Conserved charged residues in the leucine-rich repeat domain of the Ran GTPase activating protein are required for Ran binding and GTPase activation

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GTPase activating proteins (GAPs) for Ran, a Ras-related GTPase participating in nucleocytoplasmic transport, have been identified in different species ranging from yeast to man. All RanGAPs are characterized by a conserved domain consisting of eight leucine-rich repeats (LRRs) interrupted at two positions by so-called separating regions, the latter being unique for RanGAPs within the family of LRR proteins. The cytosolic RanGAP activity is essential for the Ran GTPase cycle which in turn provides directionality in nucleocytoplasmic transport, but the structural basis for the interaction between Ran and its GAP has not been elucidated. In order to gain a better understanding of this interaction we generated a number of mutant RanGAPs carrying amino acid substitutions in the LRR domain and analysed their complex formation with Ran as well as their ability to stimulate the intrinsic GTPase activity of the G protein. We show that conserved charged residues present in the

separating regions of the LRR domain are indispensable for efficient Ran binding and GAP activity. These separating regions contain three conserved arginines which could possibly serve as catalytic residues similar to the arginine fingers identified in GAPs for other small GTPases. However, mutations in two of these arginines do not affect the GAP activity and replacement of the third conserved arginine (Arg91 in human RanGAP) severely interferes not only with GAP activity but also with Ran binding. This indicates that RanGAP-stimulated GTP hydrolysis on Ran does not involve a catalytic arginine residue but requires certain charged residues of the LRR domain of the GAP for mediating the protein–protein interaction.

Key words: arginine finger; nucleocytoplasmic transport; protein–protein interaction; small G proteins.

INTRODUCTION

The Ras-related nuclear protein Ran is a small GTPase critically important for nucleocytoplasmic transport across the nuclear pore complex (NPC). To accomplish its function Ran cycles between a GTP- and a GDP-bound conformation with RanGTP representing the active form capable of interacting with different target or effector proteins. These effectors include the Ran binding proteins RanBP1 and RanBP2 as well as the receptors recognizing nuclear import and export signals on proteins and ribonucleoproteins [1–3]. The receptors, also referred to as karyopherins, importins and exportins, are essential for signal-dependent nucleocytoplasmic transport as they bind their respective cargo in the donor compartment (cytoplasm or nucleoplasm), mediate its association with the NPC and escort the transport substrates to their final destination [4–6]. Directionality in this process is provided through the Ran GTPase cycle. In its GTP-bound state Ran triggers disassembly of the importin/substrate complex once it arrives in the nucleus. RanGTP then mediates the recycling of importins to the cytoplasm and also supports the assembly of complexes containing exportins and export cargo. In the cytoplasm removal of RanGTP from such complexes leads to liberation of the export cargo with concomitant GTP hydrolysis on Ran preventing rebinding [7,8].

The basis for this spatial directionality of the Ran GTPase cycle is an asymmetric distribution of the two auxiliary proteins controlling the GTP/GDP cycle. The factor promoting guanine nucleotide exchange (RanGEF or RCC1), i.e. the conversion of

RanGDP to RanGTP, is found in the nucleoplasm, whereas the GTPase activating protein (RanGAP) which stimulates the intrinsic GTPase of Ran by several orders of magnitude is a cytosolic protein [7–9]. The strictly compartmentalized organization of RanGEF and RanGAP guarantees a steep RanGTP/RanGDP gradient across the nuclear envelope. RanGTP is restricted to the nucleus and mediates the termination of import and the initiation of export, whereas the GAP-stimulated hydrolysis occurs at the cytosolic face of the NPC, thus terminating export.

The Ran GTPase cycle and its role in nucleocytoplasmic transport is conserved across species and genes encoding the principle regulators of the Ran GTPase cycle have been shown to be essential for viability in budding and fission yeasts [10]. In the case of RanGAP these are the *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* proteins Rna1p and rna1p, respectively. The high degree of functional conservation also reflects itself in the finding that *S. pombe* rna1p stimulates the GTP hydrolysis mediated by human Ran and complements the defect in *S. cerevisiae* *RNA1* mutant cells [11,12]. Moreover, all RanGAPs known to date share sequence similarities and two structural features, an N-terminal region of some 300 amino acids characterized by an 8-fold repetition of a leucine-rich repeat (LRR) motif and a stretch of approx. 40 amino acids which is extremely rich in acidic amino acids and immediately follows the LRRs [11,13–15]. This conservation does, however, not extend into the C-terminal sequences following the acid region. While these comprise only 12 and 15 residues, respectively, in the *S. pombe* and the *S. cerevisiae* proteins, mouse and human

Abbreviations used: DTT, dithiothreitol; EDC, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride; GAP, GTPase activating protein; GEF, guanine nucleotide exchange factor; GMPPNP, 5'-guanylimidodiphosphate; IPTG, isopropyl- β -D-thiogalactopyranoside; LRR, leucine-rich repeat; NPC, nuclear pore complex; DTE, dithioerythritol; WT, wild-type.

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RanGAP have much longer C-terminal regions of 191 and 189 residues, respectively, which carry the site for posttranslational modification with a ubiquitin-like moiety [16,17].

Although the functional importance of RanGAP in nucleocytoplasmic transport is well established, only limited data are available as to the structural basis of the Ran–RanGAP interaction. Such structural data have been obtained recently for other GAPs and their interaction with the respective G protein, e.g. RasGAP and RhoGAP [18,19]. Here the catalytic domain of the GAP is highly α -helical, with an exposed arginine residue (Arg finger) playing an important role in stabilizing the transition state of the GTP hydrolysis reaction. It is not known whether such a scenario is met in the case of RanGAPs as their principal structural element, the LRR domain, is likely to assume a different fold, most likely similar to that of the ribonuclease inhibitor, a protein composed entirely of LRRs whose three-dimensional structure has been solved by X-ray crystallography [20]. In the RNase inhibitor each LRR forms a β -strand-loop- α -helix structure, with the sum of the repeats resembling a horseshoe. While this suggests that the LRR region of RanGAP is less compact than the catalytic domains of RasGAP and RhoGAP, *in vitro* proteolysis reveals a limited stability of the LRR domain in RanGAP. In *S. pombe* rna1p, this treatment (which removes the C-terminal acidic stretch, but leaves the LRR domain intact) impairs GAP activity and Ran binding, suggesting that the C-terminal region plays an important role in mediating the interaction with Ran [21,22]. The importance of the LRR domain has, however, not been elucidated directly and it appears plausible that it cooperates with the acidic stretch in Ran binding.

To address this question we concentrated our attention on the LRR domain of RanGAP, in particular on conserved residues of positive or negative charge which occur in RanGAPs but not other LRR proteins, thus representing candidate residues for mediating specific contacts between RanGAP and Ran. A mutational analysis reveals that charged residues in these positions are required for both Ran binding and GAP activity and that this principle is conserved in human and yeast RanGAPs. This points to an important role of the LRR domain of RanGAPs in mediating specific protein–protein contacts.

MATERIALS AND METHODS

Construction of mutants

Mutant RanGAP1, rna1p and Rna1p cDNAs were constructed by oligonucleotide-directed *in vitro* mutagenesis using as templates pBluescript KS vectors containing human RanGAP cDNA inserted into the *Bam*HI site, *S. pombe* rna1p cDNA cloned via *Eco*RI and *Pst*I or a *S. cerevisiae* RNA1 *Nco*I–*Bam*HI genomic DNA fragment. Complementary pairs of the following oligonucleotides were employed in double-stranded mutagenesis (Quick Change TM Site-Directed Mutagenesis Kit, Stratagene, Heidelberg, Germany): hs1s (5'-GGCAGAA-CCGTCTGGCGAATGATGGCGCCACTGCC-3')/hs1as (5'-GGCAGTGGCGCCATCATTCGCCAGACGGTTTCTGCC-3'), hs2s (5'-GGCAGAAACCGTCTGCAGAATGATGGCGCCACTGCC-3') / hs2as (5'-GGCAGTGGCGCCATCATTCGTCAGACGGTTTCTGCC-3'), hs3s (5'-CGGGTCATCAACCTGAATGACCTTCACTGAG-3')/hs3as (5'-CTCAGTGAAGGTGTTGGCATTACAGTTGATGACCCG-3'), hs4s (5'-CGGGTCATCAACCTGAATAACAACACCTTCACTGAG-3'), hs4as (5'-CTCAGTGAAGGTGTTGTTATTCAGTTGATGACCCG-3'), hs5s (5'-GACATGTTACGGAGCGCTGCGGACCGAGATCCC-3'), hs5as (5'-GGGATCTCGGTCCGCAGCGCTCCCGTGAACATGTC-3'), sp1s (5'-GGAAGAAATCGTTTAGCAAAATGGGAGTATGAA-

AG-3')/sp1as (5'-CTTTCATACTCCCATTGCTAAACGATTTCTTCC-3'), sp2s (5'-GGAAGAAATCGTTTACAAAATGGGAGTATGAAAG-3')/sp2as (CTTTCATACTCCCATTGTTGTAACGATTTCTTCC-3'), sp3s (5'-GGTACTCGATCTTCAAGCTAACACATTTACGCAC-3')/sp3as (GTGCGTAATGTGTTAGCTTGAAGATCGAGTACC-3'), and sp4s (5'-GGTACTCGATCTTCAAAATAACACATTTACGCAC-3')/sp4as (5'-GTGCGTAAATGTGTTATTTGAAGATCGAGTACC-3'). The reactions resulted in the mutant plasmids pBSHsE193A, pBSHsE193Q, pBSHsD245A, pBSHsD245N, pBSHsR91A, pBSHsE172A, pBSspE172Q, pBSspD225A and pBSspD225N, respectively. Oligonucleotides sc1as (5'-CTCTAATCTATTTGCACCACAGATAAAAG-3'), sc2as (5'-GATCCATTCTCTAATGCATTCTACACAG-3'), sc3as (5'-CTGCGGATCCATTGCTAATCTATTTCTAC-3') and sc4as (5'-CGTGAAAGTATTGGCTTGAAGATCCAAG-5') were employed in single-strand based mutagenesis (Muta-Gene M13 *in vitro* Mutagenesis Kit, Bio-Rad, Munich, Germany) yielding the mutant plasmids pBSscR182A, pBSscR184A, pBSscE186A and pBSscD240A. The numbers indicate the positions of the amino acids exchanged in the mutant proteins. Changed triplets are underlined within the oligonucleotide sequences. Wild-type and mutant RanGAP cDNA inserts were then cloned via their *Nde*I/*Bam*HI restriction sites into the appropriately linearized expression vector pET-23a(+) (Novagen, Heidelberg, Germany). The resulting plasmids pETHsWT, pETHsR91A, pETHs193A, pETHs193Q, pETHs245A and pETHs245D were transformed into *E. coli* BL21(DE3)pLysS cells. *S. pombe* rna1p as well as *S. cerevisiae* Rna1p wild-type and mutant cDNA inserts were cloned via their *Eco*RI/*Pst*I restriction sites into the appropriately linearized expression vector pkk223-3 (Pharmacia, Freiburg, Germany). The resulting plasmids pkkspWT, pkkspE172A, pkkspE172Q, pkkspD225A, pkkspD225N, pkkscWT, pkkscR182A, pkkscR184A, pkkscE186A and pkkscD240A were transformed into *E. coli* JM105 cells.

Purification of recombinantly expressed RanGAP

BL21(DE3)pLysS cells harbouring constructs pETHsWT, pETHsR91A, pETHsE193A or pETHsD245N, respectively, were grown at 30 °C in 1 l of LB medium containing 150 μ g/ml ampicillin and 34 μ g/ml chloramphenicol. At an attenuation (D_{600}) of 0.3 IPTG was added to a concentration of 20 μ M, and the culture was incubated for another 6 h. The recombinantly expressed proteins RanGAP, R91A RanGAP, E193A RanGAP and D245N RanGAP were purified from bacterial inclusion bodies. Bacteria were harvested by centrifugation, resuspended in 50 ml buffer A [20 mM Tris/HCl, pH 7.5, 50 mM NaCl, 1 mM dithiothreitol (DTT), 1 mM PMSF] and opened by repeated (3 $\times$) freezing and thawing. After the last thawing at 30 °C 10 μ g/ml DNaseI were added. The suspension was incubated for 20 min on ice and sonicated 3 $\times$ 2 min on ice using a Branson sonifier set at 50 %. The suspension was centrifuged at 100 000 *g* for 30 min. The resulting pellet was resuspended in 50 ml buffer B (20 mM Tris/HCl, pH 7.5, 200 mM NaCl, 1 % (w/v) sodium deoxycholate, 1 % (w/v) Nonidet P40 (Sigma, Deisenhofen, Germany), 2 mM EDTA, 1 mM DTT, 1 mM PMSF) and lysis was completed using a Dounce homogenizer. The lysate was cleared at 30 000 *g* for 1 h and the pellet was washed with buffer C (0.5 % (w/v) Triton X-100, 1 mM EDTA). The resulting pellet was resuspended in 10 ml buffer D (100 mM Tris/HCl, pH 7.5, 100 mM NaCl, 8.5 M urea, 1 mM DTT, 1 mM PMSF) and sonicated 3 $\times$ 30 s at room temperature (50 % setting). Following centrifugation at 40 000 *g* for 30 min the supernatant was mixed with 0.5 volumes of Q-Sepharose (Pharmacia) equilibrated in

buffer D. The resulting suspension was gently rotated at room temperature for 3 h. The Sepharose beads were then pelleted by centrifugation (1 min at 5000 g) and incubated with buffer D containing 1 M NaCl. The supernatant containing the released RanGAP proteins was dialysed against buffer D and then incubated for 3 h at room temperature with 0.5 volumes of CM-Sepharose (Pharmacia) equilibrated in buffer D. The Sepharose beads were removed by centrifugation, and the resulting solution was diluted with buffer D to a final protein concentration of approx. 50 µg/ml. After centrifugation (30 min at 100000 g) the supernatant was dialysed twice against 20 volumes of buffer A resulting in a final urea concentration of about 20 mM. Ultra-centrifugation (20 min at 100000 g) did not result in any visible protein pellet, and the supernatant was applied to a MonoQ column equilibrated in buffer A. The column was developed with a linear salt gradient (0–1 M NaCl in buffer A) and the RanGAP proteins eluted at 0.4 M NaCl. Peak fractions were pooled, dialysed against buffer E (20 mM potassium phosphate, pH 7.4, 50 mM NaCl, 5 mM MgCl₂, 1 mM DTT, 1 mM PMSF) containing 50% glycerol, and stored at –20 °C. Purification of recombinantly expressed wild-type and mutant Rna1 proteins was carried out as described for native Rna1p/rna1p [22].

Limited proteolysis

Trypsin treatment of purified wild-type and mutant RanGAP proteins was carried out in buffer E without PMSF. The protein concentration was adjusted to 0.5 mg/ml and reactions were carried out at an enzyme/substrate ratio of 1:100. Samples were incubated at room temperature and the reaction was stopped after the times indicated by boiling in SDS sample buffer [23]. Reaction products were separated in SDS–12.5%-polyacrylamide gels and either stained with Coomassie Blue or subjected to immunoblotting.

Antibodies

The different RanGAP1 derivatives were identified by immunoblotting using an affinity purified antibody raised against amino acid residues 2–20 of the RanGAP protein (Santa Cruz, Heidelberg, Germany) or a total rabbit serum raised against a BSA-coupled peptide corresponding in sequence to residues 322–334. This serum and a monoclonal anti-Ran antibody were kindly provided by Dr Jörg Becker (MPI for Molecular Physiology, Dortmund, Germany). *S. pombe* rna1p derivatives were identified using an affinity purified antibody raised against a peptide corresponding in sequence to residues 36–49 [11]. Primary antibodies were visualized using peroxidase-coupled secondary antibodies and a chemiluminescence-based detection system (Amersham–Buchler, Brunswick, Germany).

Protein determination

Protein concentrations were determined according to Bradford [24] using bovine albumin as a standard. The amount of nucleotide bound to Ran or Gsp1p was determined by HPLC analysis and used as a measure for the concentration of active protein.

GAP assay

To measure the GAP activity of RanGAP, rna1p or Rna1p wild-type and mutant proteins, human Ran or yeast Gsp1p were loaded with [γ -³²P]GTP. Therefore 2 µM Ran in 50 mM Tris/

HCl, pH 7.5, 1 mM EDTA, 1 mM dithioerythritol (DTE), 1 mg/ml bovine serum albumin or 2 µM Gsp1p in 20 mM potassium phosphate, pH 6.0, 1 mM DTE, 1 mg/ml bovine serum albumin were incubated for 30 min at 30 °C in the presence of 10 µM GTP and 5 pM [γ -³²P]GTP (6000 Ci/mmol, ICN). The addition of an equal volume of the same buffers containing 5 mM MgCl₂ instead of EDTA terminated the nucleotide exchange reaction. Activation of the intrinsic GTPase activity was then measured at 30 °C after the addition of purified wild-type or mutant GAPs (RanGAP, R91A RanGAP, Rna1p, R182A Rna1p, R184A Rna1p, E186A Rna1p and D240A Rna1p) or of the soluble fraction of bacterial lysates containing defined concentrations of the respective wild-type or mutant GAPs (RanGAP, E193A RanGAP, E193Q RanGAP, D245A RanGAP, D245N RanGAP, rna1p, E172A rna1p, E172Q rna1p, D225A rna1p and D225N rna1p). At different reaction times aliquots of the mixture were filtered through nitrocellulose filters (BA85, Schleicher & Schüll, Dussel, Germany), and protein-bound radioactivity, i.e. the non-hydrolysed GTP-fraction, was determined. For a qualitative comparison data were fitted to a single exponential function using the computer program GraFit (Erithacus Software). A quantitative assessment used a linear fit to the initial rates of GTP hydrolysis.

For preparation of the soluble fractions containing defined amounts of GAPs, bacterial cultures carrying the respective expression plasmid were grown at 30 °C and protein synthesis was induced at a D_{600} of 0.3 using 20 µM IPTG in the case of RanGAP expressing bacteria or 500 µM in the case of rna1p expressing bacteria, respectively. Cultures were then incubated at 30 °C for another 3 h, and the cells of 1.5 ml of each suspension were pelleted by centrifugation (5 min at 30000 g) and then resuspended in 300 µl of PBS containing 1 mM DTT and 1 mM PMSF. BL21(DE3)pLysS cells carrying pET/RanGAP constructs were lysed by freezing in liquid nitrogen for three times and subsequent thawing at room temperature while JM105 cells carrying pkk-rna1p constructs were lysed by sonification using a Branson sonifier (15 × 2 s). Cellular debris and insoluble components were pelleted by ultracentrifugation for 15 min at 100000 g. In the resulting supernatant the recombinant RanGAP1/rna1p proteins were identified and quantified by comparative immunoblotting using specific antibodies and known amounts of purified wild-type proteins, respectively.

Analytical gel filtration of RanGAP proteins

Approx. 100 µg of purified RanGAP, E193A RanGAP or D245N RanGAP were applied to a Superose 12 PC 3.2/20 column connected to a FPLC system (Pharmacia) and equilibrated in 50 mM potassium phosphate, pH 7.4, 150 mM NaCl, 5 mM MgCl₂, 1 mM DTT. The retention times of the different proteins were monitored by following the absorbance at 280 nm.

Chemical cross-linking

To preload Ran with the non-hydrolysable GTP analogue GMPPNP, 500 µM Ran were treated for 16 h at 4 °C with 1 mM GMPPNP (Sigma), 20 mM potassium phosphate, pH 7.5, 2 mM MgCl₂, 1 mM DTE, 200 mM ammonium sulphate, 0.1 mM ZnCl₂ and 20 units of alkaline phosphatase (Boehringer–Mannheim, Mannheim, Germany). Unbound nucleotides were removed by gel filtration in a HiTrap desalting column (Pharmacia) equilibrated with 20 mM potassium phosphate, pH 7.5, 5 mM MgCl₂, 1 mM DTE. 2 µM wild-type or mutant RanGAP proteins and 2 µM Ran in its GDP- or GMPPNP-bound form were mixed and 2.6 mM 1-ethyl-3-[dimethylaminopropyl]car-

Figure 1 Sequence comparison of human RanGAP (*hum.*), *S. pombe* rna1p (*S.p.*) and *S. cerevisiae* Rna1p (*S.c.*)

The sequences are arranged in blocks to illustrate the repeat organization. Eight LRRs (residues 24–48, 86–112, 113–141, 179–206, 207–235, 236–264, 265–293 and 294–322 in *S. pombe* rna1p, respectively) are interrupted by two LRR-like separating regions (residues 49–84 and 143–178 in rna1p, respectively) and followed by a highly acidic sequence. The sequence of the long, non-homologous C-terminal region of the human protein (residues 404–587) is not listed. Blocks corresponding to the canonical LRRs are highlighted by brackets above the first and below the last LRR. Identical residues in the three GAP sequences are marked by vertical lines. Amino acids conserved in the majority of the repeats are underlined and given in bold letters with their positions marked by asterisks and plus signs above the sequences of the LRRs and separating regions, respectively. The amino acid substitutions introduced in the different GAPs are indicated.

bodiimide hydrochloride (EDC, Pierce, Rochfort, IL, U.S.A.) alone or in the presence of 2.6 mM sulpho-*N*-hydroxysuccinimide (Sulfo-NHS, Pierce) were added as cross-linking reagents. Following 90 min incubation in 20 mM potassium phosphate, pH 7.4, 50 mM NaCl, 5 mM MgCl₂ and 1 mM DTT at room temperature proteins were precipitated using methanol/CHCl₃, resuspended in SDS sample buffer, and analysed in SDS-12.5%

(or 10% for determination of higher molecular masses) polyacrylamide gels.

Miscellaneous

CD spectroscopy of wild-type and mutant RanGAP derivatives in 20 mM potassium phosphate, pH 7.4, 50 mM NaCl,

Figure 2 Purification and characterization by CD spectroscopy of RanGAP derivatives

As an example the purification of E193A RanGAP from bacterial inclusion bodies is shown in (A). Proteins present in the following fractions of the preparation (see the Materials and methods section) were separated by SDS/PAGE and stained with Coomassie Blue: lanes 2 and 3, total bacterial lysate prior to and following IPTG induction of recombinant protein expression, respectively; lane 4, material not bound to Q-Sepharose; lane 5, Q-Sepharose eluate containing the RanGAP; lane 6, proteins not binding to CM-Sepharose; lane 7, purified RanGAP eluting from the MonoQ column. Markers of the molecular masses indicated were separated in lane 1. (B) shows the CD spectra of WT (—), E193A (····) and D245N RanGAP (---), respectively.

5 mM MgCl_2 , 1 mM DTT and 25% glycerol was carried out using a Jobin Yvon CD6 dichrograph with protein concentrations adjusted to 5 μM .

RESULTS

Choice of RanGAP mutants

Previous analyses have shown that the highly acidic C-terminal region of both *S. pombe* and *S. cerevisiae*, rna1p, is necessary but not sufficient for establishing the interaction with Ran [22]. Thus, it appears likely that the LRR domain of rna1p and the other RanGAPs is also involved in mediating Ran binding, a view supported by the proposal that LRRs generally represent protein-protein interaction motifs. In the three-dimensional structure of the RNase inhibitor and the U2 snRNP protein A' the individual LRRs form β -strand-loop- α -helix folds with the conserved hydrophobic residues stabilizing the fold through internal interactions [20,25]. The unusual non-globular shape and a parallel β -sheet with one surface exposed to the solvent are characteristic features thought to mediate specificity in the interaction of LRR proteins with their partners. In the case of the RNase inhibitor and its interaction with RNase this has indeed been shown to hold true to a certain degree, although most specific contacts involve residues located in the loop connecting the C-termini of the β -strands with the N-termini of the α -helices [26].

A close inspection of the LRR domains in the different RanGAPs reveals two atypical repeat units connecting LRRs 1 and 2 as well as 3 and 4. These sequences, also referred to as separating regions, are 36 residues in length, show internal homologies and could resemble an extended LRR (Figure 1). The separating regions are unique for the RanGAPs within the

group of LRR proteins and represent prime candidates for mediating specific interactions and/or functions. Therefore, we focused our attention on these sequences and attempted to generate two sets of mutants. First, we deleted the entire separating regions 1 or 2. However, upon recombinant expression in bacteria the two mutant derivatives ($\Delta 63$ –98 and $\Delta 157$ –192 Rna1p) proved to be unstable, most likely due to improper folding (results not shown). Second, we site-specifically mutated conserved charge residues, as these could participate in mediating specific ligand contacts. In particular, we chose the three arginines at positions 91, 189 and 191 (human RanGAP sequence), as these are the only conserved arginines which could possibly serve as an Arg finger. Moreover we replaced one of the two conserved glutamate residues within the separating regions (position 193 in RanGAP) by either alanine or glutamine in human RanGAP and in the two yeast proteins. Finally, an equally conserved acidic residue in the fifth LRR (D245) was chosen for mutagenesis as an example for studying the importance of conserved charged residues present in canonical LRRs. An overview over the mutations introduced is given in Figure 1.

Preparation and characterization of RanGAP mutants

cDNAs encoding the RanGAP, rna1p and Rna1p mutants listed above were generated through oligonucleotide-directed mutagenesis and the proteins were produced using a bacterial expression system. Recombinantly expressed Rna1p derivatives were purified from the soluble bacterial lysate following a protocol described previously for *S. pombe* rna1p [22]. Wild-type (WT) RanGAP as well as the mutant proteins R91A RanGAP, E193A RanGAP and D245N RanGAP remained insoluble and were purified from bacterial inclusion bodies (see the Materials

Figure 3 Limited proteolysis of WT RanGAP and mutant derivatives

WT RanGAP (A–III), E193A RanGAP (B–III) or D245N RanGAP (C–III) were incubated with trypsin at an enzyme:substrate ratio of 1:100. Aliquots of the reaction were removed after 1 (lanes 3), 5 (lanes 4), 10 (lanes 5), 30 (lanes 6) and 60 min (lanes 7), respectively, and digestion in these aliquots was stopped by the addition of hot SDS sample buffer. The reaction products were resolved in SDS gels and identified by Coomassie Blue staining (I) or immunoblotting with anti-RanGAP antibodies raised against synthetic peptides corresponding in sequence to residues 2–20 (II) or 322–334 (III) of the human protein. Markers of the molecular masses indicated were separated in lanes 1 whereas the non-digested proteins are shown in lanes 2. Note that the digestion patterns of the mutant RanGAPs are indistinguishable from those of the WT protein.

and methods section). The purification involved solubilization in 8.5 M urea, Q- and CM-Sepharose chromatography and renaturation of the purified proteins at low concentration. As judged by biochemical criteria (CD spectroscopy, limited proteolysis) this approach was previously shown to yield correctly folded protein in the case of *S. pombe* rna1p derivatives [22]. The RanGAP enrichment in subsequent steps of the purification protocol is shown in Figure 2A for E193A RanGAP as an example. A fraction of all WT and mutant GAP derivatives was always recovered from the soluble bacterial lysate and lysates containing the recombinantly expressed proteins were also employed in the GAP assays described below.

To elucidate whether the point mutations introduced led to significant conformational changes most likely affecting the biochemical/enzymatic properties, we employed CD spectroscopy and compared the secondary structures of the mutant derivatives with those of the respective WT proteins. As an example, the spectra of WT, E193A and D245N RanGAP are shown in Figure 2B. The results are representative for the mutants generated and reveal no significant differences in the overall shape and thus the ratio of secondary structure elements

in the folded molecules. Another means of probing secondary and tertiary structure is limited proteolysis of the folded molecules. Often such a treatment yields relatively stable intermediates probably representing more compact domains within the molecule. A time course of trypsin digestion and an analysis of the products by SDS/PAGE and subsequent Coomassie staining or probing with two different antibodies is depicted in Figure 3 again for WT, E193A and D245N RanGAP as examples. Two major fragments resistant to limited trypsin treatment are generated in the case of WT as well as the mutant RanGAPs. The larger one of approx. 48 kDa most likely represents the entire LRR domain as it reacts with a peptide antibody directed against residues 2–20 of human RanGAP as well as with another peptide antibody recognizing residues 322–334 of the protein. The smaller trypsin-generated fragment has an apparent molecular mass of approx. 20 kDa and is not recognized by the two anti-RanGAP antibodies. Thus it most likely represents the C-terminal region of the protein, indicating that this region which includes the SUMO modification site also forms a domain of limited proteolytic stability. Given the lack of specific peptide antibodies recognizing the acidic stretch, its presence in the 20 kDa fragment

Figure 4 GAP activities of WT and mutant RanGAP derivatives

1 μ M [γ - 32 P]GTP-loaded Ran were incubated at 30 °C with buffer ($\square$), with 0.1 nM of WT RanGAP ($\bullet$), or with varying amounts of mutant GAPs provided as purified protein (**C**) or as bacterial lysates containing a defined amount of recombinant protein (**A** and **B**). Human Ran was titrated in (**A**) with 0.1 nM E193A RanGAP ($\square$), 1 nM E193A RanGAP ($\blacksquare$), 0.1 nM E193Q RanGAP ($\triangle$), or 1 nM E193Q RanGAP ($\blacktriangle$), respectively. In (**B**) reactions contained 0.1 nM D245A RanGAP ($\square$), 1 nM D245A RanGAP ($\blacksquare$), 0.1 nM D245N RanGAP ($\triangle$), or 1 nM D245N RanGAP ($\blacktriangle$), respectively. (**C**) shows the stimulatory effect of 0.1 ($\square$), 1 ($\blacksquare$) and 10 nM ($\triangle$) R91A RanGAP on human Ran. GTPase activity was determined as the decrease of nitrocellulose-bound [γ - 32 P]GTP, and results are given as percentage of radioactivity bound to Ran 30 s after addition of the respective GAP.

cannot be elucidated. However, as this acidic region is susceptible to limited protease digestion and thus most likely is rather unstructured in the yeast Rna1 proteins [22], it is probably not a part of the stable C-terminal tryptic fragment. A close inspection of the proteolysis pattern reveals the generation at later time points of an approx. 46 kDa band which reacts with the more C-terminal but not with the N-terminal peptide antibody. Thus, removal of a 2 kDa piece from the N-terminus of the 48 kDa fragment most likely has produced the 46 kDa fragment suggesting the presence of a trypsin-sensitive site located in the first LRR or prior to the first separating region. Moreover, cleavage at this site leaves the remainder of the LRR domain relatively intact. As the trypsin cleavage patterns look identical for the mutant proteins generated we conclude that the mutations introduced do not cause significant conformational changes.

GAP activity and Ran binding of RanGAP mutants

The effect of different RanGAP/rna1p/Rna1p mutants on the GTPase activity of human Ran or *S. cerevisiae* Gsp1p was assessed by determining the GTP hydrolysis in mixtures containing 1 μ M Ran or Gsp1p and increasing amounts of the GAPs. Gsp1p was chosen in the case of the *S. cerevisiae* Rna1p derivatives, whereas human RanGAP and *S. pombe* rna1p acted upon human Ran. The various WT or mutant GAPs were either employed as purified proteins or as cleared bacterial lysates containing a defined concentration of the recombinantly expressed protein which was determined by semi-quantitative immunoblotting. Comparative analyses employing the WT proteins had revealed that a given amount of GAP is equally active in the assay, regardless of whether the protein is supplied in a purified form or in a total lysate from bacteria expressing the GAP (results not shown). Moreover, assuming saturation, k_{cat} values of the recombinantly expressed and purified GAPs can be

Table 1 Initial hydrolysis rates of RanGTPase activating wild-type and mutant proteins

1 μ M [γ - 32 P]GTP-loaded Ran or Gsp1p were incubated in the absence (w/o GAP) or presence of different concentrations of human RanGAP-(RG), *S. pombe* rna1p- or *S. cerevisiae* Rna1p-derivatives, respectively. GTPase activity was determined as decrease of protein-bound radioactivity 0.5, 5 and 10 min following the addition of the GAP and initial rates of GTP hydrolysis were calculated from a linear fit to these data points. The wild-type human RanGAP proteins were either resolubilized from bacterial inclusion bodies (RGWT_{ib}) or were present in a total bacterial lysate (RGWT_{lysate}).

GAP	GAP concentration [nM]	Initial hydrolysis rate [μ mol/min]	GAP	GAP concentration [nM]	Initiation hydrolysis rate [μ mol/min]
w/o GAP		0.0031	w/o GAP		0.0028
RGWT _{ib}	0.1	0.073	rna1p WT	0.1	0.085
RGWT _{lysate}	0.1	0.080	rna1p E172A	0.1	0.087
RG E193A	0.1	0.0046	rna1p E172Q	0.1	0.087
RG E193A	1	0.0051	rna1p D225A	0.1	0.0030
RG E193Q	0.1	0.023	rna1p D225A	1	0.0044
RG E193Q	1	0.075	rna1p D225N	0.1	0.0009
RG D245A	0.1	0.0037	rna1p D225N	1	0.0027
RG D245A	1	0.0013	w/o GAP		0.0015
RG D245N	0.1	0.0019	Rna1p WT	0.2	0.088
RG D245N	1	0.0043	Rna1p E186A	1	0.014
w/o GAP		0.0042	Rna1p E186A	10	0.085
RG R91A	0.1	0.0041	Rna1p D240A	10	0.0029
RG R91A	1	0.0030	Rna1p D240A	50	0.0059
RG R91A	10	0.021			

calculated from the initial rate of the GTP hydrolysis reaction. The values obtained, 12 s⁻¹ for RanGAP purified after resolubilization from inclusion bodies, 13 s⁻¹ for RanGAP present in the bacterial lysate, 14 s⁻¹ for purified rna1p and 7 s⁻¹ for purified

Figure 5 Complex formation between Ran and different RanGAP derivatives as revealed by chemical cross-linking

2 μ M RanGMPPNP (lanes 1 and 2) or RanGDP (lanes 3 and 4) were mixed with an equimolar amount of WT (A), E193A (B), D245N (C), or R91A RanGAP (D), respectively. The mixtures were treated with the cross-linkers EDC (lanes 1 and 3) or EDC plus S-NHS (lanes 2 and 4) as described in the Materials and methods section. Products of the cross-linking reactions were resolved by SDS/PAGE and analysed by immunoblotting using a Ran-specific antibody. Note that efficient complex formation depends on Ran residing in its GTP-bound conformation and that all mutant RanGAP derivatives show a significantly reduced interaction with Ran.

Rna1p, are in line with those of Ras and RhoGAPs [27,28], thus indicating that the recombinantly expressed GAPs employed in this study are fully active.

Point mutations introduced to neutralize the negative charge at positions characterized by conserved acidic side chains interfere to different degrees with the activity of the respective GAPs. Figure 4 shows representative panels of Ran GTPase stimulations induced by human RanGAP derivatives with the GTP hydrolysis data fitted to a single exponential function, whereas Table 1 summarizes the initial hydrolysis rates for all WT and mutant GAPs analysed. In the case of human RanGAP the alanine for glutamate substitution at position 193 abolishes GAP activity even when the mutant protein is used at a 10-fold molar excess as compared with WT RanGAP. The effect in the E193Q mutant is less pronounced, indicating that a hydrophilic residue at this position seems to be required (Figure 4A). At position 245, on the other hand, both alanine for aspartate as well as asparagine for aspartate replacements interfere significantly with the GAP-mediated stimulation of hydrolysis, suggesting that this conserved residue of a canonical LRR needs to carry a negative charge (Figure 4B). The scenario is very similar for the two yeast RanGAPs, rna1p and Rna1p, respectively. While a significantly reduced activity is observed in all mutants with substitutions of the conserved aspartate of the fifth LRR, the reduction in activity is less pronounced or even absent in proteins carrying mutations in the conserved glutamate of the second separating unit (Table 1). Given the importance of catalytic arginine residues in other GAPs such as RasGAP and RhoGAP, we next concentrated on the three conserved arginines present in the separating regions. Substitutions by alanine of the arginines at positions 182 and 184 (*S. cerevisiae* Rna1p counting) do not affect the GAP activity, i.e. stimulation of Gsp1p-mediated GTP hydrolysis by R182A Rna1p and R184A Rna1p is indistinguishable from that obtained with WT Rna1p (not shown). In contrast, the alanine

for arginine mutation at position 91 in human RanGAP significantly interferes with the activity of the resulting mutant in the Ran GTPase assay. The R91A RanGAP mutant protein only shows a residual stimulatory effect at 100-fold higher molar ratios as compared with the WT protein (Figure 4 and Table 1).

While the GTP hydrolysis assays argue for the importance of Arg91 (as well as Asp245 and to a lesser extent Glu193) in the enzymatic reaction, they do not allow us to distinguish between an effect of the mutations on RanGAP–Ran interaction or on the catalytic reaction itself. Therefore, we next performed direct binding assays using a chemical cross-linking approach previously developed to visualize the interaction between rna1p and human Ran [22]. Equimolar amounts of WT or mutant RanGAPs were mixed with human Ran preloaded with the non-hydrolysable analogue GMPPNP to ensure that Ran is fixed in its GTP-bound conformation, i.e. the conformation capable of interacting with the GAP. Cross-linking was carried out with the carbodiimide derivative EDC and products of the reactions were visualized by SDS/PAGE and immunoblotting using an anti-Ran antibody. Figure 5 shows the results of the cross-linking reactions for WT RanGAP and three RanGAP mutants exhibiting a reduced activity in the GAP assay. A strong cross-linking product of approx. 150 kDa is obtained in the case of the WT protein and formation of this product is greatly reduced when GDP-bound Ran is used as compared with RanGMPPNP. Comparative immunoblot analyses and densitometric scanning indicate that under the cross-linking conditions chosen approx. 10% of the Ran protein is incorporated into the 150 kDa complex. Based on its molecular mass this covalently stabilized complex could represent a trimer consisting of one Ran and two RanGAP molecules. Such a putative trimer has been observed previously in cross-linking studies employing Ran and the *S. pombe* GAP, rna1p [22]. However, the molecular details of this complex remain to be elucidated as isolated rna1p fails to

dimerize [11,22] and as dimer formation of human RanGAP although inferred from its behaviour in gel filtration chromatography [29] is not evident in our cross-linking experiments (results not shown). In the RanGAP mutants analysed formation of the 150 kDa product of the cross-linking reaction is significantly reduced, indicative of an impaired complex formation between Ran and the mutant GAPs. Densitometry reveals that this reduction is approx. 30-fold in the case of all three mutants. The residual cross-linking observed still requires Ran residing in the GTP-bound conformation, thus indicating that specificity in the recognition of the GTP- vs. the GDP-bound state of Ran is not affected by the amino acid substitutions in the mutant GAPs.

DISCUSSION

Ran is an essential component of the nucleocytoplasmic transport machinery engaged in a variety of protein-protein interactions, most of which require Ran to reside in its GTP-bound conformation. In line with this role in transport one group of Ran-binding proteins are the nuclear transport receptors shuttling between the cytosol and the nucleoplasm. The interaction of Ran with these receptors either leads to the dissociation of cargo from the import receptor (importin- β type) or is required for the assembly of export receptors (exportins) and export cargo [4,6,7,9]. While the interaction of importin- β with Ran inhibits the stimulatory effect of RanGAP on Ran-mediated GTP hydrolysis [30], a second group of Ran effectors has the opposite effect and triggers the release of Ran from transport factors of the export branch. The latter effectors are characterized by the so-called Ran binding domain (RanBD), a motif of approx. 100 residues found, e.g., in RanBP1 and RanBP2 [1,2]. The recent crystal structure determination of a complex of RanGTP with the first binding domain of RanBP2 provides a structural basis for the RanBD-mediated release of transport factors from Ran [32]. In the complex, the C-terminal region of Ran ending with the highly acidic DEDDDL motif wraps around the RanBD which thereby sequesters this motif and probably facilitates access by RanGAP. In line with this interpretation the RanBD-containing protein RanBP1 stimulates RanGAP mediated GTP hydrolysis on Ran with this stimulatory effect being abolished by a deletion of the DEDDDL motif. Although this points to a role of the DEDDDL motif in regulating the interaction of Ran with its GAP, it is most likely not part of the binding site but rather sterically or conformationally limits access of RanGAP to its binding site on Ran. This binding site could include a patch of basic residues (position 139–142) which lie in close proximity to the acidic DEDDDL sequence in RanGDP but could be freed to interact with a motif of the GAP once Ran is in the GTP-bound conformation, in particular following sequestration of the DEDDDL motif by RanBD binding [31,32]. In this respect it is interesting to note that the acidic C-terminal region in the yeast RanGAPs is required for RanGTP binding, possibly through interacting with the basic patch [22].

Although the acidic C-terminal sequence in RanGAP is required for establishing an interaction with Ran, it is not sufficient [22]. A contribution of the LRR-dominated domain has been inferred from these observations and a conformational cross-talk between the LRR domain and the acidic region within the GAP has been proposed. By characterizing the biochemical and enzymatic properties of a series of site-specifically mutated RanGAP molecules we have now addressed this problem. We identify conserved amino acid side chains of positive or negative charge which are found within the LRR domain and are indispensable for efficient Ran binding. In our analysis we

concentrated on residues of the two separating regions found within the LRR domains of all RanGAPs known so far. These sequences which comprise 36 residues separate LRRs 1 and 2 as well as 3 and 4, respectively. Like individual LRRs they contain regularly spaced hydrophobic residues but are of atypical length when compared with canonical LRRs of, e.g., the RNase inhibitor. Nevertheless, our data indicate that the separating regions are most likely integral parts of the folded molecule as complete removal of one or the other interferes with the expression of a stable protein. Of the charged residues mutated, two conserved arginines (positions 189 and 191 of human RanGAP) appear to be dispensable for catalytic activity. Mutations in the third conserved arginine (Arg91 in the human protein), on the other hand, clearly interfere with both, Ran binding and GAP activity. Likewise substitutions of the conserved acidic residue at position 193 (human RanGAP) affect the interaction with Ran at least when the charged side chain is replaced by a non-hydrophilic one. Thus, although the separating regions probably are embedded in the rest of the molecule, i.e. do not form extended loops, some residues of these sequences are likely to reside on the surface of the molecule, possibly providing contact sites for Ran. The same probably also holds true for conserved charged residues of the canonical LRRs as mutations of the conserved aspartate found in the fifth LRR significantly interfere with GAP activity even when the acidic side chain is replaced by its amide. In crystallized RNase inhibitor, the prototype of an LRR protein, the individual LRRs form β - α hairpin units with the β -strand and the α -helix orientated roughly parallel and connected by a loop [20]. Upon extrapolation of this general LRR fold on RanGAP, the conserved aspartate (Asp245 in human RanGAP) would be located in the connecting loop with its side chain possibly protruding towards the solvent. Based on the spacing of conserved hydrophobic residues it seems possible that the separating regions form a structure similar to that of the canonical LRRs. Given this assumption, the charged residues identified in our analysis as important for Ran binding and GAP activity (Arg91 and Glu193 in human RanGAP) would most likely also reside in a loop connecting a β -strand and an α -helix. This suggests that within the LRR domains of the RanGAPs the regularly spaced hydrophobic residues often found in the β -strands and α -helices form the protein core, whereas charged residues present in the connecting loops provide contact sites for the GTPase.

A particular emphasis in this study was put on conserved arginine residues as such side chains have been shown to be of critical catalytic importance in RasGAP and RhoGAP. In their interaction with Ras and Rho, respectively, the arginine is inserted into the active site of the respective GTPase, thereby stabilizing the transition state of the enzymatic reaction [33]. Mutations in these catalytic arginines lead to a significant reduction in enzymatic activity but only have a minor effect on the affinity of RasGAP and RhoGAP towards their corresponding GTPase. Although the general three-dimensional structure of RanGAP is likely to be different from that of RasGAP and RhoGAP, it seems possible that a conserved arginine in RanGAP serves a similar function as an arginine finger. Only three arginines (all of which reside in the separating regions) are conserved in all RanGAP molecules identified so far. Mutations in two of them (Arg189 and Arg191 in human RanGAP) have no effect on GAP activity. The third, Arg91 in human RanGAP, is of critical importance for GAP activity but also for establishing the interaction with Ran. Such a phenotype is, however, not expected for a typical arginine finger, which is required for efficient catalysis but not for binding of the GTPase. Thus, it appears that the mechanism of GTPase stimulation

through RanGAP differs from that mediated by RasGAP and RhoGAP. In this respect it is interesting to note that the recently established crystal structure of the Arf1–ArfGAP complex also fails to reveal in the GAP an arginine side chain protruding into the active site of the GTPase [34]. However, when coatamer as an Arf1 effector is present, the ArfGAP-mediated stimulation of GTP hydrolysis on Arf1 is accelerated a further 1000-fold possibly because coatamer now provides residues entering the active site and increasing the rate of hydrolysis [34,35]. In our analyses the k_{cat} of the RanGAP stimulated reaction is already at approx. 10 s^{-1} and a further large acceleration seems unlikely. Nevertheless, it remains to be seen whether Ran effectors such as RanBP1 or RanBP2 can function as co-activators of RanGAP by interacting with and/or altering the active site of the GTPase.

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