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Biochemistry

RanGAP1 induces GTPase activity of nuclear Ras-related Ran

(GTPase-activating protein/RCC1/TC4/G_2 checkpoint)

F. RALF BISCHOFF*†, CHRISTIAN KLEBE‡, JÜRGEN KRETSCHMER*, ALFRED WITTINGHOFER‡, AND HERWIG PONSTINGL*

*Division for Molecular Biology of Mitosis, German Cancer Research Center, D-69120 Heidelberg, Federal Republic of Germany; and ‡Abteilung Strukturelle Biologie, Max-Planck-Institut für Molekulare Physiologie, D-44139 Dortmund, Federal Republic of Germany

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ABSTRACT **The nuclear Ras-related protein Ran binds guanine nucleotide and is involved in cell cycle regulation. Models of the signal pathway predict Ran to be active as Ran·GTP at the initiation of S phase upon activation by the nucleotide exchange factor RCC1 and to be inactivated for the onset of mitosis by hydrolysis of bound GTP. Here a nuclear homodimeric 65-kDa protein, RanGAP1, is described, which we believe to be the immediate antagonist of RCC1. It was purified from HeLa cell lysates and induces GTPase activity of Ran, but not Ras, by more than 3 orders of magnitude. The Ran mutant Q69L, modeled after RasQ61L, which is unable to hydrolyze bound GTP, is insensitive to RanGAP1.**

Ras-related small guanine nucleotide-binding proteins participate in various intracellular signal pathways. As a rule, the GTP-bound form represents the active state of a signal. Their latent GTPase activity is induced upon interaction with GTPase-activating proteins (GAPs). The GDP-bound form is inactive in signaling, and nucleotide exchange factors catalyze reactivation by exchanging GDP for GTP. Both nucleotides are otherwise firmly bound in the presence of Mg^{2+}. Whereas most members of the Ras superfamily are associated with membranes, Ran (1) [or TC4, which was identified by homology screening in a human teratocarcinoma cDNA library (2)] is a soluble nuclear protein. In the GTP-bound form it is believed to be involved in inhibiting the onset of mitosis. Cells expressing mutated Ran/TC4 incapable of hydrolyzing GTP are arrested in G_2 (3). In contrast, inactivation of its nucleotide exchange factor RCC1 results in premature chromosome condensation, activation of the cdc2 kinase, and onset of mitosis in S-phase cells (4), presumably by depletion of active Ran·GTP. Additional effects of inactivating or removing RCC1 have been described in various systems: Exit from mitosis is inhibited (5), G1 cells do not enter S phase (4), synthesis of double-stranded DNA is not initiated (6), and mRNA transcripts are processed incompletely (7–9). To identify further members of this signal pathway, we have used [γ-^{32}P]GTP·Ran to monitor the isolation of a protein (RanGAP1) inducing its GTPase activity.

MATERIALS AND METHODS

Purification of RanGAP. Fifty milliliters of packed unsynchronized HeLa cells was thawed in 150 ml of lysis buffer [20 mM Bis-Tris-propane·HCl, pH 7.0/1 mM EDTA/1 mM dithiothreitol (DTT)/protease inhibitors (10)] and swollen on ice for 20 min. They were lysed by 10 strokes with an S-type Dounce homogenizer and centrifuged at 70,000 × *g* for 60 min. The pellet was extracted with 100 mM NaCl in lysis buffer and recentrifuged at 70,000 × *g* for 30 min. Supernatants were pooled and chromatographed on Fractogel EMD DMAE-650/M (Merck; Superformance, 26 × 115 mm) in 20 mM Bis-Tris-propane·HCl, pH 7.0/1 mM DTT with a linear gradient of NaCl from 0.05 M to 1 M at a flow rate of 5 ml/min. Fractions containing RanGAP were pooled and immediately applied to a hydroxylapatite column (Merck; Superformance, 10 × 150 mm) in 20 mM potassium phosphate, pH 7.0/1 mM DTT, with a linear gradient from 20 mM to 1 M phosphate at a flow rate of 2 ml/min. To fractions containing RanGAP, ammonium sulfate in 20 mM Bis-Tris-propane·HCl (pH 7.0) was added to 35% saturation within 20 min. The precipitate was removed by centrifugation for 10 min at 6000 × *g*, and the supernatant was loaded on a hydrophobic column (Merck; Fractogel EMD AFTA 650/S, Superformance, 10 × 20 mm). Chromatography was started with 35% saturated ammonium sulfate in 20 mM Bis-Tris-propane·HCl, pH 7.0/1 mM DTT at a flow rate of 1 ml/min, and a linear gradient of 35 to 0% ammonium sulfate saturation was applied in 20 mM Bis-Tris-propane·HCl, pH 7.0/1 mM DTT/100 mM NaCl. The eluate containing RanGAP was diluted 5-fold with 20 mM Tris·HCl (pH 7.5) and separated on a Mono Q (HR 5/5; Pharmacia) column at a flow rate of 1 ml/min in the same buffer containing 200 mM NaCl, with a linear gradient to 1 M NaCl. RanGAP1 was also purified from a 400 mM NaCl extract of the 70,000 × *g* pellet from HeLa cell lysate, using the same protocol.

Purification of Bacterially Expressed Ran. Ran/TC4 was expressed in *Escherichia coli* BL21(DE3) from PET3d vector (11). Bacteria were grown to an OD of 0.4 in a 2-liter culture, and expression of the protein was induced by 0.5 mM isopropyl β-D-thiogalactoside. After 3 h at 37°C cells were harvested by centrifugation for 5 min at 5000 × *g* and resuspended on ice in 8 ml of 30 mM Tris·HCl, pH 7.5/40 mM NaCl/1 mM DTT, containing lysozyme at 1 mg/ml and protease inhibitors. After 20 min on ice, cells were sonicated 15 times for 10 sec (Branson Sonifier B15, microtip, 50% cycle, output control 4) followed by a 30-min centrifugation at 30,000 × *g*. Ran was precipitated from the supernatant at 35–55% ammonium sulfate saturation and centrifuged at 70,000 × *g* for 20 min. The pellet was dissolved in 25 mM Mes, pH 6.5/50 mM NaCl/1 mM 2-mercaptoethanol/10% glycerol and purified by gel filtration on a Sephacryl S-200 (26 × 1000 mm; Pharmacia) column by using the same buffer at a flow rate of 1 ml/min. The fraction containing Ran was incubated in 10 mM EDTA with 1 mM GTP for 4 h on ice. Final purification was achieved on Fractogel EMD SO_3^- 650 (S) (Merck; 10 × 50 mm) in the same buffer with a step elution of 500 mM NaCl at a flow rate of 2 ml/min.

The Q69L substitution was introduced into Ran by site-directed mutagenesis in M13mp (Amersham), and the mutant protein was expressed and purified by the method described

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Abbreviations: DTT, dithiothreitol; GAP, GTPase-activating protein; DMAE, dimethylaminoethyl.

†To whom reprint requests should be addressed at: Division for Molecular Biology of Mitosis, German Cancer Research Center, Im Neuenheimer Feld 280, D-69120 Heidelberg, F.R.G.

by Klebe et al. (11) for recombinant wild-type Ran/TC4. RasGAP and the catalytic domain of NF1 were expressed and purified as described by Gideon et al. (12) and by Wiesmüller and Wittinghofer (13).

RanGAP Assays. Twenty-five micromolar Ran·GTP or Ras·GTP (14) was incubated for 30 min on ice with 13 μM [γ-^{32}P]GTP (15 Ci/mmol; 1 Ci = 37 GBq) in the presence of 20 mM EDTA in a final volume of 150 μl. The buffer was changed to 20 mM Hepes, pH 7.4/5 mM $MgCl_2$/0.05% hydrolyzed gelatin (incubation buffer) on a NAP-5 column (Pharmacia).

To identify RanGAP activity, dilutions of fractions in a total volume of 10 μl were added to 40 μl of 60 nM Ran·[γ-^{32}P]GTP (in incubation buffer). The GTPase reaction was stopped after shaking for 6 min at 25°C by addition of 1 ml of charcoal suspension [7% (wt/vol) charcoal/10% (vol/vol) ethanol/0.1 M HCl/10 mM KH_2PO_4 (15)]. After centrifugation at 10,000 × g, release of [^{32}P]phosphate was determined in 700 μl of the supernatant in a Packard 1500 Tri-Carb liquid scintillation counter. RanGAP activities are expressed as the percentage of Ran-bound [γ-^{32}P]GTP hydrolyzed within 6 min, corrected for buffer control. Enrichment of RanGAP activity was calculated by determining the amount of the fraction leading to 50% hydrolysis of Ran-bound [γ-^{32}P]GTP after a 2-min incubation at 25°C. Alternatively, RanGAP activity was analyzed using a filter-binding assay: 10-μl volumes of RanGAP-containing fractions were added to 300 μl of 60 nM Ran·[γ-^{32}P]GTP in incubation buffer. Forty-microliter aliquots of the reaction mixture were removed after 0.5, 1, 1.5, 2, 3, 4, and 5 min at 25°C. Two-milliliter volumes of ice-cold rinsing buffer (10 mM Tris, pH 7.5/150 mM NaCl/5 mM $MgCl_2$/1 mM DTT) were added immediately to the samples and to a control without RanGAP, followed by vacuum filtration through nitrocellulose (Schleicher & Schuell; BA85, 0.45 μm). The filters were washed three times with 2 ml of the same buffer and dried. The remaining Ran-bound radioactivity was determined by scintillation counting. cpm were plotted logarithmically versus time, and RanGAP activity was calculated from the slope of the line during the initial phase of the reaction.

Renaturation of RanGAP1 on Nitrocellulose Membrane. Purified RanGAP1 (0.55 μg) and 1 μg of ovalbumin and of bovine serum albumin as controls were separated by SDS/PAGE and transferred to nitrocellulose. The blot was stained with Ponceau S, protein bands were excised, and each piece of nitrocellulose was treated with 1 ml of 7 M guanidinium chloride in 20 mM Tris·HCl, pH 7.4/1 mM DTT/1 mM EDTA for 30 min. Protein was renatured by dropwise addition of 20 mM Tris·HCl, pH 7.4/1 mM DTT/1 mM EDTA/0.05% (vol/vol) Tween 20, within 30 min at room temperature on a rotation shaker. After three 1:10 dilution steps, the nitrocellulose pieces were blocked for 10 min in 20 mM Hepes, pH 7.4/5 mM $MgCl_2$/5% hydrolyzed gelatin and used for the RanGAP assay. After incubation in 100 μl of 70 nM Ran·[γ-^{32}P]GTP for 5 min at 25°C on a shaker, the liberated [^{32}P]phosphate in 80 μl of the reaction mixture was determined as described above. The nitrocellulose pieces were reused for further incubations for periods as indicated in Fig. 6 *Inset*.

Preparation of Subcellular Fractions. HeLa cells (2 × 10^8) arrested for 24 h with 2.5 mM thymidine in S phase or with nocodazole at 80 μg/liter in mitosis were lysed hypotonically in 20 mM Bis-Tris-propane·HCl, pH 7.0/1 mM DTT/5 mM $MgCl_2$/protease inhibitors at 4°C, leaving the nuclei morphologically intact. After adding sucrose to 15% (wt/vol), the suspension was centrifuged for 10 min at 600 × g in a swinging bucket rotor. Supernatants were removed and the plasma membrane fraction was pelleted by centrifugation for 1 h at 100,000 × g. The interphase nuclei and the mitotic pellet including the chromosomes were resuspended in lysis buffer containing 15% sucrose and layered on a saccharose step gradient (75/30%). After centrifugation for 10 min at 1200 × g, the resulting nuclei and the mitotic chromatin at the 30%/75% interphase were resuspended in lysis buffer containing 100 mM NaCl and sonicated for 10 sec as described for bacteria. The suspension was centrifuged for 10 min at 5000 × g to separate the soluble material (nucleoplasm in case of thymidine arrested cells) from the insoluble material.

RESULTS

Comparison of Ran from HeLa Cells and Recombinant TC4 as Substrates for Monitoring RanGAP Purification. Sequence alignments of all known members of the Ran/TC4 family and of most ras-related proteins showed a conserved arginine residue at the position corresponding to Arg-129 in the Ran/TC4 sequence. After reexamining our previous data (1), we could confirm Arg-129. Now all amino acid sequences obtained from HeLa Ran peptides fit the corrected reading frame of TC4 (16). Furthermore, Ran from HeLa and the human TC4 gene product expressed in *E. coli* show identical mobilities in PAGE, and RCC1-catalyzed GDP exchange (11) as well as RanGAP-stimulated GTP hydrolysis (data not shown) yielded similar results. Therefore, both proteins can be assumed to be identical. We used bacterially expressed Ran/TC4 complexed with [γ-^{32}P]GTP to monitor RanGAP activity.

Subcellular Localization of RanGAP. HeLa cells arrested in S or M phase were separated into different cellular fractions. In S-phase cells about 75% of the total RanGAP activity was detected in the nuclei, yet a significant amount of RanGAP was also found in the cytoplasmic fraction (Fig. 1). More than 50% of the nuclear RanGAP activity was released into the supernatant after disruption of the nuclear membrane by ultrasonication. We cannot rule out that RanGAP in the cytoplasmic fraction resulted from leakage of the soluble nuclear protein through the damaged nuclear membranes during preparation of nuclei.

Half of the RanGAP activity bound to the insoluble nuclear pellet could be extracted by increasing the NaCl concentra-

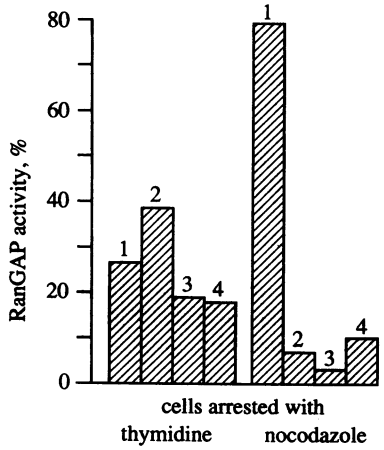

FIG. 1. Subcellular localization of RanGAP activities. Activity was determined in the cytoplasmic fraction (bar 1), the nucleoplasm (bar 2), the 400 mM NaCl extract of nuclei (bar 3), and the resuspended residual insoluble pellet (bar 4) of S-phase cells (*Left*) and in the corresponding fractions of mitotic cells (*Right*). For calculating RanGAP activity, a dilution series of every fraction was used to determine the amount of sample required to stimulate 20% hydrolysis of Ran-bound [γ-^{32}P]GTP (48 nM). Liberated [^{32}P]phosphate was quantitated as described in *Materials and Methods*. Activities are expressed as a percentage of the total RanGAP activity detected. As control, nucleotide exchange activity was determined for endogenous guanine nucleotide in the absence of added free guanine nucleotide, using Ran·[α-^{32}P]GTP as substrate.

tion to 400 mM. Treatment of the pellet with 25 mM chloroquine or digestion of DNA with DNase I at 20 μg/ml did not solubilize RanGAP, whereas RCC1 was almost quantitatively extracted under these conditions (10, 17–19). Therefore, the RanGAP activity in the insoluble HeLa pellet of interphase cells is unlikely to be bound to chromatin. No activity was associated with the plasma membrane fraction. In mitotic cell extracts, about 80% of RanGAP activity was found in the mitoplasm, the soluble fraction of mitotic cell lysates after breakdown of the nuclear membrane.

Purification of RanGAP1 from HeLa Cells. To obtain the full spectrum of cellular RanGAP activity, we subjected logarithmically growing HeLa cells (2×10^{10}) to hypotonic lysis. Both the pellet and the supernatant contained RanGAP activity, which was purified separately by identical procedures. Supernatant plus a 100 mM sodium chloride preextract of the pellet was applied to a dimethylaminoethyl (DMAE) anion-exchange column. RanGAP activity was strongly bound at pH 7.0 and eluted in a single peak at about 600 mM NaCl. The peak fraction was rechromatographed on hydroxylapatite (chromatograms not shown, see Fig. 4 and Table 1). Enzymatically active protein was further enriched in the supernatant of the eluate fraction after 35% saturation with ammonium sulfate. Since RanGAP was inactivated upon precipitation, a further increase of ammonium sulfate concentration was avoided. The supernatant was chromatographed on a hydrophobic column (Fig. 2). Interestingly, a population of RanGAP representing 6% of the activity eluted appeared in the flowthrough. The residual 94% of the activity was eluted in a single peak by reducing the ammonium sulfate concentration to 15% saturation. At this level of purification, a 65-kDa protein band in SDS gels could be correlated with RanGAP activity. Final purification of the 65-kDa RanGAP was achieved by chromatography on Mono Q (Figs. 3 and 4). It is designated RanGAP1 to distinguish it from the second activity in the flowthrough of the hydrophobic column.

To verify that the RanGAP activity results from the 65-kDa protein, the band was cut out from the nitrocellulose sheet after blotting and used for the RanGAP assay (Fig. 5 *Inset*). Significant specific activity was found in filter-bound RanGAP; however, it was reduced compared with the activity of the protein in solution. This could be due to incomplete renaturation and association with the nitrocellulose.

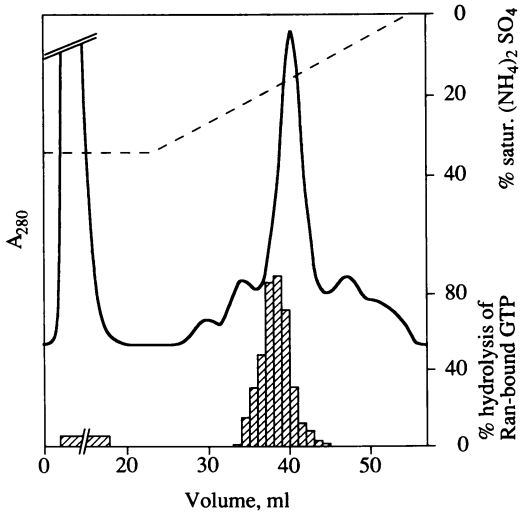

FIG. 2. Separation of two RanGAP activities by hydrophobic chromatography. Hydroxylapatite fractions containing RanGAP were brought to 35% saturation with ammonium sulfate and were chromatographed on EMD AFTA 650/S. RanGAP activity (hatched bars) is expressed as a percentage of Ran-bound [γ-^{32}P]GTP hydrolyzed within 6 min after addition of fractions.

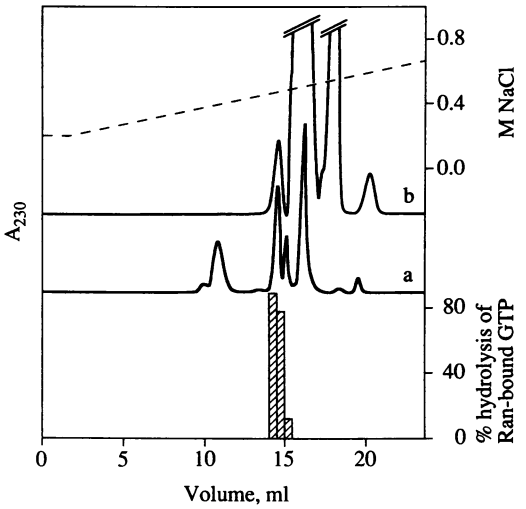

FIG. 3. Final purification of RanGAP1 on Mono Q. After hydrophobic chromatography, RanGAP-containing fractions derived from the low salt HeLa fraction (run a) and from a 400 mM NaCl extract of the crude insoluble material (run b) were subjected separately to ion-exchange chromatography on Mono Q. Hatched bars represent the RanGAP activity in fractions of run a and indicate the elution position of RanGAP1 in both profiles.

RanGAP2. The RanGAP activity in the flowthrough of the hydrophobic column was designated RanGAP2. It was eluted from Mono Q at the same concentration of sodium chloride as RanGAP1, but correlates with a 50-kDa band on SDS gels.

RanGAPs from the Pellet Fraction of HeLa Cells. We reextracted the crude HeLa pellet with 400 mM NaCl. After high-speed centrifugation, RanGAPs were purified from the supernatant using identical purification steps as for the nucleoplasmic material. Again, two RanGAPs were separated by hydrophobic chromatography. Final purification on Mono Q yielded a 65-kDa protein. After cleavage with CNBr, it displayed the same peptide pattern upon reversed-phase chromatography as RanGAP1 (data not shown). Sequence analysis of homologous peptides suggested identity with RanGAP1. Amino acid sequences derived from different CNBr and tryptic peptides of the soluble form of RanGAP1 were compared with published structures in the European Molecular Biology Laboratory and GenBank data bases. No significant homologies to known sequences were found (search of 08/13/93).

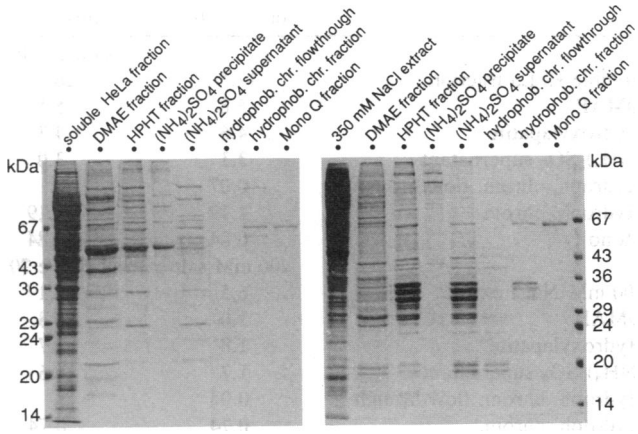

FIG. 4. SDS gel of fractions obtained from the soluble HeLa material (*Left*) and from 400 mM NaCl extract (*Right*) after each step of RanGAP purification, stained with Coomassie blue. HPHT, hydroxylapatite.

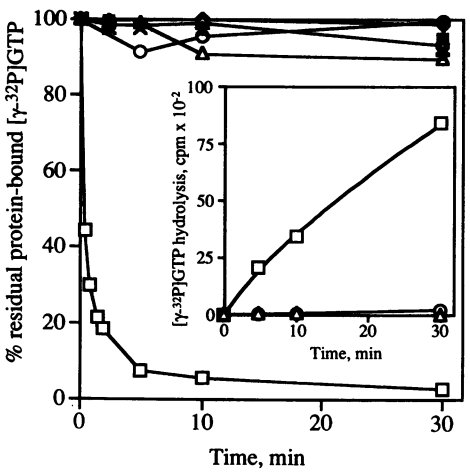

FIG. 5. Selectivity of RanGAP1 for induction of GTP hydrolysis by Ran. The GTPase reaction was started by addition of 10-μl aliquots of 40 nM (■, +) or 20 nM (□) RanGAP1, the C-terminal fragment of NF1 (○) at 1 μM, 0.8 μM RasGAP (△), or buffer as control (◆, ◇, ×) to 300 μl of 60 nM Ras·[γ-32P]GTP (closed symbols), Ran·[γ-32P]GTP (open symbols), or Q69L-Ran·[γ-32P]GTP (+, ×). After incubation at 25°C for the indicated times, protein-bound radioactivity was determined in 40-μl aliquots by the filter-binding assay as described in *Materials and Methods*. (*Inset*) Stimulation of GTPase activity of Ran by blot-purified renatured RanGAP1. Renatured RanGAP1 (□), ovalbumin (△), and bovine serum albumin (○) immobilized on nitrocellulose were sequentially incubated with 100 μl of 60 nM Ran·[γ-32P]GTP at 25°C on a rotation shaker for the times indicated, and liberated radioactive phosphate was determined.

Amount Per Cell. During purification (Table 1), the protein loses enzymatic activity to an unknown extent, and the enzymatic GAP assay used for monitoring its isolation detects active RanGAP only. With these caveats, 3×10^5 molecules per HeLa cell were calculated, in comparison to about 10^7 molecules of Ran/TC4 and 4.6×10^5 copies of RCC1 (1).

Molecular Mass Determination of RanGAPs. Fractions containing RanGAP activity were subjected to gel filtration on Superdex 200 after each step of purification. In the soluble HeLa fraction and the 400 mM NaCl extract of the nuclear pellet used as starting material for purification, RanGAP activity was split into two fractions corresponding to apparent molecular masses of roughly 150 and 850 kDa (asterisk in Fig. 6). However, after the first purification step on DMAE, only the 150-kDa form was detected. The retention time of the major RanGAP activity did not change throughout the following purification, indicating that RanGAP1 forms a homodimer. RanGAP2, which can be detected only after separation from the predominant RanGAP1 by hydrophobic chromatography, was eluted from Superdex 200 at a position corresponding to 100 kDa. Hence, dimerization can be expected for this molecule as well. To establish that the high molecular RanGAP activity also results from these two RanGAPs, we used this fraction for ion-exchange chromatography on DMAE and subjected the DMAE eluate again to gel filtration. The RanGAP activity, which was eluted at the same NaCl concentration (600 mM) as RanGAP1 and -2, was shifted to the 150- to 100-kDa position, indicating the dissociation of the high molecular mass complex on DMAE.

Specificity of RanGAP1 for Ran. $p21^{H\text{-}ras}$ was used as a control substrate for RanGAP. GTPase activity on p21 was not stimulated by the purified RanGAP1 (Fig. 5), and likewise, the GTPase activity on Ran was not significantly stimulated by the catalytical domain of the NF1 gene product or by RasGAP, even at an enzyme-to-substrate ratio of 1:2 (Fig. 5).

In the Mutant RanQ69L GTPase Activity Is Not Induced by RanGAP1. In analogy to the oncogenic mutation in Ras, glutamine residue 69 in Ran, which corresponds to Q61 in Ras, was changed to leucine by site-directed mutagenesis (C.K. and A.W., unpublished results). In $p21^{H\text{-}ras}$ this substitution prevents GTPase induction by RasGAP or the NF1 gene product. Correspondingly, in Ran Q69L no measurable stimulation of the intrinsic GTPase by RanGAP1 was observed after 30 min of incubation even at twice the GAP concentration sufficient to stimulate 80% hydrolysis of protein-bound GTP within 2 min on wild-type Ran (Fig. 5).

DISCUSSION

Ran is a very weak GTPase, hydrolyzing bound GTP with a half-life of about 6 h at 30°C (F.R.B. and H.P., unpublished results). For many Ras-related proteins, stimulators of the usually weak intrinsic GTPase activity (GAPs) have been identified. Here we report the isolation of an activity in lysates of HeLa cells that specifically stimulates the GTPase of Ran but not of the Ran mutant Q69L, which mimicks the Ras mutation Q61L rendering Ras permanently active. As

Table 1. Enrichment of RanGAP protein

	Total activity, units × 10^{-3}	Activity of RanGAP1, units × 10^{-3}	Total protein, mg	Specific activity of RanGAP1, units × 10^{-2}/mg	Enrichment factor	Yield, %
HeLa cell lysate low-salt fraction						
Soluble HeLa fraction	17.5	16.5	1550	0.1	1	73
DMAE	5.8	5.5	42	1.3	13	24
Hydroxylapatite	4.6	4.3	2.1	20	200	19
$(NH_4)_2SO_4$ supernatant	3.1	3.0	0.7	43	430	13.0
Hydroph. chrom. flowthrough	0.07		0.04			
Hydroph. chrom.	1.19	1.19	0.08	149	1490	5.3
Mono Q	0.84	0.84	0.023	365	3650	3.7
400 mM salt extract of the 70,000 × *g* pellet from HeLa cell lysate						
400 mM NaCl extract	6.5	6.1	795	0.08	1	27
DMAE	3.0	2.8	21	1.3	16	12
Hydroxylapatite	1.8	1.7	2	8.5	106	7.6
$(NH_4)_2SO_4$ supernatant	1.7	1.6	1.8	8.9	111	7.1
Hydroph. chrom. flowthrough	0.04		0.16			
Hydroph. chrom.	0.74	0.74	0.10	74	925	3.3
Mono Q	0.38	0.38	0.01	380	4750	1.7

Values refer to 50 ml of packed HeLa cells (≈2×10^{10} cells) as starting material; the total lysate contained 2900 mg of protein. RanGAP was quantitated by staining of gels with Coomassie blue. One unit is the amount of RanGAP that stimulates hydrolysis of 50% of the Ran-bound [γ-32P]GTP (18 pmol in 310 μl), corrected for buffer control, after 2 min of incubation at 25°C. Hydroph. chrom., hydrophobic chromatography.

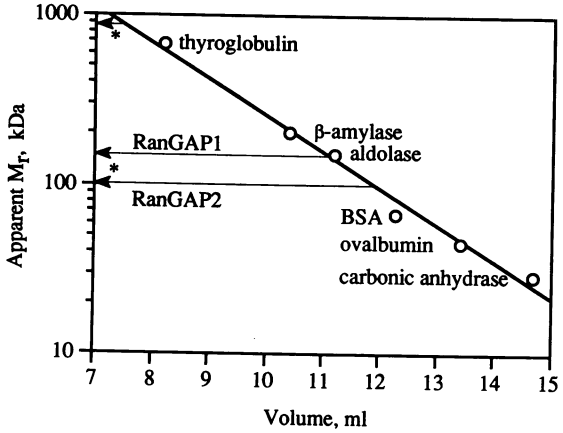

FIG. 6. Different populations of RanGAP were detected by size-exclusion chromatography. The low salt fraction of HeLa cells, the 400 mM NaCl extract, purified RanGAP1, and flowthrough from the hydrophobic chromatography containing RanGAP2 were subjected to gel filtration on Superdex 200. The retention time of the fraction with the highest GAP activity is plotted against apparent molecular mass using carbonic anhydrase (29 kDa), ovalbumin (43 kDa), bovine serum albumin (67 kDa), aldolase (165 kDa), β-amylase (200 kDa), and thyroglobulin (669 kDa) as standards. The asterisk indicates elution positions of high molecular mass RanGAP activity in crude extracts and after chromatography on DMAE-Fractogel.

expected for a regulator for the nuclear protein Ran, most of it was found in the nuclear fraction. In addition to a large proportion of nucleoplasmic RanGAP, about one-third of the total RanGAP activity was detected in the nuclear pellet, from which it was extracted with salt but not by DNase I or chloroquine treatment, indicating its association with nuclear structures differing from chromatin.

The major RanGAP activity (RanGAP1) was purified to homogeneity from the soluble fraction and from the pellet of unsynchronized cells. Purified RanGAP1 of 65 kDa was eluted from gel-filtration columns at a position corresponding to 150 kDa for globular proteins, indicative of dimerization or of a highly asymmetric shape of the molecule. Dimerization has also been shown for RapGAP (20). A small amount of RanGAP activity was separated from RanGAP1 by hydrophobic adsorption chromatography. On further purification it was found to correlate with a 50-kDa band on SDS gels and with a 100-kDa peak in gel filtration. Apart from their difference in size and hydrophobicity, the two GAPs behaved identically in all chromatographic properties and in apparent dimerization. We therefore assume that they are closely related in sequence or that RanGAP2 is derived from RanGAP1 by proteolysis.

In interplay with nucleotide exchange factors and nucleotide dissociation inhibitors, GAPs are believed to be immediate regulators of the active state of ras-related proteins. In addition, GAPs may be components of larger effector complexes; for example, RasGAP has been found to be associated with two phosphorylated proteins of 190 kDa and 62 kDa. These RasGAP-associated proteins show homologies to transcription inhibitors and heterogeneous nuclear ribonuclear proteins, respectively, which may hint at possible functions in putative effector complexes formed by binding to SH2 domains of RasGAP (21–23). We also found high molecular mass complexes of RanGAP in crude fractions (Fig. 6), from which RanGAP1 was released upon ion-exchange chromatography, but we have not established their composition or function so far.

Thus, there are now two immediate regulators of Ran activity. RCC1, the nucleotide exchange factor (24), maintains Ran in the putatively active GTP bound form, as a consequence of the higher concentration of GTP over GDP in the cell. Its antagonist RanGAP1 downregulates Ran·GTP by stimulating RanGTPase more than 1000-fold. From HeLa cell lysates, we recently isolated another factor modulating RanGTPase. The protein of 27 kDa, which specifically binds to Ran·GTP, does not induce GTPase activity on Ran by itself; however, it enhances the effect of RanGAP1 by an additional order of magnitude and inhibits RCC1-stimulated exchange of Ran-bound GTP (F.R.B. and H.P., unpublished results).

So far, there is no clear evidence of upstream regulators of RCC1 and RanGAP1, although premature induction of mitosis upon loss of RCC1, even in the presence of inhibitors of DNA synthesis, suggested a role in monitoring the completion of DNA replication. More is known about the downstream events of the signal pathway, as they are illustrated by the loss of RCC1 phenotype (see Introduction) and its suppression by overexpression of Ran (9, 25, 26). Also, Ran has recently been shown to be involved in nuclear import of proteins with nuclear localization signals (27). Translocation through the nuclear membrane is inhibited by 5′-guanylyl imidodiphosphate and thus requires GTP hydrolysis. If this hydrolysis is Ran-dependent, RanGAP should also play an active role in this process.

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