The Acidic C-terminal Domain of rna1p Is Required for the Binding of Ran-GTP and for RanGAP Activity*

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The small GTP binding protein Ran is an essential component of the nuclear protein import machinery whose GTPase cycle is regulated by the nuclear guanosine nucleotide exchange factor RCC1 and by the cytosolic GTPase activating protein RanGAP. In the yeasts Schizosaccharomyces pombe and Saccharomyces cerevisiae the RanGAP activity is encoded by the RNA1 genes which are essential for cell viability and nucleocytoplasmic transport in vivo. Although of limited sequence identity the two yeast proteins show a conserved structural organization characterized by an N-terminal domain of eight leucine-rich repeats, motifs implicated in protein-protein interactions, and a C-terminal domain rich in acidic amino acid residues. By analyzing the RanGAP activity of a series of recombinantly expressed rna1p mutant derivatives, we show that the highly acidic sequence in the C-terminal domain of both yeast proteins is indispensable for activating Ran-mediated GTP hydrolysis. Chemical cross-linking reveals that the same sequence in rna1p is required for rna1p-Ran complex formation indicating that the loss of GAP activity in the C-terminally truncated rna1p mutants results from an impaired interaction with Ran. The predominant species stabilized through the covalent cross-link is a rna1p-Ran heterodimer whose formation requires the GAP-bound conformation of Ran. As the acidic C-terminal domain of rna1p is required for establishing the interaction with Ran, the leucine-rich repeats domain in rna1p is potentially available for additional protein interactions perhaps required for directing a fraction of rna1p to the nuclear pore.

The import of karyophilic proteins into the nucleus which occurs through the nuclear pore complex (NPC) is an energy-dependent process specified by nuclear localization signals (NLSs) on the import substrate (for reviews see Refs. 1–4). Using digitonin-permeabilized HeLa cells as an in vitro transport system, it was shown that the import of substrate proteins is a multistep process depending on four cytosolic factors (5, 6).

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§ The abbreviations used are: NPC, nuclear pore complex; DST, disuccinimidyl tartarate; DTE, dithioerythritol; DTT, dithiothreitol; EDC, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide; GAP, GTPase activating protein; GMPPNP, 5′-guanylimidodiphosphate; GST, glutathione S-transferase; IPTG, isopropyl-β-D-thiogalactopyranoside; NLS, nuclear localization signal; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PMSF, phenylmethylsulfonyl fluoride; LRR, leucine-rich repeats.

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Ran is a predominantly nuclear protein with homology to Ras and other small GTPases that cycle between a GDP- and GDP-bound state (for review see Ref. 16). The GDP/GTP cycle is regulated by two auxiliary proteins, a GTPase activating protein (GAP) which stimulates the intrinsic GTPase activity of Ran by several orders of magnitude and a guanine nucleotide exchange factor which markedly accelerates the GTP for GDP exchange rate on Ran. The only guanine nucleotide exchange factor for Ran known to date is the nuclear protein RCC1, whereas the only RanGAP identified so far is a cytosolic protein (17–20). The strictly compartmentalized organization of the two principle regulators, RanGAP and RCC1, indicates that Ran shuttles between the cytoplasm and the nucleus to interact with its regulator proteins and that a gradient of Ran-GTP exists across the nuclear envelope (for review see Refs. 10, 21, and 22). Accordingly it has been suggested that Ran-GDP is the active form of the protein at the cytoplasmic side of the nuclear pore and that GTP-bound Ran is required to terminate translocation at the nuclear envelope. Evidence for this model includes the findings that Ran-GDP can bind to the NPC via its interaction with p10/NTF2, that a Ran mutant locked in the GDP-bound state fails to support nuclear protein import and prevents docking of an import substrate at the NPC, and that Ran-GTP can bind to importin β thereby inducing the dissociation of importin α from importin β and most likely the termination of translocation (15, 23, 24).

Genes encoding the principle regulators of the Ran GTPase cycle have also been shown to be essential for viability in budding and fission yeasts. In Saccharomyces cerevisiae and Schizosaccharomyces pombe, these genes encode the Ran homologues Gsp1p and spi1p (25, 26), the RCC1 homologues Prp20p and pim1p (26, 27), and the RanGAP homologues Rna1p and rna1p, respectively (28, 29). Often, mutations in these genes cause a number of different defects including an.
inhibition of both nuclear protein import and RNA export. Such pleiotropism is particularly pronounced in S. cerevisiae carrying temperature-sensitive mutations in the RNA1 gene which were first described by Hartwell (30) 3 decades ago. Only recently it was shown that Rna1p acts as a GAP for Gsp1p and participates in protein import into S. cerevisiae nuclei both in vitro and in vivo (20, 31, 32). The homologous protein from S. pombe, rnap1, possesses GAP activity for Gsp1p (31) and in contrast to S. cerevisiae Rna1p also stimulates the GTP hydrolysis mediated by human Ran (23, 31).

Structurally, all RanGAP proteins known to date, S. cerevisiae Rna1p, S. pombe rnap1, human RanGAP, and the mouse homologue Fog1, share two characteristic features as follows: an N-terminal region of approximately 310–330 amino acid residues which is characterized by an 8-fold repetition of a leucine-rich repeat (LRR) motif, and a sequence of some 40 residues which is extremly rich in acidic amino acids and follows the LRR region. In the yeast proteins, the acidic sequence is followed by a C-terminal stretch of 12 (S. pombe) or 15 (S. cerevisiae) residues capable of forming an amphipathic α-helix, whereas the C-terminal sequences in the mammalian proteins are much longer (189 and 191 residues in RanGAP and Fog1, respectively) and not related to Rna1p/rna1p (20, 28, 33). Limited proteolysis revealed that in S. pombe rnap1 the LRRs form a relatively stable domain, whereas the acidic region appears more flexible and susceptible to limited digestion (34). LRRs that are typically 22–29 amino acids in length have been identified in a number of functionally unrelated proteins and have been proposed to mediate protein-protein interactions (for review see Ref. 35).

To identify protein domains mediating RanGAP activity of the yeast rna1/rna1 proteins, we generated a series of rnap1/rna1 mutant derivatives. Biochemical analysis of these mutants reveals that the acidic C-terminal region is indispensable for full GAP activity of rnap1. Cross-linking experiments which for the first time enabled the visualization of rnap-Ran-GTP complexes show that it is this C-terminal sequence which is required for rnap1-Ran complex formation and that the LRR domain by itself is not capable of Ran binding. This unexpected finding suggests that the LRR domain of rna1 could be available for additional protein interactions thereby possibly directing the RanGAP activity to the nuclear pore.

EXPERIMENTAL PROCEDURES

Constructs Encoding Wild-type and Mutant rna1p/Rna1p as well as the Acidic C-terminal Domain of Rna1p in a Fusion Protein with GST—cDNAs encoding full-length S. pombe rnap1 and S. cerevisiae rna1p were isolated by PCR using a S. pombe RNA1 cDNA fragment (29) or a genomic S. cerevisiae RNA1 NcoI-BamHI fragment (31) as template. In the case of S. pombe RNA1 PCR employed oligonucleotide SP1 (5'-CTTCTACCAGTGATCCCTGGC-3') and antisense primer SP2 (5'-GGAAACTGCTTGCTGGTCT-3'), yielding the truncated cDNAs ΔSP375–386, ΔSP361–386, ΔSP341–386, ΔSP300–386, and ΔSP361–386/QQQD, respectively. The numbers indicate amino acid positions in the rna1 protein sequence (29) that are deleted in the mutant proteins encoded by the different cDNAs. In ΔSP361–386/QQQD, the cDNA encodes a three amino acid replacement (the glutamic acid residue at positions 351, 352, and 354 are substituted by glutamine) in the background of the ΔSP361–386 deletion. To amplify S. cerevisiae RNA1 sequences the oligonucleotide SC1 (5'-GGGAAAAT- TCCAGTGATCCCTGGCTC-3'), an EcoRI restriction site used as a sense primer in combination with the following antisense primers (a PstI restriction site is underlined): oligonucleotide SC2 (5'-CCCTCGAGAGCTTTAGGGTTTCTGACCCAG-3') resulted in the complete S. cerevisiae RNA1 cDNA, whereas PCR employing oligonucleotides SC3 (5'-GGGGATCCAG- TGGCTCCTGCAGTGTGGGCCG-3') and antisense primer SP7 (5'-GCTTCCTCCCTTCATCGTGAAGTCCTTGCC-3') yielded the full-length protein, whereas oligonucleotides SP3 (5'-CTCAGTGATCCCTGGC-3') and antisense primer SP4 (5'-GGAAACTGCTTGCTGGTCT-3') yielded the ΔSP361–386/QQQD cDNA (29) as template and the oligonucleotides SP8 (5'-CGAAGTTGCCATAGAGTCACTGCTGGTCT-3') and antisense primer SP9 (5'-CGAGGAGTGAAATTGCTCAATC-3') yielded the ΔSP361–386 deletion. To amplify for the first time enabled the visualization of rnap-Ran-GTP complexes show that it is this C-terminal sequence which is required for rnap1-Ran complex formation and that the LRR domain by itself is not capable of Ran binding. This unexpected finding suggests that the LRR domain of rna1 could be available for additional protein interactions thereby possibly directing the RanGAP activity to the nuclear pore.

The resulting supernatant was dialyzed against buffer B (20 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM DTT, 1 mM PMSF) and chromatographed on a DE52 (Whatman) column that was developed with a linear salt gradient from 0 to 1 M NaCl in buffer B. Fractions containing the wild-type or mutant rna1/Rna1 proteins were identified using specific antibodies, pooled, dialyzed against buffer B and applied on a MonoQ FPLC column (Pharmacia) equilibrated in buffer B. A linear salt gradient from 0 to 1 M NaCl in buffer B and the different rna1/Rna1 proteins eluted between 0.4 and 0.5 M salt. Relevant protein fractions (identified by SDS-PAGE and immunoblottting with rna1/Rna1 antibodies) were pooled, dialyzed against buffer B containing 50% glycerol, and stored at −20 °C.

The proteins rnap1Δ330, Rna1pΔ340, and Rna1pΔ359 encoded by the plasmids pkkSP330, pkkSC378, and pkkSC359, respectively, were purified as native polypeptides from the bacterial lysates. The same protocol was employed for wild-type S. cerevisiae Rna1p and the Rna1pΔ393 mutant protein, which were prepared from bacteria expressing the plasmids pkkSCWT and pkkSC393, respectively. Bacteria were harvested by centrifugation and washed once in ice-cold phosphate-buffered saline. The pellet was resuspended in 12 ml of buffer A (30 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM DTT, 1 mM PMSF), and cells were lysed using a French press. Subsequently, three volumes of buffer B were added, and the homogenate was clarified (60 min at 100,000 × g, 4 °C). Ammonium sulfate was added to 60% saturation, and precipitated proteins were removed by centrifugation. The resulting supernatant was dialyzed against buffer B (20 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM DTT, 1 mM PMSF) and chromatographed on a DE52 (Whatman) column that was developed with a linear salt gradient from 0 to 1 M NaCl in buffer B. Fractions containing the wild-type or mutant rna1/Rna1 proteins were identified using specific antibodies, pooled, dialyzed against buffer B and applied on a MonoQ FPLC column (Pharmacia) equilibrated in buffer B. A linear salt gradient from 0 to 1 M NaCl in buffer B and the different rna1/Rna1 proteins eluted between 0.4 and 0.5 M salt. Relevant protein fractions (identified by SDS-PAGE and immunoblottting with rna1/Rna1 antibodies) were pooled, dialyzed against buffer B containing 50% glycerol, and stored at −20 °C.
0.5 volume of CM-Sepharose (Pharmacia) equilibrated in buffer B. The Sepharose beads were removed by centrifugation, and the resulting supernatant was added dropwise to 10 volumes of buffer B while stirring. After dialysis against buffer B the material was applied to a MonoQ column equilibrated in the same buffer. The column was developed with a linear NaCl gradient (0 to 0.6 M NaCl) and the chymotryptic rna1p protein eluted at approximately 0.3 M NaCl. The peak fractions containing the individual rna1/Rna1 proteins eluted between 0.5 and 0.6 M NaCl in buffer B. Undigested rna1p eluted at approximately 0.4 M NaCl. The chymotryptic fragment of 36.5 kDa eluted at approximately 0.3 M NaCl. The peak fractions containing the individual polypeptides were pooled separately, dialyzed against buffer B containing 50 mM Tris-HCl, pH 8.0, containing 10 mg/ml reduced glutathione (GSH). The eluate was dialyzed against 20 mM potassium phosphate, pH 7.4, 5 mM MgCl₂, 1 mM DTG, and 50% glycerol and stored at -20 °C.

Purification of GST and of the Fusion Protein—Purification of GST and the Acidic C-terminal Domain of rna1p—BL21(DE3)pLyS8 cells containing the unmodified pGEX-KG vector (for expression vector) or the construct pGEX-SPTC (for expression of the fusion protein) were grown at 30 °C in 1 liter of 2xTY medium containing 150 μg/ml ampicillin. At an A₆₀₀ of 0.7 IPTG was added to a final concentration of 0.25 mM, and the culture was incubated for another 4 h. Bacteria were then harvested by centrifugation, and the pellet was suspended in 40 ml of ice-cold PBS containing 1 mM DTT and 1 mM PMSF. The cells were lysed on ice using a Branson sonifier (6× 30 min in the presence of 5 mM EDTA and 5 μM [α-32P]GTP instead of EDTA, respectively) and applied to a Superose 12 PC 3.2/30 column connected to a SMART dichrograph.

Protein Cross-linking—Wild-type and mutant rna1 proteins as well as Ran in its GDP- or GMPNP-bound form were dialyzed against 20 mM potassium phosphate, pH 7.5, 5 mM MgCl₂, 1 mM DTG and diluted to concentrations of 2 μM in the same buffer. Proteins were then mixed as indicated and 5.2 μM 1-ethyl-3-[3-dimethylaminopropyl]-carbodiimide hydrochloride (EDC, Pierce) or 4.4 μM disuccinimidyl tartarate (DST, Pierce), respectively, were added as cross-linking reagent. Following 90 min incubation at room temperature proteins were precipitated using methanol/CHCl₃ (42), resuspended in SDS sample buffer, and analyzed in SDS-12.5% gel (or 10% for determination of higher molecular masses) polyacrylamide gels (43).

Miscellaneous—The authenticity of the purified Rna1p derivatives and of the proteolytic fragment resulting from digestion by chymotryptic cleavage was verified by automated N-terminal sequence analysis on an Applied Biosystems gas phase sequencer (model 470 A). The molecular mass of the chymotryptic rna1p fragment was determined by mass spectroscopy using an IR MALDI mass spectrocope. CD spectroscopy of wild-type and mutant rna1p derivatives was carried out on a Jobin Yvon CD6 dichrograph.

RESULTS

Although the actual sequence identity between the two yeast RanGAPs, S. pombe rna1p and S. cerevisiae Rna1p, is limited to 42%, expression of the S. pombe protein in the rna1-1 S. cerevisiae mutant strain complements the temperature-sensitive defect in the RNA1 gene (29). This functional equivalence is reflected by the highly conserved domain structure of the two proteins as both comprise (from N- to C-terminal end) the LRR motif, the acidic C-terminal domain, the highly conserved region, and a short amphipathic α-helix. To probe this domain structure of the yeast rna1 proteins with respect to its importance for the function of the proteins, we generated a series of rna1p mutant derivatives and analyzed their GAP activity and their mode of interaction with Ran. In this analysis we concentrated on features of the C-terminal domains of proteins by progressively truncating C-terminal sequences (Fig. 1).

Preparation of C-terminally Truncated rna1p/Rna1p Derivatives—The rna1/Rna1 mutant proteins were expressed and purified following a protocol developed for wild-type S. pombe rna1p (29, 34). Purification involved ammonium sulfate fractionation and chromatography on DE52 and MonoQ (Fig. 2).
Due to progressive deletions of the acidic C-terminal domain in the shorter rna1p/Rna1p derivatives, the respective mutant proteins showed different chromatography properties and were eluted from the MonoQ resin at lower salt concentrations. Three of the mutant proteins, S. pombe rna1pD330, as well as S. cerevisiae Rna1pD378 and Rna1pD359 remained insoluble after expression in bacteria and were purified from inclusion bodies (see “Experimental Procedures”). This involved solubilization in 8.5 M urea and subsequent renaturation of the proteins. To ensure that this treatment did not lead to any major conformational alterations in the refolded molecule, the truncated rna1pD330 derivative was also generated by limited proteolysis.
teolysis of native *S. pombe* rna1p. 5 min digestion of the wild-type protein with α-chymotrypsin at an enzyme:substrate ratio of 1:100 yielded a stable 36.5-kDa fragment that could be separated from the undigested protein by ion exchange chromatography (Fig. 2C). The 36.5-kDa fragment was shown by N-terminal sequence analysis to contain the authentic rna1p N terminus. Moreover, mass spectroscopy revealed a molecular mass of 36,590 Da for the stable fragment. Collectively, these findings show that the chymotryptic fragment comprises the N-terminal 329 amino acids of the protein thus showing the same C-terminal end as the rna1p330 mutant. Since generation of the 36.5-kDa chymotryptic fragment did not involve any urea treatment, the purified 36.5-kDa fragment served as a control in our studies with recombinantly expressed mutant proteins purified from inclusion bodies. In the analyses described below the chymotryptic 36.5-kDa fragment showed properties indistinguishable from those of the recombinantly expressed rna1p330 mutant, thus indicating that the urea treatment and the subsequent renaturation of rna1p330 does not result in significant conformational alterations. This was corroborated by CD spectroscopy of the different mutant derivatives that revealed no significant differences in the degree of α-helicity, e.g. between rna1p375 purified from the soluble protein fraction and rna1p330 mutant purified from inclusion bodies (not shown). Since the differences between these mutant proteins solely lie in the length of their acidic C-terminal extension, the CD analyses also indicate that the C-terminal extension does not contribute significantly to the α-helical content of the entire rna1p molecule.

The Activation of Ran-mediated GTP Hydrolysis by Different rna1p Mutants—The effect of the different *S. pombe* rna1p derivatives on the GTPase activity of Ran was analyzed by comparing the GTP hydrolysis in mixtures containing 1 μM Ran and increasing amounts of the different rna1p mutants (Fig. 3). In line with our previous observation (31), 0.1 nM wild-type rna1p increases the GTP hydrolysis by a factor of 40 over the intrinsic rate when initial rates are compared (Fig. 3A). This value is not affected in the rna1p375 mutant (Fig. 3A), i.e. an rna1p derivative devoid of 12 residues at the C-terminal end which most likely form an amphipathic α-helix (29). Likewise, Ran-mediated GTP hydrolysis is accelerated to the same extent in the presence of 0.1 nM rna1p361 mutant protein indicating that the C-terminal 30% of the acidic extension and the C-terminal helix are dispensable for full GAP activity (Fig. 3B). However, when additional portions of the highly acidic region are removed, the ability of rna1p to increase the intrinsic GTP hydrolysis of Ran is significantly impaired. In the case of rna1p341 a 100-fold higher concentration of the mutant derivative as compared with wild-type rna1p (10 nM compared with 0.1 nM) is required to induce measurable GTP hydrolysis over a period of 60 min (Fig. 3C). This effect is even more pronounced in the shortest mutant, rna1p330, whose remaining activity in this GTP hydrolysis assay is almost negligible and indistinguishable from that of the chymotryptic 36.5-kDa fragment (Fig. 3D).

Similar C-terminal truncations were also analyzed using the *S. cerevisiae* Rna1p as a GAP. Fig. 4 shows that the GTPase activity of Gsp1p, the *S. cerevisiae* homologue of Ran, is also affected by C-terminal truncations in *S. cerevisiae* Rna1p. In line with the results obtained with the *S. pombe* protein the GAP activity of *S. cerevisiae* Rna1p remains unchanged when the C-terminal α-helix is removed in the Rna1p375 mutant (Fig. 4A). A truncation of increasing portions of the acidic region, however, results in moderately to greatly reduced GAP activities (in the Rna1p378 and Rna1p359 mutant proteins; Fig. 4B and C). Taken together these results indicate that the activation of Ran-mediated GTP hydrolysis by rna1p/Rna1p requires the presence of a substantial portion of the highly acidic region in the unique C-terminal extension of the respective RanGAP.

We next analyzed whether the rna1p mutant derivatives displaying markedly reduced GAP activities had an inhibitory
Fig. 5. GAP activity of *S. pombe* rna1p in the presence of increasing concentrations of the C-terminally truncated mutant protein rna1pΔ341. 1 μM Ran loaded with [γ-32P]GTP was incubated at 30 °C with buffer (○), 0.025 nm rna1p (■), 0.05 nm rna1p (△), or 0.1 nm rna1p (▲). In the latter case (1 μM Ran, 0.1 nm rna1p) the reaction was carried out in the absence (○) or presence of 0.025 nm rna1pΔ341 (△), 0.05 nm rna1pΔ341 (▲), or 0.1 nm rna1pΔ341 (▲). The individual experiments were carried out at least three times, and curves of a representative experiment are shown. Note that the addition of increasing amounts of the rna1pΔ341 does not affect the GAP activity of 0.1 nm wild-type rna1p. All curves characterizing the reactions containing 1 μM Ran and 0.1 nm rna1p are nearly identical and partially mask one another.

Effect on wild-type rna1p in the Ran-GAP reaction. Due to the high molar excess of Ran over rna1p in the GTP hydrolysis assay such an inhibition would be indicative of a functionally important rna1p dimerization, a property suggested for the human RanGAP based on gel filtration analysis (19). In a series of competition assays we added increasing amounts of the truncated rna1p mutants to mixtures containing 1 μM Ran and 0.1 nm wild-type rna1p and determined the rate of Ran-mediated GTP hydrolysis. Fig. 5 shows representative examples, in this case for competition assays with the rna1pΔ341 mutant. The GAP activity of wild-type rna1p remains unchanged even in the presence of a 200-fold molar excess of rna1pΔ341. This indicates that the truncated mutant protein does not interfere with wild-type rna1p or act on preformed rna1p-Ran complexes in a manner resulting in functional interference.

**Complex Formation between Ran and rna1p Is Inhibited in C-terminal rna1p Mutants with Impaired GAP Activity**—Two possible models could explain the findings that C-terminal truncations removing most of the highly acidic region of rna1p markedly impair the ability to active Ran-mediated GTP hydrolysis. First, complex formation between Ran and the rna1p mutant derivative could be impaired and second, complexes between Ran and rna1p mutants form but are functionally inhibited because the C-terminal truncations render the rna1p derivatives enzymatically inactive. To distinguish between these possibilities we analyzed the interaction between Ran and wild-type or mutant rna1p since this interaction should be affected in the case of the former but not the latter model.

In a first approach we characterized hydrodynamic properties of rna1p, Ran, and rna1p/Ran mixtures. In this and all subsequent protein interaction analyses we used GMPPNP-loaded Ran, i.e. a Ran molecule fixed in its GTP-bound conformation, as we predicted that only GTP-bound Ran interacts with rna1p. Analytical gel filtration reveals that rna1p shows migration properties corresponding to those of a globular protein of approximately 90 kDa, whereas the migration position of Ran corresponds to that of a globular protein of approximately 20 kDa (not shown). When the two proteins are mixed at an equimolar ratio prior to the molecular sieving chromatography, the migration positions remain unchanged and no other species corresponding to rna1p-Ran complex is observed (Fig. 5A). Moreover, anti-rna1p and anti-Ran immunoblot analysis of the fractions obtained after the gel filtration reveals that Ran is absent from the rna1p peak and vice versa (Fig. 5B). The migration properties of rna1p are somewhat unusual for a 43-kDa protein and are indicative of either a non-globular shape of the protein or rna1p dimerization. Our previous sedimentation equilibrium measurements of authentic rna1p purified from *S. pombe* only revealed the existence of a monomeric species (29). Moreover, the GAP competition experiments did not yield any indication of direct rna1p-rna1p interactions (Fig. 5). Thus, we concluded that the atypical behavior in gel filtration is not due to dimerization but stems from unusual molecular sieving properties of rna1p. To corroborate this and to analyze whether the highly acidic C-terminal domain confers such properties, we subjected the C-terminal truncation mutants to analytical gel filtration. Fig. 6B reveals that progressive truncations of the acidic sequence lead to mutant derivatives with significantly altered migration positions. The shorter the acidic stretch the closer the migration properties resemble those of a globular protein of the same length. In fact, the rna1pΔ341 mutant, in which most of the C-terminal domain has been removed, elutes at a position corresponding to that of a globular protein of the same size, i.e. 38 kDa. This gradual increase in the retardation times of the progressively truncated rna1p mutants argues against a dimer to monomer transition between the longer (GAP active) and the shorter (GAP inactive) derivatives. More likely, the stretch of highly acidic residues in the C-terminal extension of rna1p induces atypical molecular...
Fig. 7. Chemical cross-linking of wild-type and mutant rna1 proteins to Ran-GMPPNP. 2 μM rna1p (lanes 1 and 2), rna1pΔ375 (lanes 3 and 4), rna1pΔ361 (lanes 5 and 6), rna1pΔ341 (lanes 7 and 8), or rna1pΔ330 (lanes 9 and 10) were mixed with 2 μM Ran-GMPPNP (lanes 1, 3, 5, 7, and 9) or with 2 μM Ran-GDP (lanes 2, 4, 6, 8, and 10) and incubated in the presence of 5.2 mM EDC (A and B) or 4.4 mM DST (C and D). Reaction products were separated by SDS-PAGE, transferred to polyvinylidene difluoride membrane, and probed with anti-Ran antibodies (A and C) or anti-rna1p antibodies (B and D). Note that both cross-linking reagents yield covalently linked products between Ran and rna1p, rna1pΔ375, and rna1pΔ361, respectively, with apparent molecular masses of approximately 68, 67, and 65 kDa, respectively (A–D; lanes 1–3). In contrast, no cross-linking is observed between Ran and the shorter rna1p mutants, rna1pΔ341 and rna1pΔ330. Molecular masses of marker proteins are indicated on the left.

Fig. 8. Cross-linking of wild-type and mutant rna1 proteins to GMPPNP- and GDP-loaded Ran. 2 μM rna1p (lanes 1 and 2), rna1pΔ375 (lanes 3 and 4), rna1pΔ361 (lanes 5 and 6), rna1pΔ341 (lanes 7 and 8), or rna1pΔ330 (lanes 9 and 10) were mixed with 2 μM Ran-GMPPNP (lanes 1, 3, 5, 7, and 9) or with 2 μM Ran-GDP (lanes 2, 4, 6, 8, and 10) and incubated in the presence of 5.2 mM EDC. Products of the cross-linking reactions were separated by SDS-PAGE, transferred to polyvinylidene difluoride membrane, and probed with anti-Ran antibodies (A) or anti-rna1p antibodies (B). Note that cross-linked products with molecular masses of approximately 68, 67, and 65 kDa, respectively, which contain Ran and rna1p, rna1pΔ375, and rna1pΔ361, respectively, are solely formed in the case of GMPPNP-loaded Ran (lanes 1, 3, and 5). Molecular masses of marker proteins are given on the left.

...sieving properties, possibly due to the high concentration of negative charges.

Transient, unstable, or relatively weak protein-protein interactions are frequently not detected by gel filtration. Therefore, we employed chemical cross-linking as a different means for visualizing the postulated rna1p-Ran interaction. Equimolar amounts of rna1p and GMPPNP-bound Ran were treated with the cross-linking agents EDC (a carbodiimide derivative) and DST (a succinimide derivative), respectively, and the reaction products were then analyzed by immunoblotting with rna1p and Ran antibodies, respectively. Fig. 7 (lanes 1) shows that treatment with both cross-linkers yields a 68-kDa species, which is recognized by both rna1p and Ran antibodies. Based on its apparent molecular mass this product most likely represents a dimer of covalently linked rna1p and Ran-GMPPNP. In addition to this dimer cross-linking with DST also leads to the formation of a polypeptide of 109 kDa that also reacts with the two antibodies.

Next, we used the same cross-linking protocol to characterize the interaction of C-terminally truncated rna1p mutants with Ran-GMPPNP. Complex formation with Ran is observed in the case of rna1pΔ375 and rna1pΔ361, the two rna1p mutants displaying GAP activities indistinguishable from that of the wild-type protein (Fig. 7, lanes 2 and 3). In fact, the yield of covalently cross-linked products even appears somewhat higher in the case of these mutants when compared with wild-type rna1p. In contrast, the mutants showing severely impaired GAP activity, rna1pΔ341 and rna1pΔ330, fail to yield cross-linking products with Ran-GMPPNP (Fig. 7, lanes 4 and 5). Thus, the major part of the acidic C-terminal sequence which is indispensable for full GAP activity of rna1p is also required for rna1p-Ran complex formation.

A specific, functionally meaningful interaction between rna1p and Ran would require that rna1p only forms a complex with Ran in its GTP-bound and not its GDP-bound conformation. To test this prediction we performed EDC cross-linking experiments with mixtures containing rna1p and either GMPPNP- or GDP-loaded Ran. Fig. 8 shows that chemical cross-linking between Ran and wild-type rna1p, rna1pΔ375, and rna1pΔ361 is only observed when Ran is fixed in its GTP-bound conformation through loading with GMPPNP. GDP-bound Ran is unable to form complexes with wild-type rna1p or either of the rna1p mutant derivatives.

The Acidic C-terminal Domain of rna1p Is Not Sufficient for Ran Binding—So far our analyses have revealed that the Ran-GAP activity of rna1p required the integrity of a major part of the C-terminal domain and that this acidic C-terminal sequence is indispensable for the physical interaction between rna1p and Ran-GTP. To analyze in detail the contribution of the acidic domain to establishing a functional interaction of rna1p with Ran, we generated and characterized two additional rna1p mutant derivatives. First, we replaced in a stretch of five consecutive acidic residues (Glu351–Glu355) (Fig. 1) the three glutamic acids at positions 351, 352, and 354 by glutamines. These mutations were chosen since a similar stretch of five acidic amino acids appears to be a crucial although not sufficient part of the Ran binding motif in several nuclear pore proteins (44). Moreover, to elucidate specifically the importance of this sequence for the rna1p activity, the mutation was introduced in the rna1pΔ361 mutant, i.e., the shortest derivative still displaying GAP activity and Ran-GTP binding. The second mutant protein generated in this series was a GST fusion protein, in which the acidic C-terminal domain encompassing residues 323–386 was covalently attached to GST-GST-rna1pCT. The characterization of such a mutant should directly answer the question whether the acidic domain itself is sufficient for mediating the rna1p activities analyzed here. Fig. 9 reveals that both mutant proteins could be purified to homogeneity by following the procedure developed for bacterially expressed wild-type rna1p (rna1pΔ361/QQDQ; Fig. 9A) or by employing a glutathione-Sepharose affinity matrix (GST-rna1pCT; Fig. 9B).

The purified proteins were first analyzed for their ability to stimulate the intrinsic GTPase activity of Ran. As shown in Fig. 10A, rna1pΔ361/QQDQ displays a RanGAP activity which is indistinguishable from that of wild-type rna1p and the rna1pΔ361 mutant, respectively. In contrast, the GST-rna1pCT mutant does not stimulate the Ran GTPase over intrinsic levels, even when employed at a 500-fold molar excess...
similar products in the EDC reaction (Fig. 11).

Antibody directed against GST. However, formation of these
species which is recognized by both Ran and rna1p antibodies,
mutant proteins was analyzed by EDC-mediated cross-linking.

A protein consisting of GST and the 64 C-terminal amino acids of rna1p
was prepared from bacteria transformed with the respective expres-
sion plasmids. The purifications were carried out as described under
“Experimental Procedures,” and proteins present in the different frac-
tions were analyzed by SDS-PAGE. A, bacterial cells expressing
rna1pΔ361/QQDQ were lysed using a French press, and soluble pro-
teins (lane 2) were fractionated by ammonium sulfate precipitation.
Proteins soluble in the presence of 60% ammonium sulfate (lane 3) were
chromatographed on a DE52 column. Fractions containing the
rna1pΔ361/QQDQ protein (lane 4) were pooled and chromatographed
on a MonoQ column to yield the pure protein (lane 5). B, bacterial cells
harboring the pGEX-SPCT expression plasmid (a total protein extract
of these cells is shown in lane 2) were induced to express the recom-
binant protein by the addition of IPTG (a total protein extract after IPTG
induction is shown in lane 3). Subsequently, the cells were lysed by
sonication, and soluble proteins (lane 4) were incubated with glutathi-
one-Sepharose. Pure GST-rna1pCT protein was then eluted by the
addition of reduced glutathione (lane 5). Molecular mass markers are
shown in lanes 1 of the Coomassie Blue-stained gels.

FIG. 10. GAP activities of the rna1pΔ361/QQDQ and GST-
RNA1pCT mutant proteins. A, 1 μM Ran complexed with γ-32P-GTP
was incubated at 30 °C with buffer alone (○), or with 0.1 nM rna1p (●),
with 0.1 nM rna1pΔ361 (□), or with 0.1 nM rna1pΔ361/QQDQ (△). B,
mixtures consisted of 1 μM γ-32P-GTP-loaded Ran and buffer alone (○),
0.1 nM GST (●), 0.1 nM GST-rna1pCT (□), or 50 nM GST-
RNA1pCT (△). The specific activities were determined as the decrease
of radioactivity bound to GTPase activity of the radioactive protein complexed to Ran after the addition of buffer, rna1p,
RNA1pΔ361, RNA1pΔ361/QQDQ, GST, or GST-RNA1pCT, respectively. All curves characterizing reactions containing GST or GST fusion proteins are
indistinguishable from the control with buffer alone.

A similar picture was obtained when Ran binding to the mutant proteins was analyzed by EDC-mediated cross-linking.

Heterodimer formation, i.e. the appearance of a 65-kDa protein
species which is recognized by both Ran and rna1p antibodies,
is not affected in the rna1pΔ361/QQDQ mutant when compared with
rna1pΔ361 (Fig. 11A). The GST-rna1pCT derivative, on the other hand, fails to show any binding to Ran (Fig. 11B).

Some self-dimerization/oligomerization of GST-rna1pCT
is observed when the cross-linking products are probed with an
antibody directed against GST. However, formation of these
homodimers/oligomers is most likely mediated through the
GST part of the fusion protein as purified GST alone yields
similar products in the EDC reaction (Fig. 11B). Taken to-
tgether these analyses show that the acidic C-terminal domain
of rna1p alone is not capable of binding to and stimulating the
intrinsic GTPase activity of Ran. Moreover, a specific sequence
encompassing five acidic amino acids within this C-terminal
domain which shares homologies with other Ran binding pro-
teins is dispensable for Ran binding and activation.

DISCUSSION

The role of the small GTPase Ran as an essential component
of the nuclear import machinery has been well established
through the use of permeabilized cell systems capable of trans-
porting karyophilic proteins and through the use of genetic
approaches in yeast (for recent reviews see Refs. 9, 11, and 22).

Importantly, non-hydrolyzable analogues of GTP block nuclear
import of NLS containing cargo in in vitro systems, and Ran
has been shown to be the only or the major GTPase involved in
this process (24, 45). Most likely, Ran undergoes at least one
entire GTPase cycle for nuclear import to be completed, and
directionality in this process is provided by the distinct spatial
localization of the two principal Ran effector proteins regulat-
ing the nucleotide-bound state of the GTPase (23). Although human RanGAP and its yeast homologues
S. pombe Rna1p and S. cerevisiae Rna1p are cytosolic proteins possibly enriched at
the nuclear periphery, the exchange factor RCC1 is restricted
to the nucleus and might also bind to a component(s) of the
nuclear pore (17, 29, 46–48). Thus, the ratio of GDP-
bound versus GTP-bound Ran is high in the cytosol and low in
the nucleoplasm, and it is conceivable that this difference
determines vectoriality. In line with this interpretation, Ran-GDP
appears to be the import competent form of the GTPase in the
cytosol where it is required for initial stages of nuclear
import protein, whereas Ran-GTP can displace importin a and
the NLS-containing transport substrate from importin β
thereby terminating transport at the nucleoplasmic side of the
nuclear pore (23, 24, 49). Activation of the intrinsic GTPase
activity of Ran in the cytoplasm is therefore essential to lower
the concentration of Ran-GTP in the cytoplasm which would
otherwise disrupt the NLS-substrate-importin a/β complex
already at the cytoplasmic side of the nuclear pore, thus termi-

nating the transport prematurely.

This model underscores the importance of the RanGAP ac-

tivity being localized in the cytoplasm. The mutagenesis ap-

proach presented here describes for the first time a structural
basis for this RanGAP activity. All RanGAP molecules identified
so far share two distinct structural features, an 8-fold
repetition of the LRR motif which is followed by an extremely
The structure of the former most likely resembles that of a similar LRR domain in the RNase inhibitor which was shown by x-ray crystallography to have a horseshoe-like shape with the individual LRRs forming β-sheets structural units (50). These units are arranged in a way that they form solvent-exposed parallel β-sheets, and crystal structure analysis of the RNase inhibitor in a complex with RNase A revealed that the solvent-exposed β-sheets and the flexibility of the entire LRR domain structure are used in the interaction with RNase A (51). Since LRRs of varying lengths and numbers are present in a variety of functionally diverse proteins all shown or expected to engage in protein-protein interactions, the solvent-exposed parallel β-sheets have been proposed to represent versatile protein binding motifs (52). In the case of the LRR proteins Rna1p and rna1p, the S. cerevisiae and S. pombe RanGAPs, it was therefore proposed that it is the LRR domain that mediates the interaction with a cellular protein target (29). In contrast to this speculation we show here that the functional interaction of rna1p/Rna1p with Ran, the only target protein identified so far, requires a major portion of the C-terminal acidic domain. This stretch of acidic amino acids is essential for establishing both the physical interaction between rna1p and Ran and the full RanGAP activity of rna1p with the latter most likely being a consequence of the former. The functional importance of the highly acidic sequence is also underscored by in vivo data using gene replacement in yeast. Deletion of the sequence encoding amino acid residues 359–397 in the S. cerevisiae RNA1 gene, i.e. the region also removed in our inactive Rna1359 mutant, caused temperature-sensitive growth defects in the respective mutant cells (28). Our results now provide a satisfactory mechanistic explanation for these in vivo findings.

Although the LRR domain alone is incapable of binding Ran, we cannot exclude its participation in rna1p/Ran complex formation. Conceptually, the acidic stretch could induce a certain conformation in the LRRs which is in turn required in Ran binding. However, a direct interaction between the acidic domain of rna1p and Ran could also be envisaged, in particular since acidic amino acids appear to be a crucial although not sufficient part of the Ran binding motif found in several nucleic acid-containing proteins (for review see Ref. 44). Nevertheless even if the acidic C-terminal domain binds directly to Ran, it requires the presence of the LRRs for this activity since it is not capable of Ran binding when provided in a fusion protein with GST (Figs. 10 and 11). A close spatial relation and possibly a conformational cross-talk between the LRRs and the acidic domain is also supported by the finding that the acidic region encompassing residues 341–361 although being required for Ran-GTP binding is not involved in forming the covalent DST cross-link to Ran-GTP (note that no basic amino acid residue required for DST modification is found in this sequence, Fig. 1).

Our data show that the GTP-bound conformation of Ran is required for establishing a physical Ran-rna1p interaction. Although the crystal structure of Ran has been determined, this is only the case for the GDP-bound form (53). We can therefore only speculate which region in Ran-GTP interacts with rna1p, in particular since major conformational changes are likely to occur in Ran upon GTP binding (53). One possible candidate region is a cluster of basic amino acids between Lys127 and Lys134 which potentially could engage in electrostatic interactions with the acidic stretch found in the Ran binding motif and in rna1p.

Although the cross-linking approach revealed the existence of heterodimeric rna1p-Ran-GTP complexes, it remains to be elucidated whether this is the functionally active entity showing accelerated Ran-mediated GTP hydrolysis. In this respect it is interesting to note that DST cross-linking of Ran-GTP with wild-type and GAP active rna1p mutants in addition to the heterodimer also yields species with apparent molecular masses of 109, 106, and 103 kDa (Fig. 7). Based on these masses these species could correspond to heterotrimeric complexes consisting of one Ran (24 kDa) and two rna1p, rna1pΔ375, or rna1pΔ361 molecules (each 43, 42, or 40 kDa), respectively. The molecular nature of such putative heterotrimers is completely unknown. Ran could possibly harbor two rna1p binding sites although there is no experimental evidence supporting this view. On the other hand a trimeric species could represent an rna1p dimer with only one subunit linked to a Ran molecule. However, our previous sedimentation analysis (29) and chemical cross-linking experiments with rna1p alone (not shown) reveal no evidence for the existence of an rna1p dimer, at least in the absence of Ran. All rna1p-Ran-GTP complexes have only been identified following covalent stabilization through chemical cross-linking. Molecular sieving chromatography, on the other hand, failed to give an indication of complex formation (Fig. 6) suggesting that the interaction between Ran-GTP and rna1p is rather transient and/or unstable. This interpretation is supported by the fact that two-hybrid screens using Ran as a bait and probing of expression libraries with labeled Ran-GTP so far failed to identify RanGAP as a ligand (54, 55).

Most likely, this transient/unstable interaction is not stabilized by other cytosolic factors since gel filtration chromatography of total S. pombe cytosol reveals a single rna1p species migrating at a position indistinguishable from that of purified rna1p. This migration position corresponds to that of a globular protein of 90 kDa. However, our sedimentation equilibrium analyses (29) and the gel filtration chromatography of C-terminally truncated molecules (Fig. 6) show that rna1p is most likely a monomeric molecule whose atypical behavior in gel filtration stems from the highly acidic C-terminal domain. This could also explain the gel filtration properties of human RanGAP, which shows a similar structural organization as the yeast rna1 proteins, i.e. an LRR and a highly acidic domain, but which is extended C-terminally by an unrelated sequence of some 180 residues when compared with the yeast proteins (20). The human RanGAP polypeptide of 587 amino acid residues migrates upon gel filtration analysis at a position corresponding to that of a globular protein of 150 kDa (19). While this has been interpreted as an indication of RanGAP dimerization, it could also stem from an atypical gel filtration behavior mediated through the highly charged acidic domain that has been documented here for rna1p. Alternatively, the 189 amino acids that follow the acidic domain in human RanGAP but are absent in the yeast proteins could be required for dimer formation.

An argument in favor of the former interpretation is the existence of a second RanGAP species in HeLa cell extracts which shows highly similar properties when compared with the 65-kDa RanGAP but correlates with a 50-kDa band in SDS gels and with a migration peak in gel filtration corresponding to 100 kDa for a globular protein (19). The latter is reminiscent of rna1p and could therefore indicate that the second human RanGAP species is the result of limited proteolysis which has cleaved off the unique C-terminal 140 amino acids leaving the LRR and the acidic domain intact. On the basis of this assumption it appears that an atypical gel filtration behavior of the human RanGAP is also carried through the highly acidic domain and does not depend on the C-terminal extension and a potential dimerization mediated through this sequence. On the other hand, it also remains possible that the gel filtration properties of human RanGAP are altered by post-translational modifications, e.g.
the attachment of a ubiquitin-like moiety described recently (56, 57). Detailed structural analyses, e.g. crystal structure determination of rna1p and RanGAP, have to settle this point.

The finding that the highly acidic domain specifies the interaction of rna1p with Ran-GTP and thus its GAP activity leaves the LRR domain potentially accessible for additional protein-protein interactions. Such interaction could direct rna1p to the cytosolic side of the nuclear envelope, possibly to nuclear pore complexes where RanGAP activity is required to suppress premature nuclear import termination (see above), e.g. after binding of Ran-GTP to the nuclear pore protein RanBP2 (55, 58). Moreover, a nuclear pore localized RanGAP would be prereq-