Mechanisms Underlying the Control of Progesterone Receptor Transcriptional Activity by SUMOylation*

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Posttranslational modification by small ubiquitin-like modifier (SUMO) is a major regulator of transcription. We previously showed that progesterone receptors (PR) have a single consensus ψKXE SUMO-conjugation motif centered at Lys-388 in the N-terminal domain of PR-B and a homologous site of PR-A. SUMOylation of the PR is hormone-dependent and has a suppressive effect on transcription of an exogenous promoter. Here we show that repression of PR activity by SUMOylation at Lys-388 is uncoupled from phosphorylation, involves synergy between tandem progesterone response elements, and is associated with lowered ligand sensitivity and slowed ligand-dependent down-regulation. However, paradoxically, cellular overexpression of SUMO-1 increases PR transcriptional activity even if Lys-388 is mutated, suggesting that the receptors are activated indirectly by other SUMOylated proteins. One of these is the coactivator SRC-1, whose binding to PR and enhancement of agonist-dependent N-/C-terminal interactions is augmented by the presence of SUMO-1. Increased transcription due to SRC-1 is independent of PR SUMOylation based on assays with the Lys-388 mutants and the pure antiprogestin ZK98299, which blocks N-/C-terminal interactions. In summary, SUMOylation tightly regulates the transcriptional activity of PR by repressing the receptors directly while activating them indirectly through augmented SRC-1 coactivation.

Human progesterone receptors (PR)2 exist as three isoforms, PR-A (~94 kDa), PR-B (~110 kDa), and PR-C (~60 kDa) (1). Like other steroid receptors (2), PR are multidomain proteins, consisting of a central DNA-binding domain (DBD); large N termini with a proximal activation function (AF-1) common to PR-A and PR-B; a distal AF-3 in the PR-B-upstream segment restricted to PR-B; and, at the C terminus of all three receptors, a nuclear localization signal in the hinge region upstream of a ligand binding domain (LBD) containing an AF-2 (1, 3–5). PR-A and PR-B are equimolar in most human tissues, but this balance is skewed in breast malignancies (6, 7). In most exogenous contexts as well as on endogenous genes, PR-B are stronger transactivators than PR-A (3, 8–10) due to synergism between AF3 and downstream AFs. However, for the most part, the two receptors regulate distinct, nonoverlapping genes and functions that are tissue-, cell-, and promoter-specific. PR-C lack the entire N terminus and first DBD zinc finger and cannot bind DNA (1). They are up-regulated in the endometrium at parturition, where they suppress the actions of PR-A and PR-B by sequestering progesterone (7).

PR-A and PR-B are transactivators that can be tethered to DNA through other transcription factors, including SP-1 (11), AP-1, the RelA (p65) subunit of NF-κB (12–14), or STAT5 (15). More commonly, however, PR bind DNA directly at progesterone-response elements (PREs). Compound PREs on exogenous promoters reportedly generate synergistic patterns of responses to PR, in which the total exceeds the sum of responses at single PREs (16).

DNA-bound PR interact with multiple transcriptional coregulators and with the basal transcriptional machinery (17). Coactivators are involved in transcriptional synergy (18) by enhancing recruitment, assembly, and stabilization of multiprotein complexes on DNA-bound receptors. The principal interaction between nuclear receptors and coactivators maps to the receptor C terminus and is hormone- and AF2-dependent. However, the p160 coactivator SRC-1 (19) and others (20, 21) also bind with lower affinity to N-terminal domains (22). Coactivator-mediated synergy is due to intensified intramolecular binding between the N- and C-terminal domains of receptors. For example, TIF-2 binds both the N- and C-terminal AFs of estrogen receptors (ER). Indirect N-/C-terminal interactions are similarly facilitated by bridging factors for other steroid receptors. These include SRC-1, SRC-2, JDP-2, and cAMP-response element-binding protein (CREB)-binding protein (CBP)/p300 for PR (19, 23–25); Ada2, TBP, and CBP for glucocorticoid receptors (GR) (26, 27); and SRC-1, SRC-2, CBP, and the RAP74 subunit of transcription factor IIF for androgen receptors (AR) (28, 29).

In sum, transcriptional synergy is highly complex and coactivator-driven and requires interaction among AFs within N and C termini of receptors, cooperative interactions among tandem receptors on compound DNA response elements, and recruitment and stabilization of multiprotein complexes on DNA (30, 31).
A growing list of transcription factors are modified by conjugation to the 97-amino acid ubiquitin-like modifier, SUMO. SUMO-1 is covalently attached by a series of enzymatic reactions to lysine residues embedded in a consensus sequence, ΨKXE (where Ψ is a large hydrophobic amino acid) of client proteins. Unlike ubiquitin, which stably attaches to proteins in multimeric chains, SUMO-1 attaches to proteins as a monomer in a dynamic, reversible process. SUMOylation influences varied processes, including protein stability, activity, cellular localization, and interactions with other proteins (32). It tends to repress transcription; hence, disruption of SUMO acceptor Lys residues generally increases activity (33–37).

Most but not all steroid receptors (the exception appearing to be ER) are targets of SUMOylation in the N-terminal AF-1 domain, a covalent modification that suppresses their transcriptional activity by preventing cooperativity at complex promoters. Mineralocorticoid receptors are SUMOylated at four acceptor sites located at GR Lys-297 and Lys-313, overlaps with the consensus SUMO attachment sites and disrupts SUMOylation. The isolated motifs inhibit GR transcription in trans at compound DNA binding sites.

The large N terminus of AR is SUMOylated in a ligand-dependent manner at Lys-385 and Lys-511 (43). Mutation of Lys-385, the major functional site, increases AR-dependent transcription from multiple hormone response elements. Thus, as in the case for mineralocorticoid receptors and GR, SUMOylation of AR suppresses their ability to synergize on compound promoters.

Whether ER are targets of SUMOylation remains unclear. Although the other steroid receptors contain clearly identifiable ΨKXE SUMO acceptor sites, neither ERα nor ERβ have sequences matching this consensus (44). This is consistent with the fact that phylogenetic and sequence alignments show that GR, mineralocorticoid receptors, AR, and PR belong to a subfamily of steroid receptors characterized by much larger N termini (ranging from 420 to 602 amino acids) than the N termini of ERα or ERβ (184 and 148 amino acids, respectively) (45, 46). Indeed, Poukka et al. (44) show that in vitro translated AR and GR, but not ERα or ERβ, are SUMOylated in the presence of the conjugating enzyme, Ubc9. Nevertheless, a recent study reported SUMOylation of ERα at hinge region Lys-266 and Lys-268. These and other hinge region mutants impaired, rather than activated, ER-driven transcription, effects of SUMOylation opposite to those observed with the large steroid receptors and other transcription factors (47).

We have shown that PR SUMOylation maps to Lys-388 in the single SUMO attachment consensus site of PR-B and a related site of PR-A in the N terminus adjacent to AF-1. SUMO-1 attached to PR covalently at this site in a ligand- and LBD-dependent manner (48). PR-C lack an N terminus and would not be SUMOylated. Like the other large steroid receptors, a single-point mutation of PR at Lys-388 increases transcriptional activity 5–10-fold over that of their wild-type counterparts on multimerized PREs but not on mouse mammary tumor virus. Daniel et al. (49) recently reported that PR SUMOylation at Lys-388 is negatively regulated by phosphorylation of Ser-294 in response to mitogenic signaling.

In sum, SUMOylation of steroid receptors suppresses their synergistic transcriptional activity on compound promoters. The present studies address mechanisms for suppression of transcriptional synergy by PR SUMOylation, focusing on effects of this covalent modification on ligand sensitivity, ligand-dependent receptor down-regulation, and N-/C-terminal interactions and the role of the coactivator SRC-1. We find that SUMOylation targets each of these steps.

**EXPERIMENTAL PROCEDURES**

**Plasmids**—The expression plasmids pSG5 hPR1 and pSG5 hPR2, encoding human PR-B and PR-A, respectively, were a gift of P. Chambon (Strasbourg, France). Cloning of pSG5 hPR1 K388R, pSG5 hPR2 K388R, pSG5 hPR1 S294A/S344A/S345A, pSG5 NT-B, and pSG5 DBD-LBD were described previously (16, 48, 50). Wild type pEGFP-SUMO-1 was a gift of J. Palvimo and O. Janne (University of Helsinki, Helsinki, Finland). pCR3.1-SRC-1 was a gift of B. O’Malley (Baylor College of Medicine, Houston, TX). pΔTAT1-Luc, pΔTAT2-Luc, and pΔTAT3-Luc reporter plasmids, containing one, two, and three PREs, respectively, were gifts of J. Iniguez-Lluhi (University of Michigan, Ann Arbor, MI).

**Cell Culture**—HeLa cells were maintained in Dulbecco’s modified Eagle’s medium with 7.5% fetal bovine serum and penicillin/streptomycin. T47D-Y, T47D-YA, and T47D-YB cells were cultured as described previously (3). T47D-YA K388R and T47D-YB K388R cells were generated from T47D-Y cells transfected with pSG5 hPR2 K388R or pSG5 hPR1 K388R, respectively, and a pSV-neo plasmid and then selected for antibiotic resistance. Electrotransfection used a Gene Pulser II (Bio-Rad). Colonies were selected as described previously (3).

**Transcription Assays**—HeLa cells were plated in minimum Eagle’s medium containing 5% fetal bovine serum (twice charcoal-stripped for experiments with full-length PR or DBD-LBD) at a density of 1.2 × 10^5 cells/60-mm dish, 1 day prior to transfection. Cells were transfected by calcium phosphate coprecipitation (16) with concentrations of expression vectors indicated in the figures. Reporter plasmids were added at 2 μg/dish. SV40- Renilla luciferase was added as an internal control at 20 ng/dish. Twenty-four hours later, cells expressing LBD-containing constructs were washed and incubated for 24 h with the synthetic progestin R5020 (Sigma) at the final concentrations indicated in the figures. Control cells received ethanol only. Cells were collected in 150 μl of lysis buffer (Promega), and 50 μl were analyzed on a dual luminometer (16). Results were normalized to Renilla luciferase activity and expressed as...
indicated in the figures. Replicate experiments were done in duplicate.

**Immunoblotting**—Whole cell extracts were prepared from HeLa cells transiently transfected with PR expression vectors as described (48). Cell were treated with 10 nM R5020 or a 100 nM concentration of the antiprogestins RU486, ZK98299, and ZK112993. Lysates containing equal protein concentrations were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with anti-PR1294 (DakoCytomation), anti-β-actin AC-74 (Sigma) monoclonal antibodies, or anti-GFP polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Bands were detected by enhanced chemiluminescence (PerkinElmer Life Sciences). For PR SUMOylation, HeLa cells cotransfected with PR and GFP-tagged SUMO-1 were collected in phosphate-buffered saline containing 20 mM N-ethylmaleimide, and cell extracts were prepared in 50 mM Tris-HCl (pH 7.8), 150 mM NaCl, 5 mM EDTA, 15 mM dithiothreitol, a protease inhibitor mixture (Roche Applied Science), and 20 mM N-ethylmaleimide. The expressed proteins were resolved on SDSPAGE, and conjugated protein was detected by immunoblotting with PgR1294. For T47D cells stably expressing PR, 5 × 10^5 cells were plated and allowed to grow for 24 h, after which 10 nM R5020 (0–16 h) were added. Cell extracts were prepared for immunoblotting.

**Mammalian Two-hybrid Assay**—Interactions between PR N-terminal fragments and the PR LBD or SRC-1 were measured with the Mammalian Matchmaker two-hybrid system (Clontech, Palo Alto, CA) as described (16), using the constructs shown in Fig. 5, and a luciferase reporter containing five copies of the Gal-4 response element (5× Gal4-Luc). In this system, full-length PR-B, the PR-B N terminus (amino acids 1–555), and PR mutants were subcloned into the pVP16 vector and expressed as VP16 activation domain fusion proteins. The hinge-LBD was subcloned into the pM vector and expressed as a fusion protein with the DNA binding domain of Gal-4. Gal4-SRC-1 and VP16-SRC-1 were gifts of B. O’Malley (Baylor College of Medicine, Houston, TX). HeLa cells were transfected with a combination of the pM and pVP16AD fusion constructs together with the Gal4-luciferase reporter and 20 ng of Renilla-luciferase vector for normalization, using calcium phosphate.

**Statistical Analysis**—Prism GraphPad software version 4 was used to determine least squares best fit of the experimental data to the theoretical dose-response curve.

**RESULTS**

**SUMOylation Slows Ligand-dependent PR Down-regulation**—Mutation of Lys-388 in the PR N terminus augments transcriptional activity of the receptors (48). Location of the single N-terminal SUMOylation site is shown in Fig. 1A. Ligand-dependent down-regulation of PR is required for transcriptional activity (50), and is mediated by ubiquitination and 26 S proteasome degradation after phosphorylation of Ser-294 in the N terminus (50). To determine whether there is a link between PR SUMOylation and ligand-dependent down-regulation, ER+, PR−T47D-Y human breast cancer cells (3) were engineered to stably express comparable levels of unliganded wild-type PR-A or PR-B or the SUMOylation-deficient PR-A Lys-388 and PR-B Lys-388 mutants (Fig. 1, B and C). The Western blots show
protein levels of wild-type and mutant PR-A (Fig. 1B) and PR-B (Fig. 1C), following vehicle or R5020 (0–16 h) treatment. Down-regulation of the SUMOylation-deficient receptors was accelerated compared with their wild-type counterparts. This was especially evident for the PR-A mutant, which was 50% down-regulated by ~1.5 h compared with ~7.0 h for wild-type PR-A (Fig. S1).

Fig. 1 also shows the transcriptional activity of a transiently expressed PRE2 reporter in cells stably expressing wild-type PR or the PR-A Lys-388 (Fig. 1D) or PR-B Lys-388 (Fig. 1E) mutants and treated with 10 nM R5020 for 24 h. Transcription by wild-type PR-A was augmented a further 5–6-fold by the Lys-388 mutant (Fig. 1D). The same pattern was observed with wild-type versus SUMOylation-deficient PR-B (Fig. 1E). Thus, impaired SUMOylation accelerates ligand-dependent down-regulation while paradoxically increasing transcription levels, demonstrating that SUMOylation dampens PR activity by suppressing down-regulation.

**Modulation of PR Transcriptional Activity by SUMO-1 Depends on the Promoter Context**—Transcription from PR-B is usually much stronger than from PR-A on synthetic (16, 48) and natural (10) promoters. This is due to synergism between AF-3 in PR-B and downstream AFs and is promoter-dependent (16). To examine the role of SUMOylation on this synergism, reporters containing zero, one, two, or three PREs were co-transfected into HeLa cells with 50 ng of cDNA encoding wild-type PR-A and PR-B, or their respective Lys-388 mutants, and R5020 was added (Fig. 2) (PR levels and down-regulation for a range of cDNA concentrations are shown in Fig. S2). PR-B–driven transcription increased 6–, 25–, and 55–fold on a PRE1, PRE2, and PRE3, respectively, a synergistic pattern of amplification. Mutation of Lys-388 failed to increase transcriptional activity of PR-B above its wild-type level on the PRE1, but a 5.5– and 6.2–fold augmentation above ligand-dependent levels were observed on PRE2 and PRE3. With regard to wild-type PR-A, their much weaker 3.0–, 3.0–, and 6.5–fold ligand-dependent activity on a PRE1, PRE2, or PRE3, was augmented 1.2–, 6.1–, and 7.5–fold with the Lys-388 mutant, equivalent to that observed with PR-B. Thus, independent of AF-3 in PR-B-upstream segment, SUMOylation limits transcriptional synergy on compound DNA response elements, which is relieved by mutation of the SUMO attachment site.

**SUMOylation and Ligand Sensitivity**—The influence of SUMOylation on PR transcriptional activity is ligand-dependent (48, 49). Effects of ligand concentration were tested using wild-type PR-B and the corresponding Lys-388 mutant. Fig. 3A compares the transcriptional activity of 10 versus 100 ng of cDNA encoding wild-type PR-B against increasing R5020 concentrations. As PR cDNA concentrations are increased, there is a 6.7–fold left shift, from an EC\(_{50}\) of 1.5 × 10\(^{-11}\) M to 2.2 × 10\(^{-12}\) M, in the R5020 dose-response curve. This is associated with an ~4–fold increase in transcriptional activity (Fig. 3A). The 10-ng cDNA dose was used to compare wild-type PR-B against the Lys-388 mutant (Fig. 3C). SUMOylation deficiency caused a left shift in the R5020-response curve from an EC\(_{50}\) of 1.5 × 10\(^{-11}\) M for wild-type PR to 2.6 × 10\(^{-12}\) M for the mutant (Fig. 3C), associated with an ~7.0–fold increase in transcriptional activity (Fig. 3D). Thus, PR SUMOylation reduces sensitivity to hormone. Taken together, the above studies indicate that SUMOylation dampens PR transcriptional activity by slowing their down-regulation, inhibiting their cooperativity on tandem DNA response elements, and reducing their sensitivity to ligand.

**PR Phosphorylation and SUMOylation Are Unconnected**—There are at least 14 phosphorylation sites on PR (51–53). Basal phosphorylation levels of multiple sites are increased in response to progestins and mixed antagonists but not to the pure antiprogestin ZK98 (54). However, ZK98 permits PR SUMOylation (see Fig. 6D), suggesting that phosphorylation at those sites is uncoupled from SUMOylation. To test this, SUMOylation by GFP-SUMO-1 of wild type PR-B and a PR-B Ser-294/344/345 phosphorylation-deficient mutant were compared (Fig. 4). R5020-dependent SUMOylation was observed on both wild-type and mutant receptors. Other phosphoryla-
tion-deficient mutants, including Ser-102 (not shown), were also appropriately SUMOylated. We conclude that PR phosphorylation and SUMOylation are uncoupled.

Recent studies reporting an association between ligand-dependent phosphorylation and SUMOylation (49) may have been influenced by experimental conditions. For example, the extent of ligand-dependent down-regulation varies with receptor concentration (Fig. S3) and film exposure time (Fig. S4). Compared with wild-type controls, phosphorylation-deficient mutants can exhibit unchanged or suppressed transcriptional activity, depending on protein concentration (Fig. S5).

**SUMO-1 Enhances PR Transcriptional Activity**

Since SUMOylation dampens PR transcriptional activity, we asked if SUMO-1 overexpression is similarly inhibitory (Fig. 5). PR-B cDNA (20 ng) was co-transfected with the PRE2 reporter plus increasing concentrations (20–200 ng) of a GFP-SUMO-1 expression vector. (SUMO expression levels at varying cDNA concentrations are shown in Fig. S6.) In the absence of SUMO-1, PR-B activates transcription 12-fold. Surprisingly, rather than inhibiting transcription, SUMO-1 increased liganded PR-B activity in a dose-dependent manner (Fig. 5A). Overexpression of SUMO-1 had no effect on a single PRE or on the mouse mammary tumor virus promoter (not shown), suggesting a possible role for SUMO-1 in cooperativity between tandem PREs. SUMO-1 G97A, a single-amino acid mutant that cannot be conjugated to target proteins, had no effect on either PR-dependent transcription or on basal activity at any concentration (not shown).

We next asked whether PR SUMOylation is responsible for the unexpected increase in transcriptional activity seen with SUMO-1 (Fig. 5B). However, in agreement with Chauchereau et al. (55), SUMO-1 also increased activity of the PR-B Lys-388 mutant. This demonstrates that the heightened transcription by SUMO-1 is not due to direct PR-B SUMOylation. Rather factor(s) that indirectly impact PR-dependent transcription must be the targets of SUMO-1. Note that the response of GR to SUMO-1 is similar to that of PR, whereas in our hands, ER are not SUMOylated (not shown).

PR SUMOylation is hormone-dependent and requires the LBD AF2 (48, 49). Mutation of Lys-388 does not influence the constitutive activity of the isolated PR-B N-terminal/DBD construct (NT-B) (Fig. S8). To investigate effects of SUMO-1 on the N and C termini of PR-B, increasing amounts of SUMO-1 were cotransfected with NT-B or DBD-LBD constructs and the
FIGURE 5. SUMO-1 expression enhances PR transcriptional activity. HeLa cells were transfected with the PRE-Luc reporter in the presence of pSV40-Renilla as internal control along with PR-B (A), PR-B K388R (B-K388R) (B), NT-B (C), or DBD-LBD (D) expression vectors and a GFP-SUMO-1 expression vector at doses of 20, 50, 100, and 200 ng of DNA or an empty vector control. Cells were treated without (−) or with (+) 10 nm R5020 for 24 h before being assayed for luciferase activity. The values are expressed as relative luciferase units normalized to Renilla controls. RLU, relative luciferase units.

PRE2 reporter. Surprisingly, rather than augmenting activity, as seen with full-length PR-B (Fig. 5A), SUMO-1 inhibited the constitutive activity of both wild-type (Fig. 5C) and K388R mutant (Fig. S8B) NT-B and had little or no effect on the ligand-dependent activity of the LBD (Fig. 5D). Taken together, the data show that only transcription by full-length PR-B is directly modulated by SUMO-1 and suggest that SUMO-1 plays a role in PR N-/C-terminal communication.

PR SUMOylation and N-/C-terminal Interactions—The mammalian two-hybrid assay was used to study this. The constructs used are shown in Fig. 6A. They include SRC-1 linked to the Gal4 DBD or the VP16 transcriptional activation domain; full-length PR-B linked to VP16; the PR LBD fused to Gal4-DBD; and the PR-B N terminus, either wild-type (VP16-NTB) or mutated at Lys-388 (VP16-NTBm), fused to VP16. A luciferase gene inserted downstream of five Gal4 DNA binding sites detected functional interactions between the Gal4 and VP16 fusion proteins in HeLa cells in the absence or presence of progesterins. Western blot analysis (not shown) confirmed that the fusion products were equally expressed and correctly sized.

First we analyzed the effects of 24-h treatment with R5020, the mixed antiprogestins RU486 and ZK112993 (ZK112), and the pure antiprogestin ZK98299 (ZK98) (Fig. 6B). Unliganded Gal4-LBD failed to interact functionally with VP16-NTB (not shown), confirming that N-/C-terminal interactions are ligand-dependent (56). R5020 and the mixed antagonists were equally robust, promoting N-/C-terminal interactions 30–40-fold, but the pure antagonist ZK98 largely prevented this. Also, unlike the mixed antagonists, ZK98 blocked the interaction enabled by R5020 (Fig. 6B), suggesting that its mechanisms of action are fundamentally different from that of the mixed antagonists. Fig. 6C shows that R5020 promotes a significant interaction between the LBD and NTB (∼28-fold) or the SUMOylation-deficient NTBm mutant (∼26-fold), demonstrating that SUMOylation of the PR N terminus is not required for its interaction with the C terminus.

We then asked the reverse. Does SUMOylation of the PR N terminus require that it be linked to the LBD (Fig. 6D)? PR-B were transiently co-expressed in HeLa cells with GFP-SUMO-1 in the presence or absence of R5020 or the antiprogestins. PR SUMOylation was assessed by Western blotting with monoclonal antibody 1294. In the absence of SUMO-1, hormones did not generate a SUMOylated PR band (lanes 2–5). (The multiple banding pattern observed is due to phosphorylation (48).) In the presence of SUMO-1 but without hormones, no SUMOylated PR were observed (lane 6). However, any ligand (the agonist R5020, the mixed antagonists RU486 and ZK112, or the pure antagonist ZK98) stimulated PR SUMOylation, as indicated by the appearance of a single, high molecular weight band in lanes 7–10. Since ZK98 blocks N-/C-terminal interactions (Fig. 6B), these data show that SUMOylation of PR does not depend on such interactions but that ligand occupancy of the LBD is required for the PR conformational changes that allow SUMO attachment at the N terminus. Mutation of AF-3 in PR-B upstream segment, which prevents N-/C-terminal interactions (16, 57), also does not prevent SUMOylation (Fig. S7), demonstrating this disconnect by an alternative method.

SUMO-1, SRC-1, and PR N-/C-terminal Interactions—The ability of SUMO-1 to augment transcription by the SUMOylation-deficient Lys-388 PR mutant (Fig. 5B) suggested a role for SUMOylation of factors other than PR, but required by PR, for efficient transcription. Since ligand-dependent PR N-/C-terminal interactions are enhanced by SRC-1 (56), we addressed the relationship between SRC-1 and SUMO-1 in this process. Fig. 7A shows that ligand-dependent PR NTB/LBD interactions are independently stimulated by SRC-1 (56) and by SUMO-1. To identify the PR domains involved, N and C termini were analyzed separately. Interestingly, the interaction between SRC-1 and liganded PR LBD is doubled by the presence of SUMO-1 (Fig. 7B), but the interaction between SRC-1 and NTB is suppressed by SUMO-1 (Fig. 7C). We therefore analyzed the effect of SUMO-1 on the interaction between SRC-1 and wild-type PR-B or the PR-Bm SUMOylation-deficient mutant (Fig. 7D). Receptor interaction with SRC-1 is doubled by SUMO-1 in both wild-type PR-B and the SUMOylation mutant, suggesting that SUMO-1 targets the SRC-1 protein complex, which then regulates PR N-/C-terminal interactions independent of PR SUMOylation. Under these conditions, SRC-1 is subjected to SUMOylation (data not shown).

DISCUSSION

Posttranslational modification by SUMO is a major regulator of transcription that increases expression from some promoters and silences others (32). Both PR isoforms have a single SUMO consensus d/KXE motif centered at Lys-388 in the N terminus of PR-B and a homologous site of PR-A. SUMO-
ylation of PR is hormone-dependent and suppresses transcription of an exogenous promoter (48, 49, 55, 58). Here we show that repression of PR activity by SUMOylation is direct and independent of phosphorylation (Fig. 4), requires two or more PREs (Fig. 2), reduces ligand sensitivity (Fig. 3), and slows ligand-dependent down-regulation (Fig. 1). Paradoxically, overexpression of SUMO-1 increases PR transcriptional activity even if Lys-388 is mutated (Fig. 5), suggesting that the receptors are...
indirectly targeted for activation by other SUMOylated proteins. One of these appears to be the coactivator SRC-1, whose binding to PR and enhancement of agonist-dependent N-/C-terminal interactions (Fig. 6) is augmented by the presence of SUMO (Fig. 7). Thus, SUMO targets the SRC-1–PR protein complex independent of PR SUMOylation. In summary, SUMOylation tightly regulates the transcriptional activity of PR by repressing the receptors directly while stimulating them indirectly through augmented SRC-1 coactivation.

PR SUMOylation and Direct Repression of Activity; Down-regulation, Phosphorylation, Ligand Binding, and PRE Synergism—Many proteins are stabilized by SUMOylation. Attachment of SUMO-1 to the inhibitor of κBα (IκBα), the negative regulator of p53 (Mdm2), or the G-protein regulator phosducin reduces their proteasomal degradation (58). Indeed, in the case of IκBα, SUMO-1 and ubiquitin compete for conjugation to the same lysine (59, 60). Data with regard to PR SUMOylation and proteasomal degradation are contradictory. Zhang et al. (61) reported that CUE-domain-containing 2 regulates PR-B ubiquitination at the Lys-388 SUMOylation site so that mutation of this site leads to resistance to progesterone-induced PR degradation in 293T human embryonic kidney cells. They argue that SUMO and ubiquitin compete for conjugation to the same lysine. However, our studies in HeLa human cervicocarcinoma and T47D breast cancer cells show that the PR K388R mutants undergo accelerated ligand-dependent down-regulation (Fig. 1, A and B) concomitant with elevated transcriptional activity. This is in agreement with Daniel et al. (49), who find that SUMOylation of PR-B retards ligand-dependent down-regulation. The discrepant results may be due to differences among cell lines, to use of fusion proteins, or to analyses limited by circumscribed protein doses or time points (61).

The connection between PR SUMOylation and phosphorylation is also of interest, since such relationships exist for other proteins. For example, activation of mitogen-activated protein kinase leads to the phosphorylation and de-SUMOylation of Elk-1 (62), resulting in transcriptional activation. Similarly, SUMOylation of p53, c-Jun, IκBα, KAP1, and promyelocytic leukemia protein is repressed by phosphorylation through unknown mechanisms (63–65). Phosphorylation also positively regulates SUMOylation of many proteins, including heat shock factor 1, HSF4b, signal transducer and activator of transcription 1, myocyte enhancer factor 2A, and globin transcription factor 1 (66). This requires the presence of a composite phosphorylation-dependent SUMOylation motif, composed of the core SUMO consensus site plus an adjacent proline-directed phosphorylation site (YKXE)(67). PR do not contain a phosphorylation-dependent SUMOylation motif, perhaps explaining why PR phosphorylation and SUMOylation are dissociated.

Multiple Ser residues of PR (51–53) are phosphorylated in response to progesterins and mixed antiprogestins but not to the pure antiprogestin ZK98299 (54). However, since ZK98299 permits PR SUMOylation (Fig. 6D), we conclude that PR phosphorylation is uncoupled from Lys-388 SUMOylation. Additionally, we observe R5020-dependent SUMOylation on both wild-type and PR Ser-294/344/345 mutant receptors (Fig. 4), another indication that PR phosphorylation and SUMOylation are uncoupled. It is possible that recent studies reporting an association between ligand-dependent phosphorylation and SUMOylation (49) were skewed by receptor dosage issues. For example, phosphorylation-deficient mutants exhibit variable transcriptional activities and inconsistent ligand-dependent down-regulation, depending on receptor concentrations (Figs. S3–S5).

Indirect Effects of SUMO; Coregulators, Transcriptional Syn-ergy, and N-/C-terminal Interactions—Here we describe two different effects of SUMOylation. On the one hand, mutation of Lys-388 clearly up-regulates transcription by the receptors (Fig. 1), demonstrating that direct conjugation of SUMO to PR is repressive. On the other hand, overexpression of SUMO-1 increases the activities of both wild-type and Lys-388 PR mutants (Fig. 5) through a mechanism that must therefore be indirect. We conclude that under these conditions, SUMO is targeting other proteins. PR-dependent gene regulation requires not only the receptors but also large, multiprotein complexes that include coregulators, the general transcriptional machinery, and chromatin components (68). Two broad
classes of nuclear receptor coregulators include the coactivators SRC-1, RIP-140, p300, and CBP and the corepressors NCoR and SMRT (22). Current models propose that varying coactivator/corepressor ratios, along with varying receptor concentrations, modulate positioning of the ligand dose-response curve for agonist-bound receptors and control tissue specificity of transcription (69). Northern blot analyses confirm the idea that different tissues and cell lines express varied transcript levels for coactivators and corepressors (70). Indeed, although corepressors were so named because they were initially thought to be restricted to unliganded or antagonist-bound receptors (71), recent studies show that coactivators and corepressors can reverse one another’s effects in both agonist- and antagonist-bound states (22).

All three members of the SRC family share two SUMOylation sites within the first nuclear receptor interaction domain, which for SRC-1 are at Lys-732 and Lys-774. SRC-1 has three additional weak affinity sites at Lys-800 and Lys-846 in the CBP/p300 interaction domain and Lys-1378 in activation domain 2 (72). SUMOylation of SRC-1 prolongs its nuclear retention and increases its interaction with PR (55).

Iñiguez-Lluhi and Pearce (42) first showed for GR, as we show here for PR (Fig. 2), that mutation of the SUMOylation motifs in nuclear receptors increases their activity from promoters bearing multiple but not single hormone response elements. Many other studies indicate that SUMO modification affects the ability of transcription factors to function synergistically (73). These effects can be explained by altered coregulator recruitment. For example, SUMOylation of Elk-1 preferentially recruits the histone deacetylase HDAC2 (74), which reduces histone acetylation and suppresses transcription. Daxx is a repressor of several SUMOylated transcription factors, including GR. It is recruited to GR-targeted promoters upon hormone treatment, a recruitment that is abrogated if GR are not SUMOylated. The relative amount of Daxx recruited to promoters depends on the GRE copy number (75).

Transcriptional synergy from multiple hormone response elements has been reported for all steroid receptors (31, 42). Proposed mechanisms include cooperative recruitment of coactivators, action at distinct rate-limiting steps in initiation, cooperative DNA binding, and/or direct protein-protein interactions between receptor dimers (56, 76). It is possible that the strong transcriptional activity of PR-B on tandem PREs is due to cooperative interactions between receptor dimers bound at adjacent PREs rather than between monomers bound at a single PRE. Consistent with this, Bain and co-workers (76) find that highly purified full-length PR-B bind cooperatively to a double PRE but noncooperatively to a single PRE. A related mechanism posits that synergy results from AF3-dependent recruitment of coactivators to PR-B bound at multiple PREs. We have shown that interaction and assembly of PR-B with SRC-1 is entirely dependent on a functionally intact PR-B-upstream segment, which contains the AF-3 (16). Since transcriptional synergy occurs with the Lys-388 mutant PR (Fig. 2), this property involves mechanisms other than direct PR SUMOylation.

Full activity by steroid receptors requires synergy between their N- and C-terminal domains. Protein-protein interaction studies using purified receptor domains show that N and C termini interact in vitro (56), and this contributes to functional synergy between AF-2 in the LBD and the upstream AFs of the N termini (56). We observe interactions between N and C termini in the presence of the mixed antiprogestins RU486 and ZK112 but not the pure antiprogestin ZK98299 (Fig. 6B). The pure antagonist nevertheless promotes PR SUMOylation (Fig. 6D), indicating that direct PR SUMOylation is not required for N-/C-terminal interactions.

However, the mechanisms by which intramolecular communication occurs within receptors are complicated by the fact that coregulators like SRC-1 also interact with both N and C termini (19). In PR, SRC-1 binds the N termini of both PR, but its binding affinity is higher for PR-B. Here we examined the effect of SUMOylation on PR/SRC-1 interactions and found that overexpression of SUMO-1 increases the interaction between SRC-1 and full-length PR-B (Fig. 6D) but also between SRC-1 and the LBD (Fig. 6B). Since the LBD lacks a SUMOylation site, this suggests that SUMO-1 overexpression does not necessarily target PR directly. We also find that SRC-1 interacts with itself (not shown), raising the possibility that PR-B-related synergy on multisite promoters involves assembly of SUMOylated SRC-1 multimers.

In summary, we show here that protein modification by SUMO has complex effects on PR-dependent transcription. Direct SUMOylation of the receptors is markedly suppressive, by reducing ligand sensitivity, slowing ligand-dependent down-regulation, and preventing synergy on tandem PREs. On the other hand, indirect effects of SUMO on coactivators like SRC-1 increase transcriptional activity by promoting PR N-/C-terminal interactions. Overall transcription levels reflect a balance between these opposing tendencies, regulated in part by SUMOylation.

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