Ubc9p and the conjugation of SUMO-1 to RanGAP1 and RanBP2

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The yeast *UBC9* gene encodes a protein with homology to the E2 ubiquitin-conjugating enzymes that mediate the attachment of ubiquitin to substrate proteins [1]. Depletion of Ubc9p arrests cells in G2 or early M phase and stabilizes B-type cyclins [1]. $p18^{Ubc9}$, the *Xenopus* homolog of Ubc9p, associates specifically with $p88^{RanGAP1}$ and $p340^{RanBP2}$ [2]. Ran-binding protein 2 ($p340^{RanBP2}$) is a nuclear pore protein [3,4], and $p88^{RanGAP1}$ is a modified form of RanGAP1, a GTPase-activating protein for the small GTPase Ran [2]. It has recently been shown that mammalian RanGAP1 can be conjugated with SUMO-1, a small ubiquitin-related modifier [5–7], and that SUMO-1 conjugation promotes RanGAP1's interaction with RanBP2 [2,5,6]. Here we show that $p18^{Ubc9}$ acts as an E2-like enzyme for SUMO-1 conjugation, but not for ubiquitin conjugation. This suggests that the SUMO-1 conjugation pathway is biochemically similar to the ubiquitin conjugation pathway but uses a distinct set of enzymes and regulatory mechanisms. We also show that $p18^{Ubc9}$ interacts specifically with the internal repeat domain of RanBP2, which is a substrate for SUMO-1 conjugation in *Xenopus* egg extracts.

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Results and discussion

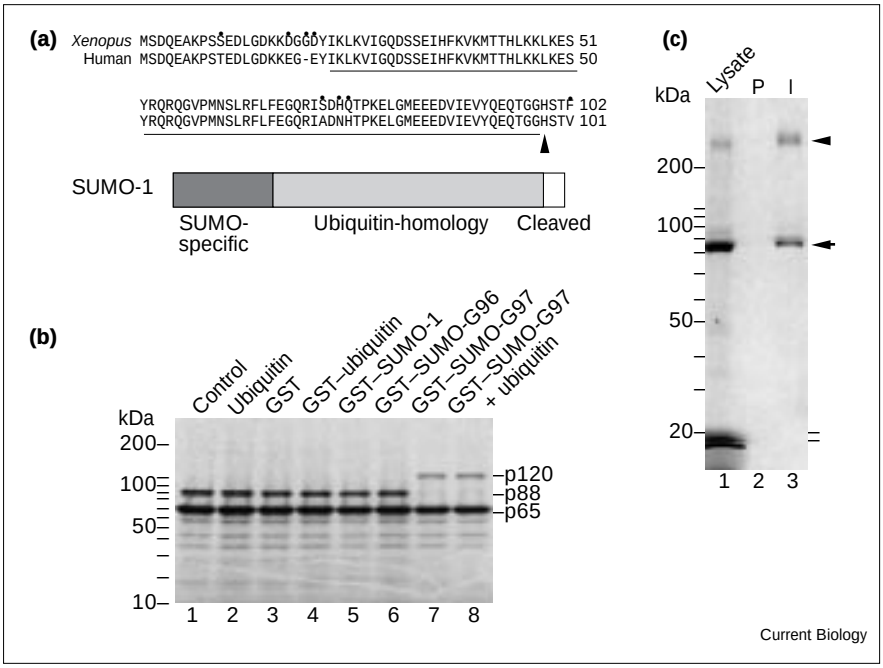
Xenopus RanGAP1 exists in egg extracts in two forms: an unmodified form ($p65^{RanGAP1}$) and a modified form ($p88^{RanGAP1}$) [2]. Two observations suggest that SUMO-1 conjugation is responsible for the conversion of $p65^{RanGAP1}$ to $p88^{RanGAP1}$ in *Xenopus*, as it is in mammalian cells. First, we isolated a *Xenopus* homolog of SUMO-1 that is highly conserved in comparison with human SUMO-1 (92% identical, Figure 1a). RNase protection assays showed the RNA to be ubiquitously expressed during early frog development (data not shown). This pattern of expression is consistent with the notion that SUMO-1 has an important role in nucleocytoplasmic trafficking in most cells.

Second, *in vitro* analysis indicated that SUMO-1 can be directly conjugated to *Xenopus* RanGAP1 (Figure 1b). We made a series of constructs encoding full-length human SUMO-1 protein, and two mutants, lacking four (SUMO-G97) and five (SUMO-G96) amino acids from the carboxyl terminus. Similar constructs were also made with a glutathione-S-transferase fusion moiety (GST–SUMO-1, GST–SUMO-G97, GST–SUMO-G96). We expected that wild-type SUMO-1 would require proteolytic processing before conjugation, whereas the mutant lacking four amino acids from its carboxyl terminus could be conjugated without processing [8]. We expected that removal of five amino acids from the carboxyl terminus would yield a protein that could not undergo conjugation [8]. Consistent with these expectations, GST–SUMO-G97 and SUMO-G97 were conjugated to target proteins in both reticulocyte lysates and *Xenopus* egg extracts (data not shown; see Figure 1). GST–SUMO-1 and SUMO-1 were processed and conjugated well only in egg extracts (data not shown), indicating that reticulocyte lysates lack the activity for the carboxy-terminal modification of SUMO-1. GST–SUMO-G96 and SUMO-G96 were not conjugated to target proteins in any of our experiments (data not shown).

When *Xenopus* RanGAP1 was expressed in a coupled transcription–translation system, two prominent ^{35}S-labeled bands were observed that co-migrated with $p88^{RanGAP1}$ and $p65^{RanGAP1}$ (Figure 1b). When we added GST–ubiquitin, GST–SUMO-1, GST–SUMO-G97 or GST–SUMO-G96 to the reaction at approximately a 100-fold excess over the level of endogenous SUMO-1, we found that GST–SUMO-G97 increased the apparent molecular weight of the upper band by 30 kDa on SDS–polyacrylamide gels (Figure 1b, lane 7). This shift is in good correspondence with the molecular weight of the GST moiety of the fusion protein, suggesting that GST–SUMO-G97 was able to compete with the endogenous SUMO-1 in reticulocyte lysates. The addition of excess ubiquitin did not reverse the shift of *Xenopus* RanGAP1, indicating that it did not compete in this reaction (Figure 1b, lane 8). This experiment confirmed that *Xenopus* RanGAP1 is subject to conjugation with SUMO-1 and suggested that SUMO-1 conjugation enzymes are distinct from the enzymes of the ubiquitin conjugation pathway (see below).

In a different approach, we added ^{35}S-labeled SUMO-1 to egg extracts and found that it was incorporated into two

Figure 1



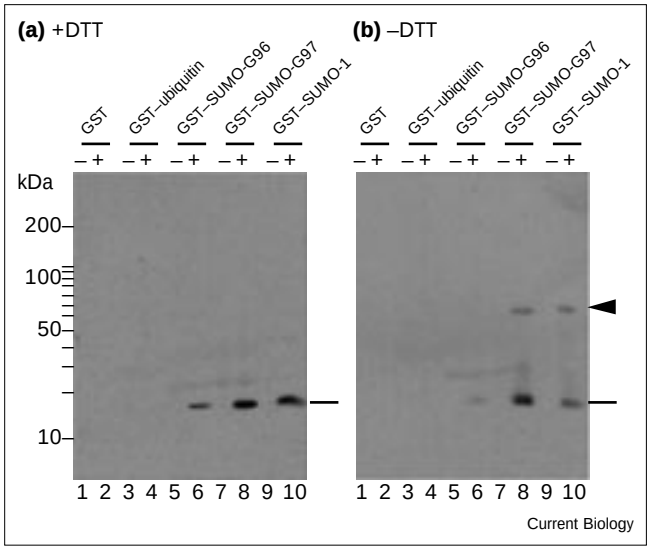
SUMO-1 is conjugated to RanGAP1 and RanBP2/Nup358 in *Xenopus laevis*. (a) *Xenopus* (upper) and human (lower) SUMO-1. Black dots indicate amino-acid substitutions. Dash indicates a gap inserted for sequence alignment. The ubiquitin homology domain is underlined. Arrowhead indicates the putative processing site. The domain structure of SUMO-1 is shown below. (b) *Xenopus* RanGAP1 labeled with ^{35}S was produced in an *in vitro* transcription–translation reaction containing exogenous proteins as indicated. The samples were subjected to SDS–PAGE and visualized by autoradiography. Positions of the protein products detected at 120 kDa, 88 kDa and 65 kDa are shown on the right. (c) SUMO-1 labeled with ^{35}S was produced in reticulocyte lysates, to which an equal volume of egg extract was added and incubated for 20 min at 30°C. 2 µl of the reaction was analyzed directly by SDS–PAGE (lane 1). 9 µl of the reaction was subjected to immunoprecipitation with either preimmune serum (lane 2) or anti-p340^{RanBP2} immune serum (lane 3). Protein products of 340 kDa (arrowhead), 88 kDa (arrow), unprocessed SUMO-1 (upper line) and processed SUMO-1 (lower line) are indicated.

major high molecular weight bands, with apparent molecular weights of 88 kDa and 340 kDa. The smaller protein co-migrated with p88^{RanGAP1}, and both bands were immunoprecipitated by anti-p340^{RanBP2} antibodies (Figure 1c), suggesting that the modified proteins were p88^{RanGAP1} and p340^{RanBP2}. On the basis of this observation and the results discussed below, we believe that p340^{RanBP2} is a SUMO-1 conjugation substrate. In this assay, *Xenopus* SUMO-1 and human SMT3a [9] behaved identically to human SUMO-1 (data not shown).

The p18^{Ubc9} protein associates tightly with p88^{RanGAP1} and p340^{RanBP2} in egg extracts [2]. We thus asked whether p18^{Ubc9} might act as an E2-like enzyme for SUMO-1 conjugation. We incubated purified recombinant GST, GST–ubiquitin or each of the three GST–SUMO fusion proteins in egg extracts. Each of the proteins and tightly bound extract proteins was purified using glutathione–Sepharose beads and analyzed by western blotting with anti-p18^{Ubc9} antibodies (Figure 2a). We found no p18^{Ubc9} associated with GST or GST–ubiquitin. In contrast, p18^{Ubc9} was clearly associated with GST–SUMO, GST–SUMO-G97 and GST–SUMO-G96. This observation suggested that p18^{Ubc9} has a higher affinity for SUMO-1 than for ubiquitin. To examine whether p18^{Ubc9} might form a thioester adduct with SUMO-1, we repeated the same experiment, omitting reducing agents during sample preparation. In this case, we observed an additional band recognized by anti-p18^{Ubc9} antibodies with an apparent molecular weight of 60 kDa (Figure 2b). This band was also recognized by anti-GST antibodies. We believe that this band is the result of thioester formation between GST–SUMO-G97 and p18^{Ubc9}, strongly supporting the idea that p18^{Ubc9} acts as an E2 conjugating subunit for SUMO-1. Because Cys93 is analogous to the cysteine residue of ubiquitin E2 enzymes used for thioester formation, we made a mutant p18^{Ubc9} with a cysteine to alanine mutation at residue 93 (p18^{Ubc9}-C93A). This did not form a thioester adduct with SUMO-1 (data not shown). This observation suggested that the mechanism whereby p18^{Ubc9} acts in SUMO-1 conjugation may be similar to that of ubiquitin E2 enzymes.

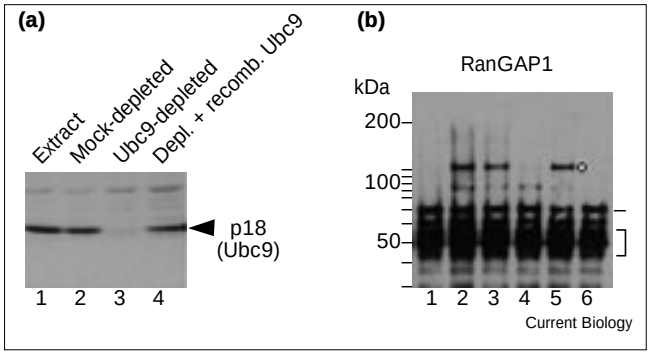
To confirm the role of p18^{Ubc9} in the conjugation of SUMO-1, we immunodepleted p18^{Ubc9} from *Xenopus* egg extracts using affinity-purified antibodies bound to protein-A–Sepharose beads (Figure 3a). Extracts lacking p18^{Ubc9} were deficient in the conjugation of SUMO-1 to bacterially expressed T7-tagged RanGAP1 (Figure 3b). The addition of bacterially expressed p18^{Ubc9} protein restored the capacity of depleted extracts to conjugate GST–SUMO-G97 to RanGAP1, demonstrating the specificity of p18^{Ubc9} depletion in our experiments. The conjugation of SUMO-1 to RanBP2 in egg extracts similarly required p18^{Ubc9} (data not shown). These results again strongly support the conclusion that p18^{Ubc9} is an E2 conjugating enzyme for SUMO-1.

Figure 2



The p18^{Ubc9} protein binds and forms a thioester adduct with SUMO-1. (**a**) In the presence of reducing agent (+DTT). GST (lanes 1,2), GST–ubiquitin (lanes 3,4), GST–SUMO-G96 (lanes 5,6), GST–SUMO-G97 (lanes 7,8) or GST–SUMO-1 (lanes 9,10) were incubated with phosphate-buffered saline (–) or *Xenopus* egg extract (+). GST pull-down assays were performed, and samples were analyzed by SDS–PAGE and western blotting with anti-p18^{Ubc9} antibodies. The line indicates p18^{Ubc9}. (**b**) Samples were prepared as in (a), but no reducing reagents (–DTT) were added during sample preparation. The arrowhead indicates the 60 kDa adduct recognized by anti-p18^{Ubc9} antibodies.

Figure 3

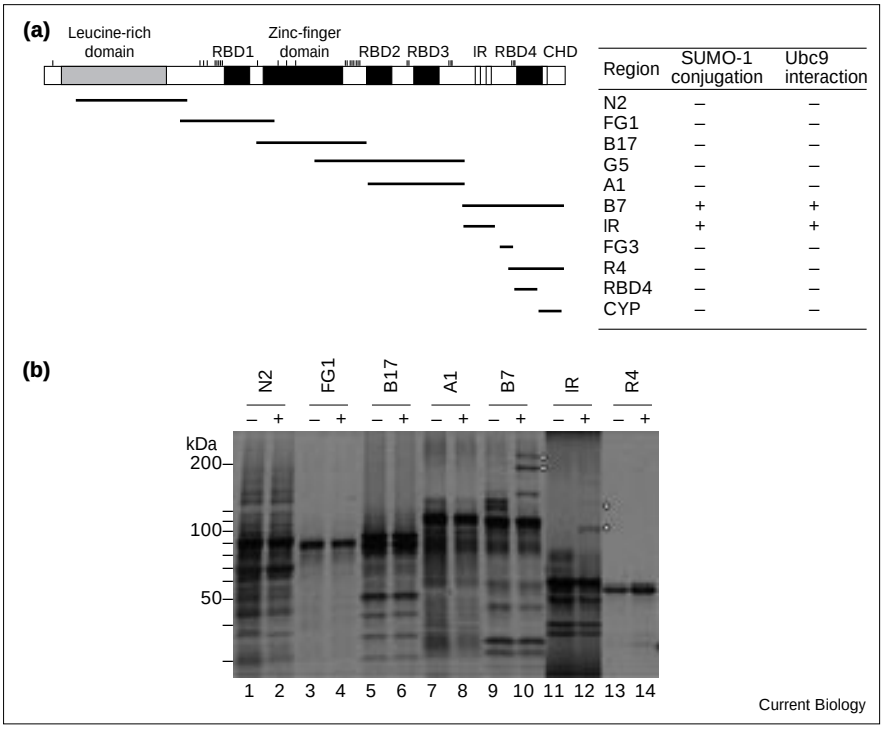


The p18^{Ubc9} protein is essential for SUMO-1 conjugation. (**a**) Equal volumes of untreated extract (lane 1), mock-depleted extract (lane 2), p18^{Ubc9}-depleted extract (lane 3) and p18^{Ubc9}-depleted extracts plus 10 ng/µl recombinant p18^{Ubc9} (lane 4) were analyzed by western blotting with anti-p18^{Ubc9} antibodies. (**b**) T7-tagged RanGAP1 protein was conjugated with GST–SUMO-G97 in reactions containing buffer (lane 1), untreated extract (lane 2), mock-depleted extract (lane 3), p18^{Ubc9}-depleted extract (lane 4), depleted extract plus recombinant p18^{Ubc9} (lane 5) or buffer plus recombinant p18^{Ubc9} (lane 6). See Supplementary material for Materials and methods. Conjugation products were analyzed by western blotting with anti-T7-tag antibodies. RanGAP1 is indicated by the lines on the right of the panel. The white dot indicates the GST–SUMO-1-RanGAP1 conjugates. Similar results were obtained using the B7 fragment (see Figure 4) of RanBP2.

Earlier reports have indicated that Ubc9p forms a thioester with ubiquitin *in vitro* [1,10]. In our hands, however, p18^{Ubc9} interacts exclusively with SUMO-1 rather than with ubiquitin. We therefore believe that p18^{Ubc9} functions preferentially as a conjugating enzyme for SUMO-1, and probably acts in the ubiquitin pathway with a very low efficiency, if at all. In the absence of Ubc9p, *S. cerevisiae* cells arrest at G_2/M and are unable to degrade B-type cyclins [1]. It was originally thought that Ubc9p might act as an E2 ubiquitin-conjugating enzyme for cyclins, but it has now been clearly demonstrated that other proteins fulfill this function [11]. A different model for Ubc9p function may involve regulation of nuclear transport. In the absence of Ubc9p, nuclear transport may arrest and thereby block the destruction of cyclins. The lack of cyclin destruction may reflect a requirement for cyclins to interact with nuclear factors in order to be destroyed. Alternatively, it may reflect an ongoing requirement for nuclear trafficking of other components required for cyclin destruction.

To better understand the interaction between p18^{Ubc9} and p340^{RanBP2}, we mapped the subdomains of RanBP2 that interact with p18^{Ubc9} and the subdomains that are subject to SUMO-1 conjugation (Figure 4a). We found co-precipitation of ^{35}S-labeled p18^{Ubc9} in B7 fragment and internal repeat (IR) domain immunoprecipitates when p18^{Ubc9} was co-expressed with these domains in reticulocyte lysates, but did not see co-precipitation with the other RanBP2 domains (data not shown; summarized in Figure 4a). When we translated the RanBP2 domains in reticulocyte lysates in the presence of [^{35}S]methionine (Figure 4b), we also noted that translation of the B7 and IR domains gave a major product of the predicted molecular weight, as well as additional species that migrated on SDS–PAGE with a 20 kDa increase in apparent molecular weight. To determine whether the upper bands could be SUMO-1 conjugates, we added excess GST–SUMO-G97 to compete with the endogenous protein. The upper bands in the B7 and IR reactions were shifted to higher molecular weight species (Figure 4b, white dots). From these results, we believe that p18^{Ubc9} associates with the IR domain of RanBP2 and that this domain of RanBP2 is subject to SUMO-1 conjugation. Other investigators have reported SUMO-1 conjugates in mammalian cells, many with apparent molecular weights above that of p88^{RanGAP1} [6,8]. However, those proteins have not been identified. At present, we do not know the functional consequences of SUMO-1 conjugation of RanBP2. It has been suggested that RanBP2 may act as a docking site for import substrates and as a scaffold for the assembly of complexes that coordinate GTP–Ran hydrolysis with the translocation of substrates into the nucleus [3,4]. If these ideas are correct,

Figure 4



Region	SUMO-1 conjugation	Ubc9 interaction
N2	–	–
FG1	–	–
B17	–	–
G5	–	–
A1	–	–
B7	+	+
IR	+	+
FG3	–	–
R4	–	–
RBD4	–	–
CYP	–	–

The IR domain of RanBP2 is conjugated with SUMO-1. (a) RanBP2 structure is shown schematically on the left [3]. In the table, the left column summarizes which RanBP2 fragments were subject to pull-downs with GST–SUMO-1; see (b); the right column summarizes which RanBP2 fragments could be co-precipitated with $p18^{Ubc9}$ when they were co-expressed with $p18^{Ubc9}$ in reticulocyte lysates (see text). (b) ^{35}S-labeled RanBP2 fragments as indicated were produced in reticulocyte lysates containing GST (–) or GST–SUMO-G97 (+) (see Supplementary material for Materials and methods), and analyzed by SDS–PAGE and autoradiography. White dots indicate supershifted B7 and IR products.

conjugation of SUMO-1 to RanBP2 may modulate its activity and thereby regulate the rate of nuclear transport.

In summary, our results suggest that the SUMO-1 conjugation pathway is biochemically similar to the ubiquitin conjugation pathway, but that it uses a distinct set of enzymes. Our results also suggest a role for $p18^{Ubc9}$ in controlling nuclear transport through $p88^{RanGAP1}$ and $p340^{RanBP2}$, and necessitate the re-evaluation of earlier data regarding the role of $p18^{Ubc9}$ in the regulation of mitosis.

Materials and methods

The plasmids used, and methods for the expression of proteins in bacteria, protein purification, preparation of *Xenopus* egg extracts, *cDNA cloning of Xenopus* SUMO-1, conjugation assay using GST–SUMO-1, conjugation assay using [^{35}S]SUMO-1, and the GST pulldown assay are given in the Supplementary material published with this paper on the internet.

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