Polypyrimidine Tract-Binding Protein-Associated Splicing Factor Is a Negative Regulator of Transcriptional Activity of the Porcine P450scc Insulin-Like Growth Factor Response Element

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The porcine P-450 cholesterol side-chain cleavage enzyme gene (P450scc) contains a 30-bp region [insulin-like growth factor response element (IGFRE)] that mediates insulin-like growth factor I (IGF-I)-stimulated gene expression and binds Sp1. In this study, we showed that polypyrimidine tract-binding protein (PTB)-associated splicing factor (PSF), an RNA-binding component of spliceosomes, binds to the IGFRE. Southwestern analysis with an IGFRE oligonucleotide showed that a protein (from Sp1-immunodepleted HeLa extract) fractionated on SDS-PAGE at 100 kDa. Microsequence analysis of 100-kDa band HeLa proteins detected PSF. DNA affinity chromatography, using an IGFRE mutant oligonucleotide that does not bind Sp1, isolated a protein that immunoreacted with PSF antibody. Deoxyribonuclease I (DNase I) footprint analysis showed recombinant PSF binds 5’ of the Sp1-binding GC box of the IGFRE, and mutant oligonucleotides further delineated this region to a palindrome, CTGAGTC. Functional analysis of these mutants by transfection experiments in a cell line overexpressing the IGF-I receptor (NWTb3) found that an inability to bind PSF significantly increased the IGFRE transcriptional activity, while retaining responsiveness to IGF-I. Moreover, transfection of expression vectors for Sp1 and PSF in porcine granulosa cells found that Sp1 expression stimulated IGFRE transcriptional activity while PSF inhibited activity even with coexpression of Sp1. In conclusion, we identified PSF as an independent, inhibitory regulator of the transcriptional activity of the porcine P450scc IGFRE. (Molecular Endocrinology 14: 774–782, 2000)

INTRODUCTION

Insulin-like growth factor-I (IGF-I) is a growth factor that is important in regulation of normal ovarian function (1, 2). IGF-I, as a local paracrine factor produced by granulosa cells, stimulates steroidogenesis (3) and increases immunoprecipitable P-450 cholesterol side-chain cleavage (P450scc) protein (4) and mRNA concentrations (5). P450scc is the rate-limiting enzyme in the steroidogenic pathway and is responsible for cleavage of the C20–C22 bond that frees the C22–C27 side chain of cholesterol (6).

The mechanisms by which IGF-I stimulates gene transcription are not understood. Studies have found IGF-I-responsive elements (IGFREs) in the chicken sl-crystallin gene (7, 8) and rat elastin gene (9–11). These regions are GC rich and bind the ubiquitous transcription factor, Sp1 (8, 11). Rat elastin gene expression is increased by IGF-I treatment in cultures of neonatal rat aortic smooth muscle cells (9). This up-regulation of elastin gene expression is mediated by the loss of binding of complexes to a GC-rich domain that functions as a negative element for gene transcription (9). Additional evidence indicates that IGF-I treatment prevents the binding of Sp3 to a retinoblastoma control
element that serves as a repressor for elastin gene transcription (12).

We previously identified a GC-rich, 30-bp IGFRE in the porcine P450scc gene (13) that binds Sp1 (14, 15). In this study, we used microsequence analysis and sequence-specific DNA affinity chromatography on HeLa cell nuclear extract protein to identify a protein, polypyrimidine tract-binding protein (PTB)-associated splicing factor (PSF), that binds to the porcine P450scc IGFRE. Moreover, using expression vectors in porcine granulosa cell transient transfection experiments, we show that Sp1 stimulates, and PSF inhibits, transcriptional activity of the porcine P450scc IGFRE.

RESULTS

Identification of a Second Protein Binding to the Porcine P450scc IGFRE

It is known that IGF-I has tissue-specific effects. Therefore, we suspected a greater degree of complexity of regulation of the porcine P450scc IGFRE than just the binding of Sp1. Using SDS-PAGE of HeLa cell nuclear extract protein and microsequence analysis, we identified a second protein binding to the porcine P450scc IGFRE. First, an Sp1 antibody was used to immunodeplete HeLa nuclear extracts of Sp1. Western blots were prepared with recombinant Sp1, HeLa nuclear extract, and Sp1-depleted HeLa extract (Fig. 1). Next, the blots were probed using conventional Southwestern methodology with labeled IGFRE oligonucleotide and the mM6 oligonucleotide, which does not bind Sp1 (14), as shown in the lower panels of Fig. 1. Both intact and Sp1-depleted extracts contained a protein that bound to wild-type IGFRE and mM6. This protein had the same SDS-PAGE electrophoretic mobility as Sp1 (100 kDa) but was not precipitated by Sp1 antibody.

Having demonstrated that Sp1 and a second protein comigrated during SDS-PAGE, and knowing that Sp1 contained a blocked N terminus (16), we microsequenced the 100-kDa band from the HeLa cell extract. Six 100-μg samples of crude HeLa cell extract were used for analysis (Materials and Methods). N-terminal sequence analysis of the 100-kDa band gave only one major sequence, shown in Table 1 under the column “N-Terminus”. Since sequence analysis of proteins, especially larger proteins, typically demonstrates some background levels of Pth-amino acids, the oc-
currence of other proteins in the 100-kDa band cannot be ruled out; however, their level of occurrence relative to the major sequence was very low, and no other identifiable sequence was evident. Furthermore, confirmation of the major sequence was also established by internal sequence analysis after hydrolysis of the 100-kDa band with trypsin, followed by capillary HPLC of the hydrolysate and sequencing of resulting peaks shown in Fig. 2 and Table 1. The protein and peptide sequences established by sequence analysis were searched in the protein database using the Intelligenetics, Inc. (Mountain View, CA) FASTDB program and revealed an exact match with PTB-associated splicing factor (PSF) reported by Patton et al. (17).

DNA Affinity Chromatography

We partially purified HeLa cell nuclear extract protein for use in affinity chromatography to determine whether PSF bound to the porcine P450scc IGFRE. HeLa cells (60 g total) were grown in suspension, and nuclear protein extract was prepared and partially purified (Materials and Methods). Partially purified HeLa cell nuclear extract proteins are shown in Fig. 3 after separation by SDS-PAGE.

The partially purified HeLa cell nuclear protein extract was applied onto a mM6 oligonucleotide column (Materials and Methods). The mM6 oligonucleotide is a mutant of the porcine P450scc IGFRE that does not bind Sp1 in gel shift assay (14). Fractions eluted with increasing salt concentrations were pooled and subjected to Western blot analysis using a polyclonal antibody to PSF. As shown in Fig. 4, the PSF antibody recognized a single protein band of 100 kDa that showed strong affinity for the column (0.6–0.8 M KCl).

DNase I Footprint Analysis of Recombinant Sp1 and PSF

Footprint analysis was done with recombinant Sp1 and PSF (Materials and Methods) to define binding regions to the porcine IGFRE. Recombinant Sp1 protected a 21-bp region partially in and 3′ of the IGFRE (Fig. 5). Sites of enhanced DNase cleavage were found directly 5′ of the footprint. A similar pattern of binding of Sp1 to the SV40 promoter has been previously described (18). The PSF footprint was 5′ to the GC box (Fig. 5).

### Table 1. Sequence Analysis of the 100-kDa HeLa Extract Protein Band

| Cycle No. | N-Terminus Residue (pmol) | Tryptic Peptidesa | Peak 4b | Peak 7a | Peak 7b |
|-----------|---------------------------|-------------------|--------|--------|--------|
| 1         | S (1.8)                   | L (0.7)           | F (0.6) | F (0.6) |
| 2         | R (0.4)                   | F (0.7)           | A (0.2) | A (0.2) |
| 3         | D (1.9)                   | V (0.5)           | T (0.6) | Q (0.6) |
| 4         | R (0.3)                   | G (0.3)           | H (0.3) | H (0.3) |
| 5         | F (1.3)                   | N (0.5)           | A (0.2) | G (0.2) |
| 6         | –                         | L (0.2)           | A (0.2) | T (0.1) |
| 7         | S (0.6)                   | P (0.2)           | A (0.1) | F (0.1) |
| 8         | –                         | A (0.1)           | L (0.6) | E (0.04) |
| 9         | G (0.4)                   | –                 | S (0.1) | Y (0.1) |
| 10        | G (0.6)                   | I (0.1)           | V (0.2) |
| 11        | G (0.7)                   | T (0.1)           | R (0.02) |
| 12        | G (0.7)                   | E (0.1)           |         |
| 13        | –                         |                   |         |
| 14        | G (0.4)                   |                   |         |
| 15        | F (0.3)                   |                   |         |
| 16        | H (0.2)                   |                   |         |
| 17        | R (0.1)                   |                   |         |
| 18        | R (0.2)                   |                   |         |
| 19        | G (0.2)                   |                   |         |
| 20        | G (0.2)                   |                   |         |

a See internal sequencing (Fig. 2).

b Corresponded to region 299–310 of PSF (17).

c This peak contained two peptides whose sequence was consistent with regions 366–376 (7a) and 480–491 (7b) of PSF (17).

d Not determined.
Identification of Nucleotides Binding PSF in the IGFRE  
Having established that PSF binds 5′ of the GC box of the IGFRE, we next defined the nucleotides that bind PSF. A protein with 71% homology to PSF, named nuclear RNA-binding protein, 54 kDa (p54nrb), binds with DNA to the nucleotides GTGAC (19). In the IGFRE a palindrome similar to these sequences was identified 5′ of the GC box, CTGAGTC. Therefore, mutant oligonucleotides of the IGFRE were made to each side of the palindrome (Table 2). These mutant oligonucleotides did not bind PSF by Southwestern analysis (Fig. 6). The mutants did bind a protein that is the same size as PSF and most likely is Sp1 (Fig. 6).

Functional Significance of PSF Binding to the IGFRE  
The functional significance of PSF binding to the IGFRE was determined with transient transfection studies in NWTb3 cells using luciferase constructs (Materials and Methods) that contained the wild-type IGFRE (mWT) and the IGFRE mutants (Table 2) that do not bind PSF. NWTb3 cells overexpress the IGF-I receptor, and the IGFRE is functional in these cells (15). This cell line was used rather than porcine granulosa cells because the only heterologous promoter we have found active in porcine granulosa cells, cytomegalovirus (CMV), is also responsive to IGF-I. As shown in Fig. 7, transient transfection experiments with luciferase constructs containing mutants of the IGFRE ligated to a SV40 promoter showed that loss of PSF binding significantly increased the transcriptional activity of the IGFRE while retaining its responsiveness to IGF-I.

To further assess the function of PSF and Sp1 binding to the porcine P450sc c IGFR, we performed transient transfection experiments in primary cultures of porcine granulosa cells with expression vectors for PSF and Sp1 that used the CMV promoter. As shown in Fig. 8, expression of Sp1 significantly increased the luciferase activity of the cotransfected porcine P450sc c IGFRE. PSF expression inhibited the activity of the IGFRE and also inhibited the IGFRE when Sp1 was concomitantly expressed (Fig. 8).

DISCUSSION  
Our previous study used deletion constructs of the porcine P-450 scc 5′-region to identify a 30-bp GC-rich domain (IGFRE) that mediates IGF-I-stimulated gene expression (13). In this study, we determined that a protein, PTB-associated splicing factor (PSF), binds to the IGFRE and acts as a negative regulator of transcriptional activity. We identified PSF by microsequence analysis and DNA affinity chromatography using HeLa cell nuclear extract protein. We then produced recombinant PSF that was used with DNase I footprint analysis and Southwestern analysis to identify a palindrome, CTGAGTC, that binds PSF 5′ of the GC box in the IGFRE. Transient transfection experiments showed that Sp1 (stimulation) and PSF (inhibition) have independent, opposite actions on transcriptional activity of the IGFRE.

PSF was isolated and cloned in 1993 by Patton et al. (17). It is a 76-kDa protein that migrates anomalously on SDS gels because it is highly basic. The protein associates with PTB to form spliceosomes for splicing of pre-mRNA. When compared with the average protein, PSF has an unusual amino acid composition with high levels of glycine (15%), proline (16%), glutamine (6.9%), and arginine (8.5%) and low amounts of hydrophobic residues (20). In particular, the N-terminal region is rich in proline and glutamine residues. Similar proline/glutamine-rich regions comprise the transactivation domains of Sp1 (21, 22). The sequence is also unusual in having many di- and multiple-repeat residues. PSF is the product of only one gene, but alternative splicing results in two isoforms that vary in length from their carboxyl terminus but retain the proline/glutamine-rich regions and two RNA-binding domains (17).  

Although the DNA-binding domain in PSF has not been identified, computer-assisted analysis of the pro-
tein’s amino acid sequence shows the highest probability of consensus DNA-binding domains such as SPXX (23) and nuclear factor I (24) are in the N-terminal region. A protein, named nuclear RNA-binding protein, 54 kDa (p54nrb), that is 71% identical to PSF has been identified (25). The DNA-binding domain of p54nrb has been localized to the N terminus while the activation domain resides in the C terminus (19). This protein has been shown to contain two RNA-binding domains similar to PSF and binds to and stimulates transcription through a DNA response element for murine intracisternal A particles (19).

In our initial identification of the porcine P450scc IGFRE, we hypothesized that additional transcription factors must bind to the IGFRE because of the cell-specific effects of IGF-I (13). In this study we report the identification of PSF as an additional protein regulating transcriptional activity of the IGFRE. However, PSF is an essential component of the spliceosome and (like Sp1) is an ubiquitously expressed cellular protein. Therefore, while we have uncovered a fascinating piece to the puzzle of cell-specific transcriptional effects of IGF-I, we do not yet understand the mechanism. The discovery of a protein (PSF) that is intimately involved in mRNA production, but can also feedback and control transcription of mRNA, presents intriguing possibilities for control of gene expression that could have much broader implications than merely IGF-I stimulation of P450scc gene expression.

The interactions of PSF and Sp1 in the porcine P450scc IGFRE are complex and cannot be determined in these experiments. Sp1 and PSF do not bind to overlapping sites within the IGFRE, but PSF inhibits Sp1-driven transcriptional activity of the IGFRE. While this is apparently incongruent data from a two-dimensional orientation, it could be explained by consideration of function of the IGFRE in three dimensions. Sp1 is a frequent proximal transcriptional enhancer of genes, and studies have shown that multiple cofactors and coactivators can assemble in complex spatial arrangements to activate RNA polymerase II for gene transcription (26). Our results indicate that PSF in some manner negatively influences such a complex for the porcine P450scc IGFRE.

Increased expression of P450scc mRNA occurs in a severe form of polycystic ovarian syndrome (PCOS), hyperthecosis (27). A hallmark of PCOS is a markedly elevated serum insulin concentration that could stimulate IGF-I receptors in the ovary (28). Several studies have proposed a genetic predisposition toward the development of PCOS (28–31). Moreover, genetic linkage stud-
ies indicate the P450scc gene as a possible candidate gene in families with PCOS (32, 33). Therefore, a mutation in the palindrome of the IGFRE that binds PSF or a mutation to the DNA-binding region of PSF could impair PSF binding and predispose a woman with such a mutation to enhanced expression of P450scc in the presence of increased insulin concentrations. Additional studies are necessary to study the interactions of Sp1 and PSF with the P450scc IGFRE and to explain how such interactions could result in abnormalities of ovarian function.

**MATERIALS AND METHODS**

**Materials**

Recombinant Sp1 protein and restriction enzymes were obtained from Promega Corp. (Madison, WI). Nitrocellulose filters were obtained from Micron Separations, Inc. (Westboro, MA). Polyvinylidene difluoride (PVDF) membranes were purchased from Bio-Rad Laboratories, Inc. (Hercules, CA). For kinase labeling, [γ-32P]-deoxyadenine-5'-triphosphate (dATP) was obtained from Amersham Pharmacia Biotech (Arlington Heights, IL). The mouse fibroblast cell line, NWTb3, was previously described (15) and was obtained from Dr. Charles Roberts (Department of Pediatrics, University of Oregon, Eugene, OR). The antibody to Sp1 was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The PSF antibody used in the affinity chromatography experiments was a gift from Dr. James Patton (Vanderbilt University, Nashville, TN). All reagents for sequence analysis were purchased from Perkin-Elmer Corp./PE Applied Biosystems Division (Norwalk, CT). Trypsin, modified sequencing grade, was a product of Promega Corp. (Madison, WI). All HPLC reagents used were of HPLC grade.

**Plasmid Constructs**

The full-length PSF cDNA clone was obtained from Dr. James Patton (Vanderbilt University) in a pET-15b expression vector.

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**Table 2. Mutant Oligonucleotides of the Porcine P450scc IGFRE with an Inability to Bind PSF**

| Oligonucleotide | Sequence |
|-----------------|----------|
| mWT (wild type) | ATCCTGAGTCTGGAGGGGCTGTGTGGGCC |
| mM18 | ATTCAGGAGTCTGGAGGGGCTGTGTGGGCC |
| mM25 | ATCCTGAAATTGGAGGGGCTGTGTGGGCC |

**Bold letters** indicate the nucleotide mutated from the wild type. **Underlined nucleotides** represent the palindrome that binds PSF.
Transfection of NWTb3 cells was done as previously described (14). The mM18 and mM25 oligonucleotide mutants of the IGFRE were cloned in pSVPLUC, a modified pGEM3 expression vector (34). These constructs were cotransfected with a promoterless luciferase vector (34). Transfection experiments in NWTb3 cells has been previously described (13). Briefly, this construct contains the entire 5'-region of P450scC including the IGFRE cloned into a promoterless luciferase vector (34).

The mWT luciferase reporter gene construct used in transfection experiments in NWTb3 cells has been previously described (14). The mM18 and mM25 oligonucleotide mutants of the IGFRE were cloned in pSVPLUC, a modified pGEM3 plasmid containing the luciferase gene and the enhancerless SV40 early region promoter (34). These constructs were made identically to mWT and verified by sequencing.

Transfection Experiments

Transfection of NWTb3 cells was done as previously described (15). Briefly, cells were cultured in DMEM + 10% FBS and 50 μg/ml Geneticin (Life Technologies, Inc., Gaithersburg, MD). Transient transfection was done by lipofection (Tfx-50 Reagent, Promega Corp.). Cells were harvested and measured for luminescence 48 h after cotransfection because this time was previously determined to show increases in P450scC mRNA after IGF-I treatment (13).

Fig. 8. Expression of PSF and Sp1 in Porcine Granulosa Cells

Porcine granulosa cells were transfected with a pcDNA3 expression vector (Control, 2 μg), an expression vector for PSF (1 μg), an expression vector for Sp1 (1 μg), or both (1 μg each). A luciferase construct of the porcine P450scC IGFRE (~2320 P450scC/luc) was cotransfected with the expression vectors. Arbitrary units are luminescence of the lysate after treatment divided by the protein concentration (13). The asterisk indicates statistical significance. The data represent the mean ± se from nine replications.

Sequence Analysis of HeLa Cell Nuclear Extract Protein

Samples of crude HeLa cell nuclear extract protein (100 μg) were fractionated by discontinuous SDS-PAGE under reducing conditions. Gel electrophoretically resolved proteins were electroblotted onto PVDF membrane using transfer buffer containing 0.05% SDS for 3.5 h. Proteins electroblotted onto PVDF were visualized by staining with Coomassie Blue G-250. Protein bands at 100 kDa were excised for both N-terminal and internal amino acid sequence analyses.

Internal sequence analysis of PVDF-blotted proteins was carried out similarly as described by Fernandez et al. (35). Excised PVDF membrane bands were cut into 1-mm² pieces and prewetted with 100% methanol. The PVDF membrane pieces were destained with 0.5 ml of 0.1% trifluoroacetic acid (TFA) in 50% actonitrile in a 2-ml polypropylene microfuge tube for 1 min followed by five washes with 0.5 ml of distilled water. Each of the aqueous washes included 5 min of sonication. An aliquot of 10 μl of hydrolysate buffer (100 mM Tris-HCl, 1% reduced Triton X-100, and 10% acetonitrile, pH 8.0) was subsequently added to the PVDF membrane pieces. Dipeptide bonds were reduced by the addition of 0 μl of 45 mM dithiothreitol and reaction for 30 min at 55 C. Alkylation was then initiated by addition of 5 μl of 100 mM iodoacetic acid followed by reaction for 30 min at 25 C in the dark. After brief centrifugation, the hydrolysate buffer was removed and the PVDF membrane pieces were washed once with 50 μl of hydrolysate buffer. Trypsin hydrolysis was then conducted in 50 μl of hydrolysate buffer to which were added 2 μl of freshly prepared trypsin (0.1 μg/μl). Trypsin hydrolysis proceeded for 4 h at 37 C, after which time another 2 μl of trypsin was added followed by incubation overnight at 37 C. After hydrolysis the reaction mixture was sonicated for 5 min and centrifuged briefly, and the supernatant was removed. The PVDF membrane pieces were treated with 3.7 μl of 5% TFA and then sonicated for 5 min after addition of 25 μl of hydrolysate buffer. The sample was centrifuged and the supernatant was saved. The PVDF sample was similarly sonicated with 25 μl of 0.1% TFA in 50% acetonitrile and centrifuged and finally sonicated in 25 μl of 0.1% TFA and centrifuged. All supernatants were combined, the volume was reduced by vacuum centrifugation to about 20 μl, and the sample was stored at –20 C.

The combined trypsin hydrolysate was subsequently fractionated by HPLC on a C18 reverse-phase capillary column (0.5 mm × 150 mm) using a Perkin-Elmer Corp./PE Applied Biosystems Division model 173A microblotter system. Peptides were eluted at a flow rate of 5 μl/min with a gradient eluant of 0.1% TFA (solvent A) and 0.085% TFA in acetonitrile (solvent B). The gradient conditions were 5% B to 45% B over 145 min. Eluted peptides were monitored at 215 nm and continuously collected on a PTFE membrane strip. An HPLC hydrolysate control was prepared exactly like the sample hydrolysate except that a blank region of the PVDF membrane was used. Peak fractions that were present in the sample hydrolysate but not in the control hydrolysate were subjected to microsequence analysis.

Selected peptides on PVDF membrane prepared as described above were subjected to automated N-terminal sequence analysis using a Perkin-Elmer Corp./PE Applied Biosystems Procise protein/peptide sequence (model 494-HT) configured with four blot cartridges. Peptide samples were
pretreated with 1–2 µl of BioBrene Plus solution (PE Applied Biosystems, Foster City, CA) [BioBrene (100 µl/ml)-0.1% TFA-methanol (2:1:7)]. Pulsed-liquid chemistry sequencing methodology was used for all samples.

**Partial Purification of Crude HeLa Cell Extract**

Crude nuclear extract from HeLa cells grown in suspension was prepared using a large-scale nuclear protein preparation method previously described (13). Approximately 700 mg of nuclear extract were precipitated by 53% saturated ammonium sulfate and centrifuged at 35,000 × g for 15 min. The pellet was resuspended in TM buffer (50 mM Tris-HCl, pH 7.9, containing 12.5 mM MgCl₂, 1 mM EDTA, 1 mM dithiothreitol, and 20% glycerol) to a final concentration of 30 mg/ml (16). The soluble protein extract was applied to an H. Prep Sephacryl S-300 High Resolution column (Pharmacia Biotech) equilibrated with TM buffer containing 0.1 M KCl. Protein elution was monitored by absorbance at 280 nm. The fractions containing Sp1 from five column runs were collected in one fraction (approx. 1 ml/ml) and reconstituted and ethanol precipitated for further purification. The major unretained protein fraction from the DEAE column was collected in one fraction (approximately 1 mg/ml).

**Sequence-Specific DNA Affinity Purification**

Preparation of DNA for coupling to Sepharose and coupling of DNA to Sepharose followed the method of Kadonaga and Tjian (36). The oligonucleotide coupled to Sepharose was the m6 oligonucleotide that in electrophoretic mobility shift assay does not bind Sp1 (14). Partially purified HeLa cell nuclear extract protein from the DEAE Sepharose column was used with 15 µg/ml of poly (dI-dC)-poly (dC-dI) as the competitor DNA. DNA affinity chromatography was performed as described by Kadonaga and Tjian (36).

**DNase I Footprint Analysis**

The porcine PSF and Porcine P450scc IGFRE Transcriptional Activity

Competent BL21DE3 plysS cells (Novagen, Madison, WI) were transformed with PSF (pET15b) DNA and used as an inoculum for an overnight culture. Expression of PSF was performed by propagation of the respective transformed host cells on super broth to an A₆₅₀ of 0.65. Isopropl-β-D-galactopyranoside was added to a final concentration of 1 mM and incubation was continued for 4 h at 37°C. Cells were collected by centrifugation at 3000 × g for 10 min at 4°C. Cells were suspended (0.2 g/ml) in 20 mM Tris HCl, pH 7.5, 500 mM NaCl, 1 mM phenylmethyl sulfonl fluoride (PMSF) (Life Technologies, Inc.), 1 µg/ml leupeptin (Amersham Pharmacia Biotech), 1 µg/ml pepstatin A (Amersham Pharmacia Biotech), and 1 µg/ml aprotonin (Amersham Pharmacia Biotech) and frozen once at −80°C. The thawed cell suspension was sonicated before centrifugation at 16,000 × g for 20 min at 4°C. The supernatant was removed and ProBond Ni-resin (Invitrogen) was added (1 ml resin/10 ml supernatant). After a 1-h incubation at 4°C with constant stirring, the resin was collected by centrifugation (2000 × g, 5 min 4°C) and suspended in 50 ml of 20 mM Tris HCl pH 7.5, 500 mM NaCl, 1 mM PMSF. The resin was collected using a 1 × 10 cm column (at 4°C) and then washed with 100 ml of 20 mM Tris HCl, pH 7.5, 500 mM NaCl, 20 mM imidazole (Sigma). A 200 ml linear gradient (20–200 mM imidazole) was used to elute the resin (16 ml/h) and 4 ml fractions were collected and analyzed (20 µl) by electrophoresis on polyacrylamide gels (10%, 1/37). PSF eluted as a broad peak between 100–150 mM imidazole. Protein concentrations were determined using Bradford reagent (Bio-Rad Laboratories, Inc.). Expressed PSF protein was isolated and given to Bio-Molecular Technology, Inc. (Frederick, MD) for generation of a polyclonal antibody to PSF.

**Statistical Analysis**

Statistical differences between transient transfection experiments in NWTb3 and porcine granulosa cells were determined by Kruskal-Wallis one-way ANOVA on ranks with Student-Newman-Keuls multiple comparison test. P values of <0.05 were considered statistically significant. Data are presented as mean ± SE.

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