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A Small RNA Derived from RNA Coactivator SRA Blocks Steroid Receptor Signaling via Inhibition of Pus1p-Mediated Pseudouridylation of SRA: Evidence of a Novel RNA Binding Domain in the N-Terminus of Steroid Receptors

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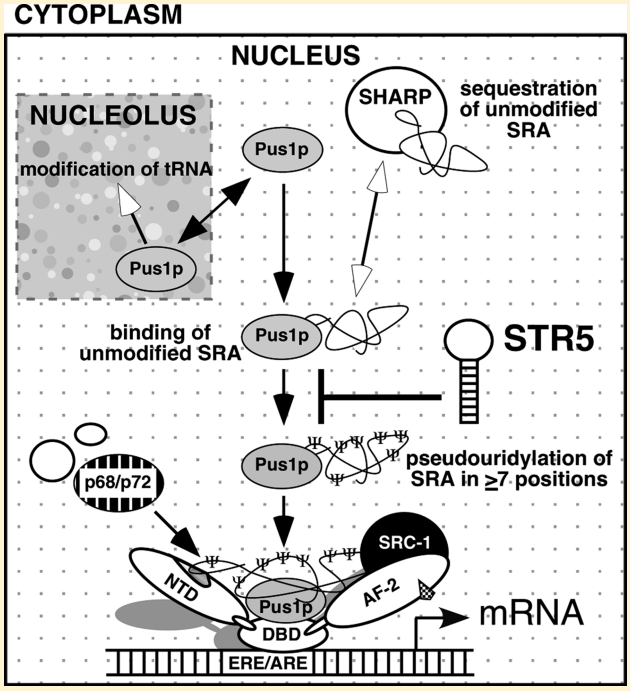
Supporting Information

ABSTRACT: Estrogen receptors (ERs) and androgen receptors (ARs) are important targets for cancer therapy; however, the efficacy of receptor antagonists is limited, and alternative strategies are needed. Steroid receptor RNA Activator (SRA) is a long, noncoding RNA coactivator (although some protein-encoding 5′ splice variants have also been reported) that requires pseudouridylation by Pus1p to stimulate steroid receptor signaling. A uridine at position 206 (U206), which is located in small hairpin structure STR5 in the conserved SRA core sequence, is a critical target for pseudouridylation. We assessed if synthetic STR5 could serve as a novel competitive inhibitor of ERα and AR signaling by disrupting the Pus1p−SRA−steroid receptor axis. STR5 specifically inhibited Pus1p-dependent pseudouridylation of SRA with higher efficiency than STR5 mutant U206A. We show that SRA binds to the N-terminal domain (NTD) of ERα and AR with high affinity despite the absence of a recognizable RNA binding motif (RBM). Finally, we show that STR5 specifically inhibits ERα- and AR-dependent transactivation of target genes in steroid-sensitive cancer cells, consistent with disruption of the targeted Pus1p−SRA pathway. Together, our results show that the NTD of ERα and AR contains a novel RBM that directly binds SRA, and that STR5 can serve as a novel class of RNA inhibitor of ERα and AR signaling by interfering with Pus1p-mediated SRA pseudouridylation. Targeting this unexplored receptor signaling pathway may pave the way for the development of new types of cancer therapeutics.



Sex steroid receptors belong to the nuclear receptor (NR) superfamily of ligand-dependent transcriptional modulators that control development, cell growth, and homeostasis in metazoa;[1] however, several also play unintentional roles in tumor development. In particular, the critical function of AR in prostate cancer[2] and ER in breast cancer[3] is well-documented. Dissection of the molecular mechanism of NR-mediated gene activation is, therefore, of major biological, clinical, and economic importance.

NRs generally contain two transcriptional activation functions (AFs) required for full receptor action. Ligand-independent AF-1 is located in the N-terminal domain (NTD), while better characterized ligand-dependent AF-2 is present in the ligand binding domain (LBD).[1] In the absence of ligand, NR-directed transcription is prevented by association with a multiprotein corepressor complex with histone deacetylase activity. When a ligand is detected, a conformational change in the LBD results in the release of corepressors and the recruitment of a coactivator complex with histone acetyltransferase and methyltransferase activities, resulting in subsequent acetylation and methylation of chromatin, respectively.[4] Coactivators essentially comprise two major

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categories: the p160/SRC-1 family, which contains SRC-1/NcoA-1/ERAP-160, TIF-2/GRIP-1/NCoA-2/SRC-2, and AIB1/p/CIP/ACTR/RAC3/SRC-3/TRAM-1, and the CBP/p300 family of transcriptional co-integrators. However, many coactivators such as SRA fall outside these groups.[5]

SRA was originally described as a long, noncoding (nc)RNA[6] that stimulates the transactivational properties of many NRs, including ERα (although not ERβ) and AR;[6,7] however, some 5′ splice variants can also encode SRAP, a protein(s) with both activator and repressor activities.[8,9] Although SRA exists in a ribonucleoprotein complex with SRC-1 at the LBD of AR,[6] a recent report demonstrated that SRA is an ERα AF-1-specific coactivator that can also associate with the NTD of AR, and possibly ERα without SRC-1.[7] SRA was also shown to bind with DEAD-box protein p68/p72 in an ERα-specific coactivator complex,[10] and NR corepressors SHARP[11] and SLIRP.[12] The SHARP−SRA association is thought to at least partially modulate ER transactivation through sequestration of SRA, while binding of SLIRP to SRA enhances corepressor NCoR promoter recruitment. Thus, SRA may serve as a scaffold for proteins involved in the modulation of NR-dependent signaling.[12] Additionally, SRA and SRAP can serve as regulators of MyoD activity in muscle cell differentiation.[13]

The ERα and AR activity enhancing properties of SRA indicate that it may play an important role in steroid-sensitive tissue tumorigenesis. This idea is supported by the overexpression of SRA in breast, uterine, and ovarian cancers.[14−16] Overexpression in mouse mammary tissue results in extensive hyperplasia of breast epithelia, which is at least partially due to increased ERα activity. Thus, SRA has proliferation promoting activities via coactivation of NR signaling[17] and is also thought to modulate tamoxifen resistance.[7,12] SRA was found to be expressed in rat prostate cancer cell lines, and its expression is readily detected in human prostate and prostate cancer cells and thus is likely to have a role in regulating AR signaling in this tissue, as well.[18,19]

Computer and experimental modeling suggests that SRA adopts a complex secondary structure.[20] While some predicted hairpin structures are important for SRA function,[12,14] contributions of higher-order RNA structures to SRA activity remained largely unknown until we showed that SRA requires posttranscriptional modification by a novel class of coactivators named Pus1p to function in NR-dependent transactivation.[21] Pus1p is a member of the pseudouridine synthase (PUS) family whose members isomerize uridine to pseudouridine (Ψ) in ncRNAs such as tRNA, rRNA, snRNA, and possibly snoRNA.[22] Pus1p and Pus3p and bacterial truA form the small truA subfamily whose products modify specific positions in many tRNAs.[23,24] Ψ has an additional imino proton that helps stabilize base stacking[25] and hydrogen bonding,[26] which in turn helps establish specific intra- and intermolecular interactions within the RNA or between RNA and RNA and between RNA and protein(s).[22] In some cases, the presence of Ψ is required for correct tRNA codon reading[27] and spliceosome assembly.[28] Aberrant PUS activity results in disorders such as dyskeratosis congenita and associated cancers, which is caused by a point mutation in Dyskerin,[29] and mitochondrial myopathy and sideroblastic anemia (MLASA), which is caused by a point mutation in Pus1p where certain abnormalities seen in these patients may be the result of defective SRA-NR signaling.[30]

Pus1p-mediated coactivator activity depends on two essential activities. First, Pus1p directly binds with the DNA binding domain (DBD) of NRs at the promoter site of target genes in a ligand-independent manner;[21] the second is the enzymatic activity of Pus1p. In SRA, at least seven pseudouridylated positions have been identified.[21,31] One position, U206, located in stem−loop structure STR5 in the SRA core, was also identified in vivo as a target for pseudouridylation.[31] Surprisingly, when U206 was mutated to A, SRA became hyperpseudouridylated, resulting in a switch from a molecule with coactivator activity to one with repressor activity. Thus, Pus1p and its target SRA play important roles in the steroid receptor signaling pathway. Here we show that a synthetic STR5 fragment blocks Pus1-mediated pseudouridylation of SRA, resulting in reduced levels of ERα and AR signaling in breast and prostate cancer cells, respectively. We also show that SRA, but not STR5, binds directly with significant affinity to the NTD of AR and ERα despite the absence of an established RNA binding motif (RBM) in these domains. Our findings may have implications for future targeting of steroid receptor activity via inhibition of this novel enzymatic pathway.

MATERIALS AND METHODS

Cell Lines, Transfection Experiments, and Luciferase Assays. ER positive breast cancer cell line MCF-7 and AR positive prostate cancer cell line LNCaP were obtained from American Tissue Culture Collection (Manassas, VA) and maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) in a humidified 37 °C incubator containing 5% CO_2. For ER transfections, 70% confluent MCF-7 cells in a 12-well plate were transfected using Lipofectamine 2000 reagent (Invitrogen). Six hours before being transfected, cells were washed twice in phosphate-buffered saline and fed with DMEM containing 10% charcoal-stripped FBS. The reporter plasmid and synthetic RNA were mixed in 100 μL of Opti-MEM (Invitrogen). Lipofectamine (3 μL) was mixed with an additional 100 μL of Opti-MEM and the mixture incubated at room temperature for 10 min before it was added to the DNA/RNA mixture. The mixture was incubated further at room temperature for an additional 30 min before being added dropwise to the cells. The AR transfections in LNCaP cells were also conducted in a similar way except that they were seeded directly in DMEM containing 10% charcoal-stripped FBS. The following day after transfection, 10 nM 17β-estradiol (E_2) or 50 nM dihydrotestosterone (DHT) was added to the MCF-7 or LNCaP cells, respectively, and after incubation for an additional 24 h, the cells were harvested for luciferase assay. Protein concentrations in the lysates were normalized before the measurement of luciferase activity. Reporter plasmid pERE-Luc or pPSA-Luc was used at a density of 200 or 400 ng/well for MCF-7 or LNCaP cells, respectively. In some experiments, a total of 250−500 ng of Pus1p or noncoding, hSRA expression plasmids was used per well. Equal amounts of DNA and RNA were always used in transfection experiments by using either empty vector or purified yeast tRNA. Reporter plasmids and expression vectors for Pus1p and SRA have been described previously.[21,31] All transfections were conducted in triplicate and repeated three times.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) Gene Expression Analysis. To assess the expression of estrogen- or androgen-dependent genes following exposure to synthetic RNA, MCF-7 or LNCaP cells were transfected using Lipofectamine 2000 reagent as described above with tRNA or synthetic RNA (STR5) in the presence or absence of E2 or DHT. Total cellular RNA from the transfected

cells was isolated with Trizol reagent according to the manufacturer's protocol (Invitrogen). RNA (5 μg) was reverse transcribed with the Superscript III first strand cDNA synthesis kit using random hexamer primers (Invitrogen). The PS2, GREB1, PSA, KLK2, or β-actin cDNA from these preparations was then amplified using SYBR green PCR amplification technology in an ABI PRISM 7500 sequence detection system (Applied Biosystems, Foster City, CA). PCR conditions were as follows: 95 °C for 10 min for one cycle and 95 °C for 15 s, 55 °C for 20 s, and 60 °C for 31 s for 40 cycles. The following primers were used in real-time PCR amplification: PS2 forward, 5′TTTGGAGCAGAGAGGAGGCAATGG; PS2 reverse, 5′TGGTATTAGGATAGAAGCACCAGGG; GREB1 forward, 5′CACCGGTGTGCACAAGTTAC; GREB1 reverse, 5′GTGCCTCCGTGTCTAGCTTC; PSA forward, 5′TGCCCACTGCATCAGGAACA; PSA reverse, 5′GTCCAGCGTCCAGCACACAG; KLK2 forward, 5′CCTGGCAGGTGGCTGTGTAC; KLK2 reverse, 5′TGTGCCGACCCAGCCA; β-actin forward, 5′GCTCGTCGTCGACAACGGCTC; β-actin reverse, 5′CAAACATGATCTGGGTCATCTTCTC. Relative quantification of gene expression was assessed by the comparative threshold method (ΔCT). Expression of the β-actin mRNA in each individual sample was used to normalize the data set.

Constructs and Generation of Protein, RNA, and DNA Fragments. To obtain recombinant AR NTD, the sequence encoding AR amino acids 1−556 was amplified by RT-PCR and cloned into bacterial expression vector pET16b (Novagen), which provides an N-terminal His tag. To express ERα NTD, the sequence encoding amino acids 1−179 was cloned into the same vector. Plasmid pGEX2T (GE Healthcare) was used to express GST control protein. The vector for GST-tagged SHARP-RRM encoding amino acids 1−668 has been described previously.[12] Proteins were isolated using standard His and GST affinity chromatography procedures (RayBiotech), and quality was checked by sodium dodecyl sulfate−polyacrylamide gel electrophoresis and/or Western blotting (not shown). With the exception of GST-SHARP, which was expressed at low levels as previously reported,[12] the purity typically reached ≥90% for GST, AR NTD, and ERα NTD (data not shown). Synthetic small RNA fragments STR5 and U206A were synthesized by Nedken (Foster City, CA). The insert containing the coding portion of human Pus1p was amplified by PCR from plasmid DNA using the following primers: 5′AACATATGGCCGGGAACGCGGAGCC and 5′GGATCCTCGAGTCCCATCGCCTCAGTCAGTG. The 1201 bp dsDNA was purified using a Qiaex II gel extraction kit (Qiagen).

Pseudouridylation Assays. Mouse Pus1p or β-Gal control proteins were expressed in bacteria and isolated as described previously.[23] The RNAs to be modified (30 fmol; ^{3}H-labeled SRA, U206A SRA, tRNA^{Ser}, tRNA^{Ala}, STR5, or U206A) were synthesized as described previously,[23,24,31] heated in reaction buffer without enzyme and DTT at 78 °C for 3 min, and allowed to cool slowly to 35 °C (at 23 °C for ∼30 min). The reaction mixture included 100 mM NH_4Cl, 10 mM DTT, 50 mM Tris-HCl (pH 7.5), and 2 mM $MgCl_2$ at 37 °C, and the concentration of Pus1p or β-Gal protein was approximately 40−50 nM. Aliquots (100 μL) were removed at the indicated time points, and the amount of Ψ formed was determined with the tritium release assay as described previously.[32] When used, competitor RNAs such as cold STR5 or U206A RNAs were added at the indicated concentration, depending on the experiment, from 100 to 800 times greater than that of the ^{3}H-labeled substrate to the reaction mixture prior to the sample being heated to 78 °C. Then the incubation and assay was conducted as described above.

For the isolation of SRA RNA or U206A SRA with or without Ψ, U-^{3}H-labeled SRA RNA was incubated in 300 μL reaction mixtures with Pus1p (for SRA with Ψ or U206A SRA with Ψ) or β-Gal (SRA without Ψ or U206A SRA without Ψ) for 2 h at 37 °C. Two aliquots (10 μL each) were removed and used to assay the amount of Ψ made (∼5.0 mol of Ψ/mol of SRA) with the tritium release assay method.[21] The remaining volume of the reaction mixture was adjusted to 0.3 M sodium acetate (pH 5.2); 10 μg of tRNA was added as a carrier, extracted once with a phenol/$CHCl_3$ mixture, and precipitated with 3 volumes of 95% ethanol at −20 °C for 16 h. The RNA pellet (14000g for 20 min) was washed once with 95% ethanol and air-dried. The pellet was dissolved in water (100 μL), and the amount of RNA recovered was determined from the counts for the two types of SRA with or without Ψ.

Protein Filter Binding Assays. Filter binding assays were conducted as described previously[33] using various protein concentrations in either 50 or 100 μL reaction mixtures with U-^{3}H-labeled ncSRA or U206A SRA (10 or 20 fmol of SRA with ∼0.3 μg of tRNA). SRA or U206A SRA with or without Ψ was incubated in 20 mM Tris (pH 8.5), 100 mM KCl, and 10% glycerol at 78 °C for 3 min and cooled to 35 °C slowly (23 °C for ∼30 min). When cool, the reaction mixtures consisted of 1 mM DTT, 0.1 mg/mL BSA [final concentration of 1.5 μM, acetylated (New England Biolabs)], and protein to be tested was added [various amounts of the proteins in storage buffers; for Pus1p, the buffer consisted of 50 mM Tris (pH 7.5), 1 mM $MgCl_2$, 50% glycerol, and 1 mM DTT; for AR NTD and ERα NTD, the storage buffer consisted of 20 sodium phosphate, 0.5 M NaCl, and 0.5 M imidazole; for SHARP-RRM and GST, the storage buffer consisted of 50 mM Tris (pH 8.0) and 10 mM reduced glutathione (pH 8.0)]. The reaction mixtures were incubated at 20 °C for 20 min, slowly filtered through nitrocellulose filters [24 mm, 0.45 μM (Schleicher and Schull)], and washed three times with 1 mL portions of 20 mM Tris (pH 8.0), 100 mM KCl, 1 mM DTT, and 10% glycerol. The filters were air-dried; scintillation fluid was added, and samples were counted. For the binding competition studies, the cold competitors [SRA, mRNA, poly(rC), poly(rG), STR5, and dsDNA] at concentrations 10−800 greater than that of U-^{3}H-labeled SRA and 0.01 μg of tRNA were added prior to the mixture being heated to 78 °C and subsequently cooled. Then the binding assay was conducted as described above.

Statistics. The data are expressed as means ± the standard deviation (SD). The Student's t test was performed to assess differences between means, and p values of <0.05 were considered significant.

RESULTS

STR5 Inhibits Pus1p-Dependent Pseudouridylation of SRA. The predicted, complex secondary structure of the SRA core sequence contains several stem−loop substructures that are thought to contribute to the total coactivator activity of SRA, as shown in Figure 1A. STR5, a predicted stable hairpin structure whose presence was confirmed by experimental RNA secondary structure analysis,[20] is likely pseudouridylated by Pus1p at U206. Mutation of U206 to A (Figure 1B) abolished SRA-mediated stimulation of ERα and AR signaling, showing that appropriate pseudouridylation of STR5 is critical for the subsequent correct pseudouridylation and functioning of

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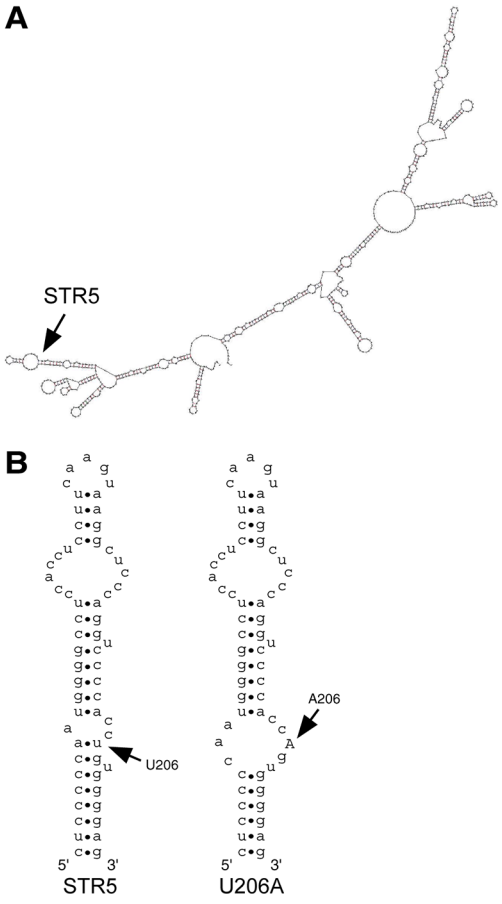

Figure 1. Secondary structure model of SRA. (A) Computer-generated RNA structure model (mfold, version 3.2) of the conserved SRA core sequence. Stem–loop structure STR5 as indicated. (B) Predicted secondary structure of STR5 and U206A RNA fragments used in our inhibitor studies. The position of U206, which is pseudouridylated in STR5 in the context of intact SRA and mutated to A in U206A, is indicated.

SRA.[31] To test whether a synthetic STR5 fragment can inhibit Pus1p-mediated pseudouridylation of SRA, we performed an in vitro pseudouridylation assay with unmodified SRA as the substrate and suboptimal amounts of Pus1p. We first measured the number of moles of Ψ generated per mole of SRA. STR5 and U206A were then added in increasing doses to the reaction mix, which was allowed to continue for rate-limiting times of 30 and 60 min. As a negative control, we used equal amounts of β-Gal with SRA for 60 min. As shown in Figure 2A, ∼2 and ∼3 mol of Ψ/mol of SRA were generated after 30 and 60 min, respectively. However, in the presence of STR5, significantly fewer Ψ residues were generated in a dose-dependent manner, reaching background levels at the largest dose. Interestingly, the U206A fragment exhibited a much reduced inhibitory activity, and even at the largest dose, only ∼40% fewer Ψ residues were generated. STR5 and U206A have similar stable secondary structures,[31] which suggests that the observed effect is related to the specific presence of uridine at position 206. We next tested if STR5 also inhibits Pus1p-dependent pseudouridylation of classic substate $tRNA^{Ile}$, which is normally modified at three positions, as shown in Figure 2B. While a 500-fold molar excess of STR5 reduced the level of pseudouridylation of SRA by 65%, it had a much weaker effect on $tRNA^{Ile}$ pseudouridylation (27% reduction) (Figure 2B). These results thus favor the intended use of STR5 as a specific inhibitor of NR signaling.

Finally, to test whether STR5 itself could act as a substrate for Pus1p, we performed a standard pseudouridylation assay with U-^{3}H-labeled STR5, U206A, and SRA as potential substrates. We included $tRNA^{Ser}$, which has only one modifiable position as a positive control, and $tRNA^{Ala}$, which is not pseudouridylated by Pus1p, as a negative control. To account for potentially slow pseudouridylation kinetics of STR5 and/or U206A, we allowed incubation reactions to continue for up to 3 h, which is sufficient to pseudouridylate much larger SRA to saturation.[21,31] As shown in Figure 2C, SRA becomes progressively more pseudouridylated over time, reaching almost 5 mol of Ψ/mol of SRA after 3 h, but neither STR5 nor U206A was pseudouridylated even after 3 h [only a very low level of nonspecific pseudouridylation (≤0.25 mol of Ψ/mol of RNA) was observed]. The single Ψ in $tRNA^{Ser}$ was generated within 60 min, and $tRNA^{Ala}$ remained negative. This shows that neither STR5 nor U206A is a substrate for Pus1p, and thus, the superior pseudouridylation inhibitory potency of STR5 versus that of U206A is not due to any differential pseudouridylation of these molecules.

Identification of RNA Binding Activity in the NTD of ERα and AR. To understand further the mechanism by which STR5 interferes with pseudouridylation of SRA by Pus1p, we analyzed the binding of SRA with transcriptional activation domains of the nuclear receptors. The AF-1 domain of AR is embedded in exon 1, and ≥70% of AF-1 transactivational properties are located within amino acids 142–485.[34] The AR NTD binds with numerous transcription factors and coregulatory proteins,[35−38] some of which have RNA binding activity.[36,39,40] The AF-1 domain of ERα is embedded in a hydrophobic, Pro-rich region between amino acids 51 and 149.[41] Like AR, ERα also associates with a large number of cofactors.[42,43] Unfortunately, it has not been clearly established whether SRA binds with the NTD in a physiologically relevant manner because no K_d values were established.[6] Moreover, the NTD of ERα and AR does not contain a recognizable RNA binding motif (RBM) such as the established RNA recognition motif (RRM) present in corepressor SHARP. This question needs to be addressed because if STR5 binds to the NTD, it may interfere with binding of SRA to this domain, which would make its mechanism of action potentially more complex.

To answer this question, we generated U-^{3}H-labeled, unpseudouridylated, and Pus1p-pseudouridylated SRA. Proteins AR NTD and ERα NTD, positive controls Pus1p and GST-SHARP-RRM, and negative control GST were also obtained. Next, excess SRA templates were incubated with increasing concentrations of proteins followed by detection of protein–SRA complexes by a filter binding assay in a high-stringency binding buffer. As shown in panels A and B of Figure 3, the NTD of ERα and AR bound unpseudouridylated SRA with intermediate and high affinity with K_d values of 600 and 45 nM, respectively. For these panels (A and B), spline curves are pictured to better represent the sigmoidal nature of the curve. As expected, the SHARP-RRM fragment bound SRA with the lowest K_d of approximately 4 nM (Figure 3C) while the K_d of Pus1p for SRA was 110 nM (Figure 3D). Negative control GST did not show any SRA binding activity (Figure 3E), and curiously, neither did GST-SLIRP (data not shown). The presence of Ψ in SRA increased the K_d by 1.5-fold for GST-SHARP-RRM and ERα NTD and 2-fold for AR NTD, suggesting that introduction of this modification has a modest

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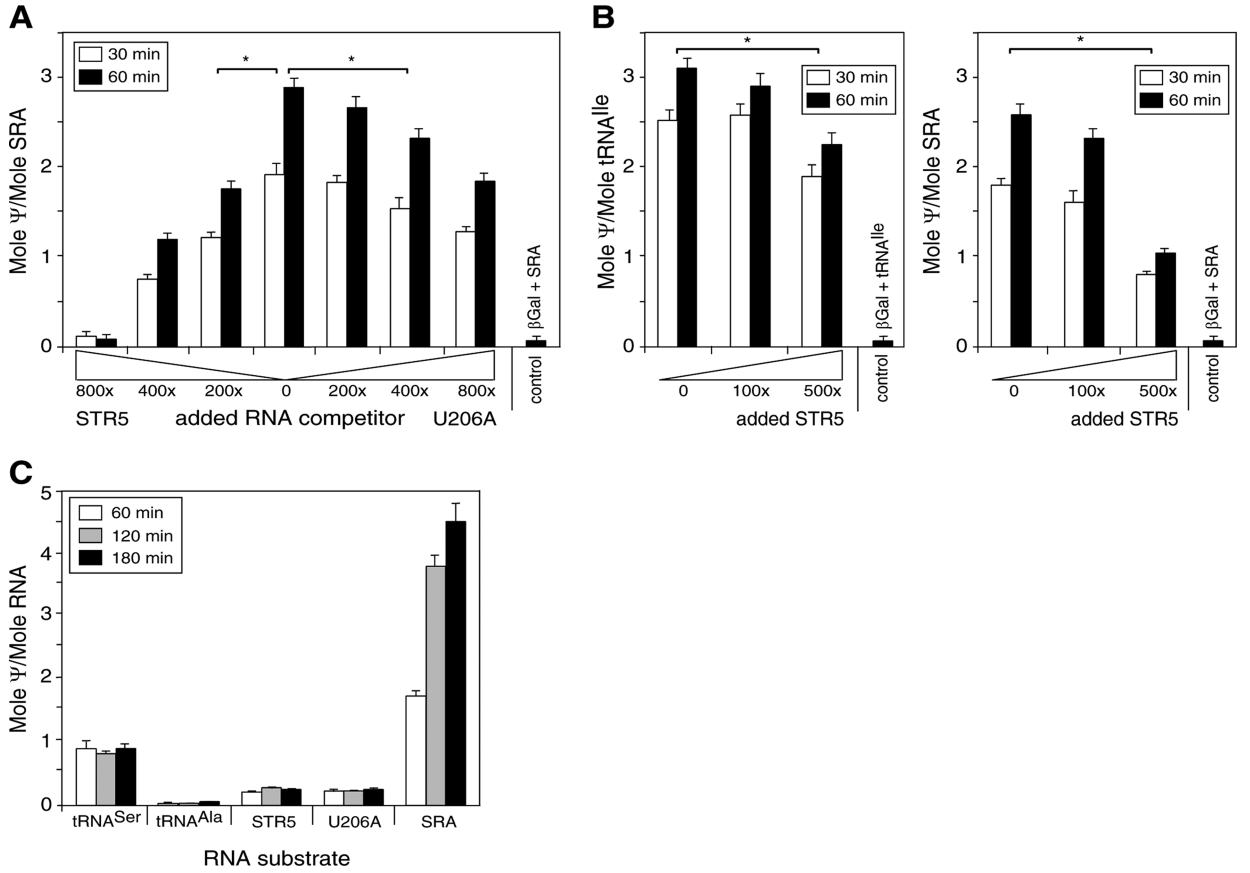

Figure 2. STR5 inhibits Pus1p-dependent pseudouridylation of SRA but is not a substrate itself. (A) Pseudouridylation assay using submaximal amounts of Pus1p with U-^{3}H-labeled SRA with or without cold STR5 or U206A inhibitors with incubation times of 30 and 60 min. STR5 inhibits pseudouridylation of SRA in a dose-dependent manner, whereas U206A has much less activity. The negative control incubation with β-Gal shows no contaminating PUS activity. (B) Similar assay showing that STR5 inhibits pseudouridylation of SRA more strongly than that of tRNA^{Ile}. (C) Pseudouridylation assay using indicated substrates at different incubation times at optimal Pus1p concentrations, as illustrated by robust time-dependent pseudouridylation of SRA. Control tRNA^{Ser} and tRNA^{Ala} are pseudouridylated with one and zero Ψ residues, respectively. Low levels (0.25 mol of Ψ/mol of RNA) of nonspecific pseudouridylation of STR5 and U206A are observed, showing that these RNA fragments do not serve as substrates for Pus1p. β-Gal negative control values have been subtracted.

but measurable effect on the higher-order structure of SRA and subsequent binding by these fragments. This is the first demonstration that both AR and ERα possess a significant SRA binding activity in their NTD despite the absence of a defined RBM, and that pseudouridylation of SRA modulates its binding properties for SRA-binding proteins. The binding of U206A SRA to ERα NTD and AR NTD was not significantly different from the binding of wild-type SRA to these proteins (Figure S1 of the Supporting Information).

Characterization of the RNA Binding Properties of the NTD of ERα and AR. To further characterize the novel RNA binding properties of the NTD of AR and ERα, competition experiments were performed using the AR NTD, ERα NTD, positive control SHARP-RRM, and submaximal amounts of U-^{3}H-labeled, un-pseudouridylated SRA as a substrate in our high-stringency buffer. Next, increasing doses of cold competitor molecules were added, and effects on SRA binding were established by a filter binding assay. As shown in the control experiment (Figure 4A), SRA competes well with itself, strongly inhibiting binding when used in an only 20-fold molar excess, suggesting that binding of SRA by all three protein fragments is specific. However, RNA binding proteins often bind RNA in a manner largely independent of its primary sequence. For instance, p68/p72 binds both sense and antisense SRA.[10] To test this, we used an mRNA of similar length that encodes Pus1p as competitor. As shown in Figure 4B, mRNA competes as effectively as SRA for NR binding. We next tested competition by long, single-stranded RNAs poly(rC) and poly(rG) (Figure 4C). Interestingly, poly(rC) did not inhibit binding of SRA even at a 100-fold molar excess; however, poly(rG) was a strong inhibitor of binding of SRA to all three proteins, preventing all binding at 10−100-fold molar excesses. Like mRNA, long, linear ds(rC+rG) also efficiently inhibited binding. These results suggest that the novel RBM in AR NTD and ERα NTD and the RRM in SHARP all prefer binding to long structured RNAs, and that interactions with guanines are critical. In contrast, STR5 RNA was unable to compete with and inhibit binding of SRA to NRs even at doses of up to 800-fold molar excess, and thus, short structured STR5 does not interfere with SRA−NR interactions (Figure 4D). Finally, we used an unrelated double-stranded (ds) cDNA fragment as a competitor, but at a 20-fold molar excess where SRA, mRNA, and poly(rG) virtually completely blocked SRA binding, no decrease in the level of SRA binding was observed (Figure 4E). These results support the existence of a novel RBM in the NTD of AR and ERα.

STR5 Inhibits ERα- and AR-Dependent Transactivation of Target Genes. In the final series of experiments, the

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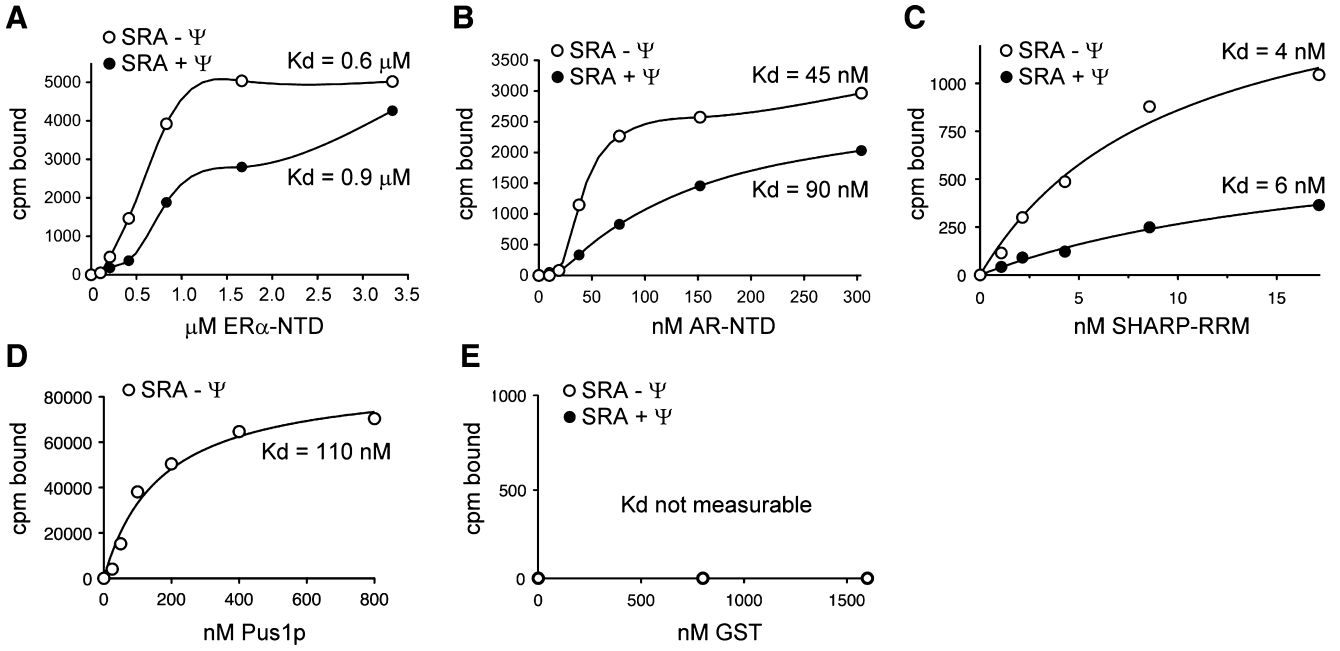

Figure 3. NTD of ERα and AR that contains a strong SRA binding activity. Results of filter binding assays establishing binding between unmodified (−Ψ) and Pus1p-pseudouridylated (+Ψ) U-^{3}H-labeled SRA and indicated proteins. Calculated K_d values for both SRA templates are shown where possible: (A) ERα NTD, (B) AR NTD, (C) positive control SHARP-RRM, (D) Pus1p, and (E) negative control GST. Representative results of experiments performed in duplicate are shown. In panels A and B, spline curves [GraphPad Prism; fit spline (cubic) with 28 segments] are pictured to better represent the sigmoidal nature of the data, whereas in panels C and D, a nonlinear best curve is represented.

effects of STR5 on the functional activity of ERα and AR in their cognate cancer cells was determined. ERα transactivational activity was determined by cotransfecting E_2-responsive Luc reporter construct ERE-Luc into MCF-7 cells, and endogenous ERα activity was established in the absence or presence of E_2. As shown in Figure 5A, Luc activity is robustly induced in the presence of ligand, as expected. However, when cells were cotransfected with STR5, a dose-dependent decrease in Luc activity was observed, resulting in completely blocked ERα-mediated activity at the largest dose. This effect was specific because cotransfected large-dose tRNA, which is similar in length and also highly structured, had no effect. Cotransfected large-dose U206A inhibited ERα-dependent transactivation to some extent, but much more weakly than STR5, which is in line with their differential effects on blocking Pus1p-mediated pseudouridylation of SRA (Figure 2A). Further, we reasoned that if STR5 inhibits Pus1p activity, and thereby the function of SRA, then an increased level of expression of either Pus1p or SRA would be expected to rescue inhibition by STR5, but if other, unknown pathways are involved, then these factors should fail to do so. To test this possibility, MCF-7 cells were again cotransfected with ERE-Luc and an intermediate inhibitory amount (200 ng) of STR5, resulting in approximately 50% inhibition of ERα activity. As shown in Figure 5A, 100 ng of cotransfected Pus1p and SRA expression vectors fully reversed the inhibitory effects of STR5, consistent with STR5 blocking the Pus1p−SRA−ERα signaling axis. Consistent with our previous studies,[21,31] rescue by overexpressed SRA also shows that STR5-mediated inhibition of transcription or translation is specific. Parallel experiments were also performed in DHT-responsive LNCaP cells, which express a mutant but functional endogenous AR. Cells were transfected with DHT-responsive PSA-Luc reporter plasmid and treated with or without DHT to determine AR-dependent transactivation. As shown in Figure 5B, reporter gene activity is strongly induced by DHT as expected but is inhibited in a dose-dependent manner by cotransfected STR5. Cotransfected U206A again has considerably weaker suppressive effects, whereas cotransfection of tRNA shows no effect at all.

To further verify the inhibitory activity of STR5 on ERα and AR signaling, we also tested regulation of endogenous hormone-responsive target genes in response to treatment with STR5. MCF-7 cells were transfected with tRNA control or STR5 and treated for 48 h with either E_2 or solvent control. Next, RNA was isolated, and the expression of established ERα target genes *pS2* and *GREB1* was assessed by qRT-PCR. As shown in panels A and B of Figure 6 in the absence of hormone, basal mRNA levels were unaffected by STR5 compared to tRNA control. When E_2 was added, both genes were upregulated by endogenous ERα in the presence of control tRNA, but here, the presence of STR5 significantly suppressed gene expression. Similar effects were observed in LNCaP cells where AR target genes *PSA* and *KLK2* were upregulated by DHT in the presence of transfected tRNA control, but here too, induction was inhibited by cotransfected STR5. Thus, these functional studies corroborate our biochemical and transfection studies and together strongly suggest that STR5 can be used to target the Pus1p−SRA−NR axis, and modulate signaling of ERα and AR in hormone-responsive cancer cells.

DISCUSSION

Earlier studies suggested that inhibition of Pus1p-mediated pseudouridylation of SRA presents a novel target for inhibition of ERα and AR signaling in breast and prostate cancer cells, respectively.[2,3] Most clinical strategies are aimed at blocking the activity of these receptors through various inhibitors and specific modulators that focus on inactivation of inducible AF-2 in the LBD, which is aided by the available atomic structure of

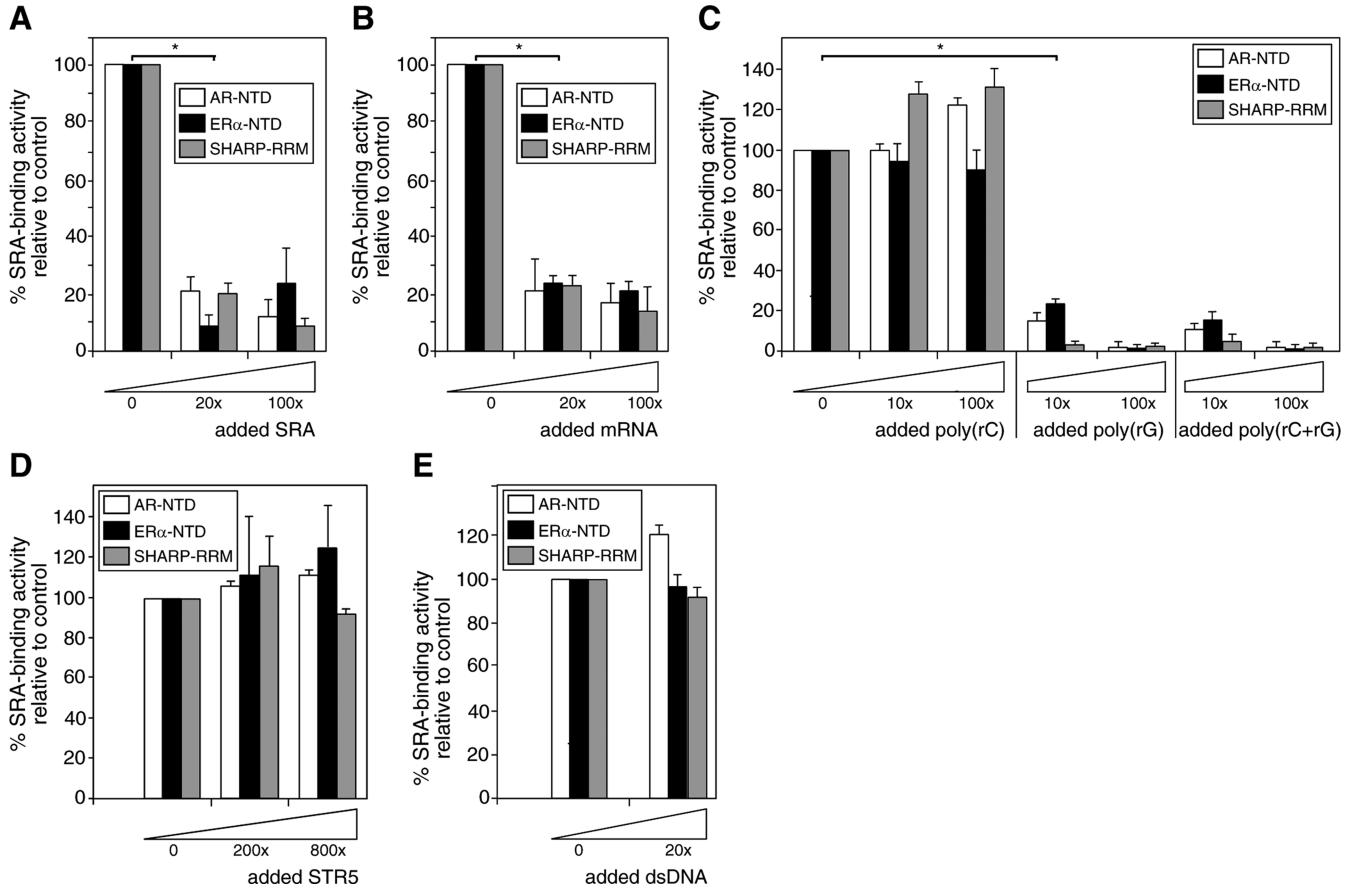

Figure 4. Characterization of the RBM in the NTD of ERα and AR. Filter binding assays with indicated proteins ERα NTD, AR NTD, positive control SHARP-RRM, and U-^{3}H-labeled SRA template (unmodified) with an increasing dose of cold competitor substrates. The level of SRA binding in the absence of a competitor is set at 100%. (A) Long, structured SRA and (B) mRNA are effective competitors. (C) Long unstructured poly(rC) does not compete, but all proteins have a high affinity for poly(rG). Long linear ds(rC+rG) also competes for binding. (D) Short structured STR5 and (E) long linear dsDNA do not inhibit binding.

this domain. However, alternative pathways that block receptor activity are still urgently needed because current drugs are not always effective or can result in unwanted side effects. We showed that STR5, a small RNA molecule representing a stable stem−loop structure in the conserved SRA core sequence that contains Pus1p target U206, can effectively inhibit Pus1p-mediated pseudouridylation of SRA. The likely mechanism is reversible competition of Pus1p because inhibition can be rescued by overexpression of either Pus1p or SRA. Curiously, the U206A mutant of STR5 does not compete as well with SRA for Pus1p-dependent pseudouridylation even though neither STR5 nor U206A is a substrate for Pus1p. It is plausible that STR5 associates more tightly with the catalytic cleft of Pus1p than U206A but lacks unknown additional structural determinants that permit pseudouridylation; however, the exact mechanism remains to be established.

In some studies, it was shown that the NTD of AR, and possibly ERα, can associate with SRA,[18,25] but because of the use of nonquantitative techniques for detecting NR−SRA complexes, the biological significance of this association remains unclear. We, for the first time, studied the putative RNA binding activity in the AR and ERα NTD in a biochemical way. The AF-1 domains of AR and ERα have been extensively studied. In AR, this modular domain can be further subdivided into transcription activation units τ-1 (amino acids 100−370) and τ-5 (amino acids 360−485),[44] but there are no structures of AF-1 available because it lacks stable secondary structure, although local folding can be induced.[45] A large number of proteins have been shown to bind to the NTD of AR, including general transcription factors TFIIH and P-TEFb, coactivators ARA160, ART27, CBP, p160 family members, AES, and EGR1, corepressors SMRT and SMAD3, transcription factors such as STAT3, and coregulatory proteins ARNIP, BRCA-1, caveolin-1, Cyclins D1 and E, and pRb.[36−38,46] Particularly noteworthy are ANT-1 (PRPF6), a coactivator of AR (but not ERα) that also binds splicing factor U5 snRNP,[36,40] and TZF, a corepressor with a potential RBD;[39] however, associations with SRA have not been tested.

Intriguing evidence has also been obtained for ERα. The AF-1 domain of ERα is embedded in a hydrophobic, Pro-rich region between amino acids 51 and 149; amino acids 51−93 and 102−149 can synergize with AF-2.[41] These regions partially or completely overlap with Box-1 (amino acids 41−64) and Box-2 (amino acids 87−108), which have been shown to be important for partial agonist activity of 4-HT, E_2-stimulated transcriptional activity,[47] and synergistic interactions with AF-2.[48] As with AR, the ERα NTD associates with a large number of cofactors.[42,43] With respect to a possible overlap between AF-1 and the novel RNA binding activity identified in our studies, an interesting result has been obtained for SRA binding protein p68. p68 is a coactivator of ERα (but not ERβ or AR) and binds AF-1, but not AF-2. This is an important observation

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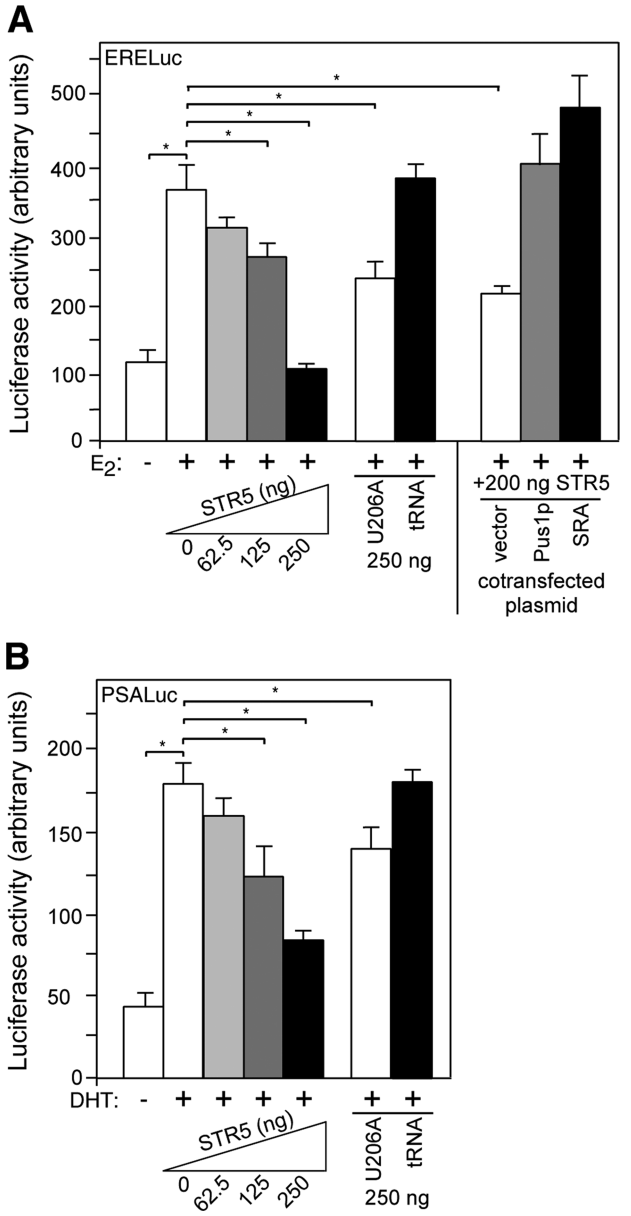

Figure 5. STR5 specifically inhibits ERα and AR signaling that can be reversed by overexpressed Pus1p and SRA. (A) The transfection assay in MCF-7 cells with the ERE-Luc reporter plasmid and cotransfected STR5 shows dose-dependent inhibition of ERα activity, whereas cotransfected U206A and tRNA have a weaker effect and no effect, respectively. STR5-mediated inhibition is reversed by cotransfected expression plasmids for Pus1p and SRA (right), consistent with their involvement in the targeted signaling pathway. (B) A similar experiment in LnCaP cells with PSA-Luc shows dose-dependent inhibition of AR signaling by STR5, but less so and none at all by U206A and tRNA, respectively. Luc activity in cell extracts was normalized and expressed as arbitrary units. Shown are results of representative experiments performed in triplicate.

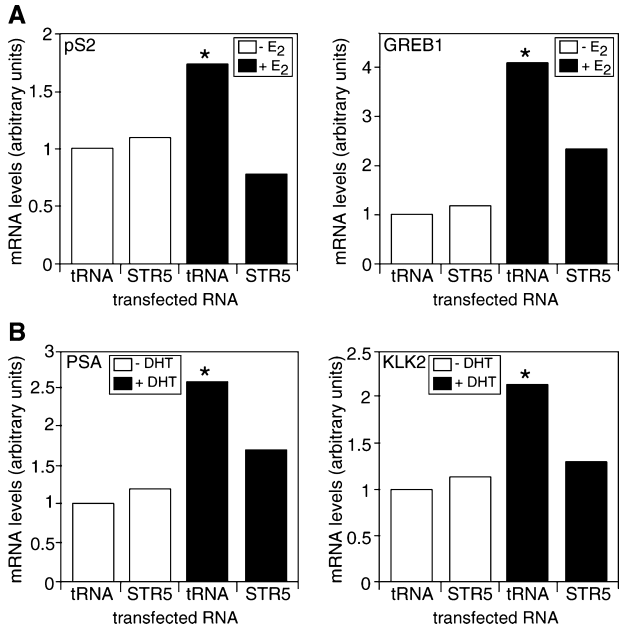

Figure 6. STR5 suppresses hormone-dependent induction of endogenous ERα and AR target genes, but not basal mRNA levels, in steroid-sensitive cancer cells. (A) MCF-7 cells grown in six-well plates were transfected with 800 ng of tRNA control or STR5 per well for 48 h with or without E_2, after which ER-regulated, endogenous pS2 and GREB1 mRNA expression levels were determined by qRT-PCR analysis. After normalization for β-actin mRNA levels, the level of expression in the absence of E2 was set to 1. (B) Similar experiment except that LNCaP cells were used and AR-regulated PSA and KLK2 gene expression was determined with or without DHT. Shown are results of representative experiments performed three times in triplicate with SD < 10%. *$P < 0.05$ (Student's t test).

because activity of ERα without AF-1 is significantly impaired.[48] These findings suggest a new mechanism by which SRA may help establish linking of AF-1 with p68. Curiously, there is little sequence homology between the AR and ERα NTDs, suggesting that either they share a similar RBM that is difficult to identify from primary sequence homology or they have completely different RNA binding folds. The latter may be more likely because AR binds SRA with an ∼10-fold higher affinity. We are currently trying to identify the precise nature of the RBM in these receptors.

Our studies show that despite the absence of a recognizable RBM in the NTD, both AR and ERα bind SRA and other long, structured RNA and linear dsRNA in a manner independent of primary sequence with surprisingly high affinity in the low to high nanomolar range. In contrast, short structured RNAs and linear dsDNA are not recognized. We also showed that this novel RBM has a high affinity for poly(rG), but not poly(rC), suggesting that critical Arg− and/or Lys−guanine interactions contribute to the novel binding fold, as has been demonstrated previously.[49] Furthermore, we found that the K_d is moderately but measurably affected by the SRA pseudouridylation status for binding by AR NTD, ERα NTD, and SHARP-RRM. We believe that pseudouridylation helps to free SRA that is sequestered by corepressor SHARP because of changes in the conformation of SRA. Indeed, we obtained evidence by RNA structure-sensitive RNase digestion analysis that pseudouridylation subtly changes the secondary structure of SRA (Figure S2 of the Supporting Information).

Taken together, we provided evidence that STR5 represents a new class of RNA inhibitor that reduces the level of steroid receptor signaling in hormone-sensitive cancer cells via an unexplored pathway that involves prevention of Pus1p-dependent pseudouridylation, and activation of coactivator SRA (summarized in our model in Figure 7). These findings indicate the possibility of a novel therapeutic strategy that combines the use of STR5 with classic receptor antagonists, and furthermore, future analysis of the novel RBMs in ERα

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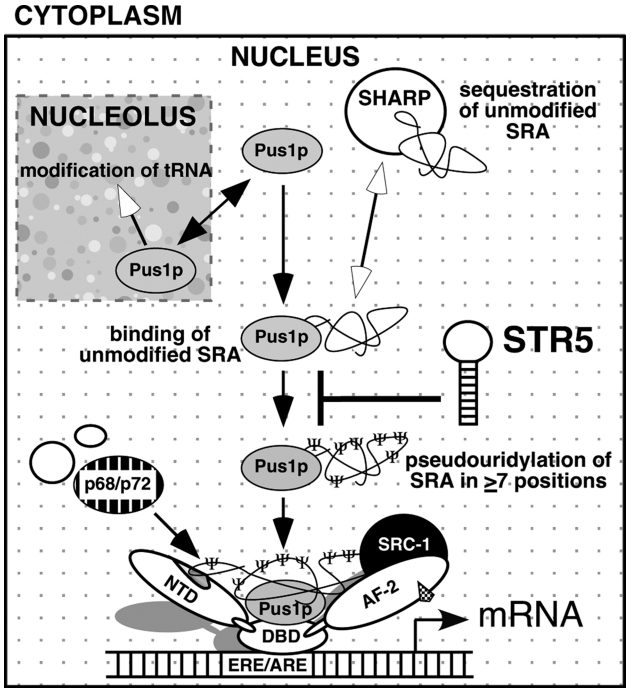

Figure 7. Proposed model of the Pus1p–SRA–steroid receptor axis and mechanism of STR5-mediated inhibition of ERα and AR signaling. Pus1p is involved in tRNA processing in the nucleolus and pseudouridylation of SRA in the nucleus. Corepressor SHARP binds unpseudouridylated SRA with high affinity and sequesters it from interacting with downstream factors. Pseudouridylation of SRA by Pus1p lowers its binding capacity, thereby allowing SRA together with Pus1p to form a complex with the NTD of ERα and AR that is bound to the ERE and ARE in target genes, respectively. This in turn results in recruitment of other proteins such as p68/p72 to SRA and coactivation of receptor signaling. STR5 competitively inhibits Pus1p-dependent pseudouridylation of SRA, preventing it from functioning as a coactivator and subsequently blocking target gene expression.

NTD and AR NTD may lead to identification of similar domains in other proteins that were never suspected to bind RNA, which may lead to many new scientific insights.

■ ASSOCIATED CONTENT

Supporting Information

Curves for binding of U206A SRA to AR NTD and ERα NTD (Figure S1) and depiction of how pseudouridylation changes the secondary structure of SRA (Figure S2). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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■ ABBREVIATIONS

AR, androgen receptor; ARE, androgen response element; DBD, DNA binding domain; DHT, dihydrotestosterone; E_2, 17β-estradiol; ER, estrogen receptor; ERE, estrogen response element; LBD, ligand binding domain; MLASA, mitochondrial myopathy and sideroblastic anemia; ncRNA, noncoding RNA; NR, nuclear receptor; NTD, N-terminal domain; PUS, pseudouridine synthase; RBM, RNA binding domain; RRM, RNA recognition motif; SRA, Steroid receptor RNA Activator.

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