Covalent Modification of PML by the Sentrin Family of Ubiquitin-like Proteins*

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PML, a RING finger protein with tumor suppressor activity, has been implicated in the pathogenesis of acute promyelocytic leukemia that arises following a reciprocal chromosomal translocation that fuses the PML gene with the retinoic acid receptor α (RARα) gene. Immunocytochemical analysis has demonstrated that PML is co-localized with a novel ubiquitin-like protein in the nuclear bodies, which could be disrupted by the PML-RARα fusion protein. The physical nature of this co-localization is unknown. Using a COS cell expression system, we show that PML is covalently modified by all three members of the sentrin family of ubiquitin-like proteins. Covalent modification of PML requires the conserved Gly residue near the C termini of sentrin proteins. Sentrinization of PML is highly specific because neither NEDD8 nor ubiquitin could modify PML. Similar specificity is also observed for the covalent modification of RanGAP1 by the sentrin member of ubiquitin-like proteins. These observations highlight the fine substrate specificity of the sentrinization pathway. In acute promyelocytic leukemia, two forms of PML-RARα fusion proteins have been reported. Remarkably, both forms of PML-RARα fusion proteins could not be sentrinized. Thus differential sentrinization of PML and PML-RARα could play an important role in regulating the biological function of PML and in the pathogenesis of acute promyelocytic leukemia.

Chromosomal translocation t(15;17), detected in the majority of patients with acute promyelocytic leukemia, generates a fusion protein composed of portions of the retinoic acid receptor α (RARα) and RING finger protein called PML (1–4). In normal myeloid cells, PML is localized to a nuclear multiprotein complex called nuclear bodies or PML oncogenic domains (5, 6). In cell lines derived from patients with acute promyelocytic leukemia, the nuclear bodies are disrupted into a microparticulate pattern, which is reversible by treatment with retinoic acid (5, 6). PML has also been shown to suppress the transformation of NIH3T3 cells by the activated neu oncogene (7). Using full-length PML as bait in a yeast two-hybrid interaction screening, Boddy et al. have isolated a novel ubiquitin-like protein called PIC1 that interacted specifically with PML in the yeast interaction assay (8). They have further shown that PIC1 was co-localized with PML to the nuclear bodies. In NB4 cells, which are derived from a patient with acute promyelocytic leukemia, there was no significant co-localization of PIC1 with PML. However, following retinoic acid treatment, a significant relocalization of PIC1 with PML was observed. These observations suggest that the association of PIC1 with PML may play an important role in the pathogenesis of acute promyelocytic leukemia.

Our laboratory has recently reported the cloning of a novel ubiquitin-like molecule, called sentrin, which interacts specifically with the death domains of both Fas and tumor necrosis factor receptor 1 (9). Overexpression of sentrin protects cells against anti-Fas or tumor necrosis factor-induced cell death. We have also demonstrated that sentrin could form covalent conjugates with other proteins in a process analogous to protein ubiquitination (10). Furthermore, the majority of the sentrinized proteins are localized to the nucleus. Remarkably, the amino acid sequences of sentrin and PIC1 are identical. Thus, it is tempting to speculate that PML could be covalently modified by sentrin (PIC1). However, Boddy et al. (8) were unable to demonstrate an in vitro interaction between PIC1 (sentrin) and PML, possibly due to technical problems in their co-precipitation assay. Using a COS cell expression system (10, 11), we demonstrated that PML is covalently modified by all members of the sentrin family of ubiquitin-like proteins. This is highly specific because neither ubiquitin nor NEDD8 could participate in this modification reaction.

EXPERIMENTAL PROCEDURES

Antibodies—16B12 (BAbCo, Richmond, CA) is mouse monoclonal antibody to the peptide sequence YPYDVPDYA of influenza hemagglutinin (HA). Mouse anti-RH (specific for the amino acid sequence, RG-SHHHH) monoclonal antibody was purchased from Qiagen. Mouse anti-PML monoclonal antibody was purchased from Santa Cruz Biotechnology. Rabbit polyclonal anti-PML antisera was kindly provided by Dr. K. S. Chang, (M. D. Anderson Cancer Center).

Plasmid Construction, Transfection, and Precipitation of RH-tagged Proteins—cDNAs of Rad51, ubiquitin, NEDD8, sentrins, RARα, and RanGAP1 were amplified by polymerase chain reaction using appropriate primers from human testis cDNA library (Life Technologies, Inc.). The cDNAs of Rad51, ubiquitin, NEDD8, and sentrins were ligated into pcDNA3/HA-N (10, 11) for the N-terminal tagging with HA epitope. The RanGAPI and RARα cDNA were inserted into pcDNA3/RH-N (11) and pcDNA3/RH-C (10), respectively. The full-length cDNA of PML was prepared from the plasmid pMAMneoPML (12) and ligated into pcDNA3/HA-N or pcDNA3/RH-C (11) to express HA-PML or PML-RH. cDNAs of PML-RARα and PML-RARα-B (4) were generated by polymerase chain reaction from cDNAs of PML and RARα and were inserted into pcDNA3/RH-C. Plasmids were transfected into COS cells using LipofectAMINE (Life Technologies, Inc.). The transfected cells were harvested for Western blotting or nickel precipitation as described previously (10, 11).

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FIG. 2. Amino acid alignment of NEDD8, ubiquitin, and the sentrin family members. Identical amino acids are printed in bold type. Lys\(^6\) of ubiquitin, critical for the formation of ubiquitin multimers, is indicated by an open triangle. The Gly residue critical for sentrinization is marked by a closed triangle. Accession numbers for human NEDD8, sentrin-1, sentrin-2, and sentrin-3 are D23662/AA484409/N24312, U83117, X95585/T08096, and X95584, respectively.

Sentrin belongs to a family of ubiquitin-like proteins (Fig. 2). Sentrin homologues have been reported from Arabidopsis thaliana to Homo sapiens, suggesting that sentrin is an evolutionary conserved protein that may perform unique functions in cellular metabolism. Further analysis of the data base revealed two additional human cDNA sequences that are highly homologous to sentrin (14, 15). This is of interest because there is only one sentrin homologue reported for all of the nonmammalian genera and species. We have renamed the original sentrin as sentrin-1 and the other two sequences as sentrin-2 and sentrin-3. It is not known whether sentrin-2 or sentrin-3 could form covalent conjugates with other proteins.

Because sentrin-1 could covalently modify PML, it is of interest to determine whether sentrin-2 or sentrin-3 could also be used as PML modifiers. For this purpose, HA tag was placed at the N termini of sentrin-1, sentrin-2, and sentrin-3 and expressed in COS cells as described previously (10). RanGAP1 was used as a positive control because RanGAP1 is covalently modified by sentrin-1 (SUMO-1) (11, 10, 11, 15). It is not known, however, whether RanGAP1 could be modified by sentrin-2 or sentrin-3. HA-tagged sentrin family members were co-expressed with RH-RanGAP1 in COS cells. Total cell lysates were incubated with nickel-charged beads to precipitate RH-RanGAP1 and its derivatives. As shown in Fig. 3A, sentrin-1, sentrin-2, or sentrin-3 could covalently modify RanGAP1 (lanes 4, 5, and 6). This is specific because Rad51 (lane 1), ubiquitin (lane 2), and NEDD8 (lane 3) could not modify RanGAP1. The purified RH-RanGAP1 derivatives were also detected utilizing anti-RH monoclonal antibody (lanes 7–12). As shown, both the unmodified RanGAP1 and sentrinized RanGAP1 were detected in all samples. It should be noted that p90 in lanes 7–9 was derived from RanGAP1 modified by native sentrin in COS cells. This is further supported by the observation of a 90-kDa doublet in the HA-sentrin-1, -2, or -3 transfected sample (lanes 10–12). The upper band of the doublet is most likely RanGAP1 modified by HA-sentrin, and the lower band is most likely RanGAP1 modified by native sentrin (lanes 10–12). It should be noted that a single 90-kDa band was observed in cells expressing HA-tagged sentrin (lanes 4–6), suggesting that RanGAP1 is modified by one sentrin molecule. These experi-

RESULTS AND DISCUSSION

HA-PML was expressed in COS cells, and total cell lysates were analyzed by Western blotting as described previously (10, 11). Three different antibodies were employed in this analysis. As shown in Fig. 1A, a mouse anti-PML monoclonal antibody detected the 70-kDa PML (arrowhead) and a 90-kDa band (lane 2). A rabbit anti-PML antiserum detected both the 70- and 90-kDa bands (lane 4). There is also a weak higher molecular mass band in the lysate and several lower molecular mass bands, which are most likely degradation products of PML (lane 4). A highly sensitive anti-HA monoclonal antibody detected several bands higher than 70 kDa (lane 6). Because the predicted molecular mass of PML is only 70 kDa, the presence of these higher molecular mass bands suggests that PML could either migrate aberrantly or could be modified by other molecules, such as sentrin or ubiquitin.

Next, PML was tagged with RH epitope (RGSHHHHHHH) at the C terminus and expressed in COS cells as described previously (11). Transfected cells were lysed with 6 M guanidine HCl to denature proteins in the lysate and to prevent any proteolysis or sentrinization. PML-RH was then precipitated with nickel-charged beads and was analyzed by Western blotting using rabbit anti-sentrin-1 antiserum (10) (Fig. 1B). Unconjugated PML was then precipitated with nickel-charged beads and analyzed by Western blotting as described previously (10, 11). Three different antibodies were employed in this analysis. As shown in Fig. 1B, three distinct bands were identified in the anti-sentrin and anti-RH lanes (lanes 2 and 4). As a control, HA-PML could not be precipitated with nickel-charged beads and could not be detected with either anti-sentrin antiserum or anti-RH antibody (lanes 1 and 3). These experiments suggest that PML is covalently modified by sentrin at multiple sites. We do not favor the possibility of modification at the single site by sentrin multimers because sentrin does not contain the conserved Lys\(^6\) equivalent required for multimer formation (11) (Fig. 2). This notion is further supported by the observation that RanGAP1 is modified by a single molecule of sentrin (SUMO-1) (11, 14, 15).
Sentrinized PML bands are marked by a bracket (arrowhead). Unmodified PML is indicated by lanes 7–12 with nickel-charged beads and analyzed by immunoblotting with an- lanes 1 controls (lanes 1–4) or anti-RH monoclonal antibody (lanes 5–8). Molecular size markers are shown on the left in kDa.

**Fig. 3. Modification of PML by sentrin-1, sentrin-2, and sentrin-3 but not by NEDD8 or ubiquitin.** A, RH-RanGAP1 was co-expressed in COS cells with HA-Rad51 (lanes 1 and 7), HA-ubiquitin (lanes 2 and 8), HA-NEDD8 (lanes 3 and 9), HA-sentrin-1 (lanes 4 and 10), HA-sentrin-2 (lanes 5 and 11), or HA-sentrin-3 (lanes 6 and 12). The RH-RanGAP1 and its derivatives were precipitated with nickel-charged beads and analyzed by immunoblotting with anti-HA monoclonal antibody (lanes 1–6) or with anti-RH monoclonal antibody (lanes 7–12). Unmodified RanGAP1, RanGAP1 modified by native COS cell sentrin, and RanGAP1 modified by HA-sentrins are indicated on the right. B, PML-RH was co-expressed in COS cells with HA-ubiquitin (lanes 2 and 8), HA-NEDD8 (lanes 3 and 9), HA-sentrin-1 (lanes 4 and 10), HA-sentrin-2 (lanes 5 and 11), or HA-sentrin-3 (lanes 6 and 12). COS cells expressing RH-RanGAP1 and HA-sentrin-1 were used as controls (lanes 1 and 7). PML-RH and its derivatives were precipitated with nickel-charged beads and analyzed by immunoblotting with anti-HA monoclonal antibody (lanes 1–6) or with anti-RH monoclonal antibody (lanes 7–12). Unmodified PML is indicated by an arrowhead. Sentrinized PML bands are marked by a bracket. WB, Western blot.

**Fig. 4. PML-RARα-A and B cannot be sentrinized.** PML (lanes 1 and 5), RARα (lanes 2 and 6), PML-RARα-A (lanes 3 and 7), and PML-RARα-B (lanes 4 and 8) were expressed with RH epitope in COS cells. Total cell lysates were precipitated with nickel-charged beads and analyzed by Western blotting (WB) using rabbit anti-sentrin antiserum (10) (lanes 1–4) or anti-RH monoclonal antibody (lanes 5–8). Molecular size markers are shown on the left in kDa.

The predicated molecular mass of PML is approximately 70 kDa. Chang and his colleagues have reported that the PML protein migrates at about 90 kDa during SDS-polyacrylamide gel electrophoresis, probably due to its acidic nature contributed by the proline-rich domain (12, 18). In view of the results presented here, the 90-kDa band observed in these reports is most likely due to modification of PML by sentrin. There is abundant desentrinizing activity in the cell lysates that could contribute to the difficulty in demonstrating that PML is covalently modified by sentrin. Our results clearly demonstrate that PML, after RanGAP1, is a second target for the sentrinization pathway. Remarkably, all of the sentrin family members could form a covalent linkage with PML via the conserved Gly residue. These results also demonstrate the difference in target specificity between the sentrinization pathway and the ubiquitination pathway because neither ubiquitin nor NEDD8 could modify PML or RanGAP1. Our recent finding of a preferential interaction between sentrin-1 and Ubc9 further supports the distinction between the ubiquitination and sentrinization pathways (19).

In acute promyelocytic leukemia, two forms of PML-RARα fusion proteins have been reported (4). The proteolytic removal of the C-terminal amino acids of PML-RARα-A and -B are due to conjugation of PML by native sentrin present in COS cells. To further confirm that PML is covalently modified by sentrin, we constructed mutants in which the conserved Gly residue involved in sentrinization (marked by a closed triangle in Fig. 2) and its adjacent C-terminal amino acids have been deleted (10). As expected, sentrin molecules lacking the conserved Gly residues were unable to modify PML (data not shown).

The inability of ubiquitin or NEDD8 to modify PML or RanGAP1 is highly specific because both modifications contribute to the difficulty in demonstrating that PML is covalently modified by sentrin. Our results clearly demonstrate that PML, after RanGAP1, is a second target for the sentrinization pathway. Remarkably, all of the sentrin family members could form a covalent linkage with PML via the conserved Gly residue. These results also demonstrate the difference in target specificity between the sentrinization pathway and the ubiquitination pathway because neither ubiquitin nor NEDD8 could modify PML or RanGAP1. Our recent finding of a preferential interaction between sentrin-1 and Ubc9 further supports the distinction between the ubiquitination and sentrinization pathways (19).
Sentrinization of PML

(lanes 7 and 8). We currently do not know why the fusion proteins could not be modified by sentrin. They may lack the sentrinization sites or binding sites for Ubc9 or E3 or are localized in a wrong cellular compartment. Elucidation of these issues will bring significant insights into the pathobiology of acute promyelocytic leukemia.

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