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Regulation of SMRT corepressor dimerization and composition by MAP kinase phosphorylation

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ABSTRACT

The SMRT (Silencing Mediator of Retinoid and Thyroid hormone receptors) corepressor mediates gene repression by nuclear receptors and other transcriptional factors. The SMRT protein serves as a key nucleating core that organizes the assembly of a larger corepressor complex. We report here that SMRT interacts with itself to form a protein dimer, and that Erk2, a mitogen-activated protein (MAP) kinase, disrupts this SMRT self-dimerization *in vitro* and *in vivo*. Notably Erk2 phosphorylation also results in a re-organization of the overall corepressor complex, characterized by a reduced sedimentation coefficient, partial release of HDAC3, TBL-1, and TBLR-1, and inhibition of transcriptional repression. We propose that SMRT dimers form the central platform on which additional corepressor components assemble, and that kinase signaling modifies the architecture, composition, and function of this complex. These observations contribute to our understanding of how the SMRT corepressor complex assembles and is regulated during cell proliferation and differentiation.

1. Introduction

The SMRT protein was originally identified based on its ability to mediate repression by thyroid hormone receptors (TRs) and retinoic acid receptors (RARs) (Chen and Evans, 1995; Sande and Privalsky, 1996). Subsequently SMRT, and its closely related paralog NCoR, were shown to serve as corepressors for additional members of the nuclear receptor family, as well as a much wider variety of transcription factors, such as PLZF, BCL-6, NF-κB, ETO-1/2, and c-Myb (Hörlein et al., 1995; Jepsen and Rosenfeld, 2002; Lazar, 2003; Lee et al., 2001; Moehren et al., 2004; Ordentlich et al., 2001; Perissi et al., 2010; Privalsky, 2004; Seol et al., 1996; Stanya and Kao, 2009; Zamir et al., 1996). Notably, SMRT and NCoR do not appear to possess intrinsic repressive properties, but instead are thought to function by recruiting additional polypeptides that mediate the actual molecular events required to inhibit gene transcription (Jepsen and Rosenfeld, 2002; Lazar, 2003; Lee et al., 2001; Moehren et al., 2004; Ordentlich et al., 2001; Perissi et al., 2010; Privalsky, 2004; Stanya and Kao, 2009). Histone deacetylases (HDACs) were among the earliest of these SMRT-tethered auxiliary proteins to be identified (Guenther et al., 2000; Heinzel et al., 1997; Huang et al., 2000; Jones et al., 2001; Kao et al., 2000; Li et al., 2000, 2002; Nagy et al., 1997; Wen et al., 2000). SMRT and NCoR are therefore viewed as molecular platforms that nucleate assembly of the overall corepressor complex, interact through additional contact surfaces with their specific transcription factor partners, and thereby tether the functional corepressor complex to specific target genes. In fact, the corepressor complexes formed in cells are quite large, with estimated sizes of 1.5–2 MDa (Guenther et al., 2000; Li et al., 2000). When purified by coimmunoprecipitation methodologies, these SMRT complexes have been shown to contain an assortment of associated proteins, including HDAC3, TBL1/TBLR1 (ubiquitin ligases implicated in the release of the corepressor complex from target genes on their activation), and GPS2 (a multifunctional protein that helps recruit TBL1/TBLR1) (Guenther et al., 2000; Guenther and Lazar, 2003; Li et al., 2000; Wen et al., 2000; Yoon et al., 2003; Zhang et al., 2002). Still additional corepressor components have been identified by various means, such as mSin3A/B, HDACs 1, 2, 4, and 5, SHARP, MeCP2, KAP-1, JMJD2A, and an assortment of ATP-dependent chromatin remodeling proteins (Jepsen and Rosenfeld, 2002; Lazar, 2003; Lee et al., 2001; Moehren et al., 2004; Ordentlich et al., 2001; Perissi et al., 2010; Privalsky, 2004; Stanya and Kao, 2009). Multiple forms of corepressor complex are likely to exist in cells (e.g. Downes et al., 2000; Jones et al., 2001; Kao et al., 2000); the stoichiometry of the corepressor components that comprise these complexes, and what defines the ability of SMRT and NCoR to assemble into different complexes, have remained incompletely resolved.

The SMRT complex is subject to multiple modes of regulation. Binding of hormone agonists by many nuclear receptors, for

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example, causes the release of SMRT (and its associated auxiliary protein entourage) from the receptor and permits subsequent coactivator recruitment, thereby switching these receptors from transcriptional repressors to activators (Chen and Evans, 1995; Glass and Rosenfeld, 2000; Hörlein et al., 1995; Sande and Privalsky, 1996; Perissi et al., 2004; Zamir et al., 1996). A variety of kinase signaling pathways also converge on the SMRT corepressor complex and modulate its activity (Privalsky, 2001, 2004). For example, stimulation of the epidermal growth factor (EGF) receptor activates a downstream MAP kinase cascade that induces phosphorylation of SMRT, causing its release from its nuclear receptor partners, its redistribution from nucleus to cytoplasm, and derepression of its target genes (Hong et al., 1998; Hong and Privalsky, 2000; Jonas and Privalsky, 2004). This same phenomenon contributes to the pro-differentiation and anti-neoplastic effects of arsenic trioxide in the treatment of acute promyelocytic leukemia (Hong and Privalsky, 2001) and may contribute to anti-androgen resistance in prostate cancer (Eisold et al., 2009).

We have recently reported that the subcellular redistribution of SMRT, and the release of this corepressor from its nuclear receptor partners are regulated by distinct tiers of this MAPK cascade (Jonas et al., 2007). The top two tiers, MEKK1 and MEK1, were found to be the primary mediators of SMRT relocalization to the cytoplasm, whereas the bottom tier kinase, Erk2, proved to be the principal effector of the inhibition of the SMRT/nuclear receptor interaction (Jonas et al., 2007). We now report that SMRT self-associates *in vitro* and *in vivo* to form SMRT:SMRT dimers, and that phosphorylation of SMRT by Erk2 not only induces release of the corepressor complex from its nuclear receptor partners, but also results in a re-organization of the corepressor complex itself. We suggest that this MAPK modulation of SMRT dimer formation is a mechanism through which the function of the SMRT complex can be regulated by pro-proliferative signals in normal and neoplastic cells.

2. Materials and methods

2.1. Molecular clones and cell culture

The origins of the pUC18, pCH110, pADH-Gal4-17-mer, pSG5-Gal4DBD, pCMV5-FLAG-ΔMEKK1, and pCMV-HA-MEK1 (R4F) plasmids were previously described (Hong et al., 1998; Hong and Privalsky, 2000; Wong and Privalsky, 1998). The pSG5-HA-Erk2 (L75P/S153D) vector, which encodes a constitutively active form of Erk2 (Emrick et al., 2001), was created by QuikChange mutagenesis of a wild-type Erk2 clone (Stratagene, La Jolla CA). The pCMV-sGFP-SMRTα (1–2470) expression vector was created by inserting HindIII–XhoI fragments from the parental full-length SMRT cloning vector into HindIII–XhoI digested pCMV-sGFP. The pSG6-Gal4DBD vector was created by inserting a synthetic oligonucleotide (Biosource International, Camarillo, CA) encoding an expanded multiple cloning site into pSG5-Gal4DBD. The pSG6-Gal4DBD-SMRTα expression vector was created by inserting a parental full-length SMRTα (1–2470) into pSG6-Gal4DBD. The pSG5-Myc-SMRTα and pSG5-HA-SMRTα constructs, used for mammalian cell expression or *in vitro* translation, were created using polymerase chain reaction to introduce approximate restriction sites (generally HindIII and XhoI) on the ends of the corresponding open reading frames and by ligating the DNA products into the pSG5-Myc or pSG5-HA vectors. A similar approach, using XbaI and XhoI sites, was used to generate the pGEX-MP constructs employed for expression of GST-fusion proteins in *Escherichia coli*. Base substitution mutations, designed to abolish MAPK sites within the SMRT sequence, were created by using QuikChange.

CV-1 cells (Jensen et al., 1964) were the generous gift of Dr. K.R. Yamamoto (University of California at San Francisco) and were propagated in Dulbecco's modified Eagle's medium (DMEM) formulated with high glucose, L-glutamine, and pyridoxine hydrochloride (Invitrogen, Carlsbad, CA) and supplemented with 5% heat inactivated fetal bovine serum (HIFBS; Hyclone, Logan, UT); cells were maintained at 37 °C in a humidified 5% CO_2 atmosphere. Transient transfections were performed using Effectene reagent, and the manufacturer's protocol (Qiagen, Valencia, CA).

2.2. Co-immunoprecipitation assays

CV-1 cells (8×10^5 cells/plate in a 10 cm plate) were transfected with various combinations of Myc-SMRTα, HA-SMRTα, a constitutively active Erk2 construct, or appropriate amounts of equivalent empty vectors using the Effectene protocol described above. Cells were collected 48 h after transfection and lysed by a 30-min incubation at 4 °C in 1 ml of immunoprecipitation buffer consisting of phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, and 1.5 mM KH_2PO_4) plus 1 mM EDTA, 1.5 mg/ml iodoacetamide, 100 μM Na_3VO_4, 0.5% Triton X-100, 20 mM glycerophosphate, 1 mM NaF, 0.2 mM phenylmethylsulfonyl fluoride, 1× complete phosphatase inhibitor mixture I (EMD Biosciences, Inc., La Jolla, CA), and 1× complete protease inhibitor mixture (Roche Applied Science, Mannheim, Germany). The cell lysates were cleared by centrifugation at 18,000 × *g* at 4 °C. A 50-μl aliquot of each cell lysate was saved, and the remaining lysate was incubated at 4 °C for 1 h with mouse anti-Myc monoclonal antiserum (diluted 1:200; Gamma One Laboratories, Lexington, KY). Next, 40 μl of a 50% slurry of protein G-Sepharose beads (GE Healthcare, Piscataway, NJ) were added, and the samples were incubated overnight at 4 °C on a rotator. The Sepharose beads and any proteins bound to them were collected by centrifugation at 1000 × *g* in a microcentrifuge at 4 °C for 2 min. The beads were washed four times with 1 ml of immunoprecipitation buffer, and any proteins remaining bound to the beads were then eluted by boiling in SDS sample buffer, were resolved by SDS-PAGE using a Tris acetate 3–12% gradient gel system; and were visualized by immunoblotting using mouse anti-HA monoclonal antibody (diluted 1:1000; Gamma One Laboratories, Lexington, KY), horseradish peroxidase-conjugated goat anti-mouse IgG antibody (diluted 1:2000; Bio-Rad, Hercules CA), and the ECL Plus Western blot detection system (GE Healthcare, Piscataway, NJ). The resulting chemiluminescent signal was detected and quantified using a Fluorchem 8900 digital detection system (Alpha Innotech, San Leandro, CA).

2.3. Glycerol gradient fractionation

CV-1 cells (8×10^5 cells/plate in a 10 cm plate) were transfected with pSG5-Myc-SMRTα, a constitutively active Erk2 construct, or appropriate amounts of equivalent empty vectors using the Effectene protocol described above. Cells were collected 48 h after transfection and lysed and cleared as described in Section 2.2. The detergent-solubilized supernatants were subjected to centrifugation in a linear glycerol gradient, consisting of 5–25% glycerol (5.25 ml total) in 10 mM Tris–HCl and 100 mM NaCl. Gradients were centrifuged at 151,000 × *g* at 4 °C for 18 h in a SW41 Ti rotor (Beckman), and fractions of 0.5 ml were collected from the bottom under gravity flow. An aliquot of each fraction was resolved by 3–12% gradient SDS-PAGE gels and analyzed by Western blotting with mouse monoclonal anti-Myc antibody (diluted 1:1000; Gamma One Laboratories) using the protocol in Section 2.2.

2.4. In vitro kinase assays

Appropriate GST-SMRT fusion proteins were expressed in *E. coli* BL21 cells and were immobilized on glutathione-agarose beads as described previously (Hong et al., 1998; Wong and Privalsky, 1998). GST-SMRT proteins were then incubated with 500 units of purified Erk2 (New England Biolabs)) for 18 h at 30 °C in 50 μl of 1× Erk2 kinase buffer (50 mM Tris–HCl, 10 mM $MgCl_2$, 1 mM EGTA, 2 mM dithiothreitol, 0.01% Brij 35, pH 7.5) containing 1 mM ATP, 1× complete phosphatase inhibitor mixture I (EMD Biosciences, Inc., La Jolla, CA), and 1× complete protease inhibitor mixture (Roche Applied Science, Mannheim, Germany). The kinase reactions were terminated by washing the beads by centrifugation four times with 1 ml of ice-cold HEMG buffer (4 mM HEPES [pH 7.8], 100 mM KCl, 0.2 mM EDTA, 5 mM $MgCl_2$, 0.1% NP-40, 10% glycerol, 1.5 mM dithiothreitol) per well. The washed beads with the bound proteins were then used in the *in vitro* protein–protein interaction assays in Section 2.5.

2.5. In vitro protein–protein interaction assay

Glutathione S-transferase (GST)-corepressor protein fusions were produced in *E. coli* strain BL-21 cells transformed by the appropriate pGEX vector (Guan and Dixon, 1991). The bacteria were lysed by sonication and the GST fusion proteins were bound to a glutathione-agarose beads as previously described (Guan and Dixon, 1991). ^{35}S-radiolabeled corepressor fragments were synthesized *in vitro* using a TnT coupled reticulocyte lysate system (Promega, Madison, WI). Each radiolabeled protein (5 μl of TnT product per reaction) was then incubated with the GST fusion protein of interest (typically 10–20 ng) immobilized on 20 μl of glutathione-agarose beads in a total volume of 200 μl of HEMG buffer (4 mM HEPES [pH 7.8], 100 mM KCl, 0.2 mM EDTA, 5 mM $MgCl_2$, 0.1% NP-40, 10% glycerol, 1.5 mM dithiothreitol) at 4 °C. All of the GST-proteins in a given experiment were used at the same concentration. The binding reactions were performed in 1.5 ml Eppendorf tubes on a rotating platform to ensure thorough mixing. After a 1 h of incubation, the beads were washed by centrifugation four times with 1 ml of ice-cold HEMG buffer, and any radiolabeled proteins remaining bound to the immobilized GST fusion proteins were eluted with 50 μl of 10 mM glutathione in 50 mM Tris–HCl [pH 7.8]. The eluted proteins were resolved by SDS-PAGE and were visualized and quantified using a Storm PhosphorImager (Amersham Biosciences, Piscataway, NJ).

2.6. Chromatin immunoprecipitation (ChIP) assays

CV-1 cells (8×10^5 cells/plate in a 10 cm plate) were transfected with 300 ng/plate of pGL4CP-TK 2xGal4, 200 ng/plate of Gal4-DBD-SMRT, 1.5 μg/plate of HA-SMRT, and 500 ng/plate of a constitutively active Erk2 construct (or equivalent empty vector) using the Effectene protocol (Qiagen, Valencia, CA). Forty-eight hours after transfection, formalin was added to the tissue culture medium to a final con-