Arsenic Trioxide Is a Potent Inhibitor of the Interaction of SMRT Corepressor with Its Transcription Factor Partners, Including the PML-Retinoic Acid Receptor α Oncoprotein Found in Human Acute Promyelocytic Leukemia

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The SMRT corepressor complex participates in transcriptional repression by a diverse array of vertebrate transcription factors. The ability to recruit SMRT appears to play a crucial role in leukemogenesis by the PML-retinoic acid receptor α (RAR α) oncoprotein, an aberrant nuclear hormone receptor implicated in human acute promyelocytic leukemia (APL). Arsenite induces clinical remission of APL through a incompletely understood mechanism. We report here that arsenite is a potent inhibitor of the interaction of SMRT with its transcription factor partners, including PML-RAR α . Arsenite operates, in part, through a mitogen-activated protein (MAP) kinase cascade culminating in phosphorylation of the SMRT protein, dissociation of SMRT from its nuclear receptor partners, and a relocalization of SMRT out of the nucleus into the cytoplasm of the cell. Conversely, inhibition of this MAP kinase cascade attenuates the effects of arsenite on APL cells. Our results implicate SMRT as an important biological target for the actions of arsenite in both normal and neoplastic cells.

Nuclear hormone receptors are hormone-regulated transcription factors that bind to cognate hormone, bind to specific DNA sequences, and regulate the expression of adjacent target genes (3, 38, 39, 61). A wide variety of nuclear hormone receptors have been identified that mediate cellular responses to an assortment of different hormone ligands, including thyroid hormone, retinoids, steroids, vitamin D₃, and a number of lipid metabolites. As a consequence, nuclear hormone receptors play key roles in many aspects of metazoan development, differentiation, and homeostasis (3, 38, 39, 61).

Many nuclear receptors are bipolar in function and are able to either repress or activate expression of target genes. Repression is conferred through the ability of nuclear receptors to recruit a complex of auxiliary proteins, designated corepressors, that mediate the molecular events necessary for transcriptional silencing (7, 10, 22, 54, 60, 66). The corepressor protein SMRT and its paralog, N-CoR, play a particularly important role in this process by serving as the principal point of contact of the corepressor complex with the nuclear receptors (6, 12, 21, 31, 50, 51, 67). Conversely, transcriptional activation is associated with release of SMRT/N-CoR from the nuclear receptor, followed by acquisition of a novel set of coactivator proteins (7, 10, 22, 25, 49, 54, 60, 66). Corepressors and coactivators regulate transcription through multiple mechanisms, including modifications of the chromatin template and interactions with the general transcriptional machinery (1, 24, 27, 32, 46, 65).

Thyroid hormone receptors (T3Rs) and retinoic acid recep-

tors (RARs) typically bind to corepressors in the absence of hormone; on addition of hormone agonists, these nuclear receptors physically release from the corepressors and recruit coactivators (23, 33, 44, 47). Intriguingly, however, nonligand signal transduction pathways also play important roles in modulating the interaction of nuclear receptors with corepressors and coactivators. Particularly notable is the ability of protein kinase signaling pathways, such as those represented by the epidermal growth factor (EGF) receptor or by protein kinase A, to interfere with the SMRT-nuclear receptor interaction and to counteract transcriptional repression (19, 30, 62). Activation of the EGF receptor, for example, virtually abolishes the ability of SMRT to interact with T3Rs and eliminates T3R-mediated repression, even in the absence of thyroid hormone (19). The inhibitory effects of EGF receptor signaling on SMRT function are also observed with RARs and are mediated, at least in part, through a mitogen-activated protein (MAP) kinase cascade that culminates in phosphorylation of the SMRT protein, dissociation of SMRT from the nuclear receptor partner, and a relocalization of SMRT out of the nucleus and into the cytoplasm of the cell (20).

Aberrations in the interaction of nuclear receptors with corepressors can result in endocrine and neoplastic disorders. For example, human acute promyelocytic leukemia (APL) is associated with chromosomal translocations that fuse ectopic open reading frames to the DNA and hormone-binding domains of RAR α (8, 11, 29, 41, 45). The most common form of translocation in APL results in the synthesis of a PML (promyelocytic leukemia)-RAR α chimeric polypeptide. The PML-RAR α chimera requires significantly higher retinoid concentrations to release from corepressor than does the wild-type RAR α (15–17, 34, 35, 42). This defect in retinoid signaling plays an important role in generating the leukemic phenotype,

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and treatment of PML-RAR α leukemic cells with supraphysiological levels of retinoic acid leads to release of corepressor from the PML-RAR α and differentiation of the leukemic cell (15–17, 18, 35).

The ability of high concentrations of retinoic acid to induce differentiation in PML-RAR α leukemias has been employed clinically to treat human APL (8, 11, 29, 41, 45). Recently, it has been recognized that arsenic trioxide acts synergistically with retinoic acid to induce long-term remissions in APL and can be effective in retinoid-resistant cases of APL (5, 14, 28, 53, 58). The precise molecular mechanisms behind the effects of arsenite in APL cells are not fully understood but are thought to involve both an apoptotic and a differentiation response (4, 14, 26, 28, 52, 59, 63, 70). Notably, arsenic trioxide is a strong inducer of MAP kinase signaling cascades in many cell contexts (2, 9, 36, 37, 40, 48). Given the ability of growth factor receptors to abolish SMRT function through a MAP kinase cascade (20), we tested whether arsenic trioxide treatment might affect SMRT function in a similar fashion. We report here that treatment with arsenic trioxide results in a profound inhibition in the ability of SMRT to interact with its transcription factor partners, such as T3Rs and RARs. At least one component of this inhibition of SMRT function appears to be mediated by an arsenite-mediated activation of a MAP kinase cascade, resulting in hyperphosphorylation of the corepressor and an alteration in its subcellular distribution. Notably, arsenite induces a similar and rapid inhibition of the ability of SMRT to interact with the PML-RAR α oncoprotein in APL cells, and the ability of SMRT to induce differentiation and apoptosis in these cells is antagonized by inhibitors of MAP kinase cascade signaling. We propose that the effects of arsenite on normal cells and in APL are, at least in part, conferred through a MAP kinase cascade-mediated inhibition of corepressor function.

MATERIALS AND METHODS

Plasmid constructs. The pCMV-SMRT-C vectors were constructed by inserting *EcoRI* fragments from the previously described pSG-5 TRAC-1 constructs into a pCR3.1 vector (Invitrogen, Carlsbad, Calif.) (50). Construction of the mammalian two-hybrid vectors for various SMRT and T3R α derivatives was as previously described (19, 20, 64). The pSG5-GAL4-activation domain (AD)-RAR α and retinoid X receptor α (RXR α) vectors were constructed by inserting *EcoRV* and *XhoI* fragments, generated by PCR, into the pSG5-GAL4-AD vector. The green fluorescent protein (GFP)-SMRT vector was constructed by inserting the *BsrGI*-to-*EcoRI*(blunt) fragment from pSG5-SMRT into the *BsrGI*-*HindIII*(blunt) sites on a CMV-GFPYGBH vector (20). Expression vectors for full-length mitogen-activated kinase kinase 1 (MEKK-1) and mitogen-activated kinase 1 (MEK-1) expression plasmids were obtained from Chris Jamieson (University of California, San Francisco).

Transient transfections. CV-1 cells transfections were performed by a Lipofectin-mediated method using the general protocol recommended by the manufacturer (GIBCO/BRL Life Technologies, Rockville, Md.). Approximately 4×10^5 cells were transfected with 50 ng of pSG5-T3R α or pSG5-*v-erbA* plasmid (designated T3R-vA), 100 ng of pCMV-lacZ or pCH110 as an internal control, and 100 ng of a ptk-luc-TRE reporter, together with expression vectors for the various signal transducers tested here (equal quantities of the equivalent empty vectors were substituted where appropriate) (20). The cells were transferred to serum-free Dulbecco's modified Eagle's medium (DMEM) 24 h after transfection and were harvested 24 h later. The luciferase and β -galactosidase assays were performed as previously described, and the relative luciferase activity (expressed as the ratio of luciferase to β -galactosidase activity) was determined (20).

Mammalian two-hybrid assays. Exponentially growing CV-1 cells (7×10^4 cells per well in 12-well culture plates) were transiently transfected by the Lipofectin method using 25 ng of the appropriate pSG5-GAL4DBD vector, 100 ng of the appropriate pSG5-GAL4AD vector, 100 ng of the pGL2-GAL4(17-mer)-luciferase reporter, 100 ng of the pCMV-lacZ internal control, and an appro-

priate expression vector for the various signal transducers tested here (or equal quantities of the equivalent empty vector where appropriate) (19, 20). The cells were transferred to serum-free DMEM 24 h after transfection and were harvested an additional 24 h later. The luciferase and β -galactosidase assays were performed as previously described, and the relative luciferase activity (expressed as the ratio of luciferase to β -galactosidase activity) was determined for each sample (19, 20).

Immunoblotting. CV-1 cells (7×10^4 per well) were transfected with the appropriate expression vectors as indicated for each experiment, harvested 48 h after transfection by scraping, and lysed by mixing the cells with sodium dodecyl sulfate (SDS)-electrophoresis sample buffer. The lysates were then sonicated to reduce viscosity, boiled for 5 min, and loaded into an SDS-7.5% polyacrylamide-0.3% bis-acrylamide gel. After electrophoresis, the proteins were electrophoretically transferred to a nitrocellulose membrane. The membrane was incubated in blocking buffer (2.5% nonfat dry milk in TBST [0.1% Tween 20, 150 mM NaCl, 10 mM Tris-Cl; pH 7.6]) for 1 h and then incubated with appropriate primary antibodies (diluted in 2.5% bovine serum albumin in TBST) for 1 h. The membranes were next washed extensively with TBST and incubated with appropriate secondary antibodies (either horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse antibody, diluted 1:2,000; Affinity BioReagents, Golden, Colo.). After extensive washing with TBST, the chemiluminescent Western detection system was employed for visualization of the immunoreactive proteins as specified by the manufacturer (NEN Life Science, Boston, Mass.).

Phosphorylation-dephosphorylation assay. CV-1 cells (2.5×10^5) were transfected with the pCMV-SMRT-C and arsenite or with the MEKK-1 expression vector, as indicated for the individual experiments. The cells were harvested 48 h later by scraping and centrifugation in 150 μ l of whole-cell extraction buffer (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, 0.5 mM phenylmethylsulfonyl fluoride, and complete protease inhibitor cocktail [Boehringer-Mannheim, Mannheim, Germany]). Lysates were then incubated in the presence or absence of 0.5 U of calf intestinal alkaline phosphatase (New England Biolabs [NEB], Beverly, Mass.) for 30 min at 30°C. The incubations were terminated by mixing the samples with SDS sample buffer; the samples were boiled for 5 min, loaded into an SDS-7.5% polyacrylamide-0.3% bis acrylamide gel, and subjected to electrophoresis and immunoblotting as described above.

Coimmunoprecipitation assay. Approximately 10^7 NB-4 cells were treated with either 1 μ M all-*trans* retinoic acid or 0.1 or 1 μ M As₂O₃ for various times. Whole-cell lysates were prepared by gently sonicating the cells in 1 ml of immunoprecipitation (IP) buffer (1 $\times$ phosphate-buffered saline, 1 mM EDTA, 1.5 mg of iodoacetamide per ml, 100 μ M Na₃VO₄, 0.5% Triton X-100, 20 mM β -glycerolphosphate, 0.2 mM phenylmethylsulfonyl fluoride, and 1 $\times$ complete protease inhibitor cocktail). After clarification by a 15-min centrifugation in a microfuge at 4°C, the resulting supernatant was incubated for an additional 3 h at 4°C with either 2.5 μ l of anti-SMRT antibody (PA1-843, diluted 1:400; Affinity Bioreagent), 2.5 μ l of anti-RAR α antibody, or 10 μ l of anti-PML antibody (Santa Cruz Biotechnology, Santa Cruz, Calif.). Fifteen microliters of either protein A-Sepharose (as a 50% slurry in IP buffer; Sigma Chemical Co., St. Louis, Mo.) or protein G-Sepharose was then added, and the samples were incubated with continuous mixing for 4 h more at 4°C. The protein A-Sepharose matrix was extensively washed with IP buffer, and any proteins remaining bound to it were eluted with SDS sample buffer and detected by Western analysis.

Laser-scanning confocal microscopy. Approximately 8×10^4 CV-1 cells were seeded in a chambered cover glass cell culture system (Nalge-Nunc, Rochester, N.Y.). The cells were then transfected with the pCMV-GFP-SMRT vector together with MEKK-1 expression vector (or an equivalent empty vector as a control) using the Effectene procedure (Qiagen, Hilden, Germany). A day after transfection the cells were transferred into serum-free DMEM containing 15 μ M As₂O₃ and were incubated for an additional 24 h. The subcellular location of the GFP-SMRT fusion polypeptide was visualized using a Leica TCS-SP UV/Ar/Kr laser-scanning confocal microscope, employing excitation at 488 nm and detection at 500 to 540 nm. The effect of arsenite on the subcellular location of TFIIB, employed as a control, was analyzed in a similar fashion but using an indirect immunofluorescence protocol: CV-1 cells were treated with arsenite for 24 h (or left untreated) as described above, washed, fixed with 3% formaldehyde, permeabilized with 0.5% Triton X-100 in 1 $\times$ PBS, and visualized using anti-TFIIB antiserum (sc-225; Santa Cruz Biotechnology) and rhodamine-conjugated anti-rabbit immunoglobulin G (Cappel, West Chester, Pa.).

Analysis of level of phosphorylated MEK-1/2. CV-1 cells were grown in a 37°C humidified atmosphere containing 5% CO₂ in DMEM supplemented with 10% fetal bovine serum. Cells were maintained in serum-free medium for at least 16 h prior to treatment with As₂O₃. As₂O₃ was added to the medium to a final concentration of 20 μ M. Cells were harvested by adding hot SDS sample buffer,

and the amount of phosphorylated MEK-1/2 was examined by Western analysis using a PhosphoPlus MEK-1/2 (Ser217/221) antibody kit (NEB).

Analysis of cell surface marker, apoptosis, and DNA content. A fluorescence-activated cell sorting (FACS) flow cytometer was calibrated with CaliBRITE beads prior to use (Becton Dickinson, Mountain View, Calif.). The amount of myeloid cell surface marker CD11c on the NB-4 cells was determined by flow cytometry analysis using phycoerythrin-conjugated antibody from Becton Dickinson. The percent positive cells was quantified by FACSscan analyzer (CellQuest program; Becton Dickinson). A terminal deoxynucleotidyl transferase labeling (TUNEL) reaction was performed on the NB-4 cells according to the manufacturer's instructions (Roche Molecular Biochemical Company, Indianapolis, Ind.) to measure apoptosis-associated DNA fragmentation. Cells were analyzed by flow cytometry using a FACSscan. All data were collected and analyzed by CellQuest software. Cells were also analyzed for DNA content by flow cytometry analysis of propidium iodide-stained nuclei.

RESULTS

Arsenic trioxide strongly and specifically interferes with the ability of SMRT to interact with nuclear receptors. We wished to determine if arsenic trioxide could alter the ability of SMRT to interact with its nuclear receptor partners. To address this question, we first employed a mammalian two-hybrid assay. For this assay, a GAL4DBD-SMRT fusion construct, a GAL4-AD-T3R fusion construct, and a luciferase reporter bearing GAL4(17-mer) binding sites were introduced separately or in combination into CV-1 cells. A strong activation of the GAL(17-mer) luciferase reporter was observed when all three constructs were introduced together, reflecting the ability of the SMRT and T3R sequences to interact and thereby reconstitute a functional GAL4 transcription factor (Fig. 1A). This two-hybrid assay appeared to be a valid measure of the SMRT-T3R interaction: (i) each of the fusion constructs was transcriptionally inactive when introduced separately; (ii) T3 thyroid hormone abolishes the T3R interaction with SMRT both in vitro and in the mammalian two-hybrid system; (iii) mutants of T3R (e.g., P156R) or other nuclear receptors, such as vitamin D3 receptor, that do not interact with SMRT in vitro do not interact in the two-hybrid system; (iv) unrelated proteins or SMRT mutants (SMRT Δ RID) that fail to interact with T3R in vitro do not interact with T3R in the two-hybrid system; and (v) mutants of T3R that bind SMRT in a hormone-independent fashion in vitro (e.g., T3R-vA) exhibit a hormone-independent interaction with SMRT in the two-hybrid assay (Fig. 1A).

As reported previously (19, 20), the ability of SMRT to interact with T3R in the mammalian two-hybrid assay was severely compromised by cointroduction of v-Erb B, a constitutively activated derivative of the EGF receptor (Fig. 1B). The inhibitory effect of v-Erb B on the SMRT-T3R interaction was also observed for the T3R-vA mutant, demonstrating that the inhibitory actions of v-Erb B do not require ligand binding by the nuclear hormone receptor (Fig. 1B). We have shown that these inhibitory effects are mediated by a MAP kinase cascade that operates downstream of the EGF receptor/v-Erb B protein, resulting in phosphorylation of the SMRT corepressor (20). The individual components of this kinase cascade, such as Ras, MEKK-1, and MEK-1, can substitute for v-Erb B in this regard and when introduced into the CV-1 cells also inhibited the SMRT-T3R interaction (Fig. 1B).

Notably, treatment of the CV-1 cells with low levels of arsenic trioxide resulted in a similar and potent inhibition of the two-hybrid interaction between SMRT and T3R (Fig. 1C). To exclude the possibility that arsenic was inhibiting the expres-

sion, stability, or function of the GAL4DBD or GAL4AD fusion, rather than interfering with the SMRT-T3R interaction itself, we assayed the effects of arsenic trioxide on the two-hybrid interaction between T3R and RXRs; RXRs are heterodimer partners for T3Rs, and the two receptor classes can physically associate in vitro and in vivo (3, 38). T3R exhibited a strong interaction with RXR in our mammalian two-hybrid system which was unaltered by arsenic trioxide treatment, in clear contrast to the arsenite-mediated inhibition observed for the T3R/SMRT interaction (Fig. 1C); note that the expression vector backbones, promoters, Kozak sequences, GAL4DBD and AD sequences, and reporter vectors were the same for both assays. Similarly, arsenic trioxide had little or no effect on expression of a β -galactosidase reporter lacking GAL4-binding sites and employed as an internal control, indicating that the effects we observed under these conditions were not simply due to a generic arsenite-mediated toxicity or an overall inhibition of transcription or translation (data not shown). An inhibition of the SMRT-T3R interaction by arsenite was also observed for the T3R-vA mutant, further suggesting that the effects of arsenite did not require ligand binding by the T3R (Fig. 1C). We conclude that the interaction between SMRT and T3R is inhibited by arsenic trioxide in a manner at least superficially resembling the inhibitory effects of the EGF receptor/MAP kinase cascade pathway.

Activation of the EGF receptor/MAP kinase cascade interferes not only with the interaction of SMRT with T3R but also with the interaction of SMRT with a variety of its other transcription factor partners, including RARs and PLZF, a nonreceptor transcriptional repressor (19, 20). Paralleling its effect on T3R, arsenic trioxide strongly interfered with the mammalian two-hybrid interaction between SMRT and RAR α but not with the control two-hybrid interaction between RXR and RAR α (Fig. 2A). The ability of SMRT to interact with PLZF in the two-hybrid system was also inhibited by arsenite treatment (Fig. 2B). We conclude that arsenic trioxide treatment, in common with MAP kinase cascade signaling, interferes with the ability of SMRT to interact with an assortment of receptor and nonreceptor transcription factors.

The inhibitory effects of arsenic trioxide on SMRT function in CV-1 cells are mediated, in part, through activation of a MAP kinase cascade and result in phosphorylation of the corepressor. In many cell types, arsenic trioxide acts as a strong inducer of MAP kinase cascade signaling (2, 9, 36, 37, 40, 48). Given the overall similarity between the inhibitory effects of arsenic trioxide and the inhibitory effects of MEKK-1 or MEK-1, we next explored whether the effects of arsenic trioxide on SMRT function were, in fact mediated through this (or an analogous) MAP kinase cascade. Consistent with this hypothesis, arsenic trioxide treatment resulted in a rapid increase in the phosphorylation state of MEK-1/2, suggestive of an activation of MAP kinase cascade signaling in these cells (Fig. 3A). We have reported that chemical inhibitors of MEK-1/2 counteract the effects of MAP kinase cascade signaling and stabilize the SMRT-nuclear receptor interaction (20). Significantly, the inhibitory effects of arsenite in the SMRT two-hybrid assay were also counteracted by MEK-1/2 inhibitors, such as PD98059 (Fig. 3B), whereas MEK-1/2 inhibitors had little or no effect on the SMRT-T3R two-hybrid interaction in the absence of arsenite (reference 20 and data not shown) and

FIG. 1. Effect of kinase signaling and As_2O_3 on the interaction between T3R α and SMRT. (A) Effect of hormone on SMRT interaction with nuclear hormone receptors in a mammalian two-hybrid assay. A pSG5 plasmid containing either GAL4DBD alone (empty DBD), a GAL4DBD fused with the receptor interaction domain of SMRT (amino acids 751 to 1495; DBD-SMRT), or a GAL4DBD fused with a region of SMRT lacking the receptor interaction domain (amino acids 751 to 1074; DBD-SMRT Δ RID) were cotransfected into CV-1 cells with a GAL4(17-mer) thymidine kinase promoter-luciferase reporter and a pSG5-GAL4AD construct by a calcium phosphate precipitation method. Cotransfected GAL4AD constructs are indicated below the panels (empty, GAL4AD domain alone; T3R, GAL4-AD fused with T3R α ligand-binding domain; P156R, GAL4-AD fused with a T3R α construct with a mutation that disrupts SMRT association; T3R-vA, GAL4-AD fused with the analogous region of the v-Erb A mutant form of T3R α ; v-Erb B, MEKK-1, and MEK-1. A pSG5 plasmid expressing a GAL4DBD alone (empty DBD) or a GAL4DBD fused with the receptor interaction domain of SMRT (DBD-SMRT) was transiently cotransfected into CV-1 cells by Lipofectin, with either an empty pSG5-GAL4AD vector, the pSG5-GAL4AD fused to the ligand-binding domain of T3R α , or T3R-vA. In addition, the cells were cotransfected with 100 ng

only a very small effect on reporter gene expression in the empty-vector system (Fig. 3B). These results suggest that the arsenite antagonizes SMRT function, at least in part, through a MAP kinase cascade involving MEK-1 or a comparable MAP kinase kinase intermediate.

SMRT is a substrate for phosphorylation by several kinases operating within the MAP kinase cascade, and these phosphorylation events are manifested as a change in the electrophoretic mobility of SMRT and a redistribution in the subcellular location of SMRT, as well as a loss in the ability of SMRT to interact with its transcription factor partners (20). We therefore examined whether SMRT was indeed phosphorylated in response to arsenic trioxide, as revealed by a change in its mobility on SDS-polyacrylamide gel electrophoresis (PAGE). Treatment of CV-1 cells with arsenic trioxide led to a reproducible decrease in the electrophoretic mobility of SMRT in a manner characteristic of phosphorylation (Fig. 4A). This arsenite-mediated change in the electrophoretic properties of SMRT was comparable to that observed in response to activation of the MAP kinase cascade by introduction of MEKK-1 (Fig. 4A) and, consistent with this SMRT modification representing phosphorylation, was reversed by treatment with calf intestine alkaline phosphatase (Fig. 4B). This presumptive phosphorylation was detectable within 15 to 30 min of arsenite addition (Fig. 4C), paralleling the rapid increase in MEK-1/2 phosphorylation seen in Fig. 3A. Although treatment with high arsenite concentrations decreased the overall level of SMRT protein expression in the CV-1 cells (data not shown), this decrease was not typically observed at the arsenite concentrations employed in these experiments; conversely, the introduction of MEKK-1, but not MEK-1, increased the levels of SMRT protein (e.g., Fig. 4A and B).

We next examined the effects of arsenite on the subcellular location of SMRT by use of a GFP-SMRT fusion construct. In agreement with prior identifications of SMRT as a nuclear protein, the GFP-SMRT protein was located virtually exclusively in the nucleus of unstimulated, transfected cells, forming a punctate pattern of fluorescent spots superimposed on a diffuse fluorescent nucleoplasm (Fig. 5A) (13, 20, 57). Coincidence of the punctate spots into a smaller number of larger dots per nucleus and, in many of these cells, a shift of the GFP-SMRT out of the nucleus and into a perinuclear or cytoplasmic distribution (Fig. 5B). Notably, arsenic trioxide treatment resulted in a similar change in GFP-SMRT localization, with a reduction in the number but an increase in the

of expression plasmid for either v-Erb B, v-Ras, a full-length MEKK-1, or a MEK-1 clone, as indicated at the bottom. The cells were incubated in the absence of T3, and the luciferase activity was determined and normalized to β -galactosidase activity for each sample. (C) Inhibition of the mammalian two-hybrid interaction between SMRT and T3R α by As_2O_3 . The protocol for panel B was repeated, but in the absence or presence or absence of 20 μM As_2O_3 , as indicated at the bottom. A control two-hybrid interaction between a DBD-SMRT construct and an AD-T3R α construct was assayed under the same conditions to detect any nonspecific effects of arsenite on the two-hybrid system (see the text). The scale for the v-Erb A interaction with SMRT (right axis) differs from that for the other three panels (left axis).

FIG. 2. Effect of As₂O₃ on the interaction between SMRT and RAR α and between SMRT and PLZF. (A) Inhibition of the mammalian two-hybrid interaction between SMRT and RAR α by As₂O₃. A protocol similar to that for Fig. 1C was used, but with pSG5-GAL4AD fused to the ligand-binding domain of RAR α in place of the T3R construct. The cells were left untreated or treated with 15 μ M As₂O₃ or with all-*trans* retinoic acid (ATRA). (B) Inhibition of the interaction between SMRT and PLZF by As₂O₃. The same protocol was used, but with pSG5-GAL4AD fused to the PLZF open reading frame in the absence or presence of 15 μ M As₂O₃. Data are averages and standard deviations of duplicate experiments.

overall size of the fluorescent nuclear dots and/or a shift to a more perinuclear or cytoplasmic distribution (Fig. 5C and D). Approximately 59% of the fluorescent-positive, arsenite-treated cells displayed this redistribution of GFP-SMRT (both responsive and nonresponsive cells are illustrated in Fig. 5C and D), whereas virtually all of the CV-1 cells transfected with MEKK-1 underwent GFP-SMRT relocalization. We do not know the molecular basis for this heterogeneity in the CV-1 cell response to arsenite but presume that it reflects some corresponding heterogeneity in the metabolic state of these cells. The overall appearance of the nuclei, and the subcellular localization of an unrelated nuclear protein, TFIIB, did not change in response to arsenite (Fig. 5E and F and data not shown), indicating that the redistribution of SMRT was a specific response to arsenic trioxide and was not due to an overall loss of nuclear integrity. Taken as a whole, our results support our hypothesis that the inhibitory effects of arsenic trioxide on SMRT are mediated, at least in part, through a MAP kinase cascade that results in phosphorylation and redistribution of the corepressor.

FIG. 3. Effect of As₂O₃ treatment on phosphorylation of MEK-1/2 (A) and effect of a MEK-1 inhibitor on SMRT-T3R α interaction (B). (A) CV-1 cells were treated with As₂O₃ for the specified times. The cells were subsequently lysed, and the extracts were resolved by SDS-PAGE and analyzed by immunoblotting using antibody specific for bulk MEK-1/2 or for phosphorylated MEK-1/2 (Ser217/221). (B) The effect of As₂O₃ on the SMRT-T3R α two-hybrid interaction was tested as described for Fig. 1C, but in the absence or presence of 5 μ M PD98059, as indicated at the bottom.

Arsenite can induce both apoptosis and an incomplete differentiation response in APL-derived NB-4 cells. The ability of arsenite to antagonize SMRT function in CV-1 cells led us to examine the effects of arsenite in APLs. We employed NB-4 cells, which are derived from a human APL and which synthesize the PML-RAR α chimeric oncoprotein. NB-4 cells can be induced to differentiate by treatment with high levels of all-*trans* retinoic acid, and this response is thought to be mediated by a hormone-mediated release of corepressor from the PML-RAR α protein (15–17, 35). Consistent with these prior observations, addition of all-*trans* retinoic acid to our NB-4 cell cultures resulted in a strong differentiation response which could be detected using FACS analysis with an antibody directed against CD-11c, an early differentiation marker (Fig. 6A), or a colorimetric assay for leukocyte alkaline phosphatase, a late differentiation marker (data not shown) (4, 14). In contrast to these robust differentiation responses, all-*trans* retinoic acid treatment induced little or no apoptosis in the NB-4 cells, as determined by TUNEL assay (Fig. 6B).

Unlike all-*trans* retinoic acid, arsenic trioxide has been reported to induce a dual response in NB-4 cells, exhibiting elements both of differentiation and apoptosis (4, 14, 26, 28, 52, 59, 63, 70). Consistent with these studies, treatment of our NB-4 cells with arsenite resulted in both a substantial increase in the number of cells expressing the CD-11c early differentiation marker (Fig. 6A) and a strong apoptotic response, with

FIG. 4. Phosphorylation of SMRT induced by As_2O_3 . (A) SMRT immunoblot of CV-1 cells after MEKK-1 and As_2O_3 treatments. pCMV-SMRT-C (a construct limited to expressing amino acids 751 to 1495 of SMRT) was introduced into the CV-1 cells together with an empty vector (None) or a MEKK-1 expression vector; alternatively, the cells were treated with $20 \mu\text{M}$ As_2O_3 . Whole-cell lysates were prepared from the cells, and the extract was analyzed by Western blotting using antibody specific for SMRT protein. (B) Phosphatase treatment of SMRT reverses the change in electrophoretic mobility induced by arsenite. CV-1 cells were transfected together with pCMV-SMRT-C (amino acids 751 to 1495) and either empty vector or the MEKK-1 expression vector. After As_2O_3 treatment as in panel A, whole-cell lysates were prepared and were incubated without or with 1 U of calf intestinal alkaline phosphatase (CIAP) for 30 min. (C) SMRT shifts in mobility rapidly after arsenite treatment. The experiment used for panel A was repeated, with samples taken and analyzed at the time points listed.

over 60% of the NB-4 cell population becoming positive in the TUNEL assay (Fig. 6B). The arsenite-mediated differentiation response was somewhat weaker than that observed with all-*trans* retinoic acid treatment (e.g., Fig. 6A) and appeared to be restricted to early differentiation: unlike all-*trans* retinoic acid, arsenite treatment did not increase expression of the late differentiation marker leukocytic alkaline phosphatase in these cells (data not shown). Combined treatment with all-*trans* retinoic acid and arsenite resulted in no alteration in the differentiation or apoptotic responses beyond that observed with these compounds when tested individually (data not shown).

The effects of arsenic trioxide on APL cell differentiation and apoptosis are mediated, in part, through a MEK-1 intermediate and may operate by disrupting the interaction between SMRT and the PML-RAR α oncoprotein. PML-RAR α -mediated leukemogenesis is closely linked to the ability of PML-RAR α to bind corepressor and to act as a dominant negative repressor of transcription; release of corepressor from PML-RAR α in response to supraphysiological concentrations of all-*trans* retinoic acid results in differentiation of the APL cells and remission of disease (15–17, 35). Might the ability of arsenite to induce APL cell differentiation reflect a corresponding ability of arsenite to inhibit the interaction of SMRT

with PML-RAR α ? We first tested whether the effects of arsenite on the NB-4 cells involve an activation of a MAP kinase cascade similar to that observed in CV-1 cells. Consistent with this hypothesis, arsenic trioxide induced a transient increase in MAP kinase cascade signaling in the NB-4 cells, as demonstrated by an increase in phosphorylation of MEK-1/2 (Fig. 6C); MEK-1/2 phosphorylation in NB-4 cells peaked somewhat more rapidly than that in CV-1 cells and then returned to baseline (Fig. 3), presumably reflecting differences in signal induction and attenuation in the two different cell lines. Is this activation of this MAP kinase cascade involved in mediating the effects of arsenite on NB-4 cell differentiation and apoptosis? Indeed, treatment of the NB-4 cells with the MEK-1/2 inhibitor U0126 significantly impaired the ability of arsenite to induce differentiation and, to a lesser degree, apoptosis in these cells (Fig. 6A and B). In contrast, U0126 did not inhibit the differentiation of NB-4 cells in response to all-*trans* retinoic acid treatment (Fig. 6A).

We next examined whether arsenic trioxide treatment of NB-4 cells results in an inhibition of the interaction of SMRT with PML-RAR α . The NB-4 cell line cannot be efficiently transfected with exogenous DNA, precluding use of a two-hybrid interaction system. We therefore employed a coimmunoprecipitation technique. We exposed NB-4 cells to a mock treatment, to arsenic trioxide, and to retinoic acid. We then lysed the cells, immunoprecipitated the lysates with SMRT-directed antisera, and analyzed the immunoprecipitates by Western analysis using antibodies to PML-RAR α . In the absence of treatment, PML-RAR α was efficiently coprecipitated with SMRT, indicative of a stable interaction between these two proteins (Fig. 7A, top). Notably, treatment of the NB-4 cells with either all-*trans* retinoic acid or with arsenite resulted in disruption of this PML-RAR α -SMRT interaction, manifested as a loss of PML-RAR α from the SMRT immunoprecipitate (Fig. 7A, top).

Arsenic trioxide treatment has been reported to induce degradation of PML-RAR α in NB-4 cells (4, 14, 43, 52, 63, 69). To test whether degradation of either PML-RAR α or SMRT could account for the loss of coimmunoprecipitation of these two proteins, we modified our immunoprecipitation-blotting procedure to examine the effects of arsenite and all-*trans* retinoic acid on the overall levels of PML-RAR α and of SMRT in the NB-4 cells. Neither arsenite nor all-*trans* retinoic acid altered the overall levels of SMRT protein under the conditions employed here (Fig. 7A, middle). However, the bulk levels of PML-RAR α in these cells did decline after extensive arsenite treatment (Fig. 7A, bottom). We therefore performed a more detailed time course experiment, comparing the effects of arsenite on the interaction of SMRT with PML-RAR α to the effects of arsenite on PML-RAR α stability (Fig. 7B and C). Consistent with prior studies, we detected a decline in the overall level of PML-RAR α in the NB-4 cells beginning at 4 h of arsenite treatment and resulting in substantial loss of this protein by 25 h. Nonetheless, the arsenite-induced dissociation of SMRT from PML-RAR α occurred extremely rapidly and clearly preceded the degradation of the PML-RAR α protein (Fig. 7B and C; compare the amount of PML-RAR α coprecipitated by the anti-SMRT antibodies to the overall levels of PML-RAR α detected by anti-PML and anti-RAR antibodies). The overall levels of SMRT were stable at these intermediate

FIG. 5. Effect of As_2O_3 treatment on subcellular location of SMRT proteins. CV-1 cells were transfected with GFP-SMRT alone, cotransfected with MEKK-1, or treated with $15 \mu M$ As_2O_3 after transfection. The subcellular location of the GFP-SMRT was subsequently visualized by confocal fluorescent microscopy. As a control experiment, the subcellular location of TFIIB under the same conditions was analyzed by immunofluorescence. (A) GFP-SMRT alone; (B) GFP-SMRT plus MEKK-1 expression vector; (C and D) GFP-SMRT plus As_2O_3 treatment; (E) TFIIB without As_2O_3 treatment; (F) TFIIB after treatment with $15 \mu M$ As_2O_3 .

time points (data not shown). Our results therefore indicate that arsenic trioxide treatment induces the release of SMRT from PML-RAR α in these cells prior to degradation of the PML-RAR α and without notable degradation of SMRT.

DISCUSSION

Arsenic trioxide is a potent inhibitor of SMRT function. The ability of nuclear receptors to modulate gene expression is mediated through the recruitment of auxiliary proteins, referred to as corepressors and coactivators (7, 10, 22, 54, 60, 66). The interactions of nuclear receptors with these auxiliary pro-

teins are tightly regulated and play a central role in determining the transcriptional properties, positive or negative, displayed by a given receptor. Hormone ligand is one important modulator of this corepressor-coactivator exchange; binding of cognate hormone is thought to induce a specific change in the conformation of the nuclear receptors that leads to a release of the corepressor complex and an acquisition of the coactivator complex (23, 33, 44, 47). However, the association of these auxiliary proteins with nuclear receptors can also be regulated in a ligand-independent manner through the effects of protein kinases (19, 62). We have previously shown that SMRT is

FIG. 6. Apoptosis and differentiation of NB-4 cells upon As₂O₃ treatment. (A) Effect of As₂O₃ and a MEK-1 inhibitor on the differentiation of NB-4 cells. NB-4 cells were left untreated (None), treated with all-*trans* retinoic acid (ATRA), or treated with As₂O₃ in the presence or absence of the MEK-1 inhibitor U0126 for 72 h. Cells were subjected to flow cytometry after staining with phycoerythrin-conjugated anti-CD11c antibody. (B) Effect of As₂O₃ and MEK-1 inhibitor on the apoptosis of NB-4 cells. NB-4 cells were treated with a 1 μ M concentration of either ATRA or As₂O₃ in the presence or absence of the MEK-1 inhibitor U0126 for 72 h. Cells were subjected to flow cytometry after TUNEL reaction. (C) Induction of MEK-1 signaling by arsenite in NB-4 cells treated with As₂O₃. The cells were lysed, and the lysate was analyzed by SDS-PAGE and immunoblotting using anti-phospho-MEK-1/2 (Ser217/221) antibody as for Fig. 3A.

phosphorylated in response to a MAP kinase cascade that is initiated by the EGF receptor and propagated through the kinase hierarchy by MEKK-1 and MEK-1 (20). As a result of these phosphorylation events, the ability of SMRT to interact with T3Rs, RARs, or PLZF is severely impaired.

Arsenic trioxide displays pleiotropic effects in many biological systems. At high concentrations, arsenic trioxide is toxic. At lower concentrations, however, arsenic trioxide can act as a carcinogen (55) or, as described here, can function conversely as a chemotherapeutic in the treatment of APL. It is this capacity of arsenite to induce remission in APL, together with the known ability of arsenite to up-regulate MAP kinases in

FIG. 7. Effect of As₂O₃ on the physical association of PML-RAR α with SMRT protein in NB-4 cells. (A) Physical association of SMRT protein with PML-RAR α in NB-4 cells. NB-4 cells were treated with all-*trans* retinoic acid (ATRA) or As₂O₃ for 24 h. Whole-cell extracts were prepared and subjected to immunoprecipitation with SMRT or PML antibody. The immunoblots were subsequently probed with the antibodies indicated on the right. (B) Loss of the physical association of SMRT with PML-RAR α occurs before degradation of PML-RAR α protein in NB-4 cells. NB-4 cells were treated with 0.1 μ M As₂O₃. Whole-cell extracts were prepared at the indicated times and subjected to immunoprecipitation with SMRT, RAR α , or PML antibody. The immunoprecipitates were then analyzed by SDS-PAGE, and the immunoblot was probed with anti-PML antibodies. The positions of SMRT, PML-RAR α , and the product of the untranslocated PML locus are indicated. (C) Quantification of data in panel B.

cells, that led us to examine whether arsenic trioxide might mediate some of its effects through an inhibition of SMRT function. As we report here, arsenic trioxide treatment transiently up-regulates MAP kinase cascade signaling in CV-1 and NB-4 cells and results in inhibition of the ability of SMRT to interact with nuclear receptors and with nonreceptor transcription factors, such as PLZF. This arsenite-mediated inhibition of SMRT function is among the most potent we have observed.

These effects of arsenite appear to be specific for the SMRT-transcription factor interaction and are not due to a general arsenic-mediated toxicity or an overall inhibition of transcription or translation.

Consistent with the proposal that at least some of the effects of arsenite are mediated through activation of a MAP kinase cascade, the SMRT protein was hyperphosphorylated and altered in its subcellular distribution in response to arsenite treatment in a fashion analogous to that observed in response to the introduction of an activated EGF receptor, MEKK-1 or MEK-1; conversely, chemical inhibitors of MAP kinase cascade signaling counteracted the effects of arsenite on SMRT function. It remains unclear, however, precisely how arsenite leads to an increase in MAP kinase cascade signaling in our system. Two targets of arsenite identified previously, the EGF receptor itself and JNK phosphatases (2, 9), do not appear to play a role in the phenomena reported here (unpublished data). It is possible that a different growth factor receptor or a component of the MAP kinase cascade itself in CV-1 and NB-4 cells may interact with and be stimulated by arsenite (e.g., see references 9, 36, 37, 40, and 48), thereby accounting for the up-regulation of MAP kinase signaling we observe. Alternatively, arsenic may operate by targeting the activity of an as-yet-undetermined phosphatase in these cells, thereby elevating the phosphorylation state, and enzymatic activity, of one or more components of the MAP kinase cascade.

Arsenite results in dissociation of SMRT from PML-RAR α and induces a mixed differentiation and apoptotic response in APL-derived cells that is counteracted by inhibitors of MEK-1 signaling. APL in humans is associated with chromosomal translocations that lead to the synthesis of aberrant x-RAR chimeric proteins (8, 11, 29, 41, 45). These x-RAR chimeras retain the ability to bind to retinoid-responsive target genes but are impaired in hormone-mediated corepressor release, and they are thought to function as dominant negative inhibitors of normal retinoid signaling. Consistent with this proposal, supraphysiological levels of retinoic acid that induce release of corepressor from PML-RAR α also induce differentiation in PML-RAR α APL cells in culture and result in clinical remission in APL patients bearing the PML-RAR α translocation (10, 15–17, 34, 35, 42).

Arsenic trioxide serves as an important adjuvant to retinoic acid-mediated differentiation therapy, and arsenic trioxide can induce remission in cases of recurrent APL that are resistant to the effects of retinoids (reviewed in references 56 and 68). The palliative effects of arsenic trioxide are, in part, attributed to a strong, arsenite-induced apoptotic response, an effect that we could reproduce in our own experiments with cultured NB-4 cells. However, we also observed in the NB-4 cell population a distinct, arsenite-induced differentiation response. This differentiation response was manifested as a substantial increase in the number of cells displaying a CD11c epitope, an early marker of macrophage/monocyte differentiation, but not as an increase in the expression of leukocyte alkaline phosphatase, a terminal granulocyte differentiation marker. A similar abortive differentiation response in arsenite-treated NB-4 cells has been reported previously (e.g., see references 4 and 14). Intriguingly, many of the NB-4 cells that display the apoptotic response to arsenite represent a distinct population from those that display the differentiation response (S. H. Hong and M. L.

Privalsky, unpublished observations). It is possible that arsenite-induced differentiation precedes apoptosis and that once the cells enter into apoptosis they lose the CD11c marker. Alternatively, these two pathways may be alternative responses to arsenite, with some of the NB-4 cells entering apoptosis and others entering differentiation.

An abnormally stable association with SMRT corepressor helps maintain the oncogenic state in APL (8, 15–17, 18, 35). Arsenite destabilizes the ability of SMRT to interact with nuclear receptors in CV-1 cells through a non-ligand-based pathway involving a MAP kinase cascade. Does arsenite induce a similar inhibition of the SMRT interaction with PML-RAR α in APL cells, and might this arsenite-mediated release of SMRT initiate the abortive differentiation phenotype? Consistent with this hypothesis, we determined that arsenite treatment induces MAP kinase signaling in the NB-4 cells, reflected as a transient increase in the phosphorylation level, and presumably activity, of MEK-1. This increase in MEK-1 activity by arsenite is paralleled by a rapid disruption of the interaction of SMRT with PML-RAR α , causing a loss of corepressor from the PML-RAR α oncoprotein similar to that observed in response to retinoic acid treatment. Conversely, the ability of arsenite to induce differentiation in NB-4 cells is blocked by U0126, a specific inhibitor of the MAP kinase cascade. The inhibitory effects of U0126 were specific for differentiation mediated by arsenite; U0126 did not alter the ability of NB-4 cells to differentiate in response to retinoic acid.

We therefore propose that arsenite induces differentiation in NB-4 cells, at least in part, through a MAP kinase cascade, leading to inhibition of SMRT function. This model may help explain why differentiation of NB-4 cells in response to arsenite is inefficient and incomplete compared to the more vigorous differentiation seen in response to retinoic acid. Arsenite induces only release of corepressor from PML-RAR α , whereas retinoic acid induces both corepressor release and coactivator acquisition. Thus, whereas the effects of arsenite treatment may be limited to reversing PML-RAR α -mediated repression, producing modest increases in the expression of differentiation-related target genes, retinoic acid would both reverse repression and induce transcriptional activation of these target genes to levels substantially above basal levels. It should be noted, however, that the effects of arsenite on NB-4 cell differentiation are complex and may involve mechanisms in addition to the phosphorylation of SMRT noted here. For example, arsenite leads to the SUMO modification of PML and of PML-RAR α , the reformation of nuclear bodies, and an eventual loss of detectable PML-RAR α protein (4, 14, 43, 52, 59, 63, 69). It is also interesting that the U0126 MEK-1 inhibitor not only blocked the partial differentiation phenotype induced in NB-4 cells by arsenic trioxide but also blocked the apoptotic response mediated by the same treatment. Precisely which of these differentiation and proapoptotic events are related to the arsenite-mediated inhibition of SMRT association by PML-RAR α , and which are independent outcomes, remains to be determined by future experiments. In addition, many cells express both SMRT and its paralog, N-CoR. RAR α and PML-RAR α have been reported to interact with SMRT in preference to N-CoR (23, 33, 44, 47), leading us to focus our present studies on the former. Nonetheless, MEKK-1 signaling can inhibit the interaction of N-CoR with its nuclear receptor part-

ners in a manner similar to what we have reported for SMRT (unpublished observations), and N-CoR may also prove to be a target of arsenite inhibition in these, or in other, cell lines.

Arsenite and cancer. Arsenic is an element that occurs in nature in many forms and exerts many pleiotropic effects on biological systems. In addition to its widely recognized, acutely toxic actions, arsenic produces many chronic effects on organisms. Somewhat paradoxically, in light of its antineoplastic effects in APL, arsenic trioxide is also known to be a powerful tumor promoter in human beings, and arsenic-mediated oncogenicity represents a significant health issue both for personnel in the chemical industry and for the human population as a whole as a result of arsenic contamination of ground water (55). Arsenic operates in biological systems through multiple mechanisms. Based on the results presented here, we suggest that a previously unrecognized effect of arsenic, the ability of arsenite to inhibit corepressor function, must be added to the mechanisms by which arsenic can perturb biological systems and should be considered in interpreting the toxic, oncogenic, and antineoplastic effects of the trivalent metalloids.

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