The ubiquitin-related protein SUMO-1 (Smt3 in yeast) is a 100-residue protein that is conjugated to substrate proteins by sequential thioester transfer reactions via specific E1 activating enzymes (Uba2/Aos1) and E2 (Ubγ9) conjugating enzyme (1, 2). SUMO is conjugated to specific lysine residues on substrate proteins typically exhibiting consensus sites hKxE, where h is a hydrophobic amino acid (3, 4). Unlike ubiquitylation, sumoylation of target proteins does not lead to proteasomal degradation but can affect diverse functions of the protein, such as subcellular localization, protein/DNA interaction, or enzymatic activity (5, 6).

Smt3 is highly conserved and essential in yeast. In addition, the conjugating and deconjugating machinery are conserved from yeast to humans and perform essential functions in yeast. The only yeast proteins known to be modified by Smt3 are the nonessential septins involved in cytokinesis and the essential Pol30, Top2, and Pds5 (7–9). A growing number of proteins that are potentially modified by Smt3. Using rapid biochemical assays, we confirmed sumoylation for some of the isolated proteins. Potential Smt3-carrying substrate proteins involved in DNA replication and repair, chromatin remodeling, transcription activation, Pol-I, Pol-II, and Pol-III transcription, pre-RNA capping, and pre-RNA processing can be identified from yeast lysates in combination with tandem liquid chromatography mass spectrometry, we have isolated potential Smt3-carrying substrate proteins involved in DNA replication and repair, chromatin remodeling, transcription activation, Pol-I, Pol-II, and Pol-III transcription, pre-RNA capping, pre-RNA processing, proteasome function, and tubulin folding. Employing tandem affinity purifications or a rapid biochemical assay referred to as “SUMO fingerprint,” we showed that several subunits of RNA polymerases I, II, and III, members of the large subunit of RNA polymerase II, and the essential Ulp1 are tethered to the nuclear pore channel via nuclear import receptors (13). The biological significance of such a tethering mechanism is poorly understood because of the lack of knowledge about its substrate proteins. Yeast has served as a powerful, rapid, genetic and in vivo biochemical model system to gain mechanistic insights into protein function in eukaryotes. Hence, we applied a proteomic approach in yeast to unravel the SUMO proteome. We have enriched sumoylated proteins from yeast cell lysates using the high affinity tag of Protein A (ProtA). In this study, we provide a list of proteins that are potentially modified by Smt3. Using rapid biochemical assays, we confirmed sumoylation for some of the isolated proteins.

**EXPERIMENTAL PROCEDURES**

**Yeast and Microbiological Methods**—Yeast strains and plasmids used in this study are listed in the supplemental information. Microbiological techniques, plasmid transformation and recovery, mating, sporulation of diploids, and tetrad analysis were done as described by Santos-Rosa et al. (17). Genomic integration of TAP (TRP1 marker) in a SMT3 shuffle strain was performed according to Rigaut et al. (18).

**Affinity Purifications and “SUMO FingerPrint”—**The ProtA-Smt3 containing the yeast strain was grown to an _A_600, nm of 3.5 in 2 liters of YPD medium (yeast extract/peptone/dextrose) at 30 °C. The cells were washed and resuspended in 10 mM Tris-HCl, pH 9.4, and 10 mM dithiothreitol. After 15 min of incubation, the cells were pelleted and resuspended in 1.2 M sorbitol, 50 mM potassium phosphate, pH 7.4. The cells were spheroplasted with 10 mg of zymolyase (20T) at 30 °C for 30 min. The pelleted spheroplasts were treated with 50 mg of iodoacetamide for 15–30 min on ice and then lysed by vortexing with glass beads in LB buffer (150 mM KCl, 5 mM MgCl2, 1% Triton X-100, 40 mM Tris- HCl). After centrifugation, the supernatant was incubated with IgG-Sepharose at 4 °C for 1 h. The beads were washed with LB buffer and increasing concentrations of MgCl2 (0.1–2 mM) followed by a wash with LB buffer and final elution with 0.5 M acetic acid (pH 3.5). The eluate was lyophilized, resuspended in SDS-loading buffer, and analyzed on a SDS 4–12% gradient polyacrylamide gel. Western blot analysis was performed using anti-ProtA antibodies (14).

**TAP purifications of RNA polymerase subunits** were performed as described previously (18), except that the cells were incubated with 10 mM N-ethylmaleimide for 30 min on ice prior to lysis. The final eluates from calmodulin-Sepharose were trichloroacetic acid-precipitated, resuspended in SDS sample buffer, and analyzed on a 4–12% gradient gel. The antibodies used were polyclonal rabbit anti-PolA horseradish peroxidase conjugate (Dako Cytomation; 1:1000), polyclonal rabbit anti-my- c (Biomol; 1:2000), and horseradish peroxidase-coupled goat anti-rabbit (Bio-Rad; 1:3000). For Western blot analysis, ECL detection was performed (Amersham Biosciences). For the SUMO fingerprint, lysates from 10 units _A_600, nm of cells were prepared using glass bead lysis in 20% cold trichloroacetic acid. The protein pellets were washed with cold ethanol, air-dried, and resus-
RESULTS

A Proteomic Approach to Identifying Sumoylated Substrate Proteins in Yeast—To enrich for sumoylated proteins by affinity purification, a yeast strain expressing ProtA-tagged Smt3 instead of endogenous Smt3 was used. This modified Smt3 version was found to be functional in the temperature range from 23 to 37 °C in a yeast strain lacking chromosomal SMT3 (Fig. 1A). Under conditions that preserve sumoylation (see “Experimental Procedures”), a cell lysate from the strain expressing ProtA-Smt3 was passed over an IgG-Sepharose column to enrich for ProtA-Smt3 conjugates. The gel lane containing purified ProtA-Smt3 conjugates remained bound to the beads (Fig. 1B). Finally, the ProtA-Smt3 conjugates were eluted from IgG-Sepharose by acetic acid and analyzed by SDS-PAGE. After staining with Coomassie Brilliant Blue, numerous bands ranging from 40 to 200 kDa became visible (Fig. 1B, lane 8; (Coomassie)). The corresponding Western blot using antibodies against the ProtA tag revealed that many of these bands carried ProtA-Smt3 (Fig. 1B, lane 8; (Western)). To elucidate the identity of these proteins, the gel lane was sliced into 48 pieces across the entire separation range to sample modified proteins without bias as to size or abundance. Each gel slice was trypsinized and subjected to mass spectrometric analysis (see “Experimental Procedures”). The data obtained are summarized in Table I. Although all of the proteins listed in Table I were unambiguously identified, we were unable to directly sequence the Smt3-modified peptides of the identified proteins. Therefore, it is possible that a fraction of the identified proteins are contaminants (e.g. abundant enzymes of metabolic/anabolic pathways or “sticky” proteins) or high affinity interactors to genuinely sumoylated proteins that were not removed by MgCl₂ treatment. However, for a representative number of identified proteins, we show that they are indeed sumoylated (see below). We have grouped the proteins isolated via ProtA-Smt3 into classes according to available functional annotations (Table I). Evidently, our proteomic approach has identified factors that are known to be sumoylated in yeast. These are the yeast septins Cdc3, Cdc11, and Sep7 (4) and Pol30 (PCNA), which is involved in DNA synthesis and repair (7).
A proteome-wide approach using affinity chromatography of ProtA-Smt3 and mass spectrometry identified potential SUMO-carrying substrates with roles in diverse cellular pathways. For further details, see “Experimental Procedures.”

| Functional category | Sumoylated substrate proteins |
|---------------------|------------------------------|
| Pol I | Rpa190, Rpa40 |
| Pol II | Rpb3 |
| Pol III | Rpet160, Rpe128, Rpe82, Rpe40, Rpe37 |
| Transcription (Pol II) | Tfg1, Tfg2, Tan1, Anc1, Med7, Mot1, Tup1, Ssn6, Pdr1, Stb3, Spt16, Caf4 |
| 5’ capping/decapping | Abd1, Cet1, Ceg1, Dcp2 |
| Sumo pathway | Uba2, Aos1 |
| Ubiquitin pathway | Cdc48, Rpn1, Rpn7, Rpn12, Pre9, Ubr1 |
| Septins | Cdc3, Cdc10, Cdc11, Sep7, Bni4 |
| Polyadenylation | Cfe2, Palb1, Ref2 |
| DNA replication/repair | Po130, Tof1 |
| Chromatin remodeling | Hda1, Rsc8, Rsc9 |
| Transport factors | Suc2, Snt3, Rna1 |
| Secretion | Sec27, Cop1, Gcs1 |
| Tubulin chaperones | Cct3, Cct8, Cct2, Tub1 |
| Meiosis | Ume1 |
| RNA metabolism | Rrn2 |
| Ribosomal proteins | Rps11, Rps6b, Rps5, Rps8 |
| tRNA synthetases | Iis1, Gsk1, Gsk1, Me1, Ded81, Yhr020w |
| Unknown | Ynl1042w (Bop3) |

Table II

| Strain name | Genotype | Origin |
|-------------|----------|--------|
| FY23        | MATa, ura3, trpl, leu2, HIS3 | Derived from S288C |
| FY86        | MATa, ura3, his3, leu2, TRPl | Derived from S288C |
| Smt3 shuffle strain | MATa trpl1 his3 leu2 lys2 ura3 smt3::kanMX4 (ARS/CEN pURA3-SMT3) | This study |
| RPC82/SMT3 shuffle strain | ura3, his3, leu2, lys2, trpl, smt3::kanMX4, rpe2::his3 (pRS16-RPC82, pRS16-SMT3) | This study |
| RPC82 K406R/myc3-SMT3 | ura3, his3, leu2, lys2, trpl, smt3::kanMX4, rpe2::his3 (pRS16-RPC82-TAP wt, pRS16-myc3-SMT3) | This study |
| RPC128 shuffle strain | ura3, his3, leu2, lys2, TRPl, rpc128::kanMX4, smt3::kanMX4 (pRS16-RPC128, pRS16-SMT3) | This study |
| RPC128 K17R/myc3-SMT3 | ura3, his3, leu2, lys2, TRPl, rpc128::kanMX4, smt3::kanMX4 (pRS16-RPC128-TAP wt, pRS16-myc3-SMT3) | This study |

Identification of sumoylated substrate proteins in yeast

| Strain name | Genotype | Origin |
|-------------|----------|--------|
| RPC128 wt/myc3-SMT3 | ura3, his3, leu2, lys2, TRPl, rpc128::kanMX4, smt3::kanMX4 (pRS16-RPC128-TAP wt, pRS16-myc3-SMT3) | This study |

Development of a SUMO Fingerprint—To confirm that ProtA-Smt3-purified proteins are sumoylated in vivo, we developed a simple verification assay, wherein a suspect target protein was genomically TAP-tagged at the C terminus to allow sensitive detection using anti-ProtA antibodies. In a next step, this TAP-tagged putative target was co-expressed with either of the detection using anti-ProtA antibodies. In a next step, this TAP-Smt3-purified proteins are sumoylated in vivo previously not known to be sumoylated in yeast. Notably, these ever, our list exhibits a vast number of proteins, which can be used to identify SUMO-targeted proteins in yeast. How-
Developing a SUMO fingerprint. Notably, mainly the larger subunits of Pol-I/II/III become sumoylated. As baits for TAP purifications, we used Rpa190 (Pol-I), Rpb2 (Pol-II), Rpb8 (common subunit of Pol-I/II/III), and Rpac40 (common subunit of Pol-I/II/III). As shown in Fig. 3B, purified RNA polymerases I and II exhibited sumoylated bands mainly in the high molecular weight range of the gel (M_r > 100,000). By comparing the different sumoylated bands in various Pol-I/II/III preparations, we concluded that Rpa190 and Rpa135 (the first and second subunit of Pol-I), Rpb1 (first subunit of Pol-II), and Rpc128 and Rpc82 (second and third subunit of Pol-III) are sumoylated. As expected, a mixed pattern of sumoylation was observed for purified Rpac40 and Rpb8, which were present in different RNA polymerases. Taken together, our data show that all three yeast RNA polymerases are targets of sumoylation.

**DISCUSSION**

Employing a powerful affinity purification of functional ProtA-tagged yeast SUMO from lysates in combination with mass spectrometry, we could isolate a large number of potential SUMO substrates and confirm sumoylation for a few of these proteins by different methods. Conspicuous targets of sumoylation are nuclear proteins, which are part of different multisubunit gene expression machineries. These include components of complexes involved in chromatin remodeling, RNA and mRNA transcription, and mRNA processing (see Table I). In agreement with these observations, both SUMO-conjugating and deconjugating enzymes are predominantly located in the nucleus and at the nuclear pore complexes (3). Moreover, our findings are consistent with data from the mammalian system, which have shown that many SUMO substrates are part of nuclear complexes with a wide role in transcription and genome organization (6). Moreover, the isolation of conjugates based on the ProtA methodology could be exploited for other ubiquitin-like proteins.

To show SUMO-1 modification, we have developed a rapid and diagnostic assay called the SUMO fingerprint. How sensi-
tive is our assay? Not all the proteins obtained in the proteomic approach exhibited the SUMO fingerprint, suggesting either the proteins represent contaminants or low abundance SUMO conjugates. Indeed, in the case of Rpc128, we were unable to obtain a SUMO fingerprint. However, upon enrichment by TAP-purification, we have been able to detect the sumoylation of Rpc128 via myc tagging of Smt3 and Western blot analysis. Moreover, Rna1 did not show the SUMO fingerprint or a myc-Smt3 Western signal when TAP-purified, suggesting that it is a very low abundant conjugate or a contaminant in our preparation. The mammalian homologue of Rna1 (RanGAP) was shown to be modified by SUMO-1 (15). Nevertheless, both the methods could be useful to analyze SUMO modification in protein complexes and follow the changes of sumoylation patterns during formation and turnover of these large multimeric assemblies. Interestingly, the human homologues of the yeast RNA polymerase subunits Rpc160, Rpc128, Rpc82, Rpc37,Rpc25, Rpac40, and Rpabc27 are found to be potentially sumoylated in a proteomic approach using mammalian cells (10). Thus, sumoylation of RNA polymerases is conserved, suggesting that this post-translational modification is important for gene expression. Whether this modification affects the catalytic activity or assembly of RNA polymerases remains to be shown.

Did our large scale approach cover the entire SUMO proteome? We did not find Top2 (topoisomerase II) and Pds5 (sister chromatin cohesion), which are known to be sumoylated.

\[^2\] V. Panse, unpublished results.
in yeast. It is possible that strong association with chromosomes (8, 9) prevented their release into the soluble cell lysates. Moreover, sumoylated proteins that were not identified in our proteomic approach may be low abundant. Although we could not unravel the entire SUMO proteome, the potent affinity purification of Smt3-ProtA in combination with mass spectrometry was able to identify several unknown SUMO substrates. Indeed similar proteomic approaches in mammalian cells lead to the isolation of a distinct set of proteins (10, 12). It is thus conceivable that only several different proteomic approaches will give a more comprehensive list of sumoylated proteins.

SUMO-1 has been implicated as a versatile regulator of protein function (16). However, two fundamental questions regarding its biological function remain to be answered. Why is SUMO-1 essential in most eukaryotic cells? What are these essential processes? The loss of Smt3 modification in Pol30, Top2, and Pds5 were shown not to be essential for the viability of yeast (7–9). Our mutational analysis, which inactivated the major sumoylation sites within Rpc128 or Rpc82, indicated that cell growth is not inhibited. Thus, the influence of SUMO-1 on Pol-III function remains unknown. It is possible that inactivation of single sumoylation sites are not sufficient to affect Pol-III assembly/function. Clearly, more work is required to understand why Pol-III, as well as Pol-I and Pol-II, transcription machineries are sumoylated in vivo. Further, insights into these issues can be gained using the power of yeast genetics. Our catalogue of sumoylated proteins in yeast can trigger further studies to reveal the essential nature of sumoylation.

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