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The Nucleoporin RanBP2 Has SUMO1 E3 Ligase Activity

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Summary

Posttranslational modification with SUMO1 regulates protein/protein interactions, localization, and stability. SUMOylation requires the E1 enzyme Aos1/Uba2 and the E2 enzyme Ubc9. A family of E3-like factors, PIAS proteins, was discovered recently. Here we show that the nucleoporin RanBP2/Nup358 also has SUMO1 E3-like activity. RanBP2 directly interacts with the E2 enzyme Ubc9 and strongly enhances SUMO1-transfer from Ubc9 to the SUMO1 target Sp100. The E3-like activity is contained within a 33 kDa domain of RanBP2 that lacks RING finger motifs and does not resemble PIAS family proteins. Our findings place SUMOylation at the cytoplasmic filaments of the NPC and suggest that, at least for some substrates, modification and nuclear import are linked events.

Introduction

SUMO1 (small ubiquitin-related modifier, also known as Pic1, Ubl1, hSmt3, or sentrin) is only 18% identical to ubiquitin but resembles its structure, its ability to be reversibly ligated to other proteins, and its mechanism of ligation. More than 30 proteins from different species have been identified as SUMOylation substrates, and available data provide compelling evidence for a role of SUMO1 in the regulation of protein-protein interactions, subcellular localization, and stability (reviewed by Hay, 2001; Melchior, 2000; Müller et al., 2001). Like ubiquitin, SUMO1 is attached to targets via an isopeptide bond between the C terminus of SUMO1 and the ϵ aminogroup of target lysine residues. Ubiquitination of a specific target requires three enzymes: an E1 activating enzyme, an E2 conjugating enzyme, and an E3 ligating enzyme (Hershko and Ciechanover, 1998). Only a single ubiquitin E1 has been identified, but multiple E2 and E3 enzymes are known. E3 ligases confer substrate specificity and are highly regulated to ensure that target degradation occurs only at the appropriate time (reviewed in Jackson et al., 2000; Joazeiro and Weissman, 2000). For SUMO1 modification, a single E1 SUMO1 activating enzyme (Aos1/Uba2) and a single E2 conjugating enzyme (Ubc9) have been identified in yeast and higher eukaryotes. In vitro, these are sufficient to modify a number of SUMO1 targets, including IκBα, RanGAP1, and p53, and it was proposed that SUMO1 modification would not require E3 ligases (references in Hay, 2001; Melchior, 2000; Müller et al., 2001). However, recently several SUMO E3-like factors were identified in yeast and mammalian cells (Johnson and Gupta, 2001; Kahyo et al., 2001; Sachdev et al., 2001; Takahashi et al., 2001). These proteins are different members of one family, the protein inhibitors of activated STAT (PIAS). *Saccharomyces cerevisiae* Siz1 is involved in septin modification, PIAS1 stimulates p53 modification, and PIASy enhances modification of Lef1.

Based on immunofluorescence studies, both subunits of the SUMO1 E1 activating enzyme reside predominantly in the nucleus (Azuma et al., 2001; Rodriguez et al., 2001). In addition, Ubc9 has been found in a complex with SUMO1-modified RanGAP1 and RanBP2 (Lee et al., 1998; Saitoh et al., 1997). Both proteins are components of the nucleocytoplasmic transport machinery (reviewed in Görlich and Kutay, 1999) and are localized to cytoplasmic filaments of nuclear pore complexes (NPCs).

Interestingly, in vivo SUMOylation of specific SUMO1 targets (Sternsdorf et al., 1999) as well as of an artificial reporter protein (Rodriguez et al., 2001) requires the presence of an intact nuclear localization signal (NLS). This NLS dependency, in conjunction with enzyme localization, has led to the common belief that SUMO1 targets need to enter the nucleus prior to their modification. However, both mamalian RanGAP1 and yeast septins are restricted to the cytoplasmic compartment and are efficient SUMO1 targets in vivo (Mahajan et al., 1997; Matunis et al., 1996; Johnson and Blobel, 1999).

This raises the question of why other targets need an NLS for steady-state modification in vivo. We could envisage three equally likely possibilities. First, SUMO1 modification of some targets may be restricted to the nuclear compartment due to the localization of their specific E3 ligases. Second, SUMO1 modification of other targets may take place in the cytoplasm, but they need to enter the nucleus in order to be protected from isopeptidases. Third, SUMO1 modification may depend on components of the nuclear import machinery.

Experiments aimed at understanding SUMO1's intranuclear localization led us to the discovery that RanBP2 has an E3-like activity in the modification of proteins with SUMO1. As RanBP2 is part of the cytoplasmic filaments of the NPC and serves as a docking site for import complexes (Ben-Efraim and Gerace, 2001; Wu et al., 1995; Yaseen and Blobel, 1999; Yokoyama et al., 1995), this suggests that NLS-containing targets for SUMO1 modification can be modified en route to the nucleus.

Results

YFP-SUMO1 Is Intranuclear In Vivo But Accumulates at the NPC In Vitro

If SUMO1 modification of intranuclear targets was restricted to the nuclear compartment, SUMO1 would en-

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ter the nucleus only in unconjugated form. Owing to the small size of SUMO1 (10 kDa), this could occur by active import or by passive diffusion. Alternatively, if SUMO1 modification can also precede nuclear import, an energy-dependent mechanism would have to contribute to SUMO1 intranuclear accumulation, because most target proteins are too large to enter by diffusion. To test this, we used three experimental approaches: first, transfection of wt and mutant SUMO1 lacking its C-terminal Gly Gly motif; second, microinjection of wt SUMO1 into HeLa cells with or without prior ATP depletion; and third, in vitro nuclear import with digitonin-permeabilized HeLa cells. For all three experiments, we chose the same reporter protein, SUMO1 fused to YFP. GFP-SUMO can replace endogeneous SUMO (pmt3) in fission yeast (Tanaka et al., 1999), indicating that a GFP-tag or the related YFP-tag does not interfere with SUMO1 function in vivo. As shown in Figure 1A, wt YFP-SUMO1 localizes exclusively in the nucleus after transfection, both diffusely distributed in the nucleoplasm and concentrated in nuclear speckles. In contrast, YFP-SUMO1 (1–95) was diffusely distributed throughout the cytoplasm and the nucleus, remarkably similarly to the localization of YFP alone. This is consistent with previous findings with HA-tagged wt and mutant SUMO1 (Mahajan et al., 1998). Immunoblotting (Figure 1B) indicated that a significant proportion of YFP-SUMO1 (1–97) was not conjugated to targets at steady-state levels. These findings suggest that an active process concentrates unconjugated YFP-SUMO1 (1–97) but not YFP-SUMO1 (1–95) in the nucleus. One model consistent with this is cytoplasmic modification of YFP-SUMO1 to targets, active import of the conjugate, and subsequent cleavage by isopeptidases known to exist in the nucleus (Hay, 2001; Melchior, 2000; Müller et al., 2001). An alternative explanation, noncovalent nuclear retention requiring the Gly Gly motif in SUMO1, was ruled out by microinjection experiments. For these, we microinjected YFP-SUMO1 (1–97) into HeLa cells and followed its localization over time (Figure 1C and Supplementary Figure S1 at http://www.cell.com/cgi/content/full/108/1/109/DC1). Only when ATP was present could YFP-SUMO1 (1–97) become strongly enriched in the nucleoplasm and in speckles. When ATP was depleted, YFP-SUMO1 was distributed throughout the cells, like mutant SUMO1 upon transfection. Our hypothesis that active import may contribute to SUMO1 intranuclear accumulation (either of free SUMO1 or of conjugates) was further supported by the kinetics of intranuclear appearance of SUMO1. With ATP, an enrichment of YFP-SUMO1 (1–97) in the nucleoplasm preceded speckle formation and was observed as early as 1 min after injection. In the absence of ATP, equal distribution between nucleus and cytoplasm was observed only significantly later (Figure 1C and Supplementary Figure S1 at http://www.cell.com/cgi/content/full/108/1/109/DC1). Finally, to investigate the possibility that SUMO1 conjugation would only take place in the cytoplasm, we also injected YFP-SUMO1 into nuclei of HeLa cells. As is revealed from ATP-dependent speckle formation indicative of conjugates, modification clearly can take place in the nucleus.

Our microinjection data suggest that YFP-SUMO1 enters the nucleus by both passive diffusion and active import. These experiments do not allow us to discriminate between active import of free or conjugated SUMO1. To investigate this, we carried out in vitro nuclear import assays (Figure 2). Consistent with our hypothesis that SUMO1 conjugates may be the imported species in vivo, YFP-SUMO1 (1–97) was not actively imported into the nucleus. Instead, it acccumulated at the nuclear envelope in a pattern reminiscent of NPC staining. Similar results were obtained with FITC-labeled SUMO (see Supplementary Figure S2 at http://www.cell.com/cgi/content/full/108/1/109/DC1). This rim staining is temperature sensitive, ATP-dependent, and saturable (unlabeled SUMO1 reduces the signal by competition; Figure 2B and data not shown). Interestingly, wheat germ agglutinin, which inhibits nuclear protein import by binding to O-glycosylated NPC proteins (Finlay et al., 1987), did not significantly inhibit rim staining (FITC-BSA-NLS import was inhibited; data not shown). This suggests that YFP-SUMO1 (1–97) accumulates at the cytoplasmic rather than the nuclear side of the NPC. Based on the ATP dependence, we speculated that YFP-SUMO1 was forming isopeptide bonds with NPC-associated proteins (thioester bonds could be excluded by the resistance of the rim staining to 50 mM DTT; see Supplementary Figure S3 at http://www.cell.com/cgi/content/full/108/1/109/DC1). Indeed, rim staining correlated under all conditions with the appearance of cell-associated YFP-SUMO1 conjugates (Figure 2C). Moreover, incubation of the cells with the *S. cerevisiae* isopeptidase Ulp1 (Li and Hochstrasser, 1999) resulted in complete loss of rim staining (Figure 2D). In summary, these data suggest that accumulation of YFP-SUMO1 at the nuclear envelope is due to isopeptide bond formation with unknown proteins. This would, however, require the presence of SUMO E1 and E2 enzymes in cytosol or at the NPC. Indeed, HeLa cytosol contains significant amounts of Aos1 and Ubc9 (see Supplementary Figure S4 at http://www.cell.com/cgi/content/full/108/1/109/DC1). Therefore, we replaced the cytosol in the assay with recombinant Aos1/Uba2 and Ubc9. Both enzymes were expressed in *E. coli* and purified to near homogeneity (Figure 2E). Addition of these enzymes together with YFP-SUMO1 (1–97) and ATP is sufficient to generate strong rim staining in semipermeable cells (Figure 2F), indicating that the modification does not require factors provided by the cytosol other than the E1 and E2 enzyme.

SUMO1 Chain Formation on RanBP2

The rapid SUMO1 modification that appears to take place at NPCs suggested the presence of a stimulatory factor, perhaps a SUMO1 E3 ligase. E3 ligases involved in ubiquitination are often characterized by stable interactions with E2 enzymes (Jackson et al., 2000; Joazeiro and Weissman, 2000), and several ubiquitin E3 ligases are themselves targets for modification (Fang et al., 2000; Nuber et al., 1998). Two NPC proteins, RanGAP1 and RanBP2, are known targets for SUMOylation (Mahajan et al., 1997; Matunis et al., 1996; Saitoh et al., 1998), and SUMOylated RanGAP1, RanBP2, and Ubc9 form a stable complex in cell extracts (Lee et al., 1998; Saitoh et al., 1997, 1998). In contrast to all other known SUMO1 targets, RanGAP1 is very efficiently modified both in vivo and in cell extracts (Mahajan et al., 1997). It is

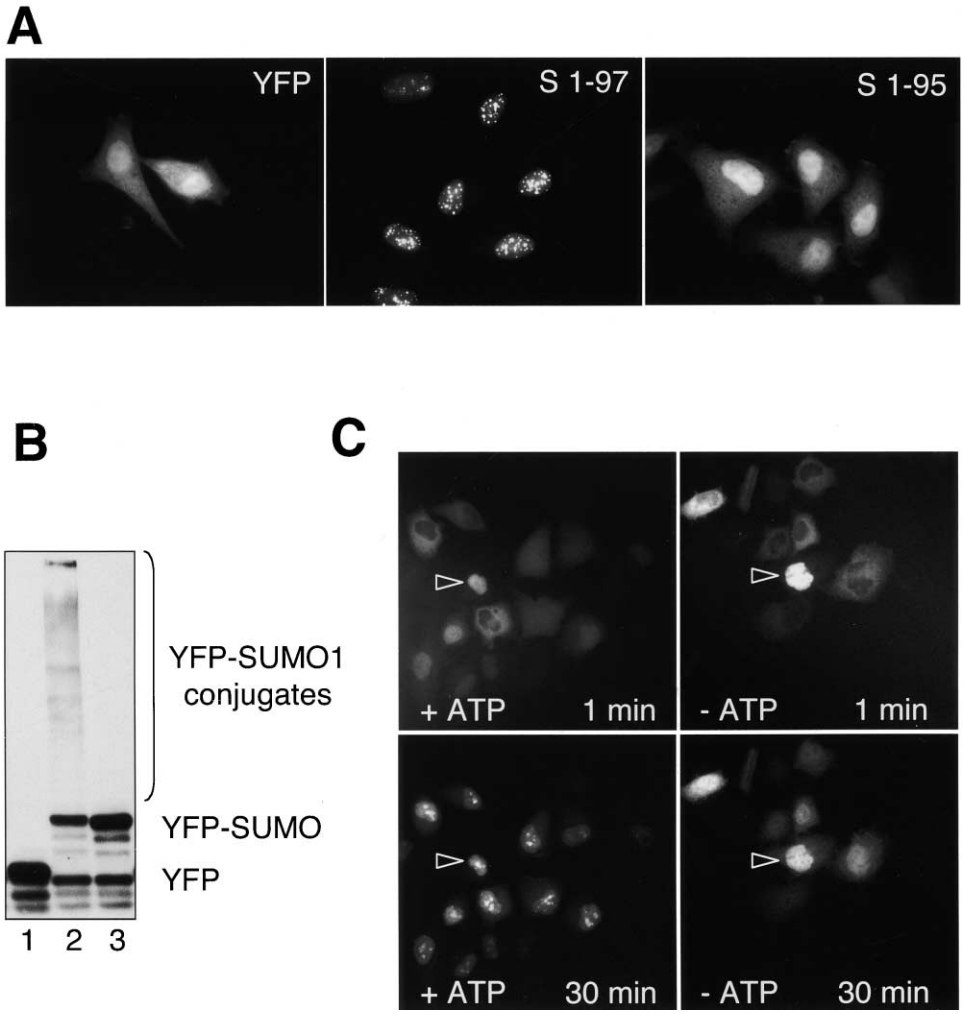

Figure 1. Passive Diffusion, Conjugation, and Active Transport Contribute to SUMO1 Intranuclear Accumulation

(A) The C terminus of SUMO1 is necessary for its accummulation in the nucleoplasm and in nuclear speckles. Adherent HeLa cells were transfected with pEYFP, pEYFP-SUMO1 (1–97), or pEYFP-SUMO1 (1–95) and fixed after 20 hr with 2% formaldehyde. YFP and YFP-fusion proteins were detected by immunofluorescence microscopy.

(B) HeLa cells transfected as in (A) were harvested after 20 hr in SDS-loading buffer, resolved on a 8% SDS gel, and analyzed by immunoblotting with α GFP antibodies.

(C) Energy depletion inhibits nuclear accumulation of YFP-SUMO1. YFP-SUMO1 (1–97) (0.4 mg/ml) was microinjected into HeLa cells with or without prior depletion of ATP. Pictures were taken at indicated time points (injections were all done within times 0 min and 1 min). Arrowheads mark nuclear injection.

also rapidly and quantitatively modified by recombinant Aos1/Uba2 and Ubc9 (see Okuma et al., 1999, and below). To test whether RanBP2 can also be modified by Aos1/Uba2 and Ubc9 alone, we generated two fragments of RanBP2 (depicted in Figure 3A) that were reported to contain the necessary determinants for SUMO1 modification as well as interactions with SUMO1*RanGAP1 and Ubc9 (Matunis et al., 1998; Saitoh et al., 1998). During the course of this study, we found that the smaller fragment (BP2ΔFG) is sufficient for the properties described below. Figure 3B shows a time course of RanGAP1 (top) and BP2ΔFG (bottom) modification with recombinant enzymes. Both proteins are clearly modified in this reaction, with RanGAP1 being converted to a single modified species within just 5 min, and BP2ΔFG being modified somewhat more slowly at multiple sites. At 30 min, a faint higher molecular weight smear was apparent, perhaps indicative of additional conjugates. To investigate this further, we repeated the time course with an excess of enzymes and SUMO1 (Figure 3C). Under these conditions, RanGAP1 was again rapidly modified to a single species. In contrast, Gst-BP2 gave rise to multiple bands that increased in molecular weight during the time course. We infer from the molar ratios used in this reaction that each Gst-BP2 is modified by up to 25 molecules of SUMO1. As shown in Figure 3D, the extent to which RanBP2 is modified depends on the molar ratio of Gst-BP2 and SUMO1. In conclusion, RanBP2 and RanGAP1 can both be efficiently modified with recombinant E1 and E2 enzymes, but in addition, RanBP2 undergoes an unusual hypermodification that leads to depletion of SUMO1 from the

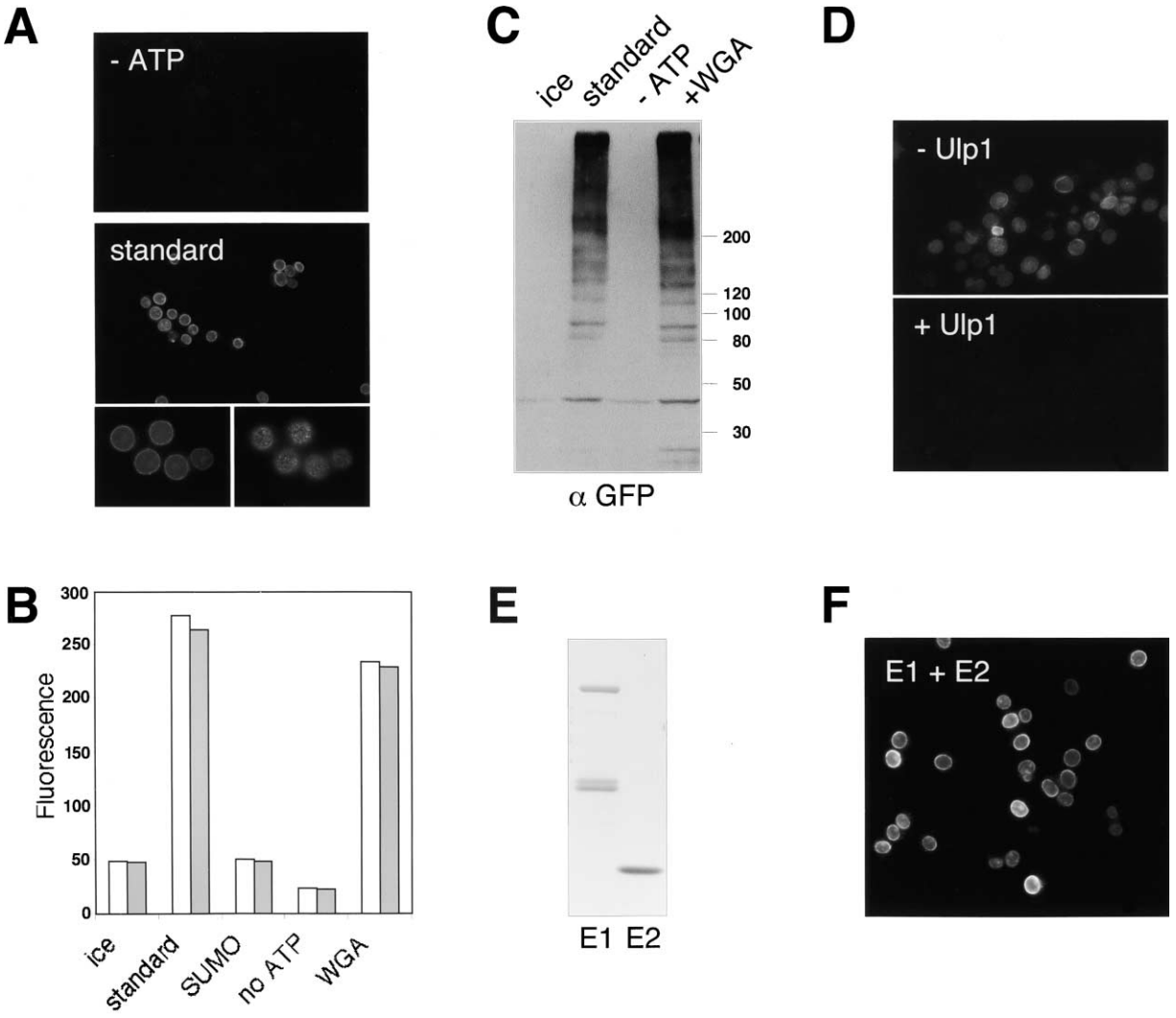

Figure 2. Covalent Modification at the NPC

(A) In vitro nuclear import assays with digitonin-permeabilized HeLa cells, cytosol, and 0.8 μg YFP-SUMO1 (1–97) were carried out in the presence (standard) or absence of ATP (addition of 16 U Hexokinase/5 mM glucose). After 30 min at 30°C, cells were washed and analyzed by immunofluorescence microscopy. Identical exposure times were used for "−ATP" and "standard" samples. Bottom: equatorial view (left) and surface view (right) of identical standard cells.

(B) In vitro reactions as in (A) but analyzed by flow cytometry. Ice, incubation at 0°C; standard, incubation at 30°C; SUMO, reaction in the presence of 2 μg nonfluorescent SUMO1 (1–97); no ATP, 16 U Hexokinase/5 mM glucose; WGA, wheat germ agglutinin (50 μg/ml final concentration) was added to inhibit transport into the nucleus. Gray and white bars indicate duplicate reactions.

(C) Reactions as in (A) but analyzed by immunoblotting. After the reaction, cells were washed twice and lysed in SDS-loading buffer. Samples were resolved on a 5%–20% SDS gel and analyzed by immunoblotting with α GFP antibodies.

(D) Standard reaction and analysis as in (A), but after washing the sample was split in half and incubated for 20 min at 37°C with or without recombinant isopeptidase (Gst-Ulp1).

(E) Recombinant SUMO1-activating enzyme (Aos1/Uba2) and SUMO1-conjugating enzyme Ubc9, resolved on a 5%–20% SDS gel and stained by Coomassie blue.

(F) Reaction and analysis as in (A), but with recombinant enzymes (18 μg/ml Aos1/Uba2 and 4 μg/ml Ubc9) instead of cytosol.

reaction mix. The high-molecular-weight RanBP2-SUMO1 conjugates could potentially form by attachment of single SUMO1 entities to multiple lysines in Gst-BP2 or could reflect the formation of SUMO1 chains via SUMO1-SUMO1 isopeptide bond formation. To distinguish between these two possibilities, we added the *S. cerevisiae* isopeptidase Ulp1 to hypermodified Gst-BP2 (Figure 3E). In one scenario (multiple attachments of single SUMO1 molecules to Gst-BP2), only monomeric SUMO1 species would be generated by Ulp1. In the second scenario (SUMO1 chains on Gst-BP2), the cleavage intermediates could also consist of SUMO1 oligomers. As is apparent from the cleavage pattern, recombinant Ulp1 rapidly generates species with apparent mobilities of ~30, ~45, and ~60 kDa, indicative of SUMO1 dimers, trimers, and tetramers, and the high-molecular-weight species disappear. This result is most consistent with the interpretation that SUMO1 forms

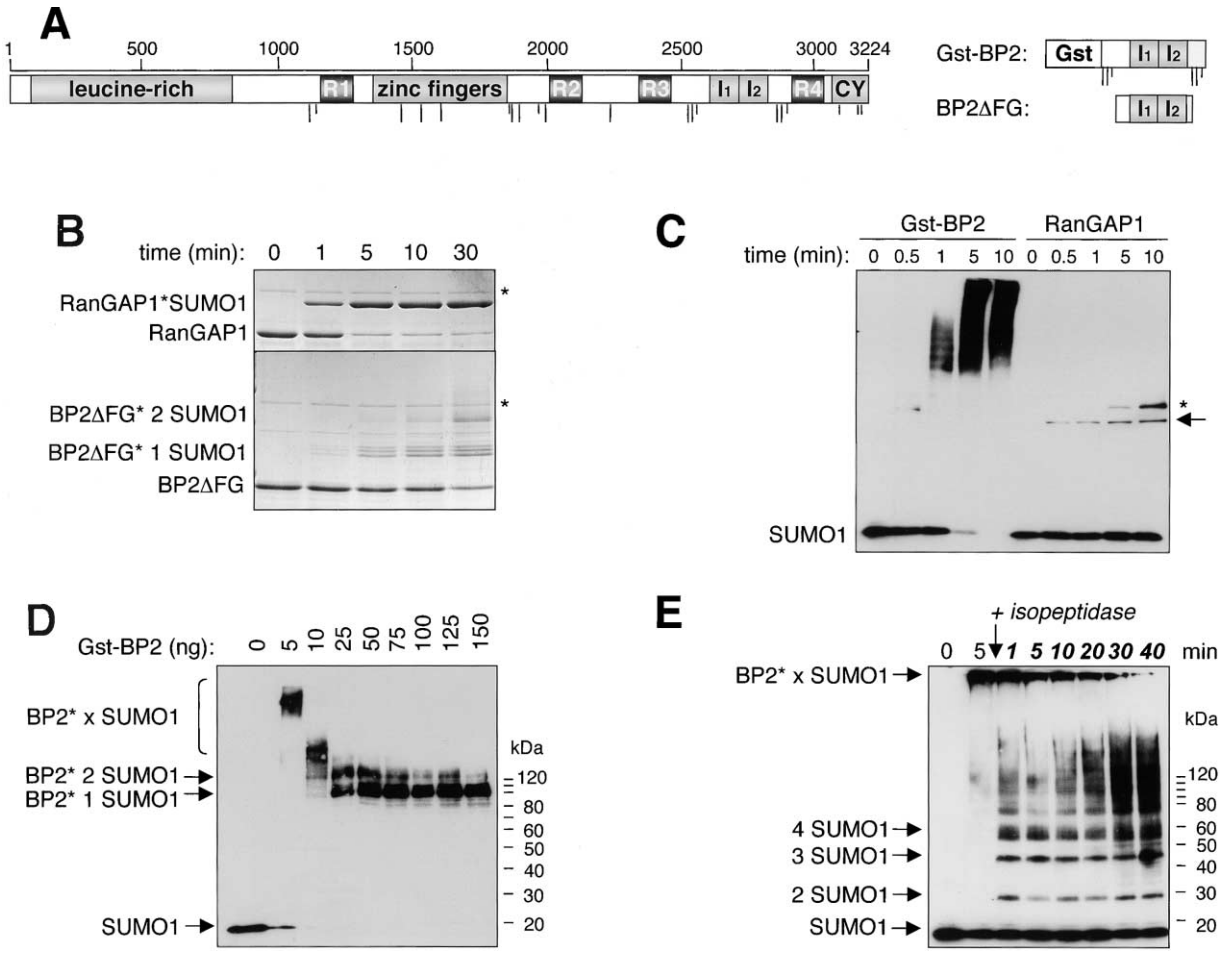

Figure 3. Hypermodification and SUMO1 Chain Formation on RanBP2

(A) Schematic diagram of RanBP2 and constructs used in this study. The 358 kDa RanBP2 contains a leucine-rich domain, a zinc finger domain, four RanGTP binding domains (R1–R4), a cyclophilin domain (CY), and several FG and FXFG repeats (indicated by short and long dashes, respectively). I_1 and I_2 indicate the presence of an internal repeat motif (Wu et al., 1995; Yokoyama et al., 1995).

(B) RanGAP1 and RanBP2 are both efficiently modified with recombinant E1 and E2 enzymes. Recombinant RanGAP1 and BP2ΔFG (500 ng each) were incubated with SUMO1 (1.5 μg), E1 (150 ng), E2 (10 ng), and 5 mM ATP at 37°C. Reactions were stopped at indicated time points with Laemmli-buffer. Proteins were separated on 5%–20% SDS-PAGE, and proteins were stained with Coomassie blue.

(C) Hypermodification of RanBP2. In vitro SUMOylation as in (B), using less substrate (5 ng Gst-BP2 or RanGAP1, 15 ng SUMO1, 150 ng E1, 10 ng E2, and 5 mM ATP) and shorter time points. Detection was by immunoblotting with α SUMO1 antibodies. The arrow indicates SUMO1-modified RanGAP1, and the asterisk indicates SUMO1-modified Uba2 (T. Büsgen, A.P., and F.M., unpublished results).

(D) Increasing amounts of Gst-BP2 (5–150 ng) were added to an otherwise constant modification mix (15 ng SUMO1, 150 ng E1, 10 ng E2, and 5 mM ATP) and incubated for 2.5 min at 37°C. Detection was by immunoblotting with α SUMO1 antibodies.

(E) Hypermodification of RanBP2 is due to SUMO1 chain formation. Gst-BP2 (5 ng) was hypermodified with 60 ng SUMO1, 150 ng E1, 10 ng E2, and 0.5 mM ATP for 5 min at 37°C. To stop the reaction, ATP was depleted with 1 U apyrase. Gst-Ulp1 isopeptidase was added and incubated for indicated time points. Reactions were separated by 5%–20% SDS-PAGE, and detection was performed using α SUMO1 antibodies.

chains on RanBP2. SUMO2 is known to form chains involving lysine 11 (Tatham et al., 2001). However, the SUMO1 chains on RanBP2 seem to be quite different, since a mutant lacking amino acids 1–20 is still competent for chain formation (M. Schergaut, A.P., and F.M., unpublished data).

Depletion of RanGAP1/RanBP2 Complexes Removes SUMOylation Activity from Cytosol

As SUMO1-SUMO1 chain formation was induced in the presence of RanBP2, we speculated that it may have an E3-like activity. To address this, we relied on our observation that addition of SUMO1 stimulates modification of endogenous proteins in HeLa cytosol. RanGAP1 and RanBP2 are both present in HeLa cytosol; RanGAP1 because it is partially cytoplasmic, and RanBP2 due to the 5% mitotic cells present in asynchronously growing cultures. If RanBP2 and/or its binding partner RanGAP1 were indeed required for SUMO1 conjugation, their immunodepletion should lead to a reduction in modification. We found that cytosol depleted with α RanGAP1 antibodies shows a significantly reduced SUMO1 modi-

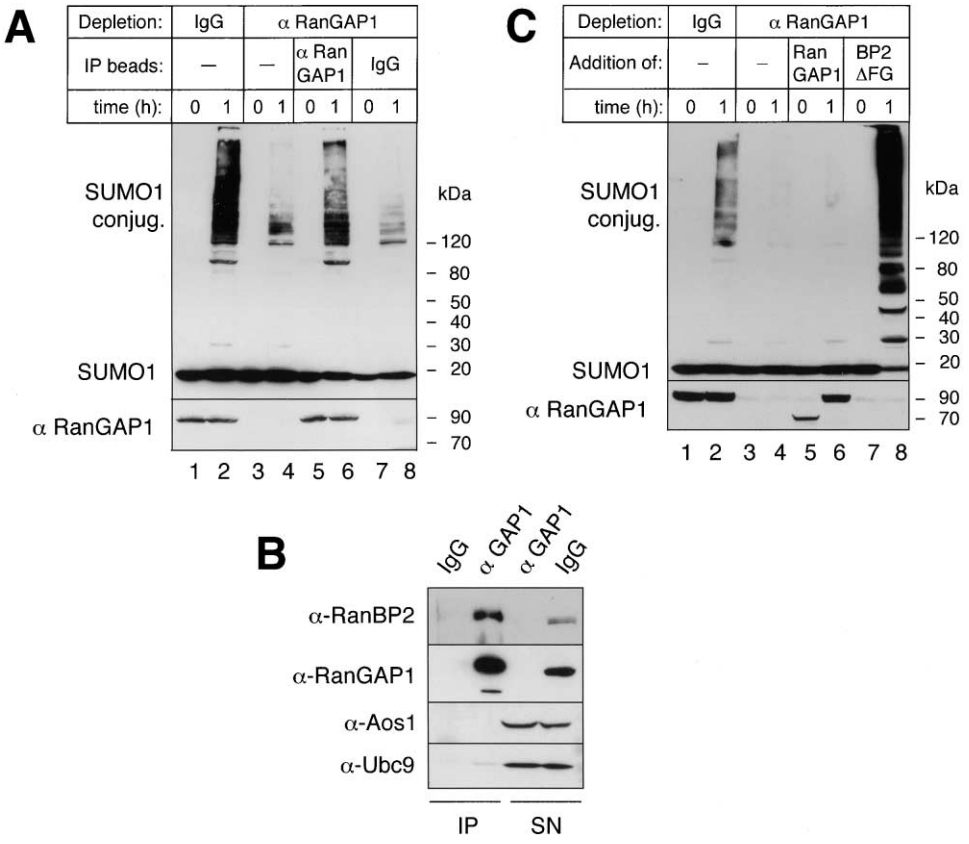

Figure 4. α RanGAP1 Antibodies Deplete a Stimulatory Activity for SUMOylation from HeLa Cytosol

(A) HeLa cytosol was incubated with α RanGAP1 antibodies or goat preimmunserum (IgG) crosslinked to protein G beads. Supernatants (30 μl) were tested for SUMOylation activity by incubation with 60 ng SUMO1 and 5 mM ATP (lanes 1–4). Samples were taken at time 0 and after 1 hr at 30°C. Lanes 5–8: α RanGAP1-depleted SN was reconstituted by adding back α RanGAP1 beads (5–6) or IgG beads (7–8) prior to the reaction. Reactions were resolved on 5%–20% SDS-PAGE and analyzed by immunoblotting with α SUMO1 (top) and α RanGAP1 antibodies (bottom).

(B) RanGAP1-depleted cytosol is also devoid of RanBP2. Beads and supernatants of the immunoprecipitation in (A) were analyzed by immunoblotting with α RanBP2, α Aos1, α Ubc9, and α RanGAP1 antibodies.

(C) Reactions were performed as in (A). Cytosol depleted with α RanGAP1 antibodies was complemented with 5 ng recombinant RanGAP1 (lanes 5–6) or 5 ng BP2ΔFG (lanes 7–8).

fication pattern compared to control cytosol (Figure 4A, lanes 2 and 4). To verify that this reduction in activity was caused by depletion rather than inactivation of factors, we added back IP beads to RanGAP1-depleted cytosol. RanGAP1 IP beads but not IgG IP beads restored most of the activity (Figure 4A, compare lanes 6 and 8). Depletion and readdition of RanGAP1 was verified by immunoblotting with α RanGAP1 antibodies (Figure 4A, bottom). As expected, removal of RanGAP1 by immunoprecipitation did result in simultaneous depletion of RanBP2, but importantly neither Ubc9 nor Aos1 levels were affected (Figure 4B). This suggested that RanGAP1, RanBP2, or an unknown associated factor could be the stimulatory activity that was depleted by the IP. We explored this possibility by adding 5 ng RanGAP1 or BP2ΔFG to our depleted cytosol (Figure 4C). RanGAP1 was quantitatively modified by the depleted extract (Figure 4C, bottom, lanes 5 and 6), confirming that E1 and E2 enzymes were still active in the extracts. However, it did not induce modification of endogenous proteins, indicating that RanGAP1 is not the stimulatory activity (Figure 4C, top, lanes 5 and 6). In contrast to RanGAP1, addition of 5 ng BP2ΔFG dramatically stimulated the appearance of SUMO1-modified bands (Figure 4C, lanes 7 and 8). However, the SUMO1 pattern induced by BP2ΔFG is more intense than, and not identical to, the pattern found in control cytosol. This is at least in part due to hypermodification of recombinant BP2ΔFG and subsequent cleavage by endogenous isopeptidases (for comparison, see Figure 3E).

RanBP2 Stimulates SUMO1 Modification of Sp100

To fully establish a role for RanBP2 as a stimulator of SUMO1 modification, we wanted to test its activity with a well-characterized SUMO1 target. For this we chose Sp100, a component of PML nuclear bodies (Sternsdorf et al., 1999). This protein seemed an ideal candidate for RanBP2-stimulated SUMOylation. It contains a classical NLS, suggesting that it encounters RanBP2 on its way into the nucleus. Importantly, this NLS is essential for modification of Sp100 at lysine 297 in vivo (Sternsdorf et al., 1999). We set up an in vitro modification assay for Sp100 with recombinant E1 and E2 and tested the effect of BP2ΔFG or full-length RanBP2 from HeLa cells

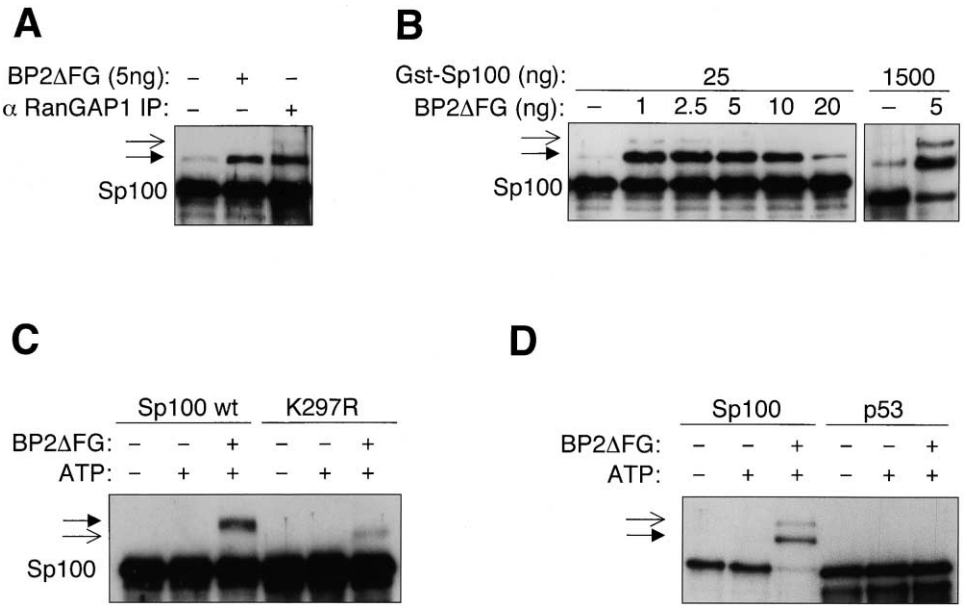

Figure 5. RanBP2 Stimulates In Vitro Modification of the SUMO1 Target Sp100 But Not p53

(A) Both BP2ΔFG and full-length HeLa RanBP2 stimulate SUMOylation of Sp100. In vitro reactions containing 25 ng Gst-Sp100ΔN, 150 ng E1, 10 ng E2, 60 ng SUMO1, and 5 mM ATP in 20 μl reaction volumes were incubated for 1 hr at 30°C in the absence or presence of 5 ng BP2ΔFG or the RanBP2-containing α RanGAP1 immunoprecipitate described in Figure 4B. Samples were analyzed by immunoblotting with α Gst antibodies. The appearance of two novel bands (arrows) indicate the presence of a major and minor SUMO1 acceptor site in Sp100.

(B) Left: In vitro reactions as in (A) in the absence or presence of increasing concentrations of BP2ΔFG. Right: Reactions with 1.5 μg Sp100ΔN, 1.5 μg SUMO1, 10 ng E2, 150 ng E1, with or without 5 ng BP2ΔFG.

(C) RanBP2 stimulates modification of Sp100 at the physiological SUMO1 acceptor site. Gst-Sp100ΔN (25 ng) or Gst-Sp100ΔN K297R were incubated with 600 ng SUMO1, 10 ng E2, 150 ng E1, and 5 mM ATP for 7 min at 30°C. Residual modification of the mutant is due to the presence of a second modification site (see [A] and [B]).

(D) RanBP2 enhances in vitro SUMOylation of Sp100 but not of p53. Reactions with 1 μg Gst-Sp100ΔN or Gst-p53, 1.5 μg SUMO1, 10 ng E2, 150 ng E1, with or without 5 ng BP2ΔFG.

(provided in the form of α RanGAP1 immunoprecipitates described in Figure 4). As expected, Aos1/Uba2 and Ubc9 modify only marginal amounts of Sp100 (Figure 5A). This can be significantly increased by the addition of either BP2ΔFG or full-length RanBP2, demonstrating that RanBP2 indeed acts as a stimulator of modification. We then compared increasing amounts of BP2ΔFG for their effect on Sp100 modification (Figure 5B, left). Already sufficient for strong stimulation was 1.5 nM BP2ΔFG (1 ng BP2ΔFG per 20 μl reaction). Surprisingly, increasing the concentration of BP2ΔFG actually decreased its efficiency (compare 1 with 20 ng). One possible explanation for this inverse dose dependence is that excess BP2ΔFG competes with Sp100 for SUMO1. This was confirmed in the next experiment (Figure 5B, right), where we repeated the experiment with a large excess of Sp100 and SUMO1 (1.5 μg each of Sp100 and SUMO1, 5 ng BP2ΔFG, 10 ng Ubc9, and 150 ng Aos1/Uba2). In the presence of BP2ΔFG, Sp100 was nearly quantitatively modified. RanBP2 stimulates Sp100 modification at its physiological SUMO1 attachment site Lys 297 (Sternsdorf et al., 1999), as is shown in Figure 5C. A faint band visible in the K297R mutant migrates with different mobility than wt SUMO1*Sp100 and reflects a second, less efficient, modification site. A hallmark for E3 ligases is that they confer substrate specificity. We therefore tested the ability of RanBP2 to stimulate SUMOylation of another well-known SUMO1 target, the tumor suppressor p53. As shown in Figure 5D, under conditions that allow efficient modification of Sp100, p53 is not SUMOylated either in the absence or presence of BP2ΔFG. Our recombinant p53 is competent for modification, as PIASy stimulates p53 SUMOylation in similar experiments (A.P., S. Sachdev, R. Grossschedl, and F.M., unpublished data). We also tested four novel SUMO1 target proteins; two of these were better modified in the presence of BP2ΔFG, and two were not affected (unpublished data). In summary, RanBP2 appears to work on several but not all SUMO1 targets. How this specificity is conferred remains to be resolved.

RanBP2 Stimulates Transfer of SUMO1 between Ubc9 and Sp100

The substrate specificity of RanBP2 already suggests that it participates in the transfer of SUMO1 from the E2 enzyme to its target. However, from our in vitro data, we could not exclude the possibility that it functions predominantly by enhancing SUMO1 transfer efficiency between the E1 and the E2 enzyme. We investigated the step at which it contributes by comparing rates of Ubc9-SUMO1 thioester bond formation in the absence or presence of BP2ΔFG (Figure 6A). SUMO1-Ubc9 thioesters are formed rapidly and efficiently with or without BP2ΔFG. We reproducibly found a modest stimulation of thioester formation with BP2ΔFG, but the reason for this remains to be determined. However, the extremely

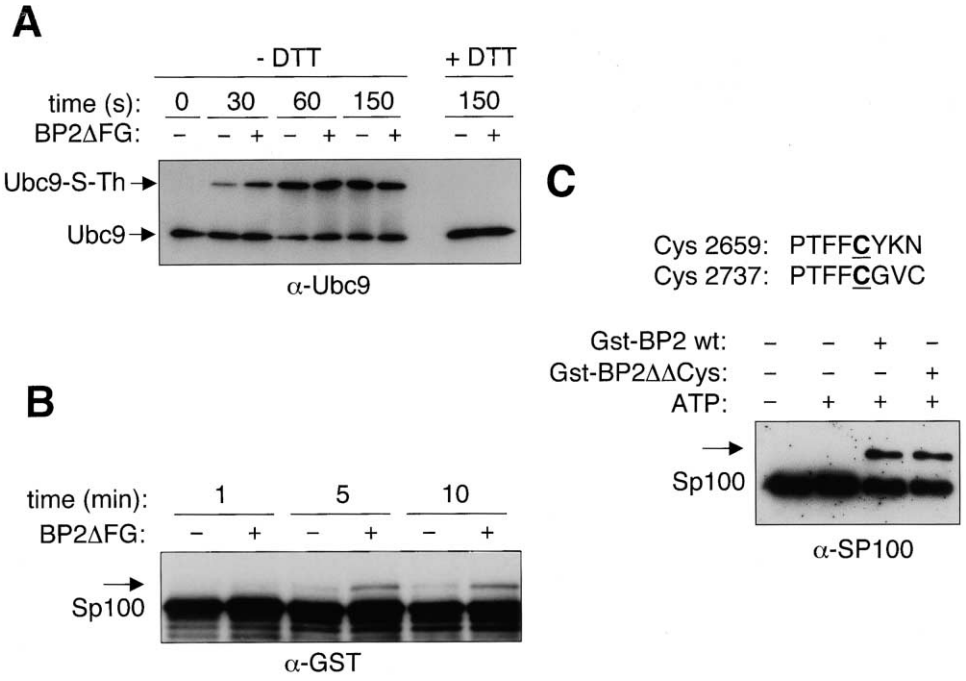

Figure 6. RanBP2 Enhances SUMO1 Transfer from Ubc9 to Sp100

(A) Ubc9-SUMO1 thioester formation in the presence or absence of RanBP2. E2 (100 ng), 150 ng E1, 100 ng SUMO1, and 5 mM ATP were incubated with or without 1 ng BP2ΔFG at 30°C. Reaction was stopped at indicated time points with a nonreducing buffer (−DTT) or with a reducing buffer (+DTT). Samples were separated on a 5%–20% SDS gel and analyzed by immunoblotting with α Ubc9.

(B) SUMO1 transfer from Ubc9-SUMO1 thioesters to Sp100 in the presence or absence of RanBP2. E2 (100 ng), 150 ng E1, 100 ng SUMO1, and 0.5 mM ATP were incubated for 30 min at 30°C. After depletion of ATP with 1 U apyrase, 200 ng Gst-Sp100ΔN and 1 ng BP2ΔFG, when indicated, were added. Reactions were stopped at indicated time points with Laemmli-buffer and separated on a 5%–20% SDS-gel.

(C) A double cysteine mutant of Gst-BP2 is still active in Gst-Sp100ΔN modification. Two cysteines, one in each of two short repeats within RanBP2, were converted to serine. Modification reactions were carried out as in Figure 5A in the presence of wt Gst-BP2 or Gst-BP2ΔΔCys. Analysis was by immunoblotting with α SP100 antibodies

rapid formation of Ubc9 thioesters indicates that this step is not rate limiting in our Sp100 modification reactions. Using preformed Ubc9-SUMO1 thioesters, we next measured the rate of SUMO1 transfer between Ubc9 and Sp100. As shown in Figure 6B, BP2ΔFG clearly enhances Sp100 modification. The low efficiency compared to the modifications shown in Figure 5 is due to the fact that Ubc9 reloading with SUMO1 was inhibited by ATP depletion. In conclusion, as expected for an E3-like activity, BP2ΔFG does stimulate the transfer of SUMO1 to its target protein. Ubiquitin E3 ligases depend on cysteines, either as catalytic residues or as part of RING finger motifs (Jackson et al., 2000; Joazeiro and Weissman, 2000). The minimal fragment used throughout this study (BP2ΔFG) contains seven cysteines but bears no resemblance to RING finger motifs. The only recognizable motif present in this domain is a short internal repeat that is conserved in RanBP2s from different species. We generated a double mutant (Gst-BP2ΔΔCys) in which one cysteine in each repeat was changed to serine. However, this double mutant still stimulated Sp100 modification (Figure 6C). Alkylating agents also did not inhibit RanBP2 function (data not shown), suggesting that free cysteine side chains are not required for its function. These findings open up the intriguing possibility that the mechanism of RanBP2 activity is different from ubiquitin E3 ligases.

RanBP2/Ubc9 Interact Transiently in Sp100 Modification

RanGAP1*SUMO1/RanBP2 and Ubc9 can be found in a stable complex (Lee et al., 1998; Saitoh et al., 1997), but it was not known whether Ubc9 binds directly to RanBP2 or SUMOylated RanGAP1. We tested this by mixing the respective proteins and applying the mixture to gel filtration (Figure 7A). Surprisingly, Ubc9 could bind stably to either protein (as well as to unmodified RanGAP1). Similar findings were also reported by others recently (Saitoh et al., 2001; Sampson et al., 2001). Considering the stable interaction of RanBP2 with Ubc9 and its ability to interact with preformed Ubc9-SUMO1 thioesters, we wanted to test whether RanBP2 functions as a stable stoichiometric cofactor for Ubc9 or whether both proteins undergo cycles of association and dissociation during Sp100 modification. In the first scenario, addition of a stoichiometric complex between RanBP2 and Ubc9 should abolish the need for additional Ubc9 under all conditions. In the second scenario, free Ubc9 concentrations could influence the reaction rate by determining the efficiency of reassociation. We generated two different complexes: a complex consisting just of BP2ΔFG and Ubc9 (F13), and a complex that also included RanGAP1*SUMO1 to mimic the physiological situation at the NPC. We first tested 1 μl of each complex (in F9 was an estimated amount of 15 ng BP2ΔFG, 5

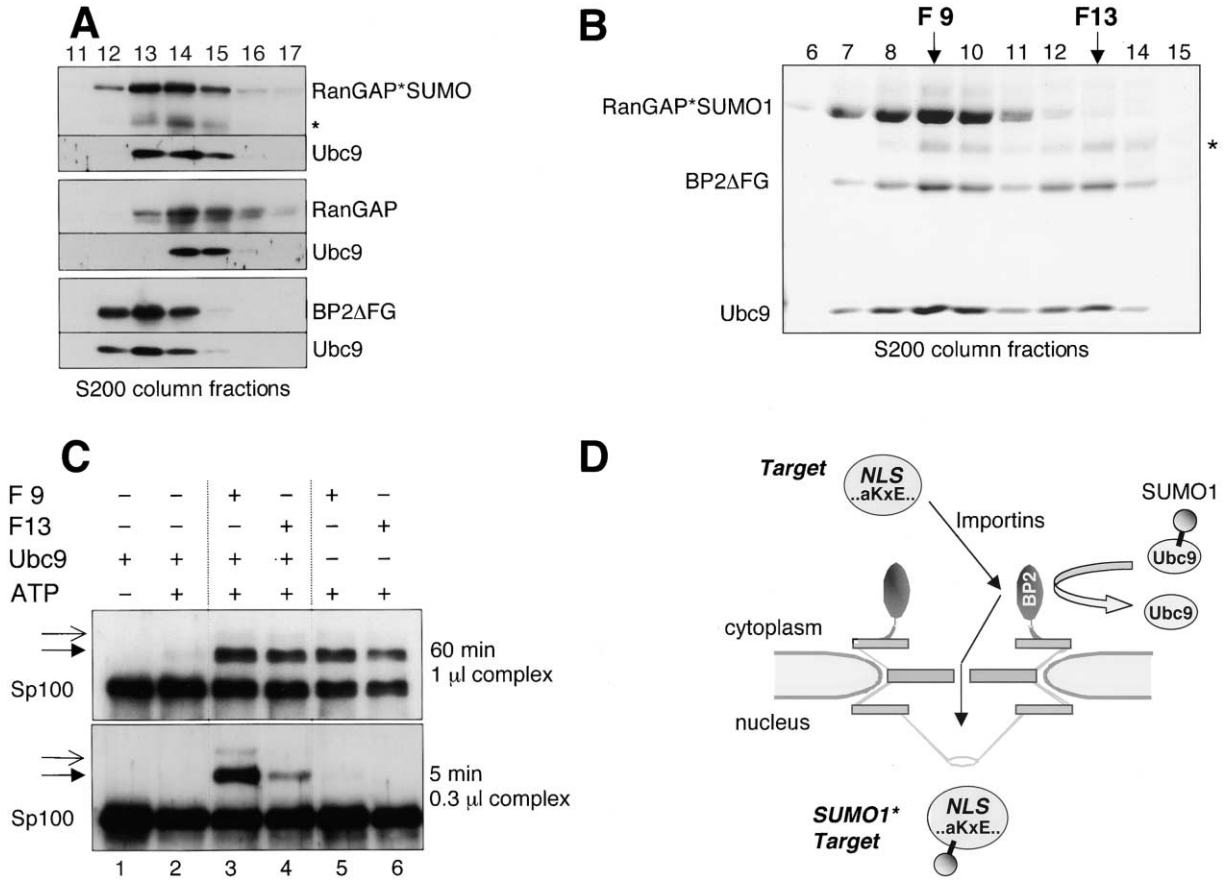

Figure 7. RanBP2 and Ubc9 Interact Only Transiently during Sp100 SUMOylation

(A) Ubc9 binds directly to RanGAP1, SUMO1*RanGAP1, and RanBP2. SUMOylated RanGAP1 (43 μg), unmodified RanGAP1 (40 μg), or BP2ΔFG (20 μg) were each mixed with an excess of Ubc9 (50 μg). After incubation for 1 hr, samples were separated by gel filtration on Superdex 200. Fractions were analyzed by SDS-PAGE and immunostaining. The asterisk marks residual unmodified RanGAP1.

(B) RanGAP1 (100 μg) was modified with SUMO and ATP depleted using a desalting column. RanGAP1*SUMO1 was then incubated with BP2ΔFG (40 μg) and Ubc9 (100μg), and the mixture was separated by gel filtration on Superdex 200. Fractions were analyzed by SDS-PAGE and Coomassie staining. The asterisk marks SUMOylated BP2ΔFG that was generated due to incomplete depletion of ATP. F9, RanGAP1*SUMO1/BP2ΔFG/Ubc9 complex; F13, BP2ΔFG/Ubc9 complex.

(C) Top: Sp100 modification reactions with 25 ng Gst-Sp100ΔN, 60 ng SUMO1, 150 ng E1, and 5 mM ATP and incubation for 1 hr in the presence or absence of 1 μl fraction 9 (F9) or fraction 13 (F13), with or without addition of 10 ng Ubc9. Bottom: Sp100 modification reactions with 25 ng Gst-Sp100ΔN, 120 ng SUMO1, 150 ng E1, and 5 mM ATP and incubation for 5 min in the presence or absence of 0.3 μl F9 or F13, with or without addition of 10 ng Ubc9. Analysis was by immunoblotting with α Gst antibodies.

(D) Model: RanBP2 functions as an E3 SUMO1 ligase that couples modification with nuclear import.

ng Ubc9) for its effect on Sp100 modification in 1 hr reactions. Both complexes efficiently stimulated Sp100 modification in the absence of additional Ubc9 (Figure 7C, top, compare lanes 3 and 5 or lanes 4 and 6), indicating that the Ubc9 provided as part of either complex is catalytically active. We then repeated the experiment with less complex (0.3 μl) and a much shorter incubation time (5 min). Strikingly, under these conditions, the reaction was clearly dependent on the addition of free Ubc9 (Figure 7C, bottom, compare lanes 3 and 5 or 4 and 6). The different efficiencies of F9 and F13 appear to be due to different levels of BP2ΔFG and Ubc9 in these fractions, rather than a contribution by RanGAP1 (titration experiments, data not shown). In summary, these findings are not consistent with a model in which Ubc9 and RanBP2 function as a stable complex, but rather suggest that Ubc9 and RanBP2 undergo cycles of association and dissociation during the reaction. One model consistent with these observations is that SUMO1-loaded Ubc9 binds to RanBP2, transfers SUMO1 to its target, and dissociates again prior to its reloading by Aos1/Uba2 (Figure 7D).

Discussion

RanBP2 Is an E3 SUMO1 Ligase

We have demonstrated here that both full-length RanBP2 and a 33 kDa fragment of RanBP2 strongly enhance SUMO1 modification of Sp100 in the presence of recombinant E1 and E2 enzymes. RanBP2 clearly functions catalytically, as 5 ng BP2ΔFG are sufficient to induce conversion of a 300-fold molar excess of Sp100 (Figure 5B, right). Does this make RanBP2 a SUMO1 E3 ligase? Ubiquitin E3 ligases are defined as "enzymes

that bind, directly or indirectly, specific protein substrates and promote the transfer of ubiquitin, directly or indirectly, from a thioester intermediate to amide linkages with proteins or polyubiquitin chains" (Hershko and Ciechanover, 1998). RanBP2 interacts directly with Ubc9 and promotes SUMO1 modification of Sp100 by stimulating transfer of SUMO1 from Ubc9 to Sp100. RanBP2 does not interact directly with Sp100 in pull-down experiments (data not shown) but certainly does interact indirectly via Ubc9. Importantly, endogenous RanBP2 at the NPC will probably also interact with Sp100 by a second mechanism, due to its role as a docking site in nuclear protein import. This interaction would be mediated by import receptors that interact with RanBP2 via FG repeat domains directly flanking its E3-like domain (Figure 3; Ben-Efraim and Gerace, 2001; Stewart et al., 2001; Yaseen and Blobel, 1999). Finally, RanBP2 appears to confer substrate specificity, since it does stimulate modification of some but not all SUMO1 targets. Thus, RanBP2 fulfills the functional criteria for being an E3 ligase.

RanBP2 and PIAS SUMO1 E3 Ligases

Ubiquitin E3 ligases can be classified into two groups according to their mode of action (Hershko and Ciechanover, 1998; Jackson et al., 2000): HECT domain E3 ligases are true enzymes, because they form a thioester bond with ubiquitin before they transfer it to their target; a second class of E3 ligases, either in the form of individual proteins or as multiprotein complexes, serve as adaptors between the E2 enzyme and the target. These E3 ligases contain RING finger motifs essential for their function. RanBP2 does not appear to fall into either group. Treatment of the recombinant protein with alkylating agents does not inhibit its stimulatory effect on Sp100 modification (data not shown), suggesting that thioester bond formation is not required for its activity (due to RanBP2's rapid hypermodification, it has not been possible to directly test for the appearance of a thioester bond with SUMO1). On the other hand, the catalytic domain of RanBP2 (BP2ΔFG) lacks the consensus sequence for RING finger domains (Jackson et al., 2000), and mutagenesis of two out of seven cysteines had no effect (no histidines are present in this domain). Full-length RanBP2 may serve in part as an adaptor, bringing together NLS-containing target proteins and Ubc9. This does not, however, explain the dramatic effect of the isolated BP2ΔFG domain. We consider it most likely that BP2ΔFG functions allosterically by increasing Ubc9's affinity for specific targets or by facilitating SUMO1 transfer to specific lysine residues.

RanBP2 is clearly distinct from PIAS E3 ligases (Johnson and Gupta, 2001; Kahyo et al., 2001; Sachdev et al., 2001; Takahashi et al., 2001), both by amino acid sequence and due to the fact that PIAS—but not RanBP2—catalytic activity depends on a RING-like domain.

Common to both is their ability to stimulate SUMO chain formation. RanBP2 forms chains on itself, while Siz1 induces free SUMO chains as well as chains on targets (our data; Johnson and Gupta, 2001). The physiological relevance for this, however, is not yet clear. An exciting function for RanBP2 chain formation would be autoinhibition of its catalytic activity. However, in vitro, at least, this is not the case, since hypermodified BP2ΔFG still stimulates Sp100 modification (data not shown). Interestingly, both classes of SUMO1 E3 ligases seem to function on many different target proteins. We find that RanBP2 stimulates modification of three out of six tested proteins, and gene disruption of *Siz1* and its relative *Siz2* abolishes modification of most targets in yeast (Johnson and Gupta, 2001). Whether this indicates that SUMOylation involves fewer distinct E3-like factors than ubiquitination remains to be seen.

RanBP2 May Coordinate SUMO1 Modification and Nuclear Import

RanBP2 is part of the nucleocytoplasmic transport machinery (reviewed in Görlich and Kutay, 1999) and serves as a docking factor for import complexes on their way into the nucleus. Its dual role in nuclear import and as an E3 ligase agrees well with the observation that a functional NLS is required for SUMOylation of several targets in vivo (Rodriguez et al., 2001, and references therein). Since RanBP2 is restricted to the NPC in interphase cells, a functional NLS would be required for efficient target contact with RanBP2.

SUMO1 modification can clearly take place in the nucleus, so why would SUMO1 modification also require an E3 ligase at the cytoplasmic phase of the NPC? We can envision two different possibilities. SUMOylation may be mechanistically involved in the translocation through the NPC. Although intriguing, there is currently no evidence for such a function. Alternatively, SUMOylation at the NPC may serve as a mechanism to switch proteins from a cytoplasmic to a nuclear mode of action. Placing this at the NPC rather than into the nucleus would be more effective and may be particularly important for SUMO1 targets that shuttle rapidly between both compartments.

What are the in vivo targets for RanBP2? For two reasons, we consider it possible that RanBP2 may serve to modify many targets on their way into the nucleus. First, most of the known SUMO1 targets contain a classical NLS and will therefore encounter RanBP2 during their translocation into the nucleus. Second, SUMOylation of three out of six proteins was stimulated by BP2ΔFG in vitro (Figure 6 and unpublished data). Upon translocation of modified targets into the nucleus, their SUMOylation status could be further regulated by isopeptidases as well as by E1, E2, and E3 modifying enzymes that reside inside the nucleus.

In conclusion, we have provided compelling evidence that the NPC protein RanBP2 has SUMO1 E3 ligase activity. Together with RanBP2's well-established role as a docking site for transport complexes, this suggests that, at least for some SUMO1 targets, modification and nuclear import are coordinated events.

Experimental Procedures

Plasmids

pEYFP-SUMO (1–97) and pEYFP-SUMO (1–101) were generated by amplification of SUMO1 expression plasmids (Mahajan et al., 1998) and subsequent cloning into the BamHI/KpnI sites of pEYFP (Clontech). pEYFP-SUMO (1–95) was made by introduction of a stop codon in pEYFP-SUMO (1–101) through mutagenesis. For protein expression, YFP-SUMO (1–97) was recloned into the NcoI/BamHI

site of pET11d (Novagen). For expression of untagged SUMO1 (1–97), SUMO1 (1−97) cDNA was amplified by PCR and cloned into the NdeI and BamHI sites of pET11a. Human Uba2 cDNA was amplified by PCR from EST clone DKFZp434DO717 and cloned into NcoI and BamHI sites of pET11d. Human Aos1 cDNA was PCR amplified from EST clone DKFZp434J0913 and cloned into NheI and BamHI sites of pET28a. Mouse Ubc9 cDNA was amplified by PCR from EST clone number IMAGp998A061122 and cloned into NdeI and BamHI sites of pET23a. Expression clones for Gst-RanBP2 (aa 2503–2893) and Gst-RanBP2ΔFG (aa 2553–2838) were obtained through PCR from human RanBP2 cDNA (a kind gift by Dr. Takeharu Nishimoto) and cloning into the BamHI/EcoRI sites of pGEX3X (Amersham Pharmacia Biotechnology). A plasmid for Gst-RanBP2ΔΔCys (Cys2659Ser and Cys2737Ser) was obtained through site-directed mutagenesis. A plasmid for Gst-SP100 (aa 187–480) was obtained by cloning a MscI-EcoRI fragment from SP100 cDNA (Seeler et al., 2001) into the SmaI and EcoRI sites of pGEX-3X. Gst-SP100 (187–480, K297R) was obtained by site-directed mutagenesis. pET11d-RanGAP1 was described previously (Mahajan et al., 1997). Expression plasmids for Gst-Ulp1 and GST-p53 were kindly provided by Dr. Mark Hochstrasser (Li and Hochstrasser, 1999) and Dr. Moshe Oren, respectively.

Expression and Purification of Recombinant Proteins

Unless stated otherwise, all protein purification protocols involved IPTG-induced expression in *E. coli* BL21 gold (Stratagene), bacterial lysis with lysozyme, and a 100,000 × g spin for 1 hr to collect soluble proteins. Each buffer contained 1 μg/ml each of leupeptin, pepstatin, and aprotinin, and 1 mM DTT (or β-mercapto ethanol); lysis buffers also contained 0.1 mM PMSF. After specific purification steps described below, proteins were aliquoted, flash frozen, and stored at −80°C. The final buffer in each protocol was transport buffer (TB: 20 mM HEPES, 110 mM K-acetate, 2 mM Mg-acetate, 0.5 mM EGTA). Gst-fusion proteins followed standard protocols and included dialysis against TB as the final step. Pure BP2ΔFG and BP2ΔΔCys were obtained by factor Xa cleavage according to the manufacturer's protocol (Novagen), removal of Gst by glutathion sepharose, and molecular sieving (Superdex 200, Pharmacia). Purification of RanGAP1 was as described previously (Mahajan et al., 1997). SUMO1 purification involved bacterial lysis in 50 mM Tris/HCl (pH 8), 50 mM NaCl by sonification, preclearing of the 100,000 × g supernatant with Q sepharose (SIGMA), concentration, and subsequent gel filtration. YFP-SUMO1 (1–97) purification involved lysis in 50 mM Tris/HCl (pH 8), 100 mM NaCl, 1 mM EDTA, ultracentrifugation, ion exchange chromatography (HightrapQ, Amersham Pharmacia), and molecular sieving. Purification of SUMO E1 enzyme involved coexpression of His-Aos1 and Uba2, bacterial lysis in 50 mM Na-phosphate (pH 8), 300 mM NaCl, 10 mM imidazol, purification on ProBond Resin (Invitrogen), molecular sieving (Superdex 200), and ion exchange chromatography (Mono Q, Pharmacia Biotech). Ubc9 purification involved lysis in 50 mM Na-phosphate (pH 6.5), 50 mM NaCl, incubation of the 100,000 × g supernatant with SP-sepharose beads (SIGMA), elution of Ubc9 from the beads with 50 mM Na-phosphate (pH 6.5), 300 mM NaCl, and molecular sieving (Superdex 200).

Antibodies

α RanBP2 antibodies were raised in rabbit; α Ubc9, α Aos1, and α RanGAP1 antibodies were raised in goat. Injection procedures and affinity purification were essentially as in Mahajan et al. (1997). Gst-SP100 proteins were detected with rabbit α Gst (a kind gift by Dr. Ludger Hengst) or with mouse monoclonal α Sp100 antibody (J.S.S. and A.D., unpublished data). Rabbit α GFP was from Santa Cruz Biotechnology, mouse monoclonal α SUMO1 was from Zymed Laboratories, and α Rcc1 antibody was kindly provided by Dr. Takeharu Nishimoto. Secondary antibodies were from Jackson Laboratories and Molecular Probes.

Cytosolic Extracts and Immunoprecipitation

Cytosol preparation for in vitro import reactions was as follows. HeLa suspension cells were collected by centrifugation at 250 × g for 10 min, washed, resuspended in 2 cell volumes TB, and permeabilized with digitonin. Centrifugation at 250 × g for 10 min and ultracentrifugation for 1 hr followed. Aliquots were stored at −80°C. Cytosol preparation for in vitro modification assays and immunoprecipitations involved cell lysis by hypotonic swelling and douncing and was essentially as in Melchior (1998). The final step involved chromatography over a desalting column (PD10, Amersham Pharmacia) in TB.

For immunoprecipitation, 1 mg affinity-purified goat α RanGAP1 antibody and goat IgGs were each crosslinked to 0.5 ml Ultralink TM Immobilized Protein G Plus beads (PIERCE) using dimethylpimelimidate (PIERCE) as crosslinker. After 1 hr preclearing with preimmunserum, 1 ml cytosolic extracts were either incubated with 100 μl crosslinked anti-RanGAP1 or 100 μl crosslinked IgG for 2 hr at 4°C. After centrifugation, beads were washed four times with TB containing protease inhibitors and 1 mM DTT.

In Vitro SUMOylation Assay

SUMO1 modification assays were performed in a total volume of 20 μl in TB with protease inhibitors and 1 mM DTT. Reactions containing low concentrations of recombinant enzymes were supplemented with 0.05% Tween and 0.2 mg/ml Ovalbumin Grade VI (SIGMA). Reactions were incubated at 30°C or 37°C and were stopped by addition of Laemmli-buffer. Protein concentrations were as indicated in the figure legends. Thioester reactions were performed in 20 mM Tris (pH 7.6), 50 mM NaCl, 10 mM $MgCl_2$, protease inhibitors, and 0.1 mM DTT. Reactions were terminated by 1:1 dilution with 2× nonreducing sample buffer (50 mM Tris [pH 6.8], 2% SDS, 4 M urea, 10% glycerol).

In Vitro Nuclear Import, Transfection, and Microinjection

Cultivation of HeLa suspension cells, in vitro import assays, and quantitation by flow cytometry were essentially as described (Melchior, 1998). YFP-SUMO1 (1–97) (0.8 μg) was used per 40 μl reaction. Pictures were taken using a Zeiss Axioskop II and a MicroMax CCD camera (Princeton Instruments). Transfection of adherent HeLa cells was carried out with Superfect transfection reagent according to manufacturer's instructions (Qiagen). After 20 hr, cell were fixed with 2% paraformaldehyde for 10 min and analyzed by immunofluorescence, or where harvested, were lysed with SDS-loading buffer and analyzed by immunoblotting. Microinjection of YFP-SUMO1 (1–97) was into adherent HeLa cells. ATP depletion was accomplished by incubating the cells for 30 min in glucose-free DMEM (GIBCO) supplemented with 10% FBS, 6 mM 2-deoxyglucose, and 10 mM sodium azide. Cells were kept at 37°C, and pictures were taken at different times after injection using an inverted microscope with CCD camera (Olympus IX70).

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