Published in final edited form as:

Mol Cell Endocrinol. 2012 April 4; 351(2): 306–316. doi:10.1016/j.mce.2012.01.006.

SMRT ϵ , a corepressor variant, interacts with a restricted subset of nuclear receptors, including the retinoic acid receptors α and β

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Abstract

The SMRT and NCoR corepressors bind to, and mediate transcriptional repression by, many nuclear receptors. Both SMRT and NCoR are expressed by alternative mRNA splicing, generating a series of structurally and functionally distinct corepressor “variants.” We report that a splice variant of SMRT, SMRT ϵ , recognizes a restricted subset of nuclear receptors. Unlike the other corepressor variants characterized, SMRT ϵ possesses only a single receptor interaction domain (RID) and exhibits an unusual specificity for a subset of nuclear receptors that includes the retinoic acid receptors (RARs). The ability of the single RID in SMRT ϵ to efficiently interact with RARs appears to be enhanced by a recently recognized β -strand/ β -strand interaction between corepressor and receptor. We suggest that alternative mRNA splicing of corepressors can restrict their function to specific nuclear receptor partnerships, and we propose that this may serve to customize the transcriptional repression properties of different cell types for different biological purposes.

Keywords

Corepressors; nuclear receptors; alternative-mRNA splicing; SMRT; retinoic acid receptors; β -strand

1. INTRODUCTION

Nuclear receptors are a large family of ligand regulated transcription factors that control key aspects of animal reproduction, development, and homeostasis (e.g. (Flamant et al., 2006, Glass and Saijo, 2011, Hager, 2001, McEwan, 2009, Sonoda et al., 2008)). Nuclear receptors bind to specific DNA sequences, bind to small, hydrophobic ligands, and regulate transcription of nearby target genes. Nuclear receptors arose early in the evolution of

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animals and appear to have gained their current diversity through cycles of gene duplication and divergence (Bertrand et al., 2004, Bertrand et al., 2007). Over 48 different nuclear receptors have been identified in the human genome (49 in the mouse genome), and include receptors for traditional endocrine and paracrine hormones, for intracellular metabolic intermediates, and for xenobiotics (Flamant et al., 2006, Glass and Saijo, 2011, Hager, 2001, McEwan, 2009, Sonoda et al., 2008). Additional “orphan” members of the nuclear receptor family either appear to bind ligand constitutively or may not bind any ligand (Benoit et al., 2006).

Many of the nuclear receptors are bimodal in their transcriptional regulatory properties, and they can either enhance or suppress expression of a given target gene by recruiting auxiliary proteins that serve as coactivators or as corepressors (Buranaprast and Chakravarti, 2009, Lonard and O'Malley B, 2007, Savkur et al., 2004, Tsai and Fondell, 2004). Coactivators place activating marks in the chromatin, help recruit components of the general transcriptional machinery, and/or alter nucleosome structure through ATP-dependent chromatin remodeling. Corepressors possess (or tether) reciprocal enzymatic activities that render chromatin a less effective substrate for transcription and/or interfere with the general transcriptional machinery.

SMRT and NCoR are genetic paralogs that mediate transcriptional repression by many members of the nuclear receptor family (Jones and Shi, 2003, Lazar, 2003, Moehren et al., 2004, Ordentlich et al., 2001, Perissi et al., Privalsky, 2004, Stanya and Kao, 2009). The SMRT and NCoR proteins serve as crucial nucleating molecules that recruit an array of enzymatic and architectural proteins into a larger corepressor assembly. These additional corepressor subunits include histone deacetylase 3 (HDAC3), TBL1, TBLR1, and GPS2, and they bind primarily to interaction surfaces within the N-terminal and central regions of NCoR and SMRT (Guenther and Lazar, 2003, Li et al., 2000, Nagy et al., 1997, Wen et al., 2000, Yoon et al., 2003, You et al., 2010). The corepressor holo-complex is then tethered to its nuclear receptor partners through receptor interaction domains (RIDs) that map primarily to the SMRT and NCoR C-terminal region (Cohen et al., 2001, Downes et al., 1996, Li et al., 1997, Muscat et al., 1998, Seol et al., 1996, Wong and Privalsky, 1998). Each RID possess an α -helical CoRNR box that can dock with a hydrophobic groove found on the surface of its nuclear receptor partners; the sequence of the CoRNR boxes and flanking sequences in each RID, therefore, determine whether the corepressor holocomplex can interact with, and mediate repression by, a given nuclear receptor or not (Burke et al., 1998, Hodgson et al., 2008, Hu et al., 2001, Makowski et al., 2003, Marimuthu et al., 2002, Perissi et al., 1999, Webb et al., 2000, Xu et al., 2002).

Many nuclear receptors are expressed from multiple, interrelated genetic loci and by alternative mRNA splicing to produce a series of protein variants or isoforms that differ in their biochemical properties *in vitro* and in their biological functions in cells (Flamant et al., 2006, Glass and Saijo, Hager, 2001, McEwan, 2009, Sonoda et al., 2008). Similar diversification exists among the coregulator families that mediate the actions of these receptors. SMRT and NCoR, for example, are expressed from two distinct genes and share approximately 50% identity at the amino acid level (Horlein et al., 1995, Chen and Evans, 1995, Ordentlich et al., 1999, Sande and Privalsky, 1996, Seol et al., 1996). Alternative mRNA splicing at both corepressor loci generates additional diversity, yielding multiple SMRT and NCoR protein variants that differ in their amino acid sequence, in their preference for specific nuclear receptor partners, in their response to kinase regulatory pathways, and in their tissue expression patterns (Faist et al., 2009, Goodson et al., 2005a, Goodson et al., 2005b, Jonas et al., 2007, Malartre et al., 2004, Muscat et al., 1998, Short et al., 2005).

Most nuclear receptors bind to their target genes as protein dimers (although other modes are known, including monomers, oligomers, and tethering through other transcription factors). It has been generally accepted that each receptor in the dimer contacts a separate RID domain on a single SMRT or NCoR protein; by this model, two RID domains should be required for efficient recruitment of the corepressor complex (e.g. (Cohen et al., 1998, Downes et al., 1996, Ghosh et al., 2002, Hu et al., 2001, Machado et al., 2009, Makowski et al., 2003, Zamir et al., 1997b)). Consistent with this, the majority of known SMRT and NCoR splice variants possess at least two RIDs. However, one recently recognized SMRT splice variant, denoted SMRT ϵ in our nomenclature, possesses only one RID (Faist et al., 2009, Goodson et al., 2005a, Short et al., 2005), and would therefore be predicted to be unable to bind to nuclear receptors by the prevailing interaction model. We report here that SMRT ϵ binds to a relatively limited subset of nuclear receptors in solution, including retinoic acid receptors (RARs), thyroid hormone receptors (TRs), Rev-Erba, and chicken ovalbumin upstream promoter transcription factor (COUP-TF) II. Notably, only RARs retain the strong SMRT ϵ interaction when these receptors are further allowed to assemble into dimers on their cognate DNA response elements. The interaction of SMRT ϵ with RARs appears to be enhanced by a recently elucidated β -strand/ β -strand contact surface that works in addition to the previously identified α -helical/groove contacts (le Maire et al., 2010, Phelan et al., 2010). We propose that SMRT ϵ is a specialized corepressor splice variant customized during evolution to function with a limited subset of nuclear receptors, and that alternative mRNA splicing in different tissues creates cell-specific, receptor-specific repression programs.

2. MATERIALS AND METHODS

2.1. DNA Constructs

The C-terminal amino acids of SMRT ϵ (amino acids 1851–2393) were subcloned in frame to the GST gene in pGEX in order to express GST-SMRT ϵ as a fusion protein. The GST-SMRT ϵ β -strand mutation, V2141P, CoNR Box mutation of amino acids 2092–2096 from IXXII to IXXAA, the RAR α -I396E mutation, and the TR α 1-L386E mutation were created using the Quick Change site-directed mutagenesis protocol from Stratagene (La Jolla, CA).

2.2. Reverse Transcriptase-Polymerase Chain Reaction (rtPCR)

RT-PCR was performed using primer pairs spanning the alternatively-spliced exon 44. This resulted in three distinctly sized DNA products, with the relative abundance of each reflecting the relative abundance of the corresponding alternatively spliced exon (44-, 44b-, and 44b+) in the mRNA population. The bands were resolved by agarose gel electrophoresis, stained by ethidium bromide, and quantified by image analysis (Mengeling et al., 2011). The relative ratios of the different PCR products, being internally controlled, was reproducible over a range of input dilutions and for different primer pairs.

2.3. Protein-Protein Interaction Assays

Nuclear receptors were transcribed and translated *in vitro* in the presence of 35 S-methionine using either the TNT T7 Quick Coupled Transcription/Translation System or the TNT SP6 High-Yield Protein Expression System following the manufacturer's protocols (Promega, Madison, WI). Glutathione-S-Transferase-corepressor fusion proteins (GST-CoR) encompassing the C-terminal receptor interaction domains of the different corepressor variants were expressed from baculovirus in Sf9 cells for 72 h at 28 °C, and cell lysates were isolated as previously described (Mengeling et al., 2011). Alternatively, for analysis of the β -strand/ β -strand interactions, GST-CoR fusions were expressed in BL-21 *E. coli* overnight at 16 °C after induction with 1 mM IPTG at an A600 value of 0.8–1.0 and cell lysates were made as described (Goodson et al., 2007). GST pulldown assays were adapted to a

microplate format (Goodson et al., 2007). Briefly, radiolabeled NR (typically 1–3 μ l of TNT reaction product per assay) was incubated at 4 °C with equal mass amounts of immobilized GST-CoR in 100 μ l of Binding Buffer A (Goodson et al., 2007). The binding reactions were carried out in 96-well multiscreen filter plates (Millipore, Bedford, MA). After a 45 min incubation with end-over-end rotation at 4 °C, the filter wells were washed 3 times with 200 μ l of ice-cold Buffer A, and bound radiolabeled proteins were then eluted with 50 μ l of 20 mM glutathione in 100 mM Tris-HCl, pH 8 at 4 °C for 45 min with end-over-end rotation. The eluted proteins were resolved by SDS-PAGE and were visualized and quantified using a PhosphorImager/STORM system (GE Healthcare) and the GraphPad Prism 5 statistical/plotting package (La Jolla, CA).

2.4. Repression Assays

CV-1 cells were grown in Dulbecco's modified Eagle medium (DMEM) formulated with high glucose and L-glutamine (Invitrogen, Carlsbad, CA), and supplemented with 5% heat-inactivated FBS (PAA, Dartmouth, MA). Cells were buffered with a bicarbonate/5% CO₂ system and maintained at 37 °C. Transfections were performed in DMEM, 5% FBS (hormone-stripped) using 24-well culture plates and Effectene (QIAGEN, Valencia, CA) per the manufacturer's instructions. Each transfection employed 3×10^4 cells, plated the day prior to transfection; 30 ng of pGL4-CMV-2X-17mer luciferase reporter containing 2 iterations of the Gal4 DNA binding sequence; 10 ng of pSG5-GBD, pSG5GBD-RAR α (LBD), pSG5GBD-LXR α (LBD), pSG5GBD-TR β 1(LBD), or pSG5GBD-Rev-Erb α (LBD) wherein the "ligand-binding" E/F domain of each nuclear receptor is fused in frame to the DNA binding domain of Gal4; 200 ng of pSG5HA-SMRT ϵ , pSG5HA-NCoR ω or empty pSG5HA vector; and 10 ng of pCMV-LacZ as an internal transfection efficiency control. A pSG5HA-SMRT ϵ bearing mutations in either the α -helical CoNR box or the adjacent β -strand region was used instead of the wild-type construct in several experiments. The medium was replaced 24h later with fresh DMEM, 5% hormone-stripped FBS. After a further 24 h, the cells were washed and lysed. Luciferase activity was measured using the Luciferase Assay System (Promega, Madison, WI) with a Turner Design 20/20 luminometer. β -Galactosidase activity was measured using a chlorophenol-red- β -D-galactopyranoside (CPRG) substrate (Roche, Indianapolis, IN) and a Molecular Devices SpectraMax 250 microplate reader.

2.5. Electrophoretic Mobility Supershift Assays

Oligonucleotide probes were for RARs and COUP TF II: consensus DR5 (top strand: 5'-agctA AAGGT CAgA AAGGT CAgca gga-3' bottom strand: 5'-tcgat cctgc TGACC TTTAT CTGAC CTTT-3'); for TRs: cLys F2 (top strand: 5'-tcgac ttatTGACCC cagctgAGGTC Aagttag-3' bottom strand 5'-gaataA CTGGGgt cgacTCCA GTcaatcagct-3'); for RevErb α : RevDR2 (Zamir et al., 1997a) (top strand: 5'-agctc caact AGGTC ACTAG GTCAa aggga -3' bottom strand: 5'-tcgat ccctt TGACC TAGTG ACCTa gttgg-3')(IDT, Coralville, IA). They were annealed and radiolabeled by Klenow polymerase fill-in using ³²P- α -dGTP (3000 Ci/mmol, Perkin-Elmer, Wellesley, MA) plus the three remaining unlabeled dNTPs. RARs and Rev-Erb α were expressed using the TNT SP6 High-Yield Protein Expression System following the manufacturer's instructions (Promega, Madison, WI). Three micrograms of plasmid were used per translation. hTR α 1 and TR β 1 were expressed from recombinant baculovirus in Sf9 cells, and nuclear extracts were made as previously described (Mengeling et al., 2005, Phan et al. 2010). Electrophoretic mobility shift assays (EMSAs) contained the nuclear receptor of interest, 1 pmol of radiolabeled probe, and the specified amount of GST-corepressor in 20 μ l binding buffer per reaction (Mengeling et al., 2011). Protein/DNA complexes were resolved by native electrophoresis at 200 V for 90 min. through a 4% polyacrylamide gel polymerized over an 8% polyacrylamide gel cushion, both buffered in 0.5X TBE (45 mM Tris-borate, 1 mM EDTA).

Gels were visualized by Storm phosphorimager analysis (GE Healthcare Bio-Sciences Corp., Piscataway, NJ).

3. RESULTS

3.1. SMRT ϵ is expressed in a variety of tissues by alternative splicing

SMRT ϵ is generated by utilization of an mRNA splice donor that excises exon 44 and produces an in-frame deletion of 228 nucleotides, representing 76 codons (Fig. 1A). This results in the complete loss of the C-terminal-most RID (denoted RID1 in Fig. 1A), a domain otherwise found in the much more widely studied SMRT α and SMRT τ splice forms (Goodson et al., 2005a, Short et al., 2005, Malartre et al., 2004). No equivalent splice form has been detected in the NCoR paralog ((Goodson et al., 2005a, Malartre et al., 2004) and MLG data not shown).

We examined the abundance of the SMRT ϵ splice variant in a variety of tissues and cell lines by utilizing rtPCR and oligonucleotide primers that span the splice site (Fig. 1B). We found that the 44- splice form is expressed in all mouse tissues examined, and represents 5 to 38% of the total SMRT mRNA. The highest SMRT ϵ mRNA levels were observed in lung, spleen, skeletal muscle, and in white and brown adipose tissue. A similar range of abundance was observed in a variety of established human cell lines, with the exception of the human acute promyelocytic cell line NB4, where the 44- splice form represented a narrow majority of the total SMRT mRNA. These results indicate that SMRT ϵ represents a significant percent of the total SMRT corepressor present in a wide variety of cell types.

3.2. SMRT ϵ interacts with a distinct subset of nuclear receptors

We next surveyed the ability of SMRT ϵ to bind to a variety of nuclear receptors by a GST pulldown protocol. GST-SMRT ϵ fusion constructs were synthesized in a recombinant baculovirus system, purified by binding to glutathione-agarose, and then tested for the ability to bind to a series of radiolabeled nuclear receptors. Equivalent GST-SMRT α and GST-NCoR ω fusions, representing the corepressor variant prototypes first published and/or most widely studied (Horlein et al., 1995, Chen and Evans, 1995, Ordentlich et al., 1999), were tested in parallel. Background binding to a GST-only negative control was uniformly very low for all nuclear receptors tested (Fig. 2A, with additional statistical analysis in Supplemental Table 1). The GST-SMRT α construct displayed a relatively broad specificity, binding strongly to RAR α , RAR β , TR α 1, TR β 1, LXR α , and Rev-Erb α , moderately to PPAR α , PPAR γ , and PPAR δ , and weakly to RAR γ and COUP-TFII (Fig. 2B). The GST-NCoR ω construct displayed a specificity that largely paralleled the SMRT α specificity (Fig. 2C). The SMRT ϵ construct, however, recognized a clearly distinct set of these receptors, retaining the ability of SMRT α to bind strongly to RAR α , RAR β , TR α 1, TR β 1, and Rev-Erb α , but losing the ability to bind to LXR α , PPAR α , PPAR β/δ , and PPAR γ , while gaining an enhanced ability to bind COUP-TFII and (to a much lesser extent) RAR γ and VDR (Fig. 2D). All three corepressor variants interacted only weakly with Dax-1 and GR (Fig. 2A–D).

We conclude that SMRT ϵ recognizes a distinct subset of nuclear receptors compared to SMRT α or NCoR ω . Reciprocally, certain nuclear receptors tested appeared to be relatively specific for a given corepressor variant in the GST-pulldown assay, whereas others were broader in their binding abilities. We therefore expanded this comparison for RAR α , RAR β , RAR γ , and LXR α to additional corepressor splice variants. Notably RAR α and RAR β had a broad specificity and interacted strongly with all the corepressor variants tested, RAR γ reacted weakly with all, whereas LXR α displayed a more selective specificity, interacting most strongly with SMRT τ and NCoR δ , more weakly with SMRT α , SMRT γ , NCoR ω , and (as noted previously) not detectably with SMRT ϵ (Fig. 3).

3.3. A β -strand motif in SMRT ϵ helps define acceptable nuclear receptor partnerships

The nuclear receptors that bind to SMRT ϵ in our GST-pulldown assay must do so by interacting with RID2 (all other known RIDs are absent from this construct). RID2 contains a well-characterized α -helical “CoRNR box motif” that can interact with a matching hydrophobic groove present on the surface of many nuclear receptors (Fig. 4). Discovered only recently, however, is a β -strand region present in RID2 that can interact with a complementary β -strand motif in the RARs and Rev-Erbs (Fig. 4A); this anti-parallel β -strand/ β -strand interaction helps stabilize corepressor recruitment by these two receptors ((le Maire et al., Phelan et al.). These prior studies did not perform a broad survey of corepressor variants/receptors. Therefore, we examined if the same phenomenon contributed to the interaction of the SMRT ϵ variant with several of the receptors tested here. Disrupting either the β -strand motif of the corepressor, or the β -strand motif of RAR α by use of appropriate amino acid substitutions severely inhibited the interaction of SMRT ϵ with RAR α (Fig. 4B). Similarly, disruption of the α -helical CoRNR box motif in SMRT ϵ also destabilized RAR α binding (Fig. 4B). These results are consistent with the proposal that both the β -strand/ β -strand and α -helical/hydrophobic groove interactions are required for high affinity RAR α binding by the SMRT ϵ RID2.

Unexpectedly, the ability of SMRT ϵ to bind to TR α was also decreased by mutagenesis of the β -strand motif in SMRT ϵ , although to a lesser extent than seen for RAR α (Fig. 4B). The same was true of a mutation in the region of TR α 1 that corresponds to the β -strand region of RAR α (Fig. 4B). In contrast, disruption of the α -helical CoRNR box motif in the RID2 of the SMRT ϵ construct completely abrogated TR α binding (Fig. 4B). These experiments suggest that nuclear receptors, such as TR α 1, previously thought to dock to SMRT exclusively through α -helical/hydrophobic groove contacts, are likely to make additional, β -strand/ β -strand contacts that further stabilize their binding to RID2.

3.4. Binding to DNA narrows further the nuclear partner specificity of SMRT ϵ

DNA binding restricts the ability of many nuclear receptors to recruit SMRT and NCoR, at least in part due to differences in the corepressor contacts made with the receptor dimers that form on DNA versus receptor monomers in solution. PPAR γ , for example, binds NCoR ω in solution, but not when bound to DNA in the form of a receptor dimer (Zamir et al., 1997b). TRs interact with SMRT α and NCoR ω both in solution and when bound to DNA as TR homodimers, but not when bound to the same DNA sequence as TR/RXR heterodimers (Cohen et al., 1998, Cohen et al., 2000, Machado et al., 2009, Yoh and Privalsky, 2001). We therefore used an electrophoretic mobility shift/supershift assay (EMSA) to test whether the nuclear receptors that bound SMRT ϵ in our prior GST-pulldown protocol retained that ability when bound to DNA. We previously have shown that RXR heterodimer formation strongly interferes with corepressor recruitment (Mengeling et al., 2011, Yoh et al., 2001), so we focused on receptor homodimers in these assays.

RAR α readily bound to a cognate DR5 DNA response element (a direct-repeat of AGGTCA with a 5 base spacer) as a receptor homodimer (Fig. 5); the identity of these complexes has been extensively characterized (Phan et al. 2010). These RAR α homodimer/DNA complexes were efficiently bound (supershifted) by all three corepressors tested: SMRT ϵ , SMRT α , and NCoR ω (Fig. 5). RAR β homodimers, assembled on the same DR5 element, were also efficiently supershifted by all three corepressors tested, whereas RAR γ homodimers on the DR5 element were supershifted by these corepressors very weakly or not at all (Fig. 5). RARs also bind to DR2 DNA response elements (Claessens and Gewirth, 2004), and the RAR homodimers that formed on a DR2 element were supershifted by SMRT ϵ to a similar extent as were the RAR homodimers that formed on a DR5 element (quantified in Supplemental Fig. 2).

Although TR α 1, TR β 1, Rev-Er α and COUP-TFII also formed receptor homodimers on their cognate DNA response elements, these receptor/DNA complexes were not significantly supershifted by SMRT ϵ . Nonetheless, TR α 1, TR β 1 were both strongly supershifted by SMRT α and by NCoR ω , and consistent with prior studies, Rev-Er α was supershifted by NCoR ω , but not by SMRT α (Fig. 6A). No corepressor binding was detected for COUP-TFII by EMSA supershift (Fig. 6B).

We quantified the results of multiple RAR and TR EMSA supershifts (Fig. 7). RAR α bound most efficiently to SMRT ϵ , and SMRT α , and more weakly to NCoR ω by this method (Fig. 7A–C). RAR β bound slightly less efficiently to SMRT ϵ than did RAR α (most observable at lower SMRT ϵ concentrations) but was near equal to RAR α in binding to SMRT α and NCoR ω (Fig. 7A–C). RAR γ bound weakly to SMRT ϵ and SMRT α , but not to NCoR ω (Fig. 7A–C). TR α 1 and TR β 1 displayed virtually no ability to bind SMRT ϵ , but bound to both SMRT α and NCoR ω , with the latter significantly stronger than the former (Fig. 7D–F); the overall affinities of TR α 1 and TR β 1 for NCoR ω and SMRT α were also somewhat lower than observed for RAR α and RAR β . We conclude that SMRT ϵ displays high specificity for RAR α and, to a slightly lesser extent, RAR β , when assayed in the context of a cognate DNA response element, and binds poorly or not at all to the other nuclear receptors tested under the same conditions.

3.5. SMRT ϵ can help enhance repression by RAR α in cells

We next examined if the ability of SMRT ϵ to bind RAR α *in vitro* was reflected in its ability to participate in RAR α -mediated transcriptional repression *ex vivo*. We introduced a Gal17-mer-luciferase reporter into CV-1 cells together with a Gal4-DNA binding domain GBD-RAR α construct, a GBD-LXR α construct, or a GBD-only construct. In the absence of ligand, both the GBD-RAR α and the GBD-LXR α constructs repressed reporter gene expression compared to the empty GBD (Fig. 8A). Coexpression of either SMRT ϵ or NCoR ω in these cells resulted in more severe reporter gene repression by the GBD-RAR α construct. In contrast, only NCoR ω , and not SMRT ϵ , enhanced repression by the GBD-LXR α construct. Consistent with our *in vitro* binding experiments, these experiments suggest that SMRT ϵ can partner with RAR α , but not LXR α , to mediate transcriptional repression in cells, whereas NCoR ω can partner with a broader range of nuclear receptors, including both LXR α and RAR α .

We expanded this assay to include GBD-TR β 1 and GBD-RevEr α constructs, representing nuclear receptors that interacted with SMRT ϵ in our GST-pulldowns but not in the EMSA supershift protocol. The GBD-TR β 1 fusion repressed reporter gene expression in the absence of an ectopic corepressor construct, and this repression was more severe in the presence of either SMRT ϵ or NCoR ω . Repression by the GBD-RevEr α construct was also enhanced by expression of NCoR ω and possibly by SMRT ϵ (although the latter was not statistically significant). We note that our reporter assay utilized GAL4 binding sites whereas our EMSA-supershifts employed native receptors and consensus response elements; taken together with our GST-pulldown data, these results indicate that the ability of the different corepressor splice forms to physically and functionally interact with specific nuclear receptors can depend on context.

Finally we examined if the β -strand/ β -strand interaction necessary for efficient interaction of the RARs and TRs with SMRT ϵ *in vitro* was also required for SMRT ϵ function in these reporter gene assays. Notably, amino acid substitutions designed to disrupt either the β -strand or the CoNR box α -helical regions in SMRT ϵ impaired the ability of this corepressor variant to enhance repression by either GBD-RAR α or GBD-TR β 1 in our transfection assay (Figure 8B). Our results are consistent with both of these motifs

contributing to the ability of SMRT ϵ to partner functionally, as well as physically, with these two nuclear receptors.

4. DISCUSSION

4.1. Alternative mRNA splicing creates corepressor variants selective for specific nuclear receptors

We report here a characterization of SMRT ϵ , an unusual, alternatively-spliced form of the SMRT corepressor that possesses only a single RID domain (RID2) within its C-terminal region (Short et al., 2005). The alternative splice event that generates SMRT ϵ is conserved in both humans and mice and is observed in many mammalian tissues and established cell lines ((Faist et al., 2009, Goodson et al., 2005a, Malartre et al., 2004, Short et al., 2005) and MLG unpublished observations). Interestingly, although SMRT itself is found throughout the vertebrate lineage, the SMRT ϵ splice product can not be detected in *Xenopus* or *Danio* by RT-PCR or by EST database inspection, indicating that SMRT ϵ is a relatively recent evolutionary development ((Faist et al., 2009, Goodson et al., 2005a, Malartre et al., 2004, Short et al., 2005) and MLG unpublished observations). Consistent with this interpretation, although alternative mRNA splicing at the NCoR locus often parallels that at the SMRT locus, an ϵ -like form of NCoR has not been identified. Instead all known NCoR splice variants encode both RID1 and RID2. Taken as a whole, these observations suggest that the alternative splicing event that generates SMRT ϵ arose after the evolutionary duplication and divergence that produced the distinct SMRT and NCoR loci, and after the divergence of mammals from teleost and amphibian lineages.

In an immobilized protein-protein interaction assay, SMRT ϵ interacts strongly with RAR α , RAR β , TR α 1, TR β 1, Rev-Erba, and COUP-TFII, but binds weakly, or not at all, to RAR γ , LXR α , Dax1, GR, PPAR α , PPAR γ , and PPAR δ . In contrast, the SMRT α splice variant, which encodes both RID2 and RID1, also binds PPAR α , PPAR γ , PPAR δ , and LXR α , but fails to bind COUP-TFII. NCoR ω , which encodes all three RIDs, largely parallels SMRT α in the same assay. It is interesting to note that the insertion of a RID through alternative splicing can either increase or decrease the interaction of corepressor with a given nuclear receptor; for example, the presence of RID3 in SMRT α increases its binding to LXR α , yet decreases its binding to COUP-TFII. It is possible that the presence of a “wrong” RID in a corepressor splice variant can interfere allosterically, or otherwise, with the ability of the “correct” RID to interact productively with a given nuclear receptor partner.

DNA binding by nuclear receptors further restricts their ability to recruit corepressors. SMRT ϵ , when tested on receptor homodimers assembled on their cognate DNA response elements, became specific for RAR α and (to a lesser extent) RAR β , and lost the ability to interact efficiently with any of the other nuclear receptors tested. Unlike SMRT ϵ , the SMRT α and NCoR ω variants remained able to recognize a broad spectrum of DNA-bound nuclear receptors, with RAR α , RAR β , TR α 1, and TR β 1 recognized by both SMRT α and NCoR ω , with RAR γ recognized by SMRT α , and with Rev-Erba recognized by NCoR ω . These results are consistent with a prior EMSA supershift study that demonstrated the SMRT ϵ variant can bind to RAR α , but not to PPAR γ (Faist et al., 2009), although our results and those of the previous study differ in a number of details relating to the relative ability of different receptors to interact with different SMRT variants. Notably the prior study focused on RAR/RXR heterodimers, which bind to corepressors much more weakly than do the RAR (and TR) homodimers analyzed in our own experiments (Cohen et al., 1998, Cohen et al., 2000, Ghosh et al., 2002, Mengeling et al., 2011, Yoh and Privalsky, 2001, Zubkova and Subauste, 2004). Although we favor the concept that homodimers of TRs and of RARs are important mediators of repression *in vivo* (e.g. (Machado et al., 2009)), heterodimers of these receptors clearly contribute to transcriptional regulation, and it

is plausible that these heterodimers contribute to transcriptional repression in certain contexts.

Consistent with our GST-pulldown and EMSA *in vitro* receptor interaction assays, SMRT ϵ enhanced repression by a GBD-RAR α construct, but not by a GBD-LXR α construct in transfected cells, whereas NCoR ω enhanced repression by both nuclear receptors. TR β 1 and Rev-Erb α interact with SMRT ϵ by GST-pulldown, but fail to do so by EMSA supershift; intriguingly, repression by GBD-TR β 1 and by GBD-Rev-Erb α also appeared to be enhanced by co-expression of SMRT ϵ or NCoR ω (although the effect of SMRT ϵ on GBD-Rev-Erb α did not achieve statistical significance). It appears likely that the nature of receptor/DNA interaction interface, the sequence of the DNA binding site itself, and/or the presence of bridging proteins and other transcription factors bound to a particular target gene in cells can further influence the corepressor interactions observed using consensus response elements and purified proteins *in vitro*. It is therefore likely that both the GST-pulldown and EMSA supershift methodologies reveal biologically relevant, if distinct, features of the corepressor/receptor interaction. These issues are described in more detail in Section 4.3. Although it would be informative to examine SMRT ϵ occupancy on different target genes using chromatin immunoprecipitation methods, there are significant technical limitations to such a strategy, including the difficulty of obtaining specific antisera to a protein that can be distinguished from other SMRT splice variants only by the absence of certain epitopes.

Taken as a whole, our results suggest that alternative mRNA splicing can tailor corepressor specificity to a particular subset of nuclear receptor partners, and this tailoring can impact repression more than does the genetic locus of origin. SMRT γ and NCoR ω , for example, despite being generated from different loci, possess similar arrangements of RIDs (Suppl. Fig 1), are enriched in mouse brain and testes, and target extensively overlapping nuclear receptor partners. In contrast, although originating from an identical locus, SMRT γ and SMRT ϵ display very different RID organizations, tissue distributions, and nuclear receptor specificities (Goodson et al., 2005a, Malartre et al., 2004, Short et al., 2005). Many studies of SMRT and NCoR, performed before recognition of the extensive splicing at these loci, compared the properties of SMRT α to those of NCoR ω . Current insight indicates a more apt comparison would be SMRT α to NCoR δ , or SMRT γ to NCoR ω , and would likely reveal more similarities than previously recognized.

4.2. SMRT ϵ requires a recently recognized β -strand motif to bind to its nuclear receptor partners

Each CoRNR box motif in the SMRT and NCoR corepressors can form an extended α -helix that can dock with a matching hydrophobic groove on the surface of an appropriate nuclear receptor partner. Differences in the amino acid sequence in the CoRNR boxes themselves, or in flanking regions, help determine which CoRNR box, and therefore which corepressor, can interact with a given nuclear receptor. It was recently discovered that a β -strand motif adjacent to the RID2 CoRNR box itself also contributes to corepressor docking by RARs and Rev-Erb α (le Maire et al., 2010, Phelan et al. 2010). A portion of helix 11 in these two receptors can assume a β -strand conformation that in turn interacts with the β -strand in the corepressor to further stabilize the complex. Our studies confirm that both the α -helix and β -strand motifs in the SMRT ϵ RID2 domain contribute to the ability of this splice variant to tether RARs in the immobilized protein assay and to function with RAR α to mediate repression in our reporter gene assay. Notably, the β -strand mutation in SMRT ϵ also compromises the ability of TR α 1 to interact with SMRT ϵ , although not to the same extent as in RAR α or the mutation in CoRNR box α -helix sequence; both the β -strand and CoRNR box α -helix contributed to the ability of SMRT ϵ to enhance TR-mediated repression. We suggest that a wider variety of nuclear receptors than previously reported employ the β -strand surface in RID2 to stabilize their recruitment of corepressor, and that this mode of

corepressor interaction plays a particularly important role in SMRT ϵ binding due to the absence of RID1 and RID3 from this splice variant.

4.3. Nuclear receptor binding to DNA modulates the corepressor partnership

As noted, the panel of nuclear receptors recognized by SMRT ϵ was further restricted when tested by EMSA using cognate DNA response elements, with RAR α and RAR β the only permissive partners detected. This type of DNA-mediated restriction has been reported before for other nuclear receptors and for other corepressor splice variants (e.g. (Cohen et al., 1998, Cohen et al., 2000, Ghosh et al., 2002, Machado et al., 2009, Yoh and Privalsky, 2001, Zamir et al., 1997b, Zhang et al., 1999)), yet the molecular basis remains incompletely understood. As observed here for TR α 1 and TR β 1, the DNA-bound receptor can lose the ability to bind to SMRT ϵ , yet retain the ability to bind to other SMRT or NCoR splice variants. This suggests that DNA binding can impair the ability of certain nuclear receptors to bind to RID2, but remain permissive for receptor binding to RID1 or RID3. It has been proposed that each nuclear receptor dimer, when bound to DNA, recruits corepressors by contacting two RID domains simultaneously on a single SMRT or NCoR molecule (Cohen et al., 1998, Downes et al., 1996, Ghosh et al., 2002, Hu et al., 2001, Machado et al., 2009, Makowski et al., 2003, Zamir et al., 1997b). In this model, the spacing and orientation of the RIDs in a given corepressor variant must match the topology of the corresponding corepressor binding surfaces on the receptor dimer. Although this model may explain why SMRT ϵ , restricted to a single RID2 domain, fails to bind TRs, Rev-Erb α , and COUP-TFII in the EMSA supershift, it does not account for why SMRT ϵ retains the ability to bind to RAR α and β under the same circumstances.

We considered other possibilities. Perhaps the RAR α dimer recruits one molecule of SMRT α , but two molecules of SMRT ϵ ? Arguing against this concept, the mobilities of the SMRT ϵ /RAR α and SMRT α /RAR α complexes were the same in our EMSA supershifts, suggesting the stoichiometry was the same for both splice variants. Alternatively, RID-like motifs have been mapped to the SMRT and NCoR N-termini (Varlakhanova et al., 2010, Wu et al., 2006, Zhang et al., 2009); these “nRIDs” hypothetically could substitute for the RID1 absent from the SMRT ϵ C-terminus. However, the SMRT nRID exhibits little or no ability to interact with RARs (Varlakhanova et al., 2010), and RAR α and RAR β were both bound efficiently in our EMSA supershifts by SMRT ϵ constructs lacking nRIDs. Our results therefore indicate that one RID per corepressor molecule is indeed sufficient for efficient recruitment by certain nuclear receptor dimers when bound to their response elements. We suggest that receptor binding to a given DNA response element alters both the conformation of the individual receptors and the overall structure of the receptor dimer, and that these DNA-mediated changes do not impose a universal requirement for two RIDs per corepressor, but rather work as a whole to select for specific RID sequences and topologies among the different corepressor variants available. This is consistent with the ability of a given nuclear receptor to recognize RIDs separated by a variety of different spacings (Astapova et al., 2009).

4.4. Potential impact of other alternative splicing events on SMRT and NCoR function

In addition to SMRT ϵ , other alternative splicing events in SMRT and NCoR also influence nuclear receptor selectivity. For example, SMRT τ is a 44b+ splice variant that deletes sequences C-terminal to the RID1 CoNR box without affecting the CoNR box itself; SMRT τ exhibits a 5 to 10-fold decreased affinity for TR α 1 and TR β 1, yet a near equal affinity for RARs, compared to SMRT α (Goodson et al., 2005b). NCoR ω , generated by an alternative exon 37b+ splice that introduces a third RID, displays a significantly increased affinity for TRs, but a reduced affinity for COUP-TFII and Rev-Erb, compared to NCoR δ

(which contains only RID2 and RID1) (Bailey et al., 1997, Cohen et al., 2001, Downes et al., 1996, Makowski et al., 2003, Webb et al., 2000).

Alternative splicing events operating at multiple sites can assemble yet-additional corepressor variants. For example, combination of the 37b+ SMRT splice with the 44- SMRT splice encodes a variant (denoted SMRT λ in our nomenclature; Suppl. Fig. 1) that contains RID3 and RID2, but not RID1 (Faist et al., 2009). A SMRT λ construct binds RAR α both in our GST-pulldown experiments and in a previously reported EMSA experiment (wherein it was reported to bind more weakly than did the single RID SMRT ϵ) (Faist et al., 2009). With the exception of brain and testes, however, the 37b+ splicing event is infrequent and displays an inverse tissue distribution to that of the 44- splice; as a consequence, we estimate that SMRT λ is very rare (present at only 10% to 20% of the levels of SMRT ϵ) in the murine tissues we've examined.

There is no evidence to date for naturally-occurring splice forms of SMRT or NCoR that lack all RIDs, or that contain RID1 and/or RID3, yet lack the central RID2. Artificial SMRT mutants lacking RID2, or lacking both RID2 and RID1, lose the ability to interact with a broad sweep of nuclear receptors *in vitro*, and they display a series of developmental, endocrine, and metabolic defects, including aberrant thyroid hormone homeostasis and enhanced adipogenesis (Astapova et al., 2008, Fang et al., Nofsinger et al., 2008, Reilly et al.). It is possible that RID2, although not absolutely essential for mouse viability, is required for partnering with so many different nuclear receptors that its removal by alternative splicing would be strongly counter-selected during evolution.

In summary, alternative mRNA splicing generates a diverse series of corepressor variants, such as SMRT ϵ , that display different affinities for different nuclear receptor partners. Different splice variants also respond divergently to the kinase signals that regulate their activity (Jonas et al., 2007). Given that different tissues preferentially express different corepressor variants, we propose that organisms utilize alternative corepressor splicing to establish cell-type specific transcriptional repression programs, thereby permitting a given nuclear receptor to exert cell- and tissue-specific transcriptional regulation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors sincerely thank Liming Liu for superb technical assistance, and Meghan D. Rosen for creating the SMRT ϵ , RAR α , and TR α interaction motif mutants. This work was supported by Public Health Service/National Institute of Diabetes and Digestive and Kidney Diseases award RO1DK53528.

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HIGHLIGHTS

SMRT ϵ is an alternatively-spliced form of corepressor.

SMRT ϵ expression differs in different cell types.

SMRT ϵ displays a restricted specificity for a subset of nuclear receptor partners.

SMRT ϵ interacts with nuclear receptors by a mix of α -helical and β -strand surfaces.

SMRT ϵ is highly specific for RARs when bound to their cognate DNA response elements.

Fig. 1. SMRT ϵ is expressed in a wide variety of tissues and cell lines. **(A)** The proteins encoded by the SMRT ϵ , SMRT τ , SMRT α , and NCoR ω splice variants are depicted schematically. The protein sequences included in a given corepressor variant are shown as rectangles; sequences removed by splicing are indicated as lines. The location of the different C-terminal receptor interactions domains (RIDs) are also shown, as are the more N-terminal domains that mediate transcriptional repression by recruiting additional components of the corepressor complex. **(B)** The relative levels of expression of SMRT ϵ , τ , and α in different cell lines and in different mouse tissues was determined by rt-PCR. Skel Msc1 = skeletal muscle. WAT = white adipose tissue. BAT = brown adipose tissue. The mean and standard error from multiple independent experiments are shown.

Fig. 2.

SMRT ϵ displays a distinct specificity for nuclear receptors compared to SMRT α or NCoR ω . Proteins representing the (A) GST-only, (B) GST-SMRT α , (C) GST-NCoR ω , or (D) GST-SMRT ϵ constructs described in Materials and Methods were expressed in Sf9 cells, immobilized on glutathione-agarose beads and incubated with the radiolabeled nuclear receptors indicated below. The immobilized GST-constructs were then washed, and the nuclear receptor remaining bound to each construct was eluted and quantified (input=100%). The mean and standard error from at least three independent experiments are shown.

Fig. 3. RARs and LXR bind to differing sets of corepressor splice variants. The ability of radiolabeled (A) RAR α , (B) RAR β , (C) RAR γ , and (D) LXR α to bind to the GST-corepressor splice variants (GST-CoR) indicated below each panel was tested using the protocol in Fig. 2.

Fig. 4.

The ability of SMRT ϵ to bind to either RAR α or to TR α 1 is enhanced by β -strand/ β -strand interaction motifs. (A) The RID2 domain of SMRT ϵ is depicted schematically on top; indicated are the β -strand and α -helical domains, and the mutations used in panel B. A corresponding schematic of the β -strand and hydrophobic groove regions within the ligand binding domain (LBD) of RAR α and TR α 1, is depicted below; in the native receptor the hydrophobic groove is assembled from portions of helix 3, 4, and 5 that are brought together by protein folding. (B) Mutations that disrupt either the α -helical or β -strand motifs destabilize the interaction of SMRT ϵ with RAR α and TR α 1. Four GST-constructs were employed: GST-only, GST-SMRT ϵ wild-type, GST-SMRT ϵ bearing a β strand mutation (V2141P), or a GST-SMRT ϵ bearing an α -helix CoRNR box mutation (an II to AA substitution within amino acids 2092–2096). These constructs were tested for the ability to

bind to RAR α wild-type, RAR α bearing a β -strand mutation (I396E), TR α 1 wild-type, or TR α 1 bearing a presumptive β -strand mutation (L386E) by the protocol in Figure 2. The interactions of the wt receptors with wt SMRT ϵ , versus the wt receptors with the β -strand or α -helix SMRT ϵ mutants, were all statistically different with a p value < 0.001. Similarly, the interactions of the wt SMRT ϵ with the wt receptors, versus the β -strand mutant receptors, were all statistically different with a p value < 0.001.

Fig. 5. SMRT ϵ binds strongly to RAR α and RAR β homodimers assembled on a cognate DNA response element, despite the single RID domain in this corepressor variant. Recombinant RAR α , β , and γ proteins were incubated with a radiolabeled DNA probe containing a cognate DR5 response element, either alone (first lane of each panel), or together with increasing amounts of the corepressor constructs indicated. The resulting free probes (fp), RAR/DNA complexes ($\blacklozenge$), and supershifted corepressor/RAR/DNA complexes ($\odot$) were resolved by native gel electrophoresis and visualized by phosphorimaging. Representative phosphorimager scans are shown.

Fig. 6. SMRT ϵ fails to bind to TR α 1, TR β 1, Rev-Erba, or COUP-TFII homodimers assembled on cognate DNA response elements. Recombinant TR α 1, TR β 1, Rev-Erba, or COUP-TFII proteins were incubated with radiolabeled DNA probes containing the cognate response elements (an F2-lysozyme element for the TRs, a DR2 for Rev-Erba, and a DR4 for COUP-TFII), either alone (first lane of each panel), or together with increasing amounts of the corepressor constructs indicated. The resulting receptor/DNA complexes, either receptor monomers (●) or dimers (◆), and supershifted corepressor/receptor/DNA complexes (○) were resolved by native gel electrophoresis and visualized by phosphorimaging. The positions of free probe were deleted from these figures to save space. Representative experiments are shown.

Fig. 7.

Quantification of the EMSA supershift experiments for RARs and TRs. Multiple EMSA supershift experiments, performed as in Fig. 5 and 6, were quantified using the nuclear receptors indicated and (A) SMRT ϵ , (B) SMRT α , or (C) NCoR ω . The mean and standard error from three (RARs) or two (TRs) experiments are shown.

Fig. 8. SMRT ϵ can enhance repression by RAR α and TR β 1. (A) An expression vector encoding a Gal4 DNA binding domain (GBD) only, a GBD-RAR α (LBD) fusion, a GBD-LXR α (LBD) fusion, a GBD-TR β 1(LBD) fusion, or a GBD-Rev-Erba(LBD) fusion was transfected into CV1 cells together with a GAL-17 mer luciferase reporter. Also included was an empty expression vector (no CoR), an expression vector for full-length SMRT ϵ , or an expression vector for full length NCoR ω , as indicated. After 48 hours the cells were harvested and luciferase activity was assayed. The luciferase activity, relative to that of the GAL4DBD alone (defined as 100%), is shown for each construct. The mean and standard error from four experiments are presented. (B) The same experiment as in panel A was repeated using GBD-RAR α or GBD-TR β 1 with GST-SMRT ϵ wild-type, GST-SMRT ϵ bearing a β strand mutation (V2141P), or a GST-SMRT ϵ bearing an α -helix CoRNR box mutation (an II to AA

substitution within amino acids 2092–2096). (*) indicates a p value < 0.05; (**) indicates a p value < 0.01; (***) indicates a p value of < 0.005.