Preferential Interaction of Sentrin with a Ubiquitin-conjugating Enzyme, Ubc9*

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Limin Gong, Tetsu Kamitani, Kenichi Fujise, Laura S. Caskey, and Edward T. H. Yeh§

From the Research Center for Cardiovascular Diseases, Institute of Molecular Medicine for the Prevention of Human Diseases, and Division of Molecular Medicine, Department of Internal Medicine, The University of Texas-Houston Health Science Center, Houston, Texas 77030

Sentrin is a ubiquitin-like molecule that has been shown to interact with the death domains of Fas and tumor necrosis factor receptor 1 (TNFR1), PML, Rad51, Rad52, and RanGAP1. We have reported previously that sentrin can be conjugated to other proteins in a manner analogous to protein ubiquitination (Kamitani, T., Nguyen, H. P., and Yeh, E. T. H. (1997) J. Biol. Chem. 272, 14001–14004). Furthermore, the conserved C-terminal Gly-Gly residues are required for sentrinization to occur. To identify enzymes which play a role in sentrinization, the yeast two-hybrid system was used to screen a human placenta cDNA library using sentrin as bait. A strong positive interacting clone was found to contain a cDNA insert encoding the ubiquitin-conjugating enzyme, Ubc9. The interaction between sentrin and Ubc9 required the ubiquitin domain and the C-terminal Gly-Gly residues of sentrin. This interaction appears to be specific because sentrin could only interact weakly with UbcH5B, but could not interact with HHR6B, UbcH6 nor E2-EPF. In vitro translated sentrin could be precipitated by a GST-Ubc9 fusion protein, but not by glutathione S-transferase. A ß-mercaptoethanol-sensitive sentrin-Ubc9 conjugate could also be identified in the in vitro binding assay. Substitution of the conserved cysteine residue of Ubc9 by serine abolished the formation of the Ubc9-sentrin conjugate. Taken together, Ubc9 is a strong candidate to be the key conjugating enzyme in the sentrinization pathway.

Sentrin is a 101-amino acid ubiquitin-like protein that interacts with the death domains of Fas and TNFR1 (1), with FML, a tumor suppressor implicated in the pathogenesis of promyeloctic leukemia (2), with Rad51 and Rad52, proteins that are involved in repairing double-stranded DNA breaks (3), and with RanGAP1, a GTPase-activating protein that is critically involved in nuclear protein transport (4, 5). Using the COS cell expression system, we have shown that sentrin can be conjugated to other proteins in a manner analogous to protein ubiquitination (6). Moreover, sentrinized proteins appear to reside in the nucleus. We have also shown that the C-terminal four amino acids of sentrin, His-Ser-Thr-Val, were efficiently cleaved to allow the conjugation of sentrin to other proteins via the conserved Gly97 residue. Thus, the biochemistry of sentrinization pathway appears to be remarkably similar to that of the ubiquitination pathway.

Conjugation of ubiquitin to its target protein requires the initial activation of the conserved C-terminal Gly residue catalyzed by a specific ubiquitin-activating enzyme, E1 (7–11). An intermediate, ubiquitin adenylate, is formed by displacement of PPi from ATP and ubiquitin adenylate is then transferred to a thiol site in E1 with release of AMP. Through transacylation, ubiquitin is transferred to a ubiquitin-conjugating enzyme, E2, to form another thiol ester bond. Finally, ubiquitin is transferred from E2 to its target protein through an isopeptide linkage with the ß-amino group of the Lys residue of the target protein. The transfer of ubiquitin from E2 to the target protein requires the participation of a ligase, E3, in many instances. In recent years, ubiquitination has been shown to play a critical role in antigen processing, in the regulation of cell cycle, in receptor endocytosis, and in signal transduction (12–18). The biological specificity of the ubiquitination pathway appears to be regulated by a selective combination of E2 and E3 proteins (15). Thus far, there are more than 30 E2s identified with the conserved UBC domain that contain a cysteine residue at the active site (7–9). However, the molecular basis of this specificity has not been completely elucidated.

Since the sentrinization pathway shares many similarities with the ubiquitination pathway, it is of interest to identify specific E2 or E3 proteins that may be involved the sentrinization pathway. Using sentrin as a bait in the yeast two-hybrid screen, we identified a ubiquitin-conjugating enzyme, Ubc9, which binds to sentrin with high affinity. The interaction between sentrin and Ubc9 is much stronger than that between ubiquitin and Ubc9 or between sentrin and three other E2s. Furthermore, the conserved C-terminal Gly-Gly residues of sentrin are required for the high affinity interaction, suggesting that sentrin could form a thiol ester bond with Ubc9. This is further substantiated by an in vitro binding assay in which a ß-mercaptoethanol-sensitive sentrin-Ubc9 conjugate was observed. Thus, Ubc9 appears to be a key conjugating enzyme for the sentrinization pathway.

EXPERIMENTAL PROCEDURES

Yeast Strains and Plasmids—Yeast strains, SFY526 and HF7c, and the shuttle vectors pGB79 and pGAD424 were purchased from CLONTECH. The bait plasmid pGB79-sentrin was constructed by inserting the full-length sentrin cDNA in-frame in the BamHI site of the pGB79 vector. Deletion mutants were produced by a polymerase chain reaction-based strategy with appropriate primer sets. These inserts contained a BamHI linker at the 5′-end and a stop codon and a PstI linker at the 3′-end. The PCR products were digested with both BamHI and PstI and then ligated into the yeast two-hybrid vectors that had been predigested with the same enzymes. The full-length or truncated
human Ubc9 constructs were made by PCR amplification with appropriate primers from a plasmid containing the Ubc9 cDNA and subcloned into pGAD424. The E2 cDNAs were amplified by PCR from a human placenta cDNA library. The substitution mutant of Ubc9(C93S) was generated using QuickChange site-directed mutagenesis kit (Stratagene). The sequences of all constructs were confirmed by automated sequencing (Applied Biosystems Inc.).

**Yeast Two-hybrid Screening—** Yeast two-hybrid library screening and analysis were performed essentially as described previously (1). Briefly, the yeast strain H767 containing the bait plasmid pGBT9-sentrin was transformed with a human placenta cDNA library (from CLONTECH) using the lithium acetate method as described in the CLONTECH’s Matchmaker Library Protocol. Approximately 5 × 10^5 transformants were selected for growth on plates lacking histidine, leucine, and tryptophan. His^+ colonies were subsequently analyzed for β-galactosidase activity and retested for their specificity by using a panel of plasmids encoding different polypeptides fused to the Gal4 DNA binding domain (1).

**Expression and Purification of GST Fusion Proteins—** The full-length cDNA of UBC9 was amplified by PCR from a human placenta cDNA library. The PCR product was subcloned into pGEX-2T (Pharmacia Biotech Inc.) using BamHI and EcoRI restriction sites to generate pGEX-UBC9. Escherichia coli BL21 cells carrying pGEX-UBC9 or pGEX-2T were grown to saturation in 10 ml of LB containing 50 µg/ml ampicillin and 100 µg/ml kanamycin and induced to 0.5 OD at 600 nm with 0.1 mM IPTG. After 2 h of induction, the cells were harvested by centrifugation, and the supernatant containing the GST-fusion protein was collected. The GST-fusion protein was purified by binding to glutathione-Sepharose beads.

**Results and Discussion**

We have shown previously that sentrin can be conjugated to a number of cellular proteins in a manner analogous to ubiquitination (6). It is of interest to determine whether sentrin utilizes a similar set of enzymes in the processing of its C terminus, in the activation of the Gly residue, and in conjugation to other proteins. For this purpose, the yeast two-hybrid system was used to identify cDNA clones that encode polypeptides that are able to interact with sentrin. Yeast strain H767, which contains two Gal4-inducible reporter genes, HIS3 and lacZ, was initially transformed with the bait plasmid pGBT9-sentrin and then the resulting transformant was used as host for transformation with the placenta cDNA library. Approximately 0.5 × 10^7 primary library transformants were plated onto plates lacking histidine, leucine, and tryptophan. A total of 104 colonies appeared on the histidine dropout plates, 16 of which stained positive when tested for expression of β-galactosidase. DNA tests of the plasmid DNA of the isolated cDNAs, all of which plasmids were retransformed into the yeast strain SYP526 together with plasmids encoding other Gal4 DNA binding domain fusion proteins. All hybrid proteins were found to interact only with Gal4-sentrin. Subsequent analyses indicated that one of these colonies contained an approximately 18-kilobase pair insertion. Analysis of the cDNA sequence showed a single open reading frame of 158 amino acids. Comparison of the deduced amino acid sequence with GenBank™ revealed that the protein encoded by the newly identified cDNA is Ubc9, a human ubiquitin-conjugating enzyme that is a structural and functional homologue of ScUBC9 (19, 20).

The yeast two-hybrid system was used to further assess the structural requirement of sentrin and Ubc9 interaction. Various deletion mutants of either sentrin or Ubc9 were constructed and tested in the yeast two-hybrid assay. As shown in Fig. 1A, wild type sentrin interacted strongly with Ubc9, as evidenced by a detectable color change within 45 min. This is comparable with the interaction between SV40 large T and p53 and is stronger than the FADD/MORT1 and Fas interaction (1). Deletion of the four C-terminal amino acids had minimal effect on the interaction (3-4). Deletion of the N-terminal 23 amino acids, sentrin (24–101), reduced the interaction from 4+ to 2+. Deletion of both the N and C termini, sentrin (24–97), reduced the interaction to barely detectable. Deletion of the C-terminal 31 amino acids, sentrin (1–70), completely abolished the interaction. The N-terminal 23 amino acids also could not interact with Ubc9. Thus, the ubiquitin domain of sentrin is required for the interaction.

Since the ubiquitin domain of sentrin is required for the interaction, the interaction between ubiquitin and Ubc9 was also tested. As shown in Fig. 1B, ubiquitin did interact with Ubc9, but the interaction was much weaker than the interaction between sentrin and Ubc9. Deletion of the C-terminal 50 amino acids or the N-terminal 30 amino acids of Ubc9 also abolished these interaction. These results suggest that the N-terminal 21 amino acids of Ubc9 enhances the interaction of sentrin with Ubc9. It should be emphasized that the yeast two-hybrid results should not be taken to imply that Ubc9 prefers sentrin over ubiquitin. The process of ubiquitination and sentrinization could be influenced by the presence of additional proteins in vivo. However, these results clearly demonstrate that the interaction between sentrin and Ubc9 is highly specific and thereby biologically significant.

Ubc9 belongs to a family of ubiquitin-conjugating proteins (8). All known E2 enzymes have a conserved domain of approximately 16 kDa called the UBC domain. This domain is at least 35% identical to all known E2s and includes a centrally located cysteine residue for ubiquitin enzyme thiol ester formation (8). We have cloned four other E2s by using PCR amplification from a human placenta cDNA library. These E2s were tested for their ability to interact with sentrin. Only UbcH5B (21) had a weak interaction with sentrin. HHR6B (22), UbcH6 (23), and E2-EPF (24) were unable to interact with sentrin. These observations further demonstrate the specificity of the interaction between sentrin and Ubc9 interaction.

Our previous work demonstrates that the C terminus of sentrin is efficiently processed, and Gly97 is essential for sentrinization to occur (6). A number of C-terminal deletion and substitution mutants were constructed and tested in the yeast two-hybrid assay. As shown in Fig. 1C, deletion of Gly97, or Gly96–97, or substitution of Ala-Ala for Gly-Gly, abolished the interaction of sentrin with Ubc9. These results are consistent with our previous finding that Gly97 plays a critical role in the formation of sentrinized proteins. These results also suggest that a covalent linkage of sentrin to Ubc9 accounts for the strong interaction between these proteins in the yeast two-hybrid interaction.

To provide further evidence for the interaction between sentrin and Ubc9, a GST-Ubc9 fusion protein was engineered and expressed in E. coli. GST or GST-Ubc9 proteins were then used to precipitate in vitro transcribed and translated sentrin. As
shown in Fig. 2, GST-Ubc9 (lane 3), but not GST (lane 2), could specifically precipitate in vitro translated sentrin. In lane 3, a 60-kDa band could also be visualized. This band most likely represents sentrin conjugated to GST-Ubc9 via a thiol ester linkage, because it disappeared when the sample was reduced with 5% β-mercaptoethanol (lane 5). In separate experiments, we have shown that other GST-fusion proteins could not precipitate sentrin (data not shown). Thus, it appears that sentrin was activated by an E1 in the reticulocyte lysate after translation. The activated sentrin then bound to GST-Ubc9 via a thiol ester linkage. To further substantiate this observation, the active site cysteine residue 93 in Ubc9, which is necessary for thiol ester formation, was mutated to serine. In Fig. 3, in vitro translated sentrin could be precipitated by GST-Ubc9 (lane 3) and GST-Ubc9(C93S) (lane 4). However, the higher molecular weight band was only observed in the sample precipitated by GST-Ubc9. These results suggest that sentrin could form a thiol ester linkage with Ubc9 via the conserved cysteine residue.

ScUBC9 was first reported by Seufert and his colleagues in 1995 (20). They showed that repression of Ubc9 synthesis prevents cell cycle progression at the G2 or early M phase, causing the accumulation of large budded cells with a single nucleus, a short spindle and replicated DNA. In ubc9 mutants both CLB5, an S-phase cyclin, and CLB2, an M-phase cyclin, are stabilized (20). In wild type cells the CLB5 protein is unstable throughout the cell cycle, whereas CLB2 turnover occurs only at a specific cell cycle stage. Recently, there have been a number of reports demonstrating the association of Ubc9 with several biologically important proteins. Hateboer et al. (25) showed that murine Ubc9 binds to the CR2 of adenovirus E1A protein. Gottlicher et al. (26) demonstrated that human Ubc9 interacts with c-Jun and the glucocorticoid receptor. Jiang and Koltin (27) showed that scUbc9 interacts with cbf3p subunit of the Saccharomyces cerevisiae centromere DNA-binding core complex. Wang et al. (28) reported that human Ubc9 associates with the negative regulatory domain of the Wilms’ tumor gene product (WT1). Yasugi and Howley (19) also showed that human Ubc9 interacts with the human papillomavirus type 16 E1 replication protein. Kho et al. (29) also reported that the rat Ubc9 interacts with the helix-loop-helix E2A proteins. Three additional re-
ports warrant special attention. Wright et al. (30) reported that human Ubc9 is associated with the signal-competent form of human Fas. In light of our finding that Fas binds to sentrin (1) and sentrin binds to Ubc9, these results could indicate that either Fas is a target for sentrinization or Fas is part of a novel sentrinization complex that includes sentrin and Ubc9. The association of both sentrin and Ubc9 with other proteins is not limited to Fas. Kovalenko and his colleagues reported that human Ubc9 interacts with human Rad51 (31). Furthermore, Saitoh et al. (32) reported that RanBP2 associates with Ubc9 and a sentrin-modified form of RanGAP1. The interaction of sentrin and/or Ubc9 with other proteins is listed in Table I to facilitate comparison. It is not clear which protein listed in Table I is sentrinized or ubiquitinated utilizing Ubc9 as a specific E2. At present, RanGAP1 is the only protein that is known to be sentrinized (4–6). It is also possible that some of these proteins could either interact with other sentrinized protein or function as an E3 in facilitating ubiquitination or sentrinization.

In conclusion, our results suggest that Ubc9 is a strong candidate to be the key conjugating enzyme in the sentrinization pathway. Whether additional conjugating enzymes, such as UbcH5B, may also play a role in the sentrinization pathway remains to be determined.

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