Modification of Ran GTP-activating Protein by the Small Ubiquitin-related Modifier SUMO-1 Requires Ubc9, an E2-type Ubiquitin-conjugating Enzyme Homologue*

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The transport of selected proteins and nucleic acids across the nuclear pore complex (NPC)† is regulated by the small GTPase Ran/TC4 (4, 5). Ran alternates between a GTP-bound state and a GDP-bound state, a transition facilitated by a nuclear GTP-exchange factor (RCC1) and a cytoplasmic GTP-activating protein (RanGAP1) (reviewed in Refs. 6 and 7). The differential binding of Ran-GTP and Ran-GDP to cargo proteins (e.g. importins, transportin, and NTF2) and NPC proteins provides a mechanism that allows Ran to regulate the transport of cargo across the nuclear pore complex. In this system, the subcellular localization of RCC1 and RanGAP1 are major determinants of directed nuclear transport.

The localization of RanGAP1 to the nuclear pore complex is imparted by its specific association with Nup358, a component of the cytoplasmic fibrils emanating from the nuclear pore complex (1, 2). In Xenopus egg extracts, Nup358 can be found in a complex with Ubc9 (3), a structural homologue of the E2-type ubiquitin-conjugating enzyme (Ubc9). Here we show that a subset of the human homologue of Ubc9 (HsUbc9) colocalizes with RanGAP1 at the nuclear envelope. HsUbc9 forms thiolester conjugates with recombinant SUMO-1, but not with recombinant ubiquitin, indicating that it is functionally distinct from E2-type UBCs. Finally, HsUbc9 is required for the modification of RanGAP1 by SUMO-1. These results suggest that HsUbc9 is a component of a novel enzymatic cascade that modifies RanGAP1, and possibly other substrates, with SUMO-1.

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† The abbreviations used are: NPC, nuclear pore complex; RanGAP1, Ran GTP-activating protein; SUMO-1, small ubiquitin-related modifier; Ubc, ubiquitin-conjugating enzyme; NE, nuclear envelope; GST, glutathione S-transferase; HsUbc9, human homologue of Ubc9; PBS, phosphate-buffered saline; RIPA, radioimmuneprecipitation buffer; PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol; BSA, bovine serum albumin.

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MATERIALS AND METHODS

Preparation of Affinity Purified Antibodies—Rabbit antiserum raised against Escherichia coli-derived recombinant HsUbc9 was affinity purified by passage over a CNBr-activated Sepharose B matrix coupled to E. coli-derived recombinant HsUbc9 at a final antigen concentration of ~2–5 mg/ml, using methods described (14). Monoclonal antibodies to RanGAP1 (19C7) and SUMO-1 (21C7) were previously described (2). Rabbit anti-BSA antibody preparations were obtained from Calbiochem (San Diego, CA), and IgGs were purified by chromatography on protein G-Sepharose.

Immunofluorescence Cell Microscopy—HeLa cells were plated on coverslips and cultured for 2 days in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. When the culture reached 50–75% confluence, the cells were fixed in 2% paraformaldehyde for 10 min at room temperature and methanol at −20 °C for 10 min. The fixed cells were blocked for 1 h in 5% normal goat serum in PBS at room temperature and then incubated for 1 h at room temperature with a 1:3000 dilution of 1 mg/ml affinity purified polyclonal antisera against HsUbc9 or with a 1:2000 dilution of monoclonal antibodies raised against RanGAP1 (19C7) in 2.5% normal goat serum. Cells were washed in PBS and then incubated with goat anti-rabbit IgG-rhodamine or with goat anti-mouse IgG-fluorescein diluted 1:400 in 2.5% normal goat serum. Cells were washed in PBS and mounted for immunofluorescence microscopy.

Preparation of Cell Extracts and Immunoprecipitation—Rat liver subcellular fractions and nuclear envelopes (NEs) were prepared as described (15). For immunoprecipitations, NEs were solubilized at 100 A260 μg/ml in RIPA buffer (50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 1% Nonidet P40, 0.5% deoxycholate, 0.1% SDS, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 1 μg/ml each of leupeptin, pepstatin A, and aprotinin) for 2 h on ice and centrifuged at 100,000 × g for 30 min. 10-μg antibodies prebound to 5-μl protein A-Sepharocider beads (Pierce) were added per 100 μg solubilized NEs and incubated overnight on ice. After three washes in RIPA buffer, antigen-antibody complexes were solubilized in SDS-PAGE loading buffer.
To prepare cell extracts for the RanGAP1 modification assay, ~1 × 10^6 HeLa cells were washed and resuspended in 700 μl of buffer A (20 mM HEPES, pH 7.3, 110 mM potassium acetate, 5 mM sodium acetate, 2 mM magnesium acetate, 1 mM EGTA, 2 mM DTT) containing 100 μg/ml digitonin, 1 mM phenylmethylsulfonyl fluoride, and 1 μg/ml aprotinin and left on ice for 15 min. Cells were centrifuged at 13,000 rpm for 15 min at 4 °C, and the supernatant was transferred to a fresh tube.

**In Vitro Translation and SUMO-1 Conjugation—**HsUbc9 cDNA was cloned into the pSP65 expression vector. Mutants of HsUbc9 cDNA were generated by PCR-mediated site-directed mutagenesis (14). Linearized plasmids were transcribed in vitro using SP6 polymerase, and newly synthesized RNAs were briefly treated with DNase. RNAs were translated in vitro using rabbit reticulocyte lysate (Promega) and [35S]methionine (10 mCi/ml). In vitro translated proteins were tested for their ability to form thioester intermediates with ubiquitin (Sigma) or recombinant GST-SUMO-1. GST-SUMO-1 (SUMO1ΔC4) was synthesized with the four carboxyl-terminal amino acids (HSTV) deleted, exposing two glycines at the carboxyl terminus (15). To test for SUMO-1 conjugating activity, in vitro translated HsUbc9 was incubated with ~0.5 μg of recombinant SUMO-1 or ubiquitin in reaction buffer (50 mM Tris, pH 7.6, 5 mM MgCl₂, 0.1 mM DTT, 4 mM ATP) for 1 h at 25 °C. For the ATP depletion experiment, 10 units of hexokinase (Sigma) and 5 mM glucose were added to, and ATP omitted from, the reaction. After completion of the conjugation reaction, 5 mM dithiothreitol was added to the sample buffer were added to the indicated samples, and the sample was boiled for 5 min. Samples which were not treated with dithiothreitol were incubated at room temperature for 20 min after addition of sample buffer. Reaction products were analyzed by SDS-PAGE followed by autoradiography.

Expression vectors encoding wild-type and mutant RanGAP1 constructs (16) were linearized and transcribed in vitro using T7 polymerase, and transcripts were translated using a rabbit reticulocyte lysate system as above. To create a RanGAP1 mutant that is unable to form a conjugate with SUMO-1, the codon corresponding to lysine at position 526 was mutated to encode arginine. The K526R (Lys→Arg) RanGAP1 conjugate with SUMO-1, the codon corresponding to lysine at position 526 was mutated to encode arginine. The K526R (Lys→Arg) RanGAP1 may be a substrate for SUMO-1 modification by HsUbc9. Furthermore, the similar localization of RanGAP1 and Ubc9 in the nuclear envelope indicates that more of the protein resides in the nucleus than is indicated by immunoblot analysis, it is possible that some of the Ubc9 diffuses out of the nucleus during the subcellular fractionation due to its small size (18 kDa). DNase and RNase digestion of the nuclei, which releases many nuclear components into the supernatant, failed to liberate Ubc9 (Fig. 2A, lane 6), indicating that a significant fraction of the nuclear Ubc9 is associated with the nuclear envelope. The insoluble nuclear envelope fraction that remained after two nuclease digests of nuclei also contained Ubc9 (Fig. 2A, lane 7). The nuclear envelope fraction represents ~0.3% of the total protein, yet contains an estimated 5–10% of the total Ubc9. This indicates that Ubc9 is concentrated at the nuclear envelope and is consistent with the immunofluorescence data shown in Fig. 1.

To test if RanGAP1 associates with Ubc9 at the NPC, the nuclear envelope fraction was solubilized and immunoprecipitated using protein A-Sepharcl in the absence or presence of antibodies reactive with BSA (as a specificity control), RanGAP1, or Ubc9. Immunoprecipitates were separated on SDS-polyacrylamide gels, transferred to nitrocellulose, and blotted with antibodies reactive with RanGAP1 and Ubc9 (Fig. 2B, bottom panel). Immunoprecipitates prepared using antibodies reactive with RanGAP1 included both Ubc9 (Fig. 2B, lane 3, top panel) and RanGAP1 (Fig. 2B, lane 3, bottom panel), neither of which were detected in control precipitates (Fig. 2B, lanes 1 and 2, top and bottom panels). The two isoforms of RanGAP1 were previously shown to represent unmodified and SUMO-1 modified RanGAP1 (1, 2). In addition, RanGAP1 was present in immunoprecipitates prepared using antibodies reactive with Ubc9 (Fig. 2B, lane 7). Comparison of immunoprecipitates and supernatants after immunodepletion using control antibodies or antibodies reactive with RanGAP1 revealed that similar fractions of RanGAP1 and Ubc9 were depleted by the antibodies (Fig. 2B, lanes 1–6). Taken together, these data indicate that most, if not all, Ubc9 found at the nuclear envelope is present in a complex with RanGAP1.

**FIG. 1. Localization of HsUbc9 and RanGAP1 at the nuclear envelope.** HeLa cells were fixed and stained with antibodies reactive against HsUbc9 or RanGAP1 and analyzed by immunofluorescence microscopy. A, HsUbc9; B, RanGAP1; bar, 10 μm.
addition of SUMO-1 to specific substrates, it should form a thiolester conjugate. In vitro, recombinant HsUbc9, consistent with the formation of a HsUbc9:GST-SUMO-1 conjugate. Just as the formation of E2-type UBC:ubiquitin conjugates is ATP-dependent, the putative HsUbc9:SUMO-1 conjugate was not observed in lysates depleted of ATP by hexokinase treatment (Fig. 3A, lane 7). Just as the thiolester bond between E2-type UBCs and ubiquitin is disrupted in the presence of reducing agents, the putative HsUbc9:SUMO-1 conjugate was not observed in samples treated with dithiothreitol (Fig. 3A, lane 8). Interestingly, both ATP depletion and dithiothreitol treatment also abolished the presence of the 36-kDa HsUbc9 species (Fig. 3A, lanes 7 and 8), suggesting that this species represents a thiolester intermediate between HsUbc9 and endogenous SUMO-1. The apparent increase in the intensity of the 36-kDa band in the presence of ubiquitin (Fig. 3A, lane 5) may be due to a contaminant in the ubiquitin preparation used in the experiment, as the overall background signal is higher in those samples supplemented with ubiquitin (Fig. 3A, lanes 2 and 5).

Sequence comparison of HsUbc9 with known E2-type UBCs suggests that the cysteine residue at position 93 is the active site for thiolester conjugation (19), a conclusion that is supported by genetic reconstitution analysis of Ubc9 mutants (12, 13). This prediction was tested by comparing the electrophoretic migration of [35S]labeled recombinant wild-type (W) or mutant (M) HsUbc9 translated in reticulocyte lysates supplemented with buffer control (Fig. 3B, panel a), GST-SUMO-1 (Fig. 3B, panel b), or ubiquitin (Fig. 3B, panel c). The mutant HsUbc9 contains a cysteine to alanine substitution at position 93. Whereas the addition of ubiquitin had no effect on the

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**Fig. 2.** A fraction of Ubc9 is present in isolated nuclear envelopes and co-immunoprecipitates with RanGAP1. A, subcellular localization of Ubc9. Subcellular fractions of rat liver (equivalent to \( 1 \times 10^8 \) nuclei) were resolved on a 15% SDS-polyacrylamide gel and immunoblotted with anti-Ubc9 antibodies. Lane 1, total homogenate of rat liver (29 \( \mu \)g of protein); lane 2, pellet of first low speed centrifugation (11 \( \mu \)g of protein); lane 3, supernatant of first low speed centrifugation (18 \( \mu \)g of protein); lane 4, cytosolic fraction is the supernatant of high speed centrifugation of SN1 fraction (17 \( \mu \)g of protein); lane 5, purified nuclei (0.6 \( \mu \)g of protein); lane 6, DNase SUP is the supernatant of the first nuclease digest of the purified nuclei (0.55 \( \mu \)g); and lane 7, NE is the final nuclear envelope fraction (0.09 \( \mu \)g of protein). B, co-immunoprecipitation of Ubc9 and RanGAP1. NEs were solubilized in RIPA buffer and incubated overnight with anti-BSA antibodies (lanes 2 and 5), RanGAP1 antibodies (lanes 3 and 6), or anti-Ubc9 antibodies (lane 7) preadsorbed to protein A beads or with protein A beads only (lanes 1 and 4). Equivalent amounts of antibody-antigen complexes (lanes 1–3 and 7) and the immunodepleted supernatants (lanes 4–6) were separated on a 15% SDS-polyacrylamide gel and immunoblotted with 1.5 \( \mu \)g/ml anti-Ubc9 antibodies (top panel) or separated on an 8% SDS-polyacrylamide gel and immunoblotted with 0.5 \( \mu \)g/ml anti-RanGAP antibodies (bottom panels). *, IgG heavy and light chains.
migration of wild-type HsUbc9 (Fig. 3B, panel c), the addition of GST-SUMO-1 resulted in the appearance of a labeled protein migrating with a molecular mass of \(-75 \text{ kDa}\), consistent with the formation of a HsUbc9-GST-SUMO-1 conjugate (Fig. 3B, panel b, W). Substitution of cysteine with alanine at position 93 of HsUbc9 abolished its ability to form thiolester intermediates with GST-SUMO-1 (Fig. 3B, panel b, M), suggesting that the active site for thiolester conjugation to both SUMO-1 and ubiquitin are conserved between members of this structural family. Moreover, the 36-kDa species present in wild-type HsUbc9 translation reactions is not formed when mutant HsUbc9 is synthesized \textit{in vitro}, indicating that newly synthesized wild-type HsUbc9 but not mutant HsUbc9 is able to utilize SUMO-1 found endogenously in the rabbit reticulocyte lysate (Fig. 3B, panel A).

We used a similar approach to compare the modification of \textit{in vitro} translated wild-type and mutant RanGAP1 with SUMO-1 (Fig. 4). \textit{In vitro} translation of wild-type RanGAP1 produced the full-length recombinant protein (\(-70 \text{ kDa}\)) as well as the RanGAP1-SUMO-1 conjugate (migrating at \(-90 \text{ kDa}\)) (Fig. 4, lane 3; Ref. 16). The 90-kDa species was not observed when a K526R RanGAP1 mutant (Lys \rightarrow Arg mutant), which is incapable of conjugating to SUMO-1, was translated \textit{in vitro} (Fig. 4, lane 1; Ref. 16). The conversion of the unmodified form of RanGAP1 to the SUMO-1 conjugated from rabbit reticulocyte lysates was incomplete (Fig. 4, lane 3). We therefore added HeLa cell extracts to \textit{in vitro} translated RanGAP1, which was previously shown to contain all the necessary components for the modification reaction (1). The addition of HeLa cell extracts to the \textit{in vitro} translation system facilitated the conversion of RanGAP1 to the RanGAP1-SUMO-1 conjugate (Fig. 4, lane 4), an effect that was not observed with the Lys \rightarrow Arg mutant (Fig. 4, lane 2). To determine whether HsUbc9 can catalyze the conjugation of RanGAP1 with SUMO-1, HeLa cell extracts were immunodepleted using pre-imune control sera (Fig. 4, lane 10) or antibodies reactive with HsUbc9 (Fig. 4, lanes 5–7) or SUMO-1 (Fig. 4, lanes 8 and 9). Immunodepletion of either HsUbc9 (Fig. 4, lane 5) or SUMO-1 (Fig. 4, lane 8) specifically prevented the ability of HeLa extracts to support the conjugation of RanGAP1 with SUMO-1. Importantly, recombinant HsUbc9 (Fig. 4, lane 6), but not HsUbcH5 (Fig. 4, lane 7) reconstituted the conjugation reaction in HsUbc9-depleted extracts. Similarly, recombinant SUMO-1 (HISTV deletion mutant) reconstituted the conjugation reaction in SUMO-1-depleted extracts (Fig. 4, lane 9). These results strongly support the requirement for HsUbc9 in the covalent conjugation of RanGAP1 with SUMO-1.

**FIG. 4.** Modification of RanGAP1 requires HsUbc9 activity. HeLa cell extracts were immunodepleted with antibodies against HsUbc9 or SUMO-1 prior to use in \textit{in vitro} modification assay. \textsuperscript{3}S-labeled RanGAP1 translated \textit{in vitro} in rabbit reticulocyte lysate was incubated with HeLa cell extracts for 10 min at room temperature and resolved on a 6% SDS-polyacrylamide gel and transferred to nitrocellulose prior to autoradiography. Lane 1, mutant (K526R) RanGAP1, no extract; lane 2, mutant RanGAP1 + HeLa extract; lane 3, wild-type RanGAP1, no extract; lane 4, wild-type RanGAP1 + HeLa extract; lane 5, wild-type RanGAP1 + HeLa extract, HsUbc9 depleted; lane 6, wild-type RanGAP1 + HeLa extract, HsUbc9 depleted + recombinant HsUbc9; lane 7, wild-type RanGAP1 + HeLa extract, HsUbc9 depleted + recombinant HsUbcH5; lane 8, wild-type RanGAP1 + HeLa extract, SUMO-1 depleted; lane 9, wild-type RanGAP1 + HeLa extract, SUMO-1 depleted + recombinant SUMO-1; and lane 10, wild-type RanGAP1 + HeLa extract, control depleted.

**DISCUSSION**

Our results suggest that while much of the total HsUbc9 present in the cell is freely soluble in the nucleus and cytoplasm, a significant fraction of HsUbc9 is also associated with the nuclear envelope, most likely as a part of a complex that includes RanGAP1. More importantly, we have shown that HsUbc9, which was previously thought to be an E2-type ubiquitin-conjugating enzyme, is required for the covalent addition of the ubiquitin-related molecule SUMO-1 to RanGAP1. Because we were unable to demonstrate the formation of HsUbc9-ubiquitin intermediates \textit{in vitro}, it is likely that HsUbc9 is a dedicated SUMO-1-conjugating enzyme and is the first characterized enzyme in this novel pathway. We initially identified HsUbc9 as a protein capable of interacting with the RRM type RNA-binding proteins TIA-1 and TIAR (20) in a yeast two-hybrid screen. In recent years, several other proteins capable of interacting with Ubc9 in the yeast two-hybrid system have also been reported (21–23). Proteins shown to interact with both Ubc9 and SUMO-1 include the cytoplasmic tail of Fas/APO-1, nuclear PML protein, and Rad52 (24–26). At present, it is unclear if HsUbc9 mediates the SUMO-1 modification of these other proteins or what effect SUMO-1 modification of these other proteins might have. Unlike ubiquitin modification, SUMO-1 modification of RanGAP1 is clearly not a signal for degradation. Rather, SUMO-1 modification of RanGAP1 has been shown to be a prerequisite for the interaction of RanGAP1 with Nup358 (1). SUMO-1 modification may therefore be a general means of regulating protein-protein interaction, function, and/or localization. For example, overexpression of SUMO-1 was shown to protect cells from Fas ligand-induced cell death, possibly by preventing the association of accessory molecules with the “death domain” of the SUMO-1 modified cytoplasmic tail of Fas (24). The majority of SUMO-1 modified proteins are localized to the nucleus (27). The localization of HsUbc9 and SUMO-1 (1, 2) to the nucleus suggests that the SUMO-1 modification machinery may preferentially target nuclear proteins. It is interesting to note that many of the proteins identified as HsUbc9-interacting proteins in yeast two-hybrid screens are also nuclear proteins (20–23, 25, 26); moreover, HsUbc9 has also been localized to the synaptonemal complex of meiotic chromatin (23). This compartmentalization may ensure that certain interactions occur only in the nucleus. The localization of a subset of HsUbc9 to the periphery of the nuclear envelope may be due to its interaction with RanGAP1, SUMO-1, or Nup358.

In yeast, Ubc9 is required for viability (10, 11). Repression of UBC9 synthesis prevents cell cycle progression at the G\(_2\) or early M phase, and in ubc9 mutants, both CLB5, an S phase cyclin, and CLB2, an M phase cyclin, are stabilized (10). These findings led to the suggestion that Ubc9 is directly involved in the ubiquitin-mediated degradation of mitotic cyclins (10, 11). However, our data as well as the findings of others indicate that Ubc9 does not function in ubiquitination but rather is required for modification of proteins with SUMO-1 in mammalian cells and with the SUMO-1 homologue Smt3p in yeast (28). We propose that Ubc9 function may therefore be required for regulating other nuclear events, including Ran-mediated nucleocytoplasmic transport. For example, mutations that abrogate Ubc9 activity in vivo may prevent the Ran-mediated import of factors into the nucleus, which are necessary to initiate specific events associated with the G\(_2\)/M progression and/or
mitosis. Because the B-type cyclins are degraded at the onset of anaphase (29), mutations which act downstream during the cell cycle may appear to result in the stabilization of these cyclins and in the delay of G2/M progression. Although yeast Ubc9p was recently shown to form thiolester intermediates with Smt3p (28), Rna1p, the yeast homologue of RanGAP1, is apparently not modified with Smt3p (30, 31). It therefore remains to be seen whether Smt3 is indeed required for nuclear import in yeast. However, other putative Smt3p-modified nuclear proteins may also play a direct or indirect role in influencing cell cycle progression and cyclin degradation. The identification of HsUbc9 as a component of the SUMO-1 conjugation pathway is therefore an important step in elucidating not only the function of this novel pathway, but also the means by which the activities of the substrates of SUMO-1/Smt3p modification may be regulated and the effect that SUMO-1/Smt3p modification may have on cellular function.

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