Identification and Characterization of the Protein-associated Splicing Factor as a Negative Co-regulator of the Progesterone Receptor

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Progesterone is essential in all species for the maintenance of pregnancy, and its withdrawal is required to activate the myometrium and to initiate labor. However, unlike most other species, progesterone levels do not fall at term in humans, raising the paradox as to how labor can occur under the continued influence of progesterone. We hypothesized that an endogenous (myometrial) repressor of the progesterone receptor (PR) could induce a functional withdrawal of progesterone and hence lead to the initiation of labor. We used the human PR as bait in a protein pull-down assay and identified polypyrimidine tract-binding protein-associated splicing factor (PSF) as a PR-interacting protein. PSF functions as a potent inhibitor of PR (but not estrogen receptor) transcriptional activity in mammalian cells. It acts through two novel mechanisms, inducing degradation of the PR through the proteasomal pathway and also interfering with binding of PR to its DNA response element. Importantly, in vivo studies in rats demonstrated a dramatic increase in myometrial PSF expression at term that was temporally associated with reduced levels of the myometrial PR. Accordingly, we propose that PSF acts as a PR corepressor and contributes to the functional withdrawal of progesterone and the initiation of human labor.

Progesterone is an essential regulator of the reproductive events associated with the establishment and maintenance of pregnancy through its ligand-activated progesterone receptor (PR)1 (1). Progesterone actions include the suppression of genes encoding contraction-associated proteins (e.g. oxytocin and prostaglandin receptors and connexin-43) that are required for myometrial activation and the onset of labor. In humans, progesterone levels remain elevated throughout labor, raising a paradox as to how labor can be initiated. Even in species in which progesterone levels fall at term, concentrations are likely sufficiently high to inhibit contraction-associated protein gene expression. This suggests there must be an active mechanism for inducing a functional withdrawal of progesterone at term. We have previously suggested that a blockade of PR signaling in the myometrium could induce a “functional withdrawal” of progesterone that would result in the initiation of labor (2). A number of mechanisms have been proposed to effect such a functional withdrawal, including changes in the expression of the PR or PR isoforms (3) as well as altered transcriptional activity of the PR as a result of changes in the expression of essential co-regulators (both coactivators and corepressors) (4).

The PR is a member of the steroid receptor superfamily of ligand-dependent transcriptional factors. In the human myometrium, the PR is transcribed as full-length PRB and an N-terminally truncated (164 amino acids) PR isoform (5). PRB is generally a weaker transcriptional activator than PRF (6–8) and can also act as a repressor of PRB as well as of other steroid receptors (9, 10). Upon ligand binding through the hormone-binding domain, the activated PR undergoes a conformational change enabling it to bind to specific progesterone response elements (PREs) through its DNA-binding domain (DBD). This in turn facilitates recruitment of the general transcriptional machinery, either directly (11) or indirectly via co-regulators (12, 13), which act to positively or negatively modulate the transcription rate of target genes. Two common transcriptional activation domains exist within PREs, a hormone-dependent activation function domain (AF2) in the C-terminal hormone-binding domain and a ligand-independent domain (AF1) in the N-terminal region (14). In addition, PRB possesses a third activation function domain (AF3) within its unique N-terminal region (15). Interactions between the N-terminal AF domains (AF1 and AF3) and the AF2 domain (either direct or indirect via co-regulators) elicit maximal hormone-dependent activity (16). Our knowledge of nuclear receptor co-regulators has increased markedly over recent years, revealing their multifaceted roles in regulating gene transcription. Besides the autonomous activation domains in steroid receptor coactivators and repression domains in the nuclear receptor corepressor and SMRT, many co-regulators possess acetylase (such as steroid receptor coactivators, p300/CREB, and p300/CREB-associated factor) or deacetylase (such as histone deacetylase-1/2) activities (17). Moreover, in the case of E6-AP and RPF-1, nuclear receptor activation by these co-regulators can be enhanced through their ubiquitin ligase activity, which is separable from their coactivation functions (18, 19).
In an effort to identify co-regulators within the myometrium that interact with the PR and modulate PR function, we used glutathione S-transferase (GST)-PR fusion proteins to pull-down protein extracts from myometrium smooth muscle cells lysate. One of the associated proteins identified (polypropiidine tract-binding protein-associated splicing factor (PSF)) was shown to inhibit the transcriptional activity of the PR by mechanisms that involve interference with PR binding to the PRE and degradation of the PR protein through the proteasomal pathway. Furthermore, the finding that the expression of PSF increased dramatically in the rat myometrium at term pregnancy in association with reduced levels of the myometrial PR led us to speculate that this novel corepressor might contribute to the functional withdrawal of progesterone and the initiation of labor.

**EXPERIMENTAL PROCEDURES**

**Materials**—DNA restriction and modification enzymes were obtained from Fermentas (Burlington, Ontario, Canada). Promega (Nepean, Ontario, and Roche (Laval, Quebec)). PCR reagents were obtained from Invitrogen (Burlington, Ontario). Progesterone (20α,21β-dihydroxy-4-pregnen-3-one) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-PSF antibody (B92) was from Sigma. Protease inhibitor mixture was purchased from Roche. Glutathione-Sepharose 4B affinity matrix was from Amersham Biosciences (Oakville).

**Plasmid Construction**—The PR expression vectors pS5G-PRA and pS5G-PRB were kindly provided by Dr. P. Chambon. With pS5G-PRB used as the template, a series of deletion mutations (PRB amino acids 1–164, 164–456, 456–556, 556–650, 556–833, and 556–933) were generated by PCR with a 5′-primer containing an EcoRI site and a 3′-primer containing a TGA stop codon and a BamHI site. Human PSF cDNA was a kind gift from Dr. J. G. Patton. Fragments were generated by PCR with a 5′-primer containing a TGA stop codon and a 3′-primer containing an XhoI site. Pretreatment of the DNA was done using the T4 DNA polymerase (Invitrogen). PCR fragments were then inserted into expression vectors pM and VP16 (Clontech) and pGEX-5X-2 (Amersham Biosciences). PR (556–933) were also inserted downstream of the T7 promoter of pcDNA3 at the EcoRI and XhoI site using high fidelity Platinum Tag DNA polymerase (Invitrogen).

**Immunoprecipitation and Western Blotting**—Co-immunoprecipitation of the transfected PR and His-PSF was performed in 293T cells. Cells were plated in 150-mm diameter dishes and grown to 60% confluence before transfection. A total of 15 μg of plasmid DNA were transfected using ExGen 500 (Fermentas). Eighteen hours after transfection, cells were washed twice with ice-cold phosphate-buffered saline and then lysed in NETN buffer plus protease inhibitor mixture. Protein concentrations of the whole cell lysate (WCL) were determined by the Bradford (45 assay, and WCL was diluted to 1 mg/ml in NETN buffer. A 900-μl aliquot of WCL was incubated overnight at 4 °C with either anti-His tag or anti-PR antibody, followed by the addition of 30 μl of protein A/G Plus-agarose beads (Santa Cruz Biotechnology, Inc.) for another 2 h at 4 °C. Resins were washed with NETN buffer, eluted with 1× Laemmli buffer, and centrifuged. The supernatant was separated by 8% SDS-PAGE, electrophoresed on polyvinylidene difluoride membrane (Millipore) and visualized by ECL. For immunoprecipitation of the endogenous PR and PSF, T47D cells cultured in 150-mm dishes were lysed in 800 μl of NETN buffer containing 150 mM NaCl plus protease inhibitor mixture. Cell lysates were then incubated overnight with 5 μg of anti-PR or anti-PSF antibody or control mouse IgG at 4 °C, followed by the addition of 30 μl of protein A/G Plus-agarose beads for another 2 h at 4 °C. Resins were washed with NETN buffer containing 250 mM NaCl and eluted with 1× Laemmli buffer. The eluted and whole cell extract were Western-blotted using anti-PR or anti-PSF antibody.

**GST Pull-down Assay**—A GST pull-down assay was performed as described previously (20). GST and its fusion proteins were first immobilized on glutathione-Sepharose 4B affinity matrix. The matrix was then incubated overnight at 4 °C with rabbit reticulocyte lysate (Promega) containing the PR or His-PSF transcribed and translated in the in vitro translation system (Promega) with vector pcDNA3-PR DBD. Full-length PR cDNA were amplified by PCR and cloned in-frame into pFLAG-CMV2 at the EcoRI and BamHI sites (Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-PSF antibody (B92) was from Sigma. Protease inhibitor mixture was purchased from Roche. Glutathione-Sepharose 4B affinity matrix was from Amersham Biosciences (Oakville).

**Cell Culture and Transient Transfection**—SHMs and 293T cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) plus 5% fetal calf serum (Sigma) as described (22). For experiments involving steroid exposure, the medium was substituted with phenol red-free DMEM containing 5% charcoal-treated fetal bovine serum (Hyclone Laboratories, Logan, UT). Transfection was performed according to the manufacturer’s protocol (Promega). Cells were seeded at a density of 5 × 10^5 to achieve 60–80% confluency the following day. The DNA and transfection reagent were mixed and added to the medium. Cell lysates were collected at least 3 h after transfection. For luciferase assay, cells were collected in 200 μl of lysis buffer (Promega), 10 μl of which were used for the luciferase and β-galactosidase activity assays, respectively. Luciferase activity was determined using the luciferin reagent (Promega) according to the manufacturer’s protocol. Transfection efficiency was normalized to β-galactosidase activity. For Western blotting, cell lysates were collected in NETN buffer plus protease inhibitor mixture. About 30 μg of protein extract were separated on SDS gel and electrophoresed, followed by Western blotting with the antibodies of interest.

**Electrophoretic Mobility Shift Assay**—The PR DBD was synthesized in rabbit reticulocyte using the TnT coupled in vitro transcription/translation system (Promega) with vector pcDNA3-PR DBD. Full-length PR cDNA (1.5 μg) were first precipitated by precipitation through GST-bound glutathione-Sepharose 4B matrix and then incubated with GST fusion proteins bound to Sepharose beads for 2 h at 4 °C. The beads were washed three times with NETN buffer and once with NETN buffer containing 100, 150, or 200 mM NaCl. The associated proteins were eluted by the addition of 20 mM glutathione and separated by 10% SDS-PAGE. Gels were stained with Coomassie Blue.

**Functional Withdrawal of Progesterone**

In rabbit reticulocyte using the TNT coupled in vitro transcription/translation system (Promega) with vector pcDNA3-PR DBD. Full-length PR cDNA (1.5 μg) were first precipitated by precipitation through GST-bound glutathione-Sepharose 4B matrix and then incubated with GST fusion proteins bound to Sepharose beads for 2 h at 4 °C. The beads were washed three times with NETN buffer and once with NETN buffer containing 100, 150, or 200 mM NaCl. The associated proteins were eluted by the addition of 20 mM glutathione and separated by 10% SDS-PAGE. Gels were stained with Coomassie Blue.
oligonucleotide G-50 columns (Roche). Binding reactions were performed in a total volume of 20 µl in 1× reaction buffer (5% glycerol, 5 mM dithiothreitol, 5 mM EDTA, 250 mM KCl, 100 mM HEPES (pH 7.5), 1 µg/ml bovine serum albumin, 1 µg of salmon sperm DNA, and 0.05% Triton X-100), 0.5 ng of labeled probe, and receptor protein. In some cases, bacterially expressed GST or GST-PSF was added as indicated. The binding reaction was allowed to proceed for 20 min at room temperature (the supershift was performed by adding 1.5 µg of anti-PR antibody for an additional 45 min) before the reaction mixtures were loaded onto 5% (60:1) nondenaturing polyacrylamide gel. After 2 h of electrophoresis in 0.5× Tris borate/EDTA at 4 °C, the gels were dried and autoradiographed.

**Tissue Collection and Northern Blotting—**Wistar rats (Charles River Laboratories, St. Constance, Canada) were housed individually under standard environmental conditions (12-h light/12-h dark cycle) and fed Purina rat chow (Ralston Purina, St. Louis, MO) and water ad libitum. Female virgin rats were mated with male Wistar rats. Day 1 of gestation was designated as the day a vaginal plug was observed. The average time of delivery under these conditions was during the morning of day 23. Our criteria for labor were based on delivery of at least one pup.

Rats were killed by carbon dioxide inhalation, and myometrial samples were collected on gestational days 6, 12, 15, 17, 19, 21, 22, and 23 or on days 1 and 4 postpartum. Tissue was collected at 10 a.m. on all days except the following exceptions: the labor sample (day 23) was collected once the animals had delivered at least one pup (n = 5). Rat myometrial tissues were placed into ice-cold phosphate-buffered saline, bisected longitudinally, and dissected away from both pups and placenta. The endometrium was carefully removed from the myometrial tissue by mechanical scraping on ice. We have previously shown that this removes the entire luminal epithelium and the majority of the uterine stroma (23). The myometrial tissue was flash-frozen in liquid nitrogen. All other tissues from female and male animals (ovary, placenta, heart, lung, small intestine, brain, kidney, skeletal muscle, and testis) were collected at the same time and flash-frozen in liquid nitrogen. All tissues were stored at −70 °C.

Total RNA was extracted from the tissues using TRIzol (Invitrogen). Northern blotting and hybridization were carried out as described (24). The probe used to detect PSF mRNA was a PCR-generated 770-bp fragment encompassing sequence 1436–2209 (GenBankTM/EBI accession number X70944). The 18 S probe (provided by Dr. David T. Denhardt, Rutgers University) was used as a control probe. A total of five complete sets of gestational profiles were analyzed by Western blotting, and the data were subjected to one-way analysis of variance, followed by pairwise multiple comparison procedures (Student-Newman-Keuls method) to determine differences between groups, with the level of significance set at p < 0.05.

The expression of the PR protein was determined by Western blotting. Frozen tissue was crushed under liquid nitrogen using a mortar and pestle. The crushed tissue was homogenized for 1 min in radioimmunoprecipitation assay buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% (w/v) Triton X-100, 1% (w/v) sodium deoxycholate, and 0.1% (w/v) SDS supplemented with 100 µM sodium orthovanadate and protease inhibitor mixture tablets). Samples were spun at 12,000 × g for 15 min at 4 °C, and the supernatant was transferred to a fresh tube to obtain a crude protein lysate. Protein concentrations were determined using Bio-Rad protein assay buffer. Protein samples (40–50 µg) were resolved by electrophoresis on 8% SDS-polyacrylamide gel. Proteins were transferred onto polyvinylidene difluoride membrane in 25 mM Tris-HCl, 250 mM glycine, and 0.1% (w/v) SDS (pH 8.3) at 30 mA for 1.5 h at 4 °C, blotted with anti-PR antibody C-20; exposed to Eastman Kodak XAR x-ray film; and analyzed by densitometry. The membrane was then stripped and blotted with anti-calponin antibody as a loading control. Four complete sets of gestational profiles were analyzed by Western blotting, and the data were subjected to one-way analysis of variance, followed by pairwise multiple comparison procedures (Student-Newman-Keuls method) to determine differences between groups, with the level of significance set at p < 0.05.

**RESULTS**

**Identification of PSF as a PR-interacting Protein.—**To identify PR-interacting proteins, GST-PR fusion proteins were bound to glutathione-Sepharose 4B matrix and incubated with either cytosolic or nuclear extracts from SHMs precleared by passage through GST-bound glutathione-Sepharose 4B matrix. Associated proteins were resolved on SDS gel and visualized by Coomassie Blue staining. Two protein bands, present only in nuclear fractions of SHMs, were identified that migrated at the same molecular mass of 100 kDa and bound to PR-(1–164) and PR-(556–933), respectively (Fig. 1A). GST-PR-(556–933) was found to bind p100 at a wider range of NaCl concentrations (100, 150, and 200 mM), whereas GST-PR-(1–164) could bind p100 only at 150 mM NaCl. This suggests that p100 may form a more stable complex with GST-PR-(556–933).

These two p100 bands were excised and processed for MALDI mass spectrometry. Four peptide sequences within the proteins were identified. Fig. 1B shows that these two p100 proteins are identical. The sequences matched perfectly within the BLAST Database to a known protein termed PSF, previously identified as an RNA-splicing factor. Two PSF protein isoforms (PSF-A and PSF-F) have been reported (Fig. 1C). These two isoforms are identical through amino acids 1–662, but diverge thereafter, with PSF-F containing 669 amino acids and PSF-A containing 707 amino acids (25). PSF contains two RNA recognition motifs (RRMs I and II, within amino acids 306–330 and 366–399, respectively) and appears to migrate anomalously as an ~100-kDa protein on SDS gel. Our MALDI mass spectrometric analysis did not detect any sequences specific to PSF-F, but the peptide FQQGAGGPVGGQGP did specifically match PSF-A.

**Confirmation of PSF Interaction with the PR in Vivo.—**We preformed immunoprecipitation to confirm the interaction be-
tween the PR and PSF in vivo. His-PSF was constructed by insertion of the PSF open reading frame into the C terminus of the His6 tag. 293T cells were transiently transfected with an expression vector for PRB and/or His-PSF for 18 h, followed by lysis in NETN buffer with 150 mM NaCl. WCL was incubated with anti-PR antibody C-20 or anti-His tag antibody H-15 using protein A/G-Sepharose as an absorbent. Resins were washed with NETN buffer containing 100 mM NaCl, and bound proteins were detected by Western blotting (WB) with antibody to the PR (C-20) or His-PSF (H-15). B, shown are Western blots of PSF (left panel) and the PR (right panel) in immunoprecipitates (IP) of control mouse IgG or mouse anti-PR (AB-52) or anti-PSF (B92) antibody from extracts of T47D cells. C, SHMs were maintained in phenol red-free DMEM plus 10% stripped fetal bovine serum and transiently transfected with the pM vector fused with or without PSF cDNA or the VP16 vector containing PRA or PRB cDNA together with the G5-luciferase reporter vector and pCH110. Four hours after transfection, the culture medium was replaced with fresh medium with or without 10 nM progesterone and maintained for at least 30 h. Luciferase (Luc) activity was measured and normalized to β-galactosidase activity. The data shown are the means ± S.E. of three separate experiments performed in triplicate.

Finally, whole cell extracts expressing PRB and/or His-tagged PSF were incubated with anti-PR antibody and then incubated with protein A/G Plus-agarose beads. The associated proteins were washed and analyzed for the presence of His-PSF. Over-expressed His-PSF was specifically co-immunoprecipitated only in the presence of the PR (Fig. 2A, upper panel). Similarly, when anti-His tag antibody was used to immunoprecipitate WCL, we observed that the association of the PR could be detected only in the presence of His-PSF. Endogenous PSF was also co-immunoprecipitated with the endogenous PR from the T47D cell extract (Fig. 2B). No immunoprecipitation of the PR
or PSF was observed when anti-PSF or anti-PR antibody was replaced with control mouse IgG. These in vivo data confirm the interaction between the PR and PSF found in the GST pull-down experiment.

Further evidence to support an in vivo interaction between PSF and the PR was obtained using the mammalian two-hybrid system (Fig. 2C). PSF was fused to the C terminus of the Gal4 DBD in the pM vector, and PRB or PRA was fused downstream of the Gal4 activation domain in the VP16 vector. When cotransfected with G5-luciferase, pM-PSF resulted in a 70% reduction in luciferase activity compared with the empty pM vector. However, cotransfection of both activation domain-tagged PRs (VP16-PRA and VP16-PRB) with pM-PSF induced a dramatic increase in luciferase activity as a result of the interaction between PSF and PRs. This interaction was ligand-independent because the addition of progesterone did not cause a significant difference in luciferase activity. These data indicate that the interaction between the PR and PSF is direct and mediated through the AF3 domain and DBD of the PR and RRM II of PSF.

Functional Consequence of the Interaction between PSF and the PR—The impact of the interaction between PSF and the PR on the transcriptional transactivation of progesterone-responsive promoters was next investigated in SHMs (Fig. 4A). We compared two promoter contexts: the mouse mammary tumor virus promoter and the artificial 3xPRE promoter linked upstream of the luciferase reporter gene. Transient transfection of PSF alone had no effect on either of these promoters. However, PSF potently inhibited both PRA and even more dramatically PRB transactivation of both promoters in a dose-dependent manner. Similar inhibitory effects were also observed in the experiments performed in 293T cells (data not shown), indicating that this effect is not dependent upon the cell or promoter context. The PSF expression vector was also cotransfected with ERα/H9251 and ERα/H9252 into SHMs (Fig. 4B) in a 3xERE promoter context. We did not observe any inhibitory effects of PSF on either ERα or ERβ, indicating that PSF selectively represses PRs rather than ERs.
PSF Enhances the Degradation of the PR Protein through the Proteasomal Pathway—To exclude the possibility that the inhibitory effects of PSF on the PR were not due to the reduced expression of PR proteins, Western blotting of WCLs was conducted following cotransfection of the PSF and PR expression vectors as described above for Fig. 4. To our surprise, we observed that increasing the dose of the PSF expression vector input (in the presence of a constant dose of the PR-expressing vector) resulted in a decrease in the levels of both PRA and PRB proteins (Fig. 5A). This interesting observation led us to investigate the possibility that PSF enhances the degradation of the PR through the 26 S proteasomal pathway. Cotransfection of PSF or PSF-(1–662) (a truncated form of PSF lacking the PSF-F-specific C-terminal domain) and PRB/PRA resulted in a total loss of PR proteins after 24 h (Fig. 5B). However, the addition of the proteasomal inhibitor MG132 at a final concentration of 60 μM for an additional 6 h blocked the loss of the PR protein. PSF-(1–662) also enhanced the degradation of PR proteins, implying that this region of PSF is sufficient to degrade the PR and that the PSF-F isoform would also have this capability.

PSF Represses PR Transactivation Domains through Two Different Mechanisms—To investigate the PSF repression of individual activation domains of the PR, we used a one-hybrid system in which each of the activation domains of the PR was fused to the C terminus of the Gal4 DBD in the pM vector and cotransfected with the G5-luciferase reporter gene. PR-(1–164) induced a 25-fold increase in luciferase activity compared with the empty pM vector (Fig. 6B). Cotransfection of increasing amounts of PSF resulted in a significant dose-dependent reduction in luciferase activity coupled with a loss of pM-PR-(1–164) protein. PSF also inhibited transactivation and enhanced the degradation of PR-(456–650) (a region containing both the AF1 domain and DBD). However, PSF had no effect on PR-(456–556) (Fig. 6, C and D), possibly because PR-(456–556) (the AF1...
domain of the PR) lacks a binding site for PSF, or on PR-(650–933) (containing only the AF2 domain) (Fig. 6F). Importantly, PSF did inhibit the transactivation of PR-(556–933) (containing the AF2 domain and DBD) without any reduction in the protein level of this domain (Fig. 6E). These data suggest that the ability of PSF to inhibit transactivation and to induce the degradation of the AF3 and AF1 domains is dependent upon the physical interaction of PSF with the AF3 domain and DBD. On the other hand, although PSF also directly interacts with PR-(556–933), inhibition of the transactivation function of this domain involves a separate mechanism distinct from degradation. Potential mechanisms might include repressive domains.
within PSF or the recruitment of other transcriptional inhibitory protein complexes. To explore this possibility, a one-hybrid system was set up in which various segments of PSF cDNA were inserted in-frame into the pM vector and cotransfected with the G5-luciferase reporter gene. Luciferase (Luc) activity was measured 30 h after transfection and normalized to β-galactosidase activity. B, the protein concentration from the cell lysate was used to normalize β-galactosidase (β-gal) activity and is plotted. The data shown are the means ± S.E. of three separate experiments performed in triplicate.

**PSF Disrupts the Interaction between the PR DBD and PRE**—The finding that the inhibition of PR-(556–933) transactivation function by PSF is not due to the degradation of this PR domain suggests that multiple mechanisms are involved in the corepression of PR signaling by PSF. The direct interaction between PSF and the PR DBD raises the possibility that PSF could block PR DBD binding to its response element. To address this question, an electrophoretic mobility shift assay was performed to study the interaction between the PR DBD and PRE. As shown in Fig. 8A (lanes 3–5), the TsT coupled in vitro transcribed/translated PR DBD formed a complex with the 32P-labeled double-stranded oligonucleotide of the PRE in a dose-dependent manner. This interaction was specific because the TsT plain lysate did not form complexes with the PRE oligonucleotide (lane 2). The addition of the GST-PSF fusion protein to the reaction resulted in decreased association of the PR DBD with the PRE (lanes 9–11), whereas GST protein alone had no effect on this interaction (lanes 6–8). To further validate the above observation, we also performed a gel shift assay with the full-length PR obtained from nuclear extraction of 293T cells transiently transfected with FLAG-PRB (Fig. 8B). The PR formed complexes with the PRE oligonucleotide in a dose-dependent manner (lanes 3 and 4). The nuclear extract of 293T cells transfected with the empty pFLAG-CMV2 vector was used as a control to determine that the protein binding to the labeled PRE was the PR protein. Further clarification of the molecular composition of the shifted band was confirmed by adding anti-PR antiserum to the incubation mixture. The band was supershifted by the addition of anti-PR antibody (lane 5). Note that the supershifted band was always diffuse and that multiple supershifted bands were observed. The addition of increasing doses of GST-PSF, but not GST, resulted in a decrease in the association of the PR with the PRE (lanes 7 and 8 and lanes 10 and 11). In the control experiments (lanes 6 and 9), GST or GST-PSF incubated with the PRE did not change the migration pattern of the PRE. Thus, blockade of PR DBD binding to the PRE represents an additional mechanism by which PSF can repress PR signaling.

**PSF mRNA Is Widely Expressed and Up-regulated in the Myometrium Prior to the Onset of Labor**—We assessed the tissue distribution of PSF in rat tissues by Northern blot analysis. PSF expression was detected in the myometrium and other tissues as two major transcripts (~2.5 and 3.0 kb in length; the relative expression of these two bands was tissue-dependent (Fig. 9A). PSF was expressed at high levels in brain, testis, and intestine; at intermediate levels in lung, kidney, ovary, and placenta; and at low but still detectable levels in liver, skeletal muscle, and non-laboring myometrium. Interestingly, PSF expression was higher in the myometrial samples from laboring and postpartum animals compared with the myometrial samples from non-pregnant animals.

To more fully define the gestational control of PSF expression, myometrial tissue was collected from pregnant rats on gestational days 6, 12, 15, 17, 19, 21, 22, and 23 (day of labor) and on days 1 and 4 postpartum (n = 5 at each time point) for Northern blot analysis. Densitometric analysis revealed that myometrial PSF mRNA was relatively low throughout early and mid pregnancy, but increased markedly on day 22 (prior to labor) and remained elevated during labor (day 23) and the immediate postpartum period (Fig. 9B). Analysis of variance revealed significant change in PSF expression during pregnancy (p = 0.03), with levels after day 22 being significantly higher than early in gestation (day 6) (p < 0.05).

**Temporal Association between the Increased Expression of PSF and Reduced PR Levels in Term Myometrium**—Based on the gestational expression profile of PSF and its ability to degrade the PR in vitro, we predicted that the increase in PSF near term would correlate with reduced myometrial PR levels. To test this prediction, myometrial tissues were collected from pregnant rats on gestational days 6, 12, 15, 17, 19, 21, 22, and 23 (day of labor) and days 1 and 4 postpartum (n = 4 at each time point) for Western blot analysis of PR expression. As predicted, densitometric analysis demonstrated an inverse relationship between PSF and PR expression, with relatively high levels of the PR throughout pregnancy (when PSF is low) and a significant decrease with the approach of term (as PSF expression increases) (p < 0.01) (Fig. 9C). These in vivo data are thus consistent with a model in which PSF induces a functional withdrawal of progesterone at term and in which at
least one mechanism involves targeting the PR for degradation through the proteasomal pathway as well as a possible action through blockade of PR binding to DNA.

**DISCUSSION**

Our study has identified PSF as a novel PR-interacting protein that is able to block progesterone signaling. Importantly, the increased myometrial expression of PSF near term coupled with reduced levels of the PR suggests that PSF may be a critical contributor to a functional withdrawal of progesterone and the initiation of labor.

PSF was first cloned and characterized by Patton et al. (25) and has been shown to be an essential component of the RNA-splicing machinery within the cell (26). PSF forms complexes with high molecular mass assembly of small nuclear ribonucleoprotein particles and other splicing factors of the SR protein and heterogeneous nuclear ribonucleoprotein families (27). Studies have demonstrated that PSF controls the expression of genes involved in cellular differentiation in higher eukaryotes through regulation of mRNA maturation (28, 29). We have provided evidence that, during pregnancy, myometrial cells undergo a program of cellular differentiation culminating in a contractile phenotype that transforms (activates) the relatively quiescent myometrium to a tissue capable of generating intense synchronous contractions of labor (1). This switch in myometrial phenotype is usually triggered by a decrease in the plasma levels of progesterone, which we have shown is able to suppress the expression of genes, within the myometrium, that are required for labor. Although progesterone withdrawal has been a widely accepted paradigm as a mechanism for labor initiation for many decades, there are several aspects that suggest that it may not fully account for the dramatic changes seen during labor. First, although progesterone levels do indeed fall in virtually all species at term, the concentration at the initiation of labor remains at a sufficiently high level (e.g. 15–40 nM in rodents) that it would be expected to saturate the myometrial PRs (Kd = 1 nM) (30). Second, and more importantly, progesterone levels do not fall prior to labor in humans, but are maintained at pregnancy levels. Nevertheless, as in other species, in humans, progesterone is required for maintenance of pregnancy, and blockade of progesterone signaling leads to the initiation of labor. Thus, administration of the PR antagonist RU486 to pregnant women results (as it does in animals) in the induction of labor (31). These observations have led us, and others, to suggest that a functional withdrawal of progesterone is a prerequisite for the initiation of human labor.

The data presented here suggest that, in addition to regulating gene expression at the level of pre-mRNA splicing, PSF can also function as a PR corepressor, thereby removing the suppressive action of this nuclear transcription factor on the expression of myometrial genes required for labor.

Accumulating evidence has suggested links between pre-mRNA splicing and gene transcription. Thus, p54nrb had been found to interact with and to coactivate the androgen receptor AF1 domain within a complex including PSF, PSP1 (garasepeckle protein-1), and PSP2, which modulate pre-mRNA processing (32). In addition, PGC-1, which was originally identified as a transcriptional coactivator of the nuclear receptor peroxisome proliferator-activated receptor-γ and of several nuclear receptors (33, 34), has been shown to interact with components of the splicing machinery, therefore allowing coordi-
nated regulation of both transcription and splicing (35). Also, ANT-1 (containing elements of sequence identity to a protein that binds to the U5 small nuclear ribonucleoprotein particle involved in the spliceosome) enhances the ligand-independent autonomous AF1 domain transactivation function of the androgen or glucocorticoid receptor, but does not enhance that of ERα (36). Other RNA-binding proteins (such as RTA, SHARP, p72/p68, TLS, and GRIP120) have been shown to either coactivate or corepress nuclear receptor (37–41). Although the detailed molecular mechanisms remain under investigation, evidence indicates an involvement in the recruitment of histone acetylase or histone deacetylase, which in turn regulates chromatin condensation. Samuels and co-workers (42) have reported that PSF can interact with Sin3A and mediates transcriptional silencing through the recruitment of histone deacetylase to the thyroid receptor DBD. Our data suggest additional mechanisms by which PSF might corepress PR transactivation, viz. enhancement of the degradation of the PR protein through the proteasomal pathway as well as interference with PR binding to the PRE.

Cotransfection of PSF and the PR resulted in decreased PR protein, and this effect could be reversed by the addition of the proteasomal inhibitor MG132, suggesting that PSF can mediate PR protein degradation through the proteasomal pathway. It is intriguing to speculate that PSF itself may possess ubiquitin-protein isopeptide ligase activity or can bridge the PR with protein complexes containing the similar function. The observation that a truncated form of PSF (PSF-(1–662)) can
More recently, Condon might induce a functional withdrawal of progesterone (3, 44). Simultaneously or sequentially remain to be determined. How act as a negative repressor of PRB within the myometrium and human pregnancy. Several groups have proposed that PRA can these mechanisms is utilized deacetylase-protein complexes. The extent to which each of with receptor/DNA binding, and recruitment of histone degradation the PR suggests that both PSF-A and PSF-F possesses PR degradation capabilities. The different localizations of PSF-F and PSF-A to the cytoplasmic and nuclear compartments, respectively, suggest that PSF-F could target the PR for degradation as early as protein synthesis in the Golgi apparatus and endoplasmic reticulum, whereas PSF-A would target the nuclear receptor. This in vitro function of PSF in targeting the PR for degradation is given increased functional significance by our in vivo data showing a correlation between the increased expression of PSF in the rat myometrium at term and reduced levels of the PR protein.

A second mechanism by which PSF might block PR signaling is through interference with PR DBD binding to the PRE as evidenced by our electrophoretic mobility shift assay. Our data suggest that, at least for the AF2 domain of the PR, this interference with DNA binding is independent of the effect of PR degradation. It remains to be determined in the context of the whole receptor whether blockade of PR binding to the PRE is a prerequisite for targeting the PR for degradation.

Our data also provide evidence that two regions within the PRB and PSF possess transcriptional inhibitory effects. This is consistent with recent published data that a C-terminally truncated form of PSF (lacking RRM5) inhibits gene transcription of the P450 cholesterol side chain cleavage enzyme by binding to a DNA sequence (CTGAGTC) within its promoter (43). Although evidence has been provided that PSF can recruit other transcriptional inhibitory protein complexes through Sin3A (42), the possibility that the N terminus of PSF possess intrinsic inhibitory functions cannot be excluded.

Our observation that PSF corepresses the transactivation function of the PR (as well as the glucocorticoid and androgen receptors), but not the ER (ERα or ERβ), suggests that this action is relatively selective. Given that the DBD of nuclear receptors has a high degree of similarity (as opposed to the N terminus), it may be that PSF binding to the DBD of PR represents a primary site of interaction. This is supported by the observation that, although PSF can interact with the PR in both the AF3 domain and DBD, the affinity of these interactions is rather different, with much stronger binding occurring between PSF and the DBD of PR. This difference may due to the AF3 domain adopting a more versatile and flexible conformation. Nevertheless, given that PRB (in contrast to PRA) contains an AF3 domain, it is possible that PSF may form a relatively stronger interaction with PRB and thus more efficiently corepress transactivation by PRB compared with PRA.

Thus, our own data, as well as those of others, suggest three potential mechanisms by which PSF may corepress steroid receptors, viz. induction of receptor degradation, interference with receptor/DNA binding, and recruitment of histone deacetylase-protein complexes. The extent to which each of these mechanisms is utilized in vivo and whether they occur simultaneously or sequentially remain to be determined. However, our data in the rat suggest an in vivo link between increased PSF expression and PR degradation.

Recent reports have suggested other potential mechanisms by which a functional progesterone withdrawal might occur in human pregnancy. Several groups have proposed that PRA can act as a negative repressor of PRB within the myometrium and that an increase in the relative expression of PRA versus PRB might induce a functional withdrawal of progesterone (3, 44).

More recently, Condon et al. (4) have reported decreased expression of steroid receptor coactivators in human fundal myometrium at term that might impair PR function. These data are not necessarily mutually exclusive. Given the critical need for progesterone at term, it is conceivable that multiple mechanisms might exist to ensure a functional progesterone withdrawal and the initiation of labor.

In summary, our investigation has identified a novel function for PSF as a PR corepressor. PSF appears to act by blocking the ability of the PR to bind to its DNA response element and to target the PR for degradation through the proteasomal pathway. Although as yet we do not have any direct evidence of an involvement of PSF in the initiation of human labor, it has been reported that there is a decrease in the binding affinity of the PR from term myometrial protein extract for the PRE, suggesting that factor(s) present in term myometrium contribute to inhibition of the interaction between the PR and PRE (45). Our results suggest that PSF may contribute to this blockade. If so, PSF may be a critical component of the mechanism(s) by which a functional withdrawal of progesterone may occur in human pregnancy and may represent a target for therapeutics aimed at controlling the process of labor both at term and preterm.

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