

Selenoprotein P regulation by the glucocorticoid receptor

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Received: 11 December 2008 / Accepted: 27 May 2009 / Published online: 10 June 2009
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Abstract Maintenance of the antioxidant activity of selenoproteins is one potential mechanism of the beneficial health effects of selenium. Selenoprotein P is the primary selenium distribution protein of the body as well as the major selenium containing protein in serum. The transcriptional regulation of selenoprotein P is of interest since the extrahepatic expression of this gene has demonstrated differentiation-dependent expression in development as well as under different disease states. SEPP1 displays patterned expression in numerous tissues during development and the loss of SEPP1 expression has been observed in malignancy. In addition, factors that influence inflammatory processes like cytokines and their regulators have been implicated in selenoprotein P transcriptional control. Herein, we identify a retinoid responsive element and describe a mechanism where the glucocorticoid receptor negatively regulates expression of selenoprotein P. Luciferase reporter assays and quantitative PCR were used to measure selenoprotein P transcription in engineered HEK-293 cells. When stimulated with ecdysone analogs, selenoprotein P expression was increased with the use of a fusion transcription factor that contains the glucocorticoid receptor DNA binding domain, an ecdysone

ligand-binding domain, and a strong transactivation domain as well as the retinoid X receptor. The native glucocorticoid receptor inhibited selenoprotein P transactivation, and selenoprotein P was further attenuated in the presence of dexamethasone. Our results may provide insight into a potential mechanism by which selenium is redistributed during development, differentiation or under conditions of critical illness, where glucocorticoid levels are typically increased.

Keywords Selenoprotein P · Glucocorticoid receptor · Glucocorticoids · Antioxidant · Retinoids

Abbreviations

Dex	Dexamethasone
DMSO	Dimethyl sulfoxide
EtOH	Ethanol
GR	Glucocorticoid receptor
FoxO1a	Forkhead box, class O1a
GRE	Glucocorticoid response element
HNF-4 α	Hepatocyte nuclear factor-4 α
PCR	Polymerase chain reaction
PGC-1 α	Peroxisomal proliferator activated receptor- γ coactivator 1 α
PonA	Ponasterone A
RRE	Retinoid responsive element
RXR	Retinoid X receptor
SeP	Selenoprotein P gene product
SEPP1	Selenoprotein P gene

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VgEcR Ecdysone-inducible fusion transcription factor

Introduction

Selenoprotein P (SeP) is an extracellular glycoprotein that carries ~40% of plasma selenium (Akesson et al. 1994). SeP is unique among the selenoproteins in that it can possess up to ten selenocysteine residues in mammals (Burk and Hill 2005). SeP primarily functions in selenium distribution (Hill et al. 2003; Renko et al. 2008), with knockout mice displaying altered selenium distribution, particularly to the testes and brain (Hill et al. 2003; Burk et al. 2006). The majority of SeP is derived from hepatic sources, however; the mRNA can be detected in almost all tissues, with appreciable concentrations observed in the kidney, heart, lung, brain, skeletal muscle, and testis (Burk and Hill 2005).

The regulation of selenoprotein P gene (SEPP1) expression is an active area of investigation with changes in SEPP1 noted under a broad spectrum of biological processes. In HepG2 cells and primary rat hepatocytes, promoter activity has been shown to be inhibited by cytokines including interleukin 1 β , tumor necrosis factor α , interferon γ , and transforming growth factor β_1 (Dreher et al. 1997; Mostert et al. 2001). This inhibition suggests that the SEPP1 gene product may function as a negative acute-phase protein in response to inflammation. Alternatively, promoter activity is stimulated in hepatic cells through the FOXO1a and HNF-4 α transcription factors (Speckmann et al. 2008; Walter et al. 2008).

In addition to inflammation, microarray analyses have revealed changes in SEPP1 expression during development and following alterations in the differentiation state of extrahepatic cells. Elegant developmental studies have demonstrated SEPP1 ortholog spatiotemporal expression in both zebrafish (Thisse et al. 2003) and murine model systems (Lee et al. 2008). Increased expression has been observed in differentiating myeloid, pulmonary, and Sertoli cells (Tabuchi et al. 2005; Ghassabeh et al. 2006; Wade et al. 2006). Conversely, SEPP1 expression is decreased with neoplastic progression from normal tissue, to carcinoma, to metastatic disease in cells of prostate origin (Dhanasekaran et al. 2001). Evaluation

of SEPP1 expression in the Oncomine database (Rhodes et al. 2004) also identifies decreased SEPP1 expression in melanoma, lung, and colon cancer compared to normal tissue suggesting that decreased SEPP1 expression may be a common feature of malignancies. Indeed, work in colorectal cancer suggests that specific selenoenzymes are reduced, indicating that changes in SEPP1 is not a general alteration in nutrition or decreased selenium (Al-Taie et al. 2004).

In the present study, we observed SEPP1 induction in human cells stably transfected with the ecdysone inducible system (VgEcR-RXR). Due to VgEcR's glucocorticoid receptor DNA binding domain, as well as evidence of SEPP1 modulation during development and inflammation, we sought to determine if SEPP1 was regulated by the glucocorticoid receptor or the retinoid X receptor. In addition, evidence exists for changes in plasma selenium levels following glucocorticoid administration, with both increases and decreases noted under different sets of conditions (Peretz et al. 1987; Marano et al. 1990; Watanabe et al. 1997). Although the effect of glucocorticoids on selenium levels has not been fully characterized, it is believed that these changes result from redistribution of selenium between tissue and plasma. It is unknown what role SeP may play in this glucocorticoid-induced selenium redistribution. Therefore, an aim of this study was to examine the glucocorticoid responsiveness of the SEPP1 promoter, and we found that the glucocorticoid receptor (GR) inhibits the expression of SEPP1.

Materials and methods

Materials

The HEK-293 cell line was purchased from American Type Culture Collection. Advanced DMEM, T4 DNA ligase, *Hind*III, *Xho*I, and *Sst*I, Accuprime Pfx DNA polymerase, SuperScript III reverse transcriptase reagents, OneShot Top 10 chemically competent cells, zeocin, geneticin, Lipofectamine 2000, and N-NTA agarose were purchased from Invitrogen. Ponasterone A (PonA) was purchased from A.G. Scientific. Dexamethasone (Dex) was purchased from EMD Chemicals. RNeasy Mini Kit and EndoFree Maxi-and Mini-prep Kits were obtained from Qiagen.

Lightcycler 480 SYBR Green I master mix was purchased from Roche Diagnostics. Biolase DNA polymerase, dNTPs, magnesium chloride, and NH_4 reaction buffer were purchased from Biotline. SYBR Green I was purchased from Cambrex. Human genomic DNA, the Dual Luciferase Reporter Assay System was purchased from Promega. NE-PER nuclear extraction reagents, Biotin 3' end DNA labeling kit, and Lightshift chemiluminescent EMSA Kit were purchased from Pierce.

Plasmids

pVgEcR (Invitrogen) encodes the fusion transcription factor used to generate ecdysone-inducible cells. pRL-RSV and pGL4.21 (Promega) were used in the luciferase reporter assays; pRL-RSV constitutively expresses *Renilla reniformis* luciferase, and SEPP1 promoter fragments were cloned into the pGL4.2.1 plasmid that contains firefly luciferase. The pLTRluc glucocorticoid reporter plasmid and pDsRed-hGR glucocorticoid receptor expression plasmid were gifts from Dr Carol Lim (University of Utah).

Cell culture

Human embryonic kidney line HEK-293, as well as all subsequently engineered cells, were cultured in Advanced DMEM medium containing 2% fetal bovine serum and 2 mM L-glutamine. Cells were maintained at 37°C in a humidified incubator with 5% CO_2 .

HEK-293 were transfected with pVgEcR and selected for Zeocin resistance to generate stable expression of the VgEcR gene product and are referred to as 293-EcR. 293-EcR cells that conditionally express 15-LOX-1 and ΔIle^{662} 15-LOX-1 were previously described (Yu et al. 2004; Cordray et al. 2007). Conditional expression of β -galactosidase in the 293-EcR was achieved using similar methods.

An expression vector, pDsRed-hGR, that constitutively expresses a DsRed2-labeled, functional human GR was generously provided by Dr Carol Lim, University of Utah. The 293-EcR cells were stably transfected with this expression vector and selected for neomycin resistance in order to study the effects of GR signaling in HEK-293 cells. These cells are referred to as EcR-GR.

Polymerase chain reaction (PCR)

The Transcription Regulatory Element Database (Jiang et al. 2007) was used to identify the ~2 kb sequence surrounding the transcriptional start site of SEPP1, promoter ID #34663 (1,770 bp upstream of start site, 300 bp downstream) and used as an electronic template to generate promoter constructs. A 1.9 kb sequence was amplified from human genomic DNA by PCR using the primer pair 5'-TAGG TACCCAGTTCTTTCCGGTGTTCA-3' and 5'-TA CTCGAGCGCACTGGGAACCTTCACCTA-3'. The PCR product was digested with *Xho*I and *Ssr*I and cloned into the pGL4.21 luciferase reporter vector. This construct is referred to as -1,652 to +247 and was utilized as template DNA in subsequent PCR reactions used to synthesize smaller fragments of the SEPP1 promoter region of interest. A *Hind*III digestion of the -1,652 to +247 construct generated -1,652 to -385 and -391 to +247 promoter fragments. The fragments were cloned into the pGL4.21 vector following *Hind*III digestion. Due to the use of the *Hind*III site in the pGL4.21 vector, the -391 to +247 fragment was only subcloned in the reverse orientation, and despite several attempts, no colonies were obtained with this fragment in the forward orientation. The -109 to +247 and the -53 to +247 fragment were generated using PCR and cloned into the pGL4.21 vector using *Xho*I and *Ssr*I digestions.

Quantitative PCR was used to assess SelP mRNA expression. 293-EcR and EcR-GR cells were treated with 10 μM ponasterone A 24 h prior to mRNA collection, and 10 nM dexamethasone was then added at 8 or 16 h prior to mRNA purification. Vehicle treatments with ethanol (EtOH) or dimethyl sulfoxide (DMSO) were used as controls. The Qiagen RNeasy Mini Kit was used to collect and purify mRNA from cells. First strand cDNA was synthesized using Superscript III reverse transcriptase and these cDNA samples were run in triplicate as 1:5 dilutions. Standards were run in duplicate at concentrations between 10^3 and 10^8 copies/ μl and β_2 microglobulin was run as a reference gene. The SEPP1 amplicon consisted of the 100 bp spanning the final intron of the genomic sequence. The primer pair 5'-TTCGGGCAG AGGAGAACA-3' and 5'-CTGGCACTGGCTTCT GTG-3' were used to amplify this region. Average threshold copy number was used to calculate changes

in expression level as compared to vehicle treated controls.

Site-directed mutagenesis

Putative response elements of interest were mutated using a PCR-based strategy. The putative GRE sequence CAAGAATGA**AC**ATTGAACT at position –87 of the SEPP1 promoter (GRE #1) was mutated to the sequence CAAGAATGA**CT**ATTGAACT using the primer 5'-GGTCACTGCAAGAATGA **CT**ATTGAACTTTGGACTATAC-3' and its complementary sequence (exchanged nucleotides are bold and underlined). The putative GRE sequence TCAG AG**TG**TGCT at position –24 of the SEPP1 promoter (GRE #2) was mutated to the sequence TCAGAG **GAT**GCT using the primer 5'-GGACTATAAATAT CAGAG **GAT**GCTGCTGTGGCTTTGTG-3' and its complementary sequence. These mutations should eliminate activity of potential GRE half sites (Nordeen et al. 1990). The putative retinoid responsive element sequence ACAT**TGA**ACTTTGG at position –73 of the SEPP1 promoter (RRE) was mutated to the sequence ACAT**CTT**ACTTTGG using the primer 5'-CTGCAAGAATGAACAT**CTT**ACTTTGGACTATACCTGAGG-3' and its complementary sequence. The FoxO1a binding sequence GTAA**ACA**A at position –46 of the SEPP1 promoter was mutated to the sequence GTAA**ATC**A using the primer 5'-CC TGAGGGGTGAGGTAA**ATC**ACAGGACTATAAA TATCAGAG-3' and its complementary sequence.

Luciferase reporter assay

Reporter assays were quantified using a Dual Luciferase reporter assay. SEPP1 promoter constructs cloned into pGL4.21 or a mouse mammary tumor virus promoter reporter construct (pLTRluc) were co-transfected with the pRL-RSV plasmid that serves as an internal control for transfection efficiency. Cells were seeded into six-well plates at a concentration of 5×10^5 cells/well. Each well was cotransfected with $\sim 1 \mu\text{g}$ of firefly reporter plasmid along with 50 ng of the pRL-RSV vector. Twenty-four hours after transfection, medium was replaced. Cells transfected with SEPP1 promoter constructs were treated with either 10 μM of the ecdysone analog ponasterone A, 10 nM dexamethasone, or a combination of both for an additional 24 h. Vehicle treatment with EtOH and/or

DMSO served as negative controls. Cells transfected with pLTRluc were treated with either DMSO or 10 nM dexamethasone for 24 h. Following treatments, cells were collected in 200 μl of Passive Lysis Buffer and stored at -80°C at least overnight to allow for cell membrane disruption. Cell lysates were diluted in Passive Lysis Buffer and each sample was quantified in triplicate on Perkin-Elmer Victor³ V plate reader. The sequential addition of Luciferase Assay Reagent II and Stop & Glo reagent allowed for the measurement of firefly and *Renilla* luciferase activity, respectively.

Immunoblotting

EcR-GR cells were supplemented with 1 μM sodium selenite and treated with EtOH as a vehicle control, 10 μM ponasterone A, 10 nM dexamethasone, or a combination of both for 24 h. Selp was partially purified from the culture medium of these cells using Ni-NTA agarose. Culture medium was mixed with the Ni-NTA agarose and the mixture was incubated on a nutating mixer at 4°C overnight. The Ni-NTA beads, along with any bound proteins, were collected by centrifugation, washed twice with 500 μl cold PBS, and then mixed with loading buffer and separated by NuPAGE 4–12% Bis-Tris gels. Proteins were transferred to a polyvinyl difluoride membrane. Membranes were blocked with 5% nonfat dry milk in TBS-T and then probed for Selp (antibody specific for Selp was a gift from Drs Kris Hill and Raymond Burk, Vanderbilt University). A peroxidase conjugated secondary antibody was used to detect chemiluminescence indicative of protein expression.

Electrophoretic mobility shift assay

Nuclear fractions were collected from 293-EcR and EcR-GR using NE-PER nuclear extraction reagents. Gel shift assays were run using the Lightshift chemiluminescent electrophoretic mobility shift assay kit. Double-stranded 5'-biotinylated oligonucleotides (5'-GGTCACTGCAAGAATGAACATTGAACTTTGGACTATAC-3') corresponding to the wild-type sequence of GRE #1 was used as a probe. Following end-labeling with biotinylated UTP, complementary oligonucleotides in equimolar amounts were heated to 95°C for 1 min, cooled to 65°C , and then stored at -20°C . Binding reactions were

performed at a 20 μ l volume containing 20 fmol labeled probe, 5 μ g nuclear proteins, 10 mM Tris, pH 7.5, 50 mM KCl, 1 mM dithiothreitol, 5 mM MgCl₂, 2.5% glycerol, 0.05% NP-40, 1 μ g herring sperm DNA, and 1 μ g bovine serum albumin. Where indicated, 4 pmol of unlabeled competitor probe was added to reactions. For supershift experiments, 1 μ g anti-glucocorticoid receptor antibody (BuGR2; Calbiochem) was added 10 min after addition of biotinylated probe and nuclear extract and incubated for an additional 20 min at room temperature. Reactions were then loaded onto an 8% TBE gel in 22.25% Tris, pH 8.4, 22.25% boric acid, 0.5 mM EDTA and electrophoresed at 22°C. DNA was transferred to a positively charged nylon membrane, UV cross-linked, probed using Lightshift chemiluminescent EMSA reagents, and detected on a Kodak Image Station 440CF.

Statistical analysis

GraphPad InStat, version 3.06, was used to evaluate the statistical significance of the results. Two-tailed student's *t*-tests were used to determine statistical significance when comparing two data sets. In cases where multiple data sets were compared, statistical significance was determined by one-way ANOVA with Tukey or Tukey–Kramer multiple comparison post hoc tests, and differences were considered significant for *P* < 0.05.

Results

Previous results using 293-EcR cells with ecdysone-inducible 15-LOX-1, when supplemented with an appropriate substrate, like arachidonate, show an inhibition of the selenoprotein thioredoxin reductase activity by ~50% (Yu et al. 2004). This raised the question of whether other selenoenzymes might demonstrate altered expression under similar conditions. Quantitative PCR experiments performed using 293-EcR cells with stable, ecdysone-inducible 15-LOX-1, as well as the control cell lines with inducible Δ Ille⁶⁶² 15-LOX-1, and β -galactosidase, demonstrated enhanced expression of SEPP1 following ponasterone A treatment (Fig. 1). Since SEPP1 demonstrated increased expression in all these cell lines, even without substrate for the 15-LOX-1, it is

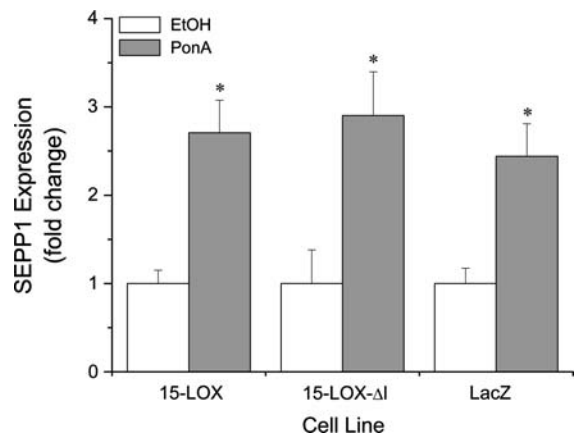


Fig. 1 Quantitative PCR analysis of SEPP1 expression in HEK-293 EcR, 15-LOX and control cell lines. Ecdysone inducible expression of 15-LOX, 15-LOX- Δ I (Δ Ille⁶⁶² 15-LOX-1), or β -galactosidase was achieved through a stable co-transfection of pVgEcR into HEK-293 cells. Cells were treated with EtOH (white) or 10 μ M PonA (grey) for 24 h prior to mRNA purification. SEPP1 expression was measured by quantitative PCR. The data are presented as the mean $\pm$ SE of relative gene expression changes observed over a minimum of three experiments and demonstrate differential expression as assessed by a two-tailed *t*-test (* *P* < 0.05)

likely that the changes in SEPP1 expression resulted from components of the ecdysone-inducible system rather than a response to 15-LOX-1 catalysis.

We examined the SEPP1 promoter, from –1,652 to +247, based on promoter ID #34663 in the Transcriptional Regulatory Element Database, to determine the region of the promoter responsible for this ecdysone-inducible transcription. Fragments of the promoter were tested using the luciferase reporter assay in the 293-EcR cells. Fragments included –1,652 to +247, –1,652 to –385, –391 to +247, –109 to +247, and –53 to +247 (Fig. 2a). The greatest level of transcriptional activation following treatment with ponasterone A was observed on the –109 to +247 fragment, suggesting that a site within this region of the promoter may bind a component of the ecdysone-inducible system and induce transcription of SEPP1 (Fig. 2b).

VgEcR is a synthetic transcription factor that is a fusion of the ligand-binding and dimerization domain of the *Drosophila* ecdysone receptor, the DNA-binding domain of the GR, and the transcriptional activation domain of herpes simplex virus VP16. This gene expression system is designed to activate transcription upon dimerization of VgEcR with the

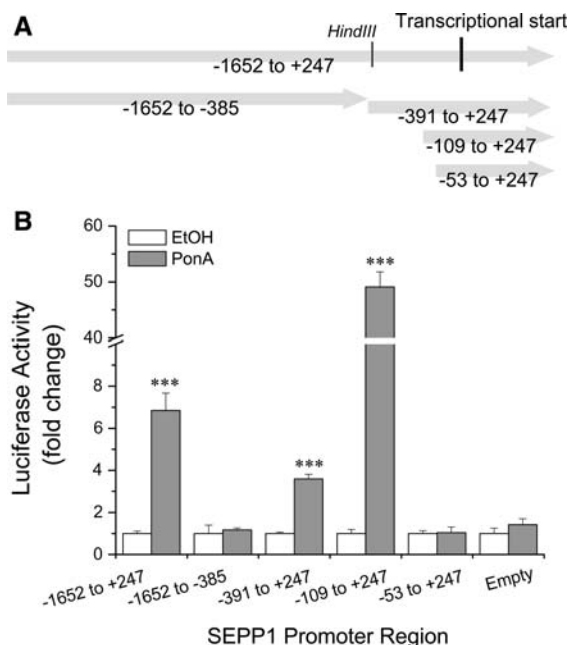


Fig. 2 *PonA* induction of SEPP1 luciferase reporter constructs. **a** Schematic of SEPP1 promoter fragments that were synthesized by PCR and cloned into the pGL4.21 vector. **b** 293-EcR were engineered through a stable transfection of pVgEcR into HEK-293 cells. 293-EcR cells were transfected with SEPP1 reporter constructs. Twenty-four hours after transfection, medium was replaced and cells were treated with EtOH (white) or 10 μ M *PonA* (grey) for an additional 24 h. Cells were lysed and relative firefly luciferase activity was measured using a Dual Luciferase reporter assay. Technical replicates were run in each experiment, and data are presented as in Fig. 1 but representing the relative activity changes observed over a minimum of three distinct biological experiments and demonstrate differential luciferase activity as assessed by a two-tailed *t*-test (***) $P < 0.001$

retinoid X receptor (RXR), and binding of the heterodimer transactivates a synthetic ecdysone-responsive element (Saez et al. 2000).

Many of the nuclear hormone receptors have similar DNA binding sites. The VgEcR-RXR binds the sequence AGTGCATTGTTCTC in the synthetic response element (the binding sites for RXR and the GR DNA binding domains are underlined), the GR binding sequence is TGT(T/C)CT(G/T/C) (Beato et al. 1989; Nordeen et al. 1990), and, for comparison, the endogenous ecdysone receptor binds the sequence (A/G)G(G/T)T(C/T)A (Vogtli et al. 1998; Panguluri et al. 2007). It is also worth noting that RXR and HNF-4 α can bind with similar affinity to direct repeats of (A/G)G(G/T)TCA with one base

spacing (Nakshatri and Chambon 1994; Nakshatri and Bhat-Nakshatri 1998). Due to the similarities in the response elements, it seemed prudent to evaluate cellular responses to both VgEcR and GR as well as to evaluate RXR DNA-binding sequences.

In order to evaluate the interplay between the VgEcR-RXR system and the GR on the SEPP1 promoter, the 293-EcR cell line was engineered to express a DsRed2-labeled, functional human GR. The pLTRluc reporter assay confirmed that the GR is activated by dexamethasone in these EcR-GR cells, with minimal activity in HEK-293 or 293-EcR cells (Fig. 3). To evaluate possible cross-talk between VgEcR-RXR and GR, ponasterone A was used to treat 293-EcR or EcR-GR cells, transiently transfected with pLTRluc, and only background reporter activity was seen (data not shown).

When the luciferase reporter assay was run in the EcR-GR cells to test activation of the SEPP1 promoter constructs, the GR exerted a repressive effect on this promoter (Fig. 4). Even in the absence of dexamethasone activation, ponasterone A-induced activity was attenuated in the EcR-GR cells, as compared to 293-EcR cells with no active GR. When the EcR-GR cells were treated with dexamethasone,

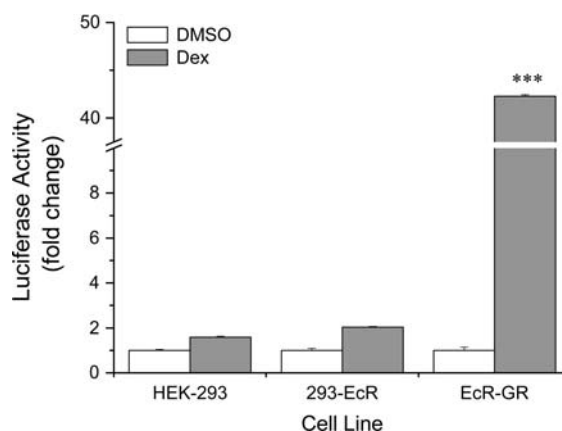


Fig. 3 Glucocorticoid receptor luciferase reporter. Stable transfection of the 293-EcR cells with the expression vector, pDsRed-hGR produced the EcR-GR cell line. HEK-293, 293-EcR, and EcR-GR cells were transfected with the mouse mammary tumor virus promoter reporter construct pLTRluc. Twenty-four hours after transfection, medium was replaced and cells were treated with DMSO (white) or 10 nM Dex (grey) for an additional 24 h. Cells were lysed and relative firefly luciferase activity was measured using a Dual Luciferase reporter assay. The data are presented as in previous figures and represent triplicate experiments (***) $P < 0.001$

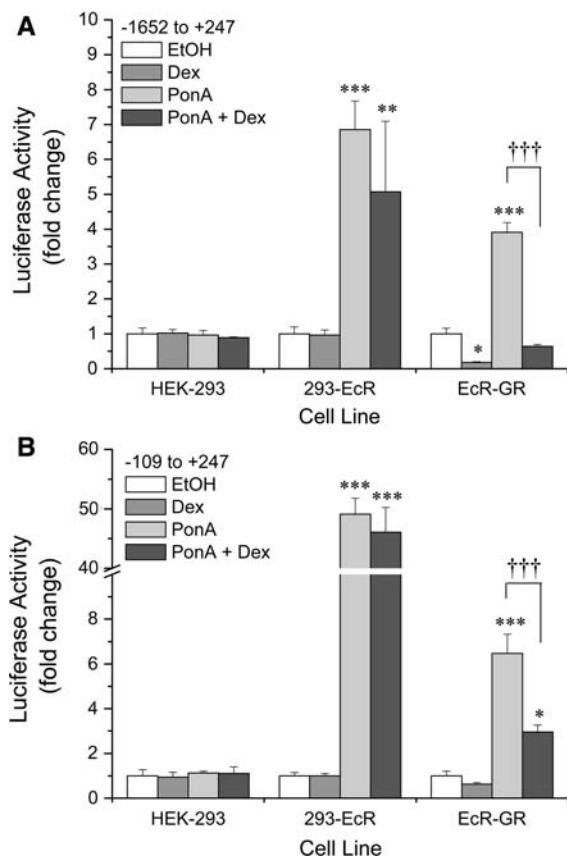


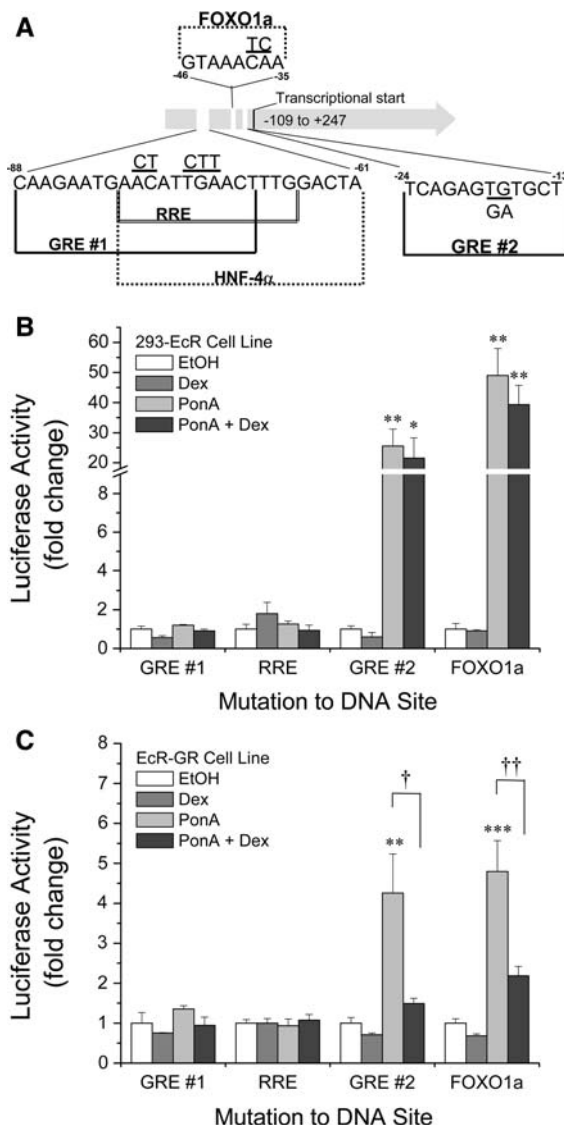
Fig. 4 Glucocorticoid responsiveness of SEPP1 luciferase reporter constructs. *HEK-293*, *293-EcR*, and *EcR-GR* cells were transfected with either (a) $-1,652$ to $+247$ SEPP1 luciferase reporter or (b) -109 to $+247$ SEPP1 luciferase reporter. Twenty-four hours after transfection, medium was replaced and cells were treated with EtOH (white), 10 nM Dex (grey), 10 μ M PonA (light grey), or a combination of 10 nM Dex and 10 μ M PonA (dark grey) for an additional 24 h. Cells were lysed and relative firefly luciferase activity was measured using a Dual Luciferase reporter assay. Triplicate samples were run in each experiment and data are presented as the mean $\pm$ SE of relative activity changes observed over at least three biological replicates. ANOVA of each cell line revealed no significant differences among the treatments in the 293 cells but highly significant, $P < 0.0001$, differences in the *EcR* and *EcR-GR* cells. Post hoc tests reveal differences from the vehicle control (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$) or differences among select treatment subsets (††† $P < 0.001$)

promoter activity was repressed by $\sim 82\%$ on the $-1,652$ to $+247$ fragment, as compared to vehicle control (Fig. 4a). Activation was repressed by $\sim 37\%$ on the -109 to $+247$ fragment under the same conditions (Fig. 4b). Simultaneous treatment of the *EcR-GR* cells with ponasterone A and dexamethasone caused attenuation of ponasterone A activity,

with an $\sim 84\%$ reduction in activation observed on the $-1,652$ to $+247$ fragment as compared ponasterone A only treatment (0.6 vs. 3.9 fold change). An $\sim 55\%$ reduction was observed on the -109 to $+247$ fragment under the same conditions (2.9 vs. 6.5 fold change). In comparison, dexamethasone treatment was unable to exert a significant influence on ponasterone A activation in the 293-*EcR* cells, with only an $\sim 26\%$ reduction in activity observed on the $-1,652$ to $+247$ fragment (5.1 vs. 6.8 fold change) and an $\sim 6\%$ reduction observed on the -109 to $+247$ fragment (46.1 vs. 49.1 fold change). Treatment with dexamethasone alone did not cause repression of promoter activity in the 293-*EcR* cells. Neither ponasterone A nor dexamethasone exerted a significant effect on the SEPP1 promoter constructs in *HEK-293* cells.

Based on the luciferase reporter assay results observed in the -109 to $+247$ region, we examined this region of the SEPP1 promoter for evidence of response elements that could potentially serve as binding sites for GR or VgEcR, as well as RXR. Two putative GREs were identified using the Transcription Element Search System (Schug and Overton 1997). These response elements are referred to as GRE #1 and GRE #2 and are found at position -87 and -24 of the SEPP1 promoter, respectively. The precise sequences suggest that these sites may not function as classical GREs but appeared to best define half-sites (Nordeen et al. 1990). In addition, a putative retinoid receptor binding site was identified at position -73 of the SEPP1 promoter, and is referred to as a putative RRE. GRE #1 and the RRE are sequential with one another and together could form a potential binding site for VgEcR-RXR. These sites also overlap with a previously characterized HNF-4 α binding site in the SEPP1 promoter (Speckmann et al. 2008; Walter et al. 2008).

In order to determine if these binding site(s) were responsible for the VgEcR-RXR and GR mediated effects, the luciferase reporter assay was repeated with SEPP1 reporter constructs in which the two putative GREs or the RRE were mutated (Fig. 5a). Despite the fact that GRE #2 was located within the -53 to $+247$ fragment that did not display any ponasterone A-induced luciferase activity in 293-*EcR* cells (Fig. 2b), a mutant form of this binding site was tested. This GRE more closely matched the consensus sequence, with an inverted repeat of the GR binding



site that could accommodate a GR homodimer, and therefore, could be involved in GR-mediated repression.

In both 293-EcR and EcR-GR cells, ponasterone A induced transactivation was completely lost upon mutation of GRE #1 or the RRE (Fig. 5b, c), with the firefly:renilla luciferase ratio being decreased by approximately ten-fold on the RRE mutant construct compared to the mutant GRE #1 reporter (data not shown). These results suggest that both of these response elements serve as binding sites for the VgEcR-RXR transcriptional activation system. Transactivation was still observed with the mutated GRE #2 (Fig. 5b, c) construct in both cell lines

Fig. 5 Site-directed mutagenesis of GRE's identified within the SEPP1 promoter. **a** Schematic of the two putative GREs and RRE identified within the -109 to +247 SEPP1 promoter fragment along with previously identified sites in the same region (*FOXO1a* and *HNF-4α*). These response elements were mutagenized, as indicated by the bases identified with a bar, using a PCR-based strategy. **b** 293-EcR, and **c** EcR-GR cells were transfected with, appropriate mutant, -109 to +247 SEPP1 reporter constructs. Twenty-four hours after transfection, medium was replaced and cells were treated with EtOH (white), 10 nM Dex (grey), 10 μM PonA (light grey), or a combination of 10 nM Dex and 10 μM PonA (dark grey) for an additional 24 h. Cells were lysed and relative firefly luciferase activity was measured using a Dual Luciferase reporter assay. Triplicate samples were run in each experiment and data are presented as the mean ± SE of relative activity changes observed over at least three biological replicates. ANOVA of each cell line revealed no significant differences when *GRE #1* or the RRE is mutated, indicating that this is the important site for transactivation in 293-EcR and EcR-GR cells, but significant, $P < 0.005$, differences in the 293-EcR and EcR-GR cells when evaluating a mutation of *GRE #2* or the *FOXO1a* binding site. Post hoc tests reveal differences from the vehicle control (EtOH) (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$) or differences among select treatment subsets († $P < 0.05$; †† $P < 0.01$)

following ponasterone A treatment; however, it was slightly reduced compared to the non-mutated form. This indicates that this element may also be involved in activation of SEPP1 through VgEcR-RXR, although to a much lesser extent than GRE #1 or the RRE. The addition of dexamethasone plus ponasterone A resulted in attenuation of ponasterone A activity on the mutated GRE #2 luciferase reporter in the EcR-GR cells but not with the mutated GRE #1 reporter, indicating GRE #2 is not involved in the GR-mediated repression. FOXO1a has previously been shown to regulate SEPP1 transcription in hepatic cells through a binding site at position -46 of the promoter (Speckmann et al. 2008; Walter et al. 2008). As this regulatory mechanism involved coordination of FOXO1a with the dexamethasone-responsive cofactor PGC-1α, we also evaluated SEPP1 transcription following mutation to the FOXO1a site. Neither a change in PonA-induced SEPP1 transactivation, nor repression by GR was observed in either 293-EcR or EcR-GR cells following mutation of the FOXO1a site (Fig. 5b, c).

Quantitative PCR results further qualified the induction of SEPP1 by VgEcR-RXR, and its repression by the GR (Fig. 6a). In 293-EcR, gene induction of approximately fivefold was observed following 24 h of ponasterone A treatment, and dexamethasone

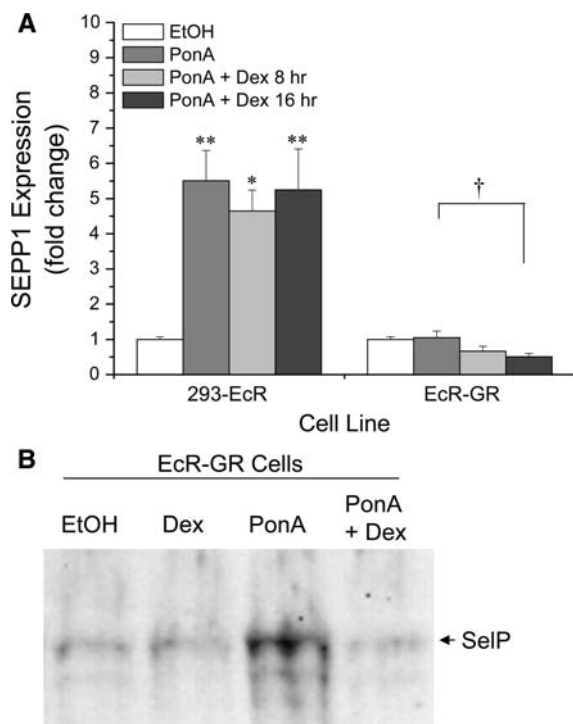


Fig. 6 Analysis of SEPP1 expression in 293-EcR and EcR-GR cells. **a** 293-EcR and EcR-GR cells were treated with EtOH or 10 μ M PonA 24 h prior to mRNA collection. Beginning 8 h after PonA was added, cells were treated with 10 nM Dex for 8 or 16 h prior to mRNA purification. SEPP1 expression was measured by quantitative PCR. Triplicate samples were run in each experiment and data are presented as the mean $\pm$ SE of relative activity changes observed over at least five biological replicates. ANOVA of each cell line revealed significant differences of SEPP1 expression in EcR and EcR-GR cells, $P < 0.05$. Post hoc tests reveal differences from the vehicle control (* $P < 0.05$; ** $P < 0.01$) or differences among select treatment subsets († $P < 0.05$). **b** SelP protein from Ni-NTA bead pull-downs from culture media demonstrate expression increases in EcR-GR cells following 24 h treatment with PonA but Dex treatment attenuated the SelP expression

treatment had no effect on this induction. Similar to the responses observed with the luciferase activity assays, the ability of ponasterone A to activate SEPP1 was attenuated in EcR-GR cells. SEPP1 expression was reduced by $\sim 80\%$ in these cells, even in the absence of dexamethasone treatment. Treatment with dexamethasone for 8 or 16 h eliminated the ability of ponasterone A to induce gene expression, and led to additional repression of SEPP1 in a time dependent manner. In addition, immunochemical analysis of SelP from a Ni-NTA bead pull-down of the media from EcR-GR cells demonstrated a similar pattern of protein expression (Fig. 6b).

To determine if the GR directly binds the GRE #1 site, we utilized electrophoretic mobility shift assays (Fig. 7). We observe a protein:DNA complex binding to the GRE #1 in both the 293-EcR and EcR-GR cells. There appears to be minimal modulation of the amount bound in the 293-EcR cells consistent with the expectation of binding by VgEcR-RXR with or without ligand present as is expected for the ecdysone-inducible system (Fig. 7, lanes 12–15); however, we observe dexamethasone-dependent inhibition of binding in the EcR-GR cells (Fig. 7, lanes 2–5, 9–11). The amount of protein:DNA complex observed appears to be consistent with the results from the heterologous reporter assays (Fig. 4). However, we were unable to demonstrate that the protein:DNA complex contains the GR as the addition of antibodies directed at the GR do not produce a supershift or substantially alter the relative levels of protein:DNA complex.

Discussion

The effects of supplemental selenium intake have been evaluated in multiple chronic and acute diseases, including cancer, cardiovascular disease, and inflammatory conditions such as sepsis, trauma, and burns (Clark et al. 1996; Mark et al. 2000; Nomura et al. 2000; Brown and Arthur 2001; Angstwurm and Gaertner 2006; Angstwurm et al. 2007). In many studies, selenium has demonstrated beneficial properties but the results of the Selenium and Vitamin E Trial (SELECT) do not support the utility of supplemental selenomethionine in prostate cancer prevention (Lippman et al. 2009). The mechanism by which selenium exerts its effects during disease conditions is not completely understood; however, it has been hypothesized to be due to the antioxidant activity of selenoproteins (Diwadkar-Navsariwala and Diamond 2004; Irons et al. 2006). These proteins contain selenium incorporated as the amino acid selenocysteine during translation of the protein (Tujebajeva et al. 2000; Small-Howard et al. 2006; Howard et al. 2007). Adequate selenium intake is important in maintaining proper translation and function of the selenoproteins (Bermano et al. 1996; Wingler and Brigelius-Flohe 1999). Therefore, maintenance of selenoprotein function may be the mechanism by which supplemental selenium intake exerts a

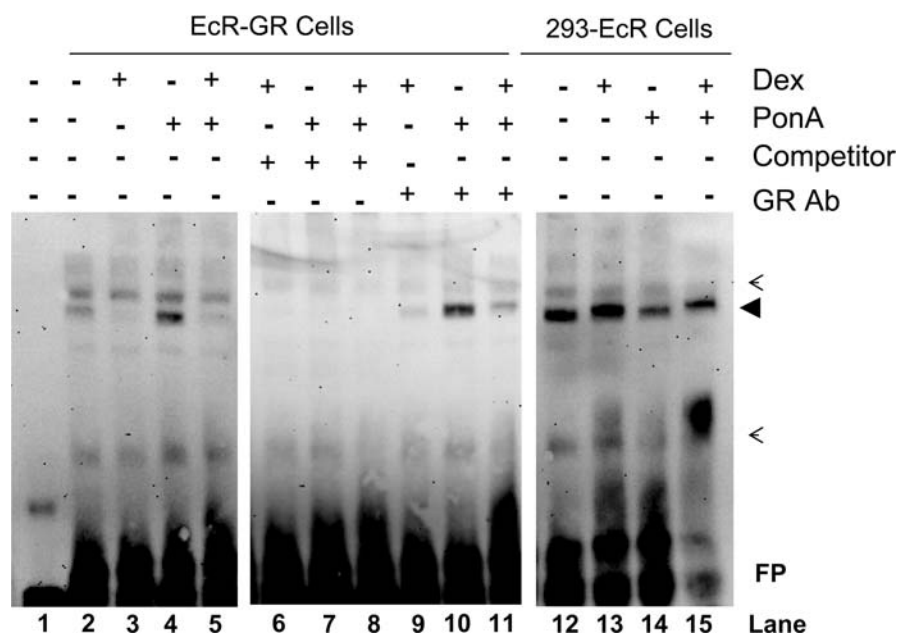


Fig. 7 Electrophoretic mobility shift assays with *GRE #1*. *Lane 1* is the labeled *GRE #1* fragment without an incubation with nuclear extract displaying the migration of the probe alone. *Lanes 2–5* are the *GRE #1* fragment with nuclear extract from *EcR-GR* cells that were treated with *EtOH*, *Dex*, *PonA*, or *PonA + Dex*, respectively. *Lanes 6–8* show the same samples (without the *EtOH* control) but excess unlabeled probe is included to identify bands that represent specific protein:DNA complexes. In *lanes 9–11* antibodies to *GR* are added to

determine if the protein:DNA complex contains *GR*; we do not observe a supershifted band. *Lanes 12–15* show the four conditions with nuclear extract from the *293-EcR* cells; all lanes display a strong protein:DNA complex. The specific complex is highlighted with the *large arrow*, the *small arrows* identify non-specific bands that are in all *lanes* with nuclear extract, and *FP* stands for the free probe at the *bottom* of the gels

beneficial health effect. In particular, the primary function of SelP is thought to be selenium distribution and the majority of the protein is synthesized in the liver for this purpose. However, most tissues can express SEPP1; suggesting alternative functions beyond selenium delivery may exist for SelP (Burk and Hill 2005) as well as the possibility of tissue selective modulation of SEPP1 expression.

While the majority of SelP is expressed in the liver of adult mammals, SEPP1 orthologs in fish and mammals demonstrate broad tissue expression. Zebrafish, who have an extensive selenoproteome, includes two SEPP1 isoforms encoded by distinct genes; one (*sepp1a*) with a selenocysteine-rich C-terminus containing 16 selenocysteine residues, and a second isoform (*sepp1b*) that lacks the selenocysteine-rich C-terminus (Kryukov and Gladyshev 2000). These genes demonstrate distinct spatiotemporal expression patterns throughout the development of the zebrafish with *sepp1a* displaying expression in multiple organs including the heart, brain and kidney,

but only limited hepatic expression, while *sepp1b* demonstrates strong hepatic expression (Thisse et al. 2003). In addition, a recent study of the expression of the murine ortholog of SEPP1 in mouse embryos also highlights a potential role of SelP in growth and developmental processes. Spatiotemporal expression of Sepp was observed in the central nervous system, limb buds, blood cells, lung, liver, intestine, testis, and developing epithelia, as well as in extraembryonic tissues, during organogenesis. The authors suggest that this increase in Sepp may provide antioxidant protection against the reactive oxygen species formed during embryogenesis, as well as provide a transplacental or intraembryonic selenium transport function (Lee et al. 2008). Additional evidence supporting a role for SelP in growth and development includes observations from the SelP knockout mouse, which displays a phenotype that includes growth retardation, neurological impairment, and male infertility (Hill et al. 2003; Schomburg et al. 2003; Renko et al. 2008). The regulatory

signals responsible for modulating SEPP1 expression for the purpose of growth and development are currently under investigation.

Recently, hepatic SEPP1 expression was shown to be controlled through coordination of the transcription factors FOXO1a and HNF-4 α by the coactivator PGC-1 α (Speckmann et al. 2008; Walter et al. 2008). Discovery of this mechanism introduces the idea that SEPP1 can be regulated in response to hormonal stimuli and may be responsive to various nuclear receptors due to the versatility of PGC-1 α .

Nuclear receptors are members of a large superfamily of proteins that function as ligand-inducible transcription factors (Germain et al. 2006; Teboul et al. 2008). This family contains steroid hormone receptors such as the glucocorticoid, estrogen, and androgen receptors, as well as receptors for thyroid hormones and retinoic acid. In addition, orphan nuclear receptors exist for which ligands have not been identified (Teboul et al. 2008). Examples of such orphan receptors include HNF-4 α and chicken ovalbumin upstream promoter-transcription factors (Benoit et al. 2006). These receptors regulate gene transcription by binding to hormone response elements in the promoter region of target genes. Most receptors bind as homo- or hetero-dimers to response elements composed of two core hexameric motifs. Consensus sequences for these motifs include AGA-ACA for steroid receptors and AG(G/T)TCA for the remaining nuclear receptors (Aranda and Pascual 2001). Multiple nuclear receptor types can bind these sequences and mediate transcriptional activity, allowing for differential control of overlapping gene networks (Bedo et al. 1989; Umesono et al. 1991). Nuclear receptors have a well established role in growth, development and homeostasis as has been reviewed (Flamant et al. 2006).

The decrease in serum selenium observed during critical illness is believed to result from redistribution of the micronutrient to high priority organs (Angsturm and Gaertner 2006). The selenium distribution (Hill et al. 2003; Renko et al. 2008) and negative acute phase functions (Dreher et al. 1997) of SelP support a potential role for this protein in selenium changes observed during critical illness. Recently, a newly developed immunoassay was used to show a decrease in SelP in the serum of septic patients (Hollenbach et al. 2008). The exact mechanism responsible for this decreased protein expression is

not known; however, the authors propose that it is due to proinflammatory cytokines that are induced as a result of the acute phase reaction occurring during sepsis, since several cytokines can repress SEPP1 expression (Dreher et al. 1997; Mostert et al. 2001). The evidence presented here also supports a potential role for the GR in regulating SEPP1 expression. Glucocorticoid responsiveness of SEPP1 could be of significance in critically ill patients, as these patients tend to have increased levels of free plasma cortisol levels (Hamrahian et al. 2004). Such regulation of SEPP1 by glucocorticoids could serve as an alternative explanation for the changes in SelP, and therefore the changes in serum selenium levels, observed during critical illness. However, a recent study demonstrates that the decrease in SelP in the acute-phase response appears to be a deficit in translation rather than a transcriptional response (Renko et al. 2009); therefore, the data herein may be more relevant for development or differentiation.

We have identified the VgEcR-RXR gene expression system as a tool for studying the expression of SEPP1. Our results indicate that once activated by ponasterone A, VgEcR-RXR is capable of inducing transcription of SEPP1 through a GRE located at position -87 or a RRE at position -73 of the promoter. In the EcR-GR cells, treatment with the GR agonist dexamethasone resulted in an attenuation of the ponasterone A-induced transcription of SEPP1 compared to ponasterone A treatment alone. This suggests that once activated by dexamethasone, the GR can travel to the nucleus and alter VgEcR-RXR binding at the site identified as GRE #1. While the EMSA failed to demonstrate GR binding by supershifting the protein:DNA complex, nuclear extract from the EcR-GR cells does display dexamethasone-dependent modulation of the protein:DNA complex that was consistent with the heterologous reporter expression assays. When a functional GR was stably integrated to make the EcR-GR cells, a generalized repression of SEPP1 was observed compared to the 293-EcR cells. This data supports the idea that the GR may indirectly regulate expression of this gene, and this effect was further validated by the evaluation of the protein levels of SelP expressed in the EcR-GR cells. An indirect mechanism of GR modulation of transcription has been described previously through the interaction with CCAAT/enhancer-binding proteins (Rudiger et al. 2002). These proteins are

involved in a broad spectrum of biological activities including development and differentiation (Ramji and Foka 2002). Whether a GR interaction with a CCAAT/enhancer-binding protein might be involved in SEPP1 regulation will require further study, and the precise cause for the repression observed in this study is unknown; however, transfection of GRs has previously been shown to be sufficient for the repression of hormone-responsive genes (Gougat et al. 2002).

The GR usually binds DNA as a homodimer; however, it has been demonstrated that monomers can bind to ‘half-sites’ and modulate transactivation when they are either close to the TATA element or can cooperate synergistically with other transcription factors (Strahle et al. 1988). The GRE #1 site we identified is 47 bp 5′ to the TATA element, and perhaps another cryptic GRE is present within this region that we have not yet identified. Another site for GR binding might explain the repression observed with dexamethasone treatment as well as the reduction of the protein:DNA complex observed in the EMSA if GR binding would modulate the occupancy of other regulators of SEPP1 expression.

The local region we identified as GRE #1 is within a region that has already demonstrated insulin-dependent attenuation of SEPP1 expression by modulation of HNF-4 α activity (Speckmann et al. 2008), and therefore, this could be a critical region that determines the expression levels of SEPP1 based on the affinity and availability of transcriptional regulators in different cell types. Other genes have HNF-4 α responsive elements that overlap with GR or RXR responsive elements and perhaps this allows for more intricate modulation of these genes in development (Crestani et al. 1998; Bailly et al. 2001). It is unlikely that the effects on transactivation we observe are related to interactions with HNF-4 α since this transcription factor is not expressed in HEK-293 cells (Lucas et al. 2005), and it is unclear how HNF-4 α -mediated SEPP1 regulation would account for alterations in serum selenium levels in critically ill patients since insulin sensitivity changes would allow for more Selp expression (Lazzeri et al. 2009).

In addition to the region we primarily focused on, the −109 to +247 fragment, it appears there are other dexamethasone-dependent repressive elements acting within the −1,652 to +247 fragment. Ponasterone A-induced activation is reduced on this fragment as

compared to the −109 to +247 fragment (Figs. 2, 4, 5). Plus, attenuation of the SEPP1 promoter was observed on the larger fragment in the EcR-GR cells following dexamethasone treatment, but was not observed on the smaller fragment (Fig. 4). *In silico* evaluation of this region identified additional potential GREs, but again, these sites are primarily half-sites and do not appear to be classical GREs. Furthermore, the region 5′ to −109 in the SEPP1 promoter appears to have additional repressive elements (Fig. 2). These elements are not well characterized and *in silico* evaluation did not reveal obvious potential repressive elements; however, one complex repeat region has demonstrated repression of SEPP1 expression with certain polymorphisms (Al-Taie et al. 2002). This region overlaps the 5′ end of the promoter reporter construct −391 to +247 we used in this study, and perhaps was responsible for the attenuated response we observed compared to the −109 to +247 promoter construct.

Finally, despite the fact that the VgEcR-RXR system is not expected to transactivate host genes by itself, changes in endogenous gene levels have been previously observed in mammalian cells treated with ecdysone receptor ligands (Oehme et al. 2006; Panguluri et al. 2007). In the experiments described here, activation of the transcriptional machinery was shown to be sufficient for changes in expression of at least one host gene, SEPP1. Due to the complex nature of selenoprotein translation (Tujebajeva et al. 2000; Small-Howard et al. 2006; Howard et al. 2007), many cell lines that are commonly used express selenoproteins poorly; however, HEK-293 cells have been successfully used in other studies for the expression of selenoenzymes (Madeja et al. 2005; Squires et al. 2007). Therefore, this 293-EcR system may function as a particularly effective system for the study of Selp transcription and translation process. While serving as a beneficial tool in the studies presented herein, the potential for this system to transactivate host genes may be considered as a possible limitation to the use of this inducible gene expression system in other studies.

In conclusion, we provide data supporting alternative mechanisms for extrahepatic regulatory mechanisms of SEPP1 expression that may help explain SEPP1 expression in inflammation, development and differentiation. We took advantage of an engineered, fusion transcription factor that contains the GR’s

DNA binding domain coupled with a strong transactivation domain, along with RXR, to identify the site responsible for the induction of SEPP1 expression. However, our studies revealed that the native GR inhibits the expression of SEPP1 through an indirect mechanism. Therefore, the ability of corticosteroids, and perhaps retinoids, to modulate SEPP1 expression may be a mechanism that could result in altered tissue selenium distribution since SeP is the major carrier of selenium.

Acknowledgments This work was supported by CA115616 (PJM) from the National Cancer Institute and P30 CA042014 to Huntsman Cancer Institute for support of core facilities. We thank Drs Hill and Burk for providing the anti-SeP antiserum. We also thank Drs Carol Lim, Michael Franklin, and the reviewers for their helpful suggestions on this article.

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