Retinoic Acid (RA) Regulates 17β-Hydroxysteroid Dehydrogenase Type 2 Expression in Endometrium: Interaction of RA Receptors with Specificity Protein (SP) 1/SP3 for Estradiol Metabolism

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Context: The enzyme 17β-hydroxysteroid dehydrogenase type 2 (HSD17B2) exerts a local antiestrogenic effect by metabolizing biologically active estradiol to inactive estrone in endometrial epithelial cells. Retinoic acid (RA) induces HSD17B2 expression, but the underlying mechanism is not known.

Objective: Our objective was to elucidate the molecular mechanisms responsible for HSD17B2 expression in human endometrial cells.

Method: Human endometrial Ishikawa and RL95–2 cell lines were cultured in the presence or absence of RA to analyze endogenous HSD17B2 expression, transcription factor complex formation, and promoter activity.

Results: RA induced HSD17B2 mRNA levels in a dose- and time-dependent manner in endometrial cells. The RA antagonist ANG11273 abolished RA-induced HSD17B2 expression. Small interfering RNA ablation of RA receptor (RAR)α or retinoid X receptor (RXR)α completely blocked RA-induced HSD17B2 gene expression. Analysis of serial deletion and site-directed mutants of the HSD17B2 promoter fused to a reporter gene indicated that RA induction requires a *cis*-regulatory sequence that binds the specificity protein (SP) class of transcription factors. Chromatin-immunoprecipitation-PCR and gel-shift assays showed that RARα/RXRα and SP1/SP3 interact with this HSD17B2 promoter sequence. Small interfering RNA ablation of SP1 and SP3 expression markedly decreased HSD17B2 basal expression and blocked RA-induced expression. Finally, immunoprecipitation-immunoblotting demonstrated RA-induced interactions between RARα/RXRα and SP1/SP3 in intact endometrial cells.

Conclusions: In endometrial epithelial cells, RA stimulates formation of a multimeric complex comprised of RARα/RXRα tethered to transcription factors SP1 and SP3 on the HSD17B2 promoter. Assembly of this transcriptional complex is necessary for RA induction of HSD17B2 expression and may be an important mechanism for local estradiol inactivation in the endometrium. **(*J Clin Endocrinol Metab* 93: 1915–1923, 2008)**

ESTROGEN and progesterone play important roles in the regulation of endometrial function and the pathogenesis of endometrial cancer (1). The biologically potent estrogen, estradiol (E_2), causes thickening of endometrium through epithelial proliferation that is abruptly blocked and switched to a state of stromal-epithelial differentiation upon the addition of progesterone. This influence of progesterone in creating a secretory phenotype is essential for implantation of the embryo and pre-

0021-972X/08/$15.00/0

Printed in U.S.A.

doi: 10.1210/jc.2007-1536 Received July 11, 2007. Accepted February 6, 2008.

First Published Online February 12, 2008

Abbreviations: ChIP, Chromatin immunoprecipitation-PCR; E_2, estradiol; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GRE, glucocorticoid response element; HSD, hydroxysteroid dehydrogenase; HSD17B2, 17β-hydroxysteroid dehydrogenase type 2; IB, immunoblotting; IP, immunoprecipitation; nt, nucleotide; RA, retinoic acid; RAR, retinoic acid receptor; RARE, RAR response element; RXR, retinoid X receptor; siRNA, small interfering RNA; SP, specificity protein.

vention of malignant transformation of endometrial epithelium under unopposed estrogenic action. Progesterone opposes estrogenic action via a number of mechanisms in the endometrium (1), including inactivation of E_2 via rapid conversion to estrone (2). In endometrial epithelial cells, the enzyme 17β-hydroxysteroid dehydrogenase type 2 (HSD17B2) is responsible for the rapid conversion of E_2 to estrone by oxidation at the C17 position (3, 4). By decreasing the local levels of biologically active estrogen, HSD17B2 may play a critical role in limiting the local action of estrogen in target tissues (2).

Previous studies have shown that the HSD17B2 gene is much more highly expressed within the glandular epithelium than in stromal cells within human endometrial tissue, and the expression of HSD17B2 is markedly enhanced by progesterone (3). Furthermore, Yang *et al.* (2) demonstrated that progesterone indirectly induced HSD17B2 expression in human endometrium via a paracrine mechanism whereby stromal cells secrete factors that induce HSD17B2 transcription within epithelial cells. Moreover, recent studies indicated that retinoids are a component of the hormonal cocktail secreted by endometrial stromal cells that regulates endometrial epithelial gene expression (5, 6). However, the mechanisms responsible for retinoic acid (RA)-dependent regulation of the HSD17B2 gene remain to be identified.

RA, the most active form of vitamin A, plays a critical role in the development and homeostasis of a variety of vertebrate tissues through its regulatory effects on cell differentiation, proliferation, and apoptosis (for review, see Refs. 7–9). Numerous experiments have demonstrated that RA is essential for the maintenance of differentiated phenotypes of many epithelial tissues. In rat endometrium, RA deficiency leads to widespread hyperkeratinization, whereas high concentrations of retinoids promote secretory characteristics (10, 11). Furthermore, retinol deficiency in rat leads to irregular estrous cycles, morphological changes in the uterine epithelium, failure to establish or complete pregnancy, and fetal malformations. RA treatment restores normal uterine epithelium and maintains fertility (10, 11).

The physiological effects of RA are mediated by members of two families of nuclear receptors, the RA receptors (RARs) (12) and the retinoid X receptors (RXRs) (7–9). All-*trans* RA is a ligand for RAR, whereas the isomer 9-*cis* RA can bind either RAR or RXR. There are three subtypes of each receptor (α, β, and γ) and several isoforms that differ in tissue distribution. Upon binding of RA, RAR and RXR form the functional heterodimers and bind to RA receptor response elements (RAREs) within target gene promoters to modulate gene transcription. Northern blotting analysis indicated that mRNA transcripts of RARα, RARβ, RARγ, and RXRα were present in normal human endometrium (12) and endometrial carcinoma tissues (13). Immunohistochemistry showed that RARα, β, and γ, and RXRα, β, and γ were all detectable in human endometrium tissue throughout the phases of the menstrual cycle, and RARα and RXRα were predominantly expressed in both endometrial epithelial and stromal cells (14). Recent studies from Li (15) and Ito (14) *et al.* indicate that RA stimulates the expression of the HSD17B2 gene in the endometrial RL-95 cell line. Furthermore, Su *et al.* (16) reported that RA up-regulated HSD17B2 mRNA and enzymatic activity in human placental cells. However, the *cis*-DNA sequence(s) and transactivating factor(s) involved in retinoid-induced HSD17B2 expression have not yet been identified. Here, we used the well-differentiated malignant endometrial epithelial cell lines that endogenously express HSD17B2 as model systems to elucidate the mechanisms responsible for RA-dependent induction of HSD17B2 in the endometrium.

Materials and Methods

Plasmid constructs

Luciferase reporter plasmids containing various fragments of the HSD17B2 promoter were prepared as previously described (2). Oligonucleotide-directed mutagenesis of a half glucocorticoid response element (GRE) and specificity protein (SP) 1 motifs in pHSD(−150/−1)-Luc and pHSD(−100/−1)-Luc plasmids were performed using the Quick Change Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA). Oligonucleotides used were: half GRE mutant, GGGTGAGTATGTGCATGTacTaTGTATGCAGGGGTGGGGA; SP1 single mutant, ATGTGTTGTGTATGCAGaaGaTaaGaAGGGGGCGAGAGCGGTC; and SP1 double mutant, ATGTGTTGTGTATGCAGaaGaTaaGaAaaGaCGAGAGCGGTCAAATAGG. The mutated oligonucleotides are shown in lower case letters. Mutated base pairs were confirmed by DNA sequencing.

Cell culture

The human endometrial epithelial Ishikawa cell line was a kind gift from Dr. Masato Nishida (Kasumigaura National Hospital, Tsuchiura, Ibaraki, Japan) (2). The RL95–2 (CRL-1671) cell line was obtained from the America Type Culture Collection (Manassas, VA). Both cell lines were grown in a mixture of DMEM and F12 (1:1) medium (Invitrogen Life Technologies Inc., New York, NY) with 2.5 mM L-glutamine, 1.2 g/liter sodium bicarbonate, 1.5 mM HEPES, and 10% fetal bovine serum. Ishikawa and RL95–2 cells express endogenous estrogen receptor-α, progesterone receptor, and HSD17B2 (17, 18). Previous studies indicated that Ishikawa cells treated with estrogen and progesterone were responsible via estrogen receptor-α and progesterone receptor (2).

Transfection studies

Transient transfection studies were performed in triplicate in six-well plates by the liposome method described by Cheng and Handwerger (19). Each well contained 5 μg reporter plasmid and 0.5 μg pRL-TK-Luc (Promega Corp., Madison, WI). Cell extracts were prepared using the luciferase cell lysis reagent (Promega Corp.). Results represent the average of three independent transfection assays normalized to pRL-TK-Luc using a dual luciferase reporter assay system (Promega Corp.).

Small interfering RNA (siRNA) transfection

RNA oligonucleotides directed against RARα (sense sequences: GGUAUUAAUUCUCGCUGGUtt), RXRα (sense sequences: GGAGAUGCAUCUAUUUUAAtt), SP1 (sense sequences: GGAUGGUUCUGGUCAAAUAtt), and SP3 (sense sequences: GGGAUAGGAACUGUUAAUAtt) were purchased from Ambion, Inc. (Austin, TX). After transfected with siRNA, the Ishikawa and RL95–2 cells were incubated with vehicle or all-*trans* RA. A negative control siRNA was purchased from Ambion; the control sequence had no matches in the human genome.

RT and real-time quantitative PCR

Total RNA was isolated from Ishikawa or RL95–2 cells. First-strand cDNA synthesis was performed using SuperScript III Reverse Transcrip-

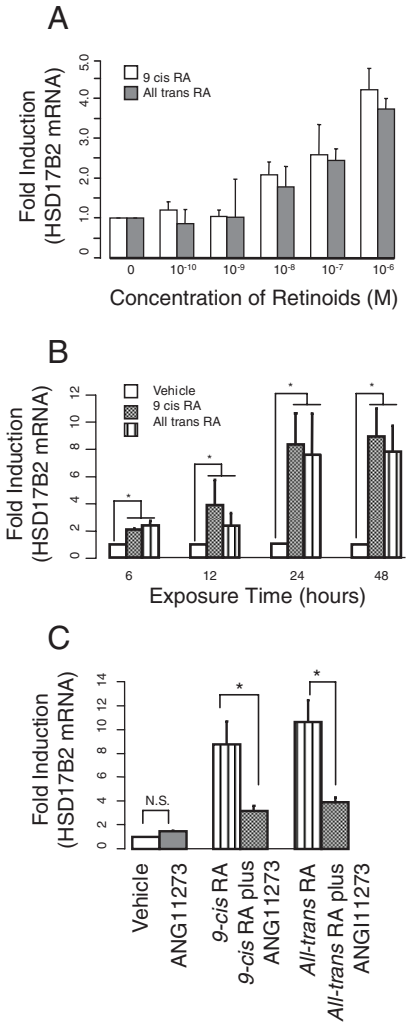

FIG. 1. Retinoids up-regulate HSD17B2 mRNA levels in human endometrial epithelial cells. A, Ishikawa cells were exposed to either vehicle (0) or the indicated concentration of retinoids for 24 h. B, Ishikawa cells were exposed to 10^{-7} mol/liter retinoids for the indicated time. C, Ishikawa cells were exposed to vehicle, 10^{-5} mol/liter ANG11273, 10^{-7} mol/liter retinoids, or retinoids plus ANG11273 for 24 h. Cells were then harvested for RNA extraction. First-strand cDNA was prepared from RNA, and equal amounts were subjected to real-time quantitative PCR analysis. Levels of specific mRNAs were normalized to the amount of GAPDH in the same sample. Results are expressed as the fold increase in mRNA levels relative to the control sample. Data reported are representative of three independent experiments, each performed in triplicate. *, $P < 0.01$. N.S., Not significant.

tase (Invitrogen Life Technologies) according to the manufacturer's protocol. Real-time quantitative PCR was performed using a 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) using a SYBR green Master Mix (Applied Biosystems). The sequences of primers used for detection of specific transcripts were: HSD17B2-f, GCCAGTGCAGATAAAAGATGC; HSD17B2-r, ATAAGAAGAAGCTCCCCATCAG; RARα-f, AGCATCCAGAAGAACATGGTG; RARα-r, TTCGGTCGTTTCTCACAGACT; RXRα-f, CAGCTC AGCTCACCTATGAA; RXRα-r, AGGAAGCCATGTTTCCTGAG; SP1-f, CTTGGTATCATCACAAGCCAGTT; SP1-r, TCCCTGATGATCCACTGGTAGTA; SP3-f, TTGACTACATCTAGTGGGCAGGT; SP3-r, TACAACAGGCTGTGCTGTAGAAA; glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-f, CCATGGAGAAGGCTGGGG; and GAPDH-r, CAAAGTTGTCATGGATGACC. The amount of RT-PCR product for the gene of interest was normalized to the amount of GAPDH in the same sample.

Preparation of nuclear extracts

Nuclear extracts were prepared from Ishikawa or RL95–2 cells using the NE-PER nuclear extraction kit (Pierce, Rockford, IL). Protein concentration was determined by Bradford assay (Bio-Rad Laboratories, Inc., Hercules, CA) using BSA as a standard.

Immunoblotting

Equal amounts of nuclear extract from Ishikawa or RL95–2 cells were subjected to 7.5% SDS-PAGE. Immunoblotting studies were performed as described previously (19). Antisera used were rabbit polyclonal IgGs raised against RARα, RXRα, SP1, or SP3 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). To confirm equal loading of the samples, the immunoblots were stripped and reprobed with a monoclonal mouse antibody specific for β-actin (CHEMICON International, Inc., Temecula, CA) or GAPDH (Ambion, Inc.). Quantification of the blots was performed with Quantity One software (Bio-Rad, München, Germany).

EMSAs

EMSAs were performed as described previously (19). The double-stranded oligonucleotides used for the assays were 5′-TATGCAGGGGGTGGGGAGGGGCGAGAG-3′, identical to a 27-bp sequence [nucleotide (nt) −87/−60] within the regulatory region of the HSD17B2 gene that contains two overlapping SP motifs (at nt −82/−72 and nt −76/−65). For competition assays, 100-fold molar excess of unlabeled oligonucleotide was incubated with the nuclear extracts before the addition of labeled probe. For supershift analysis, nuclear extracts were incubated with rabbit polyclonal IgGs raised against RARα, RARγ, RXRα, RXRγ, SP1, and SP3 before the addition of the radiolabeled probe. Normal rabbit IgG was used as a control. Binding reactions were resolved in a 5% nondenaturing polyacrylamide gel.

Chromatin immunoprecipitation-PCR (ChIP) assays

ChIP assays were performed using a kit following the manufacturer's instructions (Upstate Biotechnology, Inc., Lake Placid, NY). Ishikawa cells were transfected with pHSD(−150/−1)-Luc or pSP1dbmt-HSD(−150/−1)-Luc and incubated for 2 h with vehicle or all-trans RA, then cross-linked with 1% formaldehyde. The cell lysate was sonicated to generate DNA fragments with an average length of 200-1000 bp. Genomic and plasmid DNA enriched by antibodies against RARα or RXRα was purified by phenol extraction. The HSD17B2 promoter region was detected by PCR amplification using primers flanking the SP1 binding sites (sense 5′-TCCAGTTAGTCATCGCTCCAG-3′ and antisense 5′-TTCCTATTTGACCGCTCTGCG-3′) that yielded the predicted 128-bp product. The primers that amplify 152-bp hydroxysteroid dehydrogenase (HSD) promoter distal region (nt −2611/−2460) were used as a control; their sequences are: ACAAAGGAAACAGCCGAGTGC (sense) and AGGCACTCATGGATGGTAGG (antisense). An approximate 300-bp DNA fragment, including 150 bp of the HSD17B2 promoter that was inserted in the pGL3-Luc plasmid, was amplified by RVprimer3 and GLprimer2 (Promega Corp.).

Immunoprecipitation (IP)-immunoblotting (IB) assays

IP-IB assays were performed as described previously (19). Nuclear extracts were subjected to IP with antibodies directed against RARα or RXRα. After pull-down by protein A/G-agarose, the beads were boiled in 2× sodium dodecyl sulfate-polyacrylamide gel loading buffer and run on a 7.5% polyacrylamide gel. The gels were blotted to nitrocellulose and probed with rabbit antibodies against SP1 or SP3, or normal rabbit IgG. Immunoblots were incubated with secondary antibodies conjugated to horseradish peroxide and analyzed by chemiluminescent detection.

Statistical analysis

Statistical differences between samples were determined by ANOVA, followed by *post hoc* multiple comparison testing using the Newman-

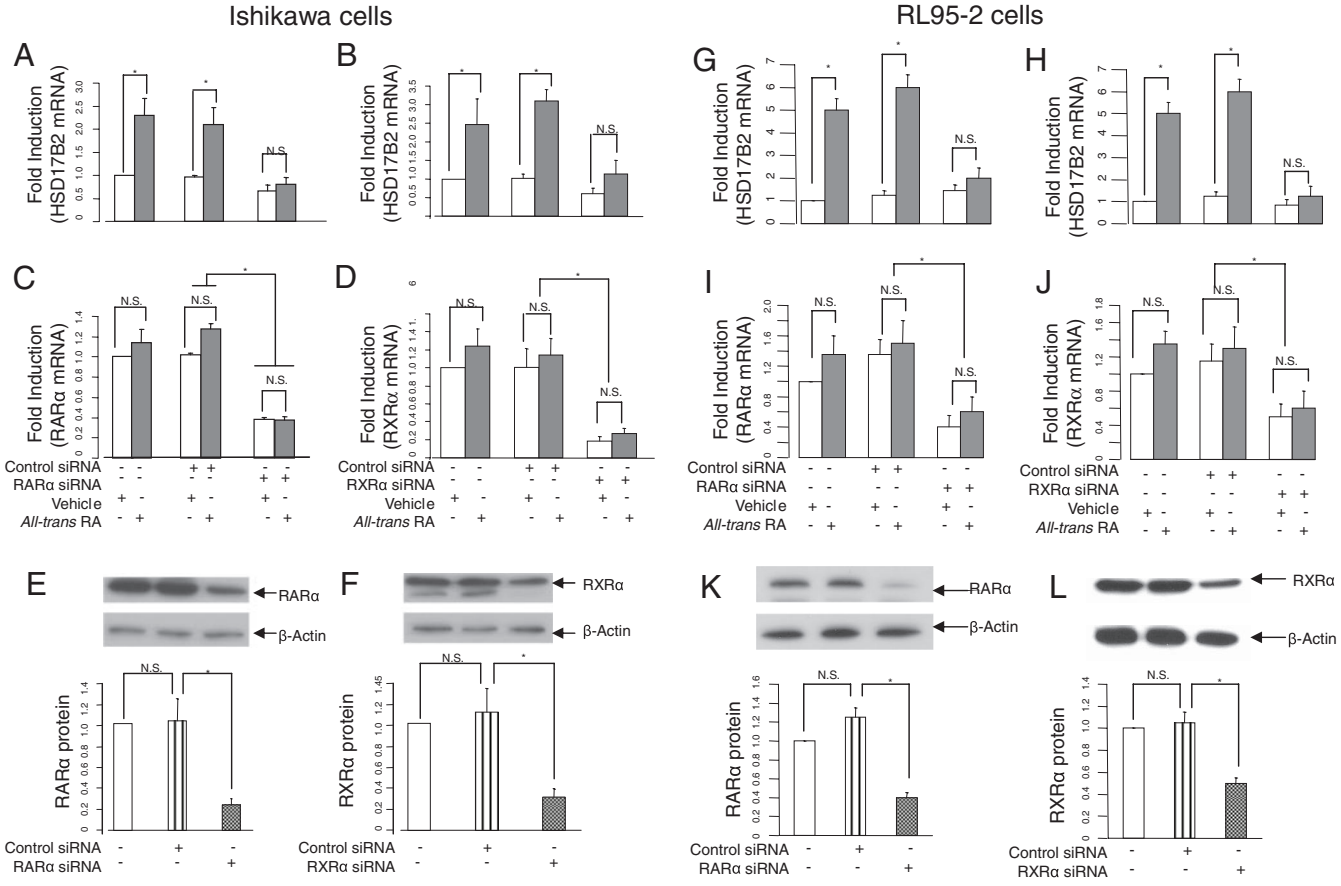

FIG. 2. Knockdown of RARα or RXRα blocked RA-mediated induction of HSD17B2 mRNA expression in Ishikawa and RL95–2 cells. Cells were transfected with siRNA targeted against RARα or RXRα and then exposed to 10^{-7} M all-trans RA for 18 h. RNA was isolated, and HSD17B2 (A, B, G, and H), RARα (C and I), or RXRα (D and J) mRNA transcripts were analyzed by quantitative RT-PCR. RARα (E and K) and RXRα (F and L) protein levels were assessed by immunoblotting. β-Actin was used as an internal control. Data are reported as the mean ± SEM of three independent experiments and are expressed as arbitrary units. *, $P < 0.01$. N.S., Not significant.

Keuls procedure. Values are expressed as mean ± SEM, and $P < 0.05$ was considered statistically significant.

Results

Retinoids up-regulated HSD17B2 expression in human endometrial epithelial cells

To understand the role of retinoids in transcriptional regulation of HSD17B2 in human endometrial cells, real-time quantitative PCR analyses were performed after Ishikawa cell exposure to increasing doses of retinoids (Fig. 1A) for 6–48 h (Fig. 1B). Both all-*trans* RA and 9-*cis* RA induced HSD17B2 mRNA levels in a dose-dependent manner in Ishikawa cells. Maximal stimulation occurred at 10^{-6} M, with a 5- to 10-fold increase in HSD17B2 mRNA levels. Increased expression of HSD17B2 mRNA could be detected after exposure to retinoids (10^{-7} M) for 6 h, and this induction continued to increase in a time-dependent manner up to 48 h.

To determine whether retinoid-mediated induction of HSD17B2 expression occurs through RARs, we used an RA antagonist, ANG11273, which previously completely blocked the activation of RXR (20, 21). ANG11273 alone had no significant effect on HSD17B2 mRNA expression but blocked both 9-*cis* and all-trans RA-induced HSD17B2 gene expression by 65% (Fig. 1C). Similar results were obtained using the RL95–2 cell line (data not shown).

Ablation of RARα and RXRα expression by siRNA blocked RA-induced HSD17B2 expression in Ishikawa cells

To examine whether retinoid induction of HSD17B2 mRNA is RAR isoform specific, Ishikawa and RL95–2 cells were transfected with siRNAs specific for RARα or RXRα. Transfected Ishikawa and RL95–2 cells were incubated for 18 h in the absence or presence of all-trans RA (10^{-7} M), followed by determination of mRNA and/or protein levels of RARα, RXRα, and HSD17B2 by real-time quantitative PCR (Fig. 2, A–D and G–J) and immunoblotting (Fig. 2, E, F, K, and L). In cells transfected with control siRNA or with transfection reagent alone, retinoid significantly stimulated HSD17B2 expression (Fig. 2, A, B, G, and H). Selective siRNA ablation of RARα or RXRα blocked RA-induced HSD17B2 expression but had no significant effects on HSD17B2 basal expression. Decreases in RARα and RXRα mRNA and protein levels confirmed the efficiency of gene silencing with siRNA (Fig. 2, C–F and I–L). Together, these results strongly suggest that RARα and RXRα are required for retinoid-induced HSD17B2 expression in human endometrium.

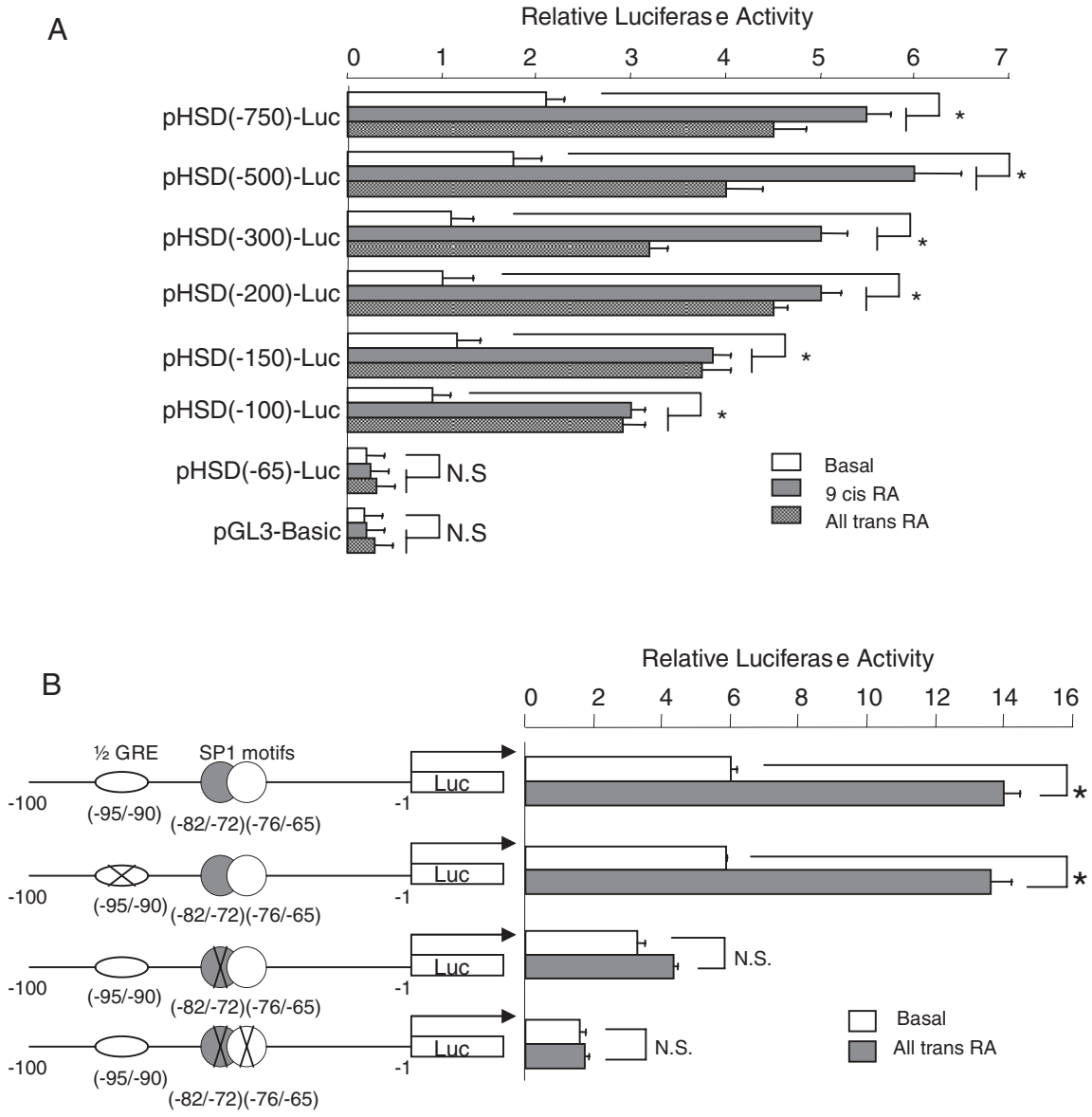

FIG. 3. Localization of the retinoid response region in the HSD17B2 promoter. A, Ishikawa cells were transfected with plasmid containing different lengths of the HSD17B2 gene promoter as shown, then exposed to 10^{-7} M retinoids or vehicle for 48 h. B, Sited-directed mutagenesis analysis of putative consensus sequences in the HSD17B2 promoter. Ishikawa cells were transiently transfected with wild-type or mutant pHSD(−100/−1)-Luc plasmids, exposed to either vehicle or 10^{-7} M retinoids for 48 h, and harvested for luciferase assay. Luciferase activity of each construct was normalized to pRL-TK-Luc. Each value represents the mean ± SEM of three separate transfections, each performed in triplicate. *, $P < 0.01$. N.S., Not significant.

Retinoids stimulated HSD17B2 promoter activity in Ishikawa cells

To determine whether DNA sequences within the 5′-flanking region of the HSD17B2 gene are involved in retinoid-mediated transcriptional regulation, transient transfection studies were performed in Ishikawa cells. Both 9-*cis* and all-*trans* RA (10^{-7} M) increased HSD17B2 promoter activity by approximately 3-fold (Fig. 3A). 5′-Deletion analysis of the HSD17B2 promoter a showed loss of retinoid responsiveness upon deletion of sequences between −100 and −65 bp.

Site-directed mutagenesis of the HSD17B2 promoter

A search of the HSD17B2 DNA sequence using the Transcription Element Search System (http://www.cbil.upenn.edu/cgi-bin/tess/tess) revealed that the region between −100 and −65 bp contains a putative half GRE and two overlapping SP1 motifs. Surprisingly, there is no consensus RARE within this region or within the full-length (nt −750/−1) HSD17B2 promoter. To identify *cis*-acting elements critical for retinoid-dependent regulation of HSD17B2 expression, we selectively mutated the half GRE (nt −95/−90) and two overlapping SP1 motifs (nt −82/−65). Retinoid stimulated wild-type promoter activity 2.5-fold (Fig. 3B). Selective mutation of the half GRE did not alter RA responsiveness, whereas mutation of the two overlapping SP1 motifs not only decreased basal HSD17B2 promoter activity ($P < 0.05$) but completely blocked RA responsiveness. Together, the results from the deletion and site-directed mutagenesis analyses strongly suggest that the two SP1 motifs between −100 and −65 bp are critical for retinoid induction of HSD17B2 transcription.

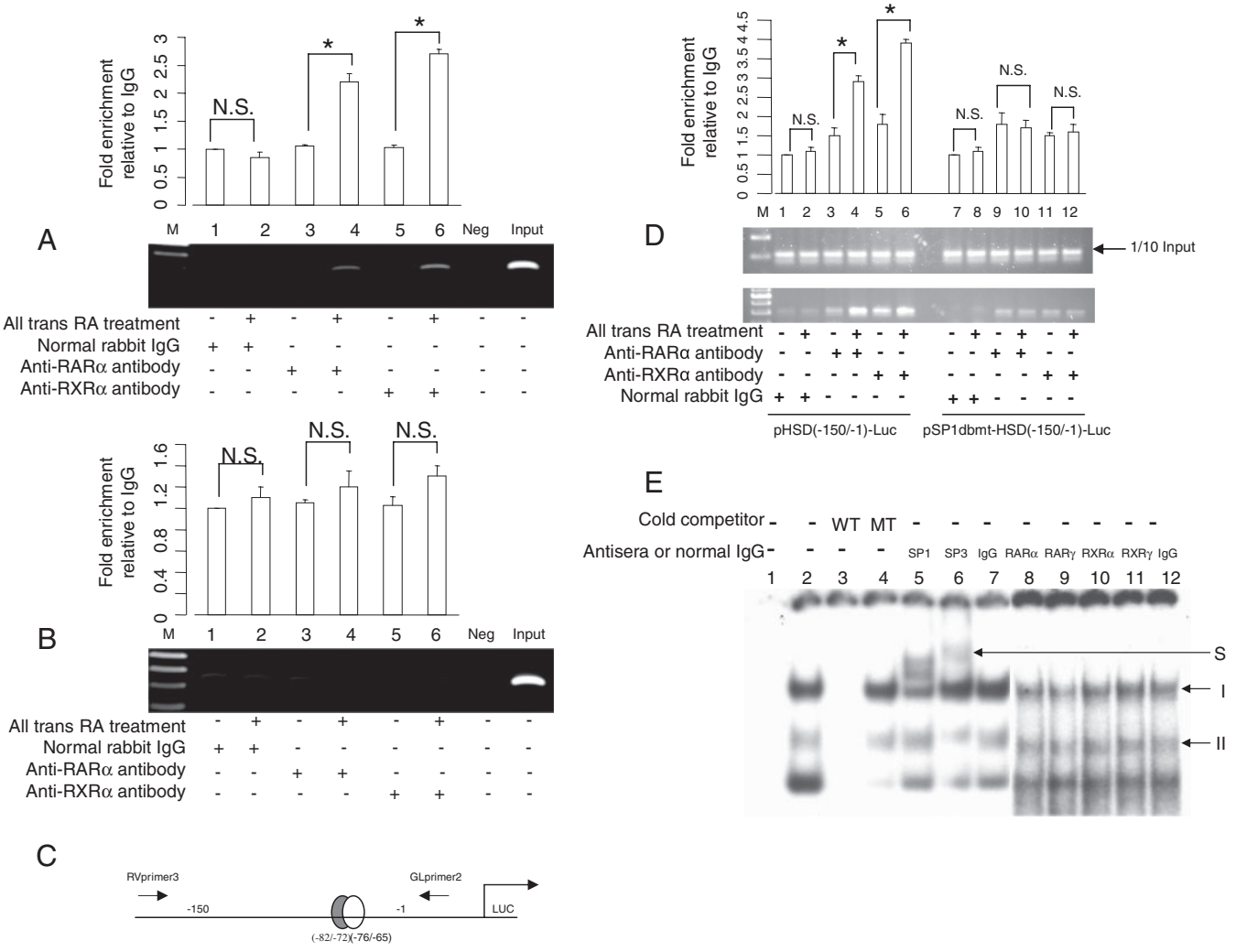

FIG. 4. Association of transcription factors with the HSD17B2 promoter. A and B, Conventional ChIP to demonstrate association of RARα/RXRα with the native promoter region. Ishikawa endometrial epithelial cells were cultured and exposed to vehicle or 10^{-7} M all-trans RA for 2 h. DNA interacting with RARα and RXRα was immunoprecipitated by anti-RARα (*lanes 3* and *4*) or anti-RXRα (*lanes 5* and *6*) antibodies and amplified for 35 PCR cycles. Nonspecific rabbit IgG was used as negative control for IP (*lanes 1* and *2*). Quantification of fold enrichment relative to IgG was shown in A, B, and D. Neg, Negative control and input, one tenth the initial volume. C and D, ChIP to demonstrate the interaction of RARα/RXRα with exogenous promoter region. C, Schematic of luciferase plasmid containing human HSD17B2 promoter (nt −150/−1). The RVprimer3 and GLprimer2 bind specifically to the plasmid DNA sequence. D, Ishikawa cells were transfected with wild-type (WT) pHSD(−150/−1)-Luc or double-mutant (MT) pSP1dbmt-HSD(−150/−1)-Luc plasmids and then exposed to vehicle or 10^{-7} mol/liter of all-trans RAs for 2 h. ChIP assays were performed with anti-RARα or anti-RXRα antibodies. The graphic results combines data from six independent experiments. The input samples (one tenth the initial volume) were used as positive controls. *, $P <$ 0.01. N.S., Not significant. E, Gel shift and supershift analysis of the HSD17B2 promoter region. Gel shift assays were performed using 5 μg nuclear extract from Ishikawa cells and radiolabeled oligonucleotide probes that contain two overlapping SP1 motifs and its flanking sequence. Competition studies were performed using a 100-fold excess of unlabeled SP1 oligonucleotide (*lane 3*) or unlabeled oligonucleotide containing mutated SP1 binding sites (*lane 4*). Supershift analyses were performed by incubating the nuclear extract from Ishikawa cells with antisera against SP1 (*lane 5*), SP3 (*lane 6*), RARα (*lanes 8*), RARγ (*lane 9*), RXRα (*lane 10*), or RXRγ (*lane 11*) before the addition of radiolabeled oligonucleotides. DNA-protein complexes are indicated by *arrows I* and *II*. Supershifted (S) bands detected with SP1 and SP3 antisera are indicated by *arrow S*. *Lanes 7* and *12* are controls: normal rabbit IgG (*lane 7*) and normal goat IgG (*lane 12*).

Retinoids stimulated interaction of RARα and RXRα with the HSD17B2 promoter

ChIP assays were performed to determine whether endogenous RARα and RXRα interact with the HSD17B2 promoter region containing the two identified SP1 motifs. Ishikawa cells were exposed to all-*trans* RA (10^{-7} M) or vehicle for 2 h. Sheared chromatin was immunoprecipitated with antibodies against RARα and RXRα, and the presence of HSD17B2 promoter DNA in the immunoprecipitate was determined by PCR. As shown in Fig. 4A, endogenous HSD17B2 DNA from Ishikawa cells was specifically immunoprecipitated with anti-RARα (*lanes 3* and *4*) or anti-RXRα (*lanes 5* and *6*), but not with nonspecific rabbit IgG (*lanes 1* and *2*). Compared with vehicle controls (Fig. 4A, *lanes 3* and *5*), retinoid treatment led to increased interaction of RARα (Fig. 4A, *lane 4*) and RXRα (Fig. 4A, *lane 6*) with the HSD17B2 promoter by 2.0- and 2.5-fold, respectively. No enrichment was observed by the antibodies against RARα or RXRα using the primers that amplify the distal region of endogenous HSD17B2 promoter (Fig. 4B).

To increase specificity, we also performed ChIP in Ishikawa

cells transfected with luciferase plasmids harboring the wild-type or mutant promoter sequence. In this instance, we amplified the promoter region using primers specific for plasmid sequences (Fig. 4, C and D). Compared with controls (Fig. 4D, *lanes 3* and *5*), retinoid treatment significantly increased interaction of RARα (Fig. 4D, *lane 4*) and RXRα (Fig. 4D, *lane 6*) with the exogenous promoter sequence containing two SP1 motifs in wild-type pHSD(150/−1)-Luc. However, mutation of the two SP motifs at nt −82/−65 reduced the binding of RARα and RXRα to this region of the promoter. These results strongly suggest that retinoids stimulate the interaction of RARα and RXRα with SP motifs within the HSD17B2 promoter.

SP1 and SP3 interact directly with the HSD17B2 promoter

To investigate whether nuclear proteins bind directly to the HSD17B2 promoter region containing the two SP1 motifs, EMSAs were performed using oligonucleotides containing intact SP1 motifs (SP1 wild type) and nuclear extracts from Ishikawa cells. As shown in Fig. 4E, two major DNA-protein complexes (I and II) were identified using a ^{32}P-labeled SP1 wild-type oligonucleotide probe (*lane 2*). Formation of the two complexes was completely prevented in the presence of 100-fold excess unlabeled wild-type oligonucleotide (*lane 3*), but not 100-fold excess of unlabeled oligonucleotide containing mutated SP1 motifs (*lane 4*). The complexes were supershifted by addition of either SP1 (*lane 5*) or SP3 antisera (*lane 6*), indicating that both SP1 and SP3 bind directly to the SP1 motifs. However, antisera to RARα (*lane 8*), RARγ (*lane 9*), RXRα (*lane 10*), or RXRγ (*lane 11*) did not cause a supershift, suggesting that RAR or RXR does not directly bind the SP motifs in the HSD17B2 promoter.

Ablation of SP1 and SP3 expression by siRNA blocked RA-induced HSD17B2 expression

To determine whether SP1 and SP3 are directly involved in RA-induced HSD17B2 expression, Ishikawa cells were transfected with SP1- or SP3-specific siRNAs and incubated with or without all-*trans* RA (10^{-7} M) for 18 h, and SP1, SP3, and HSD17B2 mRNA and protein levels were assessed by real-time quantitative PCR (Fig. 5, A–D) and immunoblotting (Fig. 5, E and F). In cells transfected with control siRNA or incubated with transfection reagent alone, RA significantly stimulated HSD17B2 expression. siRNA ablation of either SP1 or SP3 not only markedly decreased basal HSD17B2 mRNA levels but also blocked RA stimulation of HSD17B2 mRNA expression. Together, these results strongly suggest that both SP1 and SP3 isoforms are essential for basal and retinoid-induced HSD17B2 expression in human endometrium.

RARs physically interacted with SP in intact cells

To examine *in vivo* interactions between RARα/RXRα and SP1, we performed IP-IB assays using Ishikawa cells extracts, which contain endogenous RARα, RXRα, and the SP transcription factors. IP was performed using anti-RARα or anti-RXRα antibodies, or normal IgG, followed by immunoblot analysis to detect RARs and SP1. As shown in Fig. 6A, RARα (*lanes 3* and *4*) and RXRα (*lanes 5* and *6*) coprecipitated with SP1. Treatment with retinoid enhanced the interaction of SP1 with RARα (*lane 4*) and RXRα (*lane 6*). Normal rabbit IgG (*lanes 1* and *2*) was used as a negative control. Similar interactions were observed between SP3 and the RARs (Fig. 6B). Together, these data strongly suggested that SP transcription factors physically interact with RARs in intact cells.

Discussion

Cyclical endometrial proliferation (proliferative phase) and differentiation and secretion (secretory phase) during the human menstrual cycle are strictly controlled by estrogen and proges-

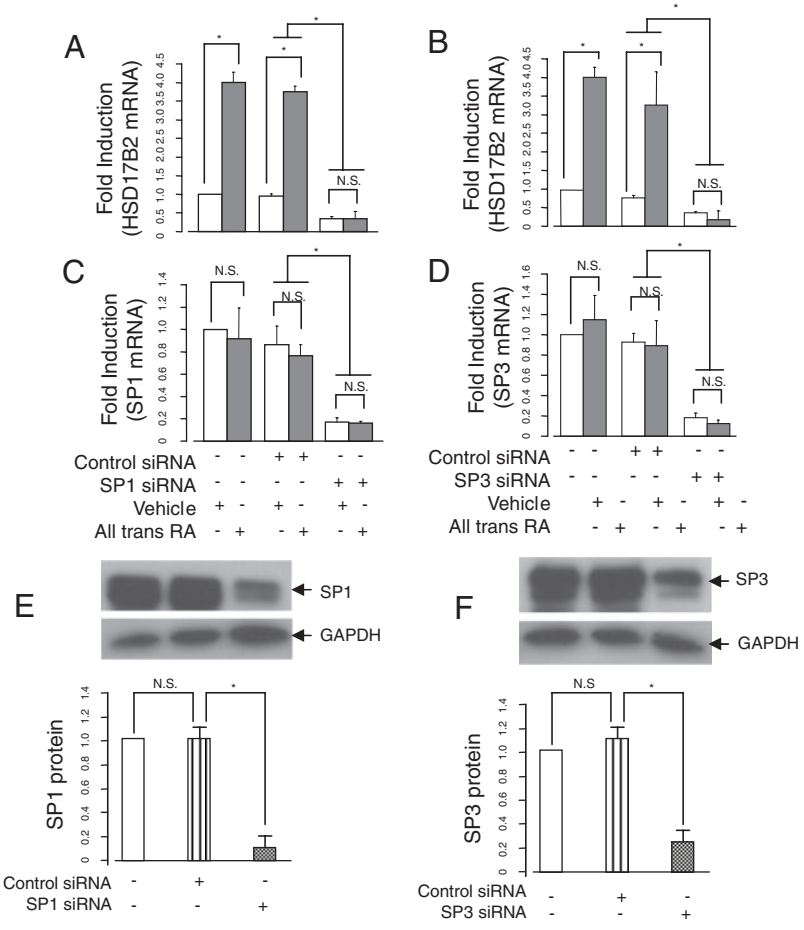

FIG. 5. siRNA mediated knockdown of SP1 or SP3 blocked RA-induced expression of HSD17B2 mRNA in Ishikawa cells. Cells were transfected with siRNA targeted against SP1 or SP3 and exposed to 10^{-7} mol/liter all-trans RA for 18 h. RNA was isolated, and the expression of HSD17B2 (A and B), SP1 (C), or SP3 (D) was analyzed by quantitative RT-PCR. SP1 (E) and SP3 (F) protein levels were assessed by immunoblotting. GAPDH was used as an internal control. Data are reported as the mean ± SEM of three independent experiments and are expressed as arbitrary units. *, $P < 0.01$. N.S., Not significant.

terone (22). The alternating dominance of E_2 and progesterone during the menstrual cycle is essential for the cyclical development of the endometrium, and HSD17B2 has been thought to play an important role in the shift from the E_2-directed endometrial proliferative phase to the progesterone-directed secretory phase (6).

We demonstrated that retinoids strongly stimulate HSD17B2 gene expression in human endometrial epithelial cells in a dose- and time-dependent manner, an effect that was blocked by retinoid antagonist ANG11273. Deletion analysis indicated that two overlapping SP binding motifs in a small region of the promoter (nt −100 to −65) are essential for RA-induced HSD17B2 gene expression in endometrial Ishikawa cells.

RA signaling via the activation of RAR/RXR is an important mediator of normal endometrial growth and differentiation. RAR/RXR-null mutants show abnormalities of fetal vitamin A deficiency syndrome, including agenesis of oviduct and uterus (23, 24). However, an increasing amount of data suggests that RA responsiveness may not always occur via direct signaling through RARs, especially if the targeted gene promoter does not contain a consensus RARE. Recent studies have shown that RARs engage in cross talk with other signaling pathways and interact with other transcription factors to regulate gene transcription (25–27). For example, RARs and SP1 cooperate to transcriptionally activate thrombomodulin (28) and retinol-binding protein (29). RARs also functionally interact with SP1 to activate CD18 (β2-leukocyte integrin) (30) and TGF-β1 (31) expression, and RAR physically interacts with SP1 on the urokinase promoter (32) and the IL-1B promoter (33). These studies suggest that RA responsiveness is mediated via SP1, and that protein-protein interactions between RARs and other transcription factors determine RA responsiveness of numerous genes. Consistent with these findings, we demonstrated that RARα and RXRα are recruited to the HSD17B2 promoter at SP binding motifs but do not directly bind these motifs (Fig. 4) to induce HSD17B expression in endometrial epithelial cells.

Transcription factor complexes containing coactivators and corepressors assemble on target promoters to modulate transcriptional activity (34, 35). We previously demonstrated that expression of the HSD17B2 gene in endometrial cells is regulated by SP1 and SP3, which directly bind to SP binding motifs in the promoter (2). We also showed that SP1 and SP3 mediated paracrine-induced HSD17B2 gene expression in human endometrium. In this study we have shown that ablation of SP1 and SP3 by siRNA blocked RA-induced HSD17B2 expression in Ishikawa cells (Fig. 5), confirming that SP1 and SP3 are involved in RA-induced expression of HSD17B2 gene in human endometrium.

One possible mechanism by which SP factors mediate RA responsiveness of the HSD17B2 promoter is through protein-protein interactions with the RARs. The transcriptional coactivators p300/CBP integrate intracellular signaling by interacting with several classes of transcription factors, including SP transcriptional factors and nuclear hormone receptors. Here, we demonstrated that RARs interact with SP transcription factors (Fig. 6).

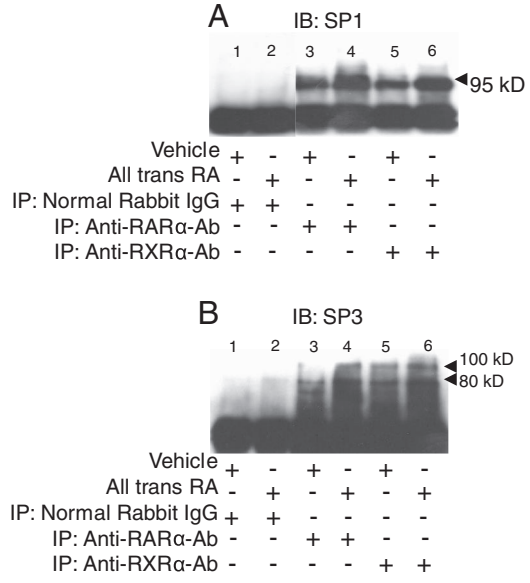

FIG. 6. Association of RARs and SP proteins in coimmunoprecipitation assays. Ishikawa cells were exposed to vehicle or 10^{-7} mol/liter all-trans RA for 2 h. Proteins from cell lysates were preincubated with normal rabbit IgG or antisera against RARα or RXRα and then immunoprecipitated with protein A/G-agarose. Protein complexes were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and probed with antiserum to SP1 (A) or SP3 (B). Data represent results from one of three independent experiments.

SP1 is a ubiquitous transcription factor in mammals, and its activity can be modulated by posttranslational modification as well as interaction with other transcription factors, including SP3 (36, 37). We demonstrated that treatment with all-trans RA markedly increases the association of RARs with SP1 and SP3 (Fig. 6), which strongly supports the notion that RA, via RAR and RXR, modulates the transcriptional activity of SP1 and SP3 to drive HSD17B2 gene expression in human endometrium.

Earlier studies in our laboratory showed that SP motifs in the HSD17B2 promoter are critical for basal and stromal factor-induced expression of HSD17B2 expression in endometrial epithelial cells (6). Furthermore, SP1 and SP3 were found to be downstream targets of progesterone-dependent paracrine signals originating from endometrial stromal cells. The present findings, that overlapping SP motifs in the HSD17B2 promoter are required for RA stimulation of HSD17B2 gene expression, indicate that RA-induced HSD17B2 gene expression in the endometrium occurs, at least in part, through the SP motif. Together, these data strongly support a model in which SP1 and SP3 act as master regulators that integrate RA signals and stromal signals to regulate HSD17B2 gene transcription and local estrogen activity in the endometrial epithelium.

Acknowledgments

We thank Dr. Rick Nicholson (Department of Endocrinology, School of Medicine and Public Health, The University of Newcastle, Callaghan, NSW, Australia) for his critical review of the manuscript.

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This work was supported by the National Institutes of Health Grant HD40093 and grants from the Friends of Prentice and the Avon Foundation.

Disclosure Statement: The authors have nothing to declare.

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