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The SMRT corepressor is a target of phosphorylation by protein kinase CK2 (casein kinase II)

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Abstract

The Silencing-Mediator for Retinoid/Thyroid hormone receptors (SMRT) interacts with, and mediates transcriptional repression by, a variety of eukaryotic transcription factors, including the nuclear hormone receptors. The ability of SMRT to function as a transcriptional ‘corepressor’ is regulated by a variety of signal transduction pathways. We report here that SMRT is a phosphoprotein *in vivo*, and is also phosphorylated *in vitro* by unfractionated cell extracts. A major site of phosphorylation of SMRT is a protein kinase CK2 motif centered on serine 1492, and located within a C-terminal SMRT domain that mediates interaction of the corepressor with the nuclear hormone receptors. Phosphorylation of SMRT by CK2 stabilizes the ability of the SMRT protein to interact with nuclear hormone receptors. Our results indicate that SMRT is a member of an expanding family of transcriptional regulators that are modified, and potentially regulated, in response to protein kinase CK2.

Keywords

corepressor; nuclear hormone receptors; protein kinase; CK2; casein kinase 2

Introduction

Nuclear hormone receptors, such as the steroid, retinoid, and thyroid hormone receptors, are hormone-regulated transcription factors that play many critical roles in vertebrate differentiation, reproduction, and homeostasis [1-5]. Nuclear hormone receptors function as ligand-regulated transcription factors, binding to the promoters of specific target genes and regulating their transcription in response to cognate hormone. Intriguingly, many nuclear hormone receptors possess bipolar transcriptional properties, and can either repress or activate expression of a given target promoter depending on the hormone status, the nature of the DNA binding site, and the impact of other signal transduction pathways operating in the cell [6-9].

The ability of nuclear hormone receptors to function in transcription in such a diverse manner is due to the ability of these receptors to recruit an array of auxiliary proteins, denoted corepressors and coactivators, that mediate the actual molecular events that lead to transcriptional regulation [10-14]. Thyroid hormone receptors (T3Rs), for example, typically bind to the SMRT (silencing mediator for retinoid/thyroid hormone receptors) and N-CoR (nuclear hormone receptor-corepressor) family of corepressor proteins in the absence of hormone, leading to repression of target gene transcription [15-19]. Conversely, the binding of hormone by T3R leads to a physical release of the corepressors from the nuclear receptor

and the acquisition of novel coactivator proteins, such as SRC-1 (Steroid Receptor Coactivator-1), p300, TRAPs (T3R-Associated Proteins), or DRIPs (vitamin D3-Receptor Interacting Proteins), which convert the T3R into a transcriptional activator [10,20-23]. The precise mechanisms by which these coactivators and corepressors regulate transcription remain incompletely understood, but appear to include both modifications of the chromatin template and interactions with components of the general transcriptional machinery [12,14,20-22, 24-31].

Cognate ligand plays a important role in regulating the transcriptional properties of the nuclear hormone receptors by regulating the interaction of these receptors with the SMRT/N-CoR corepressors [15-17,19,32-35]. However, the ability of nuclear hormone receptors to repress or activate transcription is also under tight regulation by many nonligand signal transduction pathways operating in the cell. For example, activation of the epidermal growth factor receptor, a membrane-associated tyrosine kinase, strongly interferes with transcriptional repression by T3R, apparently by interfering with the ability of corepressor to interact with the nuclear hormone receptor [36,37]. Similarly, corepressor function has also been reported to be regulated in response to cyclic AMP signaling [38]. In many cases the nuclear hormone receptor is not itself detectably modified in response to these signaling events, suggesting that it is the corepressor that is the actual target of the regulatory pathway [36,39]. We therefore have investigated if the SMRT corepressor is post-translationally modified in cells, and if these post-translational modifications can alter SMRT function. We report here that indeed SMRT is phosphorylated when expressed in mammalian cells in culture, and that SMRT can also be phosphorylated *in vitro* when incubated with nuclear extracts of these cells. A major site of SMRT phosphorylation, *in vitro* and *in vivo*, is a protein kinase CK2 phosphorylation motif located at serine 1492 within the C-terminal receptor-interaction domain of the SMRT corepressor. Phosphorylation of this site by CK2 (formally known as casein kinase II) resulted in a modest, but reproducible stabilization of the ability of SMRT to interact with thyroid hormone receptors. We conclude that SMRT is a member of the expanding class of transcription factors that are modified by protein kinase CK2, and for which this modification may play a role in function.

Materials and methods

Plasmid constructs

The pSG5-T3R α , pGEX-SMRT, pCMV-SMRT, and green fluorescent protein (GFP)-SMRT derivatives were constructed as previously described [17,39,40]. Base substitution mutations, designed to abolish potential phosphorylation sites within the SMRT sequence, were created by oligonucleotide-mediated mutagenesis using polymerase chain reaction and standard recombinant DNA methodologies [41]. Deletion mutations within the C-terminus of the pGEX-KG SMRT (1291–1495) molecular clone were created by an exonuclease III/S1 nuclease protocol, using a double-stranded nested deletion kit (Amersham Pharmacia, Piscataway, NJ, USA). The mammalian expression vector, pCMV5-CK2 α , was obtained from G.J. Rosman and A. Dusty Miller (Fred Hutchison Cancer Research Center).

In vitro kinase assays

CV-1 cells were maintained in Dulbecco-modified Eagles medium (Gibco/BRL Life Technologies, Rockville, MD, USA) supplemented with 10% fetal bovine serum and penicillin/streptomycin. Cells were harvested by mechanical scrapping and collected by centrifugation. CV-1 cell lysates were prepared by sonication of harvested cells in 25 mM HEPES pH 7.5, 300 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 1% Triton X-100, 0.1 mM dithiothreitol, and COMPLETE protease inhibitor cocktail (Berhinger-Mannheim, Germany). The GST-SMRT fusion proteins were expressed in recombinant *Escherichia coli*, immobilized

on glutathioneagarose, and repeatedly washed, as previously described [36]. The immobilized GST-SMRT proteins were then incubated at 30°C for 20 min with 30 µl of CV-1 cell lysate in 400 µl of 20 mM HEPES, pH 7.7, 20 mM MgCl₂, 20 mM β-glycerol phosphate, 2 mM dithiothreitol, 0.1 mM Na₃VO₄, and 20 µM ATP containing 5 µCi [γ -³²P] ATP. Alternatively, the immobilized GST-SMRT proteins were incubated with 500 units of purified, recombinant CK2 isolated from *E. coli* (New England Biolabs, Beverly, MA, USA) in 200 µl of 20 mM Tris-Cl, pH 7.5, 50 mM KCl, 10 mM MgCl₂, and 100 µM ATP containing 5 µCi [γ -³²P] ATP. The kinase reactions were terminated by washing the samples twice and resuspending the samples in SDS-sample buffer. The samples were then boiled for 5 min and the proteins were resolved by SDS-polyacrylamide gel electrophoresis (PAGE). Phosphorylated proteins were visualized and quantified by PhosphoImager analysis using a Storm 820 or 840 System (Molecular Dynamics, Sunnyvale, CA, USA).

Phosphorylation-dephosphorylation assays

One microgram of the pSG5-SMRT (1291–1495) expression vector was introduced into 1.5×10^5 CV-1 cells in the presence or absence of 500 ng of the pCMV5-CK2 α expression vector, using a Lipofectin protocol (Gibco/BRL Life Technologies, Rockville, MD, USA). The cells were harvested 48 h later by mechanical scraping into 1.2 ml of Tris-Buffered Saline buffer (10 mM Tris-Cl, pH 8.0, 150 mM NaCl) and concentrated by centrifugation. For the phosphorylation assay, the cells were resuspended in 100 µl of 20 mM Tris-Cl, pH 7.4, 150 mM NaCl, 1 mM Na₃VO₄, 10 mM NaF, 1 mM dithiothreitol, and COMPLETE protease inhibitor cocktail, and were lysed by sonication. Cell lysates were then analyzed by SDS-PAGE electrophoresis followed by an immunoblotting procedure using SMRT-directed antibodies [39]. Two electrophoresis systems were employed to analyze the electrophoretic shift associated with phosphorylation of the SMRT protein: a high cross-linker system (12% polyacrylamide, 0.5% bis-acrylamide) or an SDS/urea, low cross-linker system (12% polyacrylamide, 0.32% bis-acrylamide) [42]. For the dephosphorylation assay, the transfected cells were resuspended in 50 mM Tris-Cl, pH 7.9, 100 mM NaCl, 10 mM MgCl₂, and COMPLETE protease inhibitor. The cell lysates were then incubated in the presence or absence of 10 units of calf intestinal alkaline phosphatase (New England Biolabs, Beverly, MA, USA) for 10 min at 37°C. The reactions were terminated by mixing the samples with SDS-Urea sample buffer [42], and the samples were subjected to electrophoresis and immunoblotting as for the phosphorylation assay.

Thyroid hormone receptor-corepressor binding assay in vitro

The GST-SMRT (1291–1495) fusion protein was expressed in *E. coli*, was immobilized on glutathione-agarose, and was purified as described above. The immobilized GST-SMRT protein was then incubated at 30°C for 40 min with 500 units of purified, recombinant CK2 isolated from *E. coli* (New England Biolabs, Beverly, MA, USA) in 100 µl of 20 mM Tris-Cl, pH 7.5, 50 mM KCl, 10 mM MgCl₂, and 200 µM ATP, or was mock-treated in the same buffer without kinase. ³⁵S-radiolabeled T3R α protein was synthesized by a coupled *in vitro* transcription-translation system (Promega, Madison, WI, USA), and was incubated with the immobilized GST-SMRT in HEMG buffer, as described before [39]. After repeated washing, any radiolabeled T3R remaining bound to the GST-SMRT matrix was eluted, resolved by SDS-PAGE, and visualized/quantified by PhosphoImager analysis.

Laser scanning confocal microscopy

The subcellular localization of GFP-fusions of the wild-type and S1492A mutant forms of the GFP-SMRT protein was determined as previously described [39].

Transient transfections

Approximately 7×10^4 CV-1 cells per assay were transfected using the Lipofectin protocol with 100 ng of pCMV5-CK2 α vector, 20 ng of pSG5-T3R α vector, 100 ng of a thymidine kinase promoter-luciferase reporter containing a DR-4 hormone response element, and 50 ng of a pCMV-lacZ reporter (used as an internal control) [36]. Equal quantities of the empty vectors were substituted as appropriate to maintain the total amount of DNA the same in all transfections. Five hours after the transfection procedure, the cells were transferred to medium containing 100 nM of T3-thyronine hormone, or an equivalent amount of ethanol carrier, and the cells were maintained at 37°C for an additional 43 h. Luciferase and β -galactosidase assays were performed as previously described [36].

Protein stability assay

Approximately 1.5×10^5 CV-1 cells per assay were transfected with 1 μ g of pSG5-SMRT expression vector and 500 ng of pCMV5-CK2 α (or an equivalent amount of an empty expression vector). Thirty-six hours after transfection, the cells were transferred to serum-free medium containing 20 μ g/ml of cycloheximide to inhibit further protein synthesis, and samples were harvested at different time points thereafter. For each time point, cells were washed, suspended in 100 μ l of 10 mM Tris-Cl, pH 8.0, 150 mM NaCl, 1 mM DTT and COMPLETE protease inhibitor cocktail, lysed by sonication, and subjected to SDS-PAGE and immunoblotting.

Results

The SMRT corepressor is phosphorylated in vivo

We first investigated if the SMRT corepressor protein was subject to post-translational modifications in cells, either constitutively or in response to EGF-receptor signaling, that might be reflected as changes in its electrophoretic mobility. We employed SDS-polyacrylamide gel electrophoresis and Western blot analysis to detect the SMRT corepressor. Notably, the SMRT protein displayed a heterogeneous electrophoretic mobility in CV-1 cells in the absence of treatment, with a further decrease in apparent mobility observed when the cells were treated with tumor growth factor (TGF) α , an inducer of EGF-receptor signaling (Fig. 1A). The heterogeneous mobility of the corepressor, and its change in response to EGF-receptor signaling, was also observed when employing constructs restricted to the C-terminus of SMRT (Fig. 1B, and data not shown), indicating that this putative modification(s) mapped, at least in part, to within the receptor-interaction domain of the corepressor. Changes in the electrophoretic mobility of many proteins can be attributed to phosphorylation; we therefore tested the ability of alkaline phosphatase to reverse the heterogeneous mobility of the SMRT protein. Treatment with purified preparations of calf alkaline phosphatase converted the slower migrating SMRT species to a faster migrating form (Fig. 1B). We conclude that a basal level of phosphorylation occurs within the C-terminal domain of the SMRT corepressor that is augmented by EGF-signaling and reversed by phosphatase treatment.

The SMRT protein is also a substrate for phosphorylation in vitro

To identify the sites of, and the kinases responsible for, the phosphorylation of SMRT described above, we tested the ability of CV-1 cell lysates to phosphorylate *in vitro* various subdomains of SMRT (Fig. 2A). Using this method, we observed that a GST-fusion representing the C-terminal 204 amino acids of SMRT (codons 1291–1495) was strongly phosphorylated in our *in vitro* kinase assay, whereas GST-fusions representing other domains of SMRT, or the non-recombinant GST polypeptide itself, were relatively ineffective as substrates (Fig. 2B). These results indicated that the same C-terminal region of SMRT modified *in vivo* could also be phosphorylated *in vitro*.

To further delineate the site(s) of phosphorylation within SMRT, we generated a series of small deletions within the 3' end of the SMRT open reading frame, expressed the corresponding proteins as GST fusions, and tested these fusions for the ability to serve as substrates in the our *in vitro* phosphorylation assay (Fig. 2C). A SMRT 3' deletion that removed only non-coding regions of the mRNA had no effect on SMRT phosphorylation, whereas a slightly larger deletion, removing fourteen C-terminal amino acids from the corepressor ($\Delta 1482$), severely impaired the ability of the corresponding GST-SMRT fusion polypeptide to be phosphorylated by the CV-1 cell lysates (Fig. 2C). GST-fusions representing more extensive SMRT deletions were similarly unable to be phosphorylated in the kinase assay (e.g. $\Delta 1480$ and data not shown). The deletions did not significantly alter the level of expression or the stability of the resulting GST-SMRT fusion proteins, and all constructs were tested at comparable protein concentrations in the *in vitro* assay (data not shown).

Our results therefore indicated that either the actual site of phosphorylation itself, or an important recognition or conformational motif essential for kinase recognition, lay within the C-terminal 14 amino acids of the corepressor. There are five amino acids in the SMRT sequence within this region that conceivably might serve as sites of acid-stable phosphorylation, represented by three serines, one threonine, and one tyrosine (Fig. 3A, underlined). We therefore individually substituted each of these amino acids within the SMRT C-terminal domain to alanine, and tested the ability of the resulting GST-fusions to serve as substrates in the *in vitro* kinase assay (Fig. 3B). The GST-SMRT fusion with the S1492A substitution was significantly impaired in the ability to be phosphorylated in our *in vitro* assay, whereas the other four amino acid substitutions displayed wild-type, or near wild-type, substrate activity (Fig. 3B, and quantified in Fig. 3C). We conclude that the retention of serine 1492 is important for the ability of wild-type SMRT to be phosphorylated *in vitro*, and therefore probably represents the predominant site of phosphorylation by CV-1 cell extracts in our assay.

Serine 1492 of SMRT is phosphorylated by protein kinase CK2

Inspection of the SMRT amino acid sequence revealed that serine 1492, identified above, lies within a protein kinase CK2 recognition motif (S-X-X-D/E; [43]). To determine if serine 1492 was indeed a substrate for CK2, we next tested the ability of purified CK2 to phosphorylate the SMRT protein *in vitro*. The GST-SMRT-RID-2 fusion polypeptide was readily phosphorylated by CK2 under these conditions (Fig. 4A). In contrast no phosphorylation was detected employing equal or greater amounts of non-recombinant GST polypeptide, or of GST-SMRT constructs representing more N-terminal domains of corepressor (compare the phosphorylation in Fig. 4A with the Coomassie blue staining pattern in Fig. 4B). The phosphorylation of the GST-SMRT C-terminus was dependent on addition of CK2, indicating that this modification was not due to a hypothetical autophosphorylation of the GST-SMRT polypeptide or due to a contaminating bacterial kinase (Fig. 4A). Estimates based on specific activity considerations suggest that approximately one mole of phosphate could be incorporated per mole of GST-SMRT fusion protein (data not shown).

To test if serine 1492 of SMRT was the actual site of phosphorylation under these conditions, we compared the ability of our different alanine substitution mutants to serve as substrates for purified CK2 (Fig. 5). Consistent with the identification of serine 1492 as a likely CK2 recognition site by sequence analysis, the S1492A mutation severely impaired the ability of SMRT to be phosphorylated by CK2 *in vitro*. In contrast, the four other amino acid substitution mutants tested exhibited virtually wild-type levels of phosphorylation by CK2.

To determine if SMRT could be phosphorylated by CK2 *in vivo*, as well as *in vitro*, we tested the ability of an exogenously-introduced molecular clone of the CK2 α catalytic subunit to further enhance the basal levels of SMRT phosphorylation observed in transfected CV-1 cells. Co-expression of CK2 α increased the amount of SMRT displaying a retarded electrophoretic

mobility relative to SMRT isolated from mock-treated cells, and both this CK2-mediated modification, and the basal modification observed in the absence of CK2, were reversed by calf-intestine alkaline phosphatase (Fig. 6A, left panel). Similarly, a retardation in the electrophoretic mobility of SMRT could also be observed when a purified GST-SMRT construct, isolated from *E. coli*, was incubated with purified CK2 *in vitro* (Fig. 6A, right panel). Although these changes in mobility in response to the introduction of CK2 could be observed in several different electrophoretic systems, it was most clearly detected in an urea/SDS/polyacrylamide gel system in which phosphorylation results in an increased, rather than a decreased, SMRT mobility (Fig. 6B). Consistent with our interpretation that the faster mobility of SMRT in the urea gel system represents a change in phosphorylation state of serine 1492, the increased mobility associated with CK2 coexpression was reversed on alkaline phosphatase treatment and was not observed with the SMRT S1492A mutant (Figs 6C and 6D). Taken as a whole, our experiments indicate that serine 1492 is a major site of phosphorylation within the SMRT corepressor, and that this amino acid is modified by either by CK2, or by a kinase possessing a CK2 recognition specificity.

As noted previously, the basal phosphorylation of the SMRT protein in CV-1 cells was further altered in response to EGF-receptor signaling. This change in SMRT mobility in response to EGF-receptor signaling was of a greater magnitude than that observed in response to CK2, suggesting that SMRT might be modified at multiple sites, and that not all of these modifications were due to CK2 phosphorylation of serine 1492 (compare Fig. 1 to Fig. 6A). Confirming this hypothesis, although the S1492A SMRT mutant could not be phosphorylated by CK2, this mutant protein was nonetheless modified in response to EGF-receptor signaling (data not shown). In separate work, we have determined that at these additional modifications of SMRT in response to EGF-receptor signaling represent phosphorylation by components of a MAP kinase kinase kinase (MAPKKK) pathway [39].

Phosphorylation by CK2 stabilizes the ability of SMRT to interact with thyroid hormone receptor *in vitro*

We next wished to determine if phosphorylation of SMRT by protein kinase CK2 had a detectable effect on corepressor function. Given that the location of the CK2 site is within a region of SMRT involved in the interaction of corepressor with nuclear hormone receptors, we first tested if phosphorylation of this site *in vitro* altered the ability of the corepressor to bind to T3R. We employed an 'GST-pull-down' protocol to measure the ability of a GST-SMRT fusion polypeptide, synthesized in *E. coli* and immobilized on glutathione agarose, to bind to ³⁵S-radiolabeled T3R, synthesized by *in vitro* transcription and translation (Fig. 7). In the absence of CK2 treatment, the GST-SMRT fusion protein bound the ³⁵S-tagged T3R, whereas little or no binding of T3R was observed to a non-recombinant GST construct employed as a negative control (Fig. 7A). Pre-treatment of the GST-SMRT construct with CK2 led to a modest, although reproducible, enhancement of the ability of the SMRT construct to interact with T3R in this assay. This enhancement of the SMRT/T3R interaction was most clearly observed when the GST-binding assay was performed at moderate concentrations of thyroid hormone (Fig. 7A) or at high salt concentrations (Fig. 7B). High salt and thyroid hormone are both able to disrupt the SMRT/T3R interaction, and our results suggest that CK2 phosphorylation partially counteracts these destabilizing effects. It should be noted that the GST-SMRT preparation was efficiently phosphorylated in our experiments, with virtually the entire GST-SMRT polypeptide being converted by CK2 to the SDS-PAGE mobility characteristic of the phosphorylated form (Fig. 6A and data not shown).

We next examined if other properties of SMRT were detectably altered by CK2 modification. CK2 modification has been noted to alter the stability of many proteins (e.g. [44-47]). We therefore tested the turnover rate of SMRT in cells in the absence or presence of exogenously

introduced CK2 α , comparing wild-type SMRT and the S1492A mutant. Notably, no significant difference in the turnover or half-life of SMRT, could be detected in response to CK2 under these conditions (Fig. 8A). Phosphorylation by CK2 has also been reported to alter the subcellular localization of many protein substrates (e.g. [48-51]). We therefore next employed GFP-fusions of SMRT to characterize the subcellular distribution of the corepressor in the absence or presence of a CK2 phosphorylation site. As reported previously (e.g. [39,52]), wild-type SMRT, either native or expressed as a GFP-fusion, exhibits a primarily nuclear localization (Fig. 8B); within the resolution of this technique, the GFP-SMRT S1492A mutant displayed the same nuclear localization as did the GFP-SMRT wild-type (Fig. 8B).

The ability of nuclear hormone receptors to recruit corepressors or coactivators is reflected in their ability to repress or activate target gene expression in cells [9]. We therefore tested if the stabilization of the interaction of SMRT with T3R by CK2 phosphorylation, observed *in vitro*, was also manifested as a change in the repression/activation properties of the T3R when assayed in transfected cells. We employed transient transfections of CV-1 cells, introducing T3R α , a thymidine kinase promoter-luciferase reporter gene construct containing a binding site for T3R, and either the CK2 α expression vector, or equivalent amounts of empty vector (Fig. 8C). As anticipated, T3R α repressed the expression of the luciferase reporter gene in the absence of hormone. Addition of T3 hormone resulted in a dose-dependent reversal of this repression and an increased activation of reporter gene expression (Fig. 8C). Notably, however, this ability of T3R α to respond to T3 hormone by reversing repression, and by inducing expression of the reporter gene was impaired by overexpression of CK2 α (Fig. 8C, compare the solid and the dashed lines). In contrast, the expression of the reporter gene itself (i.e. in the absence of T3R α) was not influenced by CK2 expression (Fig. 8C and data not shown). Although the effect of CK2 in this assay was modest, it was reproducible, and paralleled the stabilization of the T3R/SMRT interaction we observe *in vitro* at intermediate hormone or salt concentrations. These results are consistent with the hypothesis that, by stabilizing the corepressor/nuclear receptor interaction, CK2 phosphorylation of SMRT shifts the equilibrium between corepressor and coactivator binding by T3R, resulting in enhanced corepressor occupancy at intermediate hormone concentrations, and impaired activation.

Discussion

SMRT is phosphorylated by a protein kinase CK2 activity *in vitro* and *in vivo*

We wish to better understand the mechanisms by which the transcriptional properties of nuclear hormone receptors are regulated in cells. One major aspect of this regulation is mediated by the binding of cognate hormone to the nuclear hormone receptor, which can convert the receptor from a corepressor-bound transcriptional silencer to a coactivator-bound transcriptional activator [32-35]. However, nuclear hormone receptors serve as a nexus for many different signal transduction pathways in the cell, and non-ligand-based signaling also can have profound effects on transcriptional regulation by the nuclear receptors. For example, many nuclear hormone receptors can be phosphorylated, and these phosphorylation events can mimic, enhance, or oppose the actions of the hormone ligand itself (reviewed in [53-57]). In addition, a number of the auxiliary proteins that modulate or mediate nuclear hormone receptor function appear to themselves be targets of protein kinase signal transducers [39,58]. We and others have noted previously that the ability of SMRT and N-CoR corepressors to interact with, and mediate repression by, T3Rs and retinoic acid receptors are subject to regulation by a variety of protein kinase signaling pathways in the cell, including growth factor and cyclic AMP-responsive pathways [36-38]. Aspects of this regulation suggested that the SMRT protein, not the nuclear hormone receptor, might serve as the actual target of these protein kinase-mediated regulatory pathways. In the current manuscript, we have shown that the SMRT

protein is indeed phosphorylated in cells, and that at least one component of this phosphorylation is mediated by CK2 or by a closely-related activity.

This phosphorylation of SMRT maps to the C-terminal receptor-interaction domain of the corepressor, and can be observed *in vitro* using a bacterially-synthesized SMRT-derived polypeptide as substrate and either unfractionated CV-1 cell extracts or a purified CK2, as the source of kinase. Deletion of even a small region of the SMRT C-terminus abolished the ability of the corepressor to serve as a substrate for phosphorylation, indicating that the likely site of phosphorylation was very near the corepressor C-terminus. Higher definition genetic mapping, using single amino acid substitutions, demonstrated that the integrity of serine 1492 of SMRT was crucial for phosphorylation *in vitro* by either CV-1 cells lysates or by purified CK2. Serine 1492 is embedded within a S-X-X-E amino acid motif characteristic of CK2 phosphorylation substrates, consistent with Ser 1492 representing the actual site of phosphorylation in these experiments.

The phosphorylation of SMRT at amino acid 1492 was the most prominent phosphorylation carried out *in vitro* by CV-1 cells lysates, and was also observed in transfected cells in response to the introduction of the catalytic α subunit of CK2. Nonetheless, serine 1492 is clearly not the only site of phosphorylation of SMRT either *in vitro* or *in vivo*. At least 10% of the phosphorylation of the SMRT C-terminus by CV-1 cell lysates *in vitro* was retained by the S1492A mutant, indicating that other phosphorylation sites must exist elsewhere in this protein. Similarly, the S1492A substitution altered, but did not abolish the ability of SMRT to display an altered mobility in response to EGF-receptor signaling in transfected cells; at least several of these additional modifications to SMRT represent sites of phosphorylation by the components of a MAP kinase kinase kinase cascade [39].

The studies reported here have been confined to the SMRT corepressor. N-CoR is a second member of the corepressor family, and is approximately 50% related to SMRT at the amino acid level [16,59,60]. Intriguingly, a CK2 consensus site is located in N-CoR at the equivalent position as that in SMRT (N-CoR amino acid 2449), suggesting that this post-translational modification may also be operative in N-CoR.

Phosphorylation of SMRT by CK2 alters the ability of corepressor to bind to T3R *in vitro*

We sought to determine what effect, if any, the phosphorylation of serine 1492 might have on SMRT function *in vitro* or *in vivo*. SMRT contains two adjacent, but independent protein domains, RID-1 and RID-2, that mediate the ability of the corepressor to interact with its nuclear hormone receptor partners [17-19,32,34,35,61-67]. The different RID regions display distinct affinities for different nuclear receptors: retinoic acid receptors interact primarily with RID-1, retinoid X receptors interact primarily with RID-2, and the T3Rs are able to interact with both the RID-1 and RID-2 domains of SMRT. Serine 1492 lies within the RID-2 domain; we therefore examined if phosphorylation of SMRT by CK2 could alter the ability of the corepressor to interact with nuclear hormone receptors. CK2 phosphorylation resulted in a modest, but reproducible enhancement in the interaction between GST-SMRT-RID-2 and T3R; this enhanced interaction was manifested primarily as a stabilization of the SMRT/T3R interaction against the disruptive effects of T3 hormone or high salt. Notably, the stabilizing effects of CK2 were observed when SMRT was pre-treated with CK2 and extensively washed prior to the introduction of the T3R, indicating that these effects are due to phosphorylation of SMRT, and not of T3R. Although mapping within the broadly defined RID-2 domain, Ser 1492 lies outside of an I/L-X-X-I-I motif that can function as the minimal contact site between SMRT and T3Rs [32,34,35]. The effects of Ser 1492 phosphorylation on the SMRT/T3R interaction may be mediated through hypothetical additional contacts between the nuclear receptors and SMRT RID-2 that map outside of the I/L-X-X-I-I motif itself, or conceivably, phosphorylation

of Ser 1492 may alter the overall conformation of the SMRT RID-2 domain in a manner that stabilizes the I/L-X-X-I-I contact site.

Paralleling the stabilization of the SMRT/T3R interaction we observe *in vitro*, overexpression of CK2 α in transfected cells resulted in a diminution of transcriptional activation by T3R under conditions of low to intermediate hormone concentration. We suggest that by enhancing the affinity of SMRT for T3R, CK2 phosphorylation impairs the release of corepressor from the receptor in response to hormone ligand, thereby modifying the equilibrium between T3R molecules in the population bound to corepressor, and the T3R molecules in the population bound to coactivator. This enhanced corepressor association would manifest as an attenuation of reporter gene activation (e.g. [9]), as is indeed observed. Of course, given the multiple roles of CK2 in cells, we cannot rule out that the effects of CK2 on T3R-mediated transcription regulation are mediated, at least in part, by indirect mechanisms not involving the corepressor. However, we note that the basal levels of reporter gene expression in the absence of T3R were not influenced by CK2 overexpression. Similarly, the levels of expression of a pCMV- β -galactosidase reporter, employed as an internal normalization control, were not impaired by overexpression of CK2, nor was the level of expression, subcellular distribution, or stability of SMRT detectably altered by introduction of the CK2 construct. Taken as a whole, therefore, our data suggests that CK2 phosphorylation can stabilize the physical and structural interaction of SMRT with its nuclear receptor partners.

Protein kinase CK2, corepressors, and the control of transcription

Protein kinase CK2 activity has been implicated as playing important roles in transcriptional regulation, cell cycle progression, and growth control in a variety of organisms (reviewed in [43,68-72]). CK2 is widely expressed in many different cell types, and has been reported to phosphorylate hundreds of different protein substrates. Many of these targets of CK2 phosphorylation are transcriptional regulators; for example, c-Myc, c-Myb, c-Jun, p53, serum response factor, retinoblastoma protein, and RNA polymerase I and II are all phosphorylated by CK2 [43,68-72]. In addition to these transcription factors, a variety of additional regulatory proteins, such as protein kinase A, DNA ligase, DNA topoisomerase, SV-40 large T-antigen, and several eukaryotic translational initiation factors, are also targets of protein kinase II modification [43,68-72]. Phosphorylation by CK2 has been shown to alter the properties of a number of these substrate proteins; for example, CK2 phosphorylation inhibits the ability of c-Jun and of c-Myb to bind to DNA, but enhances the nuclear uptake of SV-40 T-antigen [48,49,73-75]. Consistent with a role for casein kinase as a global regulator of cell metabolism, CK2 appears to be essential for viability in *Saccharomyces*, *Schizosaccharomyces*, and *Dictyostelium* [76-79]. However, little is known about how CK2 activity itself is regulated *in vivo*. CK2 does not appear to respond to known secondary messengers, although CK2 levels are elevated in proliferating cells and some studies have suggested that CK2 activity can be stimulated by peptide growth factors and other mitogenic stimuli [43,68,71,72]. In this context, it is intriguing that SMRT, an important transcriptional cofactor for the nuclear hormone receptors and for certain non-receptor transcription factors, such as PLZF and BCL-6 oncoproteins (e.g. [10]), is also a target of CK2 modification. We have shown here that modification of SMRT by CK2 may influence the ability of SMRT to interact with its nuclear receptor partners. It will require additional work to determine precisely how CK2 phosphorylation alters SMRT activity in the intact organism, and how this modification relates to the mechanisms by which growth stimuli and other cellular regulatory pathways modulate nuclear hormone receptor function under physiological conditions.

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References

1. Apriletti JW, Ribeiro RC, Wagner RL, Feng W, Webb P, Kushner PJ, West BL, Nilsson S, Scanlan TS, Fletterick RJ, Baxter JD. Molecular and structural biology of thyroid hormone receptors. *Clin Exp Pharmacol Physiol* 1998;25(suppl):S2–S11.
2. Mangelsdorf DJ, Thummel C, Beato M, Herrlich P, Schütz G, Umesono K, Blumberg B, Kastner P, Mark M, Chambon P, Evans RM. The nuclear receptor superfamily: The second decade. *Cell* 1995;83:835–839. [PubMed: 8521507]
3. Lazar MA. Thyroid hormone receptors: Multiple forms, multiple possibilities. *Endocr Rev* 1993;14:184–193. [PubMed: 8325251]
4. Meier CA. Regulation of gene expression by nuclear hormone receptors. *J Recept Signal Transduct Res* 1997;17:319–335. [PubMed: 9029499]
5. Tsai MJ, O'Malley BW. Molecular mechanisms of action of steroid/thyroid receptor superfamily members. *Annu Rev Biochem* 1994;63:451–486. [PubMed: 7979245]
6. Damm K, Thompson CC, Evans RM. Protein encoded by v-erbA functions as a thyroid-hormone receptor antagonist. *Nature* 1989;339:593–597. [PubMed: 2733791]
7. Sap J, Muñoz A, Schmitt J, Stunnenberg H, Vennström B. Repression of transcription mediated at a thyroid hormone response element by the v-erb-A oncogene product. *Nature* 1989;340:242–244. [PubMed: 2569164]
8. Sjöberg M, Vennstrom B. Ligand-dependent and -independent trans-activation by thyroid hormone receptor beta-2 is determined by the structure of the hormone response element. *Mol Cell Biol* 1995;15:4718–4726. [PubMed: 7651389]
9. Schulman IG, Juguilon H, Evans RM. Activation and repression by nuclear hormone receptors: Hormone modulates an equilibrium between active and repressive states. *Mol Cell Biol* 1996;16:3807–3813. [PubMed: 8668198]
10. Collingwood TN, Urnov FD, Wolffe AP. Nuclear receptors: Co-activators, corepressors and chromatin remodeling in the control of transcription. *J Mol Endocrinol* 1999;23:255–275. [PubMed: 10601972]
11. Chen JD, Li H. Coactivation and corepression in transcriptional regulation by steroid/nuclear hormone receptors. *Crit Rev Eukaryot Gene Expr* 1998;8:169–190. [PubMed: 9714896]
12. Hassig CA, Schreiber SL. Nuclear histone acetylases and deacetylases and transcriptional regulation: HATs off to HDACs. *Curr Opin Chem Biol* 1997;1:300–308. [PubMed: 9667866]
13. Horwitz KB, Jackson TA, Bain DL, Richer JK, Takimoto GS, Tung L. Nuclear receptor coactivators and corepressors. *Mol Endocrinol* 1996;10:1167–1177. [PubMed: 9121485]
14. Torchia J, Glass C, Rosenfeld MG. Co-activators and co-repressors in the integration of transcriptional responses. *Curr Opin Cell Biol* 1998;10:373–383. [PubMed: 9640539]
15. Chen JD, Evans RM. A transcriptional co-repressor that interacts with nuclear hormone receptors (see comments). *Nature* 1995;377:454–457. [PubMed: 7566127]
16. Hörlein AJ, Näär AM, Heinzel T, Torchia J, Gloss B, Kurokawa R, Ryan A, Kamei Y, Söderström M, Glass CK, Rosenfeld MG. Ligand-independent repression by the thyroid hormone receptor mediated by a nuclear receptor co-repressor (see comments). *Nature* 1995;377:397–404. [PubMed: 7566114]
17. Sande S, Privalsky ML. Identification of TRACs (T3 receptor-associating cofactors), a family of cofactors that associate with, and modulate the activity of, nuclear hormone receptors. *Mol Endocrinol* 1996;10:813–825. [PubMed: 8813722]
18. Seol W, Mahon MJ, Lee YK, Moore DD. Two receptor interacting domains in the nuclear hormone receptor corepressor RIP13/N-CoR. *Mol Endocrinol* 1996;10:1646–1655. [PubMed: 8961273]

19. Zamir I, Harding HP, Atkins GB, Hörlein A, Glass CK, Rosenfeld MG, Lazar MA. A nuclear hormone receptor corepressor mediates transcriptional silencing by receptors with distinct repression domains. *Mol Cell Biol* 1996;16:5458–5465. [PubMed: 8816459]
20. Ito M, Yuan CX, Malik S, Gu W, Fondell JD, Yamamura S, Fu ZY, Zhang X, Qin J, Roeder RG. Identity between TRAP and SMCC complexes indicates novel pathways for the function of nuclear receptors and diverse mammalian activators. *Mol Cell* 1999;3:361–370. [PubMed: 10198638]
21. Rachez C, Lemon BD, Suldan Z, Bromleigh V, Gamble M, Näär AM, Erdjument-Bromage H, Tempst P, Freedman LP. Ligand-dependent transcription activation by nuclear receptors requires the DRIP complex. *Nature* 1999;398:824–828. [PubMed: 10235266]
22. Xu L, Glass CK, Rosenfeld MG. Coactivator and corepressor complexes in nuclear receptor function. *Curr Opin Genet Dev* 1999;9:140–147. [PubMed: 10322133]
23. Chen JD. Steroid/nuclear receptor coactivators. *Vit Horm* 2000;58:391–448.
24. Ayer DE. Histone deacetylases: Transcriptional repression with SINers and NuRDs. *Trends Cell Biol* 1999;9:193–198. [PubMed: 10322454]
25. Kuo MH, Allis CD. Roles of histone acetyltransferases and deacetylases in gene regulation. *Bioessays* 1998;20:615–626. [PubMed: 9780836]
26. Lin RJ, Kao HY, Ordentlich P, Evans RM. The transcriptional basis of steroid physiology. *Cold Spring Harb Symp Quant Biol* 1998;63:577–585. [PubMed: 10384323]
27. Muscat GE, Burke LJ, Downes M. The corepressor N-CoR and its variants RIP13a and RIP13Delta1 directly interact with the basal transcription factors TFIIB, TAFII32 and TAFII70. *Nucleic Acids Res* 1998;26:2899–2907. [PubMed: 9611234]
28. Struhl K. Histone acetylation and transcriptional regulatory mechanisms. *Genes Dev* 1998;12:599–606. [PubMed: 9499396]
29. Suka N, Carmen AA, Rundlett SE, Grunstein M. The regulation of gene activity by histones and the histone deacetylase RPD3. *Cold Spring Harb Symp Quant Biol* 1998;63:391–399. [PubMed: 10384304]
30. Wong CW, Privalsky ML. Transcriptional repression by the SMRT-mSin3 corepressor: multiple interactions, multiple mechanisms, and a potential role for TFIIB. *Mol Cell Biol* 1998;18:5500–5510. [PubMed: 9710634]
31. Wu C. Chromatin remodeling and the control of gene expression. *J Biol Chem* 1997;272:28171–28174. [PubMed: 9353261]
32. Hu X, Lazar MA. The CoNRN motif controls the recruitment of corepressors by nuclear hormone receptors. *Nature* 1999;402:93–96. [PubMed: 10573424]
33. Lin BC, Hong SH, Krig S, Yoh SM, Privalsky ML. A conformational switch in nuclear hormone receptors is involved in coupling hormone binding to corepressor release. *Mol Cell Biol* 1997;17:6131–6138. [PubMed: 9315673]
34. Nagy L, Kao HY, Love JD, Li C, Banayo E, Gooch JT, Krishna V, Chatterjee K, Evans RM, Schwabe JW. Mechanism of corepressor binding and release from nuclear hormone receptors. *Genes Dev* 1999;13:3209–3216. [PubMed: 10617570]
35. Perissi V, Staszewski LM, McInerney EM, Kurokawa R, Krones A, Rose DW, Lambert MH, Milburn MV, Glass CK, Rosenfeld MG. Molecular determinants of nuclear receptor-corepressor interaction. *Genes Dev* 1999;13:3198–3208. [PubMed: 10617569]
36. Hong SH, Wong CW, Privalsky ML. Signaling by tyrosine kinases negatively regulates the interaction between transcription factors and SMRT (silencing mediator of retinoic acid and thyroid hormone receptor) corepressor. *Mol Endocrinol* 1998;12:1161–1171. [PubMed: 9717842]
37. Lavinsky RM, Jepsen K, Heinzel T, Torchia J, Mullen TM, Schiff R, Del-Rio AL, Ricote M, Ngo S, Gemsch J, Hilsenbeck SG, Osborne CK, Glass CK, Rosenfeld MG, Rose DW. Diverse signaling pathways modulate nuclear receptor recruitment of N-CoR and SMRT complexes. *Proc Natl Acad Sci USA* 1998;95:2920–2925. [PubMed: 9501191]
38. Wagner BL, Norris JD, Knotts TA, Weigel NL, McDonnell DP. The nuclear corepressors NCoR and SMRT are key regulators of both ligand- and 8-bromo-cyclic AMP-dependent transcriptional activity of the human progesterone receptor. *Mol Cell Biol* 1998;18:1369–1378. [PubMed: 9488452]
39. Hong, S-K.; Privalsky, ML. SMRT corepressor is regulated by a MEK-1 kinase pathway: inhibition of corepressor function is associated with SMRT phosphorylation and nuclear export. (in press)

40. Hong SH, David G, Wong CW, Dejean A, Privalsky ML. SMRT corepressor interacts with PLZF and with the PML-retinoic acid receptor alpha (RARalpha) and PLZF-RARalpha oncoproteins associated with acute promyelocytic leukemia. *Proc Natl Acad Sci USA* 1997;94:9028–9033. [PubMed: 9256429]
41. Ausubel, FM. Current protocols in molecular biology. Wiley-Interscience; New York: 2000.
42. Bolla, DM.; Edelman, SJ. Protein Methods. Wiley-Liss; New York: 1991.
43. Pinna LA, Meggio F. Protein kinase CK2 ('casein kinase-2') and its implication in cell division and proliferation. *Prog Cell Cycle Res* 1997;3:77–97. [PubMed: 9552408]
44. Tsurumi C, Ishida N, Tamura T, Kakizuka A, Nishida E, Okumura E, Kishimoto T, Inagaki M, Okazaki K, Sagata N, et al. Degradation of c-Fos by the 26S proteasome is accelerated by c-Jun and multiple protein kinases. *Mol Cell Biol* 1995;15:5682–5687. [PubMed: 7565719]
45. Lin R, Beauparlant P, Makris C, Meloche S, Hiscott J. Phosphorylation of IkappaBalpha in the C-terminal PEST domain by casein kinase II affects intrinsic protein stability. *Mol Cell Biol* 1996;16:1401–1409. [PubMed: 8657113]
46. Wisniewski JR, Szewczuk Z, Petry I, Schwanbeck R, Renner U. Constitutive phosphorylation of the acidic tails of the high mobility group I proteins by casein kinase II alters their conformation, stability, and DNA binding specificity. *J Biol Chem* 1999;274:20116–20122. [PubMed: 10400623]
47. Ray-Gallet D, Moreau-Gachelin F. Phosphorylation of the Spi-B transcription factor reduces its intrinsic stability. *FEBS Lett* 1999;464:164–168. [PubMed: 10618498]
48. Jans DA, Jans P. Negative charge at the casein kinase II site flanking the nuclear localization signal of the Sv40 large T-antigen is mechanistically important for enhanced nuclear import. *Oncogene* 1994;9:2961–2968. [PubMed: 8084599]
49. Oelgeschlaeger M, Krieg J, Luscherfirzla JM, Luscher B. Casein kinase II phosphorylation site mutations in C-myb affect DNA binding and transcriptional cooperativity with Nf-M. *Mol Cell Biol* 1995;15:5966–5974. [PubMed: 7565749]
50. Porter CM, Havens MA, Clipstone NA. Identification of amino acid residues and protein kinases involved in the regulation of NFATc sub-cellular localization. *J Biol Chem* 2000;275:3543–3551. [PubMed: 10652349]
51. Alconada A, Bauer U, Hoflack B. A tyrosine-based motif and a casein kinase II phosphorylation site regulate the intracellular trafficking of the varicella-zoster virus glycoprotein I, a protein localized in the trans-Golgi network. *EMBO J* 1996;15:6096–6110. [PubMed: 8947032]
52. Söderström M, Vo A, Heinzel T, Lavinsky RM, Yang WM, Seto E, Peterson DA, Rosenfeld MG, Glass CK. Differential effects of nuclear receptor corepressor (N-CoR) expression levels on retinoic acid receptor-mediated repression support the existence of dynamically regulated corepressor complexes. *Mol Endocrinol* 1997;11:682–692. [PubMed: 9171232]
53. Blok LJ, de Ruiter PE, Brinkmann AO. Androgen receptor phosphorylation. *Endocr Res* 1996;22:197–219. [PubMed: 8875135]
54. Bodwell JE, Webster JC, Jewell CM, Cidlowski JA, Hu JM, Munck A. Glucocorticoid receptor phosphorylation: Overview, function and cell cycle-dependence. *J Steroid Biochem Mol Biol* 1998;65:91–99. [PubMed: 9699861]
55. Katzenellenbogen BS. Estrogen receptors: Bioactivities and interactions with cell signaling pathways. *Biol Reprod* 1996;54:287–293. [PubMed: 8788178]
56. Smith CL. Cross-talk between peptide growth factor and estrogen receptor signaling pathways. *Biol Reprod* 1998;58:627–632. [PubMed: 9510949]
57. Weigel NL. Steroid hormone receptors and their regulation by phosphorylation. *Biochem J* 1996;319:657–667. [PubMed: 8920964]
58. Rowan BG, Weigel NL, O'Malley BW. Phosphorylation of steroid receptor coactivator-1 – identification of the phosphorylation sites and phosphorylation through the mitogen-activated protein kinase pathway. *J Biol Chem* 2000;275:4475–4483. [PubMed: 10660621]
59. Ordentlich P, Downes M, Xie W, Genin A, Spinner NB, Evans RM. Unique forms of human and mouse nuclear receptor corepressor SMRT. *Proc Natl Acad Sci USA* 1999;96:2639–2644. [PubMed: 10077563]

60. Park EJ, Schroen DJ, Yang M, Li H, Li L, Chen JD. SMRTe, a silencing mediator for retinoid and thyroid hormone receptors-extended isoform that is more related to the nuclear receptor corepressor. *Proc Natl Acad Sci USA* 1999;96:3519–3524. [PubMed: 10097068]
61. Wong CW, Privalsky ML. Transcriptional silencing is defined by isoform- and heterodimer-specific interactions between nuclear hormone receptors and corepressors. *Mol Cell Biol* 1998;18:5724–5733. [PubMed: 9742089]
62. Zamir I, Zhang J, Lazar MA. Stoichiometric and steric principles governing repression by nuclear hormone receptors. *Genes Dev* 1997;11:835–846. [PubMed: 9106656]
63. Cohen RN, Wondisford FE, Hollenberg AN. Two separate NCoR (nuclear receptor corepressor) interaction domains mediate corepressor action on thyroid hormone response elements. *Mol Endocrinol* 1998;12:1567–1581. [PubMed: 9773980]
64. Baniahmad A, Dressel U, Renkawitz R. Cell-specific inhibition of retinoic acid receptor- α silencing by the AF2/tau c activation domain can be overcome by the corepressor SMRT, but not by N-CoR. *Mol Endocrinol* 1998;12:504–512. [PubMed: 9544986]
65. Chen JD, Umesono K, Evans RM. SMRT isoforms mediate repression and anti-repression of nuclear receptor heterodimers. *Proc Natl Acad Sci USA* 1996;93:7567–7571. [PubMed: 8755515]
66. Downes M, Burke LJ, Bailey PJ, Muscat GE. Two receptor interaction domains in the corepressor, N-CoR/RIP13, are required for an efficient interaction with Rev-erbA α and RVR: Physical association is dependent on the E region of the orphan receptors. *Nucleic Acids Res* 1996;24:4379–4386. [PubMed: 8948627]
67. Li H, Leo C, Schroen DJ, Chen JD. Characterization of receptor interaction and transcriptional repression by the corepressor SMRT. *Mol Endocrinol* 1997;11:2025–2037. [PubMed: 9415406]
68. Allende JE, Allende CC. Protein kinases. 4. Protein kinase CK2: An enzyme with multiple substrates and a puzzling regulation. *FASEB J* 1995;9:313–323. [PubMed: 7896000]
69. Ghavidel A, Hockman DJ, Schultz MC. A review of progress towards elucidating the role of protein kinase CK2 in polymerase III transcription: Regulation of the TATA binding protein. *Mol Cell Biochem* 1999;191:143–148. [PubMed: 10094403]
70. Guerra B, Issinger OG. Protein kinase CK2 and its role in cellular proliferation, development and pathology. *Electrophoresis* 1999;20:391–408. [PubMed: 10197447]
71. Guerra B, Boldyreff B, Sarno S, Cesaro L, Issinger OG, Pinna LA. CK2: A protein kinase in need of control. *Pharmacol Ther* 1999;82:303–313. [PubMed: 10454208]
72. Pinna LA. Protein kinase CK2. *Int J Biochem Cell Biol* 1997;29:551–554. [PubMed: 9363631]
73. Rihs HP, Jans DA, Fan H, Peters R. The rate of nuclear cytoplasmic protein transport is determined by the casein kinase-II site flanking the nuclear localization sequence of the Sv40 T-antigen. *Embo J* 1991;10:633–639. [PubMed: 1848177]
74. Bousset K, Oelgeschlager MHH, Henriksson M, Schreek S, Burkhardt H, Litchfield DW, Luscherfirlzaff JM, Luscher B. Regulation of transcription factors C-myc, max, and C-myb by casein kinase II. *Cell Mol Biol Res* 1994;40:501–511. [PubMed: 7735324]
75. Lin AN, Frost J, Deng TL, Smeal T, Alalawi N, Kikkawa U, Hunter T, Brenner D, Karin M. Casein kinase-II is a negative regulator of c-Jun DNA binding and Ap-1 activity. *Cell* 1992;70:777–789. [PubMed: 1516134]
76. Rethinaswamy A, Birnbaum MJ, Glover CVC. Temperature-sensitive mutations of the CKA1 gene reveal a role for casein kinase II in maintenance of cell polarity in *Saccharomyces cerevisiae*. *J Biol Chem* 1998;273:5869–5877. [PubMed: 9488724]
77. Roussou I, Draetta G. The Schizosaccharomyces-Pombe casein kinase-II α -subunit and β -subunit – evolutionary conservation and positive role of the β -subunit. *Mol Cell Biol* 1994;14:576–586. [PubMed: 8264625]
78. Ospina B, Nunez A, Fernandezrenart M. Purification of a soluble casein kinase-II from dictyostelium-discoideum lacking the β -subunit – regulation during proliferation and differentiation. *Mol Cell Biochem* 1992;118:49–60. [PubMed: 1488055]
79. Kikkawa U, Mann SKO, Firtel RA, Hunter T. Molecular cloning of casein kinase-II α -subunit from dictyostelium-discoideum and its expression in the life cycle. *Mol Cell Biol* 1992;12:5711–5723. [PubMed: 1448100]

Fig. 1.

Electrophoretic mobility/phosphorylation of SMRT in CV-1 cells. (A) Heterogeneity in the electrophoretic mobility of SMRT in CV-1 cells, and its alteration in response to TGF α treatment. CV-1 cells were transfected by a pSG5-SMRT expression vector and treated or not, as indicated, with 100 ng/ml TGF α . The cells were subsequently lysed, the lysates were resolved by SDS-PAGE, and the electrophoretograms were analyzed by immunoblotting using antibodies directed against the SMRT protein. The positions of the SMRT-related proteins are indicated. (B) Alteration of the electrophoretic mobility of SMRT by alkaline phosphatase treatment. CV-1 cells were transfected with a pCMV-SMRT (751–1495) expression vector and were maintained in the absence of TGF α . The cells were subsequently harvested, and the

lysates were either mock-treated (–CIP) or incubated with 1 unit of calf alkaline phosphatase (+CIP) for 30 min prior to analysis by SDS-PAGE and immunoblotting using antibodies against SMRT. The position of the SMRT proteins are indicated.

Fig. 2.

Phosphorylation *in vitro* of different SMRT subdomains by CV-1 cell lysates. (A) Schematic representation of the SMRT corepressor. A representation of the SMRT protein is presented from N- to C- terminus. The regions involved in transcriptional silencing (Silencing Domains) and the regions involved in interactions with the nuclear hormone receptors (Receptor Interaction Domains, RID-1 and RID-2) are indicated. The amino acid sequence numbering system is derived from the first SMRT molecular clone described (15) and is shown above the schematic. (B) *In vitro* kinase assay employing CV-1 cell lysates and different domains of SMRT. GST fusion proteins representing different portions of SMRT, as indicated under the panel, were synthesized in *E. coli*, immobilized on glutathioneagarose, and incubated with [γ - 32 P] ATP in the presence of CV-1 cell lysates. Comparable amounts of total GST-fusion protein were employed in each incubation. The GST-SMRT proteins were subsequently

washed, were resolved by SDS-PAGE, and the ^{32}P -radiolabel was visualized by Phosphor-Imager analysis. The positions of molecular weight markers are indicated on the left. The most prevalent phosphorylated SMRT protein under these conditions, representing the GST-SMRT (1291–1495) construct, is indicated by an arrow on the right. (C) *In vitro* kinase assay of nested deletions of the SMRT (1291–1495) construct. The same assay as in panel B was repeated, except using as substrate a series of nested deletions of the GST-SMRT (1291–1495) construct. The extent of each deletion is indicated below the panel (3' non-coding denotes a small 3' deletion that did not impinge on the SMRT open reading frame). The GST-SMRT deletions were expressed and were employed at a protein concentration equal to that of the wild-type construct. The position of the phosphorylated SMRT protein is indicated by an arrow.

Fig. 3.

Phosphorylation *in vitro* of SMRT mutants by CV-1 cell lysates. (A) C-terminal amino acid sequence of SMRT. The amino acid sequence of SMRT is presented, using the numbering system derived from the 1495 amino acid version of SMRT as initially described [17]. The region of the SMRT C-terminus deleted from the Δ 1483 mutant is boxed, with potential sites for acid-stable phosphorylation underlined. The consensus sequence for CK2 phosphorylation is indicated by a bracket. (B) Effects of alanine substitutions on phosphorylation of SMRT by CV-1 cell lysates. The amino acids underlined in panel A were individually altered to alanines. The different mutant proteins were then expressed as GST-SMRT (1291–1495) fusions, and tested for the ability to be phosphorylated *in vitro* by incubation with CV-1 cell lysates, as

described in the legend to Fig. 2B. The proteins were subsequently resolved by SDS-PAGE and the ^{32}P -radiolabel was visualized by PhosphorImager analysis. The position of the GST-SMRT (1291–1495) protein is indicated by an arrow. 'wt' refers to the wild-type allele; 'GST' refers to a non-recombinant GST preparation used as a negative control. (C) Quantification of panel B. The ^{32}P incorporated into each SMRT protein in panel B was quantified by PhosphorImager analysis, and the relative phosphorylation, in arbitrary units, is presented.

Fig. 4. Phosphorylation *in vitro* of different SMRT subdomains by purified CK2. (A) ^{32}P -incorporation. GST fusion proteins representing different portions of SMRT, as indicated below the Fig., were synthesized in *E. coli*, were immobilized on glutathione-agarose, and were incubated with [γ - ^{32}P] ATP in the presence (right panel) or absence (left panel) of a purified protein kinase CK2 preparation obtained from recombinant *E. coli*. The proteins were resolved by SDS-PAGE and the ^{32}P -radiolabel was visualized by PhosphorImager analysis. The positions of molecular weight markers are indicated on the left. The position of the most heavily phosphorylated SMRT protein is indicated by an arrow on the right. (B) Coomassie blue staining. The same SDS-PAGE employed for panel A was stained with Coomassie blue to

visualize the locations and amounts of the different GST-SMRT polypeptides. The GST fusion polypeptides synthesized by each construct are indicated by arrows.

Fig. 5. Phosphorylation *in vitro* of SMRT mutants by purified CK2. (A) Effects of alanine substitutions on phosphorylation of SMRT by purified CK2. The different alanine substitutions of GST-SMRT (1291–1495), shown in Fig. 3A, were tested for the ability to be phosphorylated *in vitro* by incubation with purified CK2. The proteins were resolved by SDS-PAGE and the ³²P-radiolabel was visualized by PhosphorImager analysis. The position of the GST-SMRT (1291–1495) protein is indicated by an arrow. (B) Quantification of panel A. The ³²P incorporated into each SMRT protein in panel A was quantified by PhosphorImager analysis, and the relative phosphorylation, in arbitrary units, is presented.

Fig. 6.

Effect of CK2 α over-expression on the electrophoretic mobility and phosphorylation state of SMRT in transfected cells. (A) Effect of overexpression of CK2 α on the electrophoretic mobility of SMRT using SDS-PAGE. Left panel: CV-1 cells were transfected with the pSG5-SMRT (1291–1495) construct together with either pCMV5-CK2 α , or an empty vector, as indicated above the panel. The cells were subsequently lysed and the cell lysates were incubated in the absence or presence of calf alkaline phosphatase (CIP), as indicated. The proteins were resolved by SDS-PAGE and were analyzed by immunoblotting using antibody specific for the SMRT protein. Bars indicate the positions of the SMRT polypeptide before and after treatment. Right panel: a GST-SMRT (1291–1495) fusion protein, purified from *E. coli*, was incubated,

or not, with purified CK2 as indicated, was resolved by SDS-PAGE, and was visualized by Coomassie blue staining. (B) Effect of overexpression of CK2 α on the electrophoretic mobility of SMRT using urea SDS-PAGE. A similar experiment as described in panel A was performed, except that the cell lysates were resolved by in an SDS-urea PAGE system that results in phosphorylated SMRT having a higher mobility than the unmodified polypeptide. The SMRT protein was visualized by immunoblotting, as in panel A. The broken line indicates the mobility of the SMRT protein in the absence of exogenous CK2 α . (C) Alteration in the electrophoretic mobility of wild-type vs. S1492A SMRT in response to CK2 α overexpression. CV-1 cells were transfected with the wild type or S1492A mutant pSG5- SMRT (1291–1495) in the presence or absence of a CK2 α expression vector, as indicated above the panel. Cells were then analyzed as in panel B. The broken line indicates the mobility of the wild-type SMRT protein in the absence of ectopic CK2 α . n.s. refers to a non-specific immuno-reactive band observed under these conditions. (D) Alteration in the electrophoretic mobility of wild-type vs. S1492A SMRT in response to phosphatase treatment. CV-1 cells were transfected as in panel C, the cells were lysed, the cell lysates were subsequently incubated with calf intestine alkaline phosphatase (or mock treated) as indicated. The SMRT protein was resolved and visualized as described for panels B and C. The broken lines indicate the electrophoretic mobility of the SMRT proteins in the absence of CIP treatment.

Fig. 7.

Effects of CK2 phosphorylation on the interaction of SMRT with T3R α . (A) Increased binding of phosphorylated GST-SMRT to T3R α at low T3 concentrations. The GST-SMRT(1291–1495) fusion was synthesized in *E. coli*, immobilized on glutathione-agarose, and treated, or not, with purified CK2 as indicated. The immobilized GST-SMRT protein was washed, and then incubated with ^{35}S -labeled T3R α for 1 h. After additional washing, radiolabeled T3R α remaining bound to the glutathione-agarose was eluted by incubation with excess free glutathione, was resolved by SDS-PAGE, and was quantified by PhosphorImager analysis. The ability of the T3R α to bind to non-recombinant GST was tested in parallel as a negative control. Comparable data was obtained in other experiments employing intermediate hormone

concentrations. (B) Increased binding of phosphorylated GST-SMRT to T3R α at high salt concentrations. An analogous experiment as in panel A was performed in the absence of T3 hormone, but at a range of NaCl concentrations, as indicated below the panel. The average and range of replicate experiments are presented.

Fig. 8. Effects of CK2 on SMRT stability, subcellular localization, and T3R-mediated gene regulation. (A) Effect of CK2 α over-expression on the stability of SMRT protein. CV-1 cells were transfected with the wild type or S1492A mutant SMRT expression vectors, with or without co-transfection of pCMV5-CK2 α . Cycloheximide was added and samples were collected at various time points, as indicated. The cells were lysed and the SMRT protein was detected by SDS-PAGE and immunoblotting. For each time point in the 'chase,' the amount of SMRT protein was quantified by PhosphorImager analysis, and is presented relative to the amount of SMRT protein at time zero (= 100%). The data represent the average of 4 experiments. (B) Subcellular localization of the GFP-SMRT wild type vs. the GFP-SMRT (S1492A) mutant

proteins. CV-1 cells were transfected with either pCMV-GFP-SMRT wild-type or pCMV-GFP-SMRT (S1492A) mutant expression plasmids. The cells were subsequently visualized by confocal microscopy. (C) Effect of CK2 α on transcriptional regulation by T3R α . CV-1 cells were transfected with a suitable T3-responsive reporter construct, pSG5-T3R α , and either pCMV5-CK2 α (closed circles) or an empty vector (open circles). The cells were subsequently maintained in media in the presence of different concentrations of T3 hormone (as indicated below the panel). After 48 h, the cells were harvested, lysed, and the luciferase activity was determined relative to an internal β -galactosidase control. Squares indicates the relative luciferase activity observed in the absence of a pSG5-T3R α construct. Data represents the average and range of duplicate experiments.