

# A Novel Ubiquitin-like Modification Modulates the Partitioning of the Ran-GTPase-activating Protein RanGAP1 between the Cytosol and the Nuclear Pore Complex

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**Abstract.** Ran is a nuclear Ras-like GTPase that is required for the bidirectional transport of proteins and ribonucleoproteins across the nuclear pore complex (NPC). A key regulator of the Ran GTP/GDP cycle is the 70-kD Ran-GTPase-activating protein RanGAP1. Here, we report the identification and localization of a novel form of RanGAP1. Using peptide sequence analysis and specific mAbs, RanGAP1 was found to be modified by conjugation to a ubiquitin-like protein. Immunoblot analysis and immunolocalization by light and EM demonstrated that the 70-kD unmodified form

of RanGAP1 is exclusively cytoplasmic, whereas the 90-kD modified form of RanGAP1 is associated with the cytoplasmic fibers of the NPC. The modified form of RanGAP1 also appeared to associate with the mitotic spindle apparatus during mitosis. These findings have specific implications for Ran function and broad implications for protein regulation by ubiquitin-like modifications. Moreover, the variety and function of ubiquitin-like protein modifications in the cell may be more diverse than previously realized.

**T**RANSPORT of macromolecules across the nuclear envelope occurs bidirectionally through nuclear pore complexes (NPCs)<sup>1</sup>, large supramolecular assemblies that span both membranes of the nuclear envelope (Rout and Wentz, 1994). Whereas small ions and metabolites can passively diffuse through the NPC, most proteins and ribonucleoproteins are transported across the NPC by a signal- and energy-dependent mechanism. Dissection of the events culminating in nuclear import has been aided by the development of a permeabilized cell system that has made possible the identification of soluble cytosolic factors required for nuclear import (Adam et al., 1990), and more recently by the development of solution binding assays that use recombinant transport factors and nucleoporins (Rexach and Blobel, 1995).

Using the permeabilized cell assay in conjunction with biochemical fractionation of cytosolic extracts, four factors required for nuclear import have been purified and characterized (Moore and Blobel, 1993, 1994; Melchior et al., 1993; Adam and Adam, 1994; Görlich et al., 1994, 1995;

Chi et al., 1995; Enenkel et al., 1995; Imamoto et al., 1995; Moroianu et al., 1995a; Paschal and Gerace, 1995; Radu et al., 1995). Recognition of nuclear localization signal (NLS)-containing substrates in the cytoplasm is mediated by the  $\alpha$  subunit of the karyopherin- $\alpha/\beta$  heterodimer (Enenkel et al., 1995; Moroianu et al., 1995a; Weis et al., 1995), and docking of the trimeric complex to the NPC is mediated by the  $\beta$  subunit (Moroianu et al., 1995b; Radu et al., 1995). Peptide repeat-containing nucleoporins (Rout and Wentz, 1994), which bind karyopherin- $\beta$  in vitro and are components of the cytoplasmic and nucleoplasmic fibers as well as the peripheries of the central channel, have been proposed to form an array of substrate/receptor docking sites (Radu et al., 1995). The small GTPase Ran and the Ran-interactive protein p10 are believed to mediate the dissociation of substrate/receptor complexes as they dock along these sites (Melchior et al., 1993; Moore and Blobel, 1993, 1994; Paschal and Gerace, 1995). While the exact nature and molecular dynamics of translocation across the NPC remain to be elucidated, in vitro binding assays have recently revealed functional relationships among the karyopherin heterodimer, Ran, p10, and peptide repeat-containing nucleoporins. p10 binds directly to the peptide repeat-containing nucleoporins, Ran-GDP, and karyopherin- $\beta$ , and has been proposed to coordinate substrate/receptor dissociation reactions at the NPC (Nehrbass and Blobel, 1996; Paschal et al., 1996; Matunis, M.J., G. Blobel, and M. Hijikata, unpublished data). Actual release of docked substrate/receptor complexes appears to be mediated by the

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1. *Abbreviations used in this paper:* BRL, buffalo rat liver (cells); EST, expressed sequence tagged; NEM, *N*-ethylmaleimide; NPC, nuclear pore complex; PCLF, pore complex lamina formation.

binding of Ran-GTP to karyopherin- $\beta$ , which dissociates the karyopherin heterodimer (Rexach and Blobel, 1995; Moroianu et al., 1996). All these findings point to Ran and factors regulating its guanine nucleotide state as key determinants of nucleocytoplasmic transport.

Similar to other GTPases, Ran is presumed to function as a molecular switch, associating with and dissociating from interacting proteins through conformational changes driven by GTP hydrolysis or by nucleotide exchange (Bourne et al., 1991; Rush et al., 1996; Sazer, 1996). The intrinsic rates of hydrolysis and exchange by Ran are extremely low, and these reactions are therefore catalyzed by regulatory factors whose subcellular distributions define Ran's activities. Nucleotide exchange by Ran is catalyzed by the GDP-GTP exchange factor RCC1 (Bischoff and Ponsingl, 1991), and GTP hydrolysis is catalyzed by the GTPase-activating protein RanGAP1 (Bischoff et al., 1994, 1995*a,b*). RCC1 is a nuclear chromatin-associated protein, initially identified as a factor involved in the control of mitotic events, including chromatin condensation and cell cycle progression (Ohtsubo et al., 1987, 1989; Bischoff et al., 1990; Dasso, 1993). RanGAP1, on the other hand, was initially identified in *Saccharomyces cerevisiae* as *RNA1-1*, a mutant defective in RNA production, processing, and nuclear export (Hartwell, 1967; Traglia et al., 1989). Immunolocalization of Rna1p identified it as a cytoplasmic protein that is excluded from the nucleus (Hopper et al., 1990). The mammalian RanGAP1 was purified from HeLa cells as a homodimer of 65-kD subunits that specifically enhanced the rate of Ran-GTP hydrolysis by three orders of magnitude (Bischoff et al., 1994, 1995*a,b*). In general, these findings imply that Ran is converted to its GTP-bound form in the nucleus and hydrolyzed to its GDP-bound form in the cytoplasm. Two additional proteins that bind Ran-GTP and likely effect GTP hydrolysis are the cytosolic factor RanBP1 and the nucleoporin Nup358 (Coutavas et al., 1993; Wu et al., 1995; Yokoyama et al., 1995). RanBP1 and Nup358 contain domains that have been reported to enhance RanGAP1-induced Ran-GTP hydrolysis by an order of magnitude (Bischoff et al., 1995*b*; Beddow et al., 1995). The localization of Nup358 to the cytoplasmic fibers of the NPC suggests that an early step in nuclear import may involve GTP hydrolysis at or near these fibers. Consistent with this possibility, several reports have suggested a concentration of RanGAP1 at the nuclear envelope (Melchior et al., 1993; Bischoff et al., 1994; Koepp et al., 1996).

While the subcellular distributions of RanGAP1 and RCC1 seem to be strictly defined based on immunofluorescence localization and cell fractionation, it remains to be determined whether these factors are more precisely localized at the site of nuclear transport (i.e., the NPC). Targeting of a subpopulation of RCC1 and/or RanGAP1 to the NPC could potentially be regulated by modification of these factors. One modification that specifies protein targeting is ubiquitination, which is a posttranslational modification involving the covalent ligation of the carboxyl terminus of ubiquitin to internal lysine residues in a host of intracellular proteins (Hershko and Ciechanover, 1992; Wilkinson, 1995). Whereas the primary fate of ubiquitinated proteins is degradation by the 26S proteasome (Ciechanover, 1994; Jentsch and Schlenker, 1995), it has

long been recognized that ubiquitination is likely to have roles beyond proteolysis. Ubiquitin conjugation has been recently shown to act as a signal for endocytosis and vacuolar targeting (Egner and Kuchler, 1996; Galan et al., 1996; Hicke and Riezman, 1996; Roth and Davis, 1996; Strous et al., 1996) and as regulator of I $\kappa$ B kinase activity (Chen et al., 1996). In addition to functions other than proteolysis, a recurring question related to the ubiquitin system has been whether substrates other than ubiquitin may be used in parallel pathways to regulate as yet unrecognized cellular activities.

Here, we report the identification of a novel 90-kD form of the Ran-GTPase-activating protein RanGAP1. By peptide sequence analysis and using specific mAbs, the 90-kD RanGAP1 was identified as a modified form of the previously described 70-kD RanGAP1. The modification was found to consist of a covalent ligation between RanGAP1 and a novel ubiquitin-like protein, and it could be reversed by an enzymatic activity that cofractionated with the NPC. Moreover, the 90-kD modified form of RanGAP1 was itself localized to the NPC, and more specifically, to the cytoplasmic fibers of the NPC. In contrast, the 70-kD unmodified form of RanGAP1 was strictly cytoplasmic. Thus, a novel ubiquitin-like modification appears to modulate the partitioning of RanGAP1 between the cytosol and the NPC.

## Materials and Methods

### Isolation and Fractionation of Rat Liver Nuclear Envelopes

Rat liver nuclei were isolated as described (Blobel and Potter, 1966) and stored frozen at  $-80^{\circ}\text{C}$  in 100-U aliquots (1 U =  $3 \times 10^6$  nuclei). Nuclear envelopes were prepared by a modification of the procedure described by Dwyer and Blobel (1976). Nuclei were thawed and pelleted at 500 rpm in a tabletop microfuge for 1 min. After removing the supernatant, the pellet was resuspended to a final concentration of 100 U/ml by dropwise addition of cold buffer A (0.1 mM  $\text{MgCl}_2$ , protease inhibitors [0.5 mM PMSF, 1  $\mu\text{g}/\text{ml}$  leupeptin, 1  $\mu\text{g}/\text{ml}$  pepstatin A, and 18  $\mu\text{g}/\text{ml}$  aprotinin], 5  $\mu\text{g}/\text{ml}$  DNase I (Sigma Chemical Co., St. Louis, MO), and 5  $\mu\text{g}/\text{ml}$  RNase A [Sigma Chemical Co., St. Louis, MO]) with constant vortexing. The nuclei were then immediately diluted to 20 U/ml by the addition of ice-cold buffer B (10% sucrose, 20 mM triethanolamine, pH 8.5, 0.1 mM  $\text{MgCl}_2$ , 1 mM DTT, and protease inhibitors), again with constant vortexing. The suspension was dounced four times in a glass dounce homogenizer (tight pestle) and incubated at room temperature for 15 min. After the 15-min incubation, the suspension was underlaid with 5 ml of ice-cold buffer C (30% sucrose, 20 mM triethanolamine, pH 7.5, 0.1 mM  $\text{MgCl}_2$ , 1 mM DTT, and protease inhibitors) and centrifuged at 2,600 g in a swinging bucket rotor (model type HB-4; Sorvall, Wilmington, DE) for 15 min at  $4^{\circ}\text{C}$ . After removing the supernatant and sucrose cushion, the pellet was resuspended to a final concentration of 100 U/ml in ice-cold buffer D (10% sucrose, 20 mM triethanolamine, pH 7.5, 0.1 mM  $\text{MgCl}_2$ , 1 mM DTT, and protease inhibitors). The suspension was dounced as described above, and diluted to 66 U/ml by the addition of cold buffer C plus 0.3  $\mu\text{g}/\text{ml}$  heparin (Sigma Chemical Co.). The suspension was immediately underlaid with 5 ml of buffer C and pelleted as described above. The pellet resulting from this second extraction is operationally defined as the nuclear envelope fraction. The pore complex lamina fraction (PCLF) was derived from the nuclear envelope fraction by extraction with 1% Triton X-100 and 0.025% SDS, followed by an additional round of centrifugation as described above.

Conditions for Empigen BB extraction of the PCLF were as follows: The PCLF was resuspended in cold buffer D (minus DTT) at 100 U/ml and divided into two samples, one for mock *N*-ethylmaleimide (NEM) treatment and one for NEM treatment. To the mock NEM-treated sample, DTT was added to a final concentration of 10 mM, and NEM (pre-

pared as a 1 M stock in dimethyl sulfoxide immediately before use) was subsequently added to a final concentration of 5 mM. To the NEM-treated sample, NEM alone was added to 5 mM. Both samples were incubated on ice for 5 min and then extracted by dilution of the samples to 66 U/ml with buffer D containing 0.9% Empigen BB (Calbiochem/Novabiochem Corp., La Jolla, CA). Samples were incubated on ice for 5 min and subsequently pelleted for 15 min at 15,000 rpm. After precipitation with 10% TCA, the samples were resuspended in sample buffer, and the equivalent of 10 U of nuclei were analyzed by immunoblot analysis.

### Preparation of mAbs

mAbs 19C7, 19G12, and 2F1 were prepared by immunizing BALB/c mice with recombinant protein (prepared as described below) consisting of the first 203 amino-terminal residues of mouse RanGAP1. mAb 21C7 was produced by immunizing mice with the full-length recombinant GMP1, prepared as described below. Hybridoma production and screening and ascites production were performed as described previously (Choi and Dreyfuss, 1984). Antibody specificities were determined by immunofluorescence and immunoblot analysis.

### Gel Electrophoresis and Immunoblot Analysis

Proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane as described previously (Dreyfuss et al., 1984). Membranes were blocked in 5% nonfat dry milk in PBST (137 mM NaCl, 2.7 mM KCl, 1.5 mM KH<sub>2</sub>PO<sub>4</sub>, 8.1 mM Na<sub>2</sub>HPO<sub>4</sub>, 0.1% Tween 20). mAb ascites fluids were diluted 1:2,000 in PBST containing 2% BSA. Antibodies were detected using luminol-based chemiluminescence.

### Peptide Sequence Analysis

Rat liver nuclear envelopes were separated by SDS-PAGE (10% acrylamide) and transferred to a polyvinylidene difluoride membrane. The 90-kD Ran-GTP-binding protein was identified by staining with Ponceau S, cut from the membrane, and digested with endoproteinase Lys-C. Peptides were separated and sequenced as described (Fernandez et al., 1992).

### Immunofluorescence Microscopy

Buffalo rat liver (BRL) cells grown on coverslips were washed in PBS and either fixed in 2% formaldehyde/PBS for 30 min at room temperature and permeabilized with -20°C acetone for 3 min, or were permeabilized with 50 µg/ml digitonin (Aldrich Chemical Co., Milwaukee, WI) in transport buffer (20 mM HEPES-KOH, pH 7.3, 110 mM potassium acetate, 2 mM magnesium acetate, 1 mM EGTA, 2 mM DTT, and protease inhibitors) for 5 min at room temperature, followed by fixation in 2% formaldehyde/PBS for 30 min at room temperature. Primary antibodies were diluted 1:2,000 in 2% BSA/PBS and incubated with the fixed cells for 1 h at room temperature. After washing with PBS, the cells were incubated with fluorescein-conjugated goat anti-mouse (Organon/Teknika, Durham, NC) for 30 min at room temperature, washed again in PBS, and mounted in buffer containing 80% glycerol, 50 mM Tris-HCl, pH 8.0, and 0.1% *p*-phenylenediamine.

### Immunopurifications

100 U of rat liver nuclear envelopes in buffer D (200 U/ml) were solubilized by addition of SDS to a final concentration of 0.5% and by heating at 55°C for 10 min. 4 vol of RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% deoxycholate) were subsequently added to the sample followed by centrifugation at 20,000 g for 20 min. The supernatant was split into two tubes and incubated for 1 h at 4°C with 1 µl of mAb 19C7 ascites fluid or with 1 µl of ascites fluid produced by mice injected with the parent myeloma cell line SP2/0. 20 µl of protein G-Sepharose (Pharmacia Biotech Inc., Piscataway, NJ) was then added and the samples were incubated for an additional hour at 4°C. After brief centrifugations, the resins were washed four times with ice-cold RIPA buffer/0.1% SDS, one time with PBS, and then boiled in SDS sample buffer. The equivalent of 20 U of nuclei were used for immunoblot analysis.

### Bacterial Expression of Recombinant Proteins

DNA fragments encoding amino acids 1–203 of mouse RanGAP1 or the entire open reading frame of expressed sequence tagged (EST) clone 32220 (starting with the first in frame methionine) were generated by PCR

and subcloned into the NdeI and AvaI sites of the bacterial expression vector pET21a (Novagen, Inc., Madison, WI). The expression vectors were transfected into the *Escherichia coli* strain BL21 (DE3) pLysE, and recombinant proteins were induced for 3 h at 37°C by the addition of 0.2 mM isopropyl thio-β-D-galactoside (IPTG) to the culture media. Bacteria were harvested, lysed in 6 M guanidine hydrochloride, and recombinant proteins were purified by Ni-NTA agarose as recommended by the manufacturer (QIAGEN Inc., Chatsworth, CA). Purified proteins were TCA precipitated and resuspended in PBS.

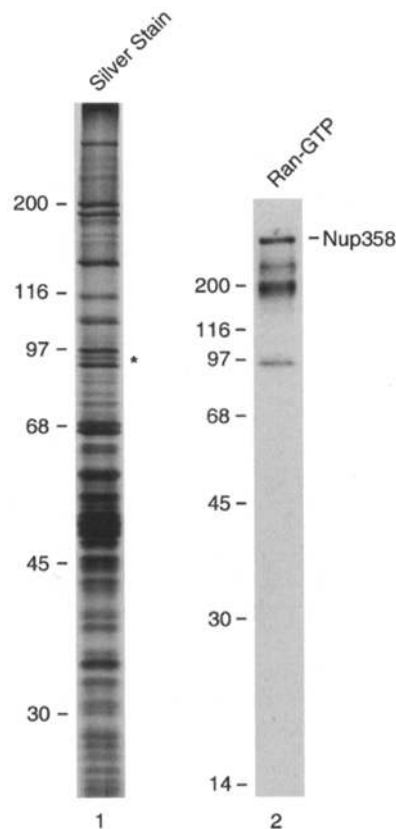
### Immunogold EM

Rat liver nuclear envelopes (prepared as described above) were processed for immunogold EM as described (Wu et al., 1995). Envelopes were incubated with a mixture of mAbs 19C7, 19G12, and 2F1, each diluted 1:1,000.

## Results

### Identification of a 90-kD Form of RanGAP1 Associated with Isolated Nuclear Envelopes

To evaluate the role of Ran in translocation of substrates through the NPC in more detail, we have characterized proteins that are associated with the NPC and that interact directly with Ran-GTP. Two elements were used in identifying proteins with these credentials: (a) a subcellular frac-



**Figure 1.** Identification of a 90-kD Ran-GTP-binding protein associated with isolated nuclear envelopes. Rat liver nuclear envelopes were isolated and separated by SDS-PAGE (10% acrylamide). Proteins were visualized by silver stain (lane 1), or were transferred to nitrocellulose membrane and probed with [<sup>32</sup>P]GTP-labeled Ran (lane 2), as described (Coutavas et al., 1993). The asterisk in lane 1 indicates the position of the 90-kD protein identified in lane 2, and the protein subjected to peptide sequence analysis.

tion highly enriched in nucleoporins; and (b) a ligand blot assay using radiolabeled Ran-GTP. Starting with purified rat liver nuclei, a highly enriched nuclear envelope fraction that contained all of the currently known nucleoporins and that was essentially free of contaminating chromatin and chromatin-associated proteins was isolated (Fig. 1, lane 1). When proteins in this fraction were transferred to a nitrocellulose membrane and probed with radiolabeled Ran-GTP, four prominent bands were detected, corresponding to proteins with apparent molecular masses of 350, 250, 180, and 90 kD (Fig. 1, lane 2). The highest molecular mass protein corresponds to the previously characterized nucleoporin, Nup358 (Wu et al., 1995; Yokoyama et al., 1995), while the proteins of 250 and 180 kD remain uncharacterized. All of the identified proteins bound specifically to Ran-GTP, since their binding was competed with excess cold Ran-GTP, but not with excess cold Ran-GDP (data not shown). Using this same assay, Lounsbury et al. (1994) have identified a similar subset of nuclear Ran-GTP-binding proteins.

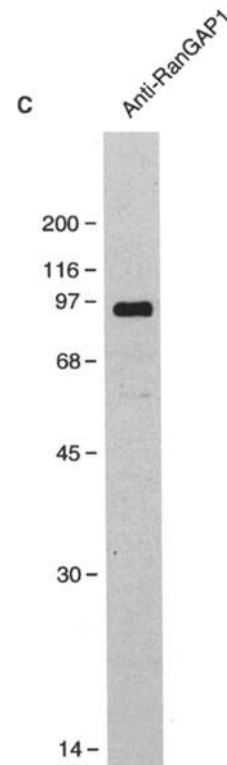
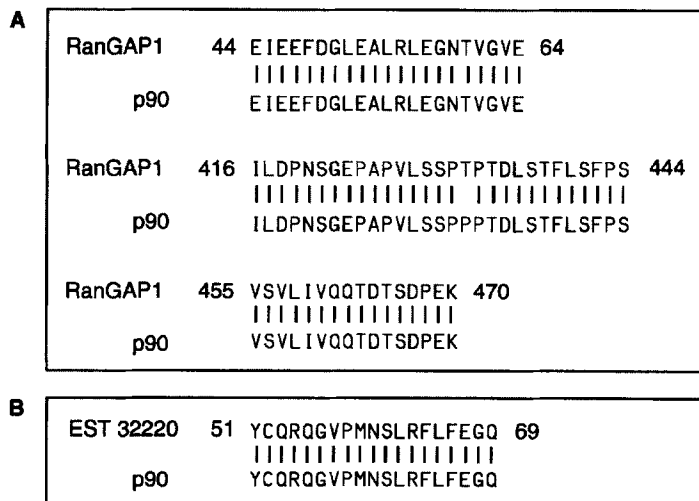
The nuclear envelope-associated Ran-GTP-binding protein migrating at 90 kD was recognized as a single protein (proteins were stained with Ponceau S and their positions were marked before incubation with Ran-GTP), and it was subjected to peptide sequence analysis. Three peptides, encompassing a total of 66 amino acids, were virtually identical to a protein that had been characterized previously as a Ran GTPase-activating protein and known as Fug1 (DeGregori et al., 1994) or RanGAP1 (Bischoff et al., 1995a) (Fig. 2 A). The peptides were derived from regions spanning nearly the entire 589 amino acids predicted for RanGAP1, ranging from amino acids 44 to 470. In addition to the three peptides identical to RanGAP1, a fourth peptide sequence was obtained that showed no homology

to the predicted amino acid sequence of RanGAP1, but that was encoded by a human EST cDNA, clone 32220 (Fig. 2 B).

cDNAs predicting a 65-kD RanGAP1 have been isolated from both human and mouse sources (DeGregori et al., 1994; Bischoff et al., 1995). Furthermore, human RanGAP1 purified from HeLa cells migrates (after eight fractionation procedures) as a 65-kD polypeptide by SDS-PAGE (Bischoff et al., 1994). To demonstrate further that the protein we identified was homologous to RanGAP1, mAbs were generated against a recombinant protein corresponding to the first 203 amino acids encoded by the mouse RanGAP1 cDNA. Hybridomas producing antibodies specific for RanGAP1 were selected by immunofluorescence and immunoblot analysis. All of the identified mAbs recognized a 90-kD protein (which on higher resolution gels appeared as three closely spaced proteins) in the nuclear envelope fraction that comigrated with the protein detected with labeled Ran-GTP (Fig. 2 C). Our identification of a 90-kD protein homologous to RanGAP1, as well as previous work identifying a 65-kD protein (Bischoff et al., 1994), suggest the existence of two highly related RanGAP1 molecules. The identification of one peptide with no homology to RanGAP1 suggested three possible origins for the 90-kD protein. These include a unique gene encoding protein highly related to RanGAP1, an alternatively spliced message derived from the RanGAP1 gene, or a posttranslational modification involving the attachment of an independently encoded polypeptide to RanGAP1.

#### Detection of Two Forms of RanGAP1 and Their Localization to Distinct Subcellular Domains

Because the nuclear envelope-associated RanGAP1 dif-

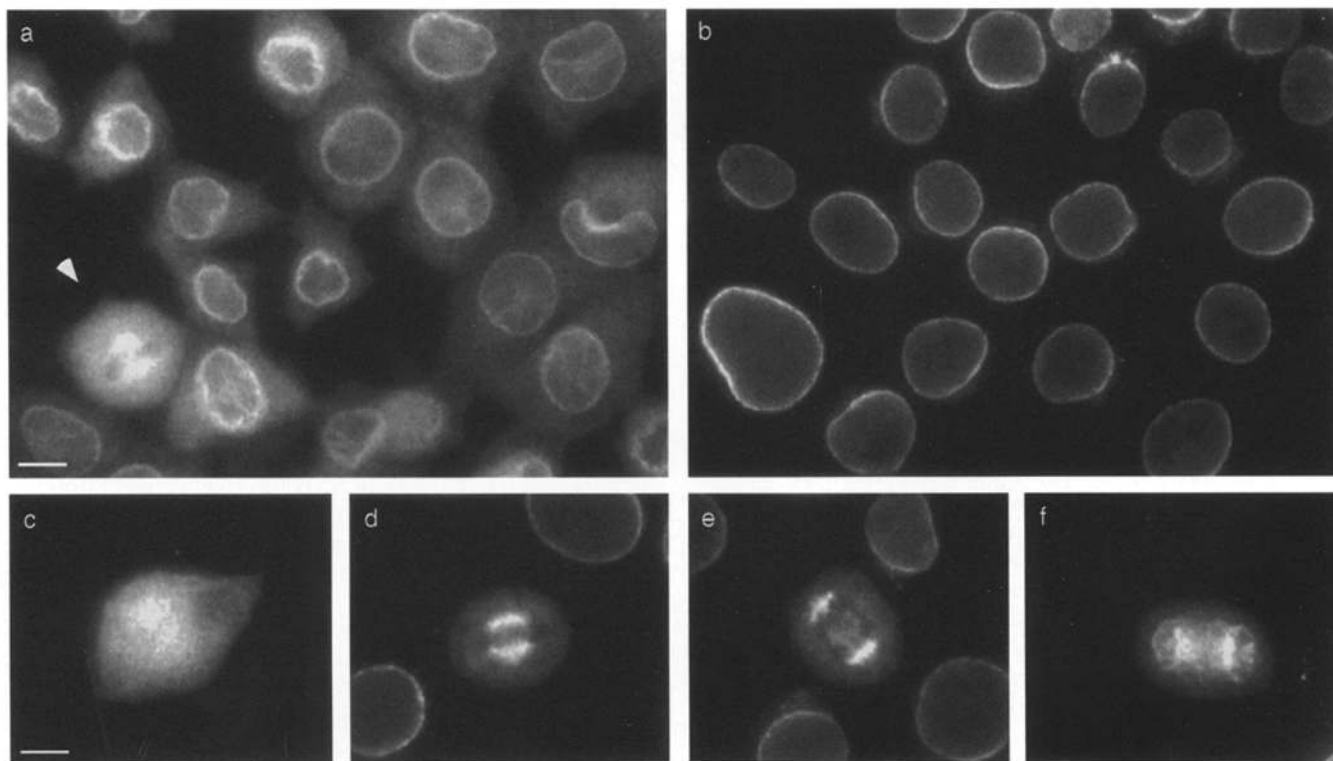


**Figure 2.** The 90-kD Ran-GTP-binding protein associated with isolated nuclear envelopes is homologous to RanGAP1. (A) Alignment of peptide sequences derived from the 90-kD Ran-GTP-binding protein (p90) with predicted amino acid sequences of mouse RanGAP1. (B) Alignment of a unique peptide sequence derived from p90 with the predicted amino acid sequence derived from EST clone 32220. (C) Immunoblot analysis of isolated nuclear envelope proteins with the RanGAP1-specific mAb 19C7.

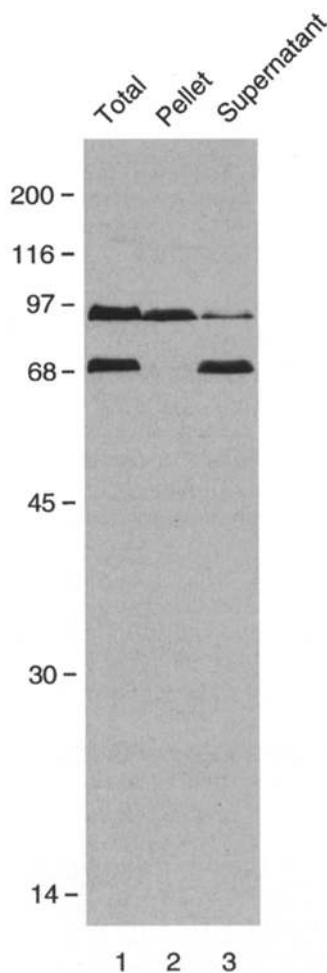
ferred in size from the previously characterized protein, we used the mAbs against RanGAP1 to characterize it in cultured cells. Immunofluorescence microscopy on formaldehyde-fixed and acetone-permeabilized BRL cells revealed a diffuse cytoplasmic signal, as well as an intense nuclear rim staining, further demonstrating an association of RanGAP1 with the nuclear envelope (Fig. 3 *a*). When cells were treated with digitonin (a detergent that specifically permeabilizes the plasma membrane, but not the nuclear envelope) before fixation, and then stained with antibodies, the cytoplasmic signal was no longer apparent, indicating an extraction of the cytoplasmic RanGAP1 (Fig. 3 *b*). Nuclear rim staining, however, was still evident and its punctate appearance was very similar to that observed with antibodies specific for nucleoporins (Davis and Blobel, 1986). Because the nuclear envelope is not permeabilized by digitonin, detection of RanGAP1 under these conditions suggests an association with the cytoplasmic side of the nuclear envelope. During mitosis, RanGAP1 was detected throughout the cell, but surprisingly, it was also found associated with the mitotic spindles. Although association with the spindles could be detected in cells fixed and then permeabilized (Fig. 3 *a*), the localization was most obvious when cells were permeabilized with digitonin before fixation (Fig. 3, *c-f*). By early telophase, RanGAP1 was detected as a halo around the surface of the newly condensed chromatin (Fig. 3 *f*).

Antibodies against RanGAP1 were next used for immunoblot analysis. When total BRL cell lysate was probed with antibodies against RanGAP1, two proteins with apparent molecular masses of 90 and 70 kD were detected (Fig. 4, lane 1), confirming the presence of two related forms of RanGAP1. Similar to the multiple bands detected in isolated nuclear envelopes, the 90-kD protein was actually three closely spaced proteins, whereas the 70-kD protein resolved into at least two proteins. As demonstrated above, the cytoplasmic pool of RanGAP1 is extracted with digitonin, leaving RanGAP1 association with the nuclear envelope intact. Immunoblot analysis of the digitonin-soluble and -insoluble fractions revealed a clear fractionation of the two forms of RanGAP1. Consistent with data presented above on isolated nuclear envelopes, only the 90-kD form of RanGAP1 was detected in the digitonin-insoluble fraction (Fig. 4, lane 2). Conversely, the 70-kD form of RanGAP1 was the predominant form detected in the soluble fraction (Fig. 4, lane 3) along with a relatively minor amount of the 90-kD form (15% of the total). Together, the immunoblot and immunofluorescence data demonstrate that the 70-kD form of RanGAP1 is completely cytoplasmic, and that the 90-kD form of RanGAP1 is associated predominantly with the nuclear envelope.

To localize further the nuclear envelope-associated 90-kD form of RanGAP1, immunogold EM was per-



**Figure 3.** RanGAP1 localizes to the cytoplasm and to the nuclear envelope in interphase cells, and during mitosis, it associates with the mitotic spindles. BRL cells were grown on coverslips, fixed, and permeabilized as indicated before incubation with mAb 19C7. (*a*) Interphase cells first fixed with formaldehyde and then permeabilized with acetone. The arrow indicates a metaphase cell in which staining of the mitotic spindles is visible. (*b*) Interphase cells first permeabilized with digitonin followed by formaldehyde fixation. (*c*) A cell in prophase, first fixed with formaldehyde and then permeabilized with acetone. (*d*) A metaphase cell first permeabilized with digitonin and then fixed with formaldehyde. (*e*) An anaphase cell first permeabilized with digitonin and then fixed with formaldehyde. (*f*) A cell in telophase, first permeabilized with digitonin and then fixed with formaldehyde. Bars: (*a* and *b*) 10  $\mu$ m; (*c-f*) 8  $\mu$ m.



**Figure 4.** Two forms of RanGAP1 are detected, and they fractionate with distinct subcellular domains. BRL cells were either lysed directly in SDS-PAGE sample buffer (lane 1), or they were fractionated into a digitonin-insoluble fraction (lane 2) and a digitonin-soluble fraction (lane 3). Proteins were separated by SDS-PAGE (12.5% acrylamide), transferred to nitrocellulose membrane, and probed with mAb 19C7.

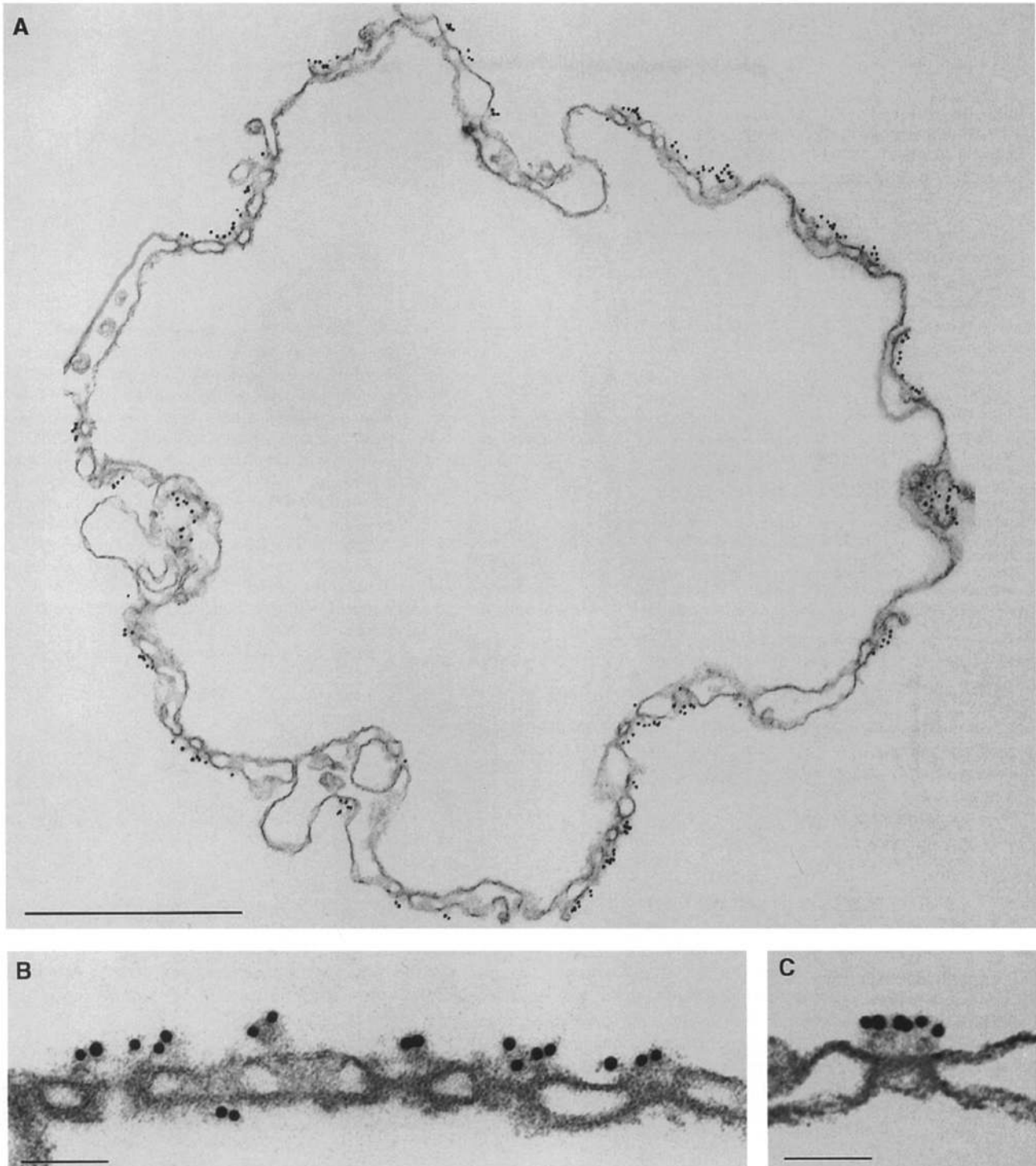
formed using isolated nuclear envelopes and RanGAP1-specific antibodies (Fig. 5). The 90-kD form of RanGAP1 localized to the NPCs (consistent with biochemical sub-fractionation; data not shown), with 95% (total = 1547) of the signal detected in association with cytoplasmic aspects of the NPC. Similar to the localization reported previously for Nup358 (Wu et al., 1995; Yokoyama et al., 1995), the 90-kD form of RanGAP1 appeared to localize at or near the tips of the cytoplasmic fibers of the NPC (Fig. 5 C). In addition to this predominant localization, 5% of the total signal was associated with the nucleoplasmic face of the NPC. The low level of signal was not a result of inaccessibility of the antibodies to the nucleoplasmic side of the nuclear envelopes, as determined by labeling with antibodies to both Nup153 and the nuclear lamins (data not shown). The 90-kD form of RanGAP1, therefore, localizes definitively to the cytoplasmic fibers of the NPC and possibly to the nucleoplasmic side of the NPC. In summary, two forms of RanGAP1 have been identified: a 70-kD form localizing specifically to the cytoplasm and corresponding to the previously characterized 65-kD RanGAP1, and a 90-kD form localizing predominantly to the cytoplasmic fibers of the NPC.

#### **Identification of a Novel Ubiquitin-like Polypeptide Associated with RanGAP1**

To further investigate the relationship between the 90-kD

form of RanGAP1 and the previously characterized 65-kD form of RanGAP1, we focused on EST clone 32220, which coded for the one novel peptide sequence described above. The 1.5-kb cDNA clone was obtained from the IMAGE consortium (Lawrence Livermore National Laboratory, Livermore, CA), and an open reading frame was identified starting at the 5' end and extending through nucleotide 386 (Fig. 6 A). A putative initiation codon begins with nucleotide 100, and surrounding nucleotides conform to the consensus for translation initiation (Kozak, 1991). Beginning with this methionine, the open reading frame codes for a protein with a predicted molecular mass of 11.5 kD. When expressed in bacteria, the recombinant protein migrated with an apparent molecular mass of 17 kD by SDS-PAGE, similar to the presumptive free form of the protein identified in rat liver nuclei (see Fig. 10; data not shown). The protein encoded by this open reading frame will be referred to as GMP1 (GAP modifying protein 1). A search of sequence data bases for proteins homologous to GMP1 revealed a family of highly related proteins in a wide range of organisms, including plants (*Oryza sativa*, *Arabidopsis thaliana*, and *Brassica campestris*), worms (*Caenorhabditis elegans*), protozoans (*Plasmodium falciparum* and *Toxoplasma gondii*), and fungi (*S. cerevisiae*). While the majority of sequences were incomplete translations predicted from EST clones, full-length open reading frames were derived from several overlapping clones (Fig. 6 B). Based on the sequence similarities between homologues, GMP1 could be divided into three domains. The amino-terminal domain (defined by the first 20 amino acids) varies greatly between homologues, while the second domain, extending from amino acid 20 to a carboxyl-terminal double glycine, is highly conserved. All of the proteins contained a third domain of variable sequence and length, extending beyond the carboxyl-terminal double glycine. Among mammals, human, mouse, and rat GMP1 were found to be 100% identical, and human cDNAs encoding two additional GMP1-related proteins were also identified. A full-length open reading frame was deduced from one of these clones, and it predicts a protein that is 45% identical to GMP1 throughout its entire length and 53% identical throughout its carboxyl-terminal domain (Mannen et al., 1996). Overall identities between GMP1 and the worm, rice, and yeast homologues are 59, 39, and 40%, respectively, whereas identities between the carboxyl-terminal domains are 64, 42, and 46%, respectively. Interestingly, the yeast homologue (Smt3p) was originally identified as a suppressor of *MIF2*, a gene encoding a centromere-associated protein required for mitotic spindle integrity (Meluh and Koshland, 1995).

The homology of most significance, however, was that between GMP1 and ubiquitin (Fig. 6 B). The homology with ubiquitin began after the divergent amino-terminal domain found in GMP1 (which is absent from ubiquitin) and extended over the entire length of the second domain. Furthermore, a signature feature of ubiquitin, an invariable double glycine at the carboxyl terminus, is conserved in GMP1 and in all of the GMP1-related proteins (Fig. 6 B). This double glycine is essential for the proteolytic processing of ubiquitin precursors and for the recycling of post-translationally synthesized polyubiquitin (Hershko and Ciechanover, 1992; Wilkinson, 1995). Similar to ubiquitin



**Figure 5.** The 90-kD form of RanGAP1 localizes to the cytoplasmic fibers of the NPC. Isolated nuclear envelopes were fixed and incubated with a combination of three RanGAP1-specific mAbs (19C7, 2F1, and 19G12) followed by incubation with goat anti-mouse IgG conjugated to 10-nm gold. Samples were processed for thin sectioning and observed by EM. (A) A section of nuclear envelope demonstrating the typical labeling pattern observed. The cytoplasmic side of the nuclear envelope (evidenced by occasional blebs in the membrane) is oriented towards the outside. (B) A high magnification view along a single length of envelope. The cytoplasmic side of the envelope is oriented towards the top. (C) A high magnification view of a single NPC, demonstrating localization to the tips of the cytoplasmic fibers. Bars: (A) 1  $\mu\text{m}$ ; (B and C) 0.1  $\mu\text{m}$ .

**A**

GGGAAGGGAGAAGGATTGCTAAACCCCGGAGCGAGGTCTGCTTACCCGAGGCCGCTGCT 60  
 GTGCGGAGACCCCGGGTGAAGCCACCGTCATCATGTCTGACCGAGGCAAAACCTTCA 120  
   M S D Q E A K P S  
 ACTGAGGACTTGGGGGATAAGAAGGAAGTGAATATATTAACCTCAAAGTCATTGGACAG 180  
 T E D L G D K K E G E Y I K L K V I G Q  
 GATAGCAGTGAGATTCACTTCAAAGTGAAGTGAACAACACATCTCAAGAACTCAAAGAA 240  
 D S S E I H F K V K M T T H L K K L K E  
 TCATACTGTCAAAGACAGGGTGTTCATGAATCACTCAGGTTTCTTTGAGGGTCAG 300  
 S Y C Q R Q G V P M N S L R F L F E G Q  
 AGAATTGCTGATAATCATACTCCAAAAGAACTGGGAATGGAGGAAGAAGATGTGATTGAA 360  
 R I A D N H T P K E L G M E E E D V I E  
 GTTTATCAGGAACAACGGGGGTCATTCAACAGTTAGATATTCTTTTATTTTTTTTT 420  
 V Y Q E Q T G G H S T V •  
 TTTTCCTCAATCCTTTTTTATTTTTAAAAATAGTCTTTTGAATGTGGTGTCAAAC 480  
 GGAATTGAAAACCTGGCACCCTATCTTTGAAACATCTGGTAATTTGAATCTAGTGCTC 540  
 ATTATTCATTATTGTTTGTTCATTGTGCTGATTTTTGGTGATCAAGCCTCAGTCCC... 598

**B**

<b>Ubiquitin</b>	M - - - - - Q I F V K	6
H. sapiens (GMP1)	M S D Q E - A K P S T E D L G D K - K E G E Y I K L K V I	27
H. sapiens-II	M A - - - D E K P K E G V K T E - - - N N D H I N L K V A	23
C. elegans	M A D D A - A Q A G D N - - - - - A E Y I K I K V V	20
O. sativa	M S A A G - E E D K K P A G G E G - - - G G A H I N L K V K	26
S. cerevisiae (Smt3p)	M S D S E V N Q E A K P E V K P E V K P E T H I N L K V -	28
<b>Ubiquitin</b>	T L T G K T I T L E V E P S D T I E N V K A K I Q D K E G	35
H. sapiens (GMP1)	G Q D S S E I H F K V K M T T H L K K L K E S Y C Q R Q G	56
H. sapiens-II	G Q D G S V V Q F K I K R H T P L S K L M K A Y C E R Q G	52
C. elegans	G Q D S N E V H F R V K Y G T S M A K L K K S Y A D R T G	49
O. sativa	G Q D G N E V F F R I K R S T Q L K K L M N A Y C D R Q S	55
S. cerevisiae (Smt3p)	S D G S S E I F F K I K K T T P L R R L M E A F A K R Q G	57
<b>Ubiquitin</b>	I P P D Q Q R L I F A G K Q L E D G R T L S D Y N I Q K E	64
H. sapiens (GMP1)	L P M N S L R F L F E G Q R I A D N H T P K E L G M E E E	85
H. sapiens-II	V S M R Q I R F R F D G Q P I N E T D T P A Q L E M E D E	81
C. elegans	V A M N S L R F L F D G R R I N D D T P K T L E M E D D	78
O. sativa	V D M N A I A F L F D G R R L R G E Q T P D E L E M E D G	84
S. cerevisiae (Smt3p)	K E M D S L R F L Y D G I R I Q A D Q T P E D L D M E D N	86
<b>Ubiquitin</b>	S T L H L V L R L R G G	76
H. sapiens (GMP1)	D V I E V Y Q E Q T G G H S T V	101
H. sapiens-II	D T I D V F Q Q Q T G G V Y	95
C. elegans	D V I E V Y Q E Q L G G - - - F	91
O. sativa	D E I D A M L H Q T G G C L P A	100
S. cerevisiae (Smt3p)	D I I E A H R E Q I G G A - T Y	101

**Figure 6.** GMP1 is homologous to ubiquitin, and it is highly conserved from human to yeast. (A) Partial DNA sequence of EST cDNA clone 32220 and the predicted amino acid sequence of GMP1. The underlined amino acids were also derived from peptide sequence analysis (see Fig. 2 B). (B) Amino acid sequence alignments between human ubiquitin, GMP1, and GMP1-related proteins. Residues identical in three or more of the aligned proteins are shaded. Asterisks indicate identical residues in GMP1 and ubiquitin. The primary sequence of human ubiquitin is as first described by Schlesinger et al. (1975). The remaining sequences, with the exception of *H. sapiens II* and the yeast homologue, were identified as predicted translations of EST clones. The human GMP1-related protein (*H. sapiens II*) was derived from the HSMT3 cDNA (Mannen et al., 1996). The *C. elegans* homologue was compiled from clones with GenBank accession Nos. D76147, D73158, and T02042, and the rice homologue (*O. sativa*) was derived from clones D15376 and D22620. The yeast sequence (*S. cerevisiae*) is derived from a cDNA clone coding Smt3p (Meluh and Koshland, 1995).

precursors (all known ubiquitins are synthesized as precursors with carboxyl-terminal extensions, or as polyubiquitin), the double glycines present in GMP1 and in the GMP1-related proteins are not at the extreme carboxyl terminus. The presence of this double glycine in the carboxyl-terminal domain of GMP1, followed by a short variable domain, suggests that GMP1 and ubiquitin may undergo similar enzymatic transformations. At the same time, the overall identity with ubiquitin is only 18%, identifying GMP1 as a novel ubiquitin-like protein.

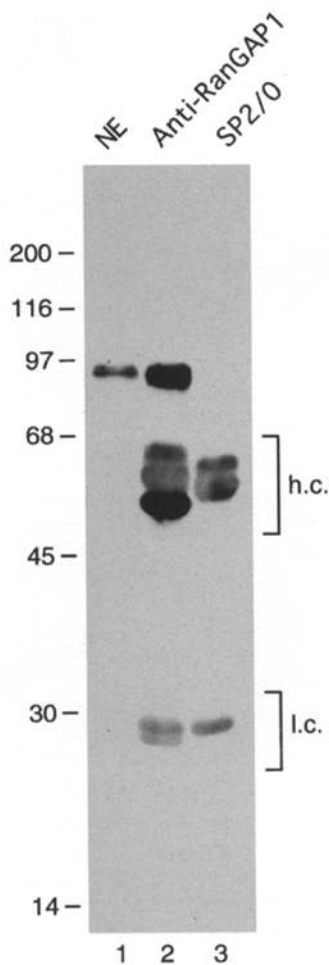
To further characterize GMP1, recombinant protein was produced in bacteria and used to generate mAbs. GMP1-specific antibodies were identified by immunofluorescence and immunoblot analysis. The antibodies were first used to demonstrate that GMP1 and the 90-kD form of RanGAP1 are covalently associated. Nuclear envelope-associated RanGAP1 was immunopurified after denaturation in SDS with a RanGAP1-specific mAb. Immunoblot analysis of the immunopurified protein was then performed using an mAb against GMP1 (the specificity of these antibodies is demonstrated in Fig. 10). Antibodies against GMP1 recognized a 90-kD protein among total nuclear envelope proteins (Fig. 7, lane 1) and a 90-kD polypeptide immunopurified with the anti-RanGAP1 antibody (Fig. 7, lane 2). No

signal was detected among proteins immunopurified with the control antibody SP2/0 (Fig. 7, lane 3). These results indicate that RanGAP1- and GMP1-specific antibodies recognize the same protein or two covalently associated proteins. The homology between GMP1 and ubiquitin is suggestive of the latter possibility. Furthermore, Northern blot analysis with probes specific for GMP1 and RanGAP1 revealed distinct mRNA transcripts of 1.4 and 3.0 kb, respectively. No indication of a single, common transcript encoding both proteins was detected by Northern blot or reverse-transcription PCR (data not shown).

**GMP1 Colocalizes to the NPC with RanGAP1 and It Is Also Present in the Nucleus**

Immunofluorescence microscopy on formaldehyde-fixed and acetone-permeabilized BRL cells detected GMP1 in the nucleus and associated with the nuclear envelope (Fig. 8 a). The intranuclear signal was largely homogeneous; however, the antigen was also concentrated in foci that varied widely in number and in intensity from cell to cell. The signal associated with the nuclear envelope was most apparent when the cells were permeabilized with digitonin before fixation and labeling, and it was very similar to the





**Figure 7.** RanGAP1 and GMP1 are covalently associated. Isolated nuclear envelopes were solubilized with SDS (lane 1), and the 90-kD form of RanGAP1 was immunopurified with the RanGAP1-specific mAb 19C7 (lane 2). A control immunopurification was performed with antibody derived from the myeloma cell line SP2/O (lane 3). Proteins were separated by SDS-PAGE (12.5% acrylamide), transferred to nitrocellulose membrane, and probed with the GMP1-specific mAb 21C7. Antibody heavy chains (*h.c.*) and light chains (*l.c.*) are indicated on the right.

signal observed with antibodies to RanGAP1 (Figs. 3 *b* and 8 *b*). Also, during mitosis, the localization of GMP1 appeared very similar to that of RanGAP1. Signal was detected throughout the cells concomitant with the breakdown of the nuclear envelope at prophase (Fig. 8 *c*), but also associated with the mitotic spindles during metaphase and anaphase (Fig. 8, *d* and *e*). By early telophase, GMP1 could be seen concentrated around the newly condensed chromatin (Fig. 8 *f*). Like the 90-kD form of RanGAP1, GMP1 was also sublocalized to the NPC by immunogold EM (data not shown).

Immunoblots of total BRL cell lysate probed with antibodies against GMP1 revealed a major signal at 90 kD, identifying the 90-kD form of RanGAP1 as the primary cellular protein associated with GMP1 (Fig. 9, lane 1). Significantly less prominent proteins were also detected, however, including a polypeptide with an apparent molecular mass of 50 kD, and a large number of proteins migrating above 116 kD. All of these proteins are presumably covalent conjugates formed between GMP1 and other cellular proteins. To determine the subcellular localization of these proteins, digitonin-soluble and -insoluble fractions (similar to those in Fig. 4) were also probed. As expected for the 90-kD form of RanGAP1, the 90-kD protein recognized by GMP1-specific antibodies was also found predominantly in the digitonin-insoluble fraction (Fig. 9, lane 2). The high molecular mass proteins fractionated exclusively

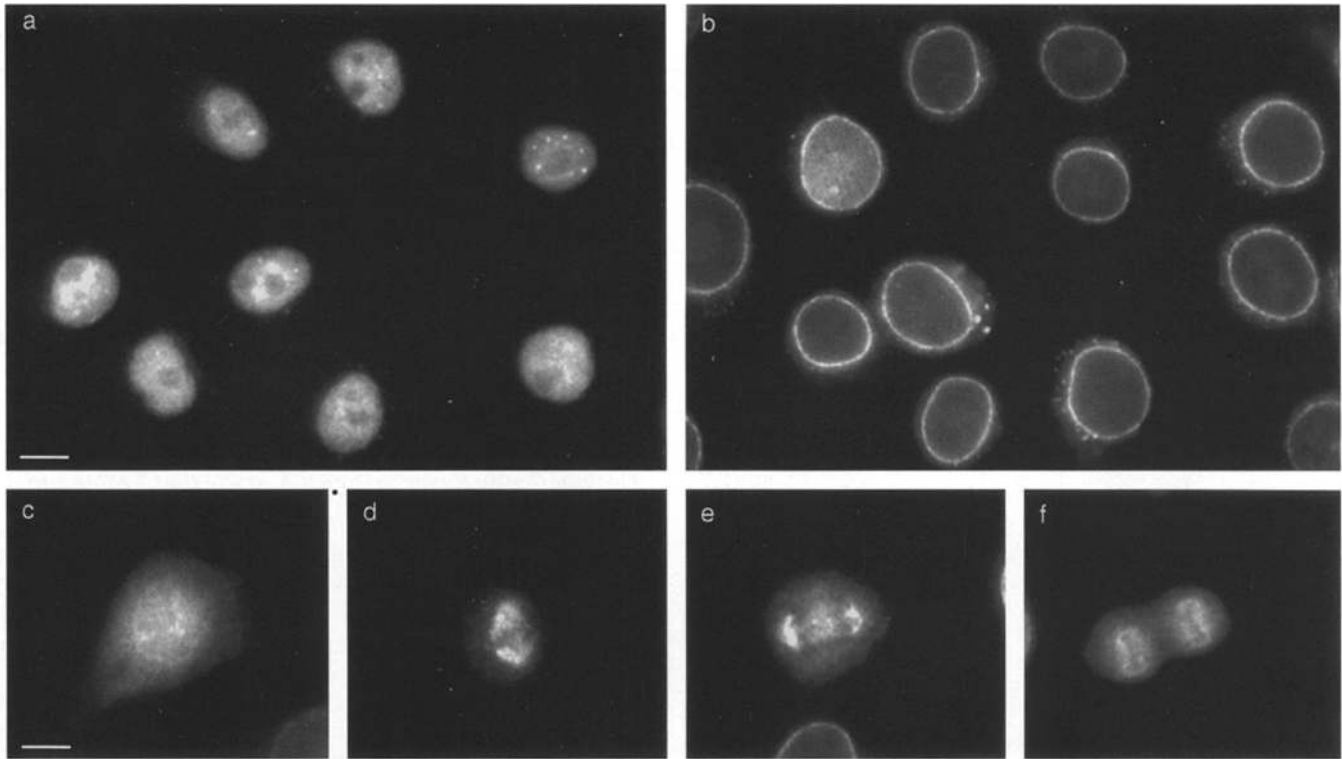
with the digitonin-insoluble fraction (Fig. 9, lane 2), and these antigens likely account for the intranuclear signal observed by immunofluorescence microscopy. The protein migrating at 50 kD, on the other hand, fractionated exclusively with the digitonin-soluble fraction (Fig. 8, lane 3) and is likely to be a cytoplasmic protein. Only a very weak signal was detected at 17 kD, presumably representing free GMP1. The levels of free GMP1 may be underrepresented, since it is possible that the antibody recognizes conjugated GMP1 better than it does free GMP1 (see Fig. 10). These data further support the association of GMP1 with RanGAP1, and they demonstrate the association of GMP1 with additional cellular proteins, predominantly in the nucleus.

### **The Association between RanGAP1 and GMP1 Can Be Enzymatically Reversed by an Activity that Fractionates with NPCs**

During the course of fractionating nuclear envelopes, it was observed that the 90-kD form of RanGAP1 consistently disappeared upon extraction of the PCLF (an insoluble fraction derived from extraction of isolated nuclear envelopes with Triton X-100) with the ionic detergent Empigen BB. Whereas the majority of nucleoporins present in the PCLF were quantitatively solubilized with Empigen BB, the 90-kD form of RanGAP1 was not detected in either the supernatant or the pellet fractions (Fig. 10, lanes 1 and 2; pellet fraction not shown). Concomitant with the disappearance of the 90-kD form of RanGAP1, however, a 70-kD protein not observed in previous fractions appeared in the Empigen supernatant (Fig. 10, lanes 1 and 2). Immunoblot analysis of the PCLF and the Empigen supernatant fractions with an antibody against RanGAP1 demonstrated that the 90-kD form of RanGAP1 had been precisely converted to a protein that comigrates with the 70-kD form (Fig. 10, lanes 3 and 4). At the same time, the antigen recognized by GMP1-specific antibodies was converted to a protein with an apparent mass of 17 kD (Fig. 10, lanes 6 and 7). Given the homologies between GMP1 and ubiquitin, this result can be interpreted as a "deubiquitination" of RanGAP1 by a specific protease that copurifies with the PCLF. To support this interpretation, the PCLF was preincubated with the sulfhydryl alkylating agent NEM before extraction with Empigen BB. NEM is a potent inhibitor of the peptidases responsible for cleaving ubiquitin/substrate isopeptide bonds (Johnson, E., personal communication). Consistent with the involvement of a specific peptidase in the release of GMP1 from RanGAP1, NEM greatly inhibited the conversions detected in mock-treated fractions (Fig. 10, lanes 5 and 8). These data are consistent with the interpretation that the 90-kD form of RanGAP1 originates through a covalent and reversible linkage to GMP1 by a pathway that is analogous to ubiquitination.

### **Discussion**

We have identified a novel ubiquitin-like modification that correlates with the partitioning of the Ran GTPase-activating protein RanGAP1 from the cytosol to the NPC. These findings have specific implications for the function



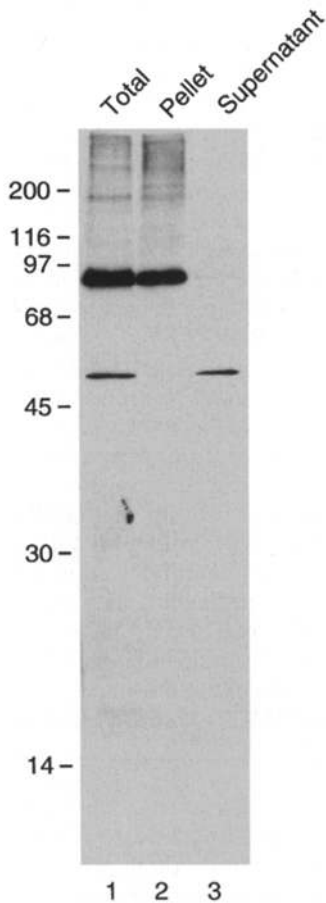
**Figure 8.** GMP1 localizes to the nucleus and the nuclear envelope during interphase, and to the mitotic spindle during mitosis. BRL cells were grown on coverslips, fixed, and permeabilized as indicated before incubation with mAb 21C7. (a) Immunofluorescence on cells fixed and permeabilized with acetone. (b) Localization of GMP1 in cells permeabilized with digitonin before fixation. (c) Localization in cells at prophase. Cells were first fixed and then permeabilized with acetone. (d) Localization in cells at metaphase. Cells were permeabilized with digitonin before fixation. (e) Localization at anaphase in cells permeabilized with digitonin before fixation. (f) Localization at telophase in cells permeabilized with digitonin before fixation. Bars: (a and b) 10  $\mu\text{m}$ ; (c–f) 8  $\mu\text{m}$ .

of Ran in mediating nuclear import, and more general implications for the diversity of ubiquitin modifications and their functions. Ubiquitin is a 76-amino acid, highly conserved protein that is universally present in eukaryotic cells. Its covalent attachment to a variety of cellular proteins is catalyzed by a family of ubiquitin-conjugating (E2) enzymes that mediate the formation of an isopeptide bond between the carboxyl-terminal glycine of ubiquitin and the  $\epsilon$  amino group of a lysine residue in an acceptor protein (Hershko and Ciechanover, 1992; Wilkinson, 1995). Equally important to their conjugation, ubiquitin subunits are also removed by ubiquitin carboxyl-terminal hydrolases (isopeptidases), making ubiquitination a reversible and regulatable posttranslational modification (Hershko and Ciechanover, 1992; Wilkinson, 1995). While ubiquitin-mediated proteolysis is the best-studied function of ubiquitin conjugation, it has also been implicated in regulating a host of other cellular processes, including endocytosis and vacuolar targeting, protein kinase activation, protein import into mitochondria, and peroxisome biogenesis (Ciechanover, 1994; Wilkinson, 1995; Chen et al., 1996; Egner and Kuchler, 1996; Galan et al., 1996; Hicke and Riezman, 1996; Roth and Davis, 1996; Strous et al., 1996). The utility of the ubiquitin system raises the question of whether parallel pathways exist, using novel ubiquitin-like proteins. At the present time, only several examples exist, including an IFN-inducible ubiquitin homologue that conjugates to a large number of intracellular proteins (Loeb

and Haas, 1992), as well as a viral-encoded ubiquitin-like protein (Haas et al., 1996). Whereas the function of the IFN-inducible protein is currently uncertain, the viral-encoded ubiquitin-like protein may function to block degradation of short-lived proteins by the host.

Here, we report the existence of a new family of ubiquitin-like proteins that likely serve novel functions through their conjugation to specific protein substrates. We identified the first member of this family as a covalent modification of the 70-kD Ran GTPase-activating protein RanGAP1, and we have named it GMP1 (for GAP modifying protein 1). Evidence for a covalent and reversible association between RanGAP1 and GMP1 included (a) derivation of peptide sequences for both RanGAP1 and GMP1 from a single protein migrating at 90 kD; (b) coimmunopurification of RanGAP1 and GMP1 antigens under protein-denaturing conditions; and (c) specific enzymatic conversion of the 90-kD form of RanGAP1 to the free 70-kD form of RanGAP1 and free GMP1. Evidence that the modification occurs posttranslationally included (a) detection of unique, nonoverlapping mRNAs coding for RanGAP1 and GMP1 by Northern blot analysis and RT-PCR; (b) overlapping but unique subcellular distributions of RanGAP1 and GMP1 in cultured cells; and (c) rapid, phosphorylation-dependent conversion of the 70-kD form of RanGAP1 to the 90-kD form in vivo (Matunis, M.J., and G. Blobel, unpublished results).

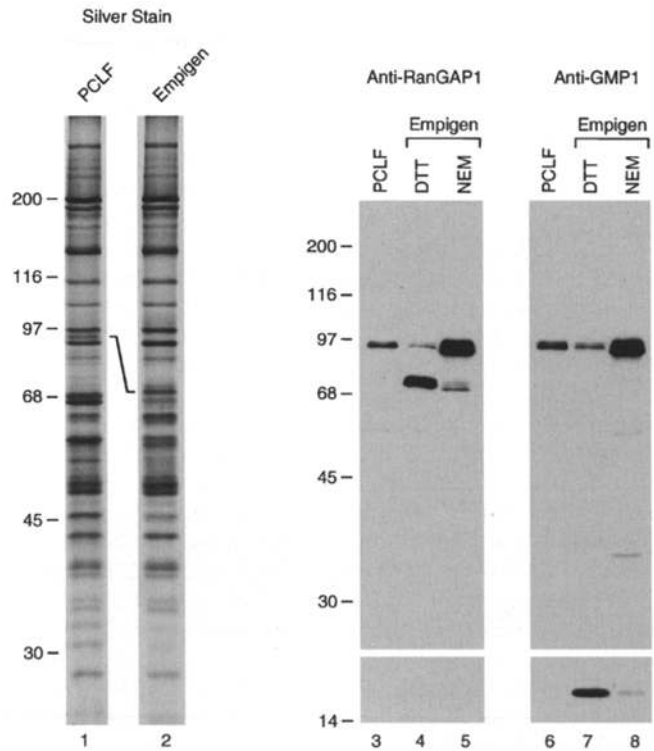
While the exact function of the ligation between RanGAP1



**Figure 9.** GMP1 associates predominantly with RanGAP1, but also with additional substrates in the nucleus and in the cytoplasm. BRL cells were either lysed directly in SDS-PAGE sample buffer (lane 1), or were fractionated into a digitonin insoluble fraction (lane 2), and a digitonin soluble fraction (lane 3). Proteins were separated by SDS-PAGE (15% polyacrylamide), transferred to nitrocellulose membrane, and probed with mAb 21C7.

and GMP1 remains to be determined, the modification correlated with localization of RanGAP1 to the NPC. Immunofluorescence and immunoblot analysis of digitonin-extracted cells revealed that while the modified form of RanGAP1 is predominantly associated with the nuclear envelope, the 70-kD unmodified form is strictly cytoplasmic. Approximately 15% of the 90-kD form of RanGAP1 was detected in the digitonin-extracted fraction; however, it remains to be determined whether this represents a true cytoplasmic pool, or more simply, protein released from the nuclear envelope during the fractionation procedure. Equivalent amounts of the 70-kD unmodified form of RanGAP1 and the 90-kD modified form of RanGAP1 were detected in whole lysates. Based on the apparent size of free recombinant GMP1 on SDS gels (17 kD), we estimate that a single copy of GMP1 is attached to RanGAP1. Unlike the majority of ubiquitin conjugates, no evidence for ligation of multiple copies of GMP1 to RanGAP1 was detected, and we have no evidence for a role in protein degradation. GMP1 may not form ubiquitin-like polymers because the lysine residues of ubiquitin that have been reported to form such chains (most commonly lysine 48) are not conserved in GMP1.

RanGAP1 has previously been purified from HeLa cells using a biochemical assay for RanGAP activity (Bischoff et al., 1994). Approximately equal levels of RanGAP activity were detected in nuclear and cytoplasmic fractions derived from interphase HeLa cells, and both activities were ultimately attributed to the 70-kD unmodified form



**Figure 10.** The 90-kD form of RanGAP1 is enzymatically processed into the 70-kD form of RanGAP1 and free GMP1. The pore complex lamina fraction (PCLF) was extracted with the ionic detergent Empigen BB after treatment with DTT and NEM, or after treatment with NEM alone. The PCLF and proteins solubilized by Empigen were separated by SDS-PAGE (10 or 12.5% acrylamide) and visualized by silver stain (lanes 1 and 2), or they were transferred to nitrocellulose membrane and probed with the RanGAP1-specific mAb 19C7 (lanes 3–5) or with the GMP1-specific mAb 21C7 (lanes 6–8). Extraction with Empigen after mock treatment with NEM resulted in conversion of the 90-kD form of RanGAP1 to the 70-kD form (lanes 3 and 4), with a concurrent shift in GMP1-reactive proteins from 90 to 17 kD (lanes 6 and 7). Conversions were inhibited by preincubation of the PCLF with the alkylating agent NEM before Empigen extraction (lanes 5 and 8). The lower portion of the immunoblots were overexposed, since the level of free GMP1 that was detected was not equivalent to the level of the 90-kD RanGAP1 (either resulting from a lower reactivity of the antibody for free GMP1 or from less free GMP1 being present on the blot).

of RanGAP1. Because our findings demonstrate that the unmodified form of RanGAP1 is strictly cytosolic, it is likely that the nuclear RanGAP1 purified in this study was converted from the GMP1-modified form to the unmodified form during the course of its purification (possibly during the 400 mM NaCl extraction of the nuclear pellet). Precedence for demodification of nuclear-associated RanGAP1 is provided by the specific release of GMP1 from RanGAP1 after detergent extraction of the isolated PCLF (Fig. 10). With this consideration, RanGAP activity detected in association with the nuclear pellet would indicate that the modified form of RanGAP1 is an active GAP. Because antibodies specific for mammalian RanGAP1 were not previously available, the 90-kD form of RanGAP1 was not detected by immunoblot analysis.

By immunogold EM, the 90-kD form of RanGAP1 was localized to the cytoplasmic fibers of the NPC. Approximately 5% of the signal detected on isolated nuclear envelopes was also localized to the nucleoplasmic side of the NPC. This relative distribution is consistent with results obtained by immunofluorescence microscopy, where the majority of the signal was detectable from the cytoplasmic side of the nuclear envelope. RanGAP1 has previously been considered a cytosolic factor, and models concerning how Ran functions to mediate nuclear import have taken this into account by postulating the existence of additional RanGAPs, or a requirement for Ran to shuttle between the nucleus and cytoplasm (Rush et al., 1996; Sazer, 1996). Localization of RanGAP1 to the cytoplasmic fibers of the NPC obviates the need for Ran to enter the cytoplasm to complete its GTP/GDP cycle, and strongly implicates the fibers as one of the major sites of RanGTPase activity and a critical regulatory point in protein import. We envision two models for Ran-mediated nuclear import based on Ran-GTP hydrolysis at this site. First, Ran-GTP hydrolysis at the cytoplasmic fibers of the NPC could be directly linked to release of docked karyopherin/substrate complexes and the commitment of these complexes to subsequent phases of translocation, as proposed recently (Melchior et al., 1995). Nup358, which is one component of the cytoplasmic fibers, potentially binds Ran-GTP and karyopherin/substrate complexes, and it would therefore be a likely site for this initial event. Subsequent steps in the translocation process would not require Ran, although Ran-GTP would have to be regenerated, presumably after translocation of Ran-GDP into the nucleus and interaction with RCC1. As an alternative model (although not necessarily exclusive), Ran-GTP hydrolysis could mediate the release and import of karyopherin/substrate complexes indirectly by simply serving to generate Ran-GDP. As recently demonstrated, the Ran-interactive protein p10 can function to coordinate dissociation of docked karyopherin receptor complexes by mediating the formation of a pentameric complex of Ran-GDP, p10, karyopherin- $\alpha$  and - $\beta$ , and nucleoporin. Dissociation of docked complexes is proposed to be mediated by the association of *in situ*-generated Ran-GTP with karyopherin- $\beta$ . According to this model, Ran would function at multiple steps in the translocation process, mediating dissociation of docked complexes along the length of the NPC. Karyopherin- $\beta$  would have to be regenerated either by GTP hydrolysis (presumably at the cytoplasmic fibers of the NPC) or by exchange of GTP for GDP in the nucleus, mediated by RCC1.

Rates of nuclear import vary depending on physiological conditions (Feldherr and Akin, 1994). According to either model presented above, the relative rates of nuclear import could be limited directly by the concentration of RanGAP1 at the NPC. In addition, the NPC-associated RanGAP1 could also be positioned to control rates of nuclear import indirectly by regulating the concentration of Ran-GTP in the cytosol. Considering the nucleus to be the primary source of Ran-GTP, both the modified 90-kD form of RanGAP1 and Nup358 could be positioned to capture and hydrolyze Ran-GTP diffusing through the NPC. Levels of Ran-GTP in the cytosol are potentially significant, since complexes formed with karyopherin- $\beta$

could inhibit nuclear import by preventing karyopherin- $\alpha/\beta$  heterodimers from forming (Rexach and Blobel, 1995). The relative concentrations of RanGAP1 at the NPC could, therefore, be used to regulate the overall rates of nuclear import both directly and indirectly. Conjugation of GMP1 to RanGAP1 correlates with its association with the NPC, and it could in turn serve to regulate the relative concentration of RanGAP1 associated with the NPC.

At least three possible scenarios can be envisioned for how GMP1 may mediate association of RanGAP1 with the NPC. First, GMP1 itself may bind directly to a component of the fibers, thereby tethering RanGAP1 to the NPC. This scenario is not immediately favored, given the detection of GMP1 both in the cytoplasm and in the nucleus, possibly ligated to additional protein substrates. Alternatively, ligation of GMP1 may function to expose a binding site on RanGAP1 that would be masked in the unmodified protein. The 70-kD unmodified form of RanGAP1 forms a homodimer (Bischoff et al., 1994), and GMP1 ligation could potentially disrupt dimer formation, allowing RanGAP1 to interact with a protein at the NPC. It is interesting to note that both RanGAP1 and Nup358 have leucine-rich motifs, which are potential protein-protein interaction domains. Finally, a combination of these two scenarios may occur, whereby ligation of GMP1 creates a binding site with residues from RanGAP1 and GMP1 contributing to association with the NPC. We are currently investigating the details of how modifications of RanGAP1, including phosphorylation and ligation of GMP1, affect the subcellular localization of RanGAP1. In addition to affecting its localization, it is also possible that ligation of GMP1 to RanGAP1 alters its enzymatic activity as a Ran-GTPase-activating protein. Biochemical analysis of the 70-kD unmodified form of RanGAP1 demonstrated it to be a potent RanGTPase activator (Bischoff et al., 1994). At the present time, we have detected significant RanGAP activity associated with isolated nuclear envelopes (Coutavas, E., and G. Blobel, unpublished data), suggesting that the 90-kD modified form of RanGAP1 is also active.

In addition to localization at the NPC, it was also observed that antibodies specific for both RanGAP1 and for GMP1 recognized the mitotic spindle apparatus in dividing cells, suggesting that the 90-kD form of RanGAP1 relocalizes from the NPC to the spindles during mitosis. This finding is particularly interesting in light of the observations that implicate Ran as a regulator of nuclear structure and entry into mitosis (Kornbluth et al., 1994; Clarke et al., 1995). Localization of RanGAP1 at the spindle implicates this structure as a point for the release of Ran-GTP from a mitotic effector molecule. Microtubules may be one such effector of the Ran pathway during mitosis, since overexpression of RCC1 in yeast suppresses mutations of  $\alpha$ -tubulin that arrest with excess microtubules (Kirkpatrick and Solomon, 1994). Regulatory interactions (direct or indirect) between Ran and microtubules could, therefore, act as one signal for cell cycle progression. A second potential target of the Ran pathway at the mitotic spindles is NuMA, a 236-kD intranuclear protein that associates with the pericentrosomal domain of the spindle apparatus during mitosis. Ectopic expression of NuMA lacking its globular head domain results in cells with a phenotype morphologically identical to that of temperature-sensitive mutants of

RCC1, and overexpression of wild-type NuMA can partially suppress the phenotype of temperature-sensitive mutant RCC1 cells (Compton and Cleveland, 1993). It remains to be determined whether these properties result strictly from a failure to reimport NuMA into the nucleus after mitosis. Finally, the yeast homologue of GMP1, Smt3p, was originally identified as a suppressor of the *MIF2* gene, whose protein product is a centromere protein required for mitotic spindle integrity (Meluh and Koshland, 1995). It is presently unknown whether Smt3p also localizes to the spindle in yeast and whether it is conjugated to the RanGAP1 homologue. At the present time, immunoblot analysis has revealed only one (apparently unmodified) form of RanGAP1 in *S. cerevisiae* (Hopper et al., 1990; Koepp et al., 1996). If the 90-kD modified form of RanGAP1 proves specifically to localize to the spindles, conjugation with GMP1 may be an important determinant. It is interesting to note that a second yeast ubiquitin-like protein, Dsk2p, has also been identified as a factor involved in mitotic spindle formation (Biggins et al., 1996).

In conclusion, our findings have specific implications for the function of Ran in mediating nuclear import, and for the use of ubiquitin-like modifications to regulate cell processes. We have characterized just one of a new family of ubiquitin-like proteins and found it to be conjugated with relative specificity to RanGAP1. In addition to GMP1, we identified mammalian cDNAs that encode two additional GMP1-related proteins. Given the indications that GMP1 ligation has specific function(s) distinct from the signaling protein degradation of RanGAP1, characterization of these homologues and their substrates will be of interest. At the present time, the enzymology of GMP1 ligation to RanGAP1 is not known, although we have identified an activity that appears to reverse the modification. Surprisingly, this activity was detected in a highly enriched pore complex lamina fraction, indicating that the activity may be associated with the NPC. Because Empigen extraction leads to the solubilization of the majority of proteins associated with the PCLF (with the exception of the lamins and several lamin-associated proteins), it is likely that the putative peptidase is solubilized along with RanGAP1. Solubilization of both substrate and enzyme could allow their interaction and may explain the observed peptidase "activation." While the significance of this peptidase activity is presently unknown, it may potentially control regulated release of RanGAP1 from the NPC. This activity and other regulators of this novel RanGAP1 modification are being investigated.

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*Note added in proof.* While this manuscript was being reviewed, another group has published the finding that GMP1 interacts with the PML component of a multiprotein complex that is disrupted in acute promyelocytic

leukemia (Boddy, M.N., K. Howe, L.D. Etkin, E. Solomon, and P.S. Freemont. 1996. *Oncogene*. 13:971-982). Consistent with our immunodetection of GMP1 in brightly stained spots in the nucleus, Boddy et al. demonstrated that GMP1 localizes to distinct structures in the nucleus known as PML nuclear bodies. At the present time, there is no evidence that RanGAP1 also localizes to these structures.

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