Chromatin Contributes to Structural Integrity of Promyelocytic Leukemia Bodies through a SUMO-1-independent Mechanism*

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Promyelocytic leukemia (PML) protein is implicated in transcriptional regulation, apoptosis, DNA repair, and tumor suppression. It is not known, however, whether PML and other components of PML bodies function within the vicinity of the bodies or elsewhere in the nucleoplasm. In this study, we demonstrate that chromatin organization around PML bodies influences their morphology, dynamics, and structural integrity by a SUMO-1-independent mechanism. Following transcriptional inhibition and during early apoptosis, chromatin retracts from the periphery of PML bodies, coinciding with the formation of new PML-containing structures through fission of supramolecular PML-containing microbodies. Both fission and fusion of microbodies with parental PML bodies indicate a loss of structural integrity of the bodies, dependent on the state of the surrounding chromatin. This is supported by the observation that treatment of live cells with DNase I could reproduce the structural instability of PML bodies. In addition, PML bodies, which are normally surrounded by chromatin and are positionally stable, become more dynamic following these treatments, presumably due to the loss of chromatin contacts. Overexpression of SUMO-1, a modification required for PML body formation, did not prevent PML body fission, indicating that chromatin-based integrity of PML body structure occurs through a SUMO-1-independent mechanism.

The promyelocytic leukemia (PML) protein was first identified as the fusion partner of the retinoic acid receptor α (RARα) in patients suffering from acute promyelocytic leukemia (1). In cells expressing the PML-RARα fusion, PML nuclear bodies, which appear as 5–20 discrete foci in normal cells, are redistributed into many punctate particles throughout the nucleoplasm. Remission of acute promyelocytic leukemia is associated with 18 U.S.C. Section 1734 solely to indicate this fact.

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translational modifications of PML protein. One of these domains is an N-terminal RING finger, which is required for protein-protein interactions. This RING finger is followed by two B boxes and, together with a central coiled-coil domain, constitutes the RBCC (RING/Bbox/coiled-coil) region found within all PML protein isoforms (23). The function of the RBCC domain has been postulated to be involved with PML homodimerization/oligomerization as well as protein-protein interactions with other PML body-associated proteins. SUMO-1 (small ubiquitin-like modifier-1) modification of the PML protein occurs at lysines 65, 160, and 490. However, Lys160 appears to be the only modification site required for the formation of PML bodies. Expression of mutant PML proteins that have an amino acid substitution for Lys160 are targeted to the nucleus but are unable to form PML bodies (24, 25). Further insights into the mechanisms of PML body assembly and disassembly have also been derived from the live cell analysis of these subnuclear domains during cellular stress. During heat shock for example, PML bodies dissociate into small microstructures, which lack normal PML body components, such as Sp100 and SUMO-1 (4, 6, 7). These small structures bleb or bud from the surface of the PML bodies and become mobile (7). The remaining PML structures, or PML body remnants, are positionally stable for several hours, as are PML bodies in unstressed interphase nuclei (7, 26). Upon recovery from stress, microstructures begin to reacquire SUMO-1 and Sp100 and can be observed to fuse with large immobile PML body remnants (7). The hypothesis that SUMO-1 is essential to the integrity of PML bodies was also supported by results demonstrating that PML bodies, in cells overexpressing SUMO-1, are resistant to the microstructure formation in heat-stressed nuclei (7).

In this study, we demonstrate that the structural integrity of PML bodies not only relies on the post-translational modification of PML by SUMO-1 but also relies on the integrity and the state of chromatin condensation. Using correlative fluorescence microscopy and electron spectroscopic imaging (ESI), we demonstrate that the integrity of PML bodies is related to the degree of association of chromatin with the protein-based cores of PML bodies. In untreated cells, PML bodies are surrounded by chromatin, which explains their positional stability. Direct physical contacts between the protein core and chromatin fibers are prevalent. Inhibition of transcription causes a reduction in these contacts through the condensation of chromatin, correlating with the fission of new PML-containing microbodies from the parental bodies. This also occurs at the early stages of apoptosis, coinciding with the cleavage of euchromatin and associated chromatin condensation (27). These newly formed microbodies dissociate from existing PML bodies and are free to diffuse through the nucleoplasm and fuse with each other or with the parental PML bodies. Finally, we confirm that PML bodies break down in response to changes in chromatin integrity by introducing exogenous nuclease (DNase I) into cells. Immunofluorescence microscopy indicates that microbodies and the PML body remnants that are generated coincidentally with these chromatin changes are biochemically indistinguishable from PML bodies in untreated cells. Further, the formation of these microbodies is not affected by SUMO-1 overexpression. We conclude that chromatin integrity and condensation state alone can influence PML body morphology and stability.

MATERIALS AND METHODS

Cell Lines—SK-N-SH cells were grown in DMEM plus 10% fetal bovine serum to the desired degree of confluence. U2OS cells stably expressing GFP-PML-IV (a gift from Dr. J. Taylor, previously described in Ref. 7) were cultured in DMEM plus 10% fetal bovine serum containing 1600 μg/ml G418 (Wisent). Transcriptional inhibition of SK-N-SH cells was accomplished by treatment with either 5 μM/ml actinomycin D (Sigma), 80 μg/ml 5,6-dichlorobenzimidazole riboside (Sigma) or 100 μg/ml α-amanitin (Sigma) at 37 °C for 2 h. Apoptosis was induced in U2OS cells expressing GFP-PML-IV or SK-N-SH cells in DMEM containing 2 μM staurosporine (Sigma) at 37 °C.

Saponin/DNase I Treatments and DNase I Transfection—U2OS or SK-N-SH cells grown on coverslips were treated on ice with 0.1% saponin/phosphate-buffered saline containing 250 μg/ml of DNase I for 5 min. Coverslips were then washed 3 times for 1 min each in ice-cold phosphate-buffered saline containing identical concentrations of DNase I. Coverslips were then incubated at room temperature in RPMI 1640 containing DNase I. For protein transfection of DNase I, either two (12 μl) or three (18 μl) reactions of Chariot™ protein transfection agent (Active Motif) were mixed with 30 μl of DNase I (5 μg/ml). The Chariot agent was allowed to couple with the DNase I for 30 min at room temperature. The mixture was placed on coverslips with serum-free media and incubated at 37 °C. After 1 h, DMEM plus 10% fetal bovine serum was added. At the specified time points of individual experiments, coverslips were fixed in 1% paraformaldehyde for 5 min at room temperature and permeabilized with phosphate-buffered saline-Triton X-100 (0.5%) for 10 min. For live cell experiments, after the appropriate incubation periods in DMEM plus 10% fetal bovine serum, coverslips were placed on an environmental chamber containing media and imaged. All fluorescence microscopy was performed using a Leica DMR upright microscope fitted with a Hamamatsu ORCA camera. Openlab version 3.1.3 (Improvision) software was used to collect fluorescence images. Images were processed using either ImageJ version 1.28 (National Institutes of Health) or Photoshop 6.0/7.0 (Adobe).

TUNEL Assay and Cell Labeling—After fixation and permeabilization, TUNEL assay labeling was performed on coverslips. 1 μl (20 units) of terminal transferase (Roche Applied Science) was mixed with 10 μl of 5× reaction buffer, 1.5 μl of CoCl2 (1 mM), 0.3 μl of Cy3-dCTP (1 mM) (Amersham Biosciences), 0.33 μl of ICFP (0.1 mM), and 3.69 μl water for each coverslip to be analyzed. The TUNEL reaction mix was placed on the coverslips and incubated in a humidity chamber at 37 °C for 1 h. The reaction was terminated by washing the coverslips in 300 mM NaCl for 15 min at room temperature. PML protein was labeled by using either SE10 antibody (1:10) (a gift from Dr. R. Van Driel) or rabbit anti-SE10 antibody (1:1000) (Chemicon). SUMO-1 and Sp100 were labeled using mouse α-GMP-1 (1:5000) (Zymed Laboratories Inc.) and rabbit-α-Sp100 (1:1000) (Chemicon), respectively. HSP70 levels were monitored by labeling with mouse α-HSP70 (1:5000) (Stressgen). ESI—After labeling, cells were fixed (2% glutaraldehyde for 5 min at room temperature) and then dehydrated in a series of graded ethanol washes (30, 50, 70, and 95%). Coverslips were then infiltrated with Quetol 651 resin (EM Science) for 2 h, and then 2× 2-h Quetol mix with 2% DMP-30 (hardener), as previously described (20). Resin containing coverslips were cured overnight at 70 °C. Blocks of resin containing cells were sectioned (Leica ultramicrotome) to 70 nm, and the sections were collected on copper grid (EM Sciences). Images were collected using a Tecnai 20 TEM (FEI) equipped with an energy-dispersive spectrometer (Gatan) (28). Nitrogen and phosphorus maps are used to distinguish protein-based from nucleic acid-based structures, without the need for exogenous stains or labels (28). The precise locations of PML bodies in images were determined by correlative fluorescence microscopy and ESI (7, 20, 29).

RESULTS

PML Bodies Make Chromatin Contacts in Both Unstressed and Stressed Nuclei—We wished to determine whether PML bodies were free to move through the nucleoplasm or whether their mobility is restricted by a nuclear component. We followed the dynamic behavior of PML bodies in live U2OS cells stably expressing GFP-PML-IV. We observed that PML bodies retain their relative positions within the nucleus over time periods of a few hours (Fig. 1A), with the exception of minor movements due to cellular shape changes. The relative sizes of the PML bodies in a given cell are conserved over several hours. In some cells, new PML bodies appear during the course of imaging, consistent with reports that PML bodies increase in size and number during interphase (29). These new structures also appear to maintain their positional stability within the nucleus.

The positional stability of PML bodies could be explained by extensive contacts with chromatin or by attachment to an underlying protein-based structure or nuclear matrix (30). To
PML Body Structure Is Linked to Chromatin Organization

Fig. 1. PML bodies remain positionally stable over several hours. A, live U2OS cells expressing GFP-PML-IV growing on coverslips were placed on an environmental chamber and imaged using fluorescence. Images were captured at the initial time point (0H), 1 h (1H), 2 h (2H), 3 h (3H), and 4 h (4H) after placing the chamber on the microscope. With the exception of global shape changes, no movement of PML bodies is seen over the duration of imaging. B, SK-N-SH cells labeled for PML were embedded and sectioned to 70 nm. ESI was used to generate elemental maps. Phosphorous (P; red) and nitrogen (N; green) were overlaid and indicate those structures that contain chromatin (yellow) or are protein-based (green). 30-nm chromatin can be seen (arrowheads) making contact with protein fibers radiating from the core of the PML body. Regions rich in RNP are located adjacent to the body (circled). C, nitrogen and phosphorus maps of heat-shocked cells were generated and superimposed to demonstrate the effects of heat stress on PML bodies (circled) and the surrounding chromatin. Fewer contacts between PML bodies and the surrounding chromatin (arrowheads) were observed. D, sections of a SK-N-SH cell heat-shocked at 43°C for 20 min were imaged using ESI. Phosphorus (P; red) represents the phosphorous-containing material. The adjacent image is the overlay of the phosphorous and nitrogen maps and indicates the position of a PML body (arrow). Boxes (i and ii) indicate regions of decondensed 10-nm chromatin. E, regions of chromatin (i and ii) are shown at a higher magnification, demonstrating the decondensation of chromatin into 10-nm fibers within the vicinity of a PML body. The arrows indicate nucleosomes, and arrowheads indicate linker DNA. The scale bar in D represents 500 nm in B and D and 320 nm in C. The scale bar in E represents 20 nm.

determine whether we could observe such a structural basis for PML body positional stability, we used correlative fluorescence microscopy and ESI (31). ESI offers the ability to distinguish between protein-, DNA-, or RNA-based structures in the nucleus (7, 20). We observed that the protein-based cores of PML bodies are completely surrounded by and make physical contacts with chromatin fibers, predominantly 30-nm fibers. Nitrogen maps (green) and phosphorus maps (red) permit us to identify chromatin, ribonucleoprotein structures (RNPs), and protein-based structures (Fig. 1B). In merged nitrogen and phosphorus maps, chromatin appears as bright yellow fibers. RNPs appear less yellow (circled RNP in Fig. 1B), due to a lower P/N ratio than chromatin. RNPs, exhibiting a distinct morphology from chromatin, consisting of a more punctuate distribution of granules between 5 and 10 nm in diameter. Protein-based structures appear as green in the merged nitrogen and phosphorus maps. The high density of chromatin (arrowheads) around the bodies provides a physical barrier that may account for their positional stability. The protein cores demonstrate radial symmetry, and images from serial sections indicate that the cores of the bodies are spherical in shape (not shown). In addition, protein-based “spokes” are frequently observed, which make physical contacts with both the cores of the bodies and the chromatin on the periphery. A final feature that is typical of PML bodies is the accumulation of RNPs near their periphery (Fig. 1B, circled).

To test whether the association of PML bodies with chromatin and RNPs may have functional consequences, we examined nuclei at the ultrastructural level from cells that had been heat-stressed. Under such conditions, most transcription is repressed, with the exception of genes involved with the heat shock response (32). We observed several major differences between PML bodies in stressed cells compared with control cells. First, the protein-based cores of over 80% of PML bodies lose their typical radial symmetry (Fig. 1C, circled structures). Instead, the protein-based cores display extended length/width ratios, confirmed in serial sections (not shown). In addition, the protein-based cores are less dense, displaying a more open and fibrous appearance (Fig. 1, compare the cores in B and C). In heat-stressed cells, it is difficult to delineate the edges of the PML accumulations from other protein-based fibers and globular depositions that are present both in the vicinity of PML bodies and throughout the nucleoplasm (within versus outside the circled regions in Fig. 1C).

A second difference we observed relates to the organization of chromatin in heat-stressed nuclei. Large blocks of condensed chromatin were observed throughout the nucleoplasm (data not shown), supporting a previous study in which heat stress led to an increase in chromatin condensation (33). In the vicinity of PML bodies, however, the chromatin is less densely distributed (Fig. 1, C and D). Instead of dense packing of 30-nm fibers, 10-nm fibers are observed, separated by large spaces. These chromatin fibers frequently make physical connections to the fibrous protein cores of PML bodies (arrowheads in Fig. 1C). The 10-nm nature of these fibers is illustrated in Fig. 1, D and E. Nucleosome-based fibers are illustrated in the higher magnification insets (i and ii). The basis for defining these structures as nucleosomes is their morphology (11-nm diameter), some displaying a doughnut-shaped appearance in phosphorus-enhanced images (consistent with en face projections) and their nitrogen and phosphorus content (34). Finally, in heat-stressed cells, we never observe clearly identifiable accumulations of RNPs on the periphery of PML bodies. This is consistent with the loss of the 5′-fluorouridine incorpo-
cells is less than 10 per cell (cell i and cell ii), whereas the number in treated cells is over 30 (cell iii and cell iv). All of the PML-containing structures contain two other PML body-associated components, Sp100 and SUMO-1, at levels indistinguishable from PML bodies in control cells (Fig. 2B). We chose these two biochemical markers, since they are labile markers following heat stress (7) and are lost in early stages of mitosis (preliminary data). We refer to these newly formed PML containing structures as “microbodies,” since they contain these characteristic markers for PML bodies. Similar results were obtained with other transcriptional inhibitors (a-amanitin and 5,6-dichlorobenzimidazole riboside) (data not shown).

Since both heat stress and transcription inhibition with drugs lead to major changes in chromatin organization in the immediate vicinity of PML bodies and the organization of PML-containing structures, we wished to address whether the loss of euchromatin in the early stages of apoptosis by endogenous nucleases (27, 35, 36) would also lead to the disruption of PML bodies. Cells expressing GFP-PML-IV were treated with the apoptotic inducer, staurosporine, and observed by fluorescence microscopy over time (Fig. 3A). Within 30 min of staurosporine treatment, new PML-containing structures begin to appear. These small PML accumulations contain significant levels of Sp100 and SUMO-1 (Supplemental Fig. 1), similar to the structures that form during transcriptional inhibition (Fig. 2A). We observed that these new structures are highly mobile, moving several hundred nanometers during 1-s intervals (Supplemental Movies 1–5). Furthermore, the large parental PML bodies present prior to exposure to staurosporine become less constrained following treatment, exhibiting an increased mobility of up to 2 body diameters (based on the GFP-PML signal) (measurement data not shown). It is possible that these changes in PML body structure, distribution, and dynamics are related to DNA damage, since the changes coincide with increased TUNEL label incorporation (Fig. 3B).

We wished to determine the origin of the new PML-containing microbodies. We observed live U2OS cells expressing GFP-PML-IV by fluorescence microscopy after induction of apoptosis by staurosporine and collected single focal plane images at 2-min intervals (Fig. 3C). We observed many fission events, demonstrating that fragments or subunits of PML were dissociating from PML body structures. Over the course of staurosporine treatment, we observed that specific PML bodies (Fig. 3C, 12, inset) formed blebs or buds on their surface (Fig. 3C, 32). As the treatment progressed, larger portions of the bodies dissociated and were released from PML body surfaces (Fig. 3C, 80, 88, and 110). These events appeared similar to the fission of microstructures from the surface of PML bodies in heat-stressed cells. Immunofluorescence analysis of the biochemical composition of microbodies demonstrated that they contain levels of Sp100 and SUMO-1 that are similar to those found in PML bodies in both treated and control cells (Supplemental Fig. 1) (7). The continuing high levels of SUMO-1 in the bodies and microbodies may account for the frequent fusion events that are also observed (Fig. 3, Supplemental Movies 6 and 7). Since both fission and fusion (Supplemental Fig. 2) events are occurring simultaneously, we conclude that the bodies have become physically unstable but do not completely disassemble.

**Transcriptional Inhibition and Apoptosis Cause Changes in PML Body Morphology**—We used correlative fluorescence microscopy and ESI to visualize the structure of PML bodies and the surrounding chromatin following transcriptional inhibition by ActD. With the aid of correlative fluorescence images, we routinely and easily recognize PML bodies in tissue culture cell lines by comparing nitrogen and phosphorus maps (20, 31).
the nucleolus. As expected for transcriptionally inactive cells, no RNP accumulations were observed in the immediate vicinity of the PML-containing structures. We conclude that the structure of PML body cores, as well as the surrounding chromatin structure, is dependent on the transcriptional activity of the nucleus. This implicates PML bodies in transcriptional activity.

We then examined the PML bodies and chromatin organization by correlative fluorescence microscopy and ESI following staurosporine treatment to determine whether structural changes similar to those observed with transcriptional inhibition were evident (Fig. 4, C and D). Similar to observations made in ActD-treated cells, large PML-containing structures are evident in immunofluorescence images of physical sections (data not shown), but these PML accumulations do not resemble PML bodies in untreated interphase nuclei when examined by ESI. PML-containing structures appeared as relatively dense accumulations of protein (nitrogen-rich, phosphorus-depleted), lacking a recognizable symmetry, or composed of small, disconnected accumulations of protein surrounded by far less chromatin than that found around PML bodies in control cells (Fig. 4, C and D, arrowheads). As observed in heat-stressed cells and cells treated with ActD, the chromatin in these nuclei was condensed into blocks (Ch) ranging in size from 500 nm to 2 μm. Few contacts were observed between the chromatin blocks and the protein-based components of the PML-containing structures.

Following heat shock, transcriptional inhibition, and induction of apoptosis, PML body integrity appears to be affected, manifested by the generation of numerous smaller PML-containing structures (observed by fluorescence microscopy) and a disruption of the protein cores of the PML bodies (observed by ESI). In all cases, the nucleoplasmic region in the immediate vicinity of PML bodies becomes depleted in chromatin relative to control cells. For example, in control cells, 52 ± 3.2% (S.E.) (n = 18 PML bodies) of the space surrounding PML bodies within 3 body diameters is occupied by chromatin (as determined by measurement of signal in phosphorus maps). However, in treated cells, only 18 ± 1.2% (S.E.) (n = 19 PML bodies) is occupied by chromatin. Our interpretation is that these treatments (heat stress, transcriptional inhibition, and apoptosis) lead to a retraction of chromatin away from the periphery of PML bodies. This may have a destabilizing effect on the integrity of the PML body core structure. This destabilizing effect is apparent in the changes of the length/width ratios of PML bodies in both ActD- and staurosporine-treated cells. In untreated cells, this ratio is 1.2 ± 0.02. However, in treated cells, this ratio increased to 2.06 ± 0.22. A change in the length/width ratio could result from fission alone or from a combination of fission and fusion events (Fig. 4, Supplementary Movie 6).

**DNase I Treatment Affects the Integrity of PML Bodies**—Whereas staurosporine treatment activates endogenous nucleases, other biochemical pathways are also affected, which may indirectly account for the observed changes in PML distribution and PML body integrity. We wished to determine whether these changes were the direct consequence of chromatin cleavage. Live U2OS cells expressing GFP-PML-IV or SK-N-SH cells were permeabilized with 0.1% saponin in phosphate-buffered saline. Cells were then washed and incubated at room temperature in normal growth media containing DNase I. To examine the effects of this treatment on PML bodies, cells were fixed, labeled for PML, and imaged. Numerous, small PML-containing structures that were not present in control cells (Fig. 5A) were seen in the nucleoplasm of cells treated with DNase I. Fluorescence microscopy indicates that the radial symmetry of
the larger PML structures was disrupted, since several bodies were elongated or had irregular protrusions (Fig. 5A, 0.1% saponin/DNase I).

We wished to observe the dynamics of the disruption of PML bodies as a consequence of chromatin cleavage. U2OS cells expressing GFP-PML-IV were again incubated in media containing saponin and DNase I on ice for 3 min. Coverslips were then placed in normal media, and fluorescence images were captured. At the first imaging time point (Fig. 5B, 5 min), large PML-containing structures are evident with normal morphology (inset). At this time point, the cells have been treated with DNase I for ~5 min. Even at this early time, small, mobile PML-containing structures are observed (arrowheads). Such structures are not seen in nuclei exposed to saponin alone (data not shown).

By the second interval of imaging (Fig. 5B, 15 min), changes in the morphology of the large parental PML-containing structures are observed (arrows). These structures continue to change by the third interval (Fig. 5B, 25 min). The fluorescence images indicate that large domains of the bodies are dissociating, leaving structures that lack radial symmetry (Fig. 5B, insets). The formation of small mobile, PML-containing structures and the deterioration of the larger PML bodies occur within minutes of cell exposure to DNase I (at the earliest recording possible), indicating that the response of PML bodies is probably due to direct actions of the nuclease rather than as a result of subsequent biochemical responses. Breakdown of PML bodies was also observed when DNase I was introduced into cells by protein transfection, no significant levels of SUMO-1 are present in the original or parental bodies and the smaller PML structures generated by transcriptional inhibition or during early apoptosis. We conclude that the changes in PML body morphology and the formation of the numerous smaller PML-containing structures are related to the integrity and condensation state of chromatin. Therefore, the PML-containing structures generated by DNase I, staurosporine, or ActD treatment arise by a different process from those generated by heat stress or Cd²⁺ exposure.

SUMO-1 is an important factor in PML body structural stability, and overexpression of SUMO-1 prevents the disruption of PML bodies following heat or heavy metal stress (7). To further strengthen our interpretation that PML body disruption may have a biophysical basis related to the chromatin environment rather than to downstream biochemical modifications of PML body components, we treated cells that were transiently overexpressing GFP-SUMO-1 with ActD and staurosporine (Fig. 8, top). We observed that SUMO-1 overexpression has no protective effect on PML body structure or stability as a result of transcription inhibition or onset of early apoptosis. Also, since all PML-containing structures contain detectable levels of SUMO-1, we conclude that the formation of the new small structures does not depend on de-SUMOylation of PML.

To provide further evidence that chromatin-based contributions to PML body stability are not related to those affected by the major cellular stress responses, we examined the levels of the stress protein HSP70 following DNA cleavage by exogenous nuclease (Fig. 8, bottom). After 2 h of DNase I treatment, introduced into cells by protein transfection, no significant increase in the nuclear levels of HSP70 was observed. In contrast, after heat stress (+HS), an increase in both the nuclear and cytoplasmic levels of HSP70 is observed. We conclude that the chromatin cleavage-based disruption of PML bodies is not the result of a stress-mediated pathway (7).

**DISCUSSION**

Post-translational modification of PML protein by SUMO-1 is required for the formation and stability of PML bodies (7, 24, 25). However, in this study, we demonstrate that chromatin organization also plays an important role in PML body integ-
PML Body Structure Is Linked to Chromatin Organization

DNase I causes the formation of small PML-containing structures and changes in PML body morphology. A, control SK-N-SH cells were treated with culture media containing 250 μg/ml DNase I at room temperature for 30 min. Cells were then fixed and labeled for PML. Immunofluorescence indicates a change in PML body morphology and distribution. B, live U2OS expressing GFP-PML-IV were treated permeabilized with 0.1% saponin in media containing DNase I for 3 min. Coverslips were transferred to an environmental chamber containing media and DNase I and moved to the microscope. The initial image (large image, left) includes the interval after removal from saponin and assembly of the environmental chamber. The arrows indicate normal appearing PML bodies, and arrowheads indicate small mobile GFP-PML-IV containing structures. The inset region of the large image is magnified to indicate the changes in PML body structure. Fifteen minutes after the initiation of imaging, PML bodies have changed morphology, which continues through the next interval of imaging. C, SK-N-SH cells were protein-transfected with 10 μl of 5 mg/ml DNase I. After 2 h of incubation (1 h at 37°C and 1 h at room temperature), cells were fixed and labeled for PML. PML bodies appear to have irregular morphologies as compared with control cells as well as having smaller PML-containing structures not present in control cells. Higher magnification images of PML bodies from this and other cells treated with DNase I via protein-transfection are indicated on the right.

Major structural changes were confirmed at the electron microscopic level; the protein cores are more elongated, less dense, taking on a more fibrous appearance, and form fewer contacts with chromatin (Fig. 4). The chromatin is also less densely packed on the periphery of the bodies, apparently having retracted to more condensed domains throughout the nucleoplasm. The disruption of the bodies was not accompanied by the induction of stress-mediated responses through HSP70 (Fig. 8, bottom). Although both heat shock and heavy metal-induced PML body instability could be blocked by overexpression of SUMO-1 (7), overexpression of SUMO-1 failed to stabilize these bodies when chromatin structure was altered by either transcriptional inhibition or DNA damage (Fig. 8, top).

We have proposed that the default organization of chromatin in the nucleus is a condensed state and that euchromatin exists under tension (28). In cells that are transcriptionally repressed through heat shock or exposure to actinomycin D, in cells with compromised chromatin integrity in early apoptosis or following DNase I treatment, and in cells where DNA has undergone double strand breaks, chromatin condenses into numerous large blocks, over and above the normal condensed regions representing heterochromatin domains (Fig. 4). As euchromatin retracts into numerous condensed chromatin domains throughout the nucleus, a few residual 10- and 30-nm extended fibers remain physically anchored to protein-based structures such as PML bodies. Thus, the loss of the majority of chromatin contacts may lead to destabilized PML bodies.

We have previously shown that highly acetylated chromatin is digested very early during apoptosis, followed by a rapid condensation of the remaining chromatin into large, dense blocks (28). Chromatin most sensitive to DNase I is enriched in expressed or potentially expressed genes, represented by chromatin that is both highly and dynamically acetylated (37). Therefore, if PML bodies form functional associations with highly acetylated chromatin, one prediction would be that changes in the morphology, number, position, or biochemical composition of PML bodies might occur during the early stages of apoptosis. (We are confident that the disruption of PML bodies is an early apoptotic event, because microbodies were observed within the first 30 min following staurosporine treatment. The first signs of nuclear blebbing did not occur until at least 3 h after the first microbodies were observed.) Although the biochemical composition of the PML bodies did not appear to change (Figs. 2 and 7), we did observe the physical destabilization of PML bodies, leading to an increase in the numbers of PML-containing microbodies. The newly formed microbodies have apparently lost their positional stability, since they move large distances through the nucleoplasm. We hypothesize that these microbodies have lost contacts with chromatin or are still attached to small digested chromatin fragments and are thus able to diffuse freely. In addition, the larger, parental PML bodies have also lost some of the interactions that previously constrained them, acquiring an irregular motion, with an amplitude up to 2 PML body diameters. Sufficient chromatin contacts remain intact, however, so that these bodies retain their positional stability within the 2-body diameter region (Fig. 3A). We propose that PML bodies may serve as anchoring sites for euchromatin. Under normal conditions, the PML bodies and the surrounding chromatin contribute to each other’s organization and stability. When chromatin is disrupted, however, the bodies are also destabilized and undergo fission, producing PML microbodies. Similar changes to PML bodies also accompanied transcription inhibition and chromatin degradation by exposure of chromatin in live cells to DNase I.

2C. H. Eskiw, G. Dellaire, and D. P. Bazett-Jones, unpublished observations.
We and others have demonstrated the positional stability of PML bodies (7, 26). In another study, it was concluded that a subset of PML bodies are highly mobile (38). The disagreement over positional stability may arise because of the use of GFP-Sp100 (38) instead of GFP-PML as a marker for the bodies. Transient expression of GFP-Sp100 can result in the formation of a subset of mobile structures that do not contain PML protein and therefore cannot be defined as PML bodies.

The results of our study are also in contrast to previous studies in which the integrity of PML bodies was shown to be independent of the integrity of DNA (39, 40). In the previous studies, DNase I digestion was performed under the relatively harsh conditions required to generate nuclear matrix preparations. The protocol used in the present study exposed chromatin to relatively low levels of DNase I and under the physiological conditions of cell culture medium such that cells could recover fully from the brief exposure to saponin required for cell permeabilization. Furthermore, we have used an even more gentle protocol, protein transfection, to introduce DNase I into cells. These results were comparable with those obtained by permeabilizing with saponin. The harsh biochemical extraction methods used in the previous studies prevent a comparison with the present study, which was carried out at close to physiological conditions in living cells.

Two possible models may account for the loss of PML body integrity and disruption into microbodies. The first model is...
biophysical, based on direct interactions between the surface of PML bodies and surrounding chromatin. Many components of PML bodies are thought to interact with chromatin, including PML, Sp100, Daxx, and CREB-binding protein. These proteins are localized on the surface of PML bodies. Following transcriptional inhibition or chromatin disruption by endogenous or exogenous nucleases, PML bodies do not break down through dissociation of their separate protein components. Instead, as chromatin retracts, supramolecular segments of the PML bodies may be pulled out from the core of the structure. Alternatively, the retraction of chromatin away from the bodies increases the volume, shifting the equilibrium toward a higher population of supramolecular subunits involved in free diffusion. This would result in the appearance of new PML-containing structures or microbodies. In a second model, the disruption of chromatin structure may activate biochemical pathways that trigger the loss of PML body integrity through modification of PML body components. Although the bodies become unstable at the earliest observed time point following DNAse I treatment and this process is independent of SUMO-1 modification (Fig. 8, top), we cannot discount the possibility that some other signaling event might occur as a result of changes to chromatin structure. For example, other types of post-translational modification such as phosphorylation of PML body components (by ATM or DNA-PK (41)) may contribute to the destabilization of PML nuclear bodies following DNA damage. Although we favor a biophysical model to explain the SUMO-1-independent disassembly of PML bodies rather than a biochemical one, the two models need not be mutually exclusive. Indeed, it is also possible that both biophysical and biochemical mechanisms together may be responsible for the disruption of PML bodies, analogous to the breakdown of the nuclear envelope through modification of both lamin proteins and tension exerted by microtubules (42).

Kentis and co-workers (43) have previously shown that proteins containing RING fingers, including PML, are capable of spontaneously forming supramolecular complexes. They observed spherical, 50-nm diameter, PML-containing structures, which form independent of SUMO-1 modifications. It is interesting that we have now observed two independent pathways that each give rise to such supramolecular PML-containing structures. Cellular stress such as heat shock yields highly mobile microstructures that are depleted in Sp100 and SUMO-1. Disruption of chromatin structure can also generate micro-PML structures, without the loss of Sp100 or SUMO-1 modifications. Thus, the breakdown of PML bodies into subunits demonstrates the inherent property of PML/RING finger proteins to self-organize into such supramolecular complexes.

This study demonstrates that when the integrity of chromatin is compromised, PML bodies are rapidly destabilized through a SUMO-1-independent mechanism. We are currently testing the hypothesis that this destabilization has functional implications for the progression of apoptosis or DNA repair.

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