Cellular stress and DNA damage invoke temporally distinct Mdm2, p53 and PML complexes and damage-specific nuclear relocalization

Sari Kurki, Leena Latonen and Marikki Laiho*

Haartman Institute and Molecular and Cancer Biology Program, Biomedicum Helsinki, University of Helsinki and Helsinki University Central Hospital, PO BOX 63, FIN-00014 Helsinki, Finland

*Author for correspondence (e-mail: marikki.laiho@helsinki.fi)

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Summary

Mdm2 is a nucleoplasmic and nucleolar protein interacting with p53 and alternative reading frame (ARF) tumor suppressor proteins. Here we demonstrate relocalization and novel interactions of Mdm2 with the promyelocytic leukemia (PML) protein following cellular stress and DNA damage. We show that Mdm2 and PML interact directly in vivo and in vitro depending on the Mdm2 RING finger domain and the PML C-terminus, and that Mdm2 is recruited to the PML nuclear bodies by overexpression of PML. Cellular stress and DNA damage caused by UV-radiation, downregulation of the proteasome and arsenic trioxide promoted Mdm2 and PML damage-specific nuclear relocalization and interaction in a p53-independent manner. However, in vitro analyses showed that PML, Mdm2 and p53 form trimeric complexes. UV-radiation caused rapid rearrangements of PML nuclear bodies and promoted PML-p53 and PML-Mdm2 complex formation, coinciding with p53 stabilization and preceding p53-Mdm2 interaction suggesting temporally distinct complexes. The results demonstrate novel associations between Mdm2 and PML and show the capacity of PML to participate in the activation and stabilization of p53 in response to cellular stress through PML interaction with Mdm2.

Key words: Mdm2, p53, Nucleolus, PML, PML nuclear bodies, DNA damage, APL

Introduction

Mdm2 (murine double minute) is a nucleoplasmic and nucleolar RING-finger protein interacting with p53 and ARF (alternative reading frame) tumor suppressor proteins and has E3 ubiquitin ligase activity towards p53 (Honda et al., 1997) and itself (Honda and Yasuda, 2000; Fang et al., 2000). Mdm2 causes p53 conjugation by ubiquitin, p53 cytoplasmic transport and degradation by the proteasome. The Mdm2 control of cellular p53 level is relieved in DNA damaged cells, leading to an increase in p53 half-life and activity (Ashcroft and Vousden, 1999). The interference of p53-Mdm2 interaction occurs through post-translational modifications and different protein-protein interactions. p53 activity is also invoked by cellular oncogenes, such as Ras and Myc. These oncogenes, however, affect p53 by increasing the levels of $p14^{ARF}$, which binds Mdm2 and sequesters it to its specific nucleolar location relieving the negative regulation of p53 imposed by Mdm2 (Weber et al., 1999; Weber et al., 2000; Lohrum et al., 2000).

Promyelocytic leukemia protein (PML) is a potential tumor suppressor protein, and its overexpression induces either growth arrest or apoptosis (Pearson and Pelicci, 2001). Seven PML isoforms have been identified (PML I-VII according to the nomenclature by Jensen et al.) (Jensen et al., 2001), and they differ only in their C-terminal sequence. PML IV interacts with p53 and recruits it to the PML nuclear bodies (PML NB), increasing p53 transcriptional activity upon γ-radiation and by oncogenic Ras (Pearson et al., 2000; Guo et al., 2000; Fogal et al., 2000). PML itself is essential for the formation of PML NBs and the association of several regulatory and transcriptional factors, such as SUMO-1, Sp100, Daxx, CBP, p53 and DNA damage repair proteins, such as Mre11, to these bodies (Zhong et al., 2000; Lombard and Guarente, 2000; Müller et al., 2001). The composition of NBs is cell-cycle regulated and also dependent on cellular stress (Everett et al., 1999; Negorev and Maul, 2001). Through its interactions, PML has been ascribed several functions, including regulation of transcription, protein degradation, cellular senescence and DNA repair (Pearson et al., 2000; Ferbeyre et al., 2000; Pearson and Pelicci, 2001; Negorev and Maul, 2001; Bischof et al., 2002; Carbone et al., 2002). Sumoylation of PML is essential for the formation of the mature PML NBs (Zhong et al., 2000), and interestingly, several other PML NB proteins are sumoylated as well, suggesting that this modification directs their localization to the PML NBs or that sumoylation takes place in the bodies. PML appears to affect the activity of several proteins either by increasing (p53) or repressing (Daxx, Rb) their activities (Zhong et al., 2000; Müller et al., 2001) possibly in the PML NBs. In acute promyelocytic leukemia (APL) the function and localization of PML is altered because of the formation of PML-RARα fusion protein (de Thé et al., 1990; Dyck et al., 1994; Mu et al., 1994; Melnick and Licht, 1999). The fusion protein blocks the expression of genes that are essential for the normal myeloid differentiation and several PML-associated apoptotic pathways. Treatment of APL cells

with As_2O_3 induces the sumoylation and relocalization of PML-RARα and PML into NBs and leads to their degradation by the proteasome pathway (Quignon et al., 1998; Zhong et al., 2000; Lallemand-Breitenbach et al., 2001; Miller et al., 2002).

Here we explore the relationship between the two regulators of the p53 pathway, Mdm2 and PML. Several studies have indicated the function of PML in regulation of the p53 pathway upon γ-radiation and by oncogenic Ras. Here we demonstrate that UV-radiation increases PML solubility and its interaction with Mdm2. We find that Mdm2 and PML interact in vitro and that in cellular stress, induced either by proteasome inhibition or UV-radiation, Mdm2 and PML form complexes in vivo and relocalize in a damage-specific manner. Moreover, we show that p53 interacts with PML rapidly after UV damage prior to its stabilization, and that this interaction preceeds p53-Mdm2 complex formation. In vitro binding analyses demonstrated that p53, Mdm2 and PML form trimeric complexes. In the trimeric complexes, the Mdm2-mediated degradation of p53 is predicted to be obstructed because of PML binding to Mdm2 RING domain. The results suggest that cellular stress, induced by UV, causes novel associations between Mdm2, PML and p53 and that PML participates in the activation of p53 through regulation of Mdm2.

Materials and Methods

Plasmids

Expression vectors for PML III (PML-L) in pSG5, PML IV (PML-3) and PML IV-3K (sumoylation-deficient triple mutant PML IV) in pCDNA3 and PML-RARα in pSG5 were kindly obtained from G. del Sal. Mdm2 deletion constructs 6-339, 1-440, Δ58-89 and Δ222-437, a kind gift from A. Levine, were cloned into pSG5 vector for in vitro studies. Mdm2 deletion mutant Δ89-222 and Mdm2 nucleolar localization-defective mutant (ΔNoLS, Δ464-471) were constructed by site-directed mutagenesis (QuickChange Site-Directed Mutagenesis Kit, Stratagene) and were verified by sequencing.

Cells and transfections

SaOS-2 osteosarcoma cells, *p53*$^{-/-}$*mdm2*$^{-/-}$ mouse embryo fibroblasts (Montes de Oca Luna et al., 1995) and WS1 human skin fibroblasts were cultured in a humidified atmosphere containing 5% CO_2 at 37°C. Cells were transfected by electroporation (Gene Pulser II, Bio-Rad) and were treated with UVC (254 nm, Stratalinker 2400, Stratagene), MG132 (10 μM, Affiniti Research Products) or As_2O_3 (1 μM, Sigma) as indicated.

Immunofluorescence

Cells were fixed with 3.5% paraformaldehyde followed by permeabilization with 0.5% NP-40. Alternatively, cells were permeabilized before fixation with 0.5% NP-40 for 5 minutes; 3% BSA was used for blocking. Primary antibodies used for detection were as follows. Mdm2, IF-2 (Oncogene Sciences), SMP-14 (Santa Cruz Biotechnology) and 2A10; PML, PG-M3 and H-238 (Santa Cruz Biotechnology), polyclonal PML IV antibody (G. del Sal); p53, DO-1, PAb421, PAb1801 and FL393 (Santa Cruz Biotechnology). Mixes of the indicated monoclonal antibodies were used for the detection of Mdm2 and p53.

In coimmunostainings, swine anti-rabbit or rabbit anti-goat FITC (DAKO), or goat anti-mouse conjugated Alexa594 or goat anti-rabbit conjugated Alexa488 (Molecular Probes) were used as fluorochromes. Absence of crossreactivity of the antibodies and conjugates was verified in separate experiments. The fluorochromes were visualized with Axioplan 2 Imaging MOT (Zeiss, Jena, Germany) equipped with appropriate filters (Chroma), and images were captured with Zeiss Axiocam CCD-video camera, followed by image processing and multilayer analysis with AxioVision program version 3.0. Confocal images were made with Bio-Rad MRC1024.

Immunoprecipitation and immunoblotting

Cellular lysates were prepared into EBC lysis buffer containing 25 mM Tris-HCl pH 8.0, 120 mM NaCl, 0.5% NP-40, 4 mM NaF, 100 μM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 100 KIU/ml aprotinin and 10 μg/ml leupeptin. Where indicated, an insoluble fraction composed of EBC lysis buffer insoluble cellular pellet was collected. Protein concentrations were determined by Bio-Rad D_c protein assay kit (Bio-Rad), and after normalization of the protein concentrations, lysates were immunoprecipitated with specific antibodies and collected on GammaBind-G Sepharose (Pharmacia Biotech). Mixes of the indicated monoclonal antibodies were used for the detection of Mdm2 and p53. Cellular lysates or immunocomplexes were boiled in Laemmli sample buffer containing dithiothreitol and were separated by 9% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membrane (Trans-Blot, Transfer Medium, Bio-Rad). Immunoblotting was performed by using antibodies listed above followed by secondary antibodies conjugated with horseradish peroxidase (HRP) and detection with enhanced chemiluminescence (ECL) (Amersham Life Sciences). Total cell lysates were extracted in Laemmli sample buffer, sonicated before boiling and analysed as above.

In vitro translation

In vitro translations of Mdm2, p53 and PML were performed with TNT Coupled Reticulocyte Lysate System (Promega) from expression vectors containing T7 promoter. Translation products were precipitated with either Mdm2, p53 or PML antibodies as above, in order to verify the direct interaction between these two proteins.

Results

Mdm2 colocalizes with PML in cellular stress and DNA damage

PML exists in cells in an insoluble form attached to the PML NBs, and as a freely detergent-soluble form present in the nucleoplasm (Müller et al., 1998; Lallemand-Breitenbach et al., 2001). The size and number of PML NBs undergo changes in response to viral infection, oncogenic Ras and in γ-irradiated cells (Pearson et al., 2000; Regad and Chelbi-Alix, 2001; Pearson and Pelicci, 2001). We first addressed the effect of UVC-radiation on PML. p53-null SaOS-2 osteosarcoma cells show an expected number (8-20) of PML NBs. Mdm2 was evenly distributed throughout the nucleoplasm (Fig. 1A). In occasional cells (<3%) Mdm2 colocalized with few PML NBs. UVC-treatment of the cells led to a rapid destruction of the nuclear architecture of the PML bodies and increased PML nucleoplasmic staining (Fig. 1A, see also Fig. 6). PML was found relocalized to perinucleolar areas, resembling a nucleolar necklace structure first described by Granick in 1975 (Granick, 1975) (Fig. 1A,B). Similar, overlapping perinucleolar staining of Mdm2 was also detected following UV-radiation, though most of the Mdm2 still remained in the nucleoplasmic fraction (Fig. 1A,B). To ascertain the solubility of the proteins, cells were shortly treated with NP-40 lysis buffer before fixation and immunostaining. In these samples insoluble Mdm2 was found in the nucleoli (Fig. 1C). In the

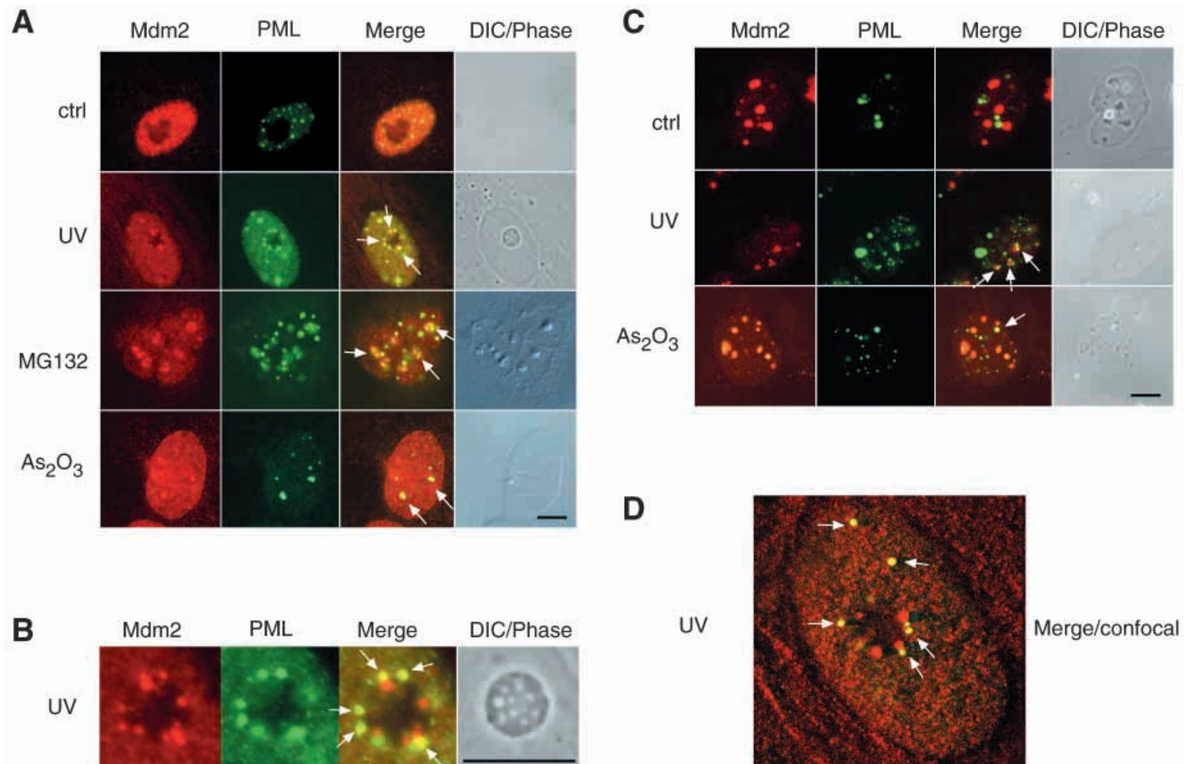

Fig. 1. Mdm2-PML colocalization. (A) SaOS-2 cells were left untreated (control) or were treated with MG132 (10 μM), As_2O_3 (1 μM) or were radiated with UVC (35 J/m^2) and incubated for 6 hours, except with As_2O_3 for 16 hours. Cells were fixed and stained for endogenous Mdm2 and PML. (B) Localizations of Mdm2 and PML in a nucleolar necklace structure after UV-treatment. The image is a 3.75× magnification from A. (C) Localizations of insoluble Mdm2 and PML. SaOS-2 cells, treated as in A, were permeabilized with 0.5% NP-40, followed by staining for endogenous Mdm2 and PML. (D) Confocal image of Mdm2-PML colocalization. WS1 cells were treated with UVC and were incubated for 6 hours and stained for endogenous Mdm2 (red) and PML (green). The localizations were visualized by confocal microscopy. A layer (0.36 μm) of the merged projection is shown. Cells were visualized by differential interference (DIC) or phase contrast. The overlay for Mdm2 and PML is shown in MERGE as yellow staining. Arrows, colocalization of Mdm2 and PML. Bars, 10 μm.

UV-treated cells, Mdm2 and PML colocalized in the detergent-insoluble PML NBs and in the perinucleolar dots, indicating that the latter were bound to nucleolar structures (Fig. 1C). Colocalization of Mdm2 and PML after UV-treatment was also confirmed by confocal microscopy (Fig. 1D).

In proteasome inhibitor-treated cells, however, PML has been found in the nucleoli (Mattson et al., 2001). Given that we and others have recently shown that Mdm2 is relocalized to the nucleoli in response to proteasome inhibitor treatment (Klibanov et al., 2001; Latonen et al., 2003), we wanted to address a possible association between Mdm2 and PML. To address the effect of downregulation of the proteasome on localizations of PML and Mdm2, we treated SaOS-2 cells with the proteasomal inhibitor MG132. Downregulation of the proteasome led to an increase in the number of PML nuclear bodies and its translocation into the nucleoli that took place within 6 hours (Fig. 1A) (Everett et al., 1998; Mattson et al., 2001). Colocalization of PML and Mdm2 was detected both in the bodies and in the nucleoli (Fig. 1A). This was not exclusive however, as both were also present in separate structures, Mdm2 in the nucleoplasm and PML in bodies devoid of Mdm2.

Arsenic trioxide recruits PML to NBs (Müller et al., 1998; Lallemand-Breitenbach et al., 2001). To test whether PML, located exclusively in the NBs after arsenic treatment, affects Mdm2 localization, we treated SaOS-2 cells with As_2O_3 for 16 hours. In As_2O_3-treated cells Mdm2 was found relocalized to large detergent-insoluble PML-bodies (Fig. 1A,C), and it also retained nucleoplasmic staining (Fig. 1A). However, the redistribution of Mdm2 following arsenic trioxide treatment was kinetically slower than the MG132-induced changes but was clearly evident after 16 hours. The results suggest that Mdm2 and PML can co-localize in response to cellular stress and DNA damage in a spatially and temporally distinct manner.

To address whether PML can affect Mdm2 localization, we ectopically expressed Mdm2 and PML III or PML IV in *p53* and *mdm2* null fibroblasts (Montes de Oca Luna et al., 1995). PML III and IV were expressed at high levels in the transfected cells, and concentrated to large nuclear bodies (Fig. 2). These structures did not counterstain for DNA or RNA (SYTO Green) and they resembled larger aggregates of natural PML bodies. Depending on the levels of the expressed proteins, Mdm2 was found to co-localize with both PML forms, either exclusively or to a large part, losing its even nucleoplasmic staining present in untreated cells and concentrated to the PML bodies (Fig. 2). The Mdm2 nucleolar localization is determined by its nuclear localization signal (NoLS) (Lohrum et al., 2000). To further address the capacity of PML to cause translocation of Mdm2, we tested whether PML can affect the localization of a mutant

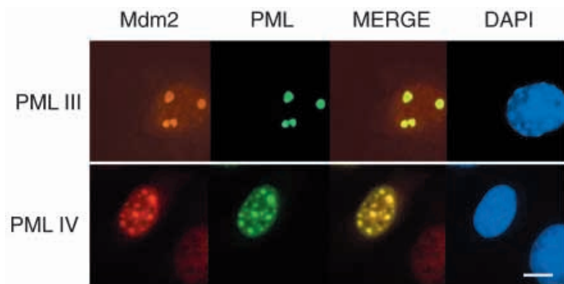

Fig. 2. PML sequesters Mdm2. *p53*$^{-/-}$*mdm2*$^{-/-}$ cells were transfected with Mdm2 and PML III or PML IV expression vectors as indicated. The cells were stained for Mdm2 (red) and PML (green). Nuclei were visualized with DAPI. The overlay for Mdm2 and PML is shown in MERGE as yellow staining. Bars, 10 µm.

Mdm2 lacking its NoLS (ΔNoLS). ΔNoLS Mdm2 was transfected into *p53*$^{-/-}$*mdm2*$^{-/-}$ fibroblasts and the cells were treated with MG132. Analysis of ΔNoLS Mdm2 transfected cells (over 200 cells from at least three separate experiments) indicated that ΔNoLS Mdm2 and PML had an overlapping staining pattern and that ΔNoLS Mdm2 was detected only in nucleoli that contained nucleolar PML (data not shown). This suggests that PML or other PML body proteins direct Mdm2 independent of the Mdm2 NoLS. The results indicate that PML has the capacity to sequester Mdm2 to the PML NBs as well as redirect Mdm2 into the nucleoli.

Mdm2 and PML interact in vitro

Direct interaction of Mdm2 and PML was verified by in vitro translation and co-immunoprecipitation analyses. The results showed that in vitro translated full-length Mdm2 interacted with PML isoforms III and IV and that the interaction was independent of PML sumoylation status (Fig. 3A). Similar results were obtained by coimmunoprecipitation analyses using an PML antibody (not shown). However, Mdm2 interaction with PML III was weaker than with PML IV. There was negligible interaction between Mdm2 and PML-RARα fusion protein, suggesting that an intact C-terminus of PML, absent in the fusion protein, is required for the interaction (Fig. 3A).

Similar analysis was performed by translating different Mdm2 deletion constructs in vitro followed by co-immunoprecipitations with in vitro translated PML IV (Fig. 3B). Mdm2 C-terminal deletion mutants (6-339 and 1-440) had significantly weaker interactions with PML IV than wild-type Mdm2 or Mdm2 deletion mutants Δ58-89, Δ89-222 and Δ222-437 (Fig. 3B). This suggests that the Mdm2 C-terminus, including the RING finger domain responsible for p53 degradation, participates in the interaction. As only a fraction of PML appears to interact with Mdm2, it is possible that additional modifications of the proteins not present in the in vitro translation products increase the efficiency of the interaction.

Mdm2 and PML interact in vivo in response to cellular stress

We further analysed the responses and interactions of endogenous Mdm2 and PML in SaOS-2 cells following treatments with either MG132 or UV-radiation. One major 92 kDa PML form was detected in the soluble and insoluble cellular fractions. Based on its molecular weight, this form could represent either PML III or IV. The PML levels increased in lysates of both UVC- and MG132-treated cells, indicating its increase in the soluble nucleoplasmic fraction (Fig. 4A). In addition, the NP-40 insoluble fraction of PML decreased in the UV-treated cells (Fig. 4A), whereas the total levels of PML were unchanged at 6 hours (data not shown, see also Fig. 5), suggesting that the increase in the soluble fraction was because of the release of PML from the PML bodies. Furthermore, PML derived from either MG132 or UV-treated SaOS-2 cells coimmunoprecipitated with endogenous Mdm2, demonstrating their interaction and suggesting that both treatments increase the availability of the interacting proteins (Fig. 4B). Although the interaction was detected in As_2O_3-treated cells as well, it did not increase as compared to the controls (not shown). The results suggest that Mdm2 and PML interact at least in the nucleoplasmic fraction. Finally, as SaOS-2 cells are null for

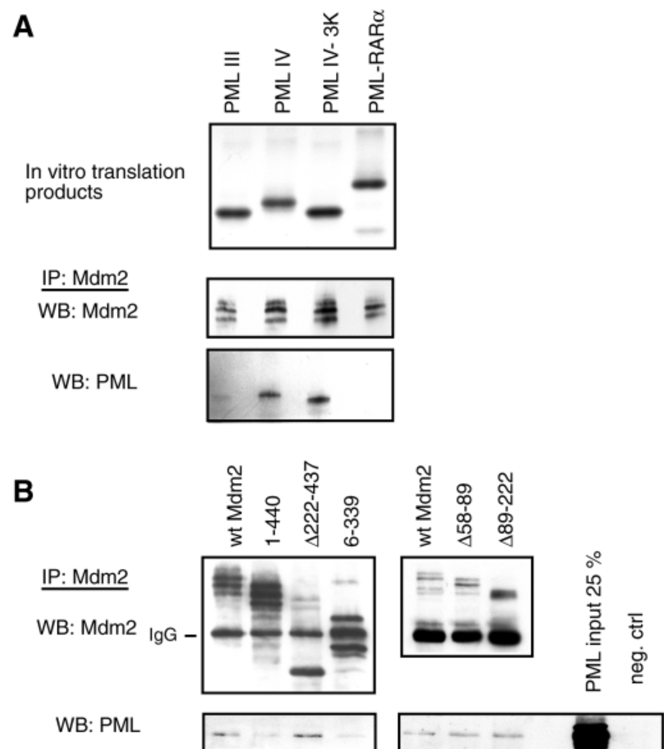

Fig. 3. Mdm2 interacts with PML in vitro. (A) PML isoforms III, IV, IV-3K and PML-RARα were translated in vitro and mixed with equal amounts of in vitro translated wild-type Mdm2. The translation products were precipitated with an Mdm2 antibody mix (IF2, 2A10, SMP14) and blotted against PML (H-238) and Mdm2. Recognition of the different PML forms by the H-238 antibody was verified in separate experiments. (B) Mdm2 C-terminus is required for PML interaction. Mdm2 deletion mutants were translated in vitro and precipitated with in vitro translated PML IV. The products were precipitated with the Mdm2 antibody mix and blotted against the indicated proteins. Deletion constructs Δ58-89 and Δ89-222 were analysed in separate experiments together with full-length Mdm2. Note that Mdm2 migrates as three bands because of alternative translation initiation. As a negative control, in vitro translated Mdm2 was omitted from the binding reaction (neg. ctrl).

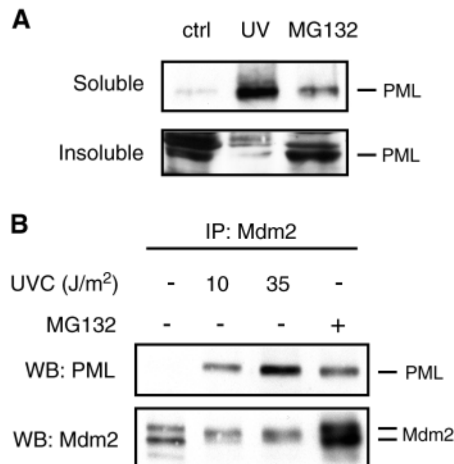

Fig. 4. UVC-radiation alters PML subcellular levels and its interaction with Mdm2. SaOS-2 cells were treated with MG132 (10 μM) or were radiated with UVC and incubated for 6 hours. (A) Soluble and insoluble PML fractions were analysed by immunoblotting. (B) Cellular lysates were immunoprecipitated with Mdm2 antibodies followed by immunoblotting as indicated.

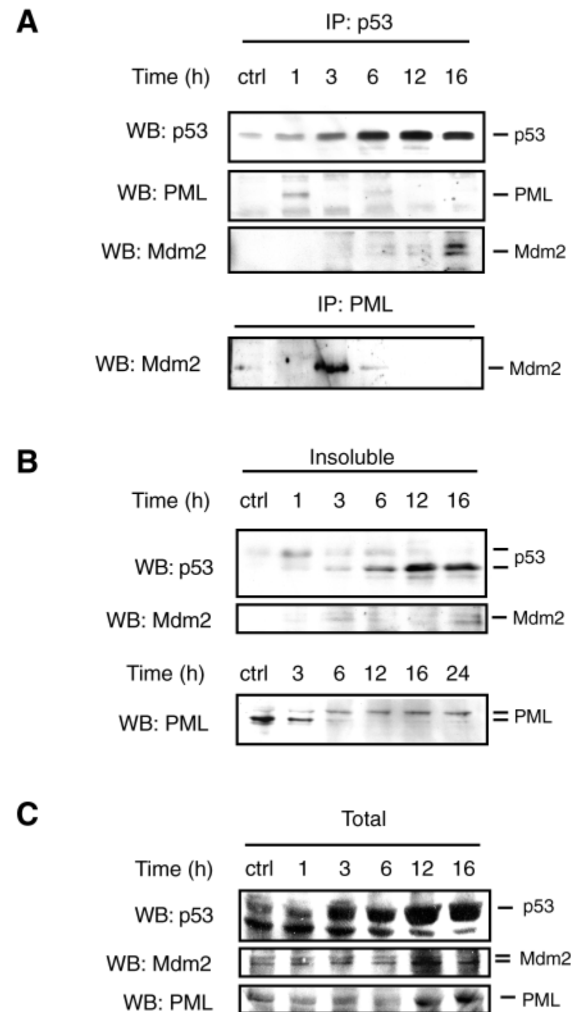

Fig. 5. Temporal complexes between p53, Mdm2 and PML. WS1 cells were treated with UVC (35 J/m^2) and were incubated for the given time. (A) Cellular lysates were precipitated with an p53 antibody mix (DO1, PAb1801, PAb421) or precipitated with a PML antibody (PG-M3). The immunoprecipitates were blotted against p53 (FL-393), Mdm2 (antibody mix of 2A10, IF2, SMP-14) or PML (H-238) as indicated. (B) Insoluble fractions of p53, Mdm2 and PML. Following UVC-treatment, the cells were lysed with NP-40 lysis buffer and the NP-40-insoluble pellet was analysed by immunoblotting as indicated. (C) WS1 cells were extracted in Laemli sample buffer and total levels of p53, Mdm2 and PML were analysed by western blotting.

p53, the Mdm2-PML interaction was clearly independent of PML-p53 interaction.

Mdm2, PML and p53 form temporal complexes in response to DNA damage

To test the timing and relationship of the complexes between Mdm2, PML and p53, we treated human fibroblasts expressing wild-type p53 with UVC and incubated the cells for different periods of time as indicated in Fig. 5. Cellular lysates were precipitated either with an p53 antibody mix or antibody against PML followed by immunoblotting. As amply demonstrated previously (Latonen et al., 2001), p53 levels increased at 3 hours after the damage during which its interaction with Mdm2 was low to negligible. p53-Mdm2 interaction increased at later timepoints coinciding with an increase in Mdm2 levels by p53 (Fig. 5A). Following UV radiation, p53 formed a complex with PML, but only at a kinetically narrow window between 1-3 hours after the damage (Fig. 5A and data not shown). Mdm2 complex formation with PML was also transient, peaking at 3 hours (Fig. 5A). Thus, p53-PML and Mdm2-PML complexes were present in UV-radiated cells early after the damage and these interactions preceeded p53 stabilization and Mdm2-p53 complex formation. Although these interactions took place in the nucleoplasmic fraction, it is possible that the interactions may take place in the PML bodies as well.

To verify the possible translocation of Mdm2, p53 and PML to the NP-40 insoluble fraction of the cells (including NBs, nucleoli, DNA bound fraction) we also performed immunoblotting analyses of the insoluble fractions. Shortly after the UV-radiation (1 hour) p53 was found in the NP-40 insoluble pellets in a slower migrating 65 kd form (Fig. 5B). As this corresponds to the size of sumoylated p53 (Rodriguez et al., 1999) (data not shown), it is possible this form represents p53 undergoing SUMO-modification. In response to UV, PML levels in the insoluble fraction initially decreased, whereas a slower migrating PML form appeared at later timepoints (Fig. 5B). There were no major changes in the insoluble fraction of Mdm2.

To correlate the changes of the respective proteins in the insoluble and soluble fractions of the cells, we also analysed total cellular lysates. This indicated, as expected from previous analyses, that total levels of p53 and Mdm2 increased at later timepoints starting from 3 and 12 hours after the UV-damage, respectively (Fig. 5C). Also, total levels of PML showed an increase 12 hours after the damage (Fig. 5C).

To address whether kinetics of p53-PML interaction correlate rapid p53 localizations to the PML bodies, we

performed immunostaining of UVC-treated WS1 cells. p53 shows gradual nucleoplasmic accumulation without specific localization to the PML NBs at any given timepoint (Fig. 6). PML present in the NBs, however, was rapidly dissociated from the NBs and was increasingly found in the nucleoplasm and in the perinucleolar regions (Fig. 6).

PML forms trimeric complexes with Mdm2 and p53 in vitro

Considering the in vitro results on the interaction domains of Mdm2, PML and p53 and the in vivo results on their temporal complex formation, we tested whether the proteins form mutually exclusive complexes. p53, Mdm2 and PML IV were translated in vitro and mixed with increasing amounts of either Mdm2 or PML IV and were tested for their capacity for complex formation (Fig. 7). Addition of increasing amounts of Mdm2 to the in vitro reactions led to an increase in p53-bound PML and vice versa, presence of increasing amounts of PML led to increased complex formation between p53 and Mdm2 (Fig. 7). This suggests that the interactions of PML with Mdm2 and p53 are not mutually exclusive, whereas PML-Mdm2 interaction promotes p53 binding. This further suggests that PML has separate binding sites for Mdm2 and p53.

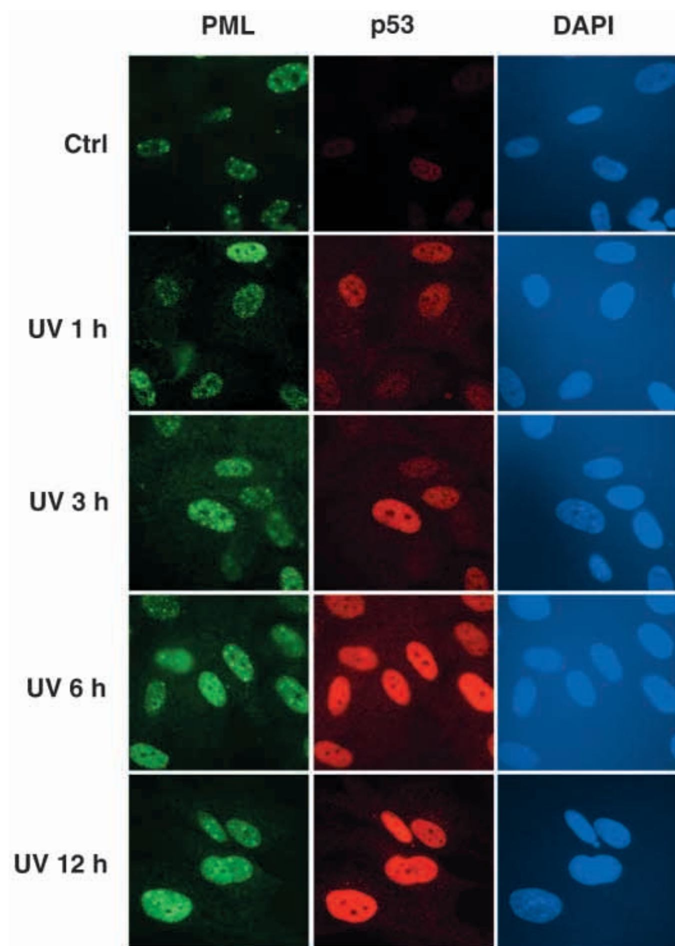

Fig. 6. Immunofluorescence staining of WS1 cells showing p53 and PML localizations following UV-irradiation. Cells were treated with UVC (35 J/m^2) and were incubated for the indicated times.

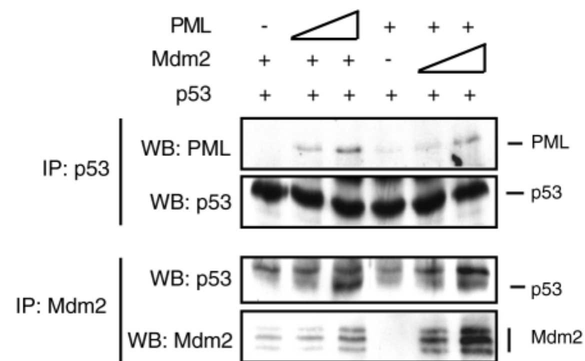

Fig. 7. PML, Mdm2 and p53 form trimeric complexes. In vitro translated p53 and increasing amounts of Mdm2 or PML were mixed followed by immunoprecipitation with an antibody against p53 (PAb421) or an Mdm2 antibody mix (IF2, 2A10, SMP14), followed by immunoblotting for PML (H-238), p53 (FL-393) and Mdm2 (IF2, 2A10, SMP14) as indicated.

Discussion

Mdm2 and PML colocalization and interaction was observed in response to diverse stimuli and insults, including DNA damage and cellular stress from proteasomal dysfunction and arsenic trioxide specifically used for the treatment of APL. Notably, the colocalization took place in distinct sites, in PML NBs and nucleoli in response to downregulation of the proteasome, in PML NBs in response to arsenic, and in the perinucleolar area in response to UVC-radiation, and as based on the co-precipitation analyses, in the nucleoplasm. Moreover, as shown by the ectopic expression, PML was found to have a profound effect on the subcellular localization of Mdm2. High PML levels caused exclusive colocalization of Mdm2 to PML NBs, indicating that PML redirects Mdm2 to the bodies. This suggests that PML can control the subcellular localization of Mdm2. In relation to its ability to regulate transcription factor functions, ectopic PML shifts the progesterone receptors and TIF1α into the PML NBs (Guiochon-Mantel et al., 1995; Zhong et al., 1999). Its ability to determine the localizations of transcription factors possibly pertains to its function as a regulator of their activities taking place within the PML NBs.

Here we demonstrate that cellular UV-damage leads to rearrangements of the PML NBs. UVC-insult led to an apparent increase in the soluble PML and small PML aggregates in the nucleoplasmic fraction, suggesting that PML NBs undergo rearrangements and more PML is found in a nucleoplasmic diffusible form. This finding is corroborated by a recent study by Seker et al. (Seker et al., 2003). A physiological release of PML from the bodies occurs during mitosis (Ascoli and Maul, 1991; Everett et al., 1999). Based on the observation that the transcriptional inhibitor actinomycin D causes scattering of the PML body components throughout the nucleoplasm (Kießlich et al., 2002), it has been suggested that transcriptional activity is required for the integrity of PML NBs. Even though the sumoylation of PML appears essential for the formation of mature PML bodies and its release from these sites is believed to involve desumoylation, other mechanisms resulting in free PML may also exist. In fact, Cd^{2+} exposure releases PML from the bodies to the nucleoplasm utilizing p38 MAPK and ERK1/2

signalling pathways (Nefkens et al., 2003). These pathways are also activated by cellular stress, including UV radiation. However, the mechanism of the UV-induced release still needs to be examined in more detail. The UV-damage-induced dispersion of PML is a novel finding, demonstrating its regulation by other types of stress besides viral infections (Zhong et al., 2000), oncogene activation (Pearson et al., 2000) or gamma-radiation (Guo et al., 2000).

In UV-treated cells, PML and Mdm2 also relocalized to perinucleolar areas. These dotted structures around the nucleoli resemble the nucleolar necklaces (Granick, 1975), which are formed in cells treated with a transcription inhibitor, DRB, and are involved in the transcription of rRNA (Panse et al., 1999). The units are composed of a small fibrillar centre surrounded by dense fibrillar component in a reorganized nucleolus, and are believed to correspond to active single gene (rRNA) transcription units (Panse et al., 1999). Nucleolar necklace also contains various transcription factors, including RNA pol I and TFIIH (Hoogstraten et al., 2002). DRB does not inhibit rRNA transcription by RNA pol I, although it causes alterations in rRNA processing and RNA pol II functions (Granick, 1975). PML also associates with RNA pol II at sites of active transcription (Kießlich et al., 2002), although the bodies themselves do not contain RNA or DNA (Grande et al., 1996). DNA helicase II (NDH II) is a component of the PML bodies in a manner dependent on active transcription. Inhibition of RNA pol II leads to its relocalization to the perinucleolar area (Fuchsová et al., 2002). The cause or purpose of the relocalization of Mdm2 and PML to these structures is presently not clear, although inhibition of RNA pol II by UV-radiation is suggestive that this could be an initiating factor for the translocation (Ljungman et al., 1999). Our findings imply a role for these proteins in the rRNA transcription unit of the nucleolus, either activatory or inhibitory.

UV-damage promotes the interaction of PML and Mdm2 despite the fact that levels of Mdm2 are slightly decreased early on after the damage (Latonen et al., 2001). Although the stress response caused by downregulation of the proteasome leads to PML-Mdm2 interaction, the distinct localizations of the proteins in response to UV-treatment as compared with proteasome dysfunction (nucleoplasm and perinucleolar dots versus nucleoli) distinguish the nature of their response. Possible bridging factors between these two proteins were excluded by in vitro translation assays in which the interaction was shown to be direct, although we cannot absolutely exclude the presence of a connecting factor in the reticulosyte lysate. In vitro analyses showed that Mdm2 binds both PML III and IV, although the in vitro interaction with PML III was much weaker. Mdm2 is subject to DNA damage-provoked phosphorylation and dephosphorylation (Khosravi et al., 1999; Blattner et al., 2002). Further studies should demonstrate whether the modifications (phosphorylation, sumoylation) of Mdm2 or alternatively, PML, affect their interactions and whether there is a preference for a certain PML isoform to bind Mdm2.

The Mdm2 binding site in PML was similar to the p53 interaction domain, as shown by their absence of binding with the PML-RARα fusion protein. These binding domains could be indicative of competitive interactions. However, although Mdm2 bound both PML III and IV, p53 interacts only with PML IV (Fogal et al., 2000). Moreover, the in vitro interaction analyses showed that increased levels of Mdm2 promoted PML-p53 interaction and vice versa, an increase in PML enhanced p53-Mdm2 complex formation. The results strongly favor the formation of a trimeric PML-p53-Mdm2 complex in which PML can accommodate the binding of both p53 and Mdm2. Mdm2, through its separate binding sites for p53 and PML, can increase the number of p53 molecules bound to PML. Furthermore, because Mdm2 interacts with PML through its RING-domain, it is unlikely that the trimeric complex would promote p53 degradation.

p53, in the nucleoplasmic fractions, interacted transiently with PML in the UV-treated cells. At the same time, insoluble p53 was found in a slower-migrating form, corresponding to its SUMO modification (Rodriguez et al., 1999). Similarly, PML present in the insoluble fraction migrated as a higher molecular weight form in UV-damaged cells whereas the PML form present in control cells decreased, suggesting that both proteins undergo modifications following UV-damage (Fig. 5C). The sumoylation of p53 affects its transcriptional activity in UV-treated cells (Rodriguez et al., 1999; Gostissa et al., 1999). The localization of p53 to NBs is not dependent on this modification (Fogal et al., 2000; Kwek et al., 2001), but the SUMO-1-conjugating enzyme, Ubc9, exists in NBs (Duprez et al., 1999). It is thus possible that sumoylation takes place in PML NBs and is a prerequisite for the activation of p53. In addition, p53 is modified in a PML-dependent manner through acetylation by CBP (Ferbeyre et al., 2000; Pearson et al., 2000), and conversely, can be deacetylated through SIRT1 deacetylase, which localizes to PML NBs (Langley et al., 2002). Following UV-damage, p53 is phosphorylated on Ser46 by HIPK2 (D'Orazi et al., 2002). Both events are presumed to increase p53 stability and enhance its transactivation function and may be mediated through events taking place in the PML NBs. Alternatively, acetylation of p53 prevents its degradation by Mdm2 because of overlapping lysines targeted by acetylation and ubiquitination (Li et al., 2002b). Mdm2 can promote p53 deacetylation by recruiting histone deacetylase-1 (HDAC1), and the deacetylation is suggested to lead to enhanced p53 degradation (Ito et al., 2002). Finally, herpesvirus-associated ubiquitin-specific protease (HAUSP) stabilizes p53 by deubiquitinating it both in vitro and in vivo (Li et al., 2002a), and interestingly, this protein also exists in PML bodies, suggesting that p53 stabilization could take place in NBs. The PML and PML NBs thus appear to be modifiers of several processes affecting p53 and its activity. Yet, evidence by Bishof et al. indicates that several p53 modifications (Ser46 phosphorylation, Lys382 acetylation) induced by PML IV-mediated senescence do not require the integrity of PML bodies (Bishof et al., 2002). We find no evidence that p53, following UV-damage, is found in the PML NBs (Fig. 6). However, the clearly evident p53-PML interactions in the nucleoplasmic fractions, and their changes in the insoluble fractions suggest that they undergo rapid dynamic interactions and possibly, modifications. We therefore propose that the capacity of PML to directly complex with Mdm2, and the formation of trimeric PML-Mdm2-p53 complexes, allow for p53 stabilization following UV-damage. In fact, PML IV overexpression has been shown to induce both p53 stabilization and acetylation (Bischof et al., 2002). The mechanism for p53 stabilization has, however, remained unknown. Our results show that one stabilizing factor, in addition to acetylation of

p53, may be interference of Mdm2 E3 ligase activity by PML. We suggest that PML inhibits Mdm2-mediated degradation of p53 by binding to the Mdm2 C-terminus, hindering its ubiquitin ligase activity in DNA-damaged cells. In addition, both PML and Mdm2 are relocalized to the perinucleolar structures. The presented results unravel complex interactions and regulation of p53, Mdm2 and PML following diverse types of cellular stress and DNA damage.

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