NH₂ terminus of PTB-associated splicing factor binds to the porcine P450scc IGF-I response element

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Urban, Randall J., Yvonne H. Bodenburg, and Thomas G. Wood. NH₂ terminus of PTB-associated splicing factor binds to the porcine P450scc IGF-I response element. Am J Physiol Endocrinol Metab 283: E423–E427, 2002. First published April 23, 2002; 10.1152/ajpendo.00057.2002.—An insulin-like growth factor (IGF) I response element (IGFRE) in the porcine P-450 cholesterol side-chain cleavage gene (P450scc) regulates transcription through the binding of two proteins, Sp1 and polypyrimidine tract-binding protein-associated splicing factor (PSF). PSF is a component of spliceosomes and contains RNA-binding domains. In this study, we localized the NH₂-terminal amino acid residues necessary for binding of PSF to the IGFRE. Three COOH-terminal truncated proteins (aa 304, 214, and 134) of PSF were designed to empirically partition the NH₂-terminal region while excluding the RNA-binding domains. Southwestern analysis showed that only the largest expressed truncated protein, P3, strongly bound the porcine P450scc IGFRE. Truncated PSF protein expression in Y1 adrenal cells showed that P3 repressed transcriptional activity of the IGFRE similar to full-length PSF, whereas P2 (minimal binding to the IGFRE) had no effect. In conclusion, the NH₂-terminal region of PSF contains the amino acid residues necessary for binding to the porcine P450scc IGFRE and repressing the transcriptional activity of the element.

Insulin-like growth factor I (IGF-I) is a growth factor that stimulates steroidogenesis in the ovary (1, 2). We determined that IGF-I increases steroidogenesis in porcine granulosa cells by increasing the expression of P-450 cholesterol side-chain cleavage (P450scc) enzyme (23). IGF-I stimulates P450scc gene expression through a 30-bp GC-rich domain (IGF-I response element, IGFRE) located ~100 bp from a classical TATA box in the P450scc gene (25). Moreover, we determined that the IGFRE binds Sp1 (21) and polypyrimidine tract-binding protein (PTB)-associated splicing factor (PSF) (22). Sp1 binds to the GC box of the IGFRE, whereas PSF binds to a nonoverlapping site upstream of the GC box, the palindromic CTGAGTC (22). Transient transfection experiments in porcine granulosa cells with an Sp1 expression plasmid stimulated IGFRE-mediated transcriptional activity of the P450scc gene, whereas PSF expression repressed transcriptional activity even during concomitant expression of Sp1 (22).

PSF was isolated and cloned by Patton in 1993 (19). 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 the splicing of pre-mRNA. It is an intriguing protein in that the amino terminus is rich in proline and glutamine residues (see Fig. 1). Similar proline/glutamine-rich regions comprise the transactivation domains of Sp1 (7, 8). PSF is the product of only one gene; however, 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 (19). A protein with 70% homology to PSF, nuclear RNA-binding protein (54 kDa) shows DNA binding in the NH₂ terminus of the protein (4). The NH₂-terminal region of PSF was shown to bind response sequences for thyroid hormone and retinoid X receptors (16). Therefore, this study focuses on determining whether the NH₂ terminus of PSF contains the amino acid residues that bind the porcine P450scc IGFRE and how this affects the function of the response element.

Studies show that the 30-bp IGFRE in P450scc serves a much broader role in controlling P450scc gene expression than just as an IGF-I response element. Stimulation of steroidogenesis by luteinizing hormone and follicle-stimulating hormone (FSH) occurs by increasing cAMP and activating protein kinase A, which can translocate to the nucleus and phosphorylate transcription factors (12). The 30-bp IGFRE of P450scc mediates a threefold stimulation of P450scc gene expression in transient transfection experiments in porcine granulosa cells treated with FSH or forskolin (25). This same region is also responsive to cAMP in bovine, rat, and human P450scc genes (3, 18). Moreover, GC-rich cAMP response regions that bind Sp1 occur in other steroidogenic enzyme genes (14) and in other cell types (27). The impact of tumor necrosis factor-α (TNF-α) on steroidogenesis is also controlled by the IGFRE. In porcine granulosa cells, TNF-α inhibits ex...
expression of P450scc mRNA concentrations stimulated by insulin or IGF-I (24, 26). The inhibition of steroidogenesis by TNF-α was mediated through the IGFR (24). Finally, there is evidence that the protein kinase C pathway can also regulate steroidogenesis by effects on FSH-stimulated steroidogenesis, indicating that this important cellular pathway may influence P450scc expression through the IGFR (10, 15). Therefore, understanding the mechanisms and interactions of PSF and Sp1 on this important 30-bp response element can result in an increased understanding of P450scc gene expression by multiple cellular pathways.

In the present study, we focused on determining the PSF amino acid residues necessary for binding to the IGFR. We used COOH-terminal truncated PSF proteins to show that the amino acid residues important to PSF binding/function were located in the NH2 terminus and did not require the RNA binding domains to repress transcriptional activity of the element.

**METHODS**

**Materials.** The antibody to PSF was made from recombinant PSF by Bio-Molecular Technology (Frederick, MD). Nitrocellulose filters were obtained from Bio-Rad (Hercules, CA). "Plasmid constructs. The PSF cdNA clone was obtained from Dr. James Patton (Vanderbilt University) in pET-15b expression vector (19). PSF truncation mutants P1-P3 were created using a PCR-based strategy. Primers introduced unique cloning sites 5’ (Ndel) and 3’ (XhoI) to facilitate construction of the expression plasmids by use of a pET-15b vector (Novagen) for bacterial expression. PCR-based strategy was also used for cloning PSF and the PSF truncation mutants P2 and P3 into the pcDNA3.1/V5-His expression vector for mammalian expression (Invitrogen). Both strategies used PCR-introduced unique cloning sites that maintained the normal reading frame for PSF and the truncation mutants. The anti-V5 antibody (Invitrogen) was used to verify protein expression of the truncation mutants in Y1 adrenal cells. The anti-V5 antibody is directed against the epitope found in the P and V proteins of the paramyxovirus, SV5, resulting in a marker of expression that is highly sensitive with low background. The plasmid pSVPLUC is a modified pGEM3 plasmid containing the luciferase gene and an enhancerless SV40 early region promoter (5). The complete sequenced upstream region of porcine P450scc (approximately ~2,000 bp including the IGFR) was used in a reporter gene construct with the pSVPLUC plasmid. The control plasmid in transfection experiments is pSV2Apap containing the SV40 early promoter enhancer region and the human placental alkaline phosphatase gene (13).

**Transient transfection in Y1 adrenal cells.** Y1 adrenal cells were cultured as previously described (25). Transient transfection was carried out by lipofection (Tfx-50 Reagent, Promega). Transfection experiments were done on 60-mm plates following the Promega protocol for Tfx-50 Reagent. A 3:1 ratio (1 μg of DNA/2.5 μl of Tfx-50) was used for each transfection. Cells were harvested, and luminescence was measured 48 h after cotransfection using Promega’s luciferase assay system.

**Southwestern analysis.** Protein samples were taken from the bacterial protein expression solutions described in Fig. 1 and were denatured for 5 min at 95°C and then loaded onto a 10% SDS-PAGE gel. Proteins were electrophoretically transferred to nitrocellulose using a 25 mM Tris, 192 mM glycine, and 20% (vol/vol) methanol for 1 h. The filter was then rinsed with TNE-50 (10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol) briefly and then blocked in Blotto (5% milk-TNE-50) for 2 h at room temperature with gentle shaking. The blots were rinsed two times in TNE-50 before being placed in hybridization buffer. (TNE-50 buffer with 1 μg/μl poly[dI-dC] and 1.5 × 106 cpm/ml labeled probe). The probe is the 30-bp porcine P450scc IGFR end-labeled with 32P. The blot was hybridized for 2–4 h at room temperature, rinsed in TNE-50 three times for 5 min at room temperature, and exposed to an autoradiogram.

**Western gel and immunoblotting.** Nuclear extract protein samples were obtained from Y1 adrenal and NWTb3 cells by use of a standard protocol to isolate nuclear protein. Samples of cell nuclear extract protein were fractionated by discontinuous 10% SDS-PAGE gel under reducing conditions. The gel was then electrophoretically transferred (TransBlot, Bio-Rad) by use of electrophoretic transfer buffer for 1 h. The blot was then blocked for 2 h in 5% milk-TBS and then incubated overnight with primary antibody, in 1% milk-TBS. The secondary 0.5-μg antibody was added to the blot and incubated for 1 h. The blot was exposed to film and developed. This standard method was used for Western analysis with antibody to PSF and V5.

**Truncated PSF protein expression in bacteria.** The truncated proteins were expressed using competent BL21DE3 plysS cells (Novagen) that were transformed with each clone. Detailed protein expression methods have been previously described (22).

**Statistical analysis.** Statistical analysis on the transient transfection experiments was done either by ANOVA on ranks with Student-Newman-Keuls multiple comparison or by paired t-test. Data are presented as means ± SE. Statistical significance is reached at P ≤ 0.05.

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RESULTS

Localization of DNA binding of PSF. Having shown previously that PSF bound to the palindrome CT-GAGTC, located 5’ of the GC box of the porcine P450sc IGFRE (22), we wanted to determine the amino acid residues of PSF necessary for binding to the porcine P450sc IGFRE. Therefore, we made three COOH-terminal truncated proteins of PSF (Fig. 1). We partitioned the NH2 terminus of the PSF protein into three regions (aa 304, 214, 134), intentionally excluding the two RNA-binding domains (Fig. 1). The NH2-terminal region is rich in prolines and glutamines, as indicated in Fig. 1. We used a PCR-based strategy to create the three truncated PSF domains, cloned the three cDNAs into the pET15b expression vector, and expressed all three truncated proteins (Fig. 1). PSF is a unique protein in that it is 76 kDa in size but runs on SDS-PAGE at 100 kDa because of the basic nature of the protein (19).

We used Southwestern analysis with the wild-type porcine P450sc IGFRE to determine whether any of the three truncated proteins would bind the IGFRE. As shown in Fig. 2, the largest truncated protein, P3, strongly bound to the IGFRE. P2 shows faint binding to the IGFRE, and no binding is seen for P1 (Fig. 2). Therefore, the NH2-terminal region contains amino acid residues that are necessary for binding of PSF to the porcine P450sc IGFRE. This occurs primarily between amino acids 214 and 304.

Truncated PSF expression in Y1 adrenal cells. We hypothesized that the truncated protein P3 could have two possible functions. It could compete with endogenous PSF and prevent repression of the transcriptional activity of the IGFRE; however, it could also function like endogenous PSF and repress the response element. We selected mouse Y1 adrenal cells to express P3 because we had previously found that this steroidogenic cell line was not responsive to IGF-I in transient transfection experiments using the P450sc IGFRE (25). We reasoned that this cell line expressed abundant amounts of PSF that would enable competition with expressed P3. Figure 3 shows the verification that PSF is expressed in Y1 adrenal cells by Western analysis with a PSF-derived antibody. Bacterially expressed PSF was used as the positive control for the Western.

PSF, P2, and P3 constructs were ligated in the pCDNA3.1/V5-His expression vector for transfection experiments (see METHODS). Figure 4 shows that PSF, P2, and P3 were expressed in Y1 cells after transient transfection with either the empty pcDNA3.1/V5 plasmid or the V5 constructs containing PSF (100 kDa), P2 (49 kDa), and P3 (65 kDa). Top: Western blot analysis showing expression of the truncated proteins by epitope labeling with an antibody to V5 (epitope found in the P and V proteins of the paramyxovirus, SV5). The arrows indicate the protein expression bands for PSF, P2, and P3. Both truncated proteins showed doublet expression, while full-length PSF expressed only 1 band. An SV40 luciferase construct of the porcine P450sc IGFRE was co-transfected with a control plasmid, pSV2Apap. Bottom: graph of transfection results expressed as means ± SE from 6 replicates. Arbitrary units are luminescence of the lysate after treatment divided by absorbance (alkaline phosphatase). *Statistical significance, P ≤ 0.05. The V5 vector was the negative control.
transfection (V5 epitope tag). Both P2 and P3 expressed as doublet bands on Western analysis at similar intensities. PSF expressed at a lower level than the two truncated proteins but as only one band. Also shown in Fig. 4 are the results of cotransfection experiments that show that P3 (strong IGFRE binding) expression represses the P450scc IGFRE similarly to full-length PSF, whereas P2 (faint IGFRE binding) expression does not influence the transcriptional activity of the IGFRE. Therefore, the RNA-binding domains and COOH-terminal region of PSF are not necessary for the inhibitory actions of the protein on the IGFRE.

DISCUSSION

In this study, we have found that the NH2 terminus of PSF contains the amino acid sequences necessary for binding to the porcine P450scc IGFRE. The truncated PSF protein alone, without the RNA-binding domains, acts on the proximal 2 kb of porcine P450scc to repress transcriptional activity of the IGFRE.

The findings in this study increase our understanding of PSF. The NH2 terminus of this protein contains many proline and glutamine residues (Fig. 1), and these basic amino acids result in a slowed migration in SDS-PAGE gels (19). The 304-amino acid truncated protein P3 contained the sequences necessary for IGFRE binding of PSF (Fig. 2) as well as maintaining repression of the transcriptional activity of the IGFRE (Fig. 4). Therefore, from our studies, PSF acts much like two distinct proteins. The NH2 terminus binds to DNA and inhibits transcription, whereas the RNA-binding domains and COOH terminus function for RNA splicing and preparation for protein translation. The physiological relevance of such a multitasking protein for ovarian steroidogenesis is yet to be determined. P450scc is the rate-limiting enzyme in the steroidogenesis such as polycystic ovary syndrome.

REFERENCES


