Role of SUMO in RNF4-mediated Promyelocytic Leukemia Protein (PML) Degradation

SUMOYLATION OF PML AND PHOSPHO-SWITCH CONTROL OF ITS SUMO BINDING DOMAIN DISSECTED IN LIVING CELLS*

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Promyelocytic leukemia protein (PML) is a tumor suppressor acting as the organizer of subnuclear structures called PML nuclear bodies (NBs). Both covalent modification of PML by the small ubiquitin-like modifier (SUMO) and non-covalent binding of SUMO to the PML SUMO binding domain (SBD) are necessary for PML NB formation and maturation. PML sumoylation and proteasome-dependent degradation induced by the E3 ubiquitin ligase, RNF4, are enhanced by the acute promyelocytic leukemia agent, arsenic trioxide (As2O3). Here, we established a novel bioluminescence resonance energy transfer (BRET) assay to dissect and monitor PML/SUMO interactions dynamically in living cells upon addition of therapeutic agents. Using this sensitive and quantitative SUMO BRET assay that distinguishes PML sumoylation from SBD-mediated PML/SUMO non-covalent interactions, we probed the respective roles of covalent and non-covalent PML/SUMO interactions in PML degradation and interaction with RNF4. We found that, although dispensable for As2O3-enhanced PML sumoylation and RNF4 interaction, PML SBD core sequence was required for As2O3- and RNF4-induced PML degradation. As confirmed with a phosphomimetic mutant, phosphorylation of a stretch of serine residues, contained within PML SBD was needed for PML interaction with SUMO-modified protein partners and thus for NB maturation. However, mutation of these serine residues did not impair As2O3- and RNF4-induced PML degradation, contrasting with the known role of these phosphoserine residues for casein kinase 2-promoted PML degradation. Altogether, these data suggest a model whereby sumoylation- and SBD-dependent PML oligomerization within NBs is sufficient for RNF4-mediated PML degradation and does not require the phosphorylation-dependent association of PML with other sumoylated partners.

Promyelocytic leukemia protein (PML) is a tumor suppressor (1) whose gene is translocated in cases of acute promyelocytic leukemia (2). PML functions as the organizer of PML NBs, which are dynamic structures harboring numerous transiently and permanently localized proteins (3). The importance of PML NB structural integrity was first revealed in acute promyelocytic leukemia because, in this malignancy, the abnormal fusion protein PML/RARα leads to NB disruption. Patient treatment with As2O3 induces the reversion of the acute promyelocytic leukemia phenotype as well as PML/RARα degradation and PML NB reformation (4).

PML is a target for the post-translational modification by SUMO, an ubiquitin-like protein that is covalently coupled to PML lysine residues 65, 160, and 490 via a process called sumoylation (5, 6). Among the four human SUMO paralogs identified, SUMO1, -2, and -3 were found to be conjugated to target proteins. It involves an enzymatic cascade for the transfer of the mature SUMO and the formation of an isopeptide bond between the COOH-terminal glycine of SUMO and a lysine from the target protein. Sumoylation is a reversible process due to the existence of several deconjugating enzymes.

PML NB formation requires both the covalent linkage (sumoylation) (reviewed in Ref. 7) and the non-covalent interactions of SUMO with PML through a SUMO binding domain (SBD also named SIM for SUMO interacting motif) (8). Interestingly, PML SBD contains specific serines, acting as substrates for the casein kinase-2 (CK2), which are implicated in PML ubiquitination and degradation (9) and which phosphorylation status could regulate the function of the SBD.

Because sumoylation of proteins is dynamic and reversible, this post-translational modification is difficult to follow in vivo and its detection mainly relies on the identification of sumoylated protein species by Western blot following cell lysis. Recently, we used bioluminescence resonance energy transfer (BRET) to detect covalent linkage of ubiquitin (ubiquitination) in living mammalian cells and in real time (10). In brief, BRET monitors the interaction...
between a protein fused to a luciferase and a protein fused to yellow or green fluorescent protein (YFP or GFP), upon addition of a luciferase substrate; it is a proximity-based assay that requires that the donor of energy (luciferase fusion) and the acceptor (YFP or GFP fusions) are within 50 to 100 Å for an efficient energy transfer (11–13). However, a demonstration that BRET may provide a method of choice to follow the dynamics of protein sumoylation in living cells is lacking. Here, we developed a sensitive and quantitative SUMO BRET assay for the detection of PML interactions with SUMO in living cells. We proved that BRET can be used to detect both SUMO covalent and non-covalent interactions with PML (model, Fig. 1H). For this purpose, we used the PMLIII isofrom in which sumoylation is induced by As2O3 and triggers a proteasome-dependent PML degradation (14); the degradation process involves the ubiquitination of poly-SUMO covalently coupled to PML by the poly-SUMO-specific E3 ubiquitin ligase RNF4 (15–17). Altogether, our BRET results indicate that, As2O3 and/or RNF4-induced PML degradation are dependent on the integrity of both PML sumoylation target sites and the PML SBD core sequence but not on the CK2 serine phosphorylation sites within the SBD. However, phosphorylation of these serines is required for most PML SBD-dependent non-covalent interactions. This phospho-regulation of PML SBD (“SBD phospho-switch”) establishes another link between the phosphorylation and SUMO, different from the phospho-sumoyl switch (18).

**EXPERIMENTAL PROCEDURES**

**Constructs and Expression Vectors**

The sequence of all cDNA constructs was confirmed by DNA sequencing.

**BRET Constructs**

**YFP-SUMO1**, **YFP-SUMO1G**, **YFP-SUMO2**, and **YFP-SUMO3** (all cloned in pEYFP C1, BD Biosciences Clontech) were a kind gift of Dr. Dasso M. (National Institutes of Health, Bethesda, MD) (19). **GFP-SUMO1** and **GFP-UBI** were previously described, respectively, in Refs. 20 and 10. The myc-tagged **SUMO1** construct used was subcloned as an Xhol fragment in the Xhol site of a myc-tagged version of pcDNA3.1 vector. **UBC9** (accession number U45328) was cloned in the SacI/KpnI site of pGFP10 C3 (Perkin Elmer Life Sciences). The NLS used for the **Luc-NLS** construct was already described (13) and **Luc-NLS** was generated by inserting the NLS sequence between the phosphorylation and SUMO, different from the phospho-sumoyl switch (18).

**PML Mutants**

PML III point mutations were generated using the QuickChange site-directed mutagenesis kit (Stratagene) starting with PML III (**PML WT**) cDNA (accession number S50913) cloned in pBluescript SK+ and the appropriate oligonucleotides (see list of primers: only sense oligonucleotides are described). **PML_3KR** was obtained by mutating sequentially Lys-65, Lys-160, and Lys-490 to Arg residues to generate **PMLK65R**, **PMLK65R,K160R**, and **PMLK65R,K160R,K490R** (PMLKBR). Then, **PML_3KR-SBD** and **PML_3KR-S560A_S562A_S563A_S565A** mutants were obtained using PML_3KR as a matrix by mutating either the four hydrophobic residues from the SBD (VVVI) (8) or the four serines serving as target for the CK2 kinase, which are within the sequence encompassing amino acids 560 to 565 (9).

**PML ACC** was constructed by replacing a 411-bp fragment coding for the PMLIII coiled-coil domain (nucleotides 669 to 1069 of PMLIII coding sequence) by a short unrelated 18-bp sequence, which includes a NotI site. The **PML ACC** sequence was generated by PCR amplification of 5’- and 3’-overlapping fragments, which both include the NotI site from the 18-bp sequence; the 5’-PCR fragment includes the initiator ATG of PMLIII and an upstream BamHI cloning site and the 3’-PCR fragment includes the PMLIII Stop codon and a downstream Xhol cloning site. The 5’ and 3’ PCR fragments (BamHI-NotI and NotI-Xhol fragments, respectively) were ligated together in a three-piece ligation reaction in the BamHI/Xhol site of pcDNA3.1 (+) (primers used: “**PML_BamHI_ATG sense**” and “**PML_ACC1_NotI antisense**” for the 5’-fragment and “**PML_ACC2_NotI sense**” and “**PML_Stop_Xhol antisense**” for the 3’-fragment).

**PML Expression Constructs**

**PML WT** and mutants were amplified by PCR from pBluescript SK+ for subcloning in appropriate vectors. To generate untagged expression constructs, **PML** PCR fragments digested with BamHI and Xhol were cloned into the BamHI/Xhol site of either pcDNA3.1 (+) for transient expression or HIV-1-based lentiviral pTRIP vector for stable expression (a gift from Dr. P. Charneau, Groupe de Virologie moléculaire et de Vectorologie, Institut Pasteur, Paris) (primers used: “**PML_BamHI ATG sense**” and “**PML_Stop_Xhol antisense**”). To generate **Luc** and **YFP** BRET constructs, **PML** PCR fragments digested with BamHI and EcoRI were cloned into XhoI terminus to **Luc** or **YFP** in the BamHI/Xhol site of pcDNA3.1 (+) as part of a three-piece ligation (primers used: “**PML_BamHI_ATG sense**” and “**PML_No Stop EcoRI_Nterm antisense**”).

**RNF4 Constructs**

pFLAG-RNF4 was a kind gift from F. J. Kaiser and B. Horstemke (22). To generate the **Luc-RNF4** BRET construct,
RNF4 was PCR amplified from pFLAG-RNF4 and cloned in-phase, as an EcoRI-XbaI fragment, COOH-terminal to Luc in the BamHI/XbaI site of pcDNA3.1 because of a three-piece ligation (primers used: “RNF4_EcoRI_ATG_C-term sense” and “RNF4_Stop_XbaI antisense”).

Primers

Luc and YFP primers were: Luc_EcoRI_ATG_C-term sense, 5’-CCGCGGATCCCTGATGGAAGCCTGCCACGATGTGAGC-3’; Luc_Stop_XhoI_C-term antisense, 5’-CTTACAGCTAGCCGGAGCTAGAACTCTTGCTCAGC-3’; Luc_BamHI_ATG_N-term sense: 5’-CTGAGCTAGCCGGAGCTAGAACTCTTGCTCAGC-3’; Luc_no Stop_EcoRI_N-term antisense, 5’-CCGCGGATCCCTGATGGAAGCCTGCCACGATGTGAGC-3’; PML_BamHI_ATG sense, 5’-GATCCATACGCTGAGTCGTGGTGG-3’; PML_No Stop_XhoI_C-term antisense, 5’-CTTACAGCTAGCCGGAGCTAGAACTCTTGCTCAGC-3’.

Antibodies

The following antibodies were used: mouse monoclonal anti-PML (clone PGM3) (used for immunofluorescence microscopy), rabbit polyclonal anti-PML (clone H-11032) (used for the Western), rabbit polyclonal anti-actin (clone C-11) were from Santa Cruz Biotechnology and mouse monoclonal anti-FLAG (clone M2) from Sigma.

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Arsenic and Epoxomicin Treatments

As₂O₃ (Sigma number A1010, 330 mM solution stock resuspended in 1 N NaOH) and Epoxomicin (Sigma number E3652, 100 μM solution stock resuspended in dimethyl sulfoxide) were used at the concentrations and for the times indicated in the text.

Stable Transduction, Transient Transfection, and Cell Culture

Stable Transduction—A stable expression of PML constructs was obtained by a lentiviral-based strategy as described (23). The efficiency of the HIV-1-based lentiviral pTRIP vector, in which PML constructs are clonobed, relies on the presence of a three-stranded DNA structure that acts as a cis-determinant of HIV-1 DNA import. The stable integration of the transgenes into the host DNA allows efficient and long term transgene expression without clone selection. Virus stock production and infection were as described in Ref. 23 to transduce and express PML wild type and mutants in the HEK293T cell line. Briefly, virus particles were produced after transient co-transfection of HEK293T cells, with the p8.91 encapsidation plasmid, the pHCMV-G vector encoding the vesicular stomatitis virus envelope and the pTRIP vector encoding PMLWT and its mutants, using a standard calcium phosphate method.

BRET² Transient Transfections—HEK293T cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with GLUTAmax (Invitrogen), 10% fetal bovine serum, 100 units/ml penicillin and streptomycin, 1 mM sodium pyruvate (all from Invitrogen). Cells were seeded at a density of 5 x 10⁵ cells per well in 6-well dishes, 24 h before transfection. Transient transfections were performed using polyethylenimine (PEI, linear, Mₑ 25,000; catalog number 23966 Polysciences, Inc., Warrington, PA) in Opti-MEM medium (Invitrogen). PEI powder was dissolved in water to a concentration of 1 mg/ml in water that was heated to 80 °C. Usually, 0.1 μg of the PML-Luc construct was transfected alone or with increasing quantities of YFP-tagged SUMO1. The amount of transfected DNA was completed to a total of 2 μg with pcDNA3.1(+) empty vector. PEI (10 μg in 100 μl of Opti-MEM medium) was added on the DNA and the samples were incubated for 20 min at room temperature. The PEI-DNA suspension was then added to the attached cells in 2 ml of fresh culture media. Following an overnight incubation, transfection medium was replaced with fresh Dulbecco’s modified Eagle’s medium for 3 h to allow cell recovery. Transfected cells (8 x 10⁶) were then detached and replated in 96-well white plates with clear bottoms (Costar) pre-treated with d-polysine (Sigma) and left in culture for 24 h before being processed for BRET² assay. When required, the plated cells were treated with As₂O₃ for the appropriate time within this 24 h.

BRET² Transient Transfections—HEK293T cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 1 mM L-glutamine, 10% fetal bovine serum (Wisent), and 100 μg/ml penicillin and streptomycin. Cells were seeded at a density of 1 x 10⁶ cells/100-mm dish, 24 h before transfection. Transient transfections of plasmids were performed using the calcium phosphate precipitation method. Usually, 1 μg of the PML-Luc construct was transfected alone or with increasing
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quantities of GFP-tagged SUMO1. The amount of transfected DNA was completed to a total of 10 μg with pGEM empty vector as described in Ref. 13. The transfection medium was replaced with the supplemented Dulbecco’s modified Eagle’s medium after 26 h and cells were left in culture for an additional 22 h before being processed for BRET² assay. When required, As₂O₃ treatment was done on the cells in suspension (see BRET² detection assay).

BRET Detection Assays

BRET¹ Assay—BRET¹ measurement was done on attached cells as previously described (21). Before measurement, culture media was replaced by PBS. Coelenterazine H (NanoLight Technology) was then added to a final concentration of 5 μM in PBS. Readings were then collected using a multidector plate reader MITHRAS LB940 (Berthold) allowing the sequential integration of the signals detected in the 480 ± 20 and 530 ± 20 nm windows, for luciferase and YFP light emissions, respectively. The BRET¹ signal was determined by calculating the ratio of the light intensity emitted by the YFP fusion over the light intensity emitted by the Luc fusion. The values were corrected by subtracting the background BRET¹ signal detected when the Luc fusion construct was expressed alone.

For BRET² titration experiments, BRET¹ ratios were expressed as a function of the [acceptor]/[donor] expression ratio (YFP/Luc). Total fluorescence and luminescence were used as a relative measure of total expression of the acceptor and donor proteins, respectively. Total fluorescence was determined with MITHRAS using an excitation filter at 485 nm and an emission filter at 535 nm. Total luminescence was measured in the MITHRAS, 10 min after the addition of coelenterazine H and the reading was taken in the absence of emission filter.

BRET² Assay—The BRET² assays were conducted on cells in suspension as previously described in Ref. 13. In brief, transiently transfected cells were resuspended in PBS. Cells were then distributed in 96-well microplates (white Optiplate from Packard) (20 μg corresponding to 1~2 × 10⁵ cells). When required, cells were treated with 10 μM As₂O₃ or vehicle for the appropriate time at 37 °C. For the BRET² assay, upon addition of the cell permeant Luciferase substrate (coelenterazine Deep Blue, PerkinElmer Life Sciences), the bioluminescence signal resulting from the degradation of the substrate was detected using a 370~450-nm band pass filter (donor emission peak 400 nm). The transferred energy resulted in a fluorescence signal emitted by the GFP acceptor (excitation peak 400 nm and emission peak 510 nm) that was detected using a 500~530-nm band pass filter. The BRET² signal was quantified by calculating the acceptor fluorescence/donor bioluminescence ratio as previously reported. Expression level of each construct was determined with MITHRAS using an excitation filter at 485 nm and donor proteins, respectively. Total fluorescence was determined with MITHRAS using an excitation filter at 485 nm and an emission filter 535 nm. The transferred energy resulted in a fluorescence signal on aliquots of transfected cell samples. The GFP total fluorescence was measured using a FluoroCount (PerkinElmer Life Sciences) with an excitation filter at 400 nm and an emission filter at 510 nm. The total luminescence was measured using the same cells incubated with coelenterazine H for 10 min (Molecular Probes) (emission peak 485 nm). The BRET² was plotted as a function of the GFP/Luc fusion protein expression ratio, both fusion protein expressions being assessed with the same cells as described above, to take into account the potential variations in the expression of individual constructs from transfection to transfection. The values were corrected by subtracting the background BRET² signal detected when the Luc fusion construct was expressed alone. All BRET¹ and BRET² results presented are derived from two to 10 independent experiments done in duplicate.

Confocal Microscopy—For confocal microscopy, 3 × 10⁵ HEK293T cells were seeded 24 h after their transfection onto glass coverslips pre-treated with poly-D-lysine (Sigma) placed within 6-well plates. Cells were then washed three times in PBS 24 h later and fixed in 4% paraformaldehyde for 10 min at room temperature. Following several washes, cells were permeabilized with blocking buffer (PBS containing 0.3% Triton X-100 and 2% bovine serum albumin (w/v)). Monoclonal anti-PML (1/250) and anti-RNF4 (1/100) antibodies were then added for 2 h in blocking buffer. Cells were washed twice and incubated for 1 h with the appropriate Alexa Fluor 488- or 594-conjugated secondary antibody (1/500 dilution, Molecular Probes, Inc.). Coverslips were mounted using Vectashield (Vector Laboratory). Confocal images were obtained on a Leica TCS-NT/SP inverted confocal laser-scanning microscope using an Apochromat ×63/1.32 oil-immersion objective. Co-localization was performed by overlay of the images using Leica Confocal Software LCS (Heidelberg, Germany). Excitation and emission filters for the different labeled dyes were as follows: YFP (green): λ_ex = 488 nm, λ_em = 540/25 nm; Texas Red (red): λ_ex = 568 nm, λ_em = 610/30 nm.

Western Blot Analysis—For total cell extracts, cells were washed in cold PBS, lysed in hot Laemmli buffer, and boiled for 10 min. Proteins were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes (Optitran BA-S 83 NC, Schleicher and Schuell). The membranes were blocked in 5% gelatin from cold water fish (Sigma) for 2 h and then incubated overnight at 4 °C with the first antibody (H-238 anti-PML, anti-FLAG, or anti-actin). Following washes, the membranes were incubated for 1 h with the appropriate Alexa Fluor 680-coupled secondary antibody (Molecular Probes) that was then revealed by an Odyssey® Infrared Imaging System (LI-COR Biosciences).

RESULTS

PML/SUMO1 Interactions Are Detected by BRET—To better understand the role of SUMO in PML function and its involvement in the dynamic of PML NB formation, we developed a BRET assay for the detection of PML interactions with SUMO in living cells. For this purpose, PMLIII was tagged at its carboxyl terminus with a luciferase (PML-WT-Luc), whereas SUMO1 was tagged at its amino terminus with YFP or its GFP green variant. We performed a classic BRET titration assay (13, 21) by co-transfecting HEK293T cells with a constant amount of PML-WT-Luc and increasing amounts of either YFP-SUMO1 (BRET¹, Fig. 1A) or GFP-SUMO1 (BRET², Fig. 1B) (differences in the BRET assay sensitivity, depending on the fluorescent protein acceptor used, were previously described (24)). In each case, the occurrence of BRET was quantified by measuring the ratio of light emitted by the acceptor (YFP or GFP) and the luciferase donor upon addition of the membrane permeable
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As shown in Fig. 1, A or B, a strong BRET signal occurred between PMLWT-Luc/YFP-SUMO1 or PMLWT-Luc/GFP-SUMO1 pairs and increased as a hyperbolic function that is indicative of a specific interaction (10). Similar results were obtained using GFP-SUMO2 as an acceptor protein (data not shown). Negative controls, which exhibited a linear nonspecific BRET signal resulting from random collision (“bystander” BRET (10)), ascertained this specificity: (i) a donor targeting Luc to the nucleus by fusion to a nuclear localization signal (Luc-NLS, Fig. 1B) and (ii) a RING finger PML mutant (PMLC57A,C60A-Luc, Fig. 1A) with altered SUMO binding properties (Fig. 3C) (8) that is unable to interact with the only known SUMO E2 ligase UBC9 (25). Altogether, these results validate the use of BRET to detect PML/SUMO1 interactions.

**Both Covalent and Non-covalent PML/SUMO1 Interactions Are Detected by BRET**—Because BRET is a proximity-based assay, the BRET signal observed with the PMLWT/SUMO1 pair may result not only from the covalent (sumoylation) but also from the non-covalent interaction between PML and SUMO (model, Fig. 1H). To evaluate the contribution of the covalent linkage of SUMO to PMLWT-Luc relative to its SUMO non-covalent interactions, we compared the BRET titration curve obtained with PMLWT-Luc with that of PML3KR-Luc, its sumoylation-less mutant (with lysines 65, 160, and 490 mutated to arginine), in pairs with YFP- or GFP-SUMO1 (Fig. 1, C and D). Because the covalent linkage of SUMO is abrogated within the PML3KR mutant, the difference in the BRET signal between the PMLWT and PML3KR saturation curves depends on PML sumoylation (Fig. 1C); furthermore, the PML3KR saturation curve mainly revealed the contribution of non-covalent interactions to the BRET signal (Figs. 1C and 7B, model). Thus, it appears that non-covalent interactions represent a major part of the BRET signal in BRET1 (Fig. 1C) and BRET2 (Fig. 1D) assays. Strikingly, a linear and low nonspecific BRET signal was obtained with PMLWT-Luc and YFP-SUMO1G, a SUMO mutant with a single glycine residue at its COOH terminus, which is unable to be covalently conjugated to proteins (19) (Fig. 1, A and C). Similar results were obtained with YFP-SUMO2G (data not shown). Interestingly, this shows that SUMO1 and -2 paralogs do not interact in their free form in vivo with PML in contrast with data obtained in vitro (8). Thus, the non-covalent PML/SUMO1 interactions detected by BRET involve SUMO1-modified proteins recruited by PML-Luc as illustrated in Fig. 7B, model.

To further establish the capacity of the BRET assay to detect variations in the sumoylation status of PML in living cells, we tested PMLWT-Luc or PML3KR-Luc/YFP (or GFP)-SUMO1 pairs in the presence of As2O3. Clearly, a significant increase in the BRET signal was observed with PMLWT upon As2O3 treatment as seen in titration (Fig. 1, C and D), time course (Fig. 1E), and dose-response (Fig. 1F) experiments. In contrast, no or very limited change in the BRET signal was observed with PML3KR as revealed by the saturation curves (Fig. 1, C and D) and confirmed by the dose-response curve (Fig. 1F). Consequently, the As2O3-enhanced BRET signal was concluded to be dependent on PML sumoylation. To evaluate the relative contribution of each of the three PML sumoylation target lysines, we compared the As2O3-induced sumoylation of single PML Lys → Arg mutants. Although K490R and K65R single mutants exhibit a partially reduced As2O3-induced sumoylation compared with PMLWT, mutation of Lys160 alone was sufficient to prevent the As2O3-induced sumoylation of PML to the same extent as the triple 3KR mutant (Fig. 1G). Thus, Lys160 is a prerequisite for As2O3-induced PML modification by SUMO1. These results are in agreement with previous Western blot data (14). Additional experiments revealed that As2O3 exhibited a similar potency to induce PML sumoylation with SUMO1 (log EC50 = −6,430 ± 0.181 μM), SUMO2 (log EC50 = −6,618 ± 0.203 μM), or SUMO3 (log EC50 = −6,626 ± 0.272 μM) as shown by comparing the EC50 in dose-response curves (Fig. 4A). Thus, we proved here that the sensitivity of both BRET1 and BRET2, which assay conditions differ as described under “Experimental Procedures,” was sufficient to monitor dynamic changes in PML sumoylation in living cells.

**PML SUMO Binding Domain Is Not Required for As2O3-induced PML Sumoylation**—The identified PML SBD, with the sequence represented in Fig. 7A, is involved in non-covalent recruitment of sumoylated proteins, among them PML itself, as demonstrated by co-immunoprecipitation studies (8). To evaluate the contribution of PML SBD in non-covalent PML/SUMO1 interactions detected by BRET, we compared the BRET signal of PMLWT with that of PMLSBD that is mutated in its SBD core sequence (VVVI hydrophobic amino acids; see Fig. 7A) (8). As seen in Fig. 2A, a significant decrease in the BRET signal was obtained with PMLSBD relative to PMLWT indicating that non-covalent PML/SUMO1 interactions are largely dependent on the PML SBD core sequence. Upon As2O3 treatment, the increase in the BRET profile was quite similar with both PMLWT and PMLSBD as shown by saturation and dose-response curves (Fig. 2, A and B). This demonstrates that SBD-dependent PML/SUMO non-covalent interactions are not required for As2O3-induced PML sumoylation.

The limited contribution of sumoylated endogenous proteins to the non-covalent BRET signal is estimated using PML3KR-Luc fusion (Fig. 1C), which can interact with endogenous sumoylated proteins via its intact SBD. Interestingly, As2O3 induced a slight increase in PML3KR-Luc BRET signal (Fig. 1C, BRET1 assay) likely resulting from the enhanced sumoylation of endogenous PML by YFP-SUMO1. In contrast, the BRET signal observed with PML3KR-Luc was not increased by As2O3 in the BRET2 assay (Fig. 1D), consistent with the fact that sensitivity of BRET2 required a much higher level of expression of the donor luciferase fusion than the BRET1 assay (24) rendering the contribution of the YFP-SUMO1-modified endogenous PML negligible.

Altogether, our results indicate that in the absence of a sumoylation inducer such as As2O3 (basal conditions), most of the PML-Luc/YFP-SUMO1 BRET signal is accounted for by the SBD-dependent non-covalent interaction of endogenous sumoylated proteins with the PML-Luc donor, whereas in the presence of the inducer, the large increase in the BRET signal only reflects a change in PML sumoylation. Our data also indi-
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green in Fig. 7A) are adjacent to the SBD hydrophobic core sequence within PML SBD (8). We hypothesized that these serines, as phosphorylation targets, could functionally regulate the interaction properties of PML SBD. To determine whether these serines may affect the binding of SUMO1-modified proteins to PML SBD, a mutant of these serines (PMLS560A,S561A,S562A,S565A-Luc, herein called PML3KR–SBD-Luc) was tested by BRET in pair with YFP-SUMO1 (Fig. 3A). Based on the titration curves (Fig. 3A, left panel), the BRET signals calculated for PMLWT–Luc and PML3KR–Luc at an identical YFP/Luc ratio were presented as a bar graph (Fig. 3A, right panel). Interestingly, a similar reduction in the BRET signal was observed with PML3KR and PML3KR–Luc when compared with PMLWT. This indicates that the serines within the SBD are important determinants for the SBD-dependent non-covalent interactions of PML with most of its SUMO-modified protein partners (if not all). Consistent with the hypothesized involvement of the phosphorylation of these serines in these interactions, a BRET signal comparable with that of PMLWT was restored when PML3KR–Luc, PML3KR–SBD–Luc, and a serine phosphomimetic mutant (herein called PML3KR–SBD560A,S561A,S562A,S565A) was used (Fig. 3B). Altogether, these results indicate that serine phosphorylation sites within the SBD regulate PML SBD-dependent interactions with SUMO1-modified proteins.

It is noteworthy that, as for PML3KR, sumoylation of the PML3KR–SBD560A,S561A,S562A,S565A mutant remains As2O3-inducible (Fig. 3A). The amplitude of the As2O3-induced signals was similar for PMLWT and PML3KR–Luc, indicating that the integrity of the polyserine motif is dispensable for As2O3-induced PML sumoylation. This is consistent with the sumoylation profile obtained by Western blot using extracts from HEK293T cells stably expressing PMLWT or its mutants (Fig. 3C). An increase in sumoylation of PMLWT–Luc and PML3KR–Luc was detected upon addition of As2O3 by a change in the sumoylation pattern toward higher molecular weight SUMO-modified species (as expected, no SUMO1-modified forms could be detected with PML3KR, PMLC57/60, and the sumoylation-deficient double mutants, PML3KR–SBD and PML3KR–SBD560A,S561A,S562A,S565A).

Interestingly, the serine phosphorylation-deficient mutant, PML3KR–SBD560A,S561A,S562A,S565A, is localized like PMLWT in multiple and dense nuclear dots characteristic of PML NBs as shown by confocal microscopy (Fig. 3D). In contrast, PML3KR is distributed in large and diffuse aggregates with disruption of PML NBs as previously described (8). This result most likely suggests that the polyserine motif (from Ser560 to Ser565), in contrast to the VVVI core sequence of the SBD, is not required for the forma-
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As suggested (26), negative charges imposed either by a stretch of neighboring acidic amino acids and/or phosphorylated serine residues within the SBD of some proteins favors the recruitment of SUMO1 but not SUMO2. Thus, we used the SUMO BRET assay to determine whether differential SBD-dependent non-covalent recruitment of sumoylated proteins by PML could be evidenced with the three SUMO paralogs. Hyperbolic curves of a similar range of amplitudes were obtained with PMLWT and the three SUMO paralogs as evidenced by BRET titration experiments (Fig. 4B). Furthermore, the BRET signal was drastically and similarly decreased with all SUMO paralogs for the PMLS560–565A mutant to a level close to those obtained with the PMLSBD mutant, as evidenced by BRET titration curves or corresponding bar graphs (Fig. 4, B and C). This indicates that comparable binding of the three SUMO paralogs occurred with PML SBD and that this binding depends on PML from Ser560 to Ser565 residues and their phosphorylation.

PML SBD and PML Sumoylation Target Sites Are Both Critical for the As2O3-induced PML Degradation—It is now known that PML is polyubiquitinated and degraded in a proteasome-dependent manner, a process that requires the Lys160 sumoylation site (14). Consequently, we studied the possible link between PML sumoylation, ubiquitination, and the As2O3-enhanced PML degradation.

In an attempt to follow the ubiquitination of PML and its mutants in basal conditions and upon exposure to As2O3, we conducted a ubiquitin BRET² assay (10) using PML-Luc fusions in pairs with GFP-UBI. As seen in Fig. 5C, the BRET titration curves obtained with PMLSBD, PML3KR, and PML3KR-SBD were similar in the presence or absence of As2O3 and show that the BRET signal increased as a function of the amount of GFP-UBI transfected. In contrast, with PMLWT and PMLS560–565A, a clear shift of the BRET curves to the right was seen upon As2O3 treatment (Fig. 5A). This is indicative of a change in the expres-
sion of the fusions in the presence of As2O3, because BRET ratios are plotted as a function of the GFP/Luc fusion protein expression. To directly assess how the BRET ratio varies in relation to the expression of PMLWT-Luc and GFP-UBI fusions, the BRET ratio, Luc expression, and GFP expression are presented as separate bar graphs for each sample (11 samples) analyzed with the ubiquitin BRET assay in the presence or absence of As2O3 (Fig. 5B). Clearly, neither the BRET signal nor the GFP-UBI expression are significantly changed upon As2O3 addition, whereas a 3–7-fold decrease in the PMLWT-Luc expression was observed. Although similar results were obtained with the PML560–565A mutant (data not shown), no significant difference in PMLSBD-Luc, PML3KR-Luc, and PML3KR-SBD-Luc expression was detected upon As2O3 treatment (Fig. 5D and data not shown). The significant decrease in PMLWT-Luc and PML560–565A-Luc expression, seen as early as 30 min after the addition of As2O3, is not normally seen in cells where GFP-UBI is not overexpressed. This indicates that overexpression of ubiquitin accelerates the kinetics of As2O3-induced PML degradation.

Consistent with the results obtained with the ubiquitin BRET assay in transiently transfected cells, PML3KR and PMLSBD stably expressed in HEK293T cells were shown to be resistant, whereas PMLWT and PML560–565A were sensitive to degradation by a long incubation (16 h) with As2O3, as revealed by Western blot (Fig. 5E). Altogether these results indicate that As2O3-induced PML degradation is not dependent on serine phosphorylation sites within the SBD but requires the integrity of both PML sumoylation target sites and the PML SBD core sequence, as also needed for PML NB formation (8).

Both PML Interaction with RNF4 and Its RNF4-induced Degradation Are Reinforced by As2O3—RNF4 is a poly-SUMO-specific E3 ubiquitin ligase involved in the control of PML stability and implicated in As2O3-induced PML degradation (15, 16). As2O3, which enhances PML sumoylation, was also suggested to favor PML SUMO-dependent polyubiquitination and proteasome-mediated degradation (15, 16).

To further study the mechanisms involved in PML degradation, we assessed the effect of RNF4 on the expression of PMLWT and its mutants exhibiting a differential sensitivity to the As2O3-induced degradation as described above (Fig. 5). First, we overexpressed RNF4 in HEK293T transiently transfected with PMLWT. Clearly, RNF4 alone induced a decrease in PML expression (Fig. 6A, lanes 1 and 4), a process accelerated by As2O3 and observed as early as 1 h after addition of this agent (Fig. 6A, lanes 4–6). In contrast, no change in PML level was observed in the absence of overexpressed RNF4 upon a similar As2O3 treatment (Fig. 6A, lanes 1–3). Then, we compared the fate of PMLWT, PML560–565A, PML3KR, or PMLSBD expressed in HEK293T cells in the presence or absence of overexpressed...
FIGURE 5. Both PML SBD and PML sumoylation target sites are critical for the As$_2$O$_3$-induced PML degradation. A–D, detection of a differential degradation of PML$_{WT}$ and its mutants induced by As$_2$O$_3$ (10 μM, 35 min exposure) in HEK293T cells transfected for expression of PML$_{WT}$-Luc or its mutants in pairs with GFP-UBI. PML-Luc fusions are divided into two groups depending on their response to As$_2$O$_3$ treatment: degradation (A and B) and no degradation (C and D). BRET$^2$ titration curves are in A and C. As confirmed by the bar graphs in E, a shift of the BRET$^2$ curves to the right seen in the presence of As$_2$O$_3$ in A is indicative of a change in the expression of the Luc fusions (change in the GFP/Luc expression ratio upon As$_2$O$_3$ treatment). Bar graphs for PML$_{WT}$-Luc (B) and PML$_{SBD}$-Luc (D) in pairs with GFP-UBI as derived from the data in A and C, respectively. For each sample treated or not with As$_2$O$_3$, the BRET$^2$ and the expression levels of the PML-Luc fusion are shown (B and D); the expression level of GFP-UBI that essentially remains unchanged upon addition As$_2$O$_3$ for all pairs tested is also shown in B for PML$_{WT}$-Luc. E, Western blot on extracts from HEK293T cells stably expressing PML$_{WT}$, PML$_{3KR}$, PML$_{SBD}$, or PML$_{S660-565A}$ and submitted or not to a 16-h treatment with 1 μM As$_2$O$_3$.
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Our results indicate that stimulation of the sumoylation or ubiquitination machinery through overexpression of SUMO1 (not shown), ubiquitin, or RNF4 ligase as well as through As$_2$O$_3$ treatment enhances PML degradation; regulation of the PML level is thus dependent on the relative abundance of the various components of the ubiquitin machinery but also, as less usual, of the SUMO machinery. We also demonstrate here that, in addition to be necessary for NB formation (8), both the covalent linkagge of SUMO to PML and the integrity of PML SBD, which mediates non-covalent interactions of PML with SUMO-modified proteins, are required for As$_2$O$_3$- and RNF4-induced PML degradation (see model in Fig. 7C). The requirement of the integrity of PML SBD for PML degradation provides an explanation for our previous data showing that a COOH terminus mutant, lacking amino acids 555 to 641 of PML and thus PML SBD, was resistant to degradation upon As$_2$O$_3$ exposure (Figs. 5 and 6C), it was unexpected to find that the interaction of PML$_{WT}$ with RNF4 was similarly increased by As$_2$O$_3$. Thus, it appears that the capacity to interact with RNF4 is not the only determinant in the differential sensitivity of PML and its mutants to RNF4-induced proteasome-dependent degradation.

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Interestingly, a functional PML SBD is, however, not required for the As$_2$O$_3$-enhanced PML sumoylation and for the basal or As$_2$O$_3$-enhanced PML/RNF4 interaction. We suggest that the increased PML interaction with RNF4 in the presence of As$_2$O$_3$ is most likely due to the As$_2$O$_3$-enhanced formation of higher molecular weight poly-SUMO chains onto PML (slower migrating species most often observed on blots upon addition of As$_2$O$_3$ data not shown) and to higher affinity of RNF4 for poly-SUMO chains over monomeric SUMO, as recently reported based on in vitro studies (16). Because inactivation studies revealed that both SUMO2/3 and SUMO1 are required for As$_2$O$_3$-induced PML degradation (15), we suggest that SUMO2/3 inactivation, by preventing the formation of the polymeric sumoylation chain onto PML, most likely impairs the PML/RNF4 interaction and thus the As$_2$O$_3$-induced PML degradation. Although As$_2$O$_3$ is suggested to increase the ratio of SUMO1 over SUMO2/3 incorporated in the sumoylated PML (15), it is not yet clear, however, how SUMO1 participates in the

To determine whether the ability of PML mutants to interact with RNF4 can explain their differential sensitivity to RNF4-induced degradation, we tested their interaction by a BRET assay using a limiting concentration of a Luc version from PML$_{WT}$ or its mutants in pairs with increasing and saturating concentrations of RNF4-YFP. Clearly, BRET titration curves indicated that PML$_{WT}$, PML$_{5560–565A}$, and unexpectedly PML$_{SBD}$ interacted with RNF4 and that their interaction was strongly increased by As$_2$O$_3$, indicating that this drug enhances the affinity of PML and its mutants for RNF4 (Fig. 6B). In contrast, lower BRET signals indicative of weak or no interaction

were obtained with PML$_{3KR}$ and these signals were only slightly increased by As$_2$O$_3$ (Fig. 6B), possibly due to the interaction of PML$_{3KR}$ with endogenous PML as previously noted (Fig. 1C). Interestingly, the differential sensitivity of PML and its mutants to RNF4-mediated degradation was also evidenced by the clear shift of PML, PML$_{5560–565A}$, and PML$_{SBD}$ BRET curves to the right upon As$_2$O$_3$ treatment. Analysis of each individual sample treated or not with As$_2$O$_3$ confirmed that these curve shifts were due in each case to a decrease in the level of expression of the Luc fusions upon As$_2$O$_3$ exposure; in the conditions used, no change in the expression of PML$_{3KR}$ was seen after As$_2$O$_3$ treatment at any of the RNF4/PML$_{3KR}$ ratios used. Considering that PML$_{SBD}$ was resistant to degradation upon As$_2$O$_3$ exposure (Figs. 5 and 6C), it was unexpected to find that the interaction of PML$_{WT}$ and PML$_{SBD}$ with RNF4 were similarly increased by As$_2$O$_3$. Thus, it appears that the capacity to interact with RNF4 is not the only determinant in the differential sensitivity of PML and its mutants to RNF4-induced proteasome-dependent degradation.

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A schematic representation of PMLIII (used in this study) and PMLIV isoforms. The three sumoylation target lysines (K), the conserved RBCC/TRIM motif (RING finger, B box 1, B box 2, coiled-coil region), the NLS, and the SBD (8) are illustrated. The whole sequence defining the SBD with its core hydrophobic amino acids (VVVI, in red) (8) and a region including adjacent phosphorylated serines (green) (9) are represented.

B, covalent (sumoylation) and SBD-dependent non-covalent interactions are illustrated as two BRET components discriminated using the PMLWT-Luc donor and its PML3KR and PMLSBD mutants in pairs with the YFP-SUMO acceptor. The black arrow indicates the BRET occurring between the Luc donor and YFP acceptor. The loss of transfer of energy resulting from mutation of the sumoylation target lysine or the SBD is highlighted by red dashed arrows.

C, model for the involvement of PML and its sumoylation, SBD, and phospho-SBD mutants in NB formation and As$_2$O$_3$- and/or RNF4-induced PML degradation. The molecular events leading to PML NB maturation are illustrated in a sequential order as an oversimplification to point to the steps prevented by the mutants used in this study and their resistance or sensitivity to As$_2$O$_3$- and/or RNF4-induced PML degradation.
As$_2$O$_3$-induced PML degradation. This may involve SUMO1 non-covalent binding to PML SBD, the coupling of SUMO1 directly to one of the PML target lysines, or its linking at the end of poly-SUMO2/3 chains to terminate chain growth. Compared with poly-SUMO2/3 chains, SUMO1-terminated poly-SUMO2/3 chains may exhibit a different substrate specificity for the cellular isopeptidases involved in depolymerizing/dismantling poly-SUMO chains (27, 28) or for RNF4.

In addition to functioning as a poly-SUMO-specific E3 ubiquitin ligase (16), RNF4 also catalyzes the ubiquitination of several PML COOH-terminal lysines in vitro and possibly the ubiquitination of PML lysine 401 in vivo (15). This could explain why a PML sumoylation-deficient mutant (PML3KR) can be down-regulated at a very high level of RNF4 overexpression; however, this degradation is not accelerated by As$_2$O$_3$ (data not shown).

A Phospho-switch Controls the PML/SBD Interaction with Sumoylated Proteins: A Possible Role in NB Formation and Maturation—Interestingly, our results also indicate that, apart from the integrity of the SBD hydrophobic core sequence, specific serines serving as targets for CK2 kinase (9) or their phosphorymimetics are required for most PML SBD-dependent non-covalent interactions with sumoylated proteins and thus presumably for progression toward normal NB late stage maturation (see model in Fig. 7C). This demonstrates that phosphorylation of a SUMO binding domain (SBD phospho-switch) can positively regulate the interaction of a SBD with its SUMO-modified protein partners. This is consistent with very recently published data showing the phospho-regulation of several SBD-containing proteins including PML (32). Notably, both localization and sensitivity to As$_2$O$_3$- and RNF4-induced degradation of PMLWT and PML560–565A are comparable but differ from those of the PMLSBD mutant. This suggests that some non-covalent interaction(s) required for localization of PML into NBs and for PML degradation are preserved in PML560–565A and lost in PMLSBD. Interestingly, we consistently observed that, for PML560–565A, increase of the SUMO BRET signal upon As$_2$O$_3$ treatment was similar to PMLWT and always slightly higher than that of PMLSBD (Fig. 3). It was thus tempting to speculate that interaction of SUMO-modified PML with PML SBD was preserved in PML560–565A. According to the PML nucleation model (8), non-covalent binding of sumoylated PML to PML SBD is determinant for nucleation events involved in NB formation and maturation. A preservation of this sumoylation- and SBD core sequence-dependent PML oligomerization could account for PML560–565A localization to PML NBs. Furthermore, it is possible that localization of PML within the NBs, a process known to be enhanced by As$_2$O$_3$ (14), facilitates its RNF4-induced degradation; this would explain why PML560–565A is more sensitive to RNF4 degradation than PMLSBD despite the fact that both interact similarly with RNF4 (model, Fig. 7C). Further studies could determine whether, as a possible mechanism, the suggested E3 ubiquitin ligase activity (29) of PML reinforces RNF4-induced PML degradation in PML NBs, where components of the ubiquitin machinery are concentrated (14, 30).

Clearly, our results demonstrate that the stretch of serines, adjacent to the SBD hydrophobic core sequence and acting as targets for CK2 kinase (9), is not required for the RNF4-induced PML degradation. Because CK2 kinase was previously found to promote ubiquitin-mediated PML degradation upon phosphorylation of these serines (9), CK2-dependent and RNF4-dependent PML degradation may represent two independent pathways regulating negatively the PML level in oncogenic and physiological conditions. Because it was recently shown that ATP depletion results in immobilization of PML at NBs, a condition that may decrease PML phosphorylation (31), it remained to be determined if cycles of phosphorylation/dephosphorylation of the serines adjacent to the SBD core sequence may be involved in regulation of PML nucleation at the NBs and/or NB maturation.

This study took advantage of the development of a sensitive and quantitative SUMO BRET assay for molecular dissection and dynamic monitoring of kinetics of protein sumoylation upon pharmacological agent treatment and non-covalent interactions with SUMO in living cells. This represents a significant advance compared with our initial description of a BRET assay to study membrane protein ubiquitination (10). In this initial study, no tools were available to determine whether covalent interaction with UBI could be discriminated by BRET from the non-covalent interaction. It is also noteworthy that the SUMO BRET assay could simultaneously detect PML/SUMO interactions and PML degradation. This represents a great advantage when strength of interactions are compared upon various pharmacological treatments or when comparing mutants with different sensitivities to degradation. Because BRET can be used in high throughput screening (24), the novel SUMO BRET assay paves the way toward large scale screening of chemical libraries for therapeutic agents modulating SUMO interactions.

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