Interaction of the Nuclear GTP-Binding Protein Ran with Its Regulatory Proteins RCC1 and RanGAP1

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ABSTRACT: The guanine nucleotide dissociation and GTPase reactions of Ran, a Ras-related nuclear protein, have been investigated using different fluorescence techniques to determine how these reactions are stimulated by the guanine nucleotide exchange factor RCC1 and the other regulatory protein, RanGAP1 (GTPase-activating protein). The intrinsic GTPase of Ran is one-tenth of the rate of $p21^{ras}$ and is even lower in the Ran(Q69L) mutant. Under saturating conditions the rate constant for the RanGAP1 stimulated GTPase reaction is 2.1 s^{-1} at 25 °C, which is a 10^5-fold stimulation, whereas RanGAP1 has no effect on Ran(Q69L). The intrinsic guanine nucleotide dissociation rates of Ran are also very low and are likewise increased 10^5-fold by the exchange factor RCC1. Methods to describe the reaction kinetically are presented. The Ran(T24N) mutant, which is analogous to the S17N mutant of $p21^{ras}$, has decreased relative affinities for both GDP/GTP and favors GDP binding. However, it was found to interact almost normally with RCC1. The combination of these properties leads to stabilization of the Ran(T24N)−RCC1 complex and may result *in vivo* in depletion of RCC1 available for stimulating guanine nucleotide exchange.

GTP binding proteins cycle between two conformational states, the GTP-bound on-state and the GDP-bound off-state [for reviews, see Bourne et al. (1990, 1991)]. The interconversion between these two states depends on the rates of guanine nucleotide dissociation with the rate constant k_{diss} and on the GTPase reaction determined by k_{cat}. Both of these reactions are intrinsically very slow, with half-lives ranging from several minutes to hours. In the cell they are catalyzed by regulatory proteins (Boguski & McCormick, 1993). The dissociation reaction is increased by guanine nucleotide exchange factors (GEFs)[1] whereas the GTPase is stimulated by GTPase-activating proteins (GAPs). In the case of $p21^{ras}$, which is a paradigm for the class of ras-related small GTP-binding proteins, the intrinsic GTPase reaction is stimulated 10^5-fold by p120-GAP and 10^4-10^5-fold by neurofibromin, another form of Ras-GAP (Trahey & McCormick, 1987; Gideon et al., 1992; Wiesmüller & Wittinghofer, 1992; Bollag & McCormick, 1991; Eccleston et al., 1993). The maximum increase of the dissociation rate of $p21^{ras}$ by the mammalian exchange factors has not been determined so far but is at least 1000-fold (Jacquet et al., 1992; Haney & Broach, 1994).

Ran (from Ras-related Nuclear) (Bischoff & Ponstingl, 1991a,b), whose gene was originally identified as TC4 (Drivas et al., 1990), is a ras-related GTP-binding protein localized preferentially in the nucleus. By genetic evidence, it has been found to be involved in the control of the cell cycle in yeast (Matsumoto & Beach, 1991; Sazer & Nurse, 1994). Recently, its role in the transport of proteins into (Moore & Blobel, 1993; Melchior et al., 1993; Dingwall et al., manuscript submitted) and the export of mRNA from the nucleus (Kadowaki et al., 1993) has been firmly established. Its regulatory protein RCC1 (Ohtsubo et al., 1989; Bischoff & Ponstingl, 1991a,b), which was identified as a regulator of entry into mitosis (Nishimoto et al., 1978, 1991; Ohtsubo et al., 1989; Matsumoto & Beach, 1991; Dasso et al., 1992), is the GEF for Ran. A GTPase-activating protein for Ran, RanGAP1, has also been identified and characterized (Bischoff et al., 1994). Here we describe the kinetics of the interaction of Ran with these two types of regulatory proteins by using fluorescent, radiolabeled, and unlabeled guanine nucleotides and the intrinsic tryptophan fluorescence of Ran. We show for the first time for a ras-related protein that both the nucleotide dissociation and the GTPase are stimulated more than 10^5-fold by the respective regulatory proteins.

Ran(Q69L) and Ran(T24N) are mutants which by analogy to the corresponding Ras mutants represent a permanently active (Q69L) and presumably permanently inactive state (T24N) of Ran. Using *Xenopus* egg extracts, Ran(T24N) was found to inhibit DNA replication and the activation of mitosis promoting factor (Kornbluth et al., 1994). Another effect was found using a permanently active double mutant Ran(G19V,Q69L), incapable of GTP hydrolysis (Coutavas et al., 1993), which arrested somatic cells in G1 and G2 (Ren et al., 1994). We studied Ran mutants T24N and Q69L in search of a biochemical basis for these biological effects.

MATERIALS AND METHODS

Cloning Methods. For site-directed mutagenesis of the coding region of Ran, a *NcoI*−*PstI* 912 bp fragment from the Ran expression vector pET-ran (Klebe et al., 1993) was

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[1] Abbreviations: Ran, protein product of the human Ran/TC4 gene; RCC1, the product of the human RCC1 (regulator of chromosome condensation) gene; Ran-GEF, guanine nucleotide exchange factor for Ran; RanGAP1, the GTPase-activating protein of Ran as described by Bischoff et al. (1994); mGDP, 2′,3′-bis-*O*-(*N*-methylanthraniloyl)-guanosine diphosphate; mGTP the corresponding triphosphate analog; mdGDP the 2′-deoxy-mGDP analog.

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subcloned into M13mp9ras (using the *Nco*I site of the ras coding sequence). Mutagenic oligonucleotides were (mutagenic base underlined) 5′-ACA GCC GGC CTG GAG AAA TTC for the Gln69 to Leu mutation, Q69L, and 5′-GAT GGT GGT ACT GGA AAA AAC ACC TTC GTG AAA CGT CAT-3′ for T24N. Mutagenesis was done using the method of Taylor et al. (1985) with the kit from Amersham Buchler. After sequencing the complete *Nco*I–*Pst*I-fragment it was recloned into the pET-ran expression vector.

Protein Methods and Buffers. Recombinant Ran was purified from *Escherichia coli* BL21(DE3) containing an expression plasmid based on the T7 polymerase driven pET3-d vector (Studier et al., 1990) as described by Klebe et al. (1993). Recombinant RCC1 was isolated from *E. coli* CK600 containing an expression plasmid based on pKK-tacI (deBoer et al., 1983). Both proteins were shock-frozen in liquid nitrogen and stored at −80 °C with no appreciable loss of activity after 6 months. RanGAP1 was purified from HeLa cells as described by Bischoff et al. (1994). Ran was isolated as a 1:1 GDP complex. In order to load it with other labeled or unlabeled nucleotides, it was incubated with excess nucleotide (20-fold mGXP, 100-fold GTP) in the presence of 5 mM EDTA for 60 min at room temperature. The excess of unbound nucleotide was removed by gel filtration over an AcA54 gel filtration column (0.5 × 30 cm) in the buffer suitable for the experiment to be carried out. Buffers used throughout this study were either phosphate buffer (30 mM potassium phosphate, pH 7.4, 2 mM β-mercaptoethanol, 5 mM $MgCl_2$) or Tris buffer (64 mM Tris-HCl, pH 7.6, 5 mM dithiotreitol, 5 mM $MgCl_2$). For the Ran GAP assays, 100 mM NaCl in phosphate buffer was used, with 5 mM $MgSO_4$ instead of $MgCl_2$.

GTPase Assays. GTPase activity was measured in Tris buffer with unlabeled GTP using either fluorescence or HPLC analysis. For long-time measurements (intrinsic GTPase) the HPLC method was more reliable than the fluorescence measurement, due to the small signal (10% change of the tryptophan fluorescence) and the long measuring time. The protein was loaded with GTP as described above. Ran·GTP (400 μM) was incubated at 37 °C. To monitor the reaction, 7.5-μL aliquots were removed and heated at 95 °C for 2 min. Six microliters were applied to a C-18 HPLC column, and the guanine nucleotides GDP/GTP were eluted from the column by isocratic elution as described before (Feuerstein et al., 1987; Klebe et al., 1993). Quantitation was done using a standard GDP/GTP solution, giving the ratio of GTP/GDP + GTP, which is c/c_o for the GTPase reaction.

RanGAP assays were performed with radiolabelled [γ-^{32}P]-GTP or by following the increase in tryptophan fluorescence from the GTP- to the GDP-bound form. In the first assay protein was loaded with the nucleotide, and the excess nucleotide was removed on a prepacked PD10 gel filtration column (Pharmacia) using phosphate buffer. The reaction was performed using either 1 μM Ran in the standard assay or varying concentrations for Michaelis–Menten kinetics. The reaction was started with 1.5 nM RanGAP1. Aliquots of the reaction mixture were filtered through nitrocellulose to determine the protein-bound radioactivity by liquid scintillation measurements. For the Michaelis–Menten kinetics the rates of GAP stimulated reaction were determined from the increase of intrinsic tryptophan fluorescence. Initial rates were determined, normalized by the Ran-GAP (enzyme) concentration, and plotted against Ran (substrate) concentration. The data were fitted nonlinearly to the Michaelis–Menten equation $v/E_o = [S]k_{cat}/K_m + [S]$.

Nucleotide Dissociation Measurements. The dissociation rates of GDP were determined using either the intrinsic tryptophan fluorescence or the fluorescence of mant nucleotides. As a control, several measurements were performed with radiolabeled [^{3}H]GDP. Ran was loaded with the respective nucleotide; in the case of GDP it was used as purified, since it is isolated as a 1:1 protein/GDP complex (Klebe et al., 1993). The exchange reaction was started by addition of 1.4 mM nucleotide, unless indicated otherwise.

When radiolabeled nucleotide was used, aliquots of the reaction mixture were filtered through nitrocellulose filters, and protein-bound radioactivity was determined. When fluorescent nucleotides bound to Ran were used, the reaction was either followed by the decrease of the mant fluorescence (30% decrease), which is not influenced by the amount of excess nucleotide, or by the increase of tryptophan fluorescence (40%) which is due to a decrease of fluorescence energy transfer from tryptophan to the mant nucleotide (Klebe et al., 1993). The formation of Ran·mant nucleotide complexes was monitored by the decrease of tryptophan fluorescence.

The observed dissociation rates were found to follow first-order kinetics. Since the rates for the dissociation of GDP are very low with a half-life of 12.8–62 h at 25 °C depending on buffer conditions, we only monitored part of the reaction and determined the amplitude of the fluorescence by adding catalytic amounts of RCC1. The dissociation of GTP is fast enough to allow nonlinear fitting of the complete reaction curve. The interaction with RCC1 was investigated by varying the substrate (Ran·nucleotide) concentration, using high excess of free nucleotide throughout. Initial reaction rates were normalized against the RCC1 concentration and plotted against the Ran-nucleotide concentration. The data were fitted nonlinearly to the Michaelis–Menten equation $v/E_o = [S]k_{cat}/K_m + [S]$.

To investigate the interaction between Ran(T24N) and RCC1, the approach described for EF-Tu by Romero et al. (1985) was used. The catalyzed reaction is comparable to that of wild-type Ran and was found to follow single-exponential decay kinetics for all Ran(T24N) concentrations. The observed first-order rate constants were plotted against Ran concentrations and fitted to the hyperbolic function given in the text.

Gel Filtration Analysis of Ran–RCC1 Interaction. Complex formation between wild-type or mutant Ran was analyzed in running buffer (50 mM potassium phosphate, pH 7.5, 5 mM $MgSO_4$, 1 mM mercaptoethanol, 0.02% sodium azide, and 100 μM GDP). Ran and RCC1 were incubated in running buffer, 200 μL was applied to a 1.6 × 60 cm column of Superdex 200 (Pharmacia), and gel filtration was carried out at 1 mL/min. Aliquots of the peak fractions were separated on a 12% SDS gel and stained with Coomassie blue. Marker proteins and their molecular masses (kDa) were as follows: aldolase, 158; bovine serum albumin, 66; ovalbumin, 45; chymotrypsinogen, 25.

Fluorescence Measurements. Either tryptophan or mant nucleotide fluorescence can be used to monitor dissociation reactions. Fluorescence with mant nucleotides is more convenient to measure because the relative fluorescence

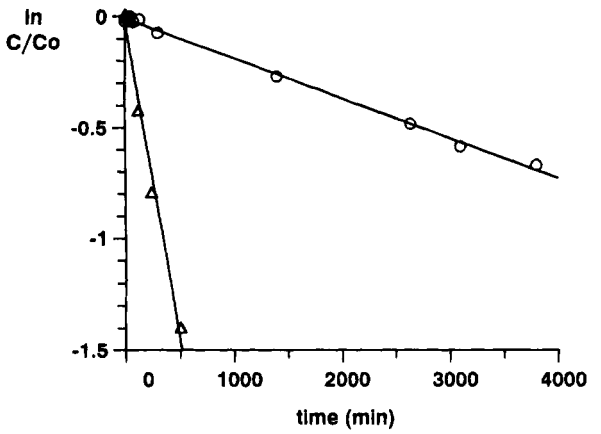


FIGURE 1: Intrinsic GTPase reactions of Ran and the Ran(Q69L) mutant. Ran•GTP was prepared by exchange of GDP against GTP as described under Materials and Methods. Ran•GTP (400 μM) was incubated at 37 °C in Tris-HCl buffer. Aliquots of the reaction mixtures were taken at the times indicated, heated for 2 min at 95 °C, and analyzed for their guanine nucleotide content on a HPLC column as described (Feuerstein et al., 1987). (△) Ran; (○) Ran (Q69L).

change, which is 30% for the mant emission signal itself (see as an example Figure 3B) and 40% for the quenching of tryptophan emission due to fluorescence energy transfer (Figure 3A), is much larger than the change in tryptophan fluorescence, which is 10% when going from the Ran−GDP to the −GTP complex [see Klebe et al. (1993)].

Static and slow time fluorescence measurements were done on an SLM 8000 or a Perkin Elmer LS 50B fluorescence spectrometer. Excitation and emission wavelengths for the tryptophan fluorescence measurements were 295 and 335 nm and 370 and 450 nm for the mant nucleotides, respectively. Fluorescent mant nucleotides, mGDP, mdGDP, mGppNHp, and mGTP (mant = *O*-[*N*-methylanthraniloyl]), were prepared as described by John et al. (1990) or kindly donated by Roger S. Goody.

RESULTS

Intrinsic and Ran-GAP Stimulated GTPase Activity. The intrinsic GTPase activity of wild-type and Q69L mutant Ran protein is shown in Figure 1. The intrinsic GTPase activity of Ran is 5.4×10^{-5} s^{-1} at 37 °C (1.8×10^{-5} s^{-1} at 25 °C), which is one-tenth of the rate of p21^{ras}. As expected, the Q69L mutation decreases the rate 18-fold, similar to the equivalent p21^{ras}(Q61L) which shows very low intrinsic GTPase activity (Der et al., 1986; Feig & Cooper, 1988) and is very weakly stimulated by GAP (Bollag & McCormick, 1991; Brownbridge et al., 1993). Bischoff et al. (1994) have recently purified a protein from HeLa cell lysate which stimulates the GTPase activity of both recombinant and HeLa cell Ran. This RanGAP1 is a homodimeric 65-kDa protein which stimulates Ran but not p21^{ras} GTPase. We measured the effect of this Ran-GAP on the GTPase activity of Ran. Figure 2A shows that 1.5 nM RanGAP1 stimulates the GTPase of 1 μM wild-type Ran (measured at 25 °C) at least 40-fold and has no effect on the reaction rate of Ran(Q69L), as shown before (Bischoff et al., 1994).

Treating the Ran-GAP stimulated GTPase as an enzymatic reaction, as shown for p21^{ras} by Gideon et al. (1992), we can observe the saturation of the enzyme (RanGAP1) with micromolar concentrations of the substrate Ran•GTP as shown in Figure 2B. Using the Michaelis−Menten equation

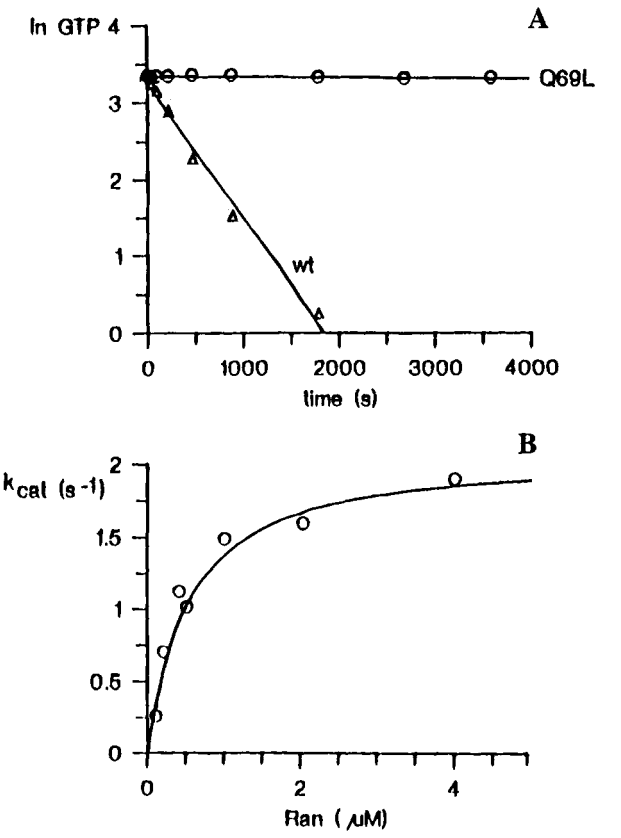


FIGURE 2: Stimulation of Ran GTPase by RanGAP1. (A) The RanGAP1 stimulated reaction was measured by incubating Ran complexed to [γ-^{32}P]GTP (prepared as described under Materials and Methods) in phosphate buffer at 25 °C in the presence of 1.5 nM RanGAP1 prepared from HeLa cells as described (Bischoff et al., 1994). Protein-bound radioactivity was determined by filtration through nitrocellulose, and the data were linearized to show that the kinetics a follow first-order reaction. (B) Michaelis−Menten kinetics of the RanGAP1 stimulated GTPase reaction. Ran-GAP (2 nM) was incubated in phosphate buffer at 25 °C with increasing concentrations of Ran•GTP, prepared as described in Methods. The GTPase reactions were monitored by following the increase of the intrinsic Trp-fluorescence (excitation wavelength 295 nm, emission at 335 nm). Initial rates were determined, divided by the RanGAP1 concentration, and plotted against substrate (Ran•GTP) concentration as shown.

to analyze the results, we obtain a K_m of 0.43 μM and a k_{cat} of 2.1 s^{-1} (at 25 °C). This means that Ran-GAP1 stimulates the reaction more than 10^5-fold. A similar stimulation has been observed for the Ras-GAP stimulated GTPase of p21^{ras} (Gideon et al., 1992; Wiesmüller & Wittinghofer, 1992; Eccleston et al., 1993).

Fluorescence Methods To Measure Dissociation Rates of Ran Nucleotides. We have shown before that Ran binds tightly to fluorescent nucleotides such as mGDP and that the binding of these nucleotides can be monitored either by a change in fluorescence of the mant nucleotide itself or by a change in the tryptophan fluorescence of the protein via fluorescence energy transfer (Klebe et al., 1993). For p21^{ras} it has been shown that the interaction of the fluorescent mant nucleotides is similar to the interaction with normal guanine nucleotides. The three-dimensional structure of mGppNHp bound to wild-type p21^{ras} has been determined, and it was shown that the interactions between nucleotide and protein are the same compared with unmodified GppNHp (Goody et al., 1992; Scheidig et al., unpublished results). For Ran we still needed to show that the mant group on the ribose does not grossly perturb the interaction between protein,

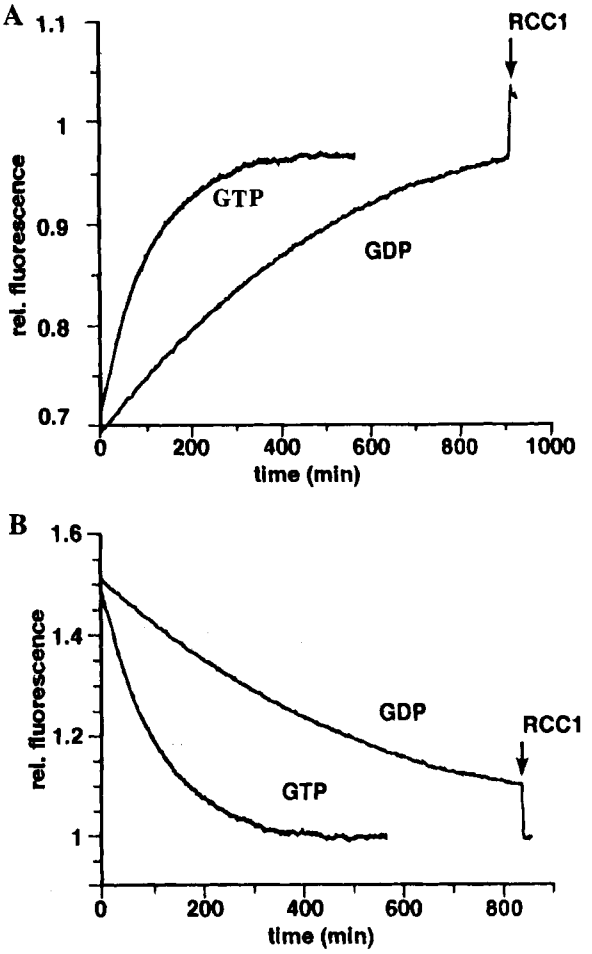


FIGURE 3: Different fluorescence measurements to determine dissociation rates of nucleotides from Ran. (A) Ran·mGDP (2 μM) was incubated in phosphate buffer at 25 °C: A 200-fold excess of GDP was added, and the increase of the intrinsic tryptophan fluorescence due to loss of energy transfer to the mant group on dissociation of the fluorescent nucleotide was followed with time. Excitation wavelength, 292 nm; emission wavelength, 335 nm. Since the reaction is very slow, it was not followed to completion. Rather, to determine the amplitude of the fluorescence change, 5 nM RCC1 was added after 900 min (approximately 75% of the reaction) to complete the reaction. The dissociation rate constant was obtained from a linearized first-order reaction, plotting ln (c/c_0) against time. Dissociation of mdGTP from Ran was measured under the same conditions except that here the complete reaction was monitored and the data were fitted nonlinearly to a first-order reaction. (B) Same experiment as in panel A, but the reactions were monitored by the decrease of the mant nucleotide signal (emission wavelength, 450 nm; excitation wavelength, 370 nm) since free nucleotide has a lower fluorescence yield as bound nucleotide. Notice that the amplitudes for the tryptophan fluorescence are different, for the mant fluorescence similar.

nucleotide, and Ran-GEF. Figure 3A shows the mdGDP dissociation from Ran, as measured by the increase in the tryptophan fluorescence at 335 nm, due to the loss of fluorescence energy transfer from protein-bound mdGDP (Klebe et al., 1993). The initial rate constant of the mdGDP dissociation is found to be 1.2×10^{-5} s^{-1} at 25 °C (Table 1). The release of GDP can be monitored spectroscopically by the reverse reaction, which monitors the release of GDP in the presence of excess mdGDP by the decrease of the tryptophan fluorescence (see Figure 4 as an example). The results, summarized in Table 1, show that mdGDP is released just as slowly as GDP.

Affinities of GTP-binding proteins for their substrates can only indirectly be measured by determining the dissociation and association rate constants (Goody et al., 1991). In the case of Ran we have not been able to measure association rate constants for the binding of nucleotides, due to the instability of nucleotide-free Ran. A concentrated solution of nucleotide-free Ran immediately precipitates on diluting it to a concentration (1−2 μM) suitable for stopped-flow kinetic measurements. Assuming that the association rate constants are always very similar, as has been found for the interaction of mutant and wild-type p21^{ras} with various guanine nucleotides and analogs thereof, we can assume that mdGDP, and probably the other mant nucleotides as well (see below), bind with similar affinity as compared to unmodified nucleotides. For a control we also determined the dissociation rate constant using radiolabeled [^{3}H]GDP. Under the same buffer conditions we find the same constant (1.4×10^{-5} s^{-1}) as observed when measuring tryptophan fluorescence.

We have observed before that Ran is stabilized against denaturation by the presence of phosphate in the buffer. Table 1 shows that in the presence of Tris buffer and in the absence of 30 mM phosphate the dissociation rate is reduced 4.5- and 5.5-fold at 25 and 37 °C, respectively. This means that high concentrations of phosphate increase the dissociation rate as has been found for p21^{ras} where sulfate and phosphate ions increase the dissociation of guanine nucleotides (Hoshino et al., 1988; Mistou et al., 1992a). However, since phosphate but not Tris buffer, for reasons unknown to us, gives a stable fluorescence signal over the time range of the dissociation reactions, all the experiments listed here have been performed with phosphate buffer.

Figure 3 also shows the dissociation of mdGTP from Ran. Since the intrinsic GTPase activity is slow enough, it does not interfere with the dissociation reaction. Table 1 shows that the GTP/mdGTP dissociation rates are again very similar, 7−11-fold faster than the dissociation rates of GDP/mdGDP. In the case of p21^{ras}, the GTP/GDP dissociation rates (ratio 1.8; John et al., 1993) were directly correlated to the different dissociation constants of these nucleotides. Assuming a similar situation for Ran, i.e., that the association rates are always very similar, we may assume that the affinity of GTP is 7−11-fold lower.

Interaction between Wild-Type Ran and RCC1. We investigated the interaction between Ran and the guanine nucleotide exchange factor RCC1. We have shown before that the interaction between Ran and RCC1 can be monitored with recombinant proteins purified from *E. coli* and that in this respect there is no difference between HeLa cell or recombinant proteins (Klebe et al., 1993). Studies on p21^{ras} showed that the interaction with exchange factors is more efficient with prenylated protein (Shou et al., 1992; Orita et al., 1993; Porfiri et al., 1994). Therefore, the Ran−RCC1 interaction is easier to study kinetically than that of other GTP-binding proteins and their GEFs, since Ran is apparently not posttranslationally modified in the cell and the interaction does not take place on the membrane surface. The reaction between Ran and RCC1 was performed as if it were an enzymatic reaction where RCC1 is the enzyme and Ran·nucleotide the substrate, as shown before by Haney and Broach (1994) in a different system, although this is formally not correct since no chemical reaction is performed. However, since an "enzyme−substrate" complex, the ternary complex Ran·nucleotide·RCC1, is an obligatory intermediate of the reaction (Romero et al., 1985; Hwang & Miller, 1985; Eccleston et al., 1988; Mistou et al., 1992b) and since the

Table 1: Dissociation Rate Constants (s^{-1}) of Various Ran−Nucleotide Complexes Determined by Fluorescence Spectroscopy in Phosphate Buffer and Nitrocellulose Filter Binding with Phosphate and Tris Buffer at Different Temperatures As Described in the Text[a]

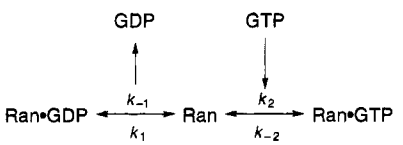
	T (°C)	GDP ($\times 10^{-5}$ s^{-1})	mdGDP ($\times 10^{-5}$ s^{-1})	GTP ($\times 10^{-5}$ s^{-1})	mdGTP ($\times 10^{-5}$ s^{-1})	GDP nitrocellulose filter (phosphate/Tris) ($\times 10^{-5}$ s^{-1})
wt	25	1.5	1.2	11	14	1.4/0.3
	37					4.7/0.85
T24N	25	790	1010			
Q69L	25	2.3	3.1	21	26	
	30	6.3	10	48	63	

[a] GDP dissociation is calculated from incorporation of mant nucleotides, dissociation of mant nucleotides is determined directly. The nitrocellulose filter assay has been used with radiolabeled nucleotides.

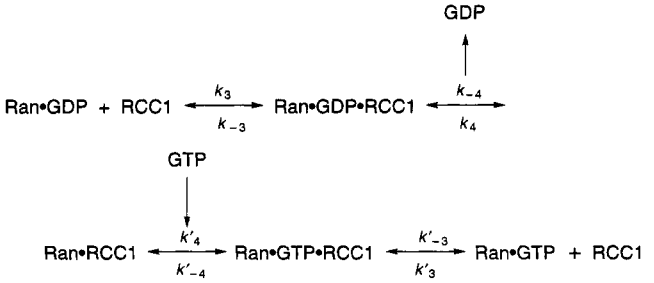
reaction is unidirectional in the presence of a high excess of unlabeled nucleotide, we can use the Michaelis−Menten equation to obtain quantitative information about the reaction.

Using 5 nM RCC1 and increasing concentrations of Ran-GDP in the presence of a large excess of mdGDP, we measured the decrease of tryptophan fluorescence during the reaction (at 335 nm) (Figure 4A). We obtained a series of fluorescence tracings which show that the exchange factor catalyzed reaction can easily be saturated at approximately 10 μM substrate, which correlates well with the physiological concentration. In Figure 4B we plot the initial rates, normalized to the RCC1 concentration, against the "substrate" Ran-GDP concentrations. Using the Michaelis−Menten equation to fit the data, we obtained half-maximal saturation, which we call K_m, at 1.1 μM Ran•GDP and a maximal rate of dissociation, called k_{cat}, of 5.0 s^{-1}. This means that the dissociation rate of GDP from the GTP-binding protein in phosphate buffer at 25 °C is stimulated 3 $\times$ 10^5-fold by Ran-GEF.

The kinetics of exchange factor catalyzed nucleotide dissociation from GTP-binding proteins has been investigated in detail for the interaction between EF-Tu and EF-Ts (Chau et al., 1981; Hwang & Miller, 1985; Romero et al., 1985; Eccleston et al., 1988). It has been described to proceed via a mechanism whereby the exchange factor forms a ternary complex EF-Tu•EF-Ts•nucleotide, from which the nucleotide is released rapidly under formation of a binary EF-Ts•EF-Tu complex (see below). This complex can form another ternary complex with excess nucleotide which releases EF-Ts. For small GTP-binding proteins such as $p21^{ras}$ (Powers et al., 1991; Mistou et al., 1992b; Haney & Broach, 1994) and Ran, one could define a similar kinetic scheme with the uncatalyzed dissociation reaction as



and the RCC1 catalyzed reaction as



From the equation it can be seen that product formation occurs only in the presence of free nucleotide. When there is a large excess of free nucleotide, the reaction is essentially irreversible toward the direction of products. According to Romero et al. (1985), the measured reaction rate of the formation of Ran-GTP is given by the sum of the two rate-limiting steps of the two independent exchange reactions

$$\frac{d[\text{Ran•GTP}]}{dt} = k_{-1}[\text{Ran•GDP}] + k_{diss}[\text{Ran•GDP•RCC1}]$$

Under saturating conditions, i.e., using a large excess of Ran-GDP over the exchange factor, the rate-limiting step of the reaction is given by either k_{-4} or k'_{-3}, either the dissociation of GDP from the ternary complex or the dissociation of RCC1 from the ternary complex upon product formation. If we assume that the formation of the enzyme−substrate complex is fast, i.e., $k_{-3} > k_{-4}$, and only one of the two ternary complexes accumulates at a relevant concentration, the observed first order reaction is given by

$$k_{obs} = k_{-1} + \frac{K_3 k_{diss}[\text{RCC1}]}{1 + K_3[\text{Ran•GDP}]}$$

where K_3 is the equilibrium binding constant of the ternary complex Ran−GDP−RCC1. If we use the equation to fit the data shown in Figure 4, we get $1/K_3 = 0.9$ μM and $k_{diss} = 5.2$ s^{-1}. This is similar to the value obtained by using the Michaelis−Menten equation and shows that the formation of the ternary complex is indeed very fast ($1/K_3 = K_m$) and that the rate-limiting reaction is either the release of GDP or RCC1 from the respective ternary complex in the reaction pathway shown above.

It has been reported that Ras-GEF preferentially increases the dissociation of GDP over GTP, thereby driving the exchange reaction into the direction of the active GTP-bound complex (Jacquet et al., 1992). It has also been reported that GTP dissociates the binary complex between $p21^{ras}$ and Ras-GEF, the intermediate in the exchange reaction, more easily than GDP (Mosteller et al., 1994). These findings seem to suggest that the activation of GTP-binding proteins is driven by the selectivity of the GEF in addition to the higher relative cellular concentration of GTP over GDP. To investigate whether RCC1 reacts differently with Ran in the GDP or GTP-bound form, we tested the Michaelis−Menten kinetics of the nucleotide exchange reaction using all possible combinations of bound and free guanine nucleotides. We used Ran which was preloaded with fluorescent mGDP or mGTP and added a large excess of unlabeled nucleotide to follow the decrease in fluorescence. Table 2 shows that the K_m (1.5 versus 1.1) and k_{cat} (3.5 versus 5.0) for the release

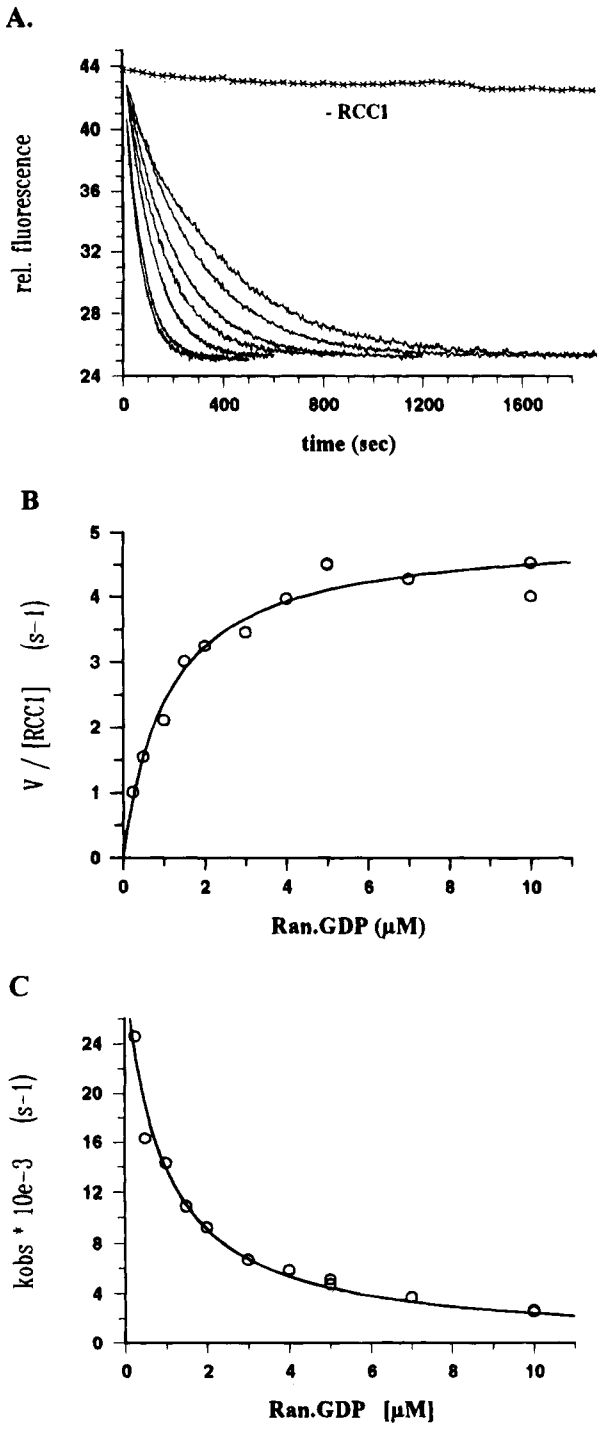


FIGURE 4: Kinetics of the RCC1 catalyzed nucleotide release from Ran. (A) Fluorescence tracings obtained when incubating 5 nM RCC1 with increasing concentration of Ran•GDP (0.5, 1, 2, 3, 5, 7, and 10 μM from right to left) in the presence of 200 μM mdGDP and in the absence of RCC1 as control, as indicated. The reaction rates were followed as the decrease in fluorescence at 335 nm (emission; excitation, 295 nm) due to energy transfer. (B) Initial rates were calculated from the slopes using the fluorescence amplitude as a signal proportional to the Ran concentration, normalized to the RCC1 concentration to give k_{diss}. These were plotted against the substrate concentration and fitted nonlinearly to the Michaelis–Menten equation to give a K_m of 1.1 μM and a k_{cat} of 5.0 s^{-1}. (C) The experimentally observed first-order rate constants (from panel A) were plotted against Ran•GDP and fitted to eq 3 shown in the text to give $1/K_3 = 0.9$ μM and $k_{diss} = 5.2$ s^{-1}.

of mGDP is very similar to the results obtained with unlabeled GDP, again supporting our notion that the fluorescent mant nucleotides provide a valuable tool for measuring kinetics of the interaction of nucleotides with GTP-binding proteins. The results of the different combinations of bound versus free nucleotide, summarized in Table 2, show no significant preference of RCC1 for either the GDP- or GTP-bound form except that the K_m for the binding of Ran-mGDP is slightly higher.

Table 2. Michaelis–Menten Kinetic Parameters of the Exchange of Ran–Nucleotide Complexes with Free Nucleotide in the Presence of RCC1[a]

		K_m (10^{-6} M)	k_{cat} (s^{-1})	k_{cat}/K_m (10^6 M^{-1} s^{-1})
wt	mGDP → GDP	1.5	3.5	2.3
	mGDP → GTP	2.4	5.3	2.2
	mGTP → GDP	0.5	3.0	6.0
	mGTP → GTP	1.0	5.9	5.9
	GDP → mdGDP	1.1	5.0	4.5
T24N	GDP → mdGDP	5.4	4.2	0.78
Q69L	GDP → mdGDP	1.1	3.7	3.3

[a] All constants were determined by the dissociation of mant nucleotides measured at 25 °C by fluorescence emission at 335 nm (excitation 295 nm). Depending on the duration of the experiment the data were fitted either to initial rates or complete reaction curves.

Nucleotide Exchange on Ran(T24N) and Ran (Q69L). The $p21^{ras}$ mutants S17N and S17A, where the Mg^{2+}-liganded Ser-17 has been replaced by Asn or Ala, behave as dominant negative suppressors of normal $p21^{ras}$ (Feig & Cooper, 1988; Szeberenyi et al., 1990; Stacey et al., 1991; Medema et al., 1991). It has been assumed that the mutant protein is locked in the GDP-bound state (Farnsworth & Feig, 1991; John et al., 1993) and binds tightly to the Ras-GEF. This notion has been supported by genetic experiments (Munder & Fürst, 1992), although a direct measurement of the interaction is still missing. The analogous mutant form of Ran, Ran(T24N), has been generated by us and others and was shown to inhibit the activation of Cdc2 kinase in *Xenopus oocyte* extracts (Kornbluth et al., 1994; Clarke et al., 1994).

To find out whether the mutant Ran protein can interact normally with its GEF, we examined the nucleotide dissociation from Ran(T24N) and its stimulation by RCC1. In analogy to the findings with $p21^{ras}$(S17N), the intrinsic GDP dissociation rate of Ran(T24N) is increased 530-fold (Figure 5A and Table 1). We were unable to load the protein with GTP and measure its dissociation rate, which indicates that the binding of GTP to Ran T24N is even weaker, which is again analogous to the S17N or the S17A mutant of $p21^{ras}$, which also have a higher affinity for GDP (Farnsworth & Feig, 1991; John et al., 1993). The dissociation of GDP from Ran(T24N) is stimulated by RCC1. As with wild-type protein, the reaction is linearly dependent on the RCC1 concentration between 0 and 30 nM (not shown). We measured the stimulation of GDP dissociation with a constant amount of RCC1 and increasing concentration of Ran T24N•GDP, as described above for wild-type protein (Figure 5B). The reaction was analyzed according to Romero et al. (1985), and $1/K_3$ (5.4 μM) and the maximally stimulated reaction rate k_{cat} (4.2 s^{-1}) are very similar to those of the wild-type protein, although the amount of stimulation is smaller due to the high intrinsic dissociation rate of Ran(T24N). We also measured the interaction of RCC1 with Ran(Q69L) and found it to behave normally (Figure 5B and Table 2).

Since Ran(T24N) has been suggested to act as a dominant negative regulator of the Ran–RCC1 interaction (Kornbluth et al., 1994), we measured the inhibition of the Ran–RCC1

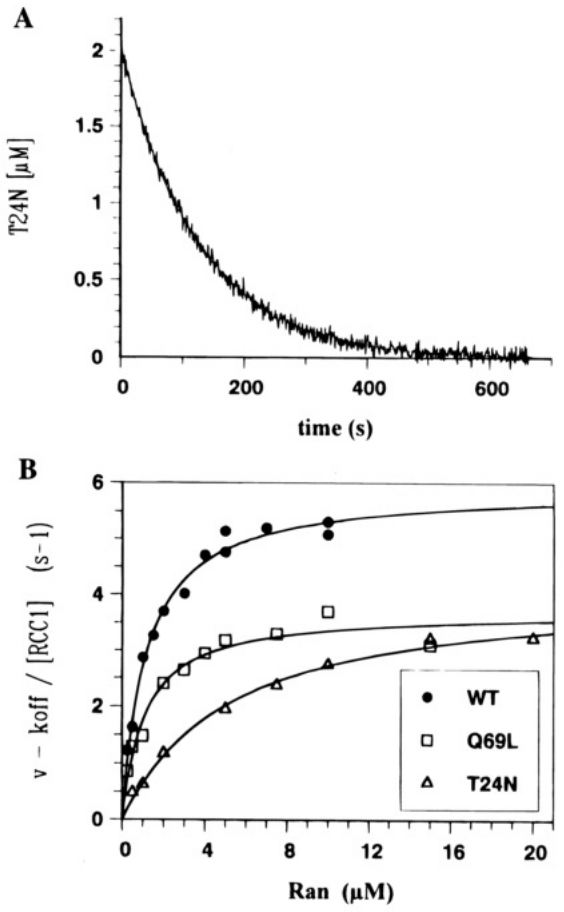


FIGURE 5: Kinetics of the intrinsic and RCC1 catalyzed nucleotide release from mutant forms of Ran. (A) The dissociation of 2 μM Ran(T24N)·GDP in the presence of 400 μM mdGDP was measured following the decrease of tryptophan fluorescence and analyzed as shown in Figure 3. Results are shown in Table 1. (B) Michaelis–Menten kinetics of the interaction of the mutants T24N and Q69L with RCC1, measured and fitted as described for wild-type in the legend to Figure 4B. The GDP dissociation rates of Ran (T24N) are corrected for the intrinsic rates as described by Romero et al. (1985). The results are shown in Table 2.

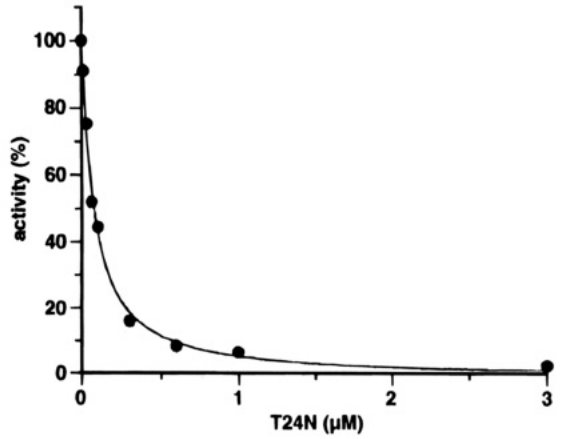


FIGURE 6: Inhibition of the interaction between RCC1 and wild-type Ran. Ran·mdGDP (1 μM) was incubated with 10 nM RCC1 and excess (1 mM) GDP in phosphate buffer at 25 °C, with increasing amounts of Ran(T24N). Release of protein-bound nucleotide was measured as the decrease of fluorescence at 440 nm. The rates were plotted against the Ran(T24N) concentration and fitted to a hyperbola with a K_i of 39 nM.

interaction by Ran(T24N). Figure 6 shows that Ran(T24N) is a very effective inhibitor (K_i of 39 nM) of the RCC1 catalyzed nucleotide exchange on Ran. This finding was surprising, considering the fact that the interaction between

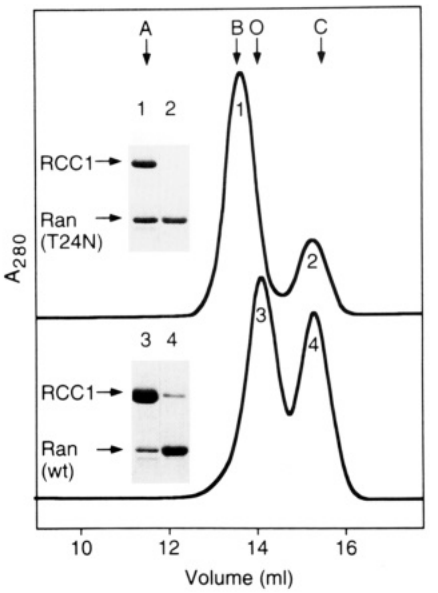


FIGURE 7: Complex formation between RCC1 and Ran(T24N) in the presence of GDP and Mg^{2+}. RCC1 (5 μM) was incubated with 10 μM Ran(T24N) (upper chromatogram) or wild-type Ran (lower) for 10 min at 25 °C in 250 μL of running buffer containing 100 μM GDP and filtrated through a Superdex 200 (Pharmacia Hiload, 1.6 × 60 cm) column in the same buffer. Aliquots of peak fractions were analyzed by SDS gel electrophoresis as shown as insets. Elution positions of marker proteins are A, aldolase, B, bovine serum albumin, O, ovalbumin and C, chymotrypsinogen.

Ran(T24N) and RCC1 as shown in Figure 5B gives a $K_m = 1/K_3$ very similar to that of wild-type. This suggests that the K_m for the mutant Ran does not reflect the affinity of the complex and that the kinetics of this interaction are different from that of wild-type Ran. We are currently trying to analyze this in more detail.

Assuming that the inhibition of the Ran–RCC1 interaction in the presence of Ran(T24N) is due to the reduced affinity of Ran(T24N) to GDP/GTP, whereas the affinity to RCC1 is unchanged, the RCC1·Ran(T24N) complex should be stable and deplete the RCC1 available for stimulating nucleotide exchange. To identify such a complex, we performed gel filtration experiments (Figure 7) in the presence of 100 μM GDP. Indeed, all the RCC1 is complexed with Ran(T24N) (peak 1 in the upper chromatogram and inset) under conditions where no such complex is formed with wild-type Ran (lower chromatogram and inset).

DISCUSSION

Here we have used intrinsic tryptophan fluorescence and fluorescence of mant nucleotides to measure kinetics of the ras-related GTP-binding protein Ran. It has low intrinsic GTPase and GDP dissociation rate constants that are of similar magnitude, 1.5×10^{-5} and 1.8×10^{-5} s^{-1}, respectively. We have shown for the first time for a ras-related GTP-binding protein that both reactions are stimulated 100 000-fold by the respective GAP and GEF, and that the maximal rates are again very similar, 2.1 and 5.0 s^{-1} for the Ran-GAP and Ran-GEF stimulated reaction rates. Whether this is coincidental or of functional significance cannot be determined at the present time. We also do not know whether this is a general phenomenon for ras-related GTP-binding proteins since to date the only other known example is the $p21^{ras}$ GTPase that it is stimulated 100 000-fold by Ras-GAP (Gideon et al., 1992; Wiesmüller & Wittinghofer, 1992; Eccleston et al., 1993). The methods that we have

used here to analyze the kinetic data should be applicable to other GTP-binding proteins, and it will be interesting to determine the nucleotide exchange and GTPase kinetics for other members of the Ras superfamily.

We have shown here that RCC1 (Ran-GEF) has almost no preference for the nucleotide state of Ran, which suggests that it is almost equally effective in releasing GDP or GTP from the protein. If it has a slight preference, it is for the GTP-bound state where a 2-fold higher k_{cat}/K_m has been found using fluorescent nucleotides. Thus there is no indication from these measurements that the RCC1 catalyzed nucleotide reaction favours loading of Ran with GTP. The equilibrium between Ran·GDP and Ran·GTP is given by the difference in ΔG between these two conformations, which in turn is given by the difference in affinity for GDP/GTP, and the concentrations of the respective nucleotides in the cell. The equilibrium should not be shifted by catalytic amounts of RCC1, which as an enzyme only lowers the activation energy for the transition. This means that Ran activation is expected to result from the 30-fold higher concentration of GTP over GDP (Trahey & McCormick, 1987), in spite of being counteracted by the 10-fold lower affinity of Ran for GTP (Table 1). This differs from the situation in the Ras system, where it has been found that the mouse exchange factors $Cdc25^{Mm}$ (Jacquet et al., 1992) and Sos (Liu et al., 1993) or the yeast factor Cdc25p (Haney & Broach, 1994) catalyze the formation of $p21^{ras}$·GTP more efficiently, probably as a reflection of the higher affinity of $p21^{ras}$ for GTP, thus acting synergistically with the effects of the higher GTP concentration in the cell.

Ran(Q69L) is the mutant analogous to $p21^{ras}$(Q61L). It has been shown that Gln61 in $p21^{ras}$ is involved in the mechanism of GTP hydrolysis, although its proposed role as a general base which activates the nucleophilic water molecule (Pai et al., 1990) could not be verified, either experimentally (Chung et al., 1993) or theoretically (Langen et al., 1992; Schweins et al., 1994). The finding that Ran(Q69L) has a drastically reduced GTPase activity which cannot be stimulated by RanGAP1 supports the notion that this residue is of importance for probably all GTP-binding proteins, whatever the mechanism of GTP hydrolysis will turn out to be. Only in the case of the corresponding rab mutants, rab3A(Q81L) and sec4p(Q79L), it has been found that the very low intrinsic GTPase can be stimulated, albeit weakly, by a corresponding GAP activity (Brondky et al., 1993; Walworth et al., 1992). Recently, Ren et al. (1994) have used a double mutant, Ran(G19V,Q69L), which also was insensitive to RanGAP induced GTPase activation (Coutavas et al., 1993), for transfection experiments. Human cells transiently expressing the transfected mutant were arrested predominantly in the G2 phase of the cell cycle, implying that onset of mitosis is inhibited by maintaining Ran in its GTP-bound state.

Ran(T24N) has a high affinity for RCC1, even though its K_m is similar to that of wild-type Ran. Since it can form a stable complex even in the presence of 100 μM GDP and its affinity to GTP is even lower, it seems likely that under physiological concentrations it is able to sequester RCC1 present in the cell. The biochemical properties of Ran(T24N) are remarkably similar to those of $p21^{ras}$(S17N), which has been postulated to sequester the Ras-GEF (Feig & Cooper, 1988; Farnsworth & Feig, 1991). Preliminary experiments similar to those reported here (Figure 6) show that $Cdc25^{Mm}$ catalyzed nucleotide exchange on $p21^{ras}$ is inhibited by $p21^{ras}$(S17N) in the same concentration range as shown for Ran(T24N) (Lenzen et al., unpublished results). It is also remarkable that the analogous mutant is inhibitory for members of the Rab (Li et al., 1994) and Rho (Ridley et al., 1992) family of ras-related proteins, strengthening the notion that the structures of the ras-related proteins should be similar to that of $p21^{ras}$ (Wittinghofer & Pai, 1991).

Since the affinity of Ran(T24N) for GTP appears to be very low, as judged from our inability to form a Ran·GTP complex, one of the effects of the mutation is that Ran(T24N) is kept in the GDP-bound, permanently inactive form of Ran. It has been used to study the role of Ran in regulating the activity of Cdc2 kinase required for entry into mitosis in *Xenopus* egg extracts (Kornbluth et al., 1994; Clarke et al., 1994). Addition of mutant Ran(T24N) to these extracts, which contain a large pool of endogenous wild-type Ran, inhibits activation of Cdc2 kinase and, as a consequence, blocks entry into mitosis. This dominant negative effect of Ran(T24N) mutant in the presence of wild-type Ran is unlikely to result primarily from its inability to bind GTP. More plausibly, the combination of the mutants low affinity for guanine nucleotides together with its presumably unchanged affinity for the nucleotide exchange factor sequesters all the RCC1 into a stable Ran(T24N)·RCC1 complex, thus rendering RCC1 unavailable for activating endogenous wild-type Ran. Together with the data on the GTPase deficient mutant, this would suggest that Ran-GTP formation together with GTP hydrolysis are required for the onset of mitosis.

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