PTB-associated splicing factor regulates growth factor-stimulated gene expression in mammalian cells

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Urban, Randall J., and Yvonne Bodenburg. PTB-associated splicing factor regulates growth factor-stimulated gene expression in mammalian cells. Am J Physiol Endocrinol Metab 283: E794–E798, 2002—An insulin-like growth factor I (IGF-I) response element (IGFRE) in the porcine P-450 cholesterol side-chain cleavage gene (P450scc) binds two transcription factors, Sp1 and polypyrimidine tract-binding protein-associated splicing factor (PSF). In this study, we investigated expression of these transcription factors in mouse Y1 adrenal cells, a cell line that does not increase P450scc expression in response to IGF-I. Western blot analysis showed a greater expression of PSF in Y1 cells when compared with a mouse fibroblast cell line (NWTb3) in which IGF-I stimulates the P450scc IGFRE. The two cell lines expressed Sp1 equally, and IGF-I did not increase expression of either transcription factor. Chromatin immunoprecipitation analysis with Y1 chromatin confirmed that PSF and Sp1 bound to the IGFRE. When increasing amounts of Sp1 were expressed in Y1 cells, the IGFRE became responsive to IGF-I. Moreover, a mutant oligonucleotide IGFRE reporter construct that lacks PSF binding was responsive to IGF-I. In conclusion, Y1 adrenal cells are a physiological example of repression of growth factor-stimulated (IGF-I) gene expression (P450scc). The dynamic nature of this repression is consistent with PSF functioning as a regulator of growth factor-stimulated gene expression in mammalian cells. Polypyrimidine tract-binding protein-associated splicing factor; P-450 cholesterol side-chain cleavage; insulin-like growth factor I; Y1 adrenal cells; Sp1

In human adrenocortical cells, IGF-I does not increase the expression of P450scc (6, 7). Moreover, when the porcine P450scc IGFRE is transfected into Y1 adrenal cells, it does not respond to IGF-I treatment (16), whereas the IGFRE is responsive to IGF-I in transient transfection experiments in a mouse fibroblast cell line (NWTb3) stably transfected with the IGF-I receptor (15). This study investigated the mechanisms responsible for the lack of an IGF-I response of the IGFRE in Y1 cells. Because PSF shows repressor activity in other cellular pathways (8), we wanted to determine whether the cell-specific loss of IGF-I response in Y1 adrenal cells was caused by enhanced PSF repression. Determining functional PSF repression would further expand our limited knowledge of physiological regulation of IGF-I by PSF.

METHODS

Materials. The mouse fibroblast cell line NWTb3 has been previously described (15) and was obtained from Dr. Charles Roberts, Department of Pediatrics, University of Oregon. The antibody to PSF was made from recombinant PSF (12) by Bio-Molecular Technologies (Frederick, MD). The Sp1 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Platinum PCR Supermix was purchased from Invitrogen (Carlsbad, CA). Chromatin immunoprecipitation (ChIP) materials used include staph A cells from Roche (Indianapolis, IN) and protease inhibitors from Sigma Chemical (St. Louis, MO). Chemicals for solutions were purchased from Sigma, as were general reagents for the ChIP assay. Reagents purchased elsewhere are indicated in the subsequent sections.

Plasmid constructs. The PSF cDNA clone was obtained from Dr. James Patton, Vanderbilt University, in a pET-15b

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expression vector (9). The cDNA was excised and cloned into a cGmegalovirus mammalian expression vector, pcDNA3 (Invitrogen), maintaining the open reading frame as previously described (12). The Sp1 expression vector pCMV-Sp1 was obtained from Dr. Robert Tjian, University of California, Berkeley. The porcine P450scc IGFRE reporter gene construct used in transfection experiments was the 

Western gel and immunoblotting. Samples of Y1 and NWTb3 nuclear extract protein collected as previously described (12) were fractionated by discontinuous 10% SDS-PAGE gel under reducing conditions. The gel was then electrophoretically transferred to nitrocellulose (TransBlot, Bio-Rad) by use of an electrophoretic transfer buffer for 1 h. The blot was then electroblotted onto nitrocellulose (TransBlot, Bio-Rad) by use of electrophoretic transfer buffer for 1 h. The blot was then electrophoretically transferred to nitrocellulose (TransBlot, Bio-Rad) by use of electrophoretic transfer buffer for 1 h.

ChIP assay. We used a modification of the technique described by Boyd et al. (3) and a protocol obtained from the Farnham laboratory website (www.mcardle.oncology.wisc.edu/farnham/). Twenty 15-cm dishes of the Y1 cells were plated at \(-2 \times 10^6\) cells/dish and incubated for 48 h in media with 2.5% fetal bovine serum. Formaldehyde was added directly to medium at a final concentration of 1%. Cross-linking was then performed on a water bath at 4°C for 1 h. After application of the ECL Western blotting detection reagent (Amersham Pharmacia Biotech, Piscataway, NJ), the blot was exposed to film and developed.

Transient transfection in Y1 adrenal cells. Y1 adrenal cells were cultured as previously described (16). Transient transfection was performed by lipofection (Tfx-50 reagent, Promega). Cells were harvested and measured for luciferase activity 48 h after transfection. For expression experiments, an SV40 construct containing 2320 bp of the upstream porcine P450scc (containing the IGFRE) was co-transfected (15).

Statistical analysis. Statistical analysis on transient transfection experiments was done by ANOVA on ranks with Student-Newman-Keuls multiple comparison. Data are presented as means ± SE.

Fig. 1. Polypyrimidine tract-binding protein (PTB)-associated splicing factor (PSF) expression in Y1 adrenal and NWTb3 cells with and without IGF-I. Western blot hybridized with an antibody to PSF described in METHODS. Recombinant PSF (0.5 μg) was run in the first lane (PSF). NWTb3 (20 μg) and Y1 (20 μg) nuclear extract protein were run in the next lanes without IGF-I treatment (control, C) or with IGF-I treatment (IGF-I, 20 nM). Shown is a representative blot of 3.

Fig. 2. Sp1 expression in Y1 adrenal and NWTb3 cells with and without IGF-I. Western blot hybridized with an Sp1 antibody. Recombinant Sp1 (0.2 μg) was run in the first lane (Sp1). NWTb3 (20 μg) and Y1 (20 μg) nuclear extract protein were run in the next lanes without IGF-I treatment (C) or with IGF-I treatment (IGF-I, 20 nM). Shown is a representative blot of 3.
RESULTS

PSF expression in Y1 adrenal cells. Previous experiments showed that IGF-I did not stimulate the porcine P450scc IGFRE in transient transfection experiments in Y1 adrenal cells (16). By use of a PSF antibody derived from recombinant protein (12), Western blot analysis was done on nuclear extract protein from Y1 adrenal and NWTb3 cells treated with and without IGF-I (Fig. 1). There was a greater amount of PSF expression in the Y1 cells compared with the NWTb3 cells. We selected the latter cell type (NWTb3) because IGF-I stimulates the IGFRE in transient transfection experiments and they are of mouse origin like the Y1 cells (15). NWTb3 cells are mouse NIH 3T3 cells stably transfected with the IGF-I receptor (5). Therefore, altered antibody recognition because of species variability is not a factor when Y1 and NWTb3 cells are compared.

Sp1 expression in Y1 cells. Western analysis was also done for Sp1 in Y1 adrenal cells and compared with Sp1 expression in NWTb3 cells treated with and without IGF-I. As shown in Fig. 2, Sp1 was expressed at similar levels in Y1 and NWTb3 cells, and Sp1 expression did not respond to IGF-I.

Fig. 3. Chromatin immunoprecipitation (ChIP) assay of Y1 nuclear extract protein for PSF and Sp1 binding to the IGF response element (IGFRE). Top: PCR analysis of ChIP reactions with Sp1 and PSF representing samples processed through the ChIP protocol with the respective antibody; (−), a sample processed through the ChIP protocol without primary antibody; Input, total chromatin before immunoprecipitation. Bottom: an ethidium-stained gel of PCR product from input material serially diluted (3-fold, lanes 1–5) to document that the amount of PCR product accurately reflects the amount of template DNA added to the PCR reaction. PCR reactions were done at 28 cycles. The ChIP assay was done 3 times.

Fig. 4. Transient transfection of PSF and Sp1 expression vectors in Y1 adrenal cells. Y1 adrenal cells were transfected with a pcDNA3 expression vector (Control, 2 μg), an expression vector for PSF (2 μg), and an expression vector for Sp1 (2 μg). An SV-40 luciferase construct of the porcine P-450 cholesterol side-chain cleavage (P450scc) IGFRE (2320 bp of 5′ sequence) and a control plasmid (pSV2Apap) were co-transfected with the expression vector. Arbitrary units are luminescence of the lysate after treatment divided by absorbance (alkaline phosphatase). *Statistical significance as determined by ANOVA (P ≤ 0.05). Data represent means ± SE from 3 experiments.

Fig. 5. Dose response of Sp1 expression on IGF-I responsiveness of the IGFRE. Y1 adrenal cells were transfected with increasing concentrations of an Sp1 expression vector and treated with IGF-I (20 nM). Co-transfection plasmids were as described in Fig. 4. *Statistical significance as determined by ANOVA, control cells at baseline vs. cells with increasing amounts of transfected Sp1 (P ≤ 0.05). **Significant increase (ANOVA) control vs. IGF-I treatment (P ≤ 0.05). Data are means ± SE from 3 experiments.

Fig. 6. Transient transfection of luciferase construct containing IGFRE mutant that binds only Sp1. Y1 adrenal cells were transfected with a luciferase construct that contains a mutation to the IGFRE such that it binds only Sp1 (mM18). A wild-type (mWT) IGFRE construct was also transfected as a positive control. Cells were studied with and without treatment with IGF-I (20 nM) for 48 h. Data are presented as described in Fig. 4. Data are means ± SE from 3 experiments. P ≤ 0.05. *Significantly increased over wild-type; **significantly greater than control in M18.
ChIP assay on Y1 nuclear extract protein. As shown in Fig. 3, ChIP assay showed that both PSF and Sp1 were able to bind to the IGFRE. This important assay shows that the transcription factors associate with chromatin and occupy the IGFRE. We are unable to successfully perform electromobility shift assays with PSF; therefore the ChIP assay is especially relevant for the transfection experiments that follow. The assay shows that, although there is an increased expression of PSF in Y1 adrenal cells, Sp1 still binds to the IGFRE in a comparable fashion to PSF.

Transient transfection of an Sp1 expression vector in Y1 adrenal cells. In porcine granulosa cells, expression of PSF repressed basal transcriptional activity of the porcine P450scc IGFRE, whereas Sp1 expression increased basal transcriptional activity of the element (12). Transient transfection experiments in Y1 cells with both expression plasmids determined that Y1 cells responded in a similar fashion to porcine granulosa cells (Fig. 4), with PSF repressing transcriptional activity of the IGFRE whereas Sp1 increased transcription.

Because Sp1 expression in Y1 cells increased IGFRE transcriptional activity, increasing concentrations of the Sp1 expression plasmid were transfected in Y1 cells. As shown in Fig. 5, the IGFRE responded to IGF-I with increasing Sp1 expression.

Transient transfection experiments with a mutant oligonucleotide that does not bind PSF. Transient transfection experiments were done in Y1 adrenal cells with a mutant oligonucleotide construct (mM18, see METHODS) that lacks PSF binding (12). As shown in Fig. 6, transient transfection experiments with this mutant oligonucleotide construct showed that IGF-I would significantly stimulate the P450scc IGFRE in the absence of PSF binding. The wild-type IGFRE (mWT) served as a positive control for this experiment.

DISCUSSION

We investigated the mechanisms responsible for the inability of IGF-I to stimulate P450scc gene expression in Y1 adrenal cells. Previous studies found that cultures of human adrenocortical cells do not increase mRNA concentrations of P450scc when treated with IGF-I (6, 7). Moreover, transient transfection studies in Y1 adrenal cells with the porcine P450scc IGFRE showed no response to IGF-I treatment (16). In this study, we investigated the expression and binding of PSF and Sp1 to the P450scc IGFRE in Y1 cells. Both of these transcription factors bind to the IGFRE, with Sp1 stimulating promoter activity and PSF functioning as a repressor of transcription (12).

In Y1 cells, PSF expression under basal conditions appeared higher compared with another mouse cell line, NWTb3, which is fibroblast in origin, is stably transfected with the IGF-I receptor, and shows an increase in the transcriptional activity of the IGFRE when treated with IGF-I (15). There was no difference in expression of Sp1 in either cell line, and neither increased its expression in response to IGF-I treatment. Transient transfection protein expression studies confirmed that PSF also functioned as a repressor in Y1 cells, indicating that the lack of response of P450scc expression in response to IGF-I cells could be the increased expression of the repressor PSF. Our findings are consistent with the developing literature that PSF is a repressor of transcription. Mathur et al. (8) identified PSF as a corepressor of the type II nuclear hormone receptors thyroid hormone receptor and retinoid X receptor. PSF bound to the DNA binding domain of these receptors and associated with Sin3A, a protein known to mediate transcriptional repression by recruitment of class I histone deacetylases (8). To add another level of complexity to PSF, a recent study found that, during apoptosis in bone marrow cells, PSF is hyperphosphorylated, dissociates from PTB, associates with new protein partners, and becomes insensitive to proteolysis (10). Therefore, PSF is a highly complex protein that may be an important component of transcriptional repression for many different genes by different mechanisms.

The present study also expands our understanding of the interactions of PSF and Sp1 on the P450scc IGFRE. From the ChIP assay, even though there was a greater expression of PSF in Y1 cells and the IGFRE was not responsive to IGF-I, there was still binding of Sp1 to the chromatin. Therefore, the repressor actions of PSF would seem to involve more than just a competition with Sp1 for occupancy of the IGFRE. However, when additional Sp1 is expressed in Y1 cells or when a reporter construct of the IGFRE that does not bind PSF is transfected in Y1 cells, the IGFRE is responsive to IGF-I treatment. This implies that binding of PSF is essential for repression of the IGFRE and that nuclear concentrations of the transcription factors are important in facilitating the IGF-I response. Such a complex interaction between these transcription factors could be regulated by their phosphorylation state.

In conclusion, Y1 adrenal cells do not increase expression of P450scc in response to IGF-I, because they have increased levels of the repressor, PSF. Although both PSF and Sp1 bind to the P450scc IGFRE in Y1 cells, increasing Sp1 levels or impairing PSF binding to the IGFRE will restore a response of the IGFRE to IGF-I. Y1 cells are a physiological model of PSF regulation of growth factor-stimulated gene expression. This model may be of physiological relevance in growth factor-mediated clinical syndromes.

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REFERENCES