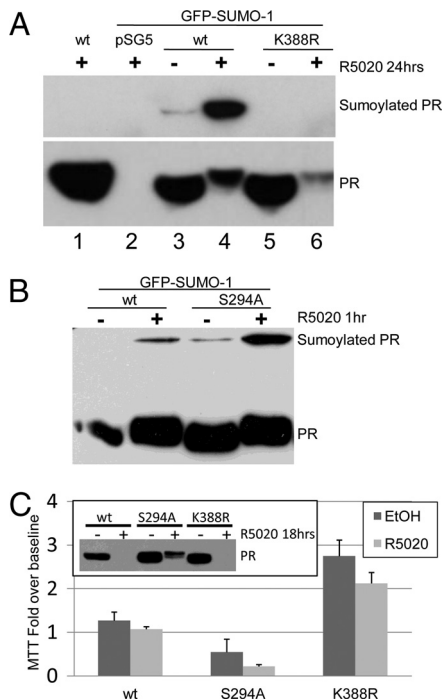


Fig. 1. Sumoylated PR represses breast cancer cell growth in the absence of progestins. (A) HeLa cells were transfected with either wt or K388R PR-B and either vector or EGFP-SUMO-1, starved for 24 h, treated with R5020 (10^{-8} M) for 24 h, and Western blotted for PR. (B) HeLa cells were transfected as in (A), except with wt or S294A PR-B, and treated with R5020 for 1 h, and Western blotted for PR. (C) Triplicate cultures of T47D cells stably expressing wt, S294A, or K388R (clone 7) PR-B were subjected to MTT assays under steroid hormone-free conditions supplemented with vehicle (EtOH) or R5020 for 6 days. Bars (y axis) represent fold increases in viable cell number over baseline (\pm SD). (Inset) T47D cells stably expressing either wt, S294A, or K388R PR-B were starved for 24 h, treated with vehicle or R5020 for 24 h, and Western blotted for PR.

S294A PR-B exhibited decreased basal proliferation relative to cells containing wt PR-B (Fig. 1C). In the absence of progestin, PR-B expression levels remained comparable between stable cell lines (Fig. 1C, inset). Liganded S294A PR-B are stabilized relative to wt or K388R PR (Fig. 1C, inset), consistent with the finding that PR-B Ser-294 phosphorylation augments ligand-induced downregulation by the ubiquitin-proteasome pathway (10).

In MTT assays (6 days), adherent T47D cells are minimally responsive to progestin (Fig. 1C), perhaps owing to the biphasic effects of progestins in short-term 2D cultures (*SI Results*) (11). We thus performed soft agar assays to examine the contribution of desumoylated PR to long-term (21 days) anchorage independent growth (Fig. S1B). Similar to MTT results, in the absence of added steroid hormone, T47D cells expressing SUMO-deficient mutant PR-B displayed significantly increased colony formation relative to wt PR-B expressing cells. In contrast, basal growth of cells expressing heavily sumoylated S294A PR-B was lower relative to wt controls, suggesting that breast cancer cell proliferation and survival in the absence of progestin is highly sensitive to the degree of PR sumoylation.

Sumoylation of PR-B Represses Transcription of a Subset of Target Genes.

To address the question of a direct role for PR sumoylation/desumoylation in breast cancer cell proliferation in the absence of progestins (Fig. 1C and S1), we investigated the regulation of selected endogenous gene targets by unliganded PR. Stanniocalcin 1 (STC1), a peptide hormone overexpressed in breast cancers, has been shown to increase cell metabolism and tumor growth (12). Gene array analysis of T47D breast cancer cells demonstrated that

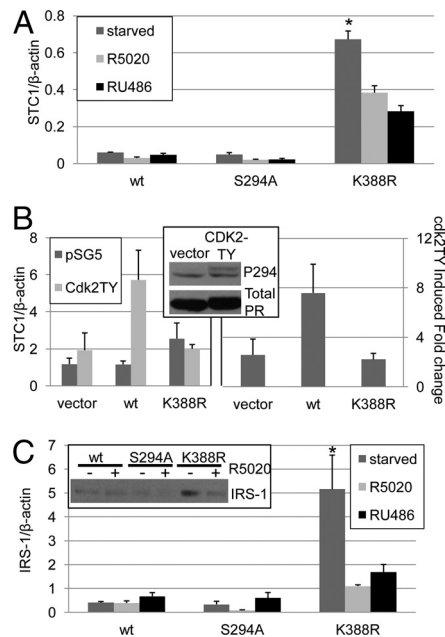


Fig. 2. Phospho-dependent regulation of PR sumoylation alters target gene levels. (A) T47D cells stably expressing wt, S294A, or K388R PR-B were plated in triplicate cultures and serum starved or treated with R5020 (10^{-8} M) or RU486 (10^{-7} M) for 48 h. Real-time PCR was performed and STC1 was normalized to β -actin (\pm SD, $*P < 0.0001$). (B) HeLa cells in triplicate cultures were transfected with vector, wt or K388R PR-B and either vector or CDK2TY. (Left panel) Real-time PCR was performed and STC1 was normalized to β -actin (\pm SD). (Right panel) Data from multiple experiments were combined and expressed as CDK2TY induced fold change over vector control (\pm SEM). (Inset) HeLa cells transfected with wt PR-B and either vector or CDK2-TY were starved for 24 h and Western blotted using phospho-S294 PR and total PR antibodies. (C) Triplicate cultures of T47D cells stably expressing wt, S294A, or K388R PR-B were serum starved or treated with R5020 (10^{-8} M) or RU486 (10^{-7} M) for 48 h, real-time PCR was performed, and IRS-1 was normalized to β -actin (\pm SD, $*P < 0.01$). (Inset) T47D cells stably expressing wt, S294A or K388R PR-B were serum starved for 24 h and treated with ethanol or R5020 (10^{-8} M) for 18 h then Western blotted for IRS-1.

STC1 expression is induced by the forced expression of unliganded PR-A or PR-B (7). We therefore examined the contribution of sumoylation to PR-B mediated STC1 regulation using real-time PCR. T47D cells stably expressing equal levels (Fig. 1C inset) of either wt, K388R or S294A PR-B were serum starved or treated with R5020 or RU486 for 48 h. In the absence of ligand, cells expressing wt or S294A PR-B exhibited low basal levels of STC1 expression and STC1 mRNA levels remained insensitive to either PR agonist (R5020) or PR antagonist/partial agonist (RU486). However, cells stably expressing K388R PR-B displayed greatly heightened STC1 transcript levels (Fig. 2A clone 7; Fig. S2 clones 24 and 28). Surprisingly, in these cells, addition of either R5020 (agonist) or RU486 (antagonist/partial agonist) for 48 h decreased basal STC1 mRNA expression by unknown mechanisms (see below), but demonstrates the specificity of STC1 regulation by desumoylated receptors. Similar results were observed following 6 h of hormone (Fig. S3). These data suggest that in the absence of ligand, STC1 is positively regulated by desumoylated PR.

PR sumoylation is negatively regulated by growth factors that input to activation of Cdk2 or MAPKs (ERK1/2) or by overexpression of these kinases (5). Although PR are multiply phosphorylated, primarily at Ser residues, the effects of these kinases on PR sumoylation mapped to phosphorylation of PR Ser-294 (5). We therefore tested the sensitivity of PR mediated STC1 expression to activated protein kinases in the absence of progestins. For these experiments, we used PR-null HeLa cells, because of their high transfection efficiency relative to T47D cells. PRs expressed in

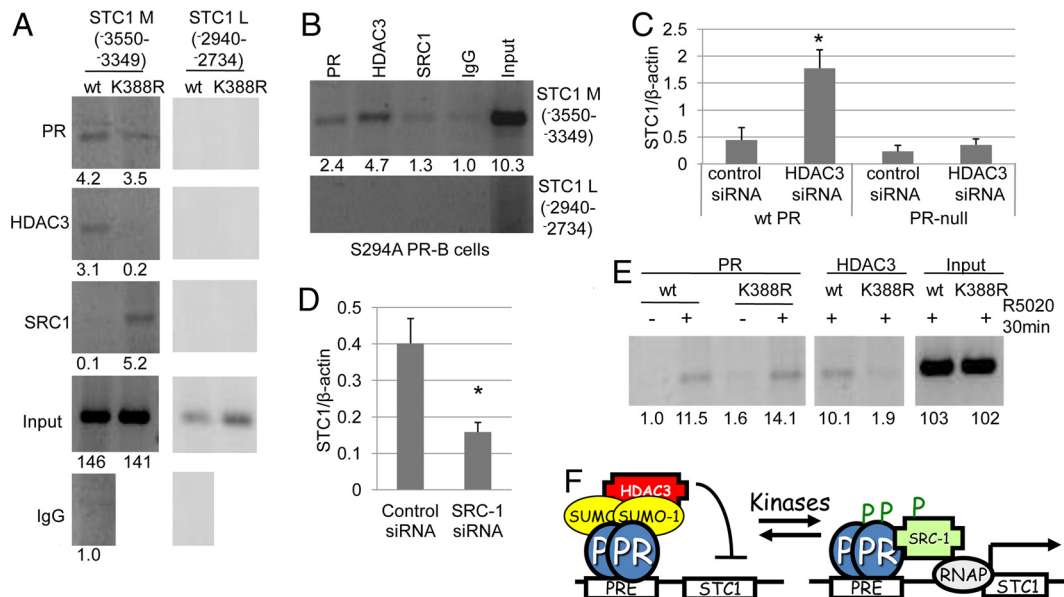


Fig. 3. PR sumoylation alters the recruitment of cofactors to PR target promoters. (A) T47D cells stably expressing wt or K388R PR-B were serum starved for 48 h and subjected to ChIP assays using antibodies against PR, HDAC3, or SRC1 (primers specific for the STC1 promoter). Normal rabbit IgG was used as an isotype control. (B) T47D cells stably expressing S294A PR-B were serum starved for 48 h and subjected to ChIP assays using antibodies against PR, HDAC3, or SRC1 or normal rabbit IgG and primers specific for the STC1 promoter. (C) PR-null HeLa cells and HeLa cells stably expressing wt PR-B were transfected with HDAC3 or negative control siRNA, and starved for 72 h, real-time PCR was performed, and STC1 was normalized to β -actin (\pm SD, $*P < 0.006$). (D) T47D cells stably expressing K388R PR-B were transfected with SRC1 or negative control siRNA, real-time PCR was performed, and STC1 was normalized to β -actin (\pm SD, $*P < 0.005$). (E) ChIP assays were performed in T47D cells stably expressing either wt or K388R PR-B using antibodies against PR and HDAC3 and primers specific for the HB-EGF promoter. Asterisk denotes statistical significance relative to cells transfected with control siRNA as determined by unpaired Student *t* tests. Clone 7 T47D K388R PR-B cells were used throughout these experiments. Bands on gels were quantified by densitometry (values are listed below each band). (F) High kinase activity in breast cancer cells blocks PR sumoylation and activates expression of growth promoting genes in the absence of progestins via altered cofactor recruitment.

expression of SRC-1. T47D cells stably expressing K388R PR-B were transiently transfected with SRC-1 or nontargeted control siRNA (Fig. S5D). As predicted, SRC-1 siRNA, but not control siRNA, reduced STC1 mRNA expression in T47D cells stably expressing SUMO-deficient K388R PR-B (Fig. 3D). These data support the conclusion that constitutive association of unsumoylated PR with SRC-1 can drive transcription (i.e., contribute to the basal expression) of novel PR target genes in the absence of progestins (Fig. 3F).

Finally, we tested HB-EGF as a progestin regulated gene whose basal expression is insensitive to PR sumoylation, but responsive to hormone addition, in part via PR phosphorylation and desumoylation (Fig. S4B). In the presence of R5020, roughly equivalent amounts of both wt and K388R PR-B were recruited to the HB-EGF promoter region -1631–1266, containing four PRE half sites (Fig. 3E). Interestingly, however, upon R5020 treatment, more HDAC3 was consistently associated with the HB-EGF promoter in cells containing wt PR-B, relative to cells containing K388R PR-B. These data suggest that PR-B sumoylation is required for efficient recruitment of HDAC3 to selected promoters (STC1 and HB-EGF); desumoylated PR-B may fail to maintain a stable complex with HDAC3 resulting in increased gene transcription (Figs. 2A and 3F and Fig. S4B).

Hormone-Independent PR Target Genes Contribute to Breast Cancer Cell Growth. Multiple PR-target genes likely contribute to increased breast cancer cell growth and survival in cells with under-sumoylated PRs (Fig. 1C and S1) (5). IRS-1 is a key mediator of IGF-1-dependent breast cancer cell proliferation (17). We reasoned that cells expressing K388R PR-B should be more responsive to IGF-1 due to PR-dependent elevated IRS-1 expression. T47D cells expressing either wt, K388R or S294A PR-B were treated for 4 or 5 days with IGF-1, and viable cells were quantified by MTT assay (as in Fig. 1C). Cells expressing wt PR-B were modestly

responsive to IGF-1 as measured by a 2- to 3-fold increased cell growth after 4–5 days (Fig. 4A). In contrast, cells expressing SUMO-deficient K388R PR-B demonstrated an increased response to this hormone (5–7-fold), whereas cells containing heavily sumoylated S294A PR remained relatively unresponsive (<2-fold) to IGF-1. These data suggest that the PR sumoylation state in the absence of progestins contributes to the sensitivity of breast cancer cells to IGF-1, in part via IRS-1 transcriptional upregulation (Fig. 2C).

In addition to IRS-1, our studies implicate PR-dependent STC1 up-regulation in the increased basal proliferation of breast cancer cells expressing SUMO-deficient K388R PR-B (Fig. 1C and S1). To directly test the contribution of STC1 to cell proliferation, T47D breast cancer cells expressing either wt or K388R PR-B were engineered to stably express either STC1 shRNA or control shRNA. In both wt and K388R PR-B expressing cell lines, we routinely observed an approximate 50% decrease in STC1 transcript levels relative to control shRNA (Fig. S6). Further knockdown of this transcript was not achievable in stable cell lines, perhaps owing to the pro-proliferative function of this gene (12). Importantly, the stable knockdown of STC1 did not alter PR-B expression in either cell line (Fig. S6 inset). The specificity of STC1 knockdown was confirmed, as measured by constant levels of STC2 (Fig. S7A), a 34% homologous STC family member that is regulated by both progestin (Fig. S7B) and estrogen (19, 20) in breast cancer cells.

We then performed MTT assays in steroid hormone-free conditions using T47D cells stably expressing either wt or K388R PR-B and control shRNA or STC1 shRNA (Fig. 4B). Consistent with previous results (Fig. 1C), cells expressing SUMO-deficient K388R PR-B and control shRNA exhibited significantly increased proliferation (day 6) relative to cells expressing wt PR-B and either shRNA. However, partial knockdown of STC1 shRNA significantly blocked excess growth of cells expressing K388R PR-B. Importantly,

Clinical Implications of PR Sumoylation/Desumoylation. High intracellular kinase activity is a hallmark of breast cancer with implications for steroid receptor action in women with and without measurable circulating hormone levels. Notably, as with ER- α , PR expression in human breast tumors is predictive of a favorable prognosis. As tumors progress toward hormone-refractory stages, they most often keep their steroid hormone receptors. In the face of elevated signal transduction from tyrosine kinase growth factor receptors, both ER and PR remain highly active, but undergo rapid protein turnover, making them more difficult to detect (10, 28). In contrast to the normal breast where PR-A and PR-B are co-expressed at equal levels, breast cancers often contain aberrant PR-A:PR-B ratios, an early event in breast carcinogenesis (29). As tumors progress, frequent high A:B ratios are most often associated with loss of PR-B protein (rather than gain of PR-A) (30). In fact, PR loss is an excellent marker of heightened protein tyrosine kinase activities and tamoxifen resistance (31). Furthermore, cyclin E/cdk2, a direct input to PR phosphorylation at sites (including Ser-294) that augment both turnover (10) and transcription (32), is associated with breast cancer progression (16). We propose that in the case of PR-B, phosphorylated and undersumoylated receptors turnover rapidly and may be difficult to detect in clinical settings (primarily conducted by immunohistochemistry analyses). These unliganded but hyperactive receptors may contribute to breast cancer proliferation by the novel mechanism of PR derepression detailed herein. Our data explain why breast cancer models often fail to respond to added progestins, but are growth inhibited by anti-progestins, and underscore the need to routinely target PR as part of endocrine-based breast cancer therapy in combination with

kinase pathway inhibitors and selective estrogen receptor modulators (SERMS) or aromatase inhibitors. This approach may be especially warranted in apparently steroid hormone receptor negative cases, where phosphorylated receptors may exhibit shortened half-lives but heightened transcriptional activities or derepression at key growth-promoting genes.

Materials and Methods

Cell Culture. T47D-Y, -YB, -K388R (clones 7, 24, 28) and -S294A cells were previously described (5, 33, 34). HeLa cells were cultured as described previously (10, 13).

Immunoblotting. SUMO assays were carried out as previously described (5). Membranes were probed with primary antibodies recognizing total PR (MS-298, Lab Vision Corp., Fremont, CA), phospho-S294 PR (Lab Vision Corp.), p44/p42 MAPK (Cell Signaling Technology, Danvers, MA), and IRS-1 (35).

RT-PCR and Real-Time Quantitative PCR. RNA isolation, cDNA synthesis, and real-time quantitative PCR were performed as previously described (*SI Materials and Methods*) (4).

Growth Assays. Soft agar and MTT assays were performed as previously described (35, 36).

Chromatin Immunoprecipitations. ChIP assays were performed as previously described (37). Antibodies used were PR (H-190), HDAC3, SRC-1, and IgG (Santa Cruz Biotechnology Inc., Santa Cruz, CA) or RNAPolII (Covance, Emeryville, CA).

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