Conjugation of the small ubiquitin-like modifier SUMO-1/SMT3C/Sentrin-1 to proteins in vitro is dependent on a heterodimeric E1 (SAE1/SAE2) and an E2 (Ubc9). Although SUMO-2/SMT3A/Sentrin-3 and SUMO-3/SMT3B/Sentrin-2 share 50% sequence identity with SUMO-1, they are functionally distinct. Inspection of the SUMO-2 and SUMO-3 sequences indicates that they both contain the sequence ψKXE, which represents the consensus SUMO modification site. As a consequence SAE1/SAE2 and Ubc9 catalyze the formation of polymeric chains of SUMO-2 and SUMO-3 on protein substrates in vitro, and SUMO-2 chains are detected in vivo. The ability to form polymeric chains is not shared by SUMO-1, and although all SUMO species use the same conjugation machinery, modification by SUMO-1 and SUMO-2/3 may have distinct functional consequences.

The small ubiquitin-like modifier SUMO-1 (also known as SMT3C, Sentrin, GMP1, UBL1, and PIC1) is a member of the ubiquitin-like protein family (1). SUMO-1 is known to be covalently conjugated to a variety of cellular substrates via a three-step enzymatic pathway analogous to that of ubiquitin conjugation. The E1-like enzymes for both SUMO-1 and the yeast homologue Smt3p exist as heterodimers known as SAE1/SAE2 and Uba2p/Aos1p, respectively (2–5). In the first step the SAE1/SAE2 heterodimer utilizes ATP to adenylate the C-terminal glycine of SUMO-1. Formation of a thioester bond between the C-terminal glycine of SUMO-1 and a cysteine residue in SAE2 is accompanied by the release of AMP. The second step is a transesterification reaction, which transfers SUMO-1 from the E1 to a cysteine residue within the SUMO-specific E2-conjugating enzyme (Cys63 in Ubc9). In the third step, Ubc9 catalyzes the formation of an isopeptide bond between the C terminus of SUMO-1 and the ε-amino group of lysine in the target protein. In contrast to the ubiquitin conjugation pathway no activity equivalent to an E3 ligase is required for SUMO-1 conjugation in vitro (2, 4), suggesting that the specificity for target proteins is conferred by Ubc9 itself or the Ubc9-SUMO-1 thioster complex. This is supported by the observations that almost all SUMO-1-conjugated proteins bind Ubc9 in two-hybrid assays, and the acceptor lysine residues on target proteins appear to exist within the consensus motif ψKXE (where ψ represents a large hydrophobic amino acid, and X represents any amino acid) (6–8). Furthermore, SUMO-1 is thought not to form SUMO-1-SUMO-1 polymers, which are characteristic of ubiquitination.

Unlike the majority of ubiquitinated proteins, acceptors of SUMO-1 modifications are not targeted for degradation. In fact, in the case of the transcriptional inhibitor IκBα the target lysine for SUMO-1 modification is the same as that of ubiquitin conjugation, thus blocking ubiquitination at that residue and stabilizing the protein (8). Transcriptional activity of specific proteins appears to be affected by SUMO-1 modification. For example, conjugation at a single site in the C terminus of p53 activates its transcriptional response (9, 10). Furthermore, SUMO-1 modification of certain substrates is also known to have implications upon subcellular localization. The interaction of RanGTPase-activating protein 1 (RanGAP1) with the Ran-GTP-binding protein 2 at the cytoplasmic face of the nuclear pore complex is dependent on SUMO-1 conjugation of RanGAP1 (11, 12). Modification of the promyelocytic leukemia protein (PML) targets it to distinct nuclear bodies (13–15) and is required for Daxx recruitment to these structures (16, 17).

Two ubiquitin-like proteins, known as SUMO-2 (SMT3A, Sentrin-3) and SUMO-3 (SMT3B, Sentrin-2), have been identified that are related to SUMO-1 but are apparently functionally distinct (18–20). SUMO-2 and SUMO-3 are very similar (95% sequence identity) but are relatively different from SUMO-1 (50% sequence identity). In vivo studies have indicated that PML is modified by SUMO-1 and SUMO-2/3, although the functional significance of SUMO-2/3 conjugation has not been revealed (21). Whether or not SUMO-2/3 conjugates to RanGAP1 in vivo may depend on the expression levels of the SUMO proteins (19, 20). Recent evidence indicates that SUMO-2/3 is more abundant than SUMO-1 in COS-7 cells and that pools of free SUMO-2/3 decrease when these cells are exposed to heat, ethanol, or hydrogen peroxide (19). Thus SUMO-2/3 may be involved in the cellular response to envi-
The ability to form polymeric chains is not shared by SUMO-1. A rat anti-FLAG M2 monoclonal antibody 12CA5 (at a 1:5000 dilution), which recognizes YPYD-HA-SUMO-3 were detected in Western blot experiments using monoclonal anti-HA (2C5) at a 1:5000 dilution, which recognizes YPYD-HA-SUMO-3. These are substrates for SUMO-1, SUMO-2, and SUMO-3, respectively. These proteins that terminate in a diglycine motif.

Formation of Poly(SUMO) Chains

The mutant SUMO-2 and SUMO-3 K11R DNAs were amplified by PCR using the same single downstream primer described above for the wild type GG constructs along with mutant upstream primers 5’-ATCCGTTGATCATGCGAGGAGAAGCCCAAGGAGGGTTGTGAGGAC-3’ and 5’-GAGGAGACTCCGGCGGGATCCATGGCCGACGAA-3’ for both with the respective upstream primer as described above. The restriction sites introduced into the primers allowed the cleavage of the cDNAs by the enzymes BamHI and EcoRI and subsequent ligation into the plasmid pCDNA3-HA (22). All SUMO proteins were expressed in E. coli strain B834, extracted, and purified as described previously (26). SUMO-2 chains are detected in vivo, and SUMO-2 chains are detected in vitro and, and SUMO-2 chains are detected in vivo. The ability to form polymeric chains is not shared by SUMO-1. Thus, although all SUMO species share the same conjugation machinery, modification by SUMO-1 and SUMO-2/3 may have distinct functional consequences.

**Experimental Procedures**

**Antibodies**—HA-SUMO-1, HA-SUMO-2, HA-K11R-SUMO-2, and HA-SUMO-3 were detected in Western blot experiments using monoclonal anti-HA (2C5) at a 1:5000 dilution, which recognizes YPYD-HA-SUMO-3. These are substrates for SUMO-1, SUMO-2, and SUMO-3, respectively. These proteins that terminate in a diglycine motif. The K59R-HDAC4 mutant DNA construct in pCDNA3.1. All cleaved SUMO protein complexes were ligated into pGEX-2T for cloning of the wt-SUMO-1-GG protein (22). All SUMO proteins were expressed in E. coli strain B834, extracted, and purified as described previously (26). SUMO-2 chains are detected in vivo, and SUMO-2 chains are detected in vitro and, and SUMO-2 chains are detected in vivo. The ability to form polymeric chains is not shared by SUMO-1. Thus, although all SUMO species share the same conjugation machinery, modification by SUMO-1 and SUMO-2/3 may have distinct functional consequences.

**Cell Culture and Transfections**—293 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. For analysis of SUMO-1/2-modified His6-HDAC4, 5-cm2 flasks of subconfluent 293 cells were transfected with 12 μg of total plasmid DNA as indicated in the figure. Lysates, protein purification, and Western blots were prepared as indicated.

**Isolation of His6 Proteins**—His6-HDAC4 proteins were purified from transfected cells by guanidine cell lysis and nickel-nitrioltriacetic acid-Sepharose (Qiagen) purification as described previously (7). Proteins eluted from the nickel-nitrioltriacetic acid-Sepharose were fractionated by electrophoresis in 8% polyacrylamide gels containing SDS before anti-HA Western blotting as described above.

**Expression and Purification of Recombinant Proteins**—GST-SAE2/SUMO-1 fusion protein was expressed in Escherichia coli B834 and purified by affinity chromatography using glutathione-Sepharose as described previously (26). The GST fusion protein was cleaved with thrombin and dialyzed to remove glutathione, and the SAE1/2 was purified by covalent affinity chromatography on a column of SUMO-1 linked to agarose as described previously (2). Expression of both proteins was confirmed by Western blotting, and the activity of recombinant protein was tested by thioste assay using 181-SUMO-1 and Ub9 (data not shown).

Recombinant Ub9 was expressed and purified as detailed previously (22). All SUMO proteins were expressed in E. coli strain B834, extracted, and purified as described previously (26). SUMO-2 proteins were bound to glutathione-Sepharose beads and either eluted with buffer containing 10 mM glutathione or cleaved by 17 unit/ml-1 thrombin while bound. Eluted GST-SUMO proteins were dialyzed against 50 mM Tris (pH 7.5), 1 mM dithiothreitol (DTT) and concentrated using 30-kDa molecular mass cut-off microcentrators to 10 mg/ml. The GST fusion protein was cleaved with thrombin and dialyzed to remove glutathione, and the SAE1/2 was purified by covalent affinity chromatography on a column of SUMO-1 linked to agarose as described previously (2). Expression of both proteins was confirmed by Western blotting, and the activity of recombinant protein was tested by thioste assay using 181-SUMO-1 and Ub9 (data not shown).

Recombinant Ub9 was expressed and purified as detailed previously (22). All SUMO proteins were expressed in E. coli strain B834, extracted, and purified as described previously (26). SUMO-2 proteins were bound to glutathione-Sepharose beads and either eluted with buffer containing 10 mM glutathione or cleaved by 17 unit/ml-1 thrombin while bound. Eluted GST-SUMO proteins were dialyzed against 50 mM Tris (pH 7.5), 1 mM dithiothreitol (DTT) and concentrated using 30-kDa molecular mass cut-off microcentrators to 10 mg/ml. The GST fusion protein was cleaved with thrombin and dialyzed to remove glutathione, and the SAE1/2 was purified by covalent affinity chromatography on a column of SUMO-1 linked to agarose as described previously (2). Expression of both proteins was confirmed by Western blotting, and the activity of recombinant protein was tested by thioste assay using 181-SUMO-1 and Ub9 (data not shown).

**Bacterial Expression of [35S]SUMO Proteins**—wt-SUMO-GG proteins were labeled by incorporation of [35S]methionine/cysteine during isopropyl-1-thio-β-D-galactopyranoside induction of bacterial cultures as described above in the presence of 35.75 μCi/ml [35S]methionine/cysteine (Amersham Pharmacia Biotech) and were then purified as outlined above for the unlabelled proteins.
radiolabeled proteins.

Radioiodination of Proteins—C52A-SUMO-1, GST-SUMO-2-FL, and GST-PML were radiolabeled with $^{125}$I using the chloramine-T method as described previously for wt-SUMO-1 (22) except that the labeled protein was dialyzed against 50 mM Tris (pH 7.5), 1 mM DTT instead of passage over a P2-acrylamide gel column.

In Vitro SUMO Conjugation Assays—Noncompetitive SUMO conjugation assays were performed in 10-$\mu$l volumes containing between 1.4 and 10 $\mu$g (as indicated) of either unlabeled or $^{35}$S-labeled SUMO proteins, an ATP-regenerating system, and buffer (50 mM Tris (pH 7.5), 5 mM MgCl$_2$, 2 mM ATP, 10 mM creatine phosphate, 3.5 units/ml creatine kinase (creatine kinase)) and 0.6 units thrombin-cleaved proteins were essentially homogeneous, and their mass, which was determined by mass spectrometry, corresponded to that predicted from the sequence. To test the possibility that SUMO-2 and SUMO-3 could be used as substrates for SUMO modification and thus form polymer (SUMO) chains. SUMO-1, SUMO-2, and SUMO-3 were therefore expressed and purified as both the full-length pro-protein precursors and the shorter active forms (exposing the C-terminal diglycine motif). GST fusion and thrombin-cleaved versions of each protein were analyzed by SDS-polyacrylamide gel electrophoresis and Coomassie Blue staining (data not shown). The thrombin-cleaved proteins were essentially homogeneous, and their mass, which was determined by mass spectrometry, corresponded to that predicted from the sequence. To test the possibility that SUMO-2 and SUMO-3 could be conjugated to themselves the purified proteins were incubated in an assay containing purified SAE1/SAE2 and Ubc9. Analysis of the reaction products by polyacrylamide gel electrophoresis and Coomassie Blue staining revealed that a consensus SUMO modification site located between amino acids 485 and 488 of PML (37). Each of the components was analyzed in a polyacrylamide gel containing SDS and was highly purified as judged by Coomassie-stained SDS-polyacrylamide gels (Fig. 1B). The specificity of the reaction was assessed by comparing modification of GST with that of the GST-PML fusion and quantitation of the $^{38}$S-labeled products by phosphorimaging. Although GST contains 21 lysine residues, none of them conform to the SUMO modification consensus $\phi$KXE, and modification of GST was less than 1% of that observed for the GST-PML fusion (Fig. 1C). The extent of modification was such that after fractionation by polyacrylamide gel electrophoresis the reaction products could be easily monitored by Coomassie Blue staining. Under these conditions the product of the reaction was GST-PML bearing a single SUMO-1 modification with no evidence for the formation of SUMO-1 multimers (Fig. 1, D and E).

Results

A Completely Characterized System for SUMO-1 Modification in Vitro—To facilitate the biochemical analysis of SUMO-1 conjugation an in vitro system has been developed that contains recombinant, bacterially produced components. The assay contains SAE1/SAE2, Ubc9, SUMO-1, and, as substrate, GST fused to an 11-amino acid sequence containing the SUMO modification consensus site located between amino acids 485 and 488 of PML (37). Each of the components was analyzed in a polyacrylamide gel containing SDS and was highly purified as judged by Coomassie-stained SDS-polyacrylamide gels (Fig. 1B). The specificity of the reaction was assessed by comparing modification of GST with that of the GST-PML fusion and quantitation of the $^{38}$S-labeled products by phosphorimaging. Although GST contains 21 lysine residues, none of them conform to the SUMO modification consensus $\phi$KXE, and modification of GST was less than 1% of that observed for the GST-PML fusion (Fig. 1C). The extent of modification was such that after fractionation by polyacrylamide gel electrophoresis the reaction products could be easily monitored by Coomassie Blue staining. Under these conditions the product of the reaction was GST-PML bearing a single SUMO-1 modification with no evidence for the formation of SUMO-1 multimers (Fig. 1, D and E).

Formation of Polymeric Chains of SUMO-2 and SUMO-3 in Vitro—Inspection of the SUMO-1, SUMO-2, and SUMO-3 sequences revealed that a consensus SUMO modification site (KXE) is present in the N-terminal regions of SUMO-2 and SUMO-3 but is absent in the sequence of SUMO-1 (Fig. 2A). This raises the possibility that SUMO-2 and SUMO-3 could be used as substrates for SUMO modification and thus form poly-SUMO chains. SUMO-1, SUMO-2, and SUMO-3 were therefore expressed and purified as both the full-length pro-protein precursors and the shorter active forms (exposing the C-terminal diglycine motif). GST fusion and thrombin-cleaved versions of each protein were analyzed by SDS-polyacrylamide gel electrophoresis and Coomassie Blue staining (data not shown). The thrombin-cleaved proteins were essentially homogeneous, and their mass, which was determined by mass spectrometry, corresponded to that predicted from the sequence. To test the possibility that SUMO-2 and SUMO-3 could be conjugated to themselves the purified proteins were incubated in an assay containing purified SAE1/SAE2 and Ubc9. Analysis of the reaction products by polyacrylamide gel electrophoresis and Coo-
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when the reactions were carried out with SUMO-2 and SUMO-3 (Fig. 4C). When the same reactions were carried out with K11R-SUMO-2 and K11R-SUMO-3 the more slowly migrating species were no longer detected, and the pattern reverted to that observed for SUMO-1 (Fig. 4C). Thus it is likely that poly(SUMO) chains of SUMO-2 and SUMO-3 are formed on protein substrates through the formation of isopeptide bonds between the C-terminal glycine residue of one SUMO molecule and lysine 11 of another molecule of SUMO-2 or SUMO-3.

Ubc9 Does Not Discriminate between SUMO-1, SUMO-2, and SUMO-3—Although the data presented in Figs. 1, 2, 3, and 4 indicate that SAE1/SAE2 and Ubc9 utilize SUMO-1, SUMO-2, and SUMO-3 for conjugation to protein substrates, it does not indicate if the enzymes preferentially utilize a particular form of SUMO. To address this question a competition analysis was carried out in which 125I-labeled C52A-SUMO-1 was incubated with GST-PML in the presence of SAE1/SAE2 and limiting Ubc9 and a range of concentrations of unlabeled SUMO-1, SUMO-2, and SUMO-3. To simplify analysis the mutant C52A-SUMO-1 was used to stop the formation of disulfide-linked dimers of SUMO-1 and the K11R mutants of SUMO-2 and SUMO-3, which cannot form poly(SUMO) chains, were used. After incubation the reaction products were separated by electrophoresis in a polyacrylamide gel, and the 125I-SUMO-1 conjugated to GST-PML was quantitated by phosphorimaging.

The addition of increasing amounts of unlabeled SUMO-1, SUMO-2, and SUMO-3 to the reaction resulted in a dose-dependent decrease in the amount of 125I-SUMO-1 conjugated to GST-PML. Analysis of the competition data indicated that each form of SUMO competes equally efficiently for formation of 125I-SUMO-1-GST-PML (Fig. 5). Thus SAE1/SAE2 and Ubc9 utilize SUMO-1, SUMO-2, and SUMO-3 for conjugation to protein substrates with little evidence that these enzymes have the ability to discriminate between the various forms of SUMO.

Formation of SUMO-2 Chains in Vivo—To provide evidence that formation of poly(SUMO) chains can occur in vivo our objective was to use a SUMO substrate that contained a single modification site and demonstrate that although SUMO-1 gave a single modified species, multiple modified species could be detected with SUMO-2. HDAC4 contains a single site of modification at lysine 559, and changing this residue to an arginine abolishes SUMO-1 modification in vivo (Fig. 6A). To examine the modification of HDAC4 with SUMO in vivo HA-SUMO-1 was cotransfected with His<sub>6</sub>-HDAC4, modified forms of HDAC4 were isolated on nickel-Sepharose, and SUMO-1 modification was assayed for their ability to form SUMO polymers in the absence of substrate (A), in the presence of 125I-GST-SUMO-2-FL (B), and in the presence of in vitro translated full-length [35S]PML (C) as outlined under “Experimental Procedures.” The unconjugated GST-SUMO-2-FL, PML, and SUMO (+1) are indicated as are the dimeric (+2), trimeric (+3), tetrameric (+4), and pentameric (+5) SUMO-2/3 polymer forms.

Fig. 3. SUMO-2 forms polymeric chains via lysine 11 and conjugates to the lysine within the SUMO consensuss motif in the GST-PML substrate. The GST-PML/SUMO-2 assay was set up as described under “Experimental Procedures.” Assays lacking GST-PML and SUMO-2, containing no GST-PML but SUMO-2, containing no SUMO-2, and containing both components were incubated for 3 h. Half of the assay mixture was separated by SDS-polyacrylamide gel electrophoresis (A); the remainder was digested with trypsin, and the resultant peptides were analyzed by MALDI-TOF mass spectrometry. Portions of the spectra obtained for the assay containing SUMO-2 without GST-PML (B) and for the assay containing both SUMO-2 and GST-PML (C) are shown. In A the asterisk (*) marks an unidentified SUMO-dependent species, and molecular weight markers are also indicated. Peptides corresponding to peaks from the mass spectrometry analysis are shown in B and C, and expected (M + H) masses for peptide fragments are 3874.09, 4644.99, 4948.36, 5049.36, and 5352.72 Da.

Fig. 4. Lysine 11 in SUMO-2 and SUMO-3 is required for polys(SUMO) formation. SUMO-1, SUMO-2, SUMO-3, and the K11R-SUMO-2/-3 mutants were assayed for their ability to form SUMO polymers in the absence of substrate (A), in the presence of 125I-GST-SUMO-2-FL (B), and in the presence of in vitro translated full-length [35S]PML (C) as outlined under “Experimental Procedures.” The unconjugated GST-SUMO-2-FL, PML, and SUMO (+1) are indicated as are the dimeric (+2), trimeric (+3), tetrameric (+4), and pentameric (+5) SUMO-2/3 polymer forms.
FORMATION OF 125I-C52A-SUMO-GST-PML WAS COMPETITIVELY INHIBITED WITH UBC9 TO PRODUCE A UBC9-DEPENDENT CONJUGATION ASSAY. THE FORMATION OF K11R-SUMO-2, HIS6-HDAC4, K559R-HIS6-HDAC4 AS INDICATED, SHOWING THAT THE K559R-HDAC4 MUTANT ELIMINATES HDAC4 (FIG. 6B) THAT IS CONSISTENT WITH THE FORMATION OF TWO LINKED SUMO-2 MOLECULES ATTACHED TO A SINGLE SITE IN HDAC4. THIS CONCLUSION WAS SUPPORTED BY COTRANSFECTION OF HIS-K559R-HDAC4 AND HA-SUMO-2, WHICH RESULTED IN LOSS OF BOTH SUMO-2-MODIFIED SPECIES (FIG. 6B), AND BY COTRANSFECTION OF HA-K11R-SUMO-2 AND HIS-HDAC4, WHICH ELIMINATED THE MORE SLOWLY MIGRATING SUMO-2-MODIFIED SPECIES. THESE DATA SUGGEST THAT SUMO-2 CHAINS CAN BE CONJUGATED TO PROTEIN SUBSTRATES IN VITRO.

**DISCUSSION**

Since their identification almost 5 years ago, the reason for the existence of these very similar ubiquitin-like proteins, SUMO-1, SUMO-2, and SUMO-3, has been unclear. Although a considerable amount of information has accumulated relating to SUMO-1, studies on SUMO-2 and SUMO-3 have been limited. Distinct roles for SUMO-1 and SUMO-2 have emerged from investigations using mammalian cells transfected with SUMO-1 and SUMO-2/-3 constructs in which differing profiles of conjugated proteins are generated when analyzed by Western blotting (19). In fact the species generated by modification with SUMO-2/-3 appear to be of a higher molecular weight than those from SUMO-1 transfection experiments. Furthermore, it was demonstrated that in HeLa cells co-transfected with plasmids expressing SUMO-1 and SUMO-2 tagged with fluorescent proteins, the modifiers largely colocalize in PML nuclear bodies, although SUMO-1 alone is found associated with the nuclear membrane (19). These data suggest that although SUMO-1 and SUMO-2/-3 may share many of the same substrates, the functional consequences of modification by SUMO-1 and SUMO-2/-3 may be quite different.

As our knowledge has developed, comparisons between ubiquitin and the SUMO proteins have revealed the extent of structural similarity but functional diversity. Like ubiquitin, it appears that SUMO conjugations can be induced by cell stress (9, 19, 28, 29). Although SUMO and ubiquitin are conjugated and deconjugated by distinct pathways, the enzymes involved in their metabolism display significant degrees of sequence, structural, and functional similarity (for review, see Ref. 30). Indeed there is convincing evidence to support the theory that the E1 enzymes for both ubiquitin and the SUMO proteins share common prokaryotic ancestors (31). It has been shown here that in contrast with their close family member SUMO-1, SUMO-2 and SUMO-3 share the capacity to form polymeric chains with their relatively distant family member ubiquitin. Tryptic digests and MALDI-TOF mass spectrometry analysis of SUMO-2 conjugation assays both in the presence and absence of a defined substrate (GST-PML) show that this self-conjugation occurs via lysine 11, which exists within a SUMO modification motif (KXE), where X is a large hydrophobic residue, K is the target lysine, and E is glutamic acid. Furthermore, mutation of lysine 11 in SUMO-2 and SUMO-3 blocks the assembly of polymeric chains in reactions either lacking or in the presence of substrate (GST-SUMO-2-FL or in vitro translated full-length PML protein). This is also recognized in vivo as the histone deacetylase protein HDAC4 that is modified by SUMO-1 at a single lysine residue (Lys635) is modified by SUMO-2 chains. Although SUMO-2 and SUMO-3 are 96% identical, SUMO-2 appears to be a better substrate for chain formation than does SUMO-3 (Figs. 2D and 4). Thus the 3-amino acid difference in the N-terminal region appears to be partially inhibitory to conjugation of SUMO-3 in comparison with SUMO-2. Whether this inhibition is due to differing secondary structures of the two modifiers in this region or disruption of specific amino acid interactions with Ubc9 remains to be determined.

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3 M. H. Tatham and R. T. Hay, unpublished results.
4 O. A. Vaughan, unpublished results.
The cellular and substrate-specific significance of these polymeric forms of SUMO-2 and SUMO-3 are not clear. One possibility is that in a manner analogous to the enhanced recognition of multiubiquitinated over monoubiquitinated proteins by 26 S protease complex proteins (for review, see Ref. 32), poly(SUMO)-modified proteins may represent a more distinct signal to proteins that bind them. Furthermore, the possibility that SUMO-1, SUMO-2, and SUMO-3 form heterotrimers is not unrealistic. In fact GST-SUMO-2-FL is conjugated by all three SUMO proteins in vitro (Figs. 2D and 4B). Thus it is possible that SUMO-1 may act as a SUMO chain terminator. This is supported by the observation that SUMO-1 and SUMO-2 appear to largely co-localize in mammalian cells, and although many of the identified SUMO-1 substrates are of relatively low molecular weight, the majority of SUMO-2 conjugated proteins found in crude cell lysates are of over 100 kDa in mass (19),3 Interestingly sequence comparison between proteins of the SUMO family from different species reveals that although the ψKXE sequence required for polymeric chain formation is present in the N-terminal region of Saccharomyces cerevisiae Smt3p, it is not conserved in all species.

To aid investigations into the dynamics of SUMO-1, SUMO-2, and SUMO-3 conjugation we developed an in vitro assay that conjugates recombinant SUMO-1, SUMO-2, or SUMO-3 onto a GST fusion substrate presenting only the 11-amino acid region surrounding one of the lysines targeted for SUMO-1 conjugation in PML (588–PRVKIMESEE–486). The assay uses recombinant Ubc9 and SAE1/2 and either 5 mM ATP or an ATP-regenerating system with 0.6 units/ml inorganic pyrophosphatase (see “Experimental Procedures” for details). Mass spectrometry and analysis of duplicate assays using GST alone as substrate revealed the specificity of the system for the 11-residue tag-GST protein and confirmed that the consensus alone as substrate revealed the specificity of the system for the SUMO conjugation system. An E2-dependent competition assay using recombinant Ubc9 was used to analyze the specificity of Ubc9 for SUMO-2/3 in comparison with SUMO-1. The data showed that wt-SUMO-1, K11R-SUMO-2, and K11R-SUMO-3 compete with 125I-C52A-SUMO-1 to approximately the same extent for conjugation to GST-PML, supporting the idea that the contrasting cellular characteristics of SUMO-1 and SUMO-2/3 are unlikely to rely solely upon Ubc9-dependent conjugation in vivo. In addition to these findings, the recent identification of SUMO isopeptidases with apparently differing specificities for SUMO-1 and SUMO-2/3 (33, 34) suggest that the overall conjugation state of SUMO-1- and SUMO-2/3-modified proteins may in fact be regulated at the level of removal rather than conjugation. While we still understand little about functional heterogeneity of SUMO-1 in comparison with SUMO-2/3, the discovery of SUMO-2 and SUMO-3 multimers adds a new dimension of complexity to the SUMO conjugation system.

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REFERENCES
1. Jentsch, S., and Pyrowolakis, G. (2000) Trends Cell Biol. 10, 335–342
2. Desterro, J. M., Rodriguez, M. S., Kemp, G. D., and Hay, R. T. (1999) J. Biol. Chem. 274, 10618–10624
3. Johnson, E. S., Schwienhorst, I., Dohmen, R. J., and Blobel, G. (1999) EMBO J. 18, 5509–5519
4. Okuma, T., Honda, R., Ichikawa, G., Tsunagari, N., and Yasuda, H. (1999) Biochem. Biophys. Res. Commun. 254, 683–688
5. Gong, L., Li, B., Millas, S., and Yeh, E. T. (1999) FEBS Lett. 485, 185–189
6. Johnson, E. S., andBlobel, G. (1999) J. Cell Biol. 147, 981–994
7. Rodriguez, M. S., Dargemont, C., and Hay, R. T. (2001) J. Biol. Chem. 276, 12654–12659
8. Desterro, J. M., Rodriguez, M. S., and Hay, R. T. (1998) Mol. Cell. 2, 233–239
9. Rodriguez, M. S., Desterro, J. M., Lain, S., Midgley, C. A., Lane, D. P., and Hay, R. T. (1999) EMBO J. 18, 6455–6461
10. Costiussa, M., Hengstermann, A., Fogal, V., Sandy, P., Schwarz, S. E., Scheffner, M., and De The, H. (1999) EMBO J. 18, 6462–6471
11. Mahajan, R., Delphin, C., Guan, T., Gerace, L., and Melchior, F. (1997) Cell 88, 97–107
12. Matunis, M. J., Coutavas, E., and Blobel, G. (1996) J. Cell Biol. 135, 1457–1470
13. Duprez, E., Saurin, A. J., Desterro, J. M., Lallemand-Breitenbach, V., Howe, K., Boddy, M. N., Solomon, E., de The, H., Hay, R. T., and Freemont, P. S. (1999) J. Cell Sci. 112, 381–393
14. Muller, S., Matunis, M. J., and Dejean, A. (1999) EMBO J. 17, 61–70
15. Sterrenstorf, T., Jensen, K., and Will, H. (1997) J. Cell Biol. 139, 1621–1634
16. Ishov, A. M., Setnikov, A. G., Negorev, D., Vladimirova, O. V., Neff, N., Kamitani, T., Yeh, E. T., Strauss, J. F., and Ullmann, G. (1999) J. Cell Biol. 147, 221–234
17. Zheng, S., Muller, S., Runchetti, S., Freemont, P. S., Dejean, A., and Pandolfo, M. P. (2000) Blood 95, 2745–2752
18. Lapenta, V., Chiurazzi, P., van der Spek, P., Pizzuti, A., Hanako, F., and Brahe, C. (1997) Genomics 40, 362–368
19. Saitoh, H., and Hinchey, J. (2000) J. Biol. Chem. 275, 6252–6258
20. Kamitani, T., Kito, K., Nguyen, H. P., Fukuda-Kamitani, T., and Yeh, E. T. (1998) J. Biol. Chem. 273, 11349–11353
21. Kamitani, T., Nguyen, H. P., Kito, K., Fukuda-Kamitani, T., and Yeh, E. T. (1998) J. Biol. Chem. 273, 3117–3120
22. Desterro, J. M., Thomson, J., and Hay, R. T. (1997) FEBS Lett. 417, 297–300
23. Aha, J. H., Xu, Y., Jang, W. J., Matunis, M. J., and Hayward, G. E. (1999) J. Viral. 75, 3859–3872
24. Dougerty, W. G., Parks, T. D., Cary, S. M., Banaz, J. P., and Flettherick, R. J. (1989) Virology 172, 302–310
25. Miska, E. A., Karleson, C., Langley, E., Nielsen, S. J., Pines, J., and Kouzrides, T. (1999) EMBO J. 18, 5099–5107
26. Jaffray, E., Wood, K. M., and Hay, R. T. (1995) Mol. Cell. Biol. 15, 2166–2172
27. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
28. Goodson, M. L., Hong, Y., Rogers, R., Matunis, M. J., Park-Sarge, O. K., and Sarge, K. D. (2001) J. Biol. Chem. 276, 18513–18518
29. Wilkinson, K. D. (1999) J. Nutr. 129, 1833–1836
30. Yeh, E. T., Gong, L., and Kamitani, T. (2000) Gene (Amst.) 248, 1–14
31. Hochstrasser, M. (2000) Nat. Cell Biol. 2, E153-E157
32. Pickart, C. M. (2000) Trends Biochem. Sci. 25, 544–548
33. Nishida, T., Tanaka, H., and Yasuda, H. (2000) Eur. J. Biochem. 267, 6423–6427
34. Gong, L., Millas, S., Mau, G. G., and Yeh, E. T. (2000) J. Biol. Chem. 275, 3355–3359