THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 279, No. 7, Issue of February 13, pp. 5374–5379, 2004

Printed in U.S.A.

The Coiled-coil Domain Is the Structural Determinant for Mammalian Homologues of *Drosophila Sina*-mediated Degradation of Promyelocytic Leukemia Protein and Other Tripartite Motif Proteins by the Proteasome*

Received for publication, June 17, 2003, and in revised form, November 24, 2003
Published, JBC Papers in Press, November 25, 2003, DOI 10.1074/jbc.M306407200

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Mammalian homologues of *Drosophila Seven in Absentia* (SIAHs) target for proteasome-mediated degradation several factors involved in cell growth and tumorigenesis. Here we show that SIAH-1/2 binds and targets for proteasome-mediated degradation the putative tumor suppressor and tripartite motif (TRIM) family member PML, leading to the loss of its transcriptional co-activating properties and a reduction in the number of endogenous PML nuclear bodies. Association with PML requires the substrate-binding domain (SBD) of SIAH-1/2 through an interacting surface apparently distinct from those predicted by the structural studies, or shown experimentally to mediate binding to SIAH-associated factors. Within PML, the coiled-coil domain is required for Siah- and proteasome-mediated degradation, and deletions of regions critical for the integrity of this region impair the ability of Siah to trigger PML-RAR degradation. Fusion of the coiled-coil domain to heterologous proteins resulted in the capacity of mSiah-2 to target their degradation. All of the TRIM proteins tested were degraded upon mSiah-2 overexpression. Finally, we show that the fusion protein PML-RAR (that retains the coiled-coil domain), which causes acute promyelocytic leukemias, is also a potential substrate of mSiah-2. As a result of mSiah-2 overexpression and subsequent degradation of the fusion protein, the arrest in hematopoietic differentiation because of expression of PML-RAR is partially rescued. These results identify PML and other TRIMs as new factors post-translationally regulated by SIAH and involve the coiled-coil region of PML and of other SIAH substrates as a novel structural determinant for targeted degradation.

SIAH[1] proteins are the mammalian homologues of *Drosophila Seven in Absentia* (*Sina*) (Ref. 1). *Sina* specifies eye cell fate by induction of proteasome-mediated degradation of the transcription factor tramtrack (2). Likewise, mammalian SIAHs behave as the RING domain-containing components of E3 ubiquitin ligase complexes and target for proteasome-mediated degradation several factors involved in transcriptional regulation, cell growth, and tumorigenesis (3–7).

SIAH-1 is a transcriptional target of p53 in human cells *in vitro*, suggesting that targeted degradation of SIAH substrates may be relevant to modulate p53 response to stress (growth arrest and/or apoptosis). The p53-SIAH connection remains to be fully clarified, because recent results suggest that *in vivo* (at least, in mouse) SIAH proteins are not induced by p53 and do not play a significant role in the p53-mediated stress response (8–9).

Among p53 coregulators, promyelocytic leukemia protein (PML) belongs to the so-called "tripartite motif" (TRIM) gene family (10). The TRIM is composed of a RING domain followed by a cysteine/histidine-rich region (B1/B2 boxes) and by a coiled-coil region mediating self-association (10, 48). PML is a nuclear protein and a structural component of the nuclear bodies (NBies) (11–12). PML is induced upon stress and facilitates post-translational modifications of p53 required for its full activity (13–14). PML-null cells are more resistant to several kinds of stresses, suggesting an important role for PML in the induction of growth arrest/apoptosis (15). Mechanistically, PML can act as a transcriptional coregulator of both transcriptional activators and repressors (16–19).

In acute promyelocytic leukemia, PML is fused to the retinoic acid receptor (RAR)α, to yield the PML-RAR fusion protein (20–21). PML-RAR inhibits hematopoietic differentiation by aberrant transcriptional repression of RAR target genes, associating with the N-CoR/histone deacetylase complex. Pharmacological doses of retinoic acid (RA) release PML-RAR from N-CoR/histone deacetylase, induce differentiation of acute promyelocytic leukemia blasts, and target PML-RAR for proteasome degradation (22–24).

In this report, we investigate the functional relationships among TRIMs, PML-RAR, and SIAHs. Our results unravel a novel structural determinant for SIAH-targeted degradation,

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[1] The abbreviations used are: SIAH, mammalian *Sina* homologue; *Sina*, *Drosophila Seven in Absentia*; Siah, mammalian *Sina* homologue genes; PML, promyelocytic leukemia protein; TRIM, tripartite motif; RA, retinoic acid; RAR, retinoic acid receptor; CC, coiled-coil; LLnL, *N*-acetyl-Leu-Leu-norleucinal; GFP, green fluorescent protein; TGF-β, transforming growth factor-β; PBS, phosphate-buffered saline; MBP, maltose-binding protein; MBP-PML, maltose-binding protein-PML fusion; NBies, nuclear bodies; GAL4, Gal4 DNA-binding domain; Gal4, Gal4 coding sequence; TK, thymidine kinase.

and suggest a potential role for SIAH proteins in the regulation of normal PML function.

EXPERIMENTAL PROCEDURES

Plasmids—Full-length hSiah-1 cDNAs (wild-type, mutant A, mutant D, and deleted of ΔRING) were cloned in the vector pcDNA3-myc as described (25). Wild-type PML224RAR-B cDNA and deletion mutants for the individual heptads of the coiled-coil region (PML273RAR-B, PML224Δ(274–300)RAR-B, PML224Δ(301–326)RAR-B, and PML-224Δ(327–360)RAR-B) were cloned in pTL2 as described (26). Coiled-coil (CC)-RAR cDNA was cloned in pSG5 as described (40).

mSiah-2 cDNA was fused in frame to the green fluorescent protein (GFP) and then cloned in the hybrid Epstein-Barr virus/retroviral PINCO vector (27). G5-TK-Luc, Gal4-PML, and pCMX-Siah-2 have been described (4).

Cell Culture and Chemicals—293T cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. *N*-acetyl-Leu-Leu-norleucinal (LLnL) was obtained from Sigma. The anti-PML (PG-M3) and anti-mSiah-2 antibodies have been described (4, 28).

Transduction of U937-PR9 Cells—U937-PR9 cells were transduced as described previously (29). Briefly, Phoenix packaging cells were transfected by calcium-phosphate with the indicated PINCO-based vectors. The supernatant from the transfections that contained the viral particles was collected, filtered, and then used to perform the infection of target cells (27). Two days after infection, cells were left untreated or placed in the presence of vitamin D-TGF-β to induce differentiation. Immunophenotypical analysis of differentiation markers was performed as described (29).

Transient Transfections, Immunoprecipitations, and Western Blotting Assays—293T cells were transfected by calcium-phosphate as indicated. For the trans-activation assays, cells were harvested 48 h after transfection and assayed for luciferase activity. Transfection efficiency was evaluated by co-transfecting 50 ng of CMV-βGal plasmid (Promega) in each sample. For the immunoprecipitation assays, transfected cells were washed in PBS, and then lysed in Nonidet P-40 buffer (50 mM Hepes, pH 7.0, 150 mM NaCl, 0.1% Nonidet P-40, 5 mM EDTA, 1 mM dithiothreitol, 2 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 10 μg/ml *N*-ethylmaleimide). 1 mg of proteins was incubated with the primary antibody for 3 h at 4 °C, followed by an additional incubation of 1 h at 4 °C with protein A-Sepharose beads. The immunoprecipitates were washed in Nonidet P-40 buffer, denatured in Laemmli buffer, and analyzed for Western blotting as described (30).

Immunofluorescence—Transduced U937 cells were fixed with 3.7% formaldehyde in PBS for 10 min and permeabilized using 0.1% Triton X-100 in PBS (2% bovine serum albumin) for 10 min. The anti-PML antibodies used for the immunostaining were dissolved in PBS (2% bovine serum albumin) and incubated for 2 h at 37 °C. After washing in PBS, the cells were incubated for 30 min with the rhodamine-conjugated secondary antibodies.

Pull-down Assays—Maltose-binding protein (MBP) and maltose-binding protein-PML fusion (MBP-PML) were expressed in bacteria and purified by amylose resin (New England Biolabs).

Equal amounts (2 μg) of MBP and MBP-PML proteins conjugated to amylose beads were incubated with *in vitro*-translated mSiah-2 or PML in 50 mM Tris-HCl, pH 7.6, 50 mM NaCl, 1% Nonidet P-40, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride for 1 h at 37 °C. Beads were washed in the same buffer, denatured in Laemmli buffer, and then analyzed by 10% SDS-PAGE followed by autoradiography.

RESULTS

Siah-1/2 Induces Degradation of PML and PML/RAR via the Proteasome—Intracellular levels of both PML and PML-RAR are regulated through pathways that are not yet fully characterized (24, 31–33). To test whether SIAH proteins are involved in their degradation, we performed transient transfection experiments in 293T cells. This approach has been used previously to demonstrate SIAH-mediated proteasomal degradation of several other proteins (4, 34–35). hSiah-1 or mSiah-2 were co-transfected with either PML, RAR, or the fusion protein PML-RAR. Western blot analysis of protein extracts from transfected cells showed marked down-regulation of PML (Fig. 1, *A* and *B*) and PML-RAR, but not of RAR (Fig. 1*A*). Incubation of transfected cells prior to harvesting with the proteasome inhibitor LLnL prevented PML and PML-RAR down-regulation, suggesting the involvement of proteasome-mediated degradation (Fig. 1*A*). Notably, overexpressed mSiah-2 (or hSiah-1) induced proteasome-dependent degradation of the portion of PML retained in the PML-RAR fusion protein (PML-P/R), indicating that SIAH-targeted degradation of PML-RAR depends on PML sequences (Fig. 1*A*, and data not shown). As observed previously (4, 37), the N-terminal RING domain of SIAH-1/2 is essential for proteasome-mediated degradation of target proteins: hSIAH-1 or mSIAH-2 ΔRING failed to induce PML degradation (Fig. 1*C*, and see Fig. 3).

We then analyzed the effects of overexpressed mSIAH-2 on endogenous PML proteins. The lack of an efficient anti-PML antibody to use in Western blot assays led us to perform immunofluorescence studies. U937 promonocytic cells were infected with a retrovirus expressing the mSiah-2 cDNA fused in frame with the GFP, or expressing only GFP as control, and stained with a monoclonal anti-PML antibody to detect the endogenous PML NBies. As shown in Fig. 1*D*, the number of NBies is significantly decreased in GFP/mSiah-2-expressing cells with respect to control cells: we counted an average of 5.6 NBies/cell in GFP-mSiah-2-infected cells against 13.1 NBies/cell in control cells not transduced or expressing GFP (Fig. 1*C*, $p < 0.05$). mSIAH-2 ΔRING failed to induce changes in NBies staining (not shown). Similar results were obtained by microinjection of mSiah-2 (not fused to GFP) or Siah-1 in WI38 cells (data not shown).

PML inhibits transcription when tethered to DNA through fusion to the GAL4 DNA-binding domain (16). We used the Gal4-PML fusion protein to repress transcription driven by the TK minimal promoter (G5-TK-Luc reporter plasmid) in transient transfection assays performed in 293T cells. Increasing amounts of mSiah-2 expression vector did not affect the TK-driven transcription (Fig. 1*E*, *bars 2* and *3*), whereas Gal4-PML-mediated repression of transcription was almost completely relieved (Fig. 1*E*, *bars 5* and *6*). Taken together, these results suggest that SIAH proteins induce proteasome-mediated degradation of PML and PML-RAR in a RING-dependent fashion, resulting in a loss of PML co-activating properties.

PML Associates with SIAH Proteins—To investigate whether PML forms a stable complex with SIAH proteins, we performed co-immunoprecipitation assays upon co-transfection of mSiah-2 (or mSiah-2 ΔRING) and PML in 293T cells. As shown in Fig. 2*A*, we detected anti-PML immunoreactive polypeptides in anti-SIAH-2 immunoprecipitates (*lane 12*), although we did not detect mSIAH-2 in anti-PML immunoprecipitates (*lane 11*). Interestingly, we could detect mSIAH-2 ΔRING protein, observed by anti-HA immunoblotting, in anti-PML immunoprecipitates, suggesting that the lack of a PML-mSiah2 association detectable by immunoprecipitation using anti-PML antibodies derives from the reduction in PML levels upon mSiah2, but not mSiah-2 ΔRING, overexpression. To investigate whether PML and mSIAH-2 associate directly, we performed *in vitro* binding experiments using a recombinant purified MBP-PML attached to amylose beads and *in vitro* translated, ^{35}S-labeled, mSIAH-2. As shown in Fig. 2*B*, MBP-PML, but not the control MBP protein, bound specifically mSIAH-2. Taken together, these results suggest that mSIAH-2 associates with PML through its C-terminal region and requires the N-terminal RING domain to target PML for proteasome-mediated degradation.

The region responsible for binding of Siah-2 (or Siah-1, data not shown) to PML corresponds to the so-called substrate-binding domain of Siah. Recently, the structure of this domain has been solved (36). Knowledge of the structure and other studies (25) indicate that Siah may bind to different Siah

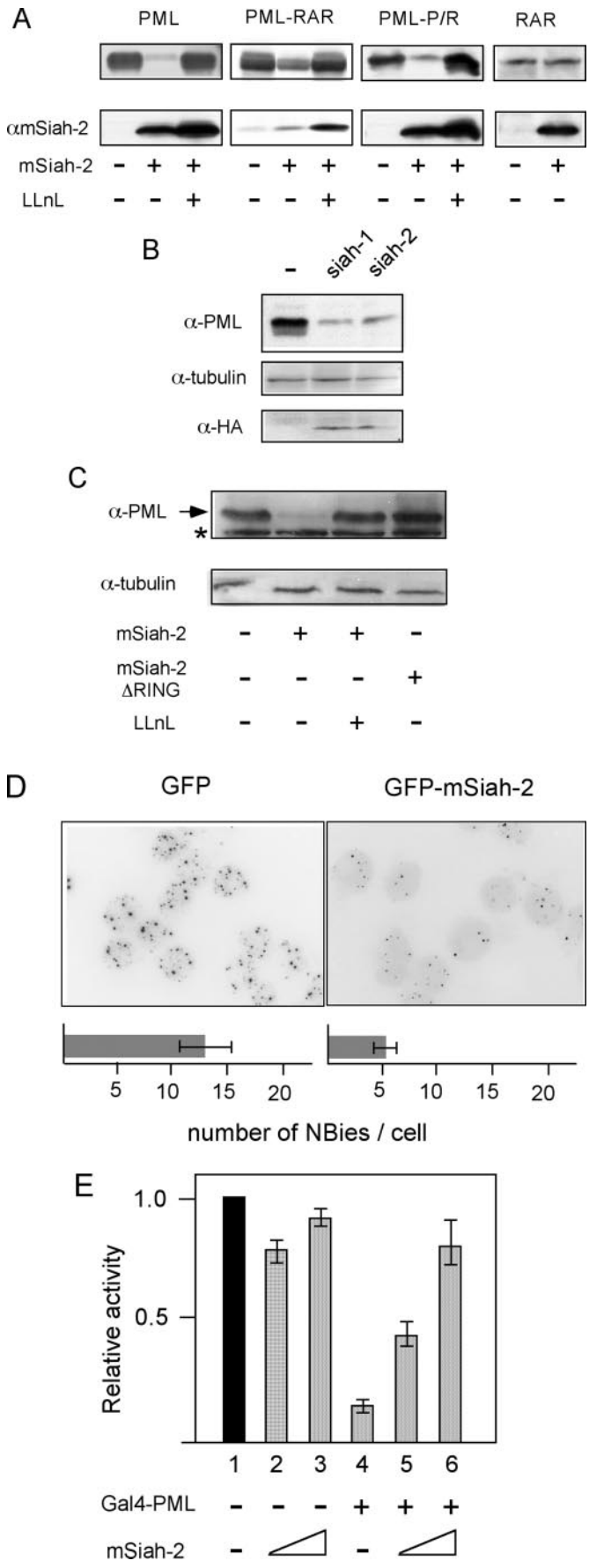

FIG. 1. **SIAH-1/2 induces proteasome-mediated degradation of PML.** *A*, 293T cells were transiently transfected with the indicated cDNAs and treated or not with the proteasome inhibitor LLnL (90 μM). Western blot analyses were carried out using anti-RARα, anti-PML, and anti-mSiah-2 antibodies (to detect RARα, PML and PML-RAR, or mSiah-2, respectively). *B*, 293T cells were transiently transfected with the indicated cDNAs. Western blot analyses were carried out using anti-PML, anti-tubulin (as a loading control), and anti-HA (to detect HA-tagged hSiah-1 and mSiah-2 proteins). *C*, 293T cells were transiently transfected with the indicated cDNAs and treated with LLnL as indicated. Western blot analyses were carried out using anti-PML or anti-tubulin antibodies (to detect PML and α-tubulin). Expression levels of mSiah-2 and mSiah-2 ΔRING were comparable (data not shown). *, an aspecific band recognized by the batch of secondary antibody used in some of the experiments shown. *D*, U937 cells were transduced using vectors expressing GFP or GFP-mSiah-2. Cells were analyzed by immunofluorescence using anti-PML antibody. *Left panel*, endogenous PML in control cells expressing GFP, showing the normal localization of PML in NBies. *Right panel*, endogenous PML in GFP-mSiah-2-positive cells. *E*, 293T cells were transfected with G5-TK-Luc (2 μg) alone or together with the Gal4-PML expression vector (1 μg). The effect of increasing doses of mSiah-2 expression vector both in the absence (*bars 2, 3*) and in the presence (*bars 5, 6*) of Gal4-PML is shown (*bars 2* and *5*, 0.5 μg; *bars 3* and *6*, 2 μg).

substrates through distinct surfaces. We evaluated whether hSiah-1, carrying mutations known to impair the association with other substrates, was able to trigger PML degradation. Interestingly, hSiah-1 mutant A (with alanine substituted for Glu-161, Asp-162, Glu-226, and Glu-237), which is mutated in a concave-negative surface required for binding to the SIAH interactor SIP (25), continued to trigger PML degradation (Fig. 3). Likewise, mutations lying in a positively charged region (Siah-1 mutant D, carrying alanine substitution for Arg-214, Arg-215, Arg-231, Arg-124, and Arg-232) proposed as a second site of protein-protein interaction (36) and required, for the *Drosophila* homologue *Sina*, to mediate association with substrates such as Phyllopod (38), did not impair the capacity of Siah to degrade PML (Fig. 3). At least another SIAH-associating protein, BAG1, does not bind Siah through either surface, suggesting that additional interaction surfaces are contained within the substrate-binding domain (25). BAG1, however, is not triggered for degradation by Siah (25); PML, therefore, apparently defines a new class of Siah substrates, which are targeted to the proteasome but do not require the two previously described SIAH surfaces required for association with interactors/substrates.

The Coiled-coil Region of PML and other TRIM Proteins Is the Structural Determinant for mSiah-2-mediated Degradation—We performed additional co-transfection studies in 293T cells to elucidate which domain(s) of PML is required for mSIAH-2-mediated proteasomal degradation.

From the preliminary analysis shown in Fig. 1, we hypothesized that the region required for mSIAH-2 binding and degradation lies in the portion of PML retained in the fusion protein. This region contains the conserved tripartite motif; therefore, we transfected PML constructs devoid of either the RING+B boxes region, or the coiled-coil region, and examined their sensitivity to mSIAH-2-mediated degradation. ΔH-PML (lacking the coiled-coil region) was not degraded by mSIAH-2 (Fig. 4*A*) and did not associate with mSiah-2 (Fig. 2*A*), whereas ΔC-PML (lacking the RING+B boxes) expression levels were drastically decreased in an LLnL-sensitive way. To establish whether the coiled-coil region was the sole element required for mSIAH-2 degradation, we used a chimeric construct where the CC domain is fused to full-length RAR (CC-RAR) (that does not behave as an mSIAH-2 substrate as shown in Fig. 1*A*). Strikingly, the CC-RAR chimera was markedly down-regulated by mSIAH-2 in a proteasome-dependent manner, whereas RAR levels were not affected or even enhanced by co-transfecting mSIAH-2 (Fig. 4*B*). These results show that the coiled-coil region of PML is necessary and sufficient for mSIAH-2 recruitment and proteasome-mediated degradation.

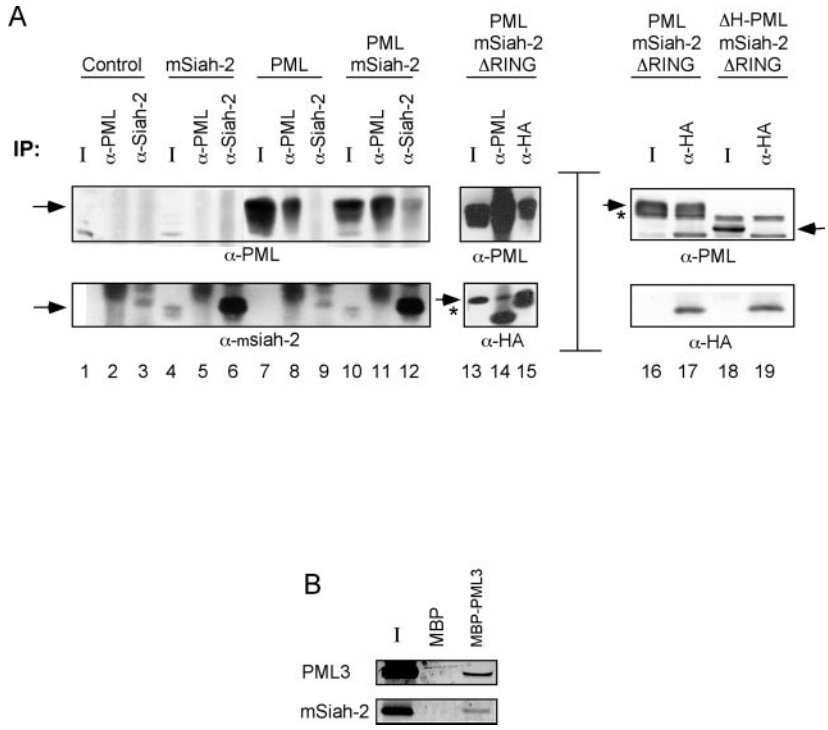

FIG. 2. **PML associates *in vivo* and *in vitro* with the C terminus of mSiah-2.** *A*, 293T cells were transfected with the indicated vectors. At the end of the transfection, whole-cell lysates were prepared. Co-immunoprecipitation studies were performed by using the indicated antibodies. The immunoprecipitates were analyzed by Western blotting using anti-PML, anti-mSiah-2, and anti-HA antibodies (to detect PML, mSiah-2, and mSiah-2 ΔRING, respectively). *IP*, *I*, input lane; *, aspecific proteins, recognized by the antibodies used. *B*, *in vitro* binding analysis performed using recombinant, purified MBP-PML (or MBP as control) attached to amylose beads and *in vitro* translated, ^{35}S-labeled mSiah-2 as mobile phase. *In vitro* translated PML served as positive control, because PML has been shown to form oligomers (48). Proteins eluted from the amylose beads were resolved by SDS-PAGE and revealed by autoradiography. 1/5 of the input material is shown for each sample.

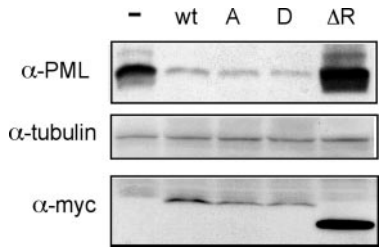

FIG. 3. **Functional analysis of the substrate-binding domain of SIAH-1: the known substrate-binding surfaces are not required for PML degradation.** 293T cells were transfected with PML and Siah-1 wild-type or mutant myc-tagged cDNAs. Mutant *A*, E161A, D162A, E226A, E237A; mutant *D*, R214A, R215A, R231A, R124A, R232A; mutant Δ*R*, deletion of N-terminal RING domain (25). Whole-cell lysates were analyzed by Western blotting using anti-PML, anti-tubulin, and/or anti-myc antibodies.

The coiled-coil region of PML has been further subdivided into four distinct subdomains (Fig. 4*C*, heptads *I–IV*); the biochemical mapping of these subdomains (in the context of the fusion protein PML-RAR) has shown that all four heptads contribute to the functionality of the coiled-coil domain (26, 30, 40, 41). Whereas deletion of each individual heptad leads to selective impairment of specific biochemical properties (formation of homodimers and hetero-oligomers with PML, binding to a DNA-response element) (26, 30, 40, 41), heptad 2 seems critical for the integrity of the coiled-coil domain, because all of the properties analyzed are impaired in constructs carrying the deletion of this region (26, 30, 40, 41). We analyzed the sensitivity of PML-RAR and deletion constructs in each individual heptad to Siah-mediated degradation (Fig. 4*D*). Deletion of heptad 1, 3, or 4 had no or minimal effect, and all of these proteins continued to be degraded by mSIAH-2 (Fig. 4*D*). In contrast, deletion of heptad 2 completely abrogated degradation by mSIAH-2. These results suggest that the integrity of the coiled-coil domain is critical for the sensitivity to SIAH.

PML (TRIM19) belongs to a conserved family of proteins, all sharing the tripartite motif, including the presence of a coiled-coil region (10). We checked whether other TRIMs may behave as mSIAH-2 substrates: co-transfection of mSiah-2 with several TRIM family members led, in all cases, to down-regulation of protein levels, showing that all TRIM proteins are potential substrates for proteasomal degradation by mSIAH-2 (Fig. 5).

mSiah-2 Partially Rescues PML/RAR-induced Differentiation Block—One of the features of PML/RAR is the capacity to block the differentiation of hematopoietic cells (42). Because mSIAH-2 induces PML-RAR degradation (Fig. 1), we investigated whether mSIAH-2 can interfere with PML/RAR biological activity. As a model system, we used hematopoietic progenitor U937-PR9 cells, expressing PML-RAR under a zinc-inducible promoter (43). In these cells, induction of PML-RAR expression leads to the blocking of differentiation induced by vitamin D/TGF-β treatment (43). PR9 cells were infected with retroviral vectors encoding GFP only (as control) or the GFP-mSiah-2 chimeric cDNA. PML/RAR expression was drastically reduced by mSiah-2 infection (Fig. 6*A*). To induce differentiation, cells were treated with vitamin D/TGF-β. Analysis of surface differentiation markers (cd14) showed that PML-RAR strongly inhibited differentiation in GFP-expressing cells (from >90% to <20%; Fig. 5*B*). mSiah-2 partially rescued the differentiation block induced by PML-RAR: >50% cells were differentiated in the presence of the fusion protein and of mSIAH-2, showing that mSIAH-2 is a negative modulator of PML-RAR function (Fig. 6*B*).

DISCUSSION

Here, we report SIAH-1/2 as a novel interactor for PML and suggest a potential role for SIAHs in the regulation of PML stability. A structural signature present in PML and in all of the other TRIM family members (the coiled-coil region) represents the critical requirement for SIAH-mediated proteasomal degradation. This region mediates self-association and the ensuing formation of oligomers in PML/TRIMs (10). Interestingly, analysis by dedicated programs (MULTICOIL) (44) of the amino-acidic sequences of several characterized mSiah-2 substrates (*e.g.* tramtrack; N-CoR) shows in these proteins stretches with a high propensity to form a coiled-coil, suggesting that this may be a more widespread structural requirement for SIAH-mediated degradation. Recently, a potential SIAH recognition motif has been described for certain SIAH substrates (45). We have failed to detect in the coiled-coil region of the TRIM proteins a conserved

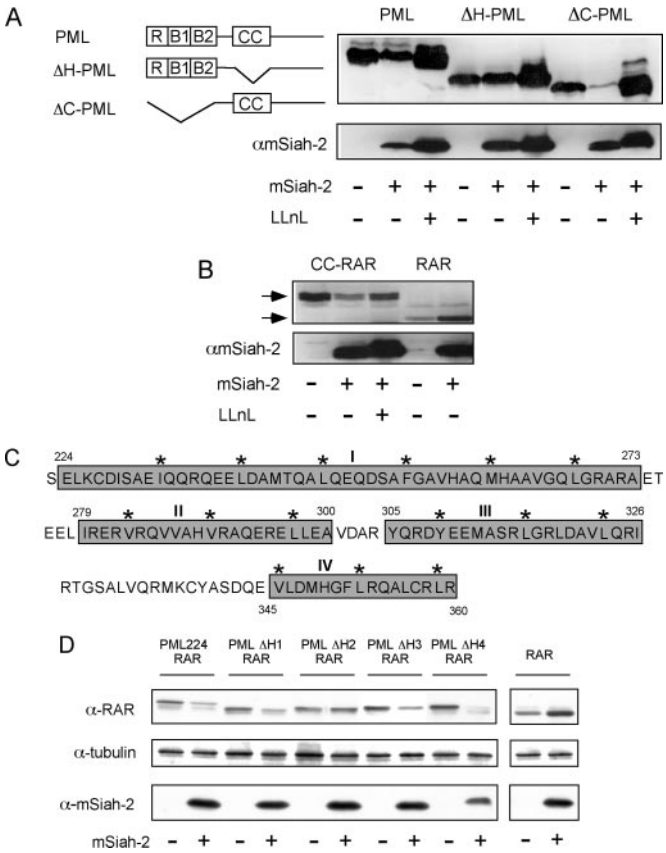

FIG. 4. **The coiled-coil domain of PML is necessary and sufficient for degradation by mSiah-2.** *A*, 293T cells were transfected with the indicated cDNAs (the scheme depicts the PML deletion constructs used). Whole-cell lysates were analyzed by Western blotting using anti-PML and/or anti-mSiah-2 antibodies. *B*, 293T cells were transfected with the indicated vectors as above in the presence or in the absence of LLnL. Whole-cell lysates were analyzed by Western blotting using anti-RAR or and anti-mSiah-2 antibodies. *C*, scheme of the PML coiled-coil domain. The four heptads are indicated with boxes numbered *I*, *II*, *III*, and *IV*. *, hydrophobic amino acids. *D*, 293T cells were transfected with the indicated PML/RAR cDNAs in the presence or in the absence of mSiah-2. The PML/RAR cDNAs are as follows: *PML224-RAR* (wild-type); *PMLΔH1-RAR* (deletion of heptad I); *PMLΔH2-RAR* (deletion of heptad II); *PMLΔH3-RAR* (deletion of heptad III), and *PMLΔH4-RAR* (deletion of heptad IV) (26). Whole-cell lysates were analyzed by Western blotting using anti-RAR, anti-tubulin, and/or anti-mSiah-2 antibodies.

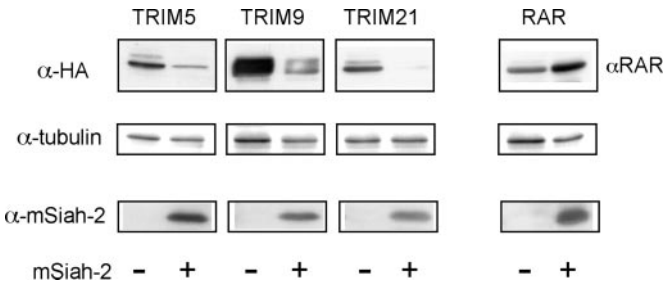

FIG. 5. **mSiah-2 targets the entire TRIM family for degradation.** 293T cells were transfected with the indicated TRIMs (HA-tagged) and RAR (as control for a protein not targeted for SIAH-mediated degradation) in the presence or in the absence of mSiah-2. Whole-cell lysates were analyzed by Western blotting using anti-HA or and anti-RAR antibodies.

motif reminiscent of this sequence, suggesting that these proteins belong to a distinct class of SIAH interactors. This possibility is strengthened by the analysis of SIAH mutants unable to associate with different categories of substrates/interactors: the finding that none of the mutations abrogates the capacity of Siah

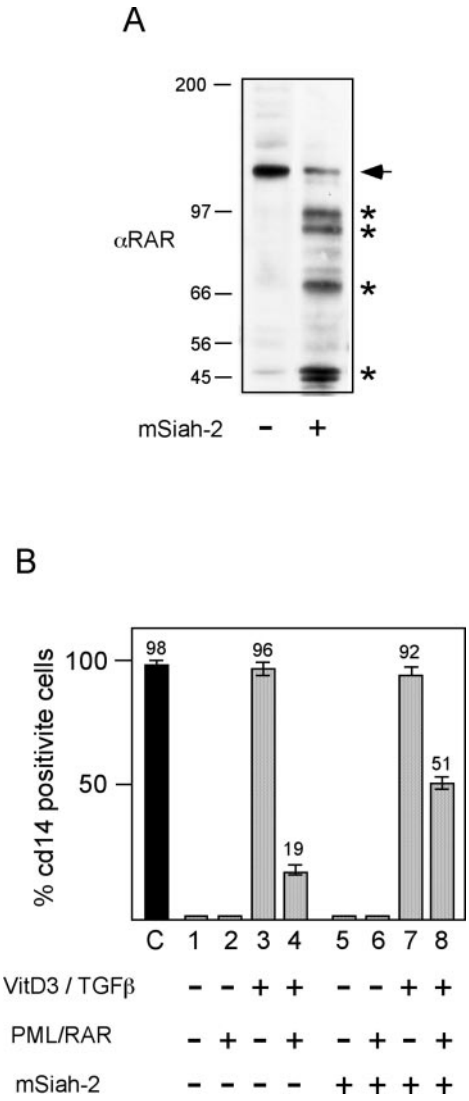

FIG. 6. **mSiah-2 induces PML-RAR degradation and partially rescues PML-RAR-mediated block of hematopoietic differentiation.** *A*, anti-RAR Western blot analysis of U937-PR9 cells (expressing PML-RAR) transduced with a viral vector encoding GFP alone (control), or GFP-mSiah-2 cDNA. *, lower molecular weight PML-RAR degradation products. *B*, differentiation analysis: U937-PR9 cells were transduced with GFP (*bars 1–4*) or GFP-mSiah-2 (*bars 5–8*). PML/RAR expression was induced by $ZnSO_4$, and differentiation was induced by VD3/TGF-β treatments. Differentiation was evaluated by FACS analysis of the differentiation marker cd14 gating the GFP-positive populations (transduced cells).

to target PML for degradation implies a novel mode of interaction. Structural studies are in progress to define precisely the PML-SIAH molecular complex.

SIAH expression (leading to both PML-RAR and N-CoR degradation) should directly target two critical leukemogenic stimuli. The differentiation block imposed by the fusion protein, however, was only partially rescued. There are several explanations for this result: (*i*) residual levels of PML-RAR/N-CoR may be sufficient to maintain the block, or (*ii*) targeting by SIAH of additional substrates is required for differentiation. Although these phenomena may both contribute to the observed resistance, we favor the hypothesis that PML-RAR may induce epigenetic alterations of the chromatin structure of its target genes (through DNA methylation and other histone modifications); once established, these modifications would no longer require PML-RAR expression to maintain a repressive pattern of gene expression stably inherited through successive

cell divisions and, therefore, maintain the differentiation block even in the absence of PML-RAR or N-CoR (46).

SIAH-1 is transcriptionally induced by p53 in human cell lines and apparently plays a positive role in the p53 stress response (8). Recent results from Siah-1/2 knock out mice suggest that this regulation might differ in murine cells or does not play a major role *in vivo*, leaving the discussion open on the significance of the Siah/p53 network (9). Nevertheless, the fact that SIAH may then contribute to the down-regulation of PML (which is itself a p53 regulator) (14, 47, 49) suggests a feedback loop to control p53 activity. This loop would reinforce the well described mdm2-p53 feed-back loop, where mdm2 is transcriptionally induced by p53, and then provokes p53 degradation through the proteasome (39). The two transcriptional p53 targets, SIAH and mdm2, might trigger degradation of two critical components of the p53 pathway (p53 itself and PML), therefore modulating its overall degree of activity. The cross-talk among mdm2 and SIAH in the p53 network is being currently investigated and may give useful insights on the cellular responses to stress and oncogenic stimuli.

Acknowledgments—We thank John C. Reed, Angela Santoni, and Mauro Magnani for reagents and their generous contribution to our work.

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The Coiled-coil Domain Is the Structural Determinant for Mammalian Homologues of *Drosophila Sina*-mediated Degradation of Promyelocytic Leukemia Protein and Other Tripartite Motif Proteins by the Proteasome

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J. Biol. Chem. 2004, 279:5374-5379.
doi: 10.1074/jbc.M306407200 originally published online November 25, 2003
