Conjugation with the ubiquitin-related modifier SUMO-1 regulates the partitioning of PML within the nucleus

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The PML protein, identified first as part of the oncogenic PML–RAR α chimera in acute promyelocytic leukemia (APL), concentrates within discrete subnuclear structures, corresponding to some types of nuclear bodies. These structures are disrupted in APL cells, and retinoic acid (RA) can trigger their reorganization, correlating with its therapeutic effect in this type of leukemia. Recently, arsenic trioxide (As₂O₃) was identified as a potent antileukemic agent which, similarly to RA, induces complete remissions in APL patients. Here we show that, in APL cells, As₂O₃ triggers rapid degradation of PML–RAR α and provokes the restoration of intact nuclear bodies. In non-APL cells, the ubiquitin-like protein SUMO-1 is covalently attached to a subset of wild-type PML in a reversible and phosphorylation-dependent manner. The unmodified form of PML is found in the soluble nucleoplasmic fraction, whereas the SUMO-1-polymodified forms of PML are compartmentalized exclusively in the PML nuclear bodies. As₂O₃ administration strikingly increases the pool of SUMO-1–PML conjugates that, subsequently, accumulate in enlarged nuclear bodies. In contrast to PML–RAR α , the overall amount of PML seems to remain unaltered up to 36 h following As₂O₃ treatment. These findings indicate that the conjugation of PML with SUMO-1 modulates its intracellular localization and suggest that post-translational modification by SUMO-1 may be more generally involved than previously suspected in the targeting of proteins to distinct subcellular structures. They provide additional evidence that the role of ‘ubiquitin-like’ post-translational modification is not limited to a degradation signal.

Keywords: arsenic/nuclear bodies/PML/post-translational modification/SUMO-1

Introduction

Acute promyelocytic leukaemia (APL) is a severe hematopoietic malignancy characterized by a specific block of differentiation of the myeloid progenitor cells at the promyelocytic stage. A unique feature of APL blasts is their ability to undergo differentiation after retinoic acid (RA) administration *in vitro* as well as *in vivo* (Huang *et al.*,

1988; Castaigne *et al.*, 1990). Thus, oral administration of RA can induce morphological complete remission of virtually all APL patients. After a disease-free interval, some patients relapse and subsequently develop resistance to RA treatment. Recently, Chen *et al.* (1996) reported that arsenic trioxide (As₂O₃) can induce remissions in both RA-sensitive and -resistant APL patients by triggering apoptosis rather than differentiation. At the molecular level, APL is characterized by a specific t(15;17) translocation which fuses the PML gene located on chromosome 15 to the retinoic acid receptor α (RAR α) gene on chromosome 17 (de Thé *et al.*, 1991; Goddard *et al.*, 1991; Kakizuka *et al.*, 1991; Pandolfi *et al.*, 1991; Kastner *et al.*, 1992). With some very rare exceptions, PML–RAR α fusion transcripts and proteins are found in all APL patients. The PML–RAR α chimera retains most of the functional domains of the parental PML and RAR α proteins. RAR α is missing its first 59 amino acids but retains the domains necessary for ligand and DNA binding. The candidate functional domains of PML, which are conserved in PML–RAR α , consist of three cysteine-rich regions, referred to as the RING finger and the B1 and B2 boxes, as well as an α -helical coiled-coil region (Reddy *et al.*, 1992). Whereas the exact function of the RING finger and B boxes are still unknown, the coiled-coil domain serves as a dimerization interface for the formation of stable PML–PML homodimers as well as PML–PML–RAR α heterodimers (Kastner *et al.*, 1992).

PML has been shown to localize to distinct subnuclear domains, the so-called PML nuclear bodies (NBs), ND 10 or PODs (Dyck *et al.*, 1994; Koken *et al.*, 1994; Weis *et al.*, 1994). These structures appear as dense spherical particles, 0.3–0.5 μ m in diameter, that are tightly associated with the nuclear matrix. Whereas normal cells contain 10–30 nuclear bodies per nucleus, in APL cells NBs are highly disorganized into numerous and aberrant microstructures containing both PML and PML–RAR α . Strikingly, RA treatment induces a drastic reorganization of the PML NBs back to their normal number and morphology, suggesting that the delocalization of PML or other nuclear body-associated proteins may play an important role in APL pathogenesis. To date, three other nuclear body antigens have been genetically characterized. The Sp100 protein has been identified using autoantibodies from patients with primary biliary cirrhosis (Szosteki *et al.*, 1990). Sp100 shows no significant homology to known proteins and its function is unknown. Another component of the nuclear bodies is NDP52 (Korioth *et al.*, 1995). As for PML (Lavau *et al.*, 1995; Stadler *et al.*, 1995) and Sp100 (Guldner *et al.*, 1992), the expression of NDP52 is strongly up-regulated by interferons (Korioth *et al.*, 1995). It contains a coiled-coil region and a LIM domain, but its biological properties are also unclear.

Recently, the putative PML-interacting clone encoding

Fig. 1. As_2O_3 induces the rapid reorganization of PML nuclear bodies in APL cells. Indirect immunofluorescence was performed with an anti-PML polyclonal antibody on untreated NB4 cells (A), on cells treated for 48 h with 1 μM RA (B) and on cells treated for 4 h with 1 μM As_2O_3 . All micrographs were obtained by confocal microscopy. The images represent a three-dimensional reconstruction of 10 optical sections.

the PIC 1 protein (now renamed SUMO-1 for small ubiquitin-related modifier) was identified in a yeast two-hybrid screen, when a human B-cell library was screened with the full-length PML protein as a bait (Boddy *et al.*, 1996). cDNAs for SUMO-1 have also been cloned independently by several other groups using different approaches. In a two-hybrid screen using the death domain of Fas/Apo-1 as a bait, SUMO-1 has been found to interact with Fas/Apo-1 and the tumor necrosis factor (TNF) receptor 1 (Okura *et al.*, 1996). Interestingly, overexpression of SUMO-1 prevents anti-Fas/Apo-1- and TNF-induced cell death. SUMO-1 has also been identified in the yeast two-hybrid system as a *bona fide* binding protein for human RAD51/52 (Shen *et al.*, 1996), the human homologs of yeast RAD51/RAD52, which are involved in the DNA double-strand break repair mechanism in yeast. SUMO-1, a polypeptide of 101 amino acids, is a member of the ubiquitin-like protein family, an expanding group of proteins which are all characterized by the presence of a UbH (ubiquitin homology) domain. Among this group, SUMO-1 shows the highest homology to human SMT3A/B (Mannen *et al.*, 1996; Lapenta *et al.*, 1997) and yeast SMT3 (Meluh and Koshland, 1995) proteins, with 47 and 52% identity, respectively. Yeast SMT3 originally has been identified as a suppressor of mutations in MIF2. The MIF2 protein is part of a centromeric multiprotein complex and is required for normal DNA replication and mitotic spindle integrity in yeast (Brown *et al.*, 1993; Meluh and Koshland, 1995). The overall identity between SUMO-1 and ubiquitin is 18%. Recently, two groups independently have shown that, like ubiquitin, SUMO-1 can be covalently attached to a target protein (Matunis *et al.*, 1996; Mahajan *et al.*, 1997). They identified as a target of SUMO-1 the Ran GTPase-activating protein RanGAP1, a component of the nuclear import machinery. In contrast to the well known function of ubiquitination in proteasomal protein degradation, modification of RanGAP1 by SUMO-1 does not lead to proteolysis, but rather seems to modulate the subcellular localization of RanGAP1. Whereas the 70 kDa unmodified form of RanGAP1 is localized exclusively in the cytosol, the SUMO-1-modified 90 kDa form is targeted to the nuclear pore complex, where it interacts with RanBP2.

Here we show that SUMO-1 is covalently conjugated with a subset of PML. The conjugation is phosphorylation

dependent and is mediated through a reversible process. The unmodified PML is found in the soluble fraction of the nucleus, whereas SUMO-1-modified PML is compartmentalized in the PML nuclear bodies, indicating that post-translational modification of proteins by SUMO-1 plays a general role in the regulation of their subcellular localization.

Results

As_2O_3 induces the rapid degradation of PML-RAR α and the reorganization of the PML nuclear bodies in APL cells

In an attempt to clarify the molecular basis for the therapeutic effect of As_2O_3 in APL, we wished to compare the effect of this compound with that of RA on PML nuclear body organization in the APL-derived NB4 cells. Figure 1A shows the typical microspeckled appearance of the PML immunolabeling in untreated NB4 cells. As described previously (Dyck *et al.*, 1994; Koken *et al.*, 1994; Weis *et al.*, 1994), treatment of the cells with 1 μM RA induces a drastic reorganization of the PML immunofluorescence pattern, with the restoration of intact nuclear bodies after 24–48 h (Figure 1B). Strikingly, treatment of NB4 cells with 1 μM As_2O_3 leads to a similar reorganization of the PML nuclear bodies (Figure 1C). However, in this case, it is a much more rapid phenomenon as a partial restoration is already detectable after 3 h and complete reorganization is seen after 4–6 h of As_2O_3 treatment. In addition, in ~30% of the cells, the PML bodies are not only reformed, but dramatically enlarged (Figure 1C). This pattern persists for up to 24–36 h and, after longer As_2O_3 exposure (~48 h), when cells are undergoing apoptosis, PML immunofluorescence staining becomes weak and more diffuse (data not shown).

It was shown previously that treatment of NB4 cells with RA leads to the degradation of the PML-RAR α fusion protein (Yoshida *et al.*, 1996). Accordingly, an immunoblot probed with an anti-RAR α antibody shows a progressive degradation of PML-RAR α after incubation of NB4 cells with 1 μM RA (Figure 2A). A decrease in the level of expression of the chimera is detectable after 12 h and the degradation is almost complete after 24 h. Examination of the PML-RAR α protein levels in NB4 cells after As_2O_3 treatment revealed that, similarly to what

Fig. 2. As_2O_3 induces the rapid degradation of PML-RAR α in APL cells. Cellular extracts from NB4 cells treated with 1 μM RA (A) or 1 μM As_2O_3 (B) for different times were prepared in SDS sample buffer. Proteins were separated on a 7.5% SDS-PAGE gel, transferred to a nitrocellulose membrane and probed with an anti-RAR α polyclonal antibody. The 120 kDa PML-RAR α and the 50 kDa RAR α proteins are indicated by arrowheads. The higher molecular weight PML-RAR α species are indicated by open triangles.

was observed for RA, As_2O_3 induces the degradation of the fusion protein (Figure 2B). However, the time course of arsenic-induced degradation is remarkably different from that of RA. After 2 h As_2O_3 treatment, the amount of PML-RAR α is already drastically diminished, and the fusion protein becomes barely detectable after 6 h. In addition to the 120 kDa chimeric protein, higher molecular PML-RAR α species are visible in untreated cell (Figure 2A and B, open triangles). After 2 h of As_2O_3 treatment, these bands are no longer detectable, but a smear of very high molecular weight bands becomes apparent after longer exposure of the blots (not shown).

As_2O_3 induces the formation of higher molecular weight PML species

Since As_2O_3 treatment had no effect on the level of expression of the endogenous RAR α in NB4 cells (Figure 2B), we wished to examine whether the PML moiety of the fusion protein might be the target of arsenic action. With this aim, we performed immunoblots on cellular extracts from HeLa cells stably overexpressing an F epitope-tagged PML protein after treatment with 1 μM RA or 1 μM As_2O_3 for different times (Figure 3). PML-overexpressing cells were used, as the endogenous PML protein is virtually not detectable by immunoblotting. PML(F) cells were lysed directly by boiling in SDS sample buffer and extracts analyzed by Western blotting. The blots were probed with a monoclonal antibody (mAb) directed against the F tag. In untreated cells, the antibody

Fig. 3. As_2O_3 induces the formation of higher molecular weight PML species in non-APL cells. Cellular extracts from HeLa cells stably overexpressing PML(F) were prepared in SDS sample buffer. Cells were treated for different times with 1 μM As_2O_3 or 1 μl of RA. Proteins were run on a 7.5% SDS-PAGE gel, transferred to a nitrocellulose membrane and the blot immunostained with a monoclonal antibody directed against the F tag. The unmodified 100 kDa PML form is indicated by an open triangle. The 120, 140 and 160 kDa forms are indicated by arrowheads and the high molecular weight species forming a smear towards the top of the gel by a square bracket. The ~68 kDa band represents a protein cross-reacting with the anti-F antibody that has been described previously (Ali *et al.*, 1993).

revealed a major PML(F) form showing an apparent M_r of 100 kDa. In addition, three larger PML species migrating between 20 and 60 kDa above this major form were detected (Figure 3, lane 1). None of these bands was detected in extracts from the parental HeLa cells (data not shown). A cross-reacting 68 kDa band, that has been observed previously (Ali *et al.*, 1993), was present in all extracts. Whereas treatment with RA did not alter the pattern of the PML-immunoreactive bands (Figure 3, compare lane 1 with lanes 6–9), unexpectedly, As_2O_3 treatment induced a dramatic shift of the 120, 140 and 160 kDa PML forms towards higher molecular weight species migrating from 160 kDa on towards the top of the gel (Figure 3, compare lane 1 with lanes 2–5). In contrast to the As_2O_3 -induced degradation of PML-RAR α (Figure 2B), no significant degradation of PML was observed upon As_2O_3 exposure, and this new pattern of bands remained unaltered up to 36 h after the beginning of the treatment. Similar results were obtained using a HeLa cell line stably overexpressing the untagged PML protein (data not shown). These data indicate that, in non-APL cells, As_2O_3 triggers post-translational modification of the wild-type PML protein.

PML is modified by conjugation to SUMO-1

The recent finding that the ubiquitin-related SUMO-1 protein interacts *in vivo* with PML (Boddy *et al.*, 1996) led us to hypothesize that the modification of PML might correspond to a covalent ligation with SUMO-1. To address this question, we looked for specific SUMO-1 immunoreactivity in PML immunoprecipitates (Figure 4A). RIPA extracts were prepared from HeLa PML(F)-expressing cells before and after a 5 h treatment with 1 μM As_2O_3 , as well as from the untreated parental HeLa cells used as a negative control. Extracts were subjected to immunoprecipitation with either the anti-F mAb or an anti-PML polyclonal antibody. Immunoprecipitates were separated by denaturing electrophoresis and analyzed by Western blotting with an anti-SUMO-1 mAb. In both the anti-F and anti-PML precipitates from untreated PML(F)

Fig. 4. PML and PML-RAR α are modified by conjugation to SUMO-1. (A and B) Cellular extracts were prepared in RIPA buffer from HeLa PML(F) cells before (lanes 2, 5, 8, 11 and 14) and after a 5 h treatment with 1 μ M As₂O₃ (lanes 3, 6, 9, 12 and 15). Cellular extracts from untreated parental HeLa cells were used as a negative control (lanes 1, 4, 7, 10 and 13). Extracts were immunoprecipitated with the antibodies as indicated. The anti-HA antibody served as an unrelated control. Immunoprecipitates were separated on a 7.5% SDS-PAGE gel and transferred to a nitrocellulose membrane. The left part of the membrane (A) was immunostained with a monoclonal anti-SUMO-1 antibody, the right part (B) with a polyclonal anti-PML antibody. The different forms of PML are indicated as described in Figure 3. Immunoglobulins are marked by an asterisk. (C) RIPA extracts from untreated NB4 cells (lanes 2 and 4) or from cells treated for 30 min with 1 μ M As₂O₃ (lanes 3 and 6) were immunoprecipitated with a polyclonal anti-PML or anti-RAR α antibody. Immunoprecipitates were separated by electrophoresis, transferred to nitrocellulose and the membrane was immunostained with a monoclonal anti-SUMO-1 antibody. Cellular extracts from untreated HL60 cells were used as a negative control (lanes 1 and 4). The 140 and 160 kDa forms of PML-RAR α are indicated by arrowheads and the high molecular weight species forming a smear towards the top of the gel by a square bracket.

cells, a major single SUMO-1-reactive band of 120 kDa was revealed (Figure 4A, lanes 2 and 5). After As₂O₃ treatment, a group of high molecular weight SUMO-1-immunoreactive bands, ranging from 120 kDa to the top of the gel, was detected in the anti-F (Figure 4A, lane 3) as well as in the anti-PML immunoprecipitates (Figure 4A, lane 6). As shown for several multi-ubiquitinated proteins (see, for example, Treier *et al.*, 1994), the slower migrating SUMO-1-reactive PML bands formed a complex pattern that transformed into a smear towards the top of the gel. No SUMO-1-immunoreactive bands were found in immunoprecipitates from the parental HeLa cells (Figure 4A, lanes 1 and 4), nor in precipitates performed with the unrelated anti-HA 'control' antibody (Figure 4A, lanes 7–9), excluding potential antibody artifacts.

To confirm these results, we performed the reciprocal experiment and probed anti-SUMO-1 immunoprecipitates with antibodies against PML. Figure 4B shows that in anti-SUMO-1 immunoprecipitates from As₂O₃-treated PML(F) cells, two PML-immunoreactive bands of 120 and 140 kDa were found (lane 12). These two SUMO-1-reactive bands co-migrated with modified forms of PML as detected using the anti-PML antibody in anti-F immunoprecipitates (Figure 4B, lane 15). On the contrary, the major unmodified 100 kDa PML form was not recognized by the anti-SUMO-1 antibody (compare lane 12 with 15). Probing a Western blot of PML(F) cell extracts directly with the anti-SUMO-1 antibody confirmed that all high molecular weight PML species observed before and after As₂O₃ treatment correspond to SUMO-1–PML conjugates (data not shown). Altogether, these data demonstrate that the modified forms of PML originate from a covalent linkage to SUMO-1. Such modified PML products pre-

exist in the cell, and As₂O₃ somehow induces the formation of multi-SUMO-1–PML conjugates similarly to a poly-ubiquitination process.

Surprisingly, the pattern of PML-reactive bands found in immunoprecipitations followed by Western blotting differs from the pattern observed in direct Western blots. Whereas in RIPA extracts from untreated PML(F) cells only a 120 kDa band (that most likely corresponds to a mono-SUMO-1-modified PML form) is detected (Figure 4B, lane 14), in direct Western blot, three PML species ranging from 120 to 160 kDa are observed (Figure 3, lane 1). The 140 and 160 kDa bands are likely to represent oligo-SUMO-1-modified PML forms consistent with the addition of two and three SUMO-1 molecules per PML, respectively. In a similar fashion, in immunoprecipitates from As₂O₃-treated PML(F) cells (Figure 4B, lane 15), the 120–160 kDa bands are the most abundant whereas in direct Western blots (Figure 3, lanes 2–5), much higher molecular weight species are detected, consistent with the addition of multiple SUMO-1 molecules. This discrepancy in the molecular weights of SUMO-1–PML conjugates recovered from immunoprecipitates, compared with those detected directly on Western blots, is observed consistently. This finding suggests that cleaving enzymes are present in RIPA extracts, which can reverse the formation of multi-SUMO-1–PML conjugates and are readily inhibited by lysing the cells directly in SDS sample buffer. A similar *in vitro* demodification has been described for RanGAP1–SUMO-1 conjugates (Matunis *et al.*, 1996; Mahajan *et al.*, 1997). These data are consistent with the interpretation that the modification of PML by SUMO-1 is reversible.

The unmodified and SUMO-1-modified forms of PML localize to distinct subnuclear compartments

As the conjugation of SUMO-1 to RanGAP1 modulates the subcellular localization of RanGAP1, we wished to determine whether the formation of SUMO-1–PML conjugates may similarly modify the localization of PML. To address this question, we took advantage of the fact that As₂O₃ dramatically increases the amount of modified PML. We thus analyzed both the PML and the SUMO-1 immunofluorescence pattern before and after As₂O₃ treatment in stably untagged PML-expressing HeLa cells as well as in wild-type HeLa cells. Double labeling with the polyclonal anti-PML antibody and the anti-SUMO-1 mAb was performed on formaldehyde-fixed and Triton-permeabilized cells. Figure 5A–I shows the confocal images obtained in the PML-overexpressing cell line. As has been observed previously, cells overexpressing PML have a higher number of nuclear bodies than normal cells and, in addition to the typical nuclear punctate staining, a nuclear diffuse PML signal is observed (Figure 5A). SUMO-1 staining reveals an intense nuclear diffuse signal, but also gives rise to a nuclear punctate labeling (Figure 5B). Note that the described SUMO-1 staining in nuclear envelopes is only visible after detergent extraction prior to fixation (Matunis *et al.*, 1996). Superimposition of the PML and the SUMO-1 signals demonstrates the co-localization of PML and SUMO-1 in almost all of the nuclear bodies (Figure 5C). After 4 h As₂O₃ treatment, both the nuclear diffuse fractions of PML (Figure 5D) and SUMO-1 (Figure 5E) have completely disappeared, and the two proteins are found exclusively together in enlarged nuclear bodies (Figure 5F). This observation suggests that As₂O₃ induces the ‘co-transfer’ of the diffuse forms of SUMO-1 and PML to the nuclear bodies. After 24 h, the number of nuclear bodies has diminished, but their size has increased further, forming sometimes large aggregates (Figure 5G–I).

Confocal images of endogenous PML and SUMO-1 localizations in wild-type HeLa cells are shown in Figure 5J–O. PML immunofluorescence exhibits the typical nuclear speckled pattern and a relatively weak nuclear diffuse signal (Figure 5J). SUMO-1 staining reveals an intense nuclear diffuse signal in all cells. In addition, in ~30% of the cells, a variable number of brighter dots is detected (Figure 5K), with most (but not all) of these dots corresponding to PML nuclear bodies (Figure 5L). Similarly to what was observed in the PML-overexpressing HeLa cells, As₂O₃ treatment for 4 h leads to the complete recruitment of the nuclear diffuse PML form to the nuclear bodies which consequently become larger (Figure 5M). In parallel, the nuclear diffuse SUMO-1 signal becomes slightly weaker and an obvious punctate pattern becomes apparent in almost every cell (Figure 5N). A clear co-localization between PML and SUMO-1 in the nuclear bodies is observed (Figure 5O), indicating that an important fraction of SUMO-1 concentrates in these structures following As₂O₃ treatment. After incubating wild-type HeLa cells for 48 h with As₂O₃, the PML staining in nuclear bodies decreased and the SUMO-1 signal became diffuse (data not shown). At this time, a large number of cells showed chromatin condensation and the formation of micronuclei, typical phenomena associated with apoptosis. As we also observed weaker staining for other nuclear body antigens

such as Sp100, we suggest that the disappearance of the PML signal is more likely to be a consequence of As₂O₃-induced cell apoptosis rather than a specific consequence of SUMO-1 conjugation.

The results from immunofluorescence microscopy demonstrate that As₂O₃ induces the recruitment of PML and SUMO-1 to the nuclear bodies. We thus wished to determine whether this concentration was due to the covalent conjugation of SUMO-1 to PML, and was not only the consequence of a protein–protein interaction between the two proteins. To address this question, cellular extracts from both untreated and As₂O₃-treated PML(F) cells were biochemically fractionated into an NP40-soluble fraction, which contains the soluble nuclear diffuse form of PML, and into an NP40-insoluble fraction, which among other insoluble cellular structures contains the nuclear matrix with the PML nuclear bodies. If the PML forms concentrating in the nuclear bodies correspond to covalent SUMO-1–PML conjugates, one would expect an accumulation of these modified PML forms in the insoluble fraction after As₂O₃ treatment. Figure 6 shows the result of a Western blot performed on the NP40-soluble and -insoluble fractions of extracts from untreated PML(F) cells or from the same cells treated for 3, 6 and 12 h with 1 μM As₂O₃. The soluble fraction from both untreated cells and cells treated for 3 h contains exclusively the unmodified 100 kDa PML form (Figure 6A, lanes 1 and 2). It is noteworthy that after 3 h As₂O₃ treatment, the amount of PML in the soluble fraction has greatly diminished and the protein becomes barely detectable after 6 h treatment (Figure 6A, lane 3). Concomitant with the disappearance of PML from the soluble fraction, the 120, 140 and 160 kDa SUMO-1-modified PML forms as well as the higher molecular weight SUMO-1–PML conjugates migrating above 160 kDa appear in the NP40-insoluble fraction (Figure 6B, lanes 6–8). As can be seen in lane 5, the 120 kDa mono-SUMO-1-modified PML form is already present in the insoluble fraction before As₂O₃ treatment. Taken together, the data of immunofluorescence and biochemical fractionation of the cells demonstrate that a nucleoplasmic unmodified form of PML can be converted into a SUMO-1-modified form concentrating in the PML nuclear bodies.

SUMO-1 conjugation to PML is regulated by phosphorylation

Ubiquitination is, in many cases, accomplished by a phosphorylation-dependent process (reviewed in Deshaies, 1997). We thus wished to investigate whether SUMO-1 modification of PML is submitted to similar types of regulation. In a recent report, it has been shown that PML is phosphorylated at serine residues (Chang *et al.*, 1995). In order to determine whether serine phosphorylation has any influence on the conjugation of SUMO-1 to PML, we treated PML(F) HeLa cells with calyculin A (a potent inhibitor of serine/threonine phosphatases 1 and 2A), As₂O₃ and a combination of the two substances. After a 45 min treatment, cells were lysed directly by boiling in SDS sample buffer and the extracts were analyzed by Western blotting for the presence of modified PML forms (Figure 7). Comparison of the pattern of PML bands in calyculin A-treated cells with non-treated controls showed that the largest (160 kDa) of the modified PML forms has

completely disappeared and that the 120 and 140 kDa forms are significantly less abundant (compare lanes 1 and 3). In consequence, the amount of unmodified PML is slightly increased. In the presence of calyculin A, As_2O_3 does not induce a significant shift of the 120, 140 and 160 kDa PML forms towards higher molecular weight species (compare lanes 2 and 4), indicating that hyperphosphorylation prevents the attachment of SUMO-1 to PML. Interestingly, SUMO-1 modification of RanGAP1 is enhanced in the presence of calyculin A (M.J.Matunis and G.Blobel, unpublished results), demonstrating that calyculin A does not down-regulate SUMO-1 modification in general. These data suggest that serine/threonine phosphorylation of PML and/or any other protein acting specifically on PML in the modification process suppresses the formation of SUMO-1–PML conjugates.

Discussion

The data presented here demonstrate the covalent linkage of the ubiquitin-like protein SUMO-1 to PML. PML is thus the second identified SUMO-1 target protein, after the Ran GTPase-activating protein RanGAP1. As previously observed for SUMO-1–RanGAP1 conjugates, we show that SUMO-1 modification of PML is implicated in protein compartmentalization, rather than protein degradation. This indicates that the subcellular partitioning of proteins through post-translational modification by SUMO-1 is of general importance. Although the modification of PML by SUMO-1 shows strong parallels to classical ubiquitination, there are also striking differences.

Ubiquitination is a post-translational modification, where single or multiple molecules of ubiquitin are covalently attached to a target protein (for review, see Ciechanover, 1994; Hochstrasser, 1995, 1996). Conjugation of ubiquitin proceeds via a three-step mechanism. Initially, ubiquitin is activated by the ATP-dependent formation of a high energy thioester intermediate between the ubiquitin-activating enzyme E1 and the C-terminus of ubiquitin (Ub). Ub is then transferred to one of several E2s (Ubc9 or Ub-conjugating enzymes), which catalyze the formation of isopeptide bonds between the C-terminus of Ub and the ϵ -group of lysines on the target protein. It is supposed that E3 (Ub-ligases) proteins serve as docking proteins for recognition of the target protein or are themselves the final intermediates in the process. A mono-ubiquitinated target protein can undergo further ubiquitination by the same cascade of events to form a protein-bound multi-ubiquitin chain. Multi-ubiquitinated proteins are recognized by the 26S proteasome complex, where they are degraded. For PML, we find a similar pattern of mono- and multi-modified SUMO-1 conjugates. However, in contrast to most ubiquitinated substrates, SUMO-1-modified PML is apparently not targeted for degradation, but rather seems to accumulate in the PML nuclear bodies. Similarly, RanGAP1 is compartmentalized in the nuclear pore complex by SUMO-1 modification, with no apparent

change in its metabolic stability. However, in contrast to what has been shown for SUMO-1–RanGAP1 conjugates, where only mono-modified RanGAP1 is found (Matunis *et al.*, 1996; Mahajan *et al.*, 1997), SUMO-1 can be attached in multiple copies to PML. Whether SUMO-1 multimers with PML are formed by targeting multiple amino acid residues of PML or by forming a SUMO-1 chain on a single amino acid residue remains to be determined. Recently it was demonstrated that in *Xenopus* extracts Ubc9, an E2 ubiquitin-conjugating enzyme that has been implicated in the regulation of the onset of mitosis (Seufert *et al.*, 1995), associates with the modified form of RanGAP1 and with RanBP2 (Saitoh *et al.*, 1997). Ubc9 is thus the candidate E2 enzyme for the formation of RanGAP1–SUMO-1 conjugates. Strikingly, using the yeast two-hybrid system, we detected a specific interaction between PML and human Ubc9, and in addition, a strong interaction between SUMO-1 and hUbc9 (S.Muller, J.Seeler and A.Dejean, unpublished results). These results strongly argue for the implication of Ubc9 in the formation of SUMO-1–PML conjugates.

Although we used the non-physiological agent As_2O_3 to induce multi-modification and relocalization of PML, we think that the interconversion of modified and unmodified PML and the shuttling of PML between the nucleoplasm and the nuclear bodies are of general importance under physiological conditions. We clearly show that, under normal conditions, both the nucleoplasmic unmodified form of PML and the nuclear bodies-associated SUMO-1–PML conjugates co-exist in the cell. Both forms are likely to be present in a dynamic equilibrium, as phosphorylation, a physiological signal, seems to influence the ratio between modified and unmodified forms. Furthermore, we observed a demodification of SUMO-1–PML conjugates *in vitro*, which may be due to the presence of a 'deubiquinating' activity in cellular extracts. It remains to be determined whether the modified or the unmodified forms of PML are the biologically active ones and what precise signals regulate the equilibrium between these two forms. One possibility is that nuclear bodies represent storage sites for PML and other nuclear factors, which can be liberated into the nucleoplasm for cellular processes such as transcription or cell division. The yeast homolog of SUMO-1, SMT3, is able to suppress mutations in the yeast MIF2 gene (Meluh and Koshland, 1995). Mutations in MIF2 lead to mitotic chromosome instability due to the formation of aberrant spindles (Brown *et al.*, 1993). Accordingly, SUMO-1 has been shown to localize to the mitotic spindle apparatus in dividing cells (Matunis *et al.*, 1996). However, until now, there is no evidence for PML or an other nuclear body antigen participating in processes such as spindle formation.

Interestingly, PML exhibits an altered nuclear distribution after infection of cells with certain DNA viruses such as herpes simplex virus (HSV) (Maul *et al.*, 1993; Everett and Maul, 1994; Everett *et al.*, 1995), cytomegalovirus (Kelly *et al.*, 1995) and adenovirus (Carvalho *et al.*, 1995;

Fig. 5. As_2O_3 induces the co-recruitment of PML and SUMO-1 to nuclear bodies. HeLa cells overexpressing an untagged PML protein (A–I) as well as wild-type HeLa cells (J–O) were subjected to double-immunofluorescence staining with a polyclonal anti-PML and a monoclonal anti-SUMO-1 antibody. Labeling was performed on untreated cells (A–C and J–L), cells treated for 4 h (D–F and M–O) or cells treated for 24 h (G–I) with 1 μ M As_2O_3 . The staining pattern was analyzed by confocal laser microscopy. The red signal (PML) is obtained with an anti-rabbit Ig Texas red-conjugated secondary antibody, the green signal (SUMO-1) with an anti-mouse Ig fluorescein-conjugated secondary antibody. Superimposing the two colors (merge) results in a yellow signal, where both proteins co-localize.

Fig. 6. SUMO-1-modified PML fractionates exclusively in the detergent-insoluble fraction. Cellular extracts from HeLa PML(F) cells treated with 1 μ M As_2O_3 for different times were separated in an NP-40-soluble (A) and an NP-40-insoluble fraction (B). Proteins were run on a 7.5% SDS-PAGE gel, transferred to a nitrocellulose membrane and the blot immunostained with a monoclonal antibody directed against the F tag. The different forms of PML are indicated as described in Figure 3.

Doucas *et al.*, 1996). It has been shown that viral immediate early proteins, such as the HSV transactivator ICP0, transiently localize to PML bodies before disrupting them. Recently the ICP0-interacting protein HAUSP (herpesvirus-associated ubiquitin-specific protease) has been identified, which functions as a ubiquitin-specific protease on model substrates (Everett *et al.*, 1997). In uninfected cells, HAUSP co-localizes with a subset of PML nuclear bodies, whereas at an early stage of HSV infection, the co-localization between HAUSP and PML is more extensive, suggesting that ICP0 is able to recruit HAUSP to nuclear bodies. In view of these observations and our present data, it is tempting to speculate that HAUSP could reverse SUMO-1 modification of PML, which subsequently disperses within the nucleus. Whether this change in PML localization is for the benefit of the virus or is part of a cellular defense mechanism is still not clear. The presence of HAUSP in a subset of nuclear bodies in non-infected cells would support the idea that there is a permanent exchange between modified and unmodified PML species under normal conditions.

In both anti-PML and anti-RAR α immunoprecipitates from As_2O_3 -treated NB4 cells, two high molecular weight SUMO-1-reactive bands migrating at 140 and 160 kDa as well as a smear migrating towards the top of the gel were detected, indicating that, similarly to PML, PML-RAR α can undergo modification by SUMO-1 (Figure 4C, lanes 3 and 6). However, in contrast to PML, PML-RAR α is degraded rapidly by As_2O_3 in APL cells. Thus, like RA, As_2O_3 induces degradation of the fusion protein and subsequent reorganization of the PML nuclear bodies. However, whereas degradation of PML-RAR α and restoration of the nuclear bodies by RA takes up to 24–48 h, arsenic provokes the same effects within 4–6 h. With both compounds, the kinetics of PML-RAR α degradation strictly correlate with the kinetics of restoration of the nuclear bodies, suggesting that nuclear body reorganization is a direct consequence of PML-RAR α destruction. Similar data were reported recently by others (Zhu *et al.*, 1997). The most simple hypothesis one may formulate to explain both RA- and As_2O_3 -induced degradation of PML-

Fig. 7. SUMO-1 conjugation to PML is regulated by phosphorylation. HeLa PML(F) cells were incubated with 1 μ M As_2O_3 (lane 2), 0.25 μ M calyculin A (lane 3) or both agents at the appropriate concentrations (lane 4). Cellular extracts were prepared by direct lysis in SDS sample buffer. Proteins were run on a 7.5% SDS-PAGE gel, transferred to a nitrocellulose membrane and the blot immunostained with a monoclonal antibody directed against the F tag. The different forms of PML are indicated as described in Figure 3.

RAR α is that binding of the ligand to the RAR α moiety in the first case and As_2O_3 -induced modification of the PML moiety in the second case alter the structure of the fusion protein. This alteration might be necessary for the degradation machinery to recognize PML-RAR α as a misfolded protein and thus change its metabolic stability.

In addition to their acute toxicity at higher doses, trivalent arsenic salts like As_2O_3 at low dose are considered as potent tumor promoters and have been shown to interfere with several important cellular pathways mostly by inhibiting enzymatic processes. As^{3+} salts have a high affinity for thiol groups in proteins and can form complexes with vicinal thiols. Interestingly, deubiquitinating enzymes (USPs) can be inhibited by thiol reagents, such as *N*-ethylmaleimide, presumably through interaction with a cysteine residue in their catalytic Cys domain (Baek *et al.*, 1997). Thus, it is reasonable to speculate that As_2O_3 might inhibit a specific SUMO-1-PML USP, which subsequently results in stabilization of the polymodified PML forms. Other well-known targets of As^{3+} salts are protein kinases or phosphatases. In view of the observation that the formation of SUMO-1-PML conjugates is prevented by serine/threonine phosphorylation, As_2O_3 might inhibit a serine/threonine protein kinase involved in the conjugation process. Understanding the exact role of As_2O_3 in the modification process of PML awaits identification and biochemical analysis of the enzymes involved.

In conclusion, our data provide evidence that covalent attachment of SUMO-1 to proteins might be a general mechanism to compartmentalize proteins in multiprotein complexes. This supports further the idea that ubiquitin or ubiquitin-like proteins have important roles beyond proteolysis. Considering that SUMO-1 is only one member of a large group of ubiquitin-like proteins, these findings open up exciting perspectives in the study of this new type of post-translational modification.

Materials and methods

Cell culture and preparation of stable cell lines

HeLa cells were grown at 37°C in 5% CO₂ in Dulbecco's modified minimal essential medium (Gibco, BRL), supplemented with antibiotics, glutamine and 10% fetal calf serum (FBS). NB4 cells (Lanotte *et al.*, 1991) were cultured in RPMI medium (Gibco, BRL) supplemented with antibiotics, glutamine and 10% FBS. All-*trans* RA (Sigma) was prepared as a 10 mM stock solution in ethanol, and As₂O₃ as a 1 mM stock solution in phosphate-buffered saline (PBS). The working concentration for both agents was 1 µM. Calyculin A (Sigma) was prepared as a 10 µM stock solution in dimethylsulfoxide (DMSO); the final concentration for cell treatment was 0.25 µM. HeLa cells stably overexpressing PML(F) were prepared by transient co-transfection of the pTL2-PML(F) vector (Kastner *et al.*, 1992), expressing an F epitope-tagged PML protein that consists of the full-length PML cDNA fused to the F region of the estrogen receptor (provided by P.Kastner and P.Chambon), and pSVneo (Clontech) at a ratio of 20:1. Neomycin-resistant clones were selected with G418 (Geneticin, Gibco, BRL) (750 µg/ml). Fifteen independent clones were tested for PML overexpression by Western blotting and immunofluorescence, and three among these were considered as positive (clones 7, 11 and 12). For all of the experiments described here, clone 11 was used. The inducible HeLa cell line stably overexpressing a full-length untagged PML protein (provided by T.Sternsdorf and H.Will) was as previously described (Sternsdorf *et al.*, 1995).

Antibodies

The polyclonal anti-PML antibody was raised against a recombinant GST-PML fusion protein and has been described previously (Weis *et al.*, 1994). The monoclonal anti-SUMO-1 antibody (21C7) was described previously (Matunis *et al.*, 1996). The anti-human RARα rabbit polyclonal antibody (RPαF-115) (Gaub *et al.*, 1993) and the anti-F antibody Ab(F3) (Ali *et al.*, 1993) ascites fluid and hybridoma culture supernatant were provided by M.-P.Gaub, D.Metzger and P.Chambon.

Preparation of cell extracts, immunoprecipitation and Western blotting

For direct Western blots, ~5×10⁶ cells were washed twice in PBS, scraped in 250 µl of SDS sample buffer and boiled for 10 min. For fractionation studies, the NP-40-soluble cellular proteins were extracted in NP-40 lysis buffer [50 mM Tris-HCl pH 8.0, 150 mM NaCl, 15 mM MgCl₂, 5 mM EDTA, 1 mM dithiothreitol (DTT)] containing protease inhibitors. Lysates were centrifuged and the remaining pellets extracted by boiling in SDS sample buffer. For immunoprecipitations, cells were lysed in RIPA buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.1% SDS, 1% NP-40, 1% sodium deoxycholate, 5 mM EDTA, 1 mM DTT) supplemented with protease inhibitors. The lysates were sonicated briefly and cleared by centrifugation. Supernatants were pre-cleared with 30 µl of protein A-Sepharose (Pharmacia) for 2 h at 4°C, centrifuged and incubated for 2 h with the appropriate antibodies. Antigen-antibody complexes were collected by adding 30 µl of protein A-Sepharose and incubation continued for an additional 1 h. The protein A-Sepharose beads were sedimented by a brief centrifugation, washed four times with ice-cold RIPA buffer and proteins recovered by boiling in SDS sample buffer. Proteins were separated by 7.5% SDS-PAGE and transferred to Hybond-C extra (Amersham) membranes. Membranes were blocked in 5% non-fat dry milk in PBST and incubated for 2 h with the various antibodies diluted in PBST. The anti-PML antibody was used at a dilution of 1:2500, the anti-F Ab(F3) hybridoma cell culture supernatant at 1:200, anti-SUMO-1 (21C7) at 1:2000 and anti-RARα (RPαF-115) at 1:500. After primary antibody incubation, blots were washed extensively in PBST and incubated for 1 h with the appropriate peroxidase-coupled secondary antibodies (Amersham). Enhanced chemiluminescence reagents (ECL, Amersham) were used for detection.

Indirect immunofluorescence and confocal laser microscopy

For immunofluorescence of adherent cells, PML-overexpressing or parental HeLa cells were grown on round coverslips in 6-well plates. Suspension cells were attached on poly-L-lysine- (Sigma) treated coverslips. Cells were fixed in 3.7% paraformaldehyde in PBS for 10 min at room temperature, and then permeabilized with 0.5% Triton X-100 in PBS for 20 min at room temperature. After fixation and permeabilization, cells were rinsed twice in PBS and once in PBS containing 0.05% Tween-20 (PBS-Tw), incubated with primary antibodies for 1 h, washed in PBS and PBS-Tw, and further incubated with the appropriate secondary antibodies conjugated with either fluorescein (Sigma) or Texas red

(Amersham). Primary antibodies were used at a dilution of 1:200 for anti-PML and 1:500 for anti-SUMO-1, secondary antibodies were used at a dilution of 1:200. After three washes in PBS, the samples were mounted in VectaShield (Vector Laboratories, Burlingame, CA). Confocal laser scanning microscopy was performed with a LEICA SM microscope, using excitation wavelengths of 488 nm (for fluorescein) and 543 nm (for Texas red). The two channels were recorded independently and pseudocolor images were generated and superimposed. The acquired digital images were processed using Adobe Photoshop v.3.1 software.

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