A Proteomic Study of SUMO-2 Target Proteins*\textsuperscript{**}

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The SUMO family in vertebrates includes at least three distinct proteins (SUMO-1, -2, and -3) that are added as post-translational modifications to target proteins. A considerable number of SUMO-1 target proteins have been identified, but little is known about SUMO-2. A stable HeLa cell line expressing His\textsubscript{n}-tagged SUMO-2 was established and used to label and purify novel endogenous SUMO-2 target proteins. Tagged forms of SUMO-2 were functional and localized predominantly in the nucleus. His\textsubscript{n}-tagged SUMO-2 conjugates were affinity-purified from nuclear fractions and identified by mass spectrometry. Eight novel potential SUMO-2 target proteins were identified by at least two peptides. Three of these proteins, SART1, heterogeneous nuclear ribonucleoprotein (RNP) M, and the US small nuclear RNP 200-kDa helicase, play a role in RNA metabolism. SART1 and heterogeneous nuclear RNP M were both shown to be genuine SUMO targets, confirming the validity of the approach.

The small ubiquitin-like modifier (SUMO)\textsuperscript{1} is linked to target proteins by post-translational conjugation and may thereby influence protein function and/or localization (1–3). The name derives from the relationship of SUMO to the better characterized post-translational modifier ubiquitin (4). They are 18% identical at the amino acid level and show a clear similarity in their respective three-dimensional structures (5). Whereas yeast and nematodes have a single SUMO gene, in humans and mice, the SUMO family consists of three members, SUMO-1, -2, and -3, which are encoded by separate genes. The mature forms of SUMO-2 and SUMO-3 are similar (~95% identical), but less closely related to SUMO-1 (~50% identical) (6–8). Despite their close similarity, there is evidence that SUMO-1 and SUMO-2/3 are preferentially conjugated to distinct sets of target proteins. Whereas RanGAP1 is modified by SUMO-1, many as yet unidentified proteins are modified by SUMO-2/3 after exposure of cells to various stress stimuli (7).

Several groups independently identified SUMO-1, which is also known as PIC1, GMP1, SMT3C, UBL1, and sentrin (9–13). In contrast to most polyubiquitinylated proteins, sumoylated proteins are not thereby targeted for degradation. SUMO-1 can even compete with ubiquitin for the same acceptor lysine in the target protein. In the case of IkBa, SUMO modification serves to protect the protein from ubiquitin-mediated degradation (14), whereas in yeast, DNA repair can proceed only after proliferating cell nuclear antigen is desumoylated to allow ubiquitination. Here, ubiquitination does not target the protein for degradation, but functions as a switch to activate the DNA repair function of proliferating cell nuclear antigen (15). Therefore, despite their structural relationship, it appears that SUMO and ubiquitin conjugation can play distinct roles in modulating target proteins. Sumoylation can also regulate the subcellular localization of target proteins. For example, SUMO conjugation is required for both the nuclear pore and kinetochore localization of RanGAP1 (16–18) and the nuclear body localization of lymphoid enhancer factor-1 and SP3, which results in transcriptional inhibition (19, 20).

Mechanistically, sumoylation is analogous to ubiquitination because it involves a set of enzymes known as E1, E2, and E3, which act sequentially to conjugate SUMO to an internal lysine residue located within a conserved motif in target proteins (1, 3, 4, 21). The human SUMO E1 enzyme comprises a heterodimer of the SAE1 and SAE2 proteins and forms a thioester bond with Gly\textsuperscript{97} of SUMO-1 (22–25). Subsequently, SUMO-1 is transferred by trans-esterification to the SUMO-specific E2-conjugating enzyme, Ubc9 (26–29). Ubc9 can directly conjugate the C-terminal glycine of SUMO to the ε-amino group of lysines in target proteins that are situated in the consensus motif ϕ(ϕXϕEϕ)(D), where ϕ stands for Val, Leu, Ile, Met, or Phe and ϕ stands for any amino acid (14, 21, 30). Recognition of target protein substrates by Ubc9 involves a group of residues, adjacent to the active-site Cys\textsuperscript{93}, that position the sumoylation motif for catalysis (31–33). Therefore, SUMO conjugation does not have an absolute requirement for a SUMO E3 ligase in vitro (23, 24), Nonetheless, proteins with SUMO E3 ligase...
activity have been characterized, including Ran-binding protein-2, Pce2, and members of the PIAS family (19, 34–41).

Sumoylation is a reversible process. In addition to the SUMO conjugation machinery, a set of specific and highly active proteases can remove SUMO from target proteins (42–49). Interestingly, these proteases exhibit unique tissue-specific expression and subcellular localization patterns, suggesting that they have non-overlapping functions (44, 45, 47–50). SUMO proteases also play a role in the maturation of SUMO precursors. All three SUMO family members are translated as larger precursors, and SUMO proteases remove C-terminal amino acids from these precursors to expose the SUMO diglycine motif required for conjugation to target proteins (22, 43, 45, 47).

The single SUMO gene in both Saccharomyces cerevisiae (51) and Caenorhabditis elegans (52, 53) is essential for viability, indicating that sumoylation is an essential process. Consistent with this, several genes encoding SUMO-conjugating enzymes and SUMO proteases are also essential (22, 42, 52, 53). Sumoylation is also likely to be an essential cellular process for higher eukaryotic cells because Ubc9 is required for the viability of chicken DT40 cells (54).

Many target proteins have been identified for SUMO-1, including RanGAP1, PML, Sp100, p53, and IxoB (9, 10, 14, 55, 56). In contrast, the only proteins reported as targets for SUMO-2 are PML, Sp3, heat shock factor-1, thymine DNA glycosylase, histone deacetylase-4, p300, and lymphoid enhancer factor-1 (8, 19, 57–62). Most recently, Xenopus topoisomerase II was shown to be a substrate for SUMO-2 specifically during M phase, thereby affecting the interaction of topoisomerase II with chromatin (63). Here, we report on a set of novel endogenous target proteins for SUMO-2, identified using proteomics coupled with an affinity selection strategy based on a stable cell line expressing low levels of His$_6$-SUMO-2.

**EXPERIMENTAL PROCEDURES**

**Plasmids Constructs and Antibodies**—The cDNA for SUMO-2 was amplified by PCR using expressed sequence tag A8143343 (Medical Research Council Rosalind Franklin Centre for Genomics Research) and specific primers with BamHI and Asp718 restriction sites on the 5’- and 3’-primers, respectively. The amplified fragment was subsequently inserted into the BamHI and Asp718 sites of pEYFP-C1 (Clontech). The previously published pCMV-SUMO-2 (59) was used to obtain a BamHI-EcoRI fragment encoding SUMO-2. This DNA fragment was cloned into similar sites of the pcDNA3-His$_6$ vector (56) to generate the pcDNA3-His$_6$-SUMO-2 construct. This vector was digested with HindIII, blunt-ended using T4 DNA polymerase, and finally digested with EcoRI. The corresponding DNA fragment encoding His$_6$-SUMO-2 was subcloned into the EcoRI and EcoRI sites of the pIRESpuro2 vector (Clontech). The cDNA for SART1 was amplified by PCR using expressed sequence tag BG257275 (Medical Research Council Rosalind Franklin Centre for Genomics Research) and specific extended primers compatible with the Gateway system (Invitrogen). The amplified fragment was subsequently inserted into pDON207, and EYFP-SART1 and T7-SART1 expression constructs were obtained using standard Gateway methods.

Peptide antibody 2277 against SUMO-2 was generated in rabbit using the sequence MEDEDTIDVFQQTQ (Eurogentec). Both serum and affinity-purified antibody were used. Polyclonal antibody NRDI.1 raised against full-length SUMO-2 was obtained from Zymed Laboratories Inc. (South San Francisco, CA). A previously published polyclonal antibody against SUMO-2 (7), generously provided by Dr. H. Saïd-K, (Picower Institute for Medical Research, Manhasset, New York), was used during initial studies. Note that the anti-SUMO-2 antibodies also recognize SUMO-3 because these SUMO family members are nearly identical. Other antibodies used were anti-PML monoclonal antibody 5E10 (a generous gift from Dr. R. van Driel, University of Amsterdam, Amsterdam, The Netherlands), anti-RbNBP M monoclonal antibody D2-1F7 (a generous gift from Dr. J.-P. Fuchs (Institut de Génétique et de Biologie Moléculaire et Cellulaire, Strasbourg, France), anti-His$_6$ monoclonal antibody (which recognizes 5 consecutive histidines, Novagen), and anti-green fluorescent protein monoclonal antibody 1814460 (which recognizes EYFP, Roche Applied Science).

**Cell Culture, Transfection, and Generation of Stable Cell Lines**—HeLa cells were grown in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal calf serum and 100 units/ml penicillin/streptomycin. Transfections in 10-cm dishes were carried out using 2 μg of plasmid construct/100-mm dish and Effectene (QIAGEN Inc.) according to the instructions of the manufacturer. The stable HeLa.EYFP-SUMO-2 cell line was selected by growth in Dulbecco’s modified Eagle’s medium supplemented with G418 at a final concentration of 200 μg/ml. The stable HeLa.EYFP-SUMO-2 cell line was obtained by transfecting HeLa cells with 15 μg of pREP4SUMO-His$_6$-SUMO-2 plasmid using FuGENE 6 reagent (Roche Applied Science) according to the manufacturer’s instructions. Selection was carried out in Dulbecco’s modified Eagle’s medium supplemented with puromycin at a final concentration of 5 μM. For all stable cell lines, colonies were subcloned and then expanded for characterization.

**Microscopy and Image Analysis**—Cells were grown on glass coverslips and fixed for 10 min in 3.7% paraformaldehyde at 37 °C in 60 mm PIPES, 25 mM Hepes, 10 mM EGTA, and 2 mM MgCl$_2$ (pH 6.9) (64). Subsequent manipulations were carried out at room temperature. Permeabilization was carried out for 20 min in phosphate-buffered saline containing 0.5% Triton X-100. Cells were blocked with 10% goat serum for 30 min if required and then incubated with primary antibodies for 1 h, washed, and incubated in secondary antibodies in 0.2% Dako Fast Red-conjugated secondary antibody (Dako) and 0.05% Triton X-100. Affinity-purified antibody against SUMO-2 were purified using a previously published method to enrich for nuclear bodies and RNA (65). 10$^6$ HeLa cells (either HeLa.EYFP-SUMO-2 or control parental HeLa) were isolated by trypsinization and washed twice with ice-cold phosphate-buffered saline. Nuclei were isolated and washed with cystoskeletal extraction buffer (10 mM PIPES (pH 6.8), 300 mM sucrose, 100 mM NaCl, 3 mM MgCl$_2$, 1 mM EGTA, and 0.5% Triton X-100). Subsequently, nuclei were digested with Dnase I (Worthington) in buffer containing 10 mM PIPES (pH 6.8), 300 mM sucrose, 50 mM NaCl, 3 mM MgCl$_2$, 1 mM EGTA, and 0.5% Triton X-100. Chromatin was extracted using buffers containing 250 mM (NH$_4$)$_2$SO$_4$ and 1 mM NaCl or 2 mM NaCl plus 10 mM PIPES (pH 6.8), 150 mM sucrose, 3 mM MgCl$_2$, and 1 mM EGTA. All steps were performed at 4 °C, and buffers contained EDTA-free complete protease inhibitor mixture (Roche Applied Science).

Subsequently, extracted fractions were solubilized in lysis buffer containing 8 μM urea, 100 mM Na$_2$HPO$_4$/NaH$_2$PO$_4$, 10 mM Tris-HCl (pH 8.0), 10 mM β-mercaptoethanol, 20 mM imidazole, and EDTA-free complete protease inhibitor mixture. His$_6$-SUMO-2 conjugates were enriched on Ni$^{2+}$-nitrilotriacetic acid-agarose beads (QIAGEN Inc.). Beads were successively washed at room temperature with 10 column volumes of first lysis buffer, then buffer A (8 μM urea, 100 mM Na$_2$HPO$_4$/NaH$_2$PO$_4$, 10 mM Tris-HCl (pH 6.3), 0.2% Triton X-100, and 10 mM β-mercaptoethanol), then buffer B lacking Triton X-100, then lysis buffer plus 0.1% Triton X-100, and finally buffer B lacking Triton X-100. Conjugates were eluted at room temperature in 0.2 μM column volumes of lysis buffer containing 200 mM imidazole. For experiments carried out to confirm the presence of NbnRBP M and SART1 in the His$_6$-SUMO-2-enriched fraction, cystoskeletal extraction buffer-isolated nuclei were lysed in lysis buffer and sonicated prior to the pull-down of His$_6$-SUMO-2 conjugates.

**Electrophoresis and Immunoblotting**—Protein samples were size-fractionated on Novex 4–12% BisTris gradient gels using MOPS buffer (Invitrogen) and subsequently transferred onto Hybond-C extra membranes (Amersham Biosciences) using a subrane machine (Invitrogen). Blots were stained for total protein using Ponceau S (Sigma). After blocking with phosphate-buffered saline containing 0.1% Tween 20 and 5% nonfat milk, blots were incubated in blocking buffer and then incubated with the following primary antibodies: anti-rabbit (1:1000), antibody 5E10 (1:100), antibody D2-1F7 (1:5000), or antibody 1814460 (1:1000). Anti-His$_6$ antibody was used according to the instructions of the manufacturer. The secondary antibodies used were anti-rabbit (1:2000) and anti-mouse (1:5000) horseradish peroxidase antibodies (Pierce). Bound antibodies were detected via chemilu-
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minescence with ECL Plus (Amersham Biosciences).

Mass Spectrometry—Both isolated SUMO conjugates and control samples (200-μl total volume) were reduced in lysis buffer lacking Triton X-100 by addition of 0.5 μg of dithiothreitol, alkylated by addition of 2 μg of iodoacetamide, and digested with endoprotease Lys-C for 4 h and with trypsin for 8 h after dilution with 50 mM NH₄HCO₃ (pH 8.0). The resulting peptide mixtures were acidified by A-buffer (0.5% acetic acid and 0.02% heptafluorobutyric acid) and desalted and concentrated using precolumns packed with poros R2 and oligo R3 reverse-phase material. The partially lyophilized eluate was reconstituted in A-buffer and pressure-loaded onto a pulled fused silica capillary with a 100-μm inner diameter and an 8-μm tip opening (New Objective) filled with Vydac 218MSB3 3-μm reverse-phase material. Peptides were eluted directly into a quadrupole time-of-flight mass spectrometer (MDS Sciex) with a 90-min linear gradient of 5–60% buffer B containing 80% acetonitrile, 0.5% acetic acid, and 0.02% heptafluorobutyric acid at a flow rate of 200 μl/min from a cross-flow splitter connected to an HPLC system (Agilent) and a high voltage power supply. Mass spectrometry and tandem mass spectrometry measurements were acquired automatically for 1 and 1.5 s, respectively. Precursor ions were dynamically excluded for 60 s. Scripts in Analyst created a peak list on the basis of the acquired product ion spectra. A combined peak list was searched against the International Protein Index Database (using the Mascot program (Matrix Science). Iterative calibration algorithms on the basis of identified peptides were used to achieve an average absolute mass accuracy of better than 20 ppm in both precursor and product ions. Identified peptides that were either not unique or had a score of <15 were removed. Proteins identified with a combined peptide score of >60 were considered significant, and lower scoring proteins were manually verified or rejected. Protein abundance ratios between the two samples were estimated from the abundance of each peptide calculated from the area of the extracted ion current peak.

SUMO Conjugation Assays—In vitro SUMO conjugation assays were performed in 10-μl volumes containing 0.11 μM SAE1/2, 2 mM ATP, 1.2 units/ml inorganic pyrophosphatase, 5 mM MgCl₂, 50 mM Tris-HCl (pH 7.5), 1 μM Ubch9, and 5 μM SUMO-2. SART1 sumoylation was carried out using in vitro transcribed and translated SART1. Assays were incubated for 3 h at 37 °C before addition of SDS sample buffer containing β-mercaptoethanol for gel analysis (59).

RESULTS

Generation and Characterization of Stable Tagged SUMO-2 Cell Lines—To characterize targets for SUMO-2 and their localization, we generated HeLa cell lines stably expressing fusions of SUMO-2 to either a His₆ tag (to purify biochemical quantities of SUMO-2 conjugates) or YFP tag (to visualize the SUMO-2 subcellular distribution in living cells). The fusions were made to the N terminus of SUMO-2 because conjugation of SUMO-2 to target proteins involves reaction of its C terminus. Plasmids pHisSUMO-2 and pEYFP-SUMO-2 (shown as a schematic in Fig. 1A) were transfected into HeLa cells, and stable clones were selected (see “Experimental Procedures”) to avoid overexpression artifacts. Following selection, representative clones were characterized to identify the expression levels and localization patterns of the respective SUMO-2 fusion proteins, and the resulting cell lines were labeled as HeLaYFP-SUMO-2 and HeLaHis-SUMO-2 (Fig. 1, B and C). In both cases, the fusion proteins were expressed at similar levels compared with endogenous SUMO-2 and showed identical subnuclear localization patterns as revealed by immunoblotting (Fig. 1, B and C) and fluorescence microscopy (Fig. 2). The tagged SUMO-2 molecules also showed similar fractionation properties compared with endogenous SUMO-2, and analysis by fluorescence-activated cell sorting further indicated that expression of tagged exogenous SUMO-2 did not compromise cell viability or progression through the cell cycle (data not shown).

It was important to verify that the tagged forms of SUMO-2 were actively conjugated to target proteins. We therefore analyzed the pattern of SUMO-2-conjugated proteins present in cell lysates prepared either from control HeLa cells or from clones HeLaYFP-SUMO-2 and HeLaHis-SUMO-2 (Fig. 1, B and C). Each lysate was separated by SDS-PAGE, transferred to a membrane, and probed with antibodies against both tags and against SUMO-2. In all cells, only low levels of unconjugated endogenous SUMO-2 were detected (Fig. 1, B and C; arrows indicate free SUMO-2). Most of the SUMO-2 signal was present in high molecular mass conjugates for the control HeLa cells and both tagged cell lines. However, we observed a higher level

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2 Available at www.ebi.ac.uk/Tools/PTI/PTIHelp.html.
of free YFP-SUMO-2 compared with free His$_6$-SUMO-2 (Fig. 1, B and C). We could not distinguish whether this was because of a higher conjugation efficiency for the smaller His$_6$-SUMO-2 construct or, conversely, because of a higher rate of deconjugation for YFP-SUMO-2.

The fluorescence microscopy analysis showed that both endogenous and exogenous SUMO-2 were predominantly nuclear and showed accumulation in subnuclear bodies (Fig. 2, arrows). The preferential nuclear localization of SUMO-2 was also supported by biochemical fractionation analysis (data not shown). A subset of the subnuclear bodies containing YFP-SUMO-2 were not labeled with anti-SUMO-2 antibody. This was not due to improperly expressed YFP species because immunoblotting analysis showed that there was little if any YFP that was not fused to SUMO-2 (Fig. 1) (data not shown). Therefore, analysis by immunofluorescence alone may not reveal the full range of nuclear structures containing SUMO-2. Most of the nuclear bodies containing SUMO-2 colocalized with PML bodies, as determined by double labeling with anti-PML antibody (Fig. 3).

In summary, we conclude that epitope-tagged forms of SUMO-2 are functional. Based upon in vivo localization patterns and the range of high molecular mass SUMO-2 conjugates detected for both tagged and endogenous forms, we anticipate that the tagged exogenous SUMO-2 molecules are conjugated to a similar range of cellular target proteins compared with endogenous SUMO-2.

Enrichment of SUMO-2 Conjugates—Our next aim was to identify cellular proteins that are conjugated to SUMO-2. To identify SUMO-2 targets, we exploited the HeLa$_{H_6}$-SUMO-2 cell line. Conjugation of His$_6$-SUMO-2 covalently linked the His$_6$ affinity tag to the target protein, thereby allowing its purification using a Ni$^{2+}$-agarose column. To enhance the purification of SUMO-2 conjugates, we took advantage of the fact that SUMO-2 accumulates in PML bodies. We therefore performed affinity purification on nuclear fractions enriched for PML bodies and other subnuclear structures (see “Experimental Procedures”). Briefly, nuclei were isolated by centrifugation and treated with DNase I to digest the chromatin, which was then removed by sequential washing with buffers of increasing salt concentrations (shown as a schematic in Fig. 4A). In parallel, we performed an identical fractionation with the HeLa$_{YFP}$-SUMO-2 cell line and analyzed the resulting fractions by fluorescence microscopy to detect YFP-SUMO-2. This showed that, in the isolated fractions, intact nuclear bodies containing YFP-SUMO-2 were present (Fig. 4B). Additionally, immunoblot analysis confirmed that PML was enriched 20-fold in the final nuclear fraction (Fig. 4C).

Large-scale Ni$^{2+}$-agarose affinity purification was then per-
formed on equal amounts of PML-enriched fractions from both the control HeLa and HeLa$^\text{His}_6$-SUMO-2 cells (Fig. 4, D and E). The affinity-purified proteins were separated by SDS-PAGE; transferred to a membrane; and probed with an antibody to detect SUMO-2, which showed that the procedure specifically enriched for SUMO-2 conjugates from the HeLa$^\text{His}_6$-SUMO-2 cell line (Fig. 4E).

**Identification of SUMO-2 Conjugates by Mass Spectrometry**—To identify endogenous SUMO-2-conjugated proteins, affinity-purified fractions from both the control HeLa and HeLa$^\text{His}_6$-SUMO-2 cells were digested with trypsin, and the resulting peptides were separated by HPLC and analyzed by tandem mass spectrometry. The control HeLa fractions were analyzed to identify fortuitously co-purifying proteins without a His$_6$-SUMO tag and hence to eliminate false positive targets. Eleven unique proteins enriched in the purified fraction derived from the HeLa$^\text{His}_6$-SUMO-2 cells were identified by at least two peptides (Table I). A list of all identified peptides from both control and His$_6$-SUMO-2 fractionations can be found in Supplemental Table A.
In principle, 10 of the proteins listed in Table I are candidate SUMO-2 targets, including both SUMO-2 and SUMO-3, due to the presence of an internal consensus sumoylation site (59). We have the most confidence in the nine proteins in this list that contain one or more consensus sumoylation motifs and that lack obvious histidine-rich regions that could result in their fortuitous co-purification (shown in boldface in Table I). However, Chromobox homolog-4 contains a histidine-rich region in addition to a consensus sumoylation motif. Nonetheless, it represents a genuine target for other SUMO family members, as recently shown (36). We identified peptides in the purified fraction from SUMO-2, as expected. The next step was to determine whether the proteins identified using this purification strategy include bona fide SUMO-2 targets.

Confirmation of SART1 and hnRNP M as Bona Fide Targets for SUMO-2 Modification—SART1 and hnRNP M were selected for further characterization because both proteins contain consensus sumoylation motifs and lack histidine-rich regions. In addition, both are RNA-binding proteins that are likely to be involved in RNA processing and/or transport, suggesting the possibility of a role for SUMO-2 modification in these processes.

A combination of in vivo and in vitro experiments were performed to assess whether SART1 and hnRNP M represent bona fide SUMO-2 targets. First, we constructed an epitope-tagged SART1 expression vector, pEYFP-SART1 (see “Experimental Procedures”), with EYFP fused to the N terminus of SART1. Both HeLaHs-cSUMO-2 and control HeLa cells were transiently transfected with pEYFP-SART1, and the His\textsubscript{8}-SUMO-2 conjugates were purified from isolated nuclei of transfected cells by affinity chromatography using Ni\textsuperscript{2+}-agarose (Fig. 5A). Proteins either present in the total nuclear lysate (lanes 1 and 2) or purified by Ni\textsuperscript{2+}-agarose chromatography (lanes 3 and 4) were separated by SDS-PAGE, transferred to a membrane, and probed using a monoclonal antibody that detects YFP. The transiently expressed YFP-SART1 construct was detected in both the control and HeLaHs-cSUMO-2 extracts as an ~100-kDa band. However, at least two slower migrating bands, consistent with the conjugation of one and two molecules of SUMO to SART1, were detected specifically in the affinity-purified fraction from the HeLaHs-cSUMO-2 cells (lane 4). Further evidence that SART1 is a target for SUMO-2 was provided by an in vitro sumoylation assay (Fig. 5B). In this assay, in vitro translated, untagged SART1 was incubated with puri-
fied recombinant conjugation enzymes and SUMO-2, which showed that SUMO-2 could be conjugated efficiently to SART1 (Fig. 5B). It is not clear from both the in vitro and in vivo assays whether SART1 uses more than one sumoylation site or, instead, if multiple SUMO molecules are added to the same lysine. However, the data strongly support that SART1 is a genuine SUMO-2 target.

We next used anti-hnRNP M monoclonal antibody to detect endogenous hnRNP M in affinity-purified fractions enriched for His$_{8}$-SUMO-2 conjugates from HeLaHissu-SUMO-2 cells (Fig. 5C). The results showed a major band of the expected size for SUMO-2-conjugated hnRNP M specifically in the SUMO-enriched fraction, as well as higher bands that likely correspond to multiply sumoylated forms of hnRNP M (lane 4). Further analysis showed that the monosumoylated band was a doublet that may correspond to the two major spliced forms of hnRNP M (which differ by 11 amino acids). These data strongly support that hnRNP M, like SART1, is a genuine SUMO-2 target. Taken together, the confirmation of both SART1 and hnRNP M as bona fide SUMO targets in vitro allows us to conclude that the affinity purification strategy we described here using HeLaHissu-SUMO-2 cells successfully identifies endogenous sumoylated targets.

**DISCUSSION**

We have constructed and characterized stable HeLa cell lines that express either YFP- or His$_{8}$-tagged SUMO-2 molecules to study the cellular localization of SUMO-2 and to identify target proteins for SUMO-2 conjugation. The data show that the tagged SUMO-2 molecules are functional and found at the same intracellular sites as endogenous SUMO-2, concentrating predominantly in the nucleus and showing enrichment in nuclear bodies. The conjugation of His$_{8}$-SUMO-2 introduced an affinity tag to the target proteins. This was exploited to enrich for SUMO-2 conjugates, which were subsequently identified by mass spectrometry. A similar approach has recently been used to identify large numbers of ubiquitin targets in yeast (66). Despite the well known problems in recovering SUMO conjugated to target proteins due to the high activity of SUMO-specific proteases, this affinity protocol results in high levels of conjugates relative to free forms of SUMO. The protocol thus provides an unbiased procedure for the systematic proteomic analysis of SUMO targets. A detailed analysis of the full range of SUMO target proteins is likely to be important for evaluating the biological significance of SUMO conjugation.

Among the novel proteins identified here as putative SUMO-2 targets, we have analyzed two in detail and confirmed that both of these, i.e. SART1 and hnRNP M, are conjugated to SUMO-2 in affinity-purified nuclear lysates. Both of these are nuclear RNA-binding proteins that may have roles in RNA processing and/or transport. For example, SART1 has been shown to be a component of the U4/U5/U6 small nuclear ribonucleoprotein subunit of the spliceosome (67). There is also evidence that hnRNP M may have a role in splicing (68), and both proteins have been detected as components of affinity-purified mammalian splicing complexes (69–71). Consistent with this, we detected exogenously expressed YFP-tagged SART1 localized in nuclear splicing speckles (data not shown). In this regard, it is interesting that the SUMO E3 ligase Pias1 was also identified co-purifying with mammalian spliceosomes (70). These results raise the possibility that sumoylation of splicing factors may influence one or more steps in the splicing mechanism.

The mass spectrometry analysis detected both SUMO-1 and SUMO-3 in the affinity-purified SUMO-2 conjugates. Due to the denaturing buffer conditions used, this implies that single target proteins can simultaneously be conjugated to multiple SUMO family members. This could occur via independent lysines on target proteins, but also via chain formation due to the presence of internal sumoylation sites in SUMO-2 and SUMO-3 (59). SUMO-1 lacks an internal sumoylation site and could therefore be present at the distal end of poly-SUMO chains.

In line with our results, SUMO-2 was recently identified in a purified SUMO-1 fraction (72). In this study, hemagglutinin-SUMO-1 was used in an unbiased approach to identify SUMO-1 targets. Many of the identified potential SUMO-1 target proteins play a role in gene expression. Interestingly, zinc finger proteins and BTB-like domain proteins were identified in both screens.

The absence of PML in the list of proteins we identified here and the fact that we have so far screened only one nuclear fraction indicate that use in the future of higher sensitivity mass spectrometry techniques and analysis of additional nuclear fractions will likely identify many additional SUMO targets. It will also be interesting in the future to extend the approach with the HeLaHissu-SUMO-2 cell line to include the analysis of target proteins that are sumoylated only under specific metabolic conditions (e.g. stress, DNA damage, etc.) or at defined stages of the cell cycle. In this regard, it is interesting that Azuma *et al.* (63) have recently identified an M phase-specific conjugation of topoisomerase II with SUMO-2 in *Xenopus*. Analysis of other subcellular fractions apart from the nuclear fractions analyzed here may also expand the number of SUMO-2 target proteins that can be identified. A similar approach can be used to detect cell type-specific factors that are SUMO-2 targets by expressing the His$_{8}$-SUMO-2 molecule in other cell lines.

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**REFERENCES**

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