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In Vitro SUMO-1 Modification Requires Two Enzymatic Steps, E1 and E2

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The SUMO-1 has been identified as a protein that is highly similar to ubiquitin and shown to conjugate to RanGAP1, PML, Sp200 and I κ B α. The conjugation steps are thought to be similar to those of ubiquitination; and human Ubc9, which is homologous to the E2 enzyme for the ubiquitin conjugation step, was identified and shown to be necessary for the conjugation of SUMO-1 to its target protein. Other essential enzymes involved in this modification, however, remain to be clarified. Here we cloned human *Sua1* (SUMO-1 activating enzyme) and *hUba2,* which are human homologs of yeast *Saccharomyces cerevisiae Aos1* and *Uba2,* respectively. The recombinant proteins, Sua1p and hUba2p, formed a complex. In this complex, hUba2 bound SUMO-1 and this complex had the activity of the SUMO-1 activating enzyme. Furthermore, in an *in vitro* system, RanGAP1 was modified by SUMO-1 in the presence of Sua1p/Uba2p and hUbc9p, showing that the modification of SUMO-1 could be catalyzed by two enzyme steps, although ubiquitination usually requires three enzyme steps. © 1999 Academic Press

SUMO-1 has been identified as a protein that is highly similar to ubiquitin (1–5). Conjugation of ubiquitin to certain proteins is a signal to promote the degradation of such proteins by proteasomes (7). However, protein modification by SUMO-1 has a role in another pathway rather than protein degradation. At first, RanGAP1 was shown to be a protein modified by SUMO-1 (4, 6). RanGAP1 associates with RanBP2/Nmp358, which is a component of the nuclear pore complex (NPC), and the stable complex is required for the import of proteins into the nucleus (8). Although RanGAP1 is predominantly localized in the cytosol, conjugation of SUMO-1 to RanGAP1 promotes the targeting of RanGAP1 to the NPC (4, 6). Conjugation of SUMO-1 to PML also modulates the cellular localization of the latter (9, 10). Thus post-translational modification by SUMO-1 seems to regulate the structure and intracelluar localization of certain proteins.

The protein modification by SUMO-1 is performed by enzymatic reactions, which seem to be similar to those involved in the ubiquitination reaction. Attachment of ubiquitin to proteins requires three enzymes, ubiquitin activating enzyme (E1), ubiquitin conjugating enzyme (E2), and ubiquitin ligase (E3) (7). If the conjugation system of SUMO-1 to proteins is similar to that of ubiquitin, there may exist E1, E2 and E3 enzymes for the SUMO-1 system. Actually, human Ubc9 is able to bind SUMO-1, not ubiquitin, through a thioester bond, suggesting that hUbc9 is a SUMO-1 conjugating enzyme (11–16). Furthermore, Smt3p, which is a SUMO-1 homolog in the budding yeast *Saccharomyces cerevisiae,* has been shown to cooperate with Aos1p/Uba2p heterodimer (17, 18). Uba2 and Aos1 are sequentially similar to the C-terminus and N-terminal of Uba1, the yeast ubiquitin activating enzyme, E1 (19), respectively. The Aos1p/Uba2p heterodimer seems to be a Smt3p activating enzyme and to promote Smt3p conjugation to other proteins. However, it is still unknown whether an E3-like molecule for Smt3 exists or not. In this study we isolated human SUMO-1 activating enzyme genes, Sua1 (SUMO-1 activating enzyme) and hUba2, and showed that RanGAP1 is modified by SUMO-1 in the presence of SUMO-1 activating enzyme (Sua1/hUba2) and hUbc9.

MATERIALS AND METHODS

Cloning and sequencing. DNA probes were phosphorylated by Megaprime DNA labelling system (Amersham, Co.) according to the manufacturer's protocol. Three million plaques of a λZAP HeLa cell cDNA library, which was kindly provided by Dr. H. Nozima, Osaka Univ., Japan, were screened by use of the probes. The positive clones obtained were converted to pSK plasmids, and both strands were sequenced with a dye termination kit and automatic sequencer ABI PRISM 310 (Applied Biosystems).

Plasmid construction and protein expression. SUMO-1 (G) cDNA, which encodes from the first methionine to glycine 97, was obtained

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by the reverse transcript polymerase chain reaction (RT-PCR) method using total RNA from HeLa cells. Human Ubc9 cDNA and RanGAP1 were also obtained by RT-PCR methods. The SUMO-1(G) cDNA was subcloned into a pET3 vector. The hUbc9 cDNA was subcloned into a pET3 vector or pET11dGST vector, the latter of which was modified pET11d vector having a glutathione-S-transferase gene. These recombinant proteins were expressed in *E.coli* (*BL21, LysS*) by the addition of isopropyl β-D(−)-thiogalactopyranoside (IPTG) to the medium. RanGAP1 cDNA, APP-BP1 cDNA (20) Sua1 cDNA and hUba2 cDNA were subcloned into pGST-FastBac or pFastBacHT vectors (GibcoBRL) and the recombinant proteins were expressed in Sf-9 cells by use of a baculovirus protein expression system. When two recombinat proteins were used together, for example when the Sua1p/hUba2p complex was used, Sf9 cells were co-infected with two kinds of viruses, one expressing Sua1p and the other, hUba2p.

Protein purification. *E. coli* (*BL21, LysS*) expressing SUMO-1 protein was disrupted by sonication in a buffer containing 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 0.1% NP40, and 1 mM phenylmethylsulfonyl fluoride (PMSF). The resultant lysate was centrifuged at 12,000g for 10 min. Ammonium sulfate was added to the supernatant at a final concentration of 80%, and the precipitated proteins were discarded. The clear supernatant was dialyzed against 50 mM Tris-HCl (pH7.4) containing 1 mM PMSF and the dialysate was loaded onto a DEAE-TOYOPEARL (TOSOH, Co.) column equilibrated with 50 mM Tris-HCl, pH7.4. The SUMO-1 protein was eluted from the column at 50 mM NaCl. The purified SUMO-1 was biotinylated as described previously (21). The recombinant GST-tagged proteins were bound to glutathione Sepharose 4B resin (Pharmacia, Co.), washed twice with a buffer containing 10 mM Tris-HCl (pH 7.4), 3 mM $MgCl_2$, 0.01% Brij 35, and 1 mM PMSF, and then eluted from the resin with 5 mM glutathione, pH 8.0. The eluted proteins were dialyzed against 10 mM Tris-HCl, pH 7.4.

In vitro SUMO-1 modification assay. For detecting of the activity of the SUMO-1 activating enzyme, Sf-9 cell lysates expressing recominant protein(s) were incubated at 25°C for 30 min in the presence of 50 mM Tris-HCl (p7.4), biotinylated SUMO-1 (G), 5 mM ATP, 10 mM $MgCl_2$, and 0.2 mM dithiothreitol (DTT). When RanGAP1 modification by SUMO-1 was tested, purified RanGAP1, Sua1p/hUba2p, and hUbc9 were incubated at 25°C for 30 min in the presence of 50 mM Tris-HCl (pH 7.4), biotinylated SUMO-1 (G), 5mM ATP, 10 mM $MgCl_2$, and 2 mM DTT. After incubation, the GST or GST-tagged protein was retrieved by glutathione sepharose 4B resin and washed twice with a buffer containing 10 mM Tris-HCl (pH7.4), 3 mM $MgCl_2$, 0.01% Brij 35, and 1 mM PMSF, and then the proteins bound to the resin were electrophoresed in SDS-polyacrylamide gel and transferred to a PVDF membrane (Millipore, Co.). The biotinylated proteins in the membrane were detected by use of peroxidase- conjugated avidin (Extravidin, Sigma, Co.) and the ECL method (Amersham, Co.) as described previously (21, 22). The proteins in the gel were stained with Coomasie brilliant blue R-250 or with silver staining (Silver Stain Plus, Bio-Rad).

Cell culture and preparation of cell lysate. HeLa S3 cells were cultured in DMEM medium supplemented with 10% newborn bovine serum. HeLa cell lysate was prepared as follows. Logarithmically growing cells (2×10^7) were suspended in 1 ml of a buffer containing 10 mM Tris-HCl (pH 7.4), 3 mM $MgCl_2$, 0.1% NP40, and 1 mM PMSF and disrupted by sonication. Then the cell suspension was centrifuged at 12, 000g for 10 min, and the supernatant was taken and used as lysate.

RESULTS

Cloning of SUMO-1 activating enzyme. The Smt3 protein in the budding yeast *Saccharomyces cerevisiae* seems to be a homolog of mammalian SUMO-1, and

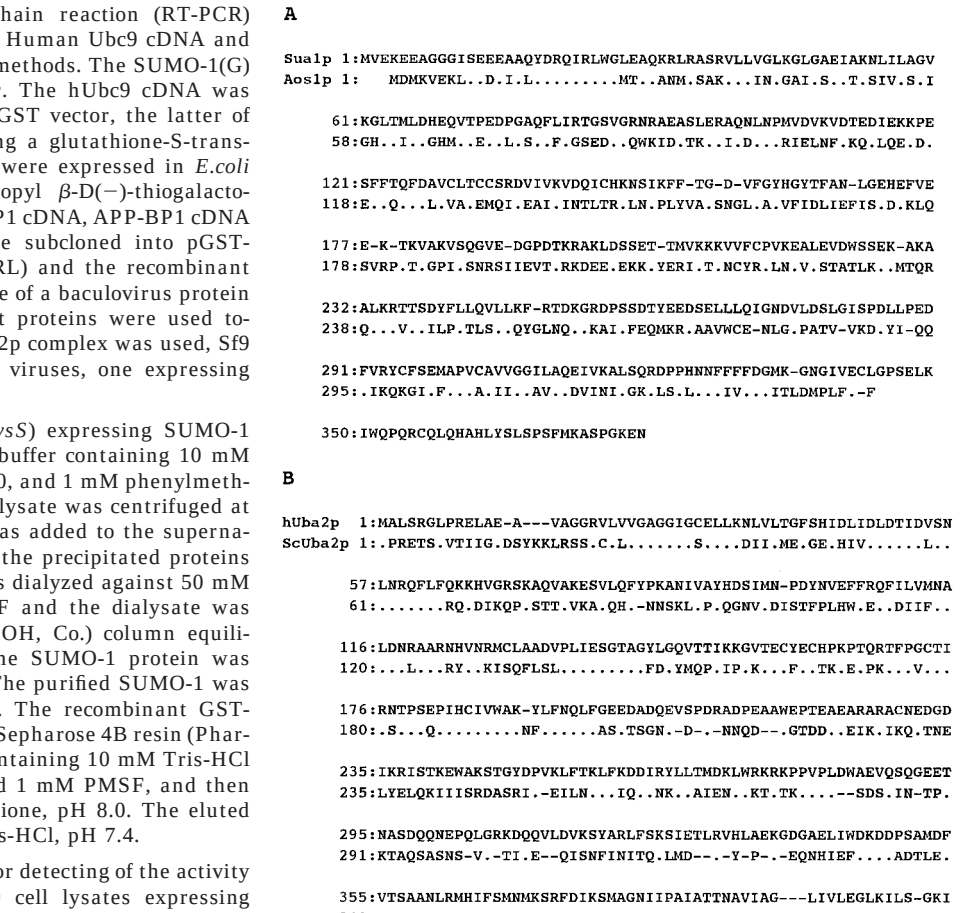

FIG. 1. Amino acid sequence alignment of Sua1p and Aos1p, and that of hUba2p and ScUba2. (A) Sequence alignment of human Sua1p (SUMO-1 activating enzyme) and budding yeast Aos1p. Dots stand for identical residues; and dashes, for gaps. Percentage of identical residues is 33%. (B) Sequence alignment of human Uba2p and budding yeast ScUba2p. Percentage of identical residues is 35%.

Smt3 activating enzyme is a heterodimer of Aos1p and Uba2p (17). When the EST public data base (GenBank) was searched by the Blast Similarity Search Program to find mammalian genes homologous to *Aos1* or *Uba2*, the clone A (GenBank accession number AA236737) or the clone B (GenBank accession number N40237) had high similarity to *Aos1* or *Uba2*, respectively. Using these two EST clones as probes, 3×10^6 phages of HeLa cDNA library were screened. Seventeen positive

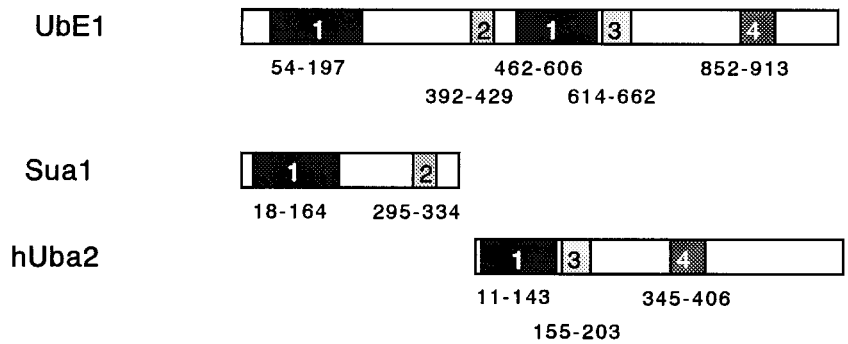

FIG. 2. Schematic representation of the similarity of domains between human E1 (UbE1) (30) and Sua1p, hUba2p. The putative active cyteine residues are located within box 3. The similarity boxes correspond to those of Johnson et al. (17).

clones and 4 positive clones were obtained for EST clone A and EST clone B, respectively. The longest clone obtained for each gene was sequenced. The lengths for the clone A* obtained using clone A as probe and the clone B* obtained using clone B as probe were 2046bp and 2648bp, respectively. When the amino acid sequences deduced from the DNA sequences of clone A* and clone B* were alined with those of *Aos1* and *Uba2*, clone A* showed 33% identity to *Aos1* and clone B*, 35% identity to *Uba2* (Fig. 1). Judging from the sequence data, clone A* seemed to be a human homolog of yeast *Aos1*, and the clone B*, that of *Uba2*. Thus, the clones A* and B* were named SUMO-1 activating enzyme 1 (*Sua1*) and human Uba2 (*hUba2*), respectively. Sua1p and hUba2p are thought to work as N-terminal domain and C-terminal domain, respectively, of the ubiquitin activating enzyme, E1 (Fig. 2).

Detection of activity of SUMO-1 activating enzyme. The cDNAs corresponding to open reading frames were subcloned into pFastBac vectors and the proteins were expressed in Sf-9 cells by means of the baculovirus protein expression system as described in Materials and Methods. Using the recombinant proteins, we tested the binding of SUMO-1 (G) protein to hUba2p. GST-Sua1p migrated at molecular weight 65K and 6xHis-tagged hUba2p did so at 97K in the SDS-7.5% polyacrylamide gel. As shown in Fig. 3A, GST-tagged hUba2p formed a complex with 6xHis-tagged Sua1p. The hUba2p in the complex bound SUMO-1 (G) but monomeric hUba2p or Sua1p did not. The APP-BP1 protein (20, 23), which works as the activating enzyme of Nedd8, another ubiquitin-like protein, by forming a complex with hUba3 protein, was also expressed in Sf-9 cells. Neither APP-BP1 nor GST formed a complex with hUba2p (data not shown).

Figure 4 shows that recombinant purified hUbc9p works as SUMO-1 conjugating enzyme to conjugate SUMO-1 in the presence of purified Sua1p/hUba2p (lane 3). Mutant hUbc9p (C93A) did not bind SUMO-1(G) in this *in vitro* system (lane 4), indicating that cysteine residue 93 must be a binding site of SUMO-1, as was shown in yeast Ubc9p (12). Actually, the binding of SUMO-1 (G) to hUbc9p was sensitive to dithiothreitol treatment, indicating that it occurs through a thioester bond (lane 5). In this system hUbc9p could not use ubiquitin in place of SUMO-1 (data not shown).

In vitro SUMO-1 modification of RanGAP1 using purified proteins. As shown above, the complex of Sua1p and Uba2p seems to be SUMO-1 activating enzyme and hUbc9, the SUMO-1 conjugating enzyme. If the SUMO-1 conjugation system is considered to be similar to the ubiquitination system, what is SUMO-1 ligase? In the *in vitro* SUMO-1 conjugation system so far reported, the cell lysate (e.g., HeLa cell lysate) was used as the source of SUMO-1 activating enzyme and SUMO-1 ligase. Here we tried to use a purified system to find out whether or not SUMO-1 ligase is necessary for RanGAP1 modification. The GST-Sua1p/hUba2p expressed in Sf-9 cells by the baculovirus expression system and GST-hUbc9p expressed in E.coli were purified by use of glutathione Sepharose 4B resin and eluted from the resin by use of glutathione. GST-RanGAP1 was purified by glutathione Sepharose 4B resin, and the RanGAP1 bound to the resin was used as the acceptor protein of SUMO-1. The SDS-7.5% polyacrylamide gel stained by Coomassie brilliant blue R-250 is shown in Figure 5A. Extra protein bands were hardly detected except for the glutathione S-transferase that originated from the Sf-9 insect cells. In the *in vitro* system using SUMO-1 (G), Sua1p/Uba2p, hUbc9 and GAP-1 as proteins, the RanGAP1 was modified by SUMO-1 as efficiently as by the HeLa cell lysate, which was used in place of Sua1p/Uba2p. This reaction was dependent on the existence of both Sua1p/Uba2p and hUbc9, though very faint modification was observed without hUba9 (Fig. 5 B, lane 3). These data clearly indicate that the modification of RanGAP1 by SUMO-1 did not require the third enzyme, rather only Sua1p/hUba2 and hUbc9p.

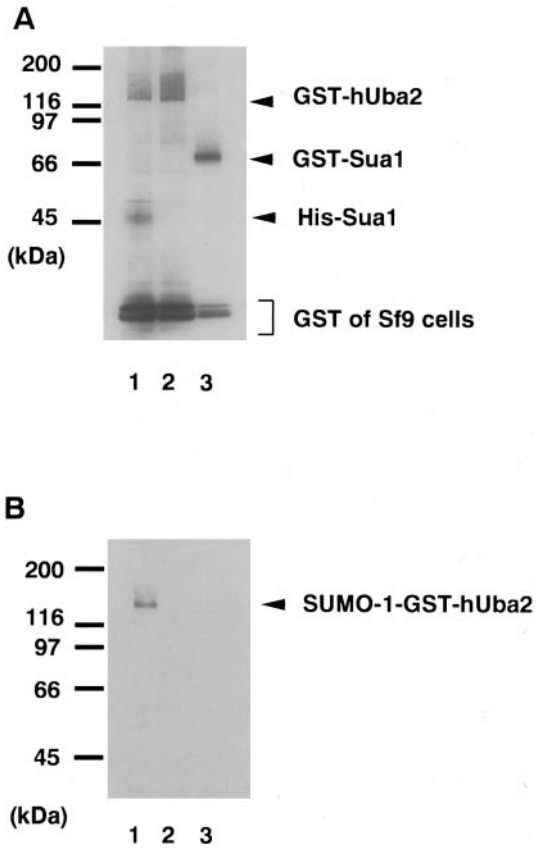

FIG. 3. Sua1p and hUba2p form a complex, and the complexed hUba2p binds SUMO-1. Sf-9 cell lysates expressing various recombinat proteins were incubated as described in Materials and Methods. After incubation, GST or GST-tagged proteins were retrieved by glutathione Sepharose 4B resin, and the *in vitro* SUMO-1 modification assay was performed as described in Materials and Methods. (A) Staining pattern of the SDS-polyacrylamide gel by silver staining. The two fastest migrating proteins are GSTs that originated from Sf-9 cells. Lane 1, GST-hUba2p/His-Sua1p; lane 2, GST-hUba2p; lane 3, GST-Sua1p; (B) ECL detection of SUMO-1 (G) binding. The binding of SUMO-1 (G) to the proteins in (A) was tested by use of biotinylated SUMO-1 (G). Lane 1, the hUba2p in the complex with Sua-1 bound biotinylated SUMO-1 (G) protein; lanes 2, 3; no binding of SUMO-1 was detected.

DISCUSSION

The modification of proteins by SUMO-1 protein was recently discovered as new protein modification. The modified proteins by SUMO-1 reported so far are RanGAP1 (4, 5), PML (9, 10), S-200 (9), and I κ B α (24). Furthermore, many proteins were shown to interact with Ubc9 in the yeast two hybrid system. These proteins include Fas (25, 26), Rad 51 (27), Rad 53, and so on (28). Though SUMO-1 is a protein having homology to ubiquitin, the modification does not seem to be related to the degradation of protein as in the case of ubiquitin. The SUMO-1 modification may have diverse functions. The RanGAP1 was changed conformationally by the modification of SUMO-1, resulting in its ability to bind RanBP2 protein (4, 6, 28). PML became localized in the nucleus after its modification by SUMO-1 (10). I κ B α was modified at the same lysine residue by SUMO-1 and ubiquitin. Once its modification by SUMO-1 took place, ubiquitination could not take place at the same residue (24). In this case, the modification by SUMO-1 resulted in the inhibition of ubiquitination.

Since the SUMO-1 is a protein with homology to ubiquitin, the conjugation system for the targeted protein might be homologous. Actually full-length SUMO-1 cannnot conjugate to the protein; but SUMO-1 (G) can, which is the truncated form of SUMO-1 having glycine at its C-terminus (5). Ubiquitin also has a glycine residue at its C-terminus. The ubiqutination system requires three steps using ubiquitin activating enzyme, E1, ubiquitin conjugating enzyme, E2, and ubiquitin ligase, E3, with one used at each step (7). SUMO-1 modification may have these three steps. The amino acid residue at the carboxyl terminus of ubiquitin is glycine, and ubiquitin is conjugated to a protein through this glycine residue as described above. However, the amino acid residue at the carboxyl terminus of SUMO-1 is tyrosine, and this form of SUMO-1 cannot conjugate to proteins (5). However, the truncated form (SUMO-1(G)), which has a glycine residue as the carboxyl teminus, is active (5). Therefore we used recombinant SUMO-1 (G) in all experiments reported here.

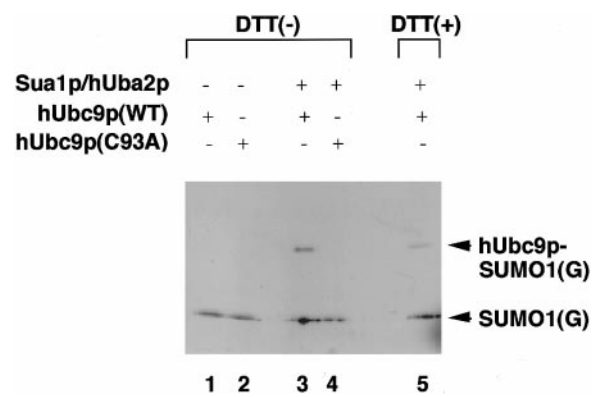

FIG. 4. Detection of the activity of SUMO-1 activating enzyme of Sua1p/hUba2p complex. *In vitro* SUMO-1 modification assay by use of purified proteins was performed as described in Materials and Methods. The conjugation of biotinylated SUMO-1 to the protein was detected by ECL method. The reaction mixture contained biotinylated SUMO-1 and lane 1, hUbc9p (wild); lane 2, hUbc9p (C93A); lane 3, Sua1p/hUba2p and hUbc9p (wild); lane 4, Sua1p/hUba2p and hUbc9p (C93A); lane 5, Sua1p/hUba2p and hUbc9p (wild). The samples in lane 1 through 4 were not treated with dithiothreitol (DTT) before being loaded onto the SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The sample in lane 5 was treated with 0.1M DTT before loading to SDS-PAGE. When Sua1p/hUba2p and hUbc9p (wild) were present, SUMO-1 modification of hUbc9p occurred (lane 3). When the sample was treated with DTT, the SUMO-1 modification of hUbc9p decreased, indicating that SUMO-1 bound hUbc9p through a thioester bond. The hUbc9p (C93A) did not bind SUMO-1.

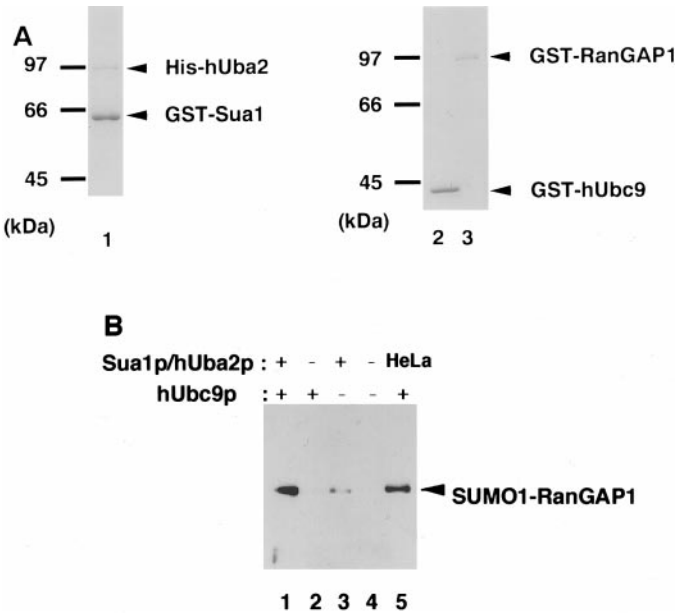

FIG. 5. SUMO-1 modification of RanGAP1 using purified proteins. (A) The purified GST-Sua1p/hUba2p (lane 1), GST-hUbc9 (lane 2) and GST-RanGAP1 (lane 3) were subjected to SDS-7.5% polyacrylamide gel electrophoresis and stained with Coomasie brilliant blue R-250. (B) By use of the proteins shown in (A), *in vitro* SUMO-1 modification of RanGAP1 was performed in the presence of both Sua1p/Uba2p and hUbc9p (lane 1), in the presence of hUbc9p (lane 2), in the presence of Sua1p/hUba2p (lane 3). In lane 4, neither Sua1p/hUba2p nor hUbc9p was present. In lane 5, HeLa cell lysate was used in place of Sua1p/hUba2p in the presence of hUbc9p.

The hUbc9 is very homologous to ubiquitin conjugating enzyme and is thought to be a SUMO-1 conjugating enzyme (14). Here we showed that the SUMO-1 activating enzyme is a complex of Sua1p/hUba2p, which covers the domains of the ubiqutin activating enzyme, E1 (Fig. 2). *Sua1* and *hUba2* are homologs of yeast *Saccharomyces cerevisiae Aos1* and*Uba2*, respectively, which seem to be the Smt3 activating enzyme (17). Smt3 is a homolog of SUMO-1 (18). A similar complex, APP-BP1/hUba3p, is thought to be involved in the modification by Nedd8 (23), another homolog of ubiquitin. Yeast *Saccharomyces cerevisiae* homolog of NEDD8, Rub1, is also activated by a complex with ULA1p and Uba3p (29). The third enzyme involved in ubiquitination is ubiquitin ligase. In the *in vitro* reconstituted system reported here, RanGAP1 was modified by SUMO-1 in the presence of Sua1p/hUba2p and hUbc9p. This system did not require any SUMO-1 ligase protein. Since the hUbc9p can bind RanGAP1, it must work as SUMO-1 ligase in this system. Finally SUMO-1 modification requires Sua1p/hUbap, hUbc9p, SUMO-1(G), and the protein to be modified. Since the SUMO-1(G) protein must be produced by proteolytic cleavage from SUMO-1 *in vivo*, the responsible proteinase may be an important key enzyme to regulate the modification of protein(s) by SUMO-1. Identification of the endoproteinase for SUMO-1 is important issue to be clarified to know the precise mechanism of regulation of protein modification by SUMO-1.

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