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Tyr39 of Ran Preserves the Ran·GTP Gradient by Inhibiting GTP Hydrolysis

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Ran is a member of the superfamily of small GTPases, which cycle between a GTP-bound "on" and a GDP-bound "off" state. Ran regulates nuclear transport. In order to maintain a gradient of excess Ran·GTP within the nucleoplasm and excess Ran·GDP within the cytoplasm, the hydrolysis of Ran·GTP in the nucleoplasm should be prevented, whereas in the cytoplasm, hydrolysis is catalyzed by Ran·GAP (GTPase-activating protein). In this article, we investigate the GTPase reaction of Ran in complex with its binding protein Ran-binding protein 1 by time-resolved Fourier transform infrared spectroscopy: We show that the slowdown of the intrinsic hydrolysis of RanGTP is accomplished by tyrosine 39, which is probably misplacing the attacking water. We monitored the interaction of Ran with RanGAP, which reveals two reactions steps. By isotopic labeling of Ran and RanGAP, we were able to assign the first step to a small conformational change within the catalytic site. The following bond breakage is the rate-limiting step of hydrolysis. An intermediate of protein-bound phosphate as found for Ras or Rap systems is kinetically unresolved. This demonstrates that despite the structural similarity among the G-domain of the GTPases, different reaction mechanisms are utilized.

Keywords: Ran; GTPase; FTIR; protein dynamics; nucleocytoplasmatic transport

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GTPases are molecular switches, characterized by the change between the inactive GDP and the active GTP-bound form. Ran, a Ras-like guanine nucleotide-binding protein, is a key regulator of nuclear transport.[1,2] Furthermore, Ran functions as a marker of chromosome positioning in spindle formation and nuclear envelope assembly of eukaryotic cells during mitosis.[3] Ran is regulated by the guanine nucleotide-exchange factor RCC1,[4] the GTPase-activating protein (GAP) Rna1p[5] and is stabilized by the Ran-binding protein 1 (RanBP1).[6] Rna1p, which we will refer to in the following as RanGAP, increases the intrinsically slow hydrolysis rate by more than 10^5-fold. RanGAP is cytosolic during the interphase and creates a Ran-GTP gradient across the nuclear membrane with RCC1,[7] which is the driving force for directed nuclear transport (Fig. 1).[8] In contrast to NF1 (a RasGAP), RanGAP has no catalytic arginine finger placed within the binding pocket by the GAP[9,10] but an asparagine interacting with the catalytic glutamine 69 of Ran.[11]

Intrinsic GTPase reaction of Ran·RanBP1

In our investigations, we always use Ran in complex with RanBP1 because Ran alone is not stable under our experimental conditions. We investigated the GTPase reaction of the Ran·RanBP1 protein–protein complex by time-resolved Fourier transform infrared spectroscopy (trFTIR)[12] by means of cagedGTP in the same way as previously published for Ras and Rap:[12–17] The respective GTPase with cagedGTP as the nucleotide is irradiated by a laser flash. Subsequently, the time-resolved changes in the trFTIR due to the GTPase

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Abbreviations used: GAP, GTPase-activating protein; RanBP1, Ran-binding protein 1; trFTIR, time-resolved Fourier transform infrared spectroscopy; pdb, protein data bank.

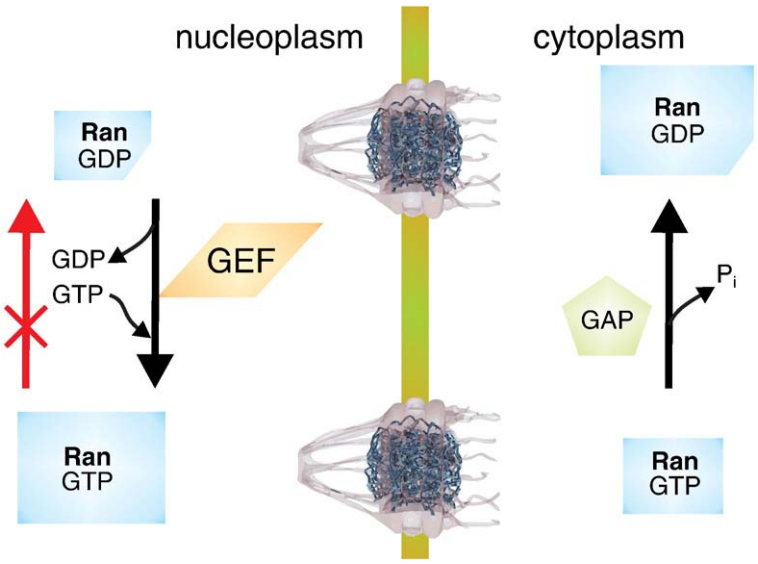
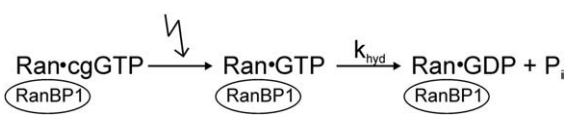


Fig. 1. The Ran gradient across the nuclear membrane with the interactions of GAP and GEF (guanine nucleotide-exchange factor) is shown schematically. In the nucleoplasm, hydrolysis should be prevented in order to efficiently enrich Ran·GTP by guanine nucleotide exchange. In the cytoplasm, the interaction with RanGAP leads to rapid hydrolysis.

reaction can be monitored. With trFTIR measurements of mutants and noninvasive group-specific isotopic labeling of proteins using stable isotopes,[18] site-specific information can be obtained, as demonstrated by, for example, threonine 35 of Ras and the arginine finger of NF1.[15,19]

As for all GTPases we investigated so far, the time course of the intrinsic GTPase reaction of Ran·RanBP1 can be described by a single exponential that characterizes the hydrolysis from Ran·RanBP1·GTP to Ran·RanBP1·GDP (Scheme 1).

By means of isotopic labeling, we assigned the phosphate absorptions of Ran·GTP and Ran·GDP (Supplemental Fig. 1). While the absorptions of GTP bound to Ras and Rap are very similar, the absorptions are shifted for Ran·GTP. A possible reason for this is a different position of the Mg^{2+} ion. In Ras[20] and Rap,[21] Mg is coordinated by β- and γ-phosphate but not by the α-phosphate. A positive countercharge (Mg^{2+}) coordinated to a phosphate will draw electrons from the bonding orbitals into the lone-pair orbitals at the oxygen. Thus, the bond order will decrease. For Ras and Rap, this is observed for the vibration of the β-phosphate but not for that of the α-phosphate. This further leads to a decoupling of the α- and β-phosphate bands. On the other hand, for the Ran·RanBP1 measurement, the positions of the asymmetric stretching vibration of the α- and β-phosphate differ by only 21 cm^{-1}, with strong coupling. This suggests that Mg^{2+} is additionally complexed by the α-phosphate. In correspondence with this, the Ran·RanBD1 structure (Protein Data Bank (pdb) code: 1rrp)[22] shows this complexation.



Scheme 1. The intrinsic GTPase reaction of Ran.

The hydrolysis rate of the slow intrinsic reaction is usually obtained by plotting the difference of the absorptions of the asymmetric α-GTP and α-GDP vibrations.[23] In the case of Ran, the α-GDP band overlaps with the β-GTP band; thus, we obtained better results using the difference of other adjacent bands of the GTP (1131 cm^{-1}) and GDP + P_i (1075 cm^{-1}) state. This method enables us to cancel out baseline drifts, which are common during long measurements. In accordance with the literature we found, compared to Ras or Rap, Ran exhibits a much slower reaction ($k = 2.5 \times 10^{-5}$ s^{-1} at 303 K).

Ras·GTP is in an equilibrium of two conformational states, one "off" state with a conformation similar to Ras·GDP and one "on" state, which is the conformation with high affinity to downstream effectors such as Raf.[17,24] For the Ran·RanBP1 complex, only one conformational state was observed by NMR spectroscopy.[25] In this state, the switch region is fixed and the binding pocket is closed. In accordance with this, we did not observe any conformational change of Ran before the hydrolysis reaction (Supplemental Fig. 2).

Rate-determining step of the GAP-catalyzed reaction

For this trFTIR investigation, we started the reaction using the complex of Ran·RanBP1·RanGAP·cagedGTP. The reaction can be fitted by a sum of two exponential functions as shown in Fig. 2a. Thus, the reaction scheme consists of Ran·RanBP1·RanGAP·GTP , an intermediate, and the final product Ran·RanBP1·RanGAP·GDP. Compared to the GAP-catalyzed reaction of Ras (Fig. 2b), this has one fewer exponential function. For Ras, the three rates are (1) conformational change from $Ras_{off}GTP$ to $Ras_{on}GTP$, (2) bond breakage, and (3) P_i release. In the intermediate formed after breakage of the phosphate ester bond, the phosphate is noncovalently bound within the binding pocket. From

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this intermediate, either GTP can be formed back or the P_i release can follow as the rate-determining step of the reaction.

The GAP-catalyzed reaction of Ran lacks a conformational change after hydrolysis, as described above for the intrinsic reaction. Furthermore, the observed intermediate still has the characteristics of GTP. As shown in Fig. 2a and c, the GTP band disappears at the same rate k_{hyd} as the GDP band appears. Thus, in this system, bond breakage is the rate-determining step and the second kinetically resolved process involves both bond breakage and P_i release. The first process described by k_1 is due to an interaction of Ran with RanGAP, because bands of both proteins are found in this rate as shown by isotopic labeling (see below).

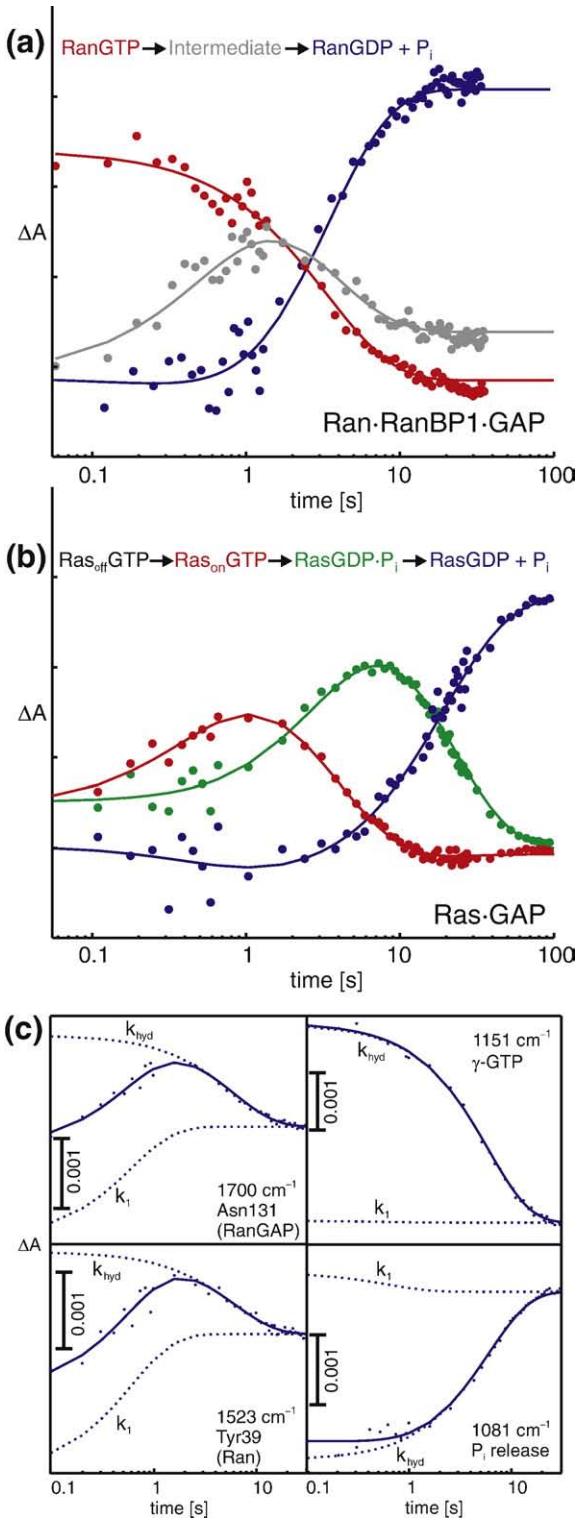


Fig. 2. (a) Time-dependent absorbance changes of marker bands of Ran·GTP (1151 cm^{-1}), an intermediate (1523 cm^{-1}), and free P_i (1081 cm^{-1}) during the GAP-catalyzed GTPase reaction of Ran·RanBP1. The continuous lines correspond to a global fit with two exponential functions. Further experimental details are given in the Supplemental Material. (b) Time-dependent absorbance changes of marker bands of $Ras_{on}GTP$ (1143 cm^{-1}, red), Ras·GDP·$H_2PO_4^-$ (1186 cm^{-1}, green), and released P_i (1078 cm^{-1}, blue) during the GAP-catalyzed GTPase reaction of Ras are shown.[16] The continuous lines correspond to a global fit with three exponential functions. (c) Time-dependent absorbance changes of marker bands during the GAP-catalyzed GTPase reaction of Ran. The amplitudes according to the global fit with two exponential functions (Scheme 2) for increasing and decreasing changes are shown as continuous lines. The dotted lines represent the two exponential components.

Tyr39 of Ran

In contrast to RasGAP and many other GAPs, RanGAP mediates GTP hydrolysis without an arginine finger.[10] An overlay of the Ran·RanGAP with the Ras·RasGAP structure (Fig. 3a) reveals that in the case of Ran·RanGAP, Tyr39 of Ran occupies the position held by the arginine finger in the Ras·RasGAP structure. A similar position is found in many Rab proteins, for example, Rab6A.[27] In order to investigate the role of Tyr39, we mutated it to Arg, Lys, and Ala. The time course of the hydrolysis reaction was investigated, and the rate constant was obtained as described for Ran wild type above. The results are shown in Fig. 3b. For the intrinsic reaction, the introduction of a positive charge adjacent to the phosphate, similar to the situation of GAP catalysis by an arginine finger, seems to accelerate the reaction. However, Ran Y39A has an even faster hydrolysis rate. Thus, we can conclude that tyrosine inhibits the reaction. In contrast, the analogous mutant of Ras (Y32A) has a similar GTPase rate as Ras wt.

We further measured the time course of the GAP-catalyzed reaction of Ran wt and the three Tyr39 mutants (Fig. 3c). In this case, the alanine mutant shows a similar rate as the wild type. Interestingly, the lysine and arginine mutants are now much slower than the wild type.

Time course of marker bands of protein absorptions

In order to assign bands of catalytically active protein side chains, we used group-specifically labeled Ran and RanGAP.[18] The band at 1523 cm^{-1} was assigned to Tyr, most likely Tyr39 of Ran, by site-specific labeling of Tyr by ^{13}C and ^{15}N (Supplemental Fig. 3). The band at 1700 cm^{-1} is

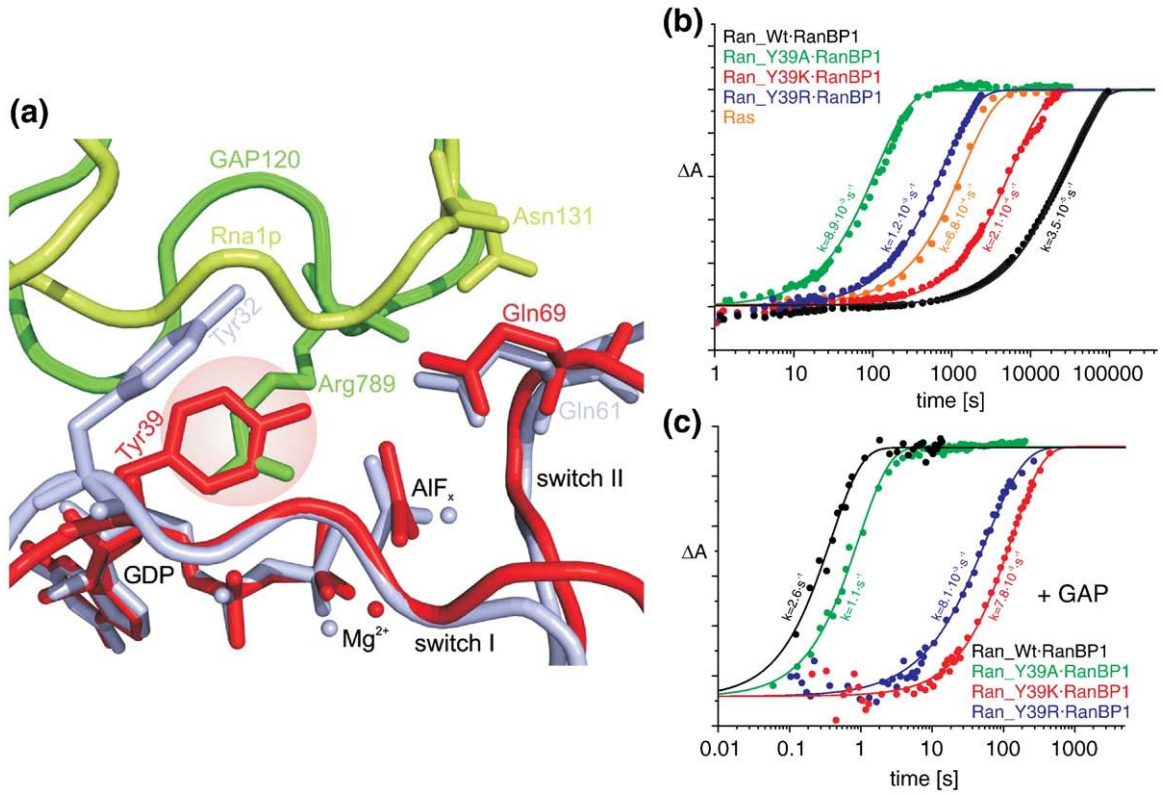
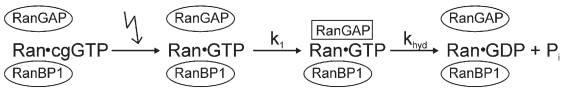


Fig. 3. (a) Comparison between the structure Ran·RanBP1·RanGAP (pdb code 1k5g,[10] brighter color) and the structure Ras·P120GAP (pdb code 1wq1,[26] darker color). The structures were fitted to the nucleotide. The catalytically important Tyr39 of Ran is located in the same position as Arg789 of P120GAP (red area). (b) Intrinsic reaction of different Ran mutants and Ras measured by time-resolved FTIR spectroscopy at 303 K in single-turnover experiments. The normalized absorbance differences of adjacent bands of the GTP and GDP state are shown as a measure for the progress of the hydrolysis. The fits to single exponential functions are shown as continuous lines, which are labeled by the resulting rate constants. (c) GAP-catalyzed reaction of different Ran mutants measured by time-resolved FTIR spectroscopy at 303 K in single-turnover experiments. The normalized absorbance differences of adjacent bands of the GTP and GDP state are shown as a measure for the progress of the hydrolysis. The fits to single exponential functions are shown as continuous lines, which are labeled by the resulting rate constants.

only present with GAP. The position of this band is indicative of asparagine. Furthermore, this band is affected by labeling of asparagine in GAP by ^{13}C and ^{15}N. Thus, we tentatively assigned this band to Asn131 of RanGAP. Even though we cannot check this assignment by a site-specific mutant, because this mutant is too invasive, it is very likely that the band is from Asn131 since this group is known to be crucial for catalysis from biochemical experiments and from the X-ray structure.[10] The kinetic behavior of both bands is similar (Fig. 2). With k_1, both bands increase due to a change in the environment: the catalytically active conformation is formed and both groups are involved in this process. In the hydrolysis rate, both bands vanish because the specific environment is lost after the bond breakage described by k_{hyd} (Scheme 2).



Scheme 2. The GAP-catalyzed GTPase reaction of Ran.

Comparing the amplitudes of k_1 for Ran with the amplitudes obtained for the "off" to "on" conformational change in Ras, where the whole switch region is moving, the changes are much smaller for Ran. This is in line with a local change at the catalytic site, that is, a movement of a water molecule from an anticatalytic to a catalytic position. Only small changes of groups of Ran and RanGAP that are in direct vicinity are seen in the amplitude spectrum.

Discussion

In Ras, catalysis by GAP is due to an increase in the entropy of activation. Our assumption is that this effect is mainly due to the displacement of ordered water molecules by the arginine finger.[16] In Ran, there is only one water molecule within the binding pocket independent of the GAP binding.[10,22] Thus, the enormous catalysis by GAP, which is even more pronounced than that of Ras, must be due to other factors. We propose that the positioning of the water molecule is the crucial factor (Fig. 4). Tyr39 holds Gln69 and the

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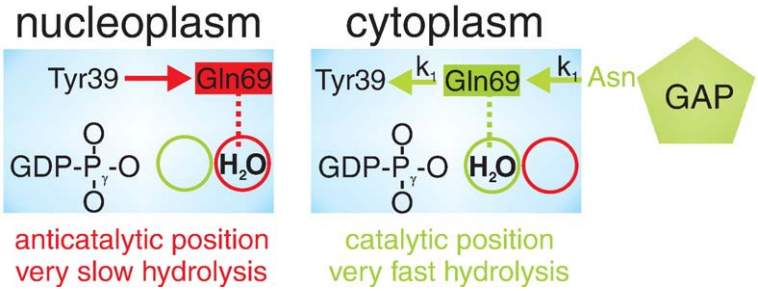

Fig. 4. The molecular interactions inside the binding niche of Ran·GTP are highlighted. In the nucleoplasm, Tyr39 prevents the positioning of the attacking water in the catalytic position. In the cytoplasm, the interaction with RanGAP enables correct positioning and rapid hydrolysis. For the kinetics of Tyr39 (Ran) and Asn131 (RanGAP) see also Fig. 2.

water in an anticatalytic position (red) where the water cannot attack the γ-phosphate. This prevents the formation of Ran·GDP in the nucleoplasm and maintains the Ran·GTP gradient. In the cytoplasm, the interaction with RanGAP is possible. Asn131 of RanGAP will interact with Gln69 of Ran, which subsequently displaces Tyr39 and the attacking water molecule to a catalytically active position (green).

In Ras, the change in the dielectricum due to the displacement of water molecules by the GAP is important for catalysis.[28] However, for Ran, the binding niche is already closed in the intrinsic case.[22] Thus, the position of the attacking water remains the most probable key to catalysis.

Interestingly, the introduction of a positive charge into the binding pocket (Y39K and Y39R) does not accelerate intrinsic hydrolysis as compared to alanine. This further substantiates the finding that for RasGAP, the charge shift due to the arginine is not important for hydrolysis either.[28] We propose that the role of arginine is to steer the catalytic loop into its active position. In the Ran Y39R mutant, the arginine is also steered towards the phosphate groups. However, in this case, it is preventing the actual catalytic machinery of RanGAP from obtaining its correct position. Our results demonstrate that, despite the structural similarity among the GTPases, different mechanisms are utilized for the reaction mechanism. Whereas for many GTPases the deactivation without GAP by intrinsic hydrolysis might be important for regulation, the intrinsic hydrolysis of Ran within the nucleoplasm would merely weaken the Ran·GTP gradient and interfere with nuclear transport. Thus, intrinsic hydrolysis of Ran is inhibited by Tyr39.

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Supplementary Data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jmb.2010.05.068

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