

# Molecular Characterization of the SUMO-1 Modification of RanGAP1 and Its Role in Nuclear Envelope Association

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**Abstract.** The mammalian guanosine triphosphate (GTP)ase-activating protein RanGAP1 is the first example of a protein covalently linked to the ubiquitin-related protein SUMO-1. Here we used peptide mapping, mass spectroscopy analysis, and mutagenesis to identify the nature of the link between RanGAP1 and SUMO-1. SUMO-1 is linked to RanGAP1 via glycine 97, indicating that the last 4 amino acids of this 101-amino acid protein are proteolytically removed before its attachment to RanGAP1. Recombinant SUMO-1 lacking the last four amino acids is efficiently used for modification of RanGAP1 in vitro and of multiple unknown proteins in vivo. In contrast to most ubiquiti-

nated proteins, only a single lysine residue (K526) in RanGAP1 can serve as the acceptor site for modification by SUMO-1. Modification of RanGAP1 with SUMO-1 leads to association of RanGAP1 with the nuclear envelope (NE), where it was previously shown to be required for nuclear protein import. Sufficient information for modification and targeting resides in a 25-kD domain of RanGAP1. RanGAP1-SUMO-1 remains stably associated with the NE during many cycles of in vitro import. This indicates that removal of RanGAP1 from the NE is not a required element of nuclear protein import and suggests that the reversible modification of RanGAP1 may have a regulatory role.

**G**TP hydrolysis by the Ras-related GTPase Ran is essential for transport of proteins into the nucleus (Melchior et al., 1993a; Moore and Blobel, 1993; for reviews on transport see Melchior and Gerace, 1995; Görlich and Mattaj, 1996; Nigg, 1997). Several different models involving one or multiple rounds of GTP hydrolysis by Ran in various regions of the nuclear pore complex (NPC)<sup>1</sup> have been proposed (e.g., Melchior et al., 1995; Rexach and Blobel, 1995; Görlich and Mattaj, 1996; Koepf and Silver, 1996). Like many other GTPases, Ran cannot hydrolyze GTP by itself at a physiologically significant rate and requires interaction with a GTPase activating protein for this to occur (Bourne et al., 1990). Therefore, the localization of the GTPase activating protein determines where GTP hydrolysis by Ran can take place. The only known GTPase-activating protein for Ran (Becker et al., 1995; Bischoff et al., 1995), known as RanGAP1 in higher eukaryotes and as Rna1p in yeast, has been directly linked to the import of proteins into the nucleus in mammalian cells (Mahajan et al., 1997) and *Saccharomyces cerevisiae* (Cor-

bett et al., 1995). Yeast Rna1p is localized predominantly in the cytoplasm, as judged by cell fractionation and immunolocalization (Hopper et al., 1990; Melchior et al., 1993b). However, yeast Rna1p may interact, at least temporarily, with the NPC, since some enrichment of Rna1p around the nuclear envelope (NE) has been observed by immunofluorescent staining in *S. cerevisiae* and *S. pombe* (Hopper et al., 1990; Melchior et al., 1993b) and particularly in the temperature-sensitive *S. cerevisiae* strain *rna1-1* (Koepf et al., 1996). In contrast, mammalian RanGAP1 is localized predominantly at the NE, where it forms a stable complex with the NPC protein RanBP2/Nup358 (Wu et al., 1995; Yokoyama et al., 1995; Matunis et al., 1996; Mahajan et al., 1997). Interestingly, the interaction of RanGAP1 with RanBP2 requires the posttranslational modification of RanGAP1 with SUMO-1 (Mahajan et al., 1997), a small ubiquitin-related protein that we and others recently identified under the names Pic1, GMP1, Sentrin, Ubl1, and SUMO-1, respectively (Boddy et al., 1996; Matunis et al., 1996; Okura et al., 1996; Shen et al., 1996a; Mahajan et al., 1997). The finding that the modification of RanGAP1 is reversible in cell extracts (Matunis et al., 1996; Mahajan et al., 1997), raises the possibility that the localization of RanGAP1 at RanBP2 may be a dynamic process involved in the mechanism or regulation of nuclear protein import.

Ubiquitination is the covalent attachment of ubiquitin to proteins via an isopeptide bond between the COOH-terminal carboxyl group of ubiquitin and the  $\epsilon$ -amino group

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1. *Abbreviations used in this paper:* GST, glutathione-S-transferase; HA, hemagglutinin; NE, nuclear envelope; NPC, nuclear pore complex; RT, room temperature; wt, wild type.

of lysines in the acceptor protein (reviewed in Hershko and Ciechanover, 1992; Ciechanover, 1994; Hochstrasser, 1995, 1996; Jennissen, 1995; Jentsch and Schlenker, 1995). This coupling reaction is ATP dependent and involves a series of enzymatic reactions in which ubiquitin is first activated by formation of a thioester bond with an E1 enzyme. The activated ubiquitin is then sequentially transferred to an E2 enzyme, to a substrate-recognizing E3 enzyme, and finally to the acceptor protein. The modification is reversible, because the ubiquitin moiety can be removed from the acceptor molecule by deubiquitinating enzymes.

Modification of proteins with ubiquitin is best known for its ability to mark substrates for regulated protein degradation by a complex called the 26S proteasome (reviewed by Ciechanover, 1994; Hochstrasser, 1995, 1996; Jentsch and Schlenker, 1995; Hilt and Wolf, 1996). Commitment of a target protein to the degradation pathway involves assembly of a polyubiquitin chain on the target, usually via isopeptide bonds between lysine 48 of one ubiquitin and the COOH-terminal glycine residue of the neighboring ubiquitin. However, it is becoming increasingly evident that the role of the ubiquitin modification is not limited to the targeting of proteins to the proteasome. For example, ubiquitination of a yeast plasma membrane receptor signals its ligand-stimulated endocytosis (Hicke and Riezman, 1996), and ubiquitination has been shown to activate a protein kinase involved in I $\kappa$ B $\alpha$  processing (Chen et al., 1996).

Recent findings suggest that ubiquitin is part of a family of related proteins involved in the covalent modification of proteins. A number of proteins related to ubiquitin (identities >35%) have been isolated over the last several years (e.g., see Kumar et al., 1993; Watkins et al., 1993; Biggins et al., 1996), among them the 15-kD interferon-inducible, ubiquitin cross-reacting protein UCRP/ISG15 (Haas et al., 1987). This protein is conjugated to unknown proteins and may serve as a *trans*-acting binding factor that directs the association of modified target proteins to intermediate filaments (Loeb and Haas, 1994). More recently, we and others identified a protein that is only very distantly related to ubiquitin (18% identical), but is nevertheless used for covalent modification. We found that this protein, which we named SUMO-1 (for small ubiquitin-related modifier), is covalently linked to RanGAP1, the GTPase-activating protein for Ran (Matunis et al., 1996; Mahajan et al., 1997). Two additional mammalian SUMO-1-related proteins have been identified in DNA-sequencing projects (Mannen et al., 1996; Lapenta et al., 1997), and the *S. cerevisiae* SMT3 protein (52% identical to SUMO-1) has been identified as a multicopy suppressor of the centromere protein Mif2 (Meluh and Koshland, 1995).

Antibodies raised against SUMO-1 recognize numerous proteins in buffalo rat liver cell extracts and isolated rat liver nuclei in addition to RanGAP1 (Matunis et al., 1996; Mahajan et al., 1997), suggesting that SUMO-1 is coupled to additional proteins. Candidate proteins are the PML protein that is linked to promyelocytic leukemia (Boddy et al., 1996), the Fas/Apo receptors involved in programmed cell death (Okura et al., 1996), and Rad51 and Rad52 that play a role in DNA repair (Shen et al., 1996), because they each interacted with SUMO-1 when used as bait in two-hybrid interaction screens. Taken together, these findings suggest

that SUMO-1, and possibly SUMO-1-related proteins, may posttranslationally modify a number of proteins.

Insofar as RanGAP1 is the first known substrate for modification by SUMO-1, biochemical and functional characterization of the RanGAP1-SUMO-1 conjugate is likely to provide a paradigm for other SUMO-1 substrates. In this study, we characterized the molecular nature of the link between RanGAP1 and SUMO-1. We found that lysine 526 in the COOH-terminal tail domain of RanGAP1 is linked to glycine 97 of SUMO-1, indicating that despite the low homology of SUMO-1 to ubiquitin, the characteristic biochemistry of the link is conserved. Mutation of lysine 526 to arginine completely abolishes modification, indicating that only a single lysine residue in RanGAP1 is available for modification with SUMO-1. We have also identified a domain within RanGAP1 that is sufficient both for modification by SUMO-1 and for targeting to the NE, and have demonstrated that modified RanGAP1 remains stably associated with the NE during the course of many cycles of nuclear protein import.

## Materials and Methods

### Immunoprecipitation and Peptide Analysis of SUMO-1-modified RanGAP1

RanGAP1 was immunoprecipitated from solubilized rat liver NEs as described (Mahajan et al., 1997). Antigen-antibody complexes were separated by SDS-PAGE and electroblotted onto polyvinylidene difluoride membrane. Unmodified and modified RanGAP1 bands were visualized with Ponceau-S stain and cut out of the membrane. Tryptic digestion and subsequent analysis of the individual bands was performed by Dr. J. Leszyk at the Worcester Foundation for Biomedical Research (Shrewsbury, MA). Proteins were digested in situ with trypsin in a digest buffer containing 100 mM ammonium bicarbonate, 10% acetonitrile, and 1% hydrogenated Triton X-100 (Fernandez et al., 1994). The digest mixture was separated on a 1  $\times$  250-mm microbore C8 column (Applied Biosystems, Inc., Foster City, CA) on a modified HPLC system (1090 M; Hewlett-Packard Co., Palo Alto, CA). Peptides were eluted using a linear gradient from 100% solvent A (0.1% trifluoroacetic acid in water) to 55% solvent B (0.08% trifluoroacetic acid in acetonitrile/water 70:30) in 30 min at a flow rate of 150  $\mu$ l/min. The eluent was monitored at 210 nm and fractions were collected manually. A 0.5- $\mu$ l aliquot of each peptide fraction was subjected to matrix-assisted laser desorption time-of-flight mass spectrometry (MALDI-TOF) using a Linear Biospectrometry Workstation (Perseptive Biosystems, Cambridge, MA) and alpha cyano-4-hydroxy cinnamic acid as the matrix. The instrument was calibrated with an external standard that consisted of angiotensin (MH<sup>+</sup> of 1,297.5 D) and Adrenocorticotropic hormone fragment (amino acids 18-39; MH<sup>+</sup> of 2,466.7 D). Edman sequence analysis was performed on a 494 Procise protein sequencer (Applied Biosystems, Inc.).

### DNA Cloning and Mutagenesis

SUMO-1 COOH-terminal deletion mutants were generated by PCR from SUMO-1 cDNA (Mahajan et al., 1997) and cloned either into pGEX-2T (Pharmacia Biotech, Inc., Piscataway, NJ) for recombinant expression and purification, or into pHHS10B (Furukawa and Hotta, 1993) for cytomegalovirus promoter-driven expression of NH<sub>2</sub>-terminal hemagglutinin (HA)-tagged proteins in transfected eukaryotic cells. Mouse RanGAP1 (fug1; DeGregori et al., 1994) cDNA constructs representing the NH<sub>2</sub>-terminal conserved domain (amino acids 1-416; RanGAP1 body; see Fig. 4) and the COOH-terminal tail domain present only in RanGAP proteins from higher eukaryotes (amino acids 400-589; RanGAP1 tail; see Fig. 4) were generated by PCR from fug1 cDNA (Mahajan et al., 1997). A single-point mutation to convert lysine 526 to arginine (K526R) was introduced into both wild-type (wt) RanGAP1 as well as into the RanGAP1 tail domain using PCR-based, site-directed mutagenesis (Higuchi, 1990). Mutagenesis reaction products were cloned into pCRII-TA vector (Invitrogen Corp., Carlsbad, CA) and sequenced at the Scripps Research Institute core facil-

ity. Wt and K526R RanGAP1, body, tail, and K526R tail were cloned into pHH10B for HA-tagged eukaryotic expression. In addition, wt and K526R RanGAP1 were cloned into the prokaryotic expression vector pET-23b vector (Novagen, Inc., Madison, WI) to express NH<sub>2</sub>-terminal T7-tagged recombinant proteins in bacteria.

### Expression and Purification of Recombinant Proteins

RanGAP1 and Gst-GAP-tail were expressed and purified as described (Mahajan et al., 1997). Wt and mutant T7-tagged RanGAP1 were expressed in *E. coli* BL21 (DE3) by induction with 0.5 mM IPTG at 30°C for 3 h. Bacteria were harvested by centrifugation, frozen once at -80°C, and resuspended to 1/20 the original culture volume in Buffer 1 (50 mM Tris-Cl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, 1 µg/ml each of leupeptin, pepstatin A, and aprotinin). After addition of 1 mg/ml lysozyme and incubation on ice for 1 h, the lysate was centrifuged at 100,000 g for 30 min. The supernatant was used for in vitro shift assays. Recombinant wt SUMO-1 and SUMOΔC4 were expressed and purified as glutathione-S-transferase (GST) fusion proteins as described for GST-SUMO-1 (Mahajan et al., 1997). Pure SUMO-1 and SUMOΔC4 were obtained by thrombin digestion of the GST-fusion proteins on glutathione beads according to the manufacturer's instructions, followed by collection of the subsequent low speed supernatant.

### Cell Culture and Transient Transfection of Cos-7 Cells

Cos-7 cells were grown in DMEM medium containing 10% FBS (HyClone, Logan, UT) and antibiotics. Transfection of cells with various plasmid constructs was carried out using the Superfect Transfection reagent (QIAGEN Inc., Chatsworth, CA) according to the manufacturer's instructions. Transfections were performed on cells seeded into 6-well tissue culture plates 12–15 h before use at a density of 2–3 × 10<sup>5</sup> cells per well. For immunofluorescence, cells were plated onto flame-sterilized cover slips. Cells were typically transfected with 2 µg of endotoxin-free DNA, incubated with the DNA suspension for 2–3 h, replenished with fresh medium and analyzed 24–48 hours later.

### Immunoblotting, Indirect Immunofluorescence Analysis, and Flow Cytometry

For Western blot analysis, transfected cells were washed once with PBS and then scraped directly in 250 µl hot 2× Laemmli buffer. Samples were boiled for 3 min, separated on SDS-polyacrylamide gels, and transferred to nitrocellulose membranes. Heterologously expressed HA-tagged proteins were identified on immunoblots by probing with an α-HA mouse monoclonal antibody (BabCO, Richmond, CA) diluted 1:2,000 in 5% milk powder in PBS, 0.2% Tween-20. Detection was by enhanced chemiluminescence (Pierce Chemical Co., Rockford, IL). Immunoblots of in vitro modification reactions were assayed using either 0.5 µg/ml α-RanGAP1 antibodies to detect recombinant untagged RanGAP1 (Mahajan et al., 1997) or an α-T7 tag mouse monoclonal antibody (Novagen, Inc.) used at 1:10,000 to detect T7-tagged wt and mutant RanGAP1. For indirect immunofluorescence using light microscopy, transfected Cos-7 cells were fixed on coverslips with 3.7% formaldehyde in PBS, 1 mM MgCl<sub>2</sub> and processed as described by Melchior et al., 1995. Expressed HA-tagged proteins were detected using an α-HA monoclonal antibody (BabCO) at a 1:1,000 dilution. Digitonin permeabilization before fixation involved incubation of the cells with 0.005% digitonin in transport buffer (110 mM KOAc, 20 mM Hepes, pH 7.3, 2 mM Mg (OAc)<sub>2</sub>, 0.5 mM EGTA, 2 mM DTT, 1 µg/ml each of leupeptin, pepstatin, and aprotinin) for 5 min on ice, and two subsequent washes. For subsequent incubation under nuclear protein import conditions the permeabilized cells were supplemented with 2.5 mg/ml HeLa cytosol, an ATP regenerating system, and FITC-BSA conjugated with peptides containing the SV-40 wt nuclear localization signal, and incubated in a humid chamber at 30°C or on ice (Adam et al., 1992). After 30 min the cells were fixed with 3.7% formaldehyde in transport buffer, permeabilized with 0.2% triton, and processed for indirect immunofluorescence as described above. For flow cytometric analysis, the cells were trypsinized to bring them into suspension, permeabilized with digitonin, and subsequently incubated in the presence of 2.5 mg/ml HeLa cytosol and ATP for 30 min. After 30 min the cells were fixed with 3.7% formaldehyde in transport buffer, stained with α-HA mouse monoclonal antibody (BabCO) and Cy5-conjugated secondary antibodies (Amersham Corp., Arlington Heights, IL), and analyzed using a FACSort® flow cytometer (Becton Dickinson, Mountain View, CA).

### In Vitro Modification of RanGAP1

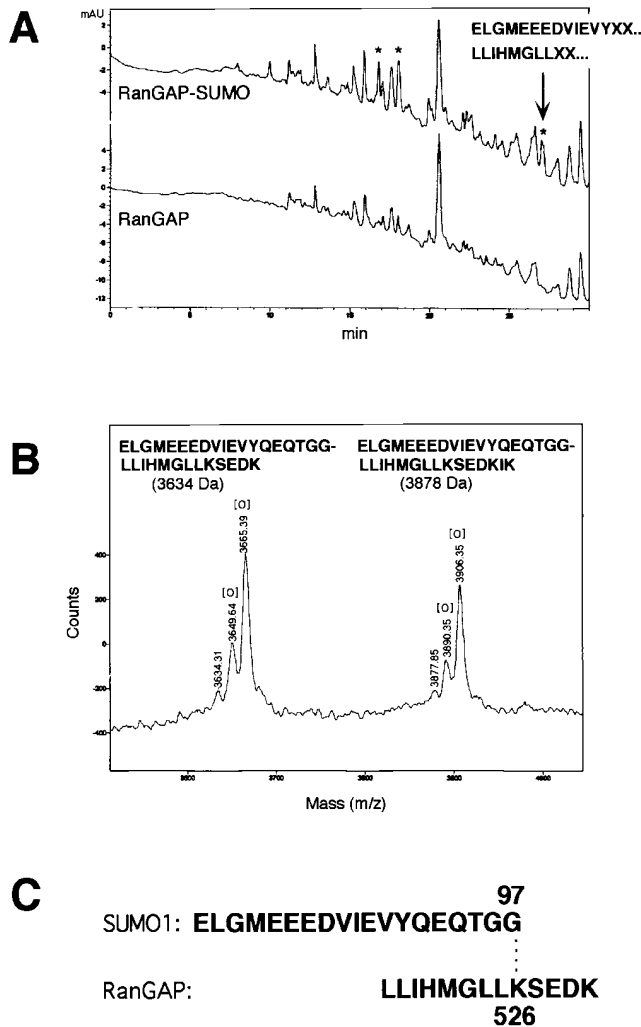
In vitro modification assays were performed as described (Mahajan et al., 1997). In brief, HeLa suspension cells were permeabilized with 0.07% digitonin in transport buffer at 4 × 10<sup>7</sup> cells/ml. This digitonin extract was supplemented with 1 mM ATP and the respective recombinant proteins or bacterial lysates and then allowed to incubate for 10 min at room temperature (RT). To examine the ability of various recombinant SUMO-1 proteins to modify RanGAP1, 1 µg of a given purified SUMO-1 protein was added to a 20-µl modification reaction along with 1 µg recombinant RanGAP1. To test for resistance of the SUMO-1–RanGAP1 bond to hydrolysis, RanGAP1 was shifted with SUMOΔC4 and the pH was either increased to 12 with NaOH or treated with 1% hydrazine at RT for 10 min. For in vitro modification of T7-tagged wt or mutant RanGAP1, 5 µl of the bacterial lysate from cells expressing either construct (see above) were added to 20 µl digitonin-extract in the presence of 1 mM ATP. Reactions were stopped by addition of 2× Laemmli buffer, and analyzed by immunoblotting.

## Results

### Identification of the Biochemical Link between RanGAP1 and SUMO-1

RanGAP1 is presently the only characterized target for modification by SUMO-1 (or by any of the other proteins distantly related to ubiquitin). To better understand the mechanism and regulation of this modification, we decided to map the residues involved in the attachment of SUMO-1 to RanGAP1. By analogy to ubiquitin, it seemed plausible that modification of RanGAP1 by SUMO-1 occurred via formation of an isopeptide bond between a COOH-terminal amino acid of SUMO-1 and the ε-amino group of a lysine in RanGAP1 (see introduction). However, considering the extremely low homology of SUMO-1 to ubiquitin (18% identity), alternative links also seemed possible, particularly since ubiquitin itself seems capable of forming alternative nonlysine links (Hodgins et al., 1996).

To identify the link between RanGAP1 and SUMO-1, we carried out peptide analysis. For this, modified and unmodified RanGAP1 obtained by immunoprecipitation from rat liver NEs were digested with trypsin, and the tryptic fragments were separated via chromatography on a C8 reversed-phase HPLC column. Fig. 1 A shows a comparison of chromatographic profiles of RanGAP1–SUMO-1 (*top line*) and unmodified RanGAP1 (*bottom line*). When chromatographic peaks unique to the RanGAP1–SUMO-1 conjugate were subjected to micropeptide sequencing, we found that one peak that migrated with a retention time of 27 min (Fig. 1 A, *arrow*) apparently contained two peptides at an equimolar ratio; one from RanGAP1 and one from SUMO-1. The sequences determined for these two peptides are displayed above the arrow in Fig. 1 A, together with additional residues that could not be unambiguously identified (indicated by an X). Since the SUMO-1 peptide was derived from the COOH-terminal end of SUMO-1, it seemed possible that the other peptide might represent the RanGAP1 region to which it was coupled by an isopeptide bond. If that were the case, one might expect to also see disappearance of a peak corresponding to the unconjugated peptide in the unmodified RanGAP1. Although this was not observed in the profiles shown here, after chromatography on a different column (capillary C18 column) we did indeed identify a peptide with the sequence LLIHMGLLK in the tryptic digest of unconjugated Ran-



**Figure 1.** Identification of a linked peptide between RanGAP1 and SUMO-1. (A) Microbore C8 column profile of tryptic digests of SUMO-1-modified (*top profile*) and unmodified (*bottom profile*) RanGAP1, obtained by immunoprecipitation from solubilized rat liver NEs. Asterisks (\*) indicate peaks unique to the SUMO-1-modified RanGAP1 that contain peptides derived from SUMO-1. Sequencing of the unique peak fraction eluting at ~27 min (*arrow*) revealed the presence of two distinct sequences, one from SUMO-1 (ELGMEEEDVIEVYXX...) and the other from RanGAP1 (LLIHGLLX...). (B) Matrix-assisted laser desorption time of flight (MALDI-TOF) mass spectrometric analysis of the 27-min peak reveals the presence of two peptides and their oxidation products (labeled with [O]) with mass values of 3,634 D (*left peaks*) and 3,877 D (*right peaks*). Based on the peptide sequences obtained in A and on the known protein sequences of RanGAP1 (Bischoff et al., 1995, DeGregori et al., 1994) and SUMO-1 (Mahajan et al., 1997), the observed mass values are consistent with two linked peptides involving the amino acid residues drawn above the peaks. (C) Depiction of the predicted isopeptide bond between the COOH-terminal glycine 97 of SUMO-1 and lysine 526 of RanGAP1.

GAP1 that was absent from conjugated RanGAP1. However, under those conditions the peak containing the potentially conjugated peptide was not resolved.

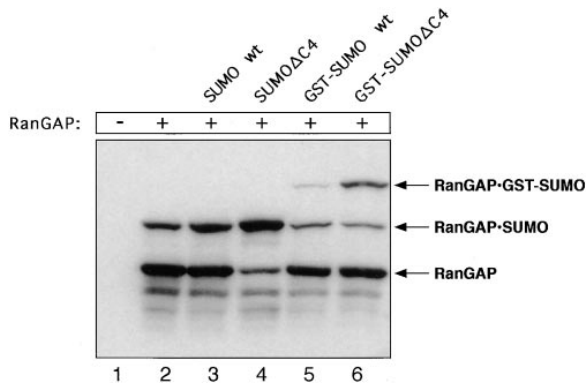
To test whether the coeluting peptides were indeed linked, we performed mass spectroscopy analysis on the

isolated peak fraction (Fig. 1 B). The mass values are significantly larger than those expected for either fragment alone and are consistent with a linked peptide. Our analysis revealed two sets of mass peaks, one with values of 3,634, 3,650, and 3,665 D; and a second with values of 3,878, 3,890, and 3,906 D. The differences in mass among the three peaks in each set suggested that the two larger peaks within a given set (labeled [O] in Fig. 1 B) are methionine-oxidation products of the smaller species that are commonly obtained in gel-purified proteins. By considering the mass values, sequence information, and predicted tryptic products of the two proteins, we determined that the observed mass values correspond to a conjugate containing a single molecule of a SUMO-1 fragment ending at glycine 97 linked via a covalent bond to a RanGAP1 tryptic fragment that includes either residues 518–530 (3,634-D peak) or residues 518–532 (3,877-D peak; Fig. 1 B). Since SUMO-1 is expressed as a 101-amino acid protein that contains four additional COOH-terminal amino acids beyond glycine 97, this demonstrates that SUMO-1 is proteolytically processed before its attachment to RanGAP1. Both RanGAP1 fragments include an internal lysine (K526) that appears to be protected from trypsin digestion, indicating that it may be involved in the formation of an isopeptide bond with SUMO-1. Theoretically, a link could also be formed via an ester bond involving serine 527. However, this possibility was ruled out by the resistance of RanGAP1–SUMO-1 to treatment with either base (pH 12) or 1% hydrazine, conditions that rapidly hydrolyze ester bonds (data not shown).

Taken together, these data strongly suggest that SUMO-1 modifies RanGAP1 via an isopeptide bond between the carboxyl group of glycine 97 of proteolytically processed SUMO-1 and the  $\epsilon$ -amino group of lysine 526 of RanGAP1, as depicted in Fig. 1 C.

### **SUMO-1 Terminating at Glycine 97 Is Fully Competent to Modify RanGAP1 In Vitro**

To confirm that the attachment of SUMO-1 to RanGAP1 involves glycine 97 and not valine 101 of SUMO-1, and to determine whether removal of the last four amino acids of SUMO-1 either precedes or is mechanistically coupled to its attachment to RanGAP1, we prepared recombinant wt SUMO-1 along with a mutant SUMO-1 lacking the COOH-terminal four residues (SUMO $\Delta$ C4). SUMO $\Delta$ C4 terminates in the glycine–glycine motif corresponding to the COOH-terminal end observed in the peptide analysis (Fig. 1 C), and represents the putative end product of the proteolytically processed protein. First, we tested the ability of the SUMO-1 proteins to modify recombinant RanGAP1 in vitro by monitoring their ability to shift the 70-kD RanGAP1 to the 90-kD modified form. Recombinant RanGAP1 was added to a digitonin lysate of HeLa cells in the presence of ATP and bacterially expressed SUMO-1 proteins (Fig. 2). After 10 min at RT, the reaction products were analyzed by immunoblotting with  $\alpha$ -RanGAP1 antibodies. Fig. 2 (lane 2) shows that in the absence of exogenously added SUMO-1, ~30–40% of the recombinant RanGAP1 was converted to the modified 90-kD species by the endogenous SUMO-1. Addition of exogenous recombinant SUMO-1 had only a negligible effect on the



**Figure 2.** SUMO-1 lacking the last four amino acids (SUMO $\Delta$ C4) is a better substrate for modification of RanGAP1 *in vitro* than wt SUMO-1. Digitonin lysates of HeLa cells were mixed with RanGAP1 and ATP, and incubated for 10 min at RT in the absence (lane 2) or presence of wt SUMO-1 (lane 3), SUMO $\Delta$ C4 (lane 4), GST-SUMO-1 (lane 5), or GST-SUMO $\Delta$ C4 (lane 6). Lane 1 contains only HeLa extract. Efficiency of conversion of the 70-kD recombinant RanGAP1 (RanGAP) to a 90-kD SUMO-1-modified form (RanGAP•SUMO) or a ~115-kD GST-SUMO-1-modified form (RanGAP•GST-SUMO) was assayed by immunoblotting with  $\alpha$ -RanGAP1 antibodies.

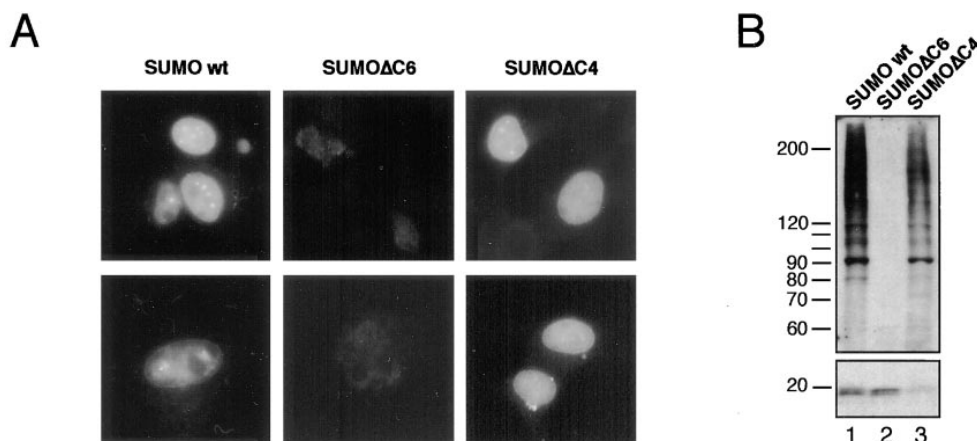
amount of RanGAP1 converted (Fig. 2, lane 3). In contrast, the addition of SUMO $\Delta$ C4 significantly increased the amount of modified RanGAP1 with a concomitant decrease in the amount of unmodified 70-kD RanGAP1 (Fig. 2, lane 4). The effect of SUMO $\Delta$ C4 was even more striking when the shift assays were performed using GST fusions of the two forms of SUMO-1. In this case, the GST-SUMO-1 converts a small amount of RanGAP1 to a unique 115 kD species (Fig. 2, lane 5). Under the same reaction conditions the GST-SUMO $\Delta$ C4 converts significantly more of the 70-kD RanGAP1 to the 115-kD form (Fig. 2, lane 6). These data demonstrate that SUMO $\Delta$ C4 can be used as a substrate for the modifying enzymes *in vitro* and indicate that proteolytic processing of SUMO-1 is not mechanistically coupled to the conjugation reaction.

Moreover, since SUMO $\Delta$ C4 is conjugated to RanGAP1 much more efficiently than wt SUMO-1, the proteolytic removal of the last four amino acids of SUMO-1 seems significantly slower than the modification reaction. It remains to be seen whether this processing activity is higher in different cell extracts.

### **Analysis of COOH-terminal Deletions of SUMO-1 *In Vivo***

To test whether SUMO-1 lacking the last four amino acids (SUMO $\Delta$ C4) is also an efficient substrate for modification of RanGAP1 and possibly other proteins *in vivo*, we expressed a series of HA epitope-tagged SUMO-1 constructs in tissue culture cells. HA-tagged wt SUMO-1 was transfected into Cos-7 cells, and the distribution of exogenously expressed SUMO-1 proteins was detected 24 h after transfection by indirect immunofluorescence microscopy using an  $\alpha$ -HA monoclonal antibody (Fig. 3 A, SUMO wt). HA-tagged SUMO-1 accumulated in intranuclear foci or speckles in addition to a diffuse nucleoplasmic distribution (Fig. 3 A). A distinct nuclear rim localization, consistent with the localization of SUMO-1-modified RanGAP1 (Matunis et al., 1996; Mahajan et al., 1997), could be observed in Cos-7 cells expressing low levels of HA-SUMO-1 (Fig. 3 A, SUMO wt, bottom). The distribution of HA-SUMO-1 was identical to the localization of endogenous SUMO-1 in untransfected cells (Boddy et al., 1996; Matunis et al., 1996; data not shown).

When HA-SUMO $\Delta$ C4 was transfected into Cos-7 cells, its distribution was indistinguishable from wt HA-SUMO-1 (Fig. 3 A, SUMO $\Delta$ C4). A strong nuclear signal was detected in >70% of the cells, with staining found diffusely in the nucleoplasm, nuclear rim, and nuclear speckles as observed for HA-SUMO-1. Cos-7 cells were also transfected with an HA-tagged SUMO-1 construct lacking six amino acids at the COOH terminus (SUMO $\Delta$ C6). This construct lacks the conserved glycine doublet required for conjugation of ubiquitin to its targets and was not expected to be conjugated to target proteins. Under conditions in which >70% of the cells were successfully transfected, SUMO $\Delta$ C6 expression was consistently barely



**Figure 3.** SUMO-1 and SUMO $\Delta$ C4, but not SUMO $\Delta$ C6, modify multiple substrates *in vivo*. (A) Immunofluorescence of Cos-7 cells transfected with HA-tagged SUMO-1 constructs and probed with an  $\alpha$ -HA monoclonal antibody. Intracellular localization of wt SUMO-1 (SUMO wt), SUMO-1 with a six-amino acid COOH-terminal deletion (SUMO $\Delta$ C6), and SUMO-1 terminating at glycine 97 (SUMO $\Delta$ C4) was analyzed. (B) Western blot analysis of SUMO-1-transfected cells. Cos-7 cells were transfected with HA-tagged wt SUMO-1 (lane 1), SUMO $\Delta$ C6 (lane 2), or SUMO $\Delta$ C4 (lane 3), and the cells were lysed in SDS gel loading buffer 24 h after transfection. Samples were electrophoresed on 8% (top) and 12.5% (bottom) polyacrylamide gels, transferred to nitrocellulose, and probed with the  $\alpha$ -HA monoclonal antibody.

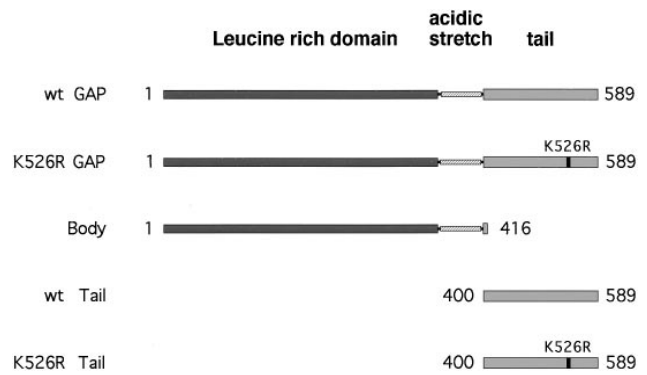
visible by immunofluorescence microscopy (Fig. 3 A, *SUMOΔC6*). What protein was detectable was not enriched in nuclear structures, but seemed to be equally distributed throughout the nucleus and cytoplasm. These findings support the notion that the intranuclear accumulation of SUMO-1 is due to SUMO-1 conjugates rather than to free SUMO-1.

This conclusion was further supported by Western blot analysis of transfected Cos-7 cells with an  $\alpha$  HA antibody (Fig. 3 B). As seen in lane 1 of Fig. 3 B, HA-tagged SUMO-1 in this cell extract was strongly represented by a 90-kD band that comigrated with modified RanGAP1 (data not shown), as well as in a number of higher molecular mass bands. We were also able to detect a band at  $\sim$ 17-kD that presumably represents monomeric HA-SUMO-1. The banding pattern for HA-SUMOΔC4 was very similar to that of the wt SUMO-1, demonstrating that SUMOΔC4 can also be used efficiently by the modification machinery in vivo (Fig. 3 B, lane 3). In contrast, HA-SUMOΔC6 was expressed only in its monomeric form (Fig. 3 B, lane 2). Similar results were recently reported by Kamitani et al. (1997).

Interestingly, the levels of unconjugated HA-SUMO-1 and HA-SUMOΔC6 in the transfected cell extracts were about the same (Fig. 3 B, bottom panel, lanes 1 and 2), even though cells contain at least an order of magnitude more of HA-SUMO-1 due to its presence in protein conjugates. The lack of higher levels of unconjugated HA-SUMOΔC6 may reflect an instability of the latter, or alternatively could indicate that the levels of unconjugated SUMO-1 are tightly regulated.

#### A Single Lysine Residue (K526) in RanGAP1 Is Modified by SUMO-1

Our peptide analysis of the modified RanGAP1 pointed to lysine 526 as a potential acceptor site for SUMO-1 in RanGAP1, since it was the only internal lysine residue in the linked peptide (Fig. 1 C). Lysine 526 resides in the 25-kD tail of RanGAP1, a domain that is unique to RanGAP proteins of higher eukaryotes (Fig. 4). To unequivocally prove the identity of the acceptor site, we mutagenized the RanGAP1 cDNA at a single base to convert lysine 526 to arginine, and expressed wt RanGAP1 and mutated K526R RanGAP1 as T7-tagged proteins in bacteria (RanGAP1 derivatives used in this study are shown in Fig. 4). We then compared the ability of the recombinant proteins to be modified by SUMO-1 in the in vitro shift assay. For this experiment, bacterial lysates from cells expressing the T7-tagged RanGAP1 proteins (Fig. 5, lanes 1 and 2) were added to digitonin lysates of HeLa cells in the presence of ATP (Fig. 5, lanes 4 and 5). After incubation at RT for 10 min, the samples were subjected to Western blot analysis. Under conditions in which  $\sim$ 90% of wt RanGAP1 was modified by SUMO-1 (Fig. 5, lane 4), RanGAP1 K526R remained completely unmodified (Fig. 5, lane 5), indicating not only that lysine 526 is the site of modification by SUMO-1, but that it also is the only site in RanGAP1 capable of being modified in this in vitro assay. Western blot analysis of the bacterial extract with  $\alpha$  T7-antibodies (Fig. 5, lanes 1 and 2) revealed the presence of a small amount of a slightly faster migrating species (Fig. 5, open arrow-

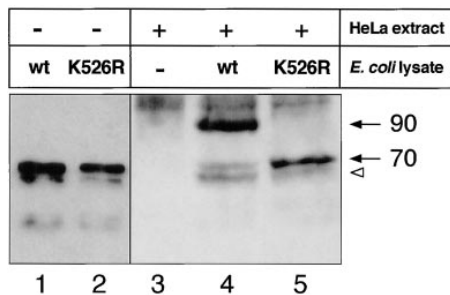


**Figure 4.** Schematic representation of RanGAP1 constructs. The schematic representation of RanGAP1 is based on the sequence of mouse RanGAP1 (DeGregori et al., 1994) that was used for this study. Full-length RanGAP1 contains three domains: a leucine-rich repeat domain, a highly acidic stretch, and a 25-kD tail domain. Although all three domains are present in RanGAP1 proteins from mouse (DeGregori et al., 1994), human (Bischoff et al., 1995), and *Xenopus laevis* (Saitoh et al., 1997), homologues from *S. cerevisiae* (Rna1p; Traglia et al., 1989) and *S. pombe* (rna1p; Melchior et al., 1993b) contain only the first two domains. In K526R GAP and K526R tail a single lysine residue in position 526 of wt RanGAP1 was altered to arginine.

head) in addition to full-length RanGAP1 protein. This species is presumably the result of proteolysis at the COOH-terminal portion of the protein, since the T7 epitope resides at the NH<sub>2</sub> terminus of the recombinant protein and was still recognized by  $\alpha$  T7 antibodies. Interestingly, this faster migrating species was not modified (Fig. 5, lane 4), suggesting that the extreme COOH terminus of RanGAP1 may contain information essential for the modification.

#### SUMO-1 Modification of RanGAP1 at K526 Is Required for Targeting RanGAP1 to the Nuclear Rim In Vivo

To verify that K526 is the only acceptor site for SUMO-1 modification, and to extend upon our previous data indicating a role for SUMO-1 modification in the targeting of RanGAP1 to the NPC (Mahajan et al., 1997), we transfected Cos-7 cells with HA-tagged wt and K526R RanGAP1 expression plasmids. Analysis of the cells by indirect immunofluorescence microscopy (Fig. 6 A) and by Western blot analysis (Fig. 6 B) showed a strikingly different distribution for the two proteins. The wt RanGAP1 was localized primarily to the nuclear rim and was also present in a diffuse cytoplasmic distribution (Fig. 6 A, wt GAP), consistent with the localization of endogenous RanGAP1 (Matunis et al., 1996; Mahajan et al. 1997). In contrast, RanGAP1 containing the lysine 526 to arginine mutation was unable to localize to the nuclear rim and instead accumulated in the cytoplasm (Fig. 6 A, K526R GAP). Western-blot analysis of the transfected cells showed that although both wt RanGAP1 and K526R RanGAP1 were expressed at equivalent levels, only the wt form of RanGAP1 was competent to be modified in vivo to the 90-kD form (Fig. 6 B). Taken together, these findings demonstrate that in vivo SUMO-1 modification of K526 in RanGAP1 is required to target RanGAP1 to the nuclear envelope.



**Figure 5.** A single lysine residue (K526) in RanGAP1 is modified by SUMO-1. Bacterial lysates of cells expressing T7-tagged wt RanGAP1 (lanes 1 and 4) or mutant RanGAP1 (lanes 2 and 5) were mixed with a digitonin lysate of HeLa cells in the presence of ATP. After a 10-min incubation at RT the reaction products were analyzed by immunoblotting using mouse monoclonal antibodies against the T7 tag. Lanes 1 and 2, bacterial lysates before shift reaction; lanes 3–5, extracts after shift reaction. Lane 3, HeLa extract; lanes 4 and 5, HeLa extract and bacterial extract. The open arrowhead marks a proteolytic fragment of RanGAP1 that apparently is not competent for modification.

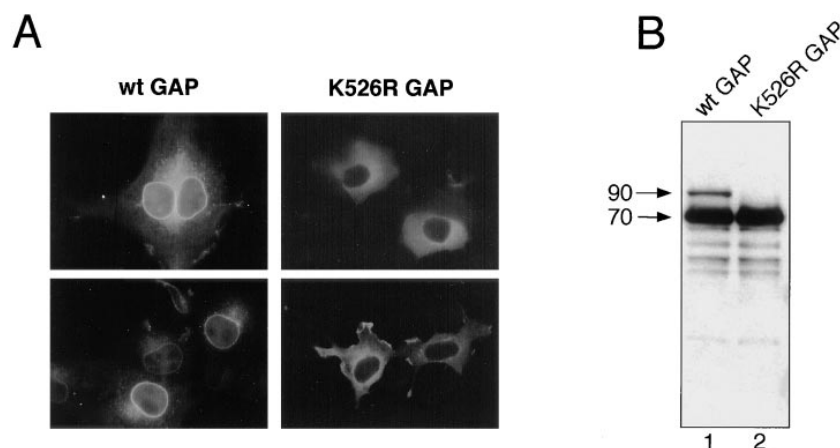
### The Tail Domain of RanGAP1 Contains Both the Modification Site as Well as the Nuclear Rim-targeting Domain

Next, we investigated the nature of the targeting signal provided by the SUMO-1 moiety. Our previous *in vitro* binding studies did not detect any significant binding of SUMO-1 alone to RanBP2 (Mahajan et al., 1997) suggesting that SUMO-1 is not simply an adaptor molecule that links RanGAP1 to RanBP2. Thus, SUMO-1 could serve to uncover a binding site in RanGAP1 that is masked in the unmodified protein. Alternatively, modification of RanGAP1 by SUMO-1 could create a composite binding site that involves both proteins. To begin to address this question, we engineered fragments of RanGAP1 (Fig. 4) as HA-tagged proteins and transfected them into Cos-7 cells (Fig. 7). The RanGAP1 body contained the first 416 NH<sub>2</sub>-terminal amino acids of RanGAP1, including both the leucine-rich repeat domain and the acidic stretch that are present in all RanGAP1 proteins from yeast to mammals (Fig. 4). Western blot analysis of transfected cells showed

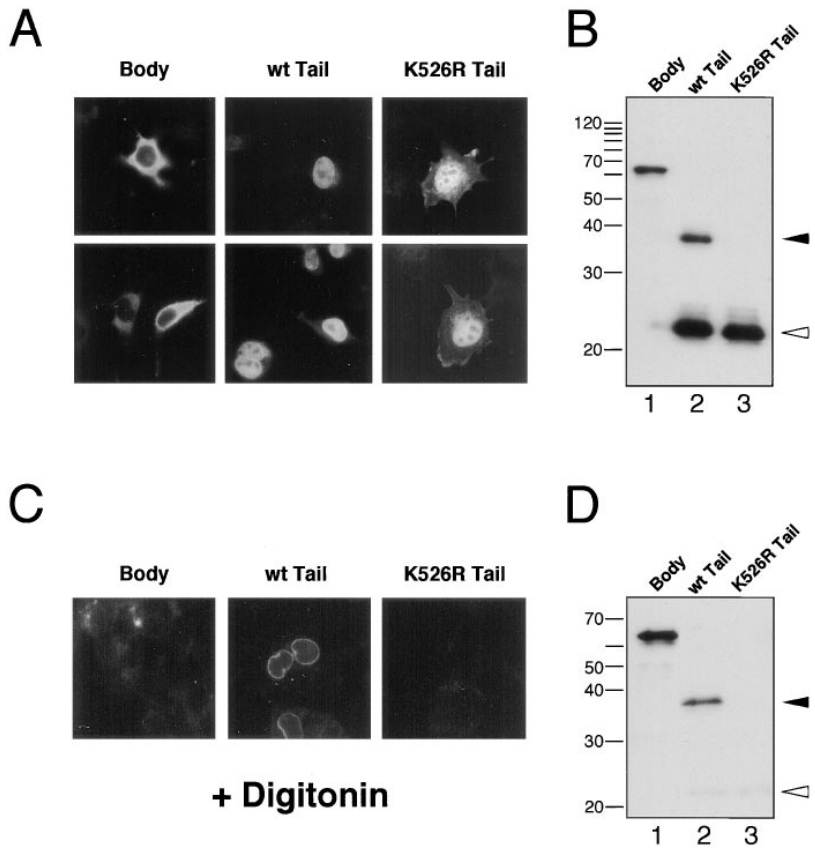
that this NH<sub>2</sub>-terminal fragment was expressed at high levels and appeared as a single band somewhat larger than the predicted molecular mass of ~43 kD (Fig. 7 B, *body*). This band did not react with α-SUMO-1 antibodies (data not shown), indicating that it is not SUMO-1 modified. Cos-7 cells expressing this construct are shown in the left panels of Fig. 7 A (*body*). This NH<sub>2</sub>-terminal fragment was clearly excluded from the nucleus and did not accumulate at the nuclear rim. Digitonin permeabilization before fixation of the cells led to the loss of most of the cytoplasmic staining and again, no accumulation at the NE was observed (see Fig. 7 C, *body*). The prominent band observed by Western blot analysis of digitonin-permeabilized cells (Fig. 7 D, *body*) is due to a low level of body that remained in the cytoplasm after permeabilization (visible in longer exposures of Fig. 7 C; not shown). Taken together these data indicate that the conserved NH<sub>2</sub>-terminal part of RanGAP1 does not contain sufficient information for targeting to the NE.

In contrast, a fragment of RanGAP1 representing the COOH-terminal domain (Fig. 4, GAP tail) exhibited a distinct nuclear rim localization in addition to intranuclear accumulation (Fig. 7 A, *wt Tail*). Digitonin permeabilization before fixation of the cells led to loss of the intranuclear staining, whereas the nuclear rim staining remained (see Fig. 7 C, *wt Tail*). Western blot analysis of transfected cells showed that the tail fragment was expressed at high levels and that a small fraction of the wt tail domain was modified by SUMO-1 (Fig. 7 B, lane 2). Modified tail, but not unmodified tail, was retained in cells after digitonin permeabilization (Fig. 7 D, lane 2), indicating that only SUMO-1–modified tail was localized to the nuclear rim.

A tail domain lacking the SUMO-1 modification site (Fig. 4, *K526R Tail*) did not localize to the nuclear rim, but accumulated within the nucleus (Fig. 7 A, *K526R Tail*). The accumulation of the tail fragments in the nucleus upon overexpression is probably not physiologically relevant, as full-length RanGAP1 is excluded from the nucleus. Western blot analysis of transfected cells showed that the mutant tail fragment was expressed at about the same level as wt tail (Fig. 7 B, lane 3). Digitonin permeabilization before fixation led to a near complete loss of the tail as judged by immunofluorescence microscopy (Fig. 7 C, *K526R Tail*) and by Western blot analysis (Fig. 7 D, lane



**Figure 6.** SUMO-1 modification of RanGAP1 at K526 is required for targeting RanGAP1 to the nuclear rim *in vivo*. HA-tagged wt RanGAP1 (*wt GAP*) and mutant RanGAP1 (*K526R GAP*) were transfected into Cos-7 cells and detected after 24 h. (A) Localization of transfected HA-tagged proteins by indirect immunofluorescence. Cells were fixed and probed with an α-HA monoclonal antibody. (B) Western blot analysis of transfected cells. Transfected cells were lysed by scraping into boiling SDS gel loading buffer, and analyzed by immunoblotting with α-HA monoclonal antibody.



**Figure 7.** The mammalian-specific tail region of RanGAP1 is both necessary and sufficient for SUMO-1 modification and nuclear rim targeting. (A) Immunofluorescence of Cos-7 cells transfected with HA-tagged RanGAP1 constructs representing the NH<sub>2</sub>-terminal 416 amino acids (*Body*), the COOH-terminal tail region containing amino acids 400-589 (*wt Tail*), and a mutant tail lacking the SUMO-1 modification site (*K526R Tail*). Transfected cells were fixed and probed with the  $\alpha$ -HA monoclonal antibody. (B) Western blot analysis of transfected cells separated on a 12.5% polyacrylamide gel. Cells were transfected as above, lysed in boiling SDS gel loading buffer and analyzed by immunoblotting with  $\alpha$ -HA monoclonal antibody. (C) Immunofluorescence of digitonin permeabilized Cos-7 cells transfected with the RanGAP1 constructs. Cells were permeabilized with 0.05% digitonin for 6 min, washed 3 $\times$  in PBS before fixation, and then processed for immunofluorescence. (D) Immunoblots of digitonin-permeabilized transfected cells. Cells were permeabilized as described for immunofluorescence analysis, lysed in SDS gel loading buffer, and analyzed by immunoblotting with  $\alpha$ -HA monoclonal antibodies. (B and D) Arrowheads indicate the positions of unmodified tail (*open arrowhead*) and modified tail (*closed arrowhead*).

3). From these experiments we concluded that (a) the tail domain contains sufficient information to be recognized by the SUMO-1 modification machinery; (b) the NE-targeting information resides in the tail domain of RanGAP1; and (c) modification with SUMO-1 is required not only for targeting of full-length RanGAP1 but also for targeting of the much shorter tail fragment.

#### ***SUMO-1-modified RanGAP1 Remains Stably Bound to the NE During In Vitro Nuclear Protein Import***

The finding that the modified tail domain remained stably associated with the NE upon digitonin permeabilization and washing of the cells (Fig. 7 C, *wt Tail* and D, lane 2) suggested that there was no rapid demodification during the treatment. To test whether this was also true for full-length RanGAP1 under conditions that allow nuclear protein import, Cos-7 cells transfected with full-length HA-tagged RanGAP1 were permeabilized with digitonin and mixed with HeLa cytosol, ATP, and a fluorescent transport substrate (FITC-BSA-NLS), and incubated in the presence or absence of recombinant untagged wt RanGAP1 (Fig. 8). After incubation of the mixture at the indicated temperature for 30 min, the cells were fixed and the amount of HA-tagged RanGAP1 in these cells was analyzed quantitatively by flow cytometry. As shown in the top panel of Fig. 8 A, which shows cells that were kept at 0°C, expression of HA-tagged RanGAP1 resulted in a large number of cells that show higher levels of HA-signal compared with background in mock-transfected cells (*bottom panel*). This is consistent with a transfection efficiency of ~50%, which is also suggested by the indirect immuno-

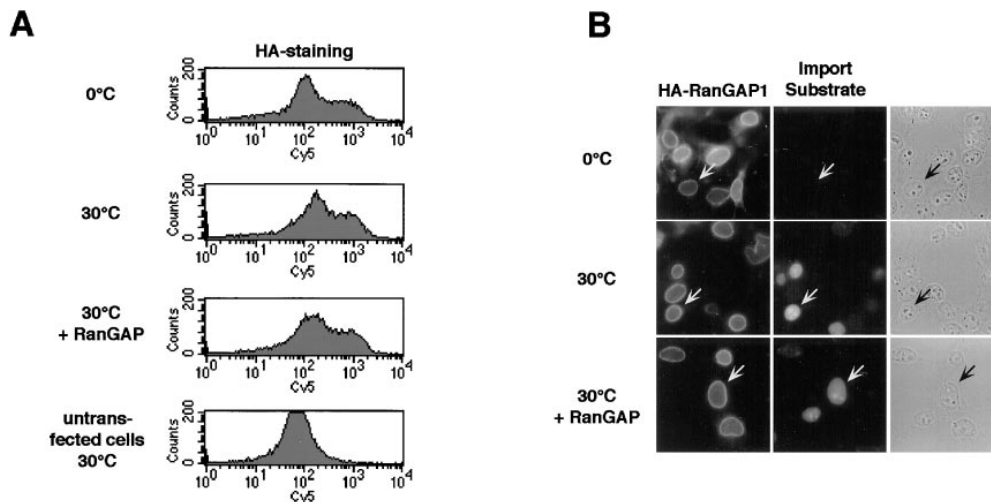
fluorescence staining shown in Fig. 8 B. Both analysis by flow cytometry and by indirect immunofluorescence showed a high variation of the HA-signal (note the logarithmic scale in Fig. 8 A), indicating a high cell to cell variability in expression levels. Incubation for 30 min at 30°C to carry out nuclear protein import did not alter the HA signal distribution, even in the presence of an excess of recombinant untagged RanGAP1. Fig. 8 B shows analysis of a similar experiment by immunofluorescence. In each field both transfected and untransfected cells are present, indicating that no redistribution of RanGAP1 occurs under conditions that clearly allow many cycles of nuclear protein import (see Discussion). Although the overall cell to cell variation in nuclear import levels was quite high both in transfected and untransfected cells, strong nuclear accumulation of transport substrate occurred in cells that retained high levels of HA-RanGAP1 at the NE (Fig. 8 B, *middle and bottom*). These findings indicate that the NE-associated RanGAP1-SUMO-1 conjugate is not rapidly turned over under in vitro import conditions, and allows the conclusion that modification and demodification of RanGAP1 is not a required element of nuclear protein import.

#### ***Discussion***

##### ***Identification of the RanGAP1-SUMO-1 Link Reveals Similarity to Ubiquitination***

We found that despite the low homology of SUMO-1 to ubiquitin, the general characteristics of SUMO-1 conjuga-





**Figure 8.** Stable association of modified RanGAP1 with the NE during nuclear protein import. (A) Analysis of Cos-7 cells transfected with HA-tagged RanGAP1 by flow cytometry. Cos7 cells transfected with HA-RanGAP1 (A, top three panels), or mock-transfected cells (bottom panel) were permeabilized with digitonin and incubated in the presence of cytosol and ATP at the indicated temperature. To one sample recombinant RanGAP1 was added (30°C + RanGAP1). After 30 min, the cells were fixed and stained with  $\alpha$ -HA monoclonal anti-

body and Cy5-conjugated secondary antibodies. The cell-associated Cy5 signal was detected by flow cytometry. (B) Analysis of nuclear import in Cos-7 cells transfected with HA-tagged RanGAP1. Cos7 cells transfected with HA-RanGAP1 were permeabilized with digitonin and incubated in the presence of cytosol, ATP, and FITC-labeled import substrate at the indicated temperature. To one sample recombinant RanGAP1 was added (30°C + RanGAP1). After 30 min, the cells were fixed, and processed for indirect immunofluorescence using  $\alpha$ -HA monoclonal antibody. Arrows indicate corresponding cells in each set and are provided for orientation.

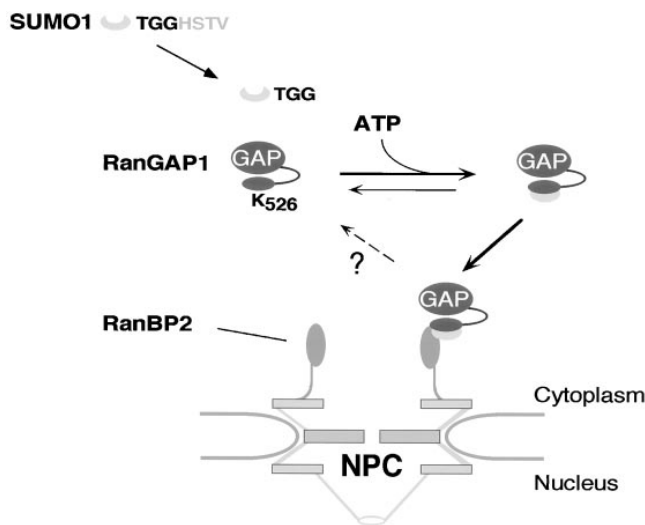
tion (summarized in Fig. 9) seem to be identical to those of ubiquitination. Ubiquitin is expressed as a polyubiquitin moiety or as an NH<sub>2</sub>-terminal fusion to unrelated proteins and must be proteolytically processed to release monomeric ubiquitin and to reveal the COOH-terminal Gly-Gly motif that is involved in coupling to targets (e.g., Ozkaynak et al., 1984; Monia et al., 1989; Callis et al., 1995). In comparison, SUMO-1 is expressed as a single 101-amino acid protein that contains 4 additional COOH-terminal amino acids beyond the Gly-Gly motif that it shares with ubiquitin (Mahajan et al., 1997). In principle, any free carboxyl group may be used for the formation of an isopeptide bond, and a ubiquitin mutant with a COOH-terminal alanine instead of glycine can be used as a substrate for ubiquitination (Hodgins et al., 1992). Therefore, it was conceivable that full-length SUMO-1 was used for modification. However, the identification of a branched peptide between RanGAP1 and SUMO-1 clearly indicates that the last four amino acids of SUMO-1 are removed to reveal the Gly-Gly motif before its attachment to RanGAP1. After proteolytic processing, the COOH-terminal glycine 97 in SUMO-1 is conjugated to a lysine residue in the acceptor protein RanGAP1 via an isopeptide bond. The similarity of the SUMO-1 modification to ubiquitination suggests that some of the multiple enzymes thought to be involved in ubiquitination and deubiquitination may use SUMO-1 or other ubiquitin-related proteins instead. The recent finding that Ubc9, an E2-like enzyme implicated in ubiquitin-mediated cyclin degradation (Seufert et al., 1995), can be found in a complex with SUMO-1-modified RanGAP1 and RanBP2 (Saitoh et al., 1997), and interacts with SUMO-1 in a two-hybrid interaction screen (Shen et al., 1996b) may support this possibility.

Proteolytic processing of SUMO-1 is not mechanistically coupled to the conjugation reaction, as a truncated

version of SUMO-1 lacking the last four amino acids is efficiently conjugated to RanGAP1 in vitro (this study) and to other unknown proteins in vivo (Kamitani et al., 1997). Interestingly, in in vitro shift assays, SUMO-1 $\Delta$ C4 is conjugated to RanGAP1 much more efficiently than wt SUMO-1, suggesting that proteolytic processing in the HeLa cell lysate is significantly slower than the enzymatic reactions leading to the formation of the isopeptide bond between SUMO-1 and RanGAP1. It remains to be seen whether the same enzyme that carries out proteolytic processing of full-length SUMO-1 is also able to cleave the RanGAP1-SUMO-1 conjugate. This dual activity has been demonstrated for at least one of the large number of deubiquitinating enzymes (Hochstrasser, 1996). If this were the case, it would seem plausible that the rate of such an enzyme is significantly lower than the rate of the modifying enzymes that lead to production of modified RanGAP1.

### Multiple Proteins Can Be Modified by SUMO-1

The overexpression of HA-tagged SUMO-1 in Cos-7 cells gives rise to multiple protein bands containing the HA epitope (this study; Kamitani et al., 1997). Since this is dependent on the presence of the double glycine motif in SUMO-1, it strongly indicates that SUMO-1 can modify multiple proteins. We cannot rule out that some of the bands may represent a single protein modified with SUMO-1 chains of various length. However, it is unclear whether SUMO-1 is capable of forming such polymeric chains, since none of the lysines shown to be used for ubiquitin-ubiquitin links (positions 6, 11, 29, 48, and 63 in ubiquitin; Arnason and Ellison, 1994; Spence et al., 1995; Baboshina and Haas, 1996) are conserved in SUMO-1. Moreover, antibodies to RanGAP1 consistently detect only a mono-modified form, even after incubation of RanGAP1 with



**Figure 9.** SUMO-1-mediated targeting of RanGAP1 to the nuclear pore complex protein RanBP2. After SUMO-1 is proteolytically processed at the COOH terminus to expose glycine 97, it serves as the substrate in the ATP-dependent formation of an isopeptide bond between the free carboxyl group of glycine 97 in SUMO-1 and the  $\epsilon$ -amino group of lysine 526 in the tail domain of RanGAP1. Modified RanGAP1 binds stably to RanBP2 at the cytoplasmic fibrils of the NPC through an interaction mediated by the SUMO-1-modified tail domain of RanGAP1. While the modification reaction is reversible, it remains to be seen whether RanGAP1 can be demodified after it has associated with RanBP2.

cell lysates in the presence of a large excess of SUMO-1 $\Delta$ C4. The finding that antibodies raised to SUMO-1 recognize numerous bands on Western blots (Matunis et al., 1996; Mahajan et al., 1997), as well as immunolocalization studies of endogenous or overexpressed HA-tagged SUMO-1 (Boddy et al., 1996; Matunis et al., 1996) also are consistent with the interpretation that other proteins besides RanGAP1 are modified. The predominantly nuclear localization of SUMO-1 and particularly its concentration in nuclear speckles and at the NE seems to be dependent on the conjugation of SUMO-1 to other proteins, since we find that free HA-tagged SUMO-1 $\Delta$ C6 lacking Gly97 is not enriched in those regions (this study). Moreover, SUMO-1 has been found to interact with a number of proteins in two-hybrid interaction screens and colocalizes with one of them, the PML protein, in nuclear bodies (Boddy et al., 1996).

Considered together, these results strongly support the notion that SUMO-1 can modify multiple proteins. However, at least two additional proteins closely related to SUMO-1 exist in mammalian cells (Mannen et al., 1996; Lapenta et al., 1997), and some of the results could be due to antibody cross-reactivity. Overexpression of SUMO-1 may also cause incorporation of SUMO-1 into proteins where a SUMO-1-related species would normally be used.

#### ***A Single Lysine in RanGAP1 Serves as an Acceptor Site for SUMO-1***

Peptide mapping and mass spectrometry strongly suggest

that the isopeptide bond between RanGAP1 and SUMO-1 is formed on lysine 526 in the tail domain of RanGAP1. Mutation of that lysine residue to arginine completely abolished the modification both in vivo and in vitro, confirming lysine 526 as the acceptor site and indicating that no alternative lysine residue in RanGAP1 can substitute as the acceptor site, despite the fact that several additional lysine residues are close by. It remains to be seen whether the acceptor site is characterized by a specific recognition site for the modifying enzyme or merely by accessibility of the lysine residue. The apparent specificity of the acceptor site is consistent with the finding that SUMO-1 serves to alter the binding properties of RanGAP1 rather than to mark it for degradation (see Fig. 9 and below).

Although only a small number of acceptor sites for ubiquitination have been identified, it seems that the ubiquitination machinery is rather promiscuous with respect to acceptor site choice (Ciechanover, 1994). Analysis of ubiquitin-mediated degradation of cyclin B (King et al., 1996) and of the T cell antigen receptor  $\zeta$  chain (Hou et al., 1994) demonstrates that multiple lysine residues can function as acceptor sites and that each is sufficient to target the protein for rapid degradation. In addition, ubiquitination of either one of two adjacent lysines in I  $\kappa$  B is sufficient for degradation (Baldi et al., 1996).

#### ***RanGAP1 Tail Is Sufficient Both for Modification by SUMO-1 and for Targeting to the NE***

RanGAP1 is localized predominantly at the NE, where it forms a complex with the nuclear pore complex protein RanBP2. We have shown previously that modified but not unmodified RanGAP1 binds to RanBP2 in vitro (Mahajan et al., 1997, see also Fig. 9). Identification of the acceptor site in RanGAP1 (K526) for SUMO-1 modification allowed us to extend those studies to in vivo experiments. We found that SUMO-1-modified wt RanGAP1 and SUMO-1-modified RanGAP1 tail, but not K526R RanGAP1, K526R tail, or GAP body are targeted to the NE upon expression in Cos-7 cells. Since SUMO-1 by itself does not bind to RanBP2 (Mahajan et al., 1997), these findings indicate that essential targeting information resides in the tail domain of RanGAP1. Whether SUMO-1 serves to unmask a binding site present in the tail, or whether a binary binding site is formed upon modification that contains elements of both the GAP tail and SUMO-1 remains to be seen. The finding that RanGAP1 lacking the tail domain is exclusively cytoplasmic is reminiscent of the cytoplasmic localization of the yeast RanGAP homologues that do not contain a homologue of the tail domain (see Introduction and Fig. 4). Although it is possible that yeast RanGAP may be able to fulfill its role in nuclear import while in the cytoplasm, we consider it more likely that a different, less stable targeting mechanism is used. One possibility is that a separate protein homologous to the tail domain of RanGAP1 serves as an adapter between Rna1p and its putative binding partner at the NPC. This protein may or may not be modified with the yeast SUMO-1 homologue SMT3. Alternatively, since no obvious homologue for RanBP2, the RanGAP1 binding partner at the NPC in higher eukaryotes, has been found in yeast, the targeting mechanisms could be quite distinct.

## A Role for the Reversible Modification of RanGAP1 with SUMO-1 in the Regulation of Nuclear Protein Import?

In analogy to ubiquitination, it is appealing to speculate that modification of RanGAP1 with SUMO-1 is reversible. In fact, demodifying activity has been observed in vitro, both in cell extracts and solubilized NEs (Matunis et al., 1996; Mahajan et al., 1997). Although it remains to be seen whether the RanGAP1-SUMO-1 associated with RanBP2 is susceptible to demodification (Fig. 9), two distinct roles could be envisioned for such a reversible SUMO-1 modification of RanGAP1. In one scenario, modification and demodification could be mechanistically linked to the nuclear import process. If this were the case, the turnover of modified RanGAP1 at the NE would have to be very rapid, since in vitro nuclear import rates are ~30 molecules per pore complex per minute (Melchior et al., 1993a). However, our data strongly argue against such a direct requirement of a modification/demodification cycle in nuclear protein import, since SUMO-1-modified RanGAP1 remains stably bound to the NE under conditions that allow many cycles of nuclear protein import. These data, together with the finding that soluble RanGAP1 is not required for in vitro nuclear import and the observation that antibody inhibition of NPC associated RanGAP1 inhibits nuclear protein import (Mahajan et al., 1997), strongly support the possibility that RanGAP1-mediated GTP hydrolysis by Ran in nuclear protein import is restricted to RanBP2.

An intriguing alternative for the function of SUMO-1 is that the reversible modification of RanGAP1 could serve to regulate nuclear protein import in response to the cellular state. Downregulation of nuclear import has been observed, for example, when cells exit the cell cycle due to contact inhibition or serum starvation (Feldherr and Akin, 1994). Since the localization of RanGAP1 at RanBP2 seems to be critical for nuclear protein import (Mahajan et al., 1997), regulated modification and demodification could directly affect the overall rate of Ran-dependent nuclear protein import by controlling the proportion of RanBP2 molecules that are complexed with RanGAP1.

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