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 ubiquitinated 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.

GTP 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) have been proposed (e.g., Melchior et al., 1995; Rexach and Blobel, 1995; Görlich and Mattaj, 1996; Koepp 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 (Corbett 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 (Koepp 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., 1996; 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 ε-amino group
activate a protein kinase involved in I
and Riezman, 1996), and ubiquitination has been shown to
receptor signals its ligand-stimulated endocytosis (Hicke
For example, ubiquitination of a yeast plasma membrane
not limited to the targeting of proteins to the proteasome.
ingly evident that the role of the ubiquitin modification is
commitment of a target protein to the degradation pathway in-
Jentsch and Schlenker, 1995; Hilt and Wolf, 1996). Com-
viewed by Ciechanover, 1994; Hochstrasser, 1995, 1996;
ubiquitin and the COOH-terminal glycine residue of the
volves assembly of a polyubiquitin chain on the target,
finally to the acceptor protein. The modification is revers-
activated ubiquitin is then sequentially transferred to an
coupling reaction is ATP dependent and involves a series
1996; Jennissen, 1995; Jentsch and Schlenker, 1995). This
Ciechanover, 1992; Ciechanover, 1994; Hochstrasser, 1995,
of lysines in the acceptor protein (reviewed in Hershko and
proteins and may serve as a
inducible, ubiquitin cross-reacting protein UCRP/ISG15
Biggins et al., 1996), among them the 15-kD interferon-
years (e.g., see Kumar et al., 1993; Watkins et al., 1993;
bigly associated with the NE during the course of many cy-
mutable deletion mutants were generated by PCR from
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
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higher eukaryotes (amino acids 400–589; RanGAP1 tail; see Fig. 4) were
conserved domain (amino acids 1-416; RanGAP1 body; see Fig. 4) and the
of 1,297.5 D) and Adreno-
corticotropic hormone fragment (amino acids 18–39; MH
Peptide Analysis of
SUMO-1–modified RanGAP1
In this study, we characterized the molecular nature of the
likely to provide a paradigm for other SUMO-1 substrates.
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lovirus promoter-driven expression of NH2-terminal hemagglutinin (HA)-
tagged proteins in transfected eukaryotic cells. Mouse RanGAP1 (fug1;
DeGregori et al., 1994) cDNA constructs representing the NH2-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.,
and sequenced at the Scripps Research Institute core facil-
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 lys-
ine 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 character-
istic biochemistry of the link is conserved. Mutation of lys-
ine 526 to arginine completely abolishes modification, in-
dicating that only a single lysine residue in RanGAP1 is
available for modification with SUMO-1. We have also iden-
tified 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 sta-
bly associated with the NE during the course of many cy-
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 de-
scribed (Mahajan et al., 1997). Antigen–antibody complexes were sepa-
rated 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.
Leszczynski at the Worcester Foundation for Biomedical Research.
(Maysville, MA). Proteins were digested in situ with trypsin in a digest buffer
containing 100 mM ammonium bicarbonate, 10% acetonitrile, and 1% hy-
drogenated Triton X-100 (Fernandez et al., 1994). The digest mixture was
separated on a 1 × 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 μl/min. The eluent was monitored at 210 nm and fractions
were collected manually. A 0.5-μl aliquot of each peptide fraction was
subjected to matrix-assisted laser desorption time-of-flight mass spec-
trometry (MALDI-TOF) using a Linear Biospectrometry Workstation
(Perspective Biosystems, Cambridge, MA) and alpha cyano-4-hydroxy cin-
namic acid as the matrix. The instrument was calibrated with an external
standard that consisted of angiotensin (MHH of 1,297.5 D) and Adreno-
corticotropic hormone fragment (amino acids 18–39; MH of 2,476.7 D).
Edman sequence analysis was performed on a 494 Procise protein se-
quencer (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
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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 pHIS101 or 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 NH2-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 TrisCl, 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 SUMOAC4 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 SUMOAC4 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 onto 6-well tissue culture plates 12–15 h before use at a density of 2.3 × 105 cells per well. For immunofluorescence, cells were plated onto flame-sterilized cover slips. Cells were typically transfected with 2 µg of eukaryotic-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 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-Ran-GAP1 antibodies to detect recombinant untagged RanGAP1 (Mahajan et al., 1997) or an α-T7 tag mouse monoclonal antibody (Novagen, Inc., Madison, WI) 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 MgCl2 at 1:10,000 to detect T7-tagged wt and mutant RanGAP1. 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 × 105 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 SUMOAC4 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. 1A 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. 1A, 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. 1A, 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-
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 ε-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 (SUMOΔC4). SUMOΔC4 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 Ran GAP1 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 α-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
amount of RanGAP1 converted (Fig. 2, lane 3). In contrast, the addition of SUMOΔC4 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 SUMOΔC4 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-SUMOΔC4 converts significantly more of the 70-kD RanGAP1 to the 115-kD form (Fig. 2, lane 6). These data demonstrate that SUMOΔC4 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 SUMOΔC4 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 (SUMOΔC4) 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 α-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-SUMOΔC4 was transfected into Cos-7 cells, its distribution was indistinguishable from wt HA–SUMO-1 (Fig. 3 A, SUMOΔC4). 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 (SUMOΔC6). 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, SUMOΔC6 expression was consistently barely

![Figure 2](image2.png)

**Figure 2.** SUMO-1 lacking the last four amino acids (SUMOΔC4) 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), SUMOΔC4 (lane 4), GST–SUMO-1 (lane 5), or GST-SUMOΔC4 (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 α-RanGAP1 antibodies.

![Figure 3](image3.png)

**Figure 3.** SUMO-1 and SUMOΔC4, but not SUMOΔC6, modify multiple substrates in vivo. (A) Immunofluorescence of Cos-7 cells transfected with HA-tagged SUMO-1 constructs and probed with an α-HA monoclonal antibody. Intracellular localization of wt SUMO-1 (SUMO wt), SUMO-1 with a six–amino acid COOH-terminal deletion (SUMOΔC6), and SUMO-1 terminating at glycine 97 (SUMOΔC4) was analyzed. (B) Western blot analysis of SUMO-1–transfected cells, Cos-7 cells were transfected with HA-tagged wt SUMO-1 (lane 1), SUMOΔC6 (lane 2), or SUMOΔC4 (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 α-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 α 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 ~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. In K526R GAP and K526R tail a single lysine residue in position 526 of wt RanGAP was altered to arginine.

**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* (r1p1; Melchior et al., 1993) 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.

**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 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.
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, D, 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 staining and again, no accumulation at the NE was observed (see Fig. 7 C, wt Tail). 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$_2$-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.

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 involving 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$_2$-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$_2$-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$_2$-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$_2$-terminal part of RanGAP1 does not contain sufficient information for targeting to the NE.

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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 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 immunofluorescence 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-
**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 monomodified 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 ε-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-1ΔC4. 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-1ΔC6 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 spectroscopy 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 ζ 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 IκB 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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