doi:10.1016/j.jmb.2006.11.022 J. Mol. Biol. (2007) 367, 983–995

JMB

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Insight into Catalysis of a Unique GTPase Reaction by a Combined Biochemical and FTIR Approach

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Rap1 and Rap2 are the only small guanine nucleotide-binding proteins of the Ras superfamily that do not use glutamine for GTP hydrolysis. Moreover, Rap1GAP, which stimulates the GTPase reaction of Rap1 10^5-fold, does not have the classical "arginine finger" like RasGAP but presumably, introduces an asparagine residue into the active site. Here, we address the requirements of this unique reaction in detail by combining various biochemical methods, such as fluorescence spectroscopy, stopped-flow and time-resolved Fourier transform infrared spectroscopy (FTIR). The fluorescence spectroscopic assay monitors primarily protein–protein interaction steps, while FTIR resolves simultaneously the elementary steps of functional groups labor-free, but it is less sensitive and needs higher concentrations. Combining both methods allows us to distinguish weather mechanistic defects caused by mutation are due to affinity or due to functionality. We show that several mutations of Asn290 block catalysis. Some of the mutants, however, still form a complex with Rap1•GDP in the presence of BeF_x but not AlF_x, supporting the notion that fluoride complexes are indicators of the ground *versus* transition state. Mutational analysis also shows that Thr61 is not required for catalysis. While replacement of Thr61 of Rap1 by Leu eliminates GTPase activation by Rap1GAP, the T61A and T61Q mutants have only a minor effect on catalysis, but change the relative rates of cleavage and (P_i^-) release. While Rap1GAP(N290A) is completely inactive on wild-type Rap1, it can act on Rap1(T61Q), arguing that Asn290 *in trans* has a role in catalysis similar to that of the intrinsic Gln in Ras and Rho. Finally, since FTIR works at high, and thus mostly saturating, concentrations, it can clearly separate effects on affinity from purely catalytic modifications, showing that Arg388, conserved between RapGAPs and mutated in the homologous RheBGAP Tuberin, affects binding affinity severely but has no effect on the cleavage reaction itself.

Keywords: Signal transduction; phosphoryl transfer; guanine nucleotide-binding protein; GTPase activation; Fourier transform infrared spectroscopy

Introduction

Rap1 is a Ras-like guanine nucleotide-binding protein (GNBP) that regulates integrin-mediated cell adhesion,[1] and might activate extracellular signal-regulated kinases.[2] The two closely related Rap1 homologues in human, Rap1A and Rap1B, share 95% sequence identity and no functional difference between them has been found. Rap1 is also a close homologue of the Ras protein, especially the switch 1 region that interacts with

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Abbreviations used: tr, time-resolved; FTIR, Fourier transform infrared spectroscopy; GNBP, guanine nucleotide-binding protein; GAP, GTPase-activating protein; P_i, organic phosphate.

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effector molecules is conserved completely. It is regulated by a specific set of guanine nucleotide-exchange factors and GTPase-activating proteins (GAPs). While guanine nucleotide-exchange factors replace GDP with GTP, thus activating the GNBP, GAP increases the otherwise slow intrinsic hydrolysis reaction by several orders of magnitude and terminates downstream signaling.

The closest homologue of Rap1GAP1 is Rap1-GAP2, which is expressed in three splice variants.[3] The family includes a PSD-95-associated RapGAP called SPAR, which regulates the morphology of dendritic spines[4] and appears to be the rat homologue of the tumor suppressor E6TP1/SpaL (E6-targeted protein, Spa1-like protein), which interacts with the E6 oncogene from high-risk papilloma viruses.[5] SpaL/SpaR is related to Spa1,[6] a negative regulator of cell adhesion, and to a number of uncharacterized cDNA clones. Moreover, Tuberin, a close homolog of Rap1GAP, is one of the key components of autosomal dominant disorder tuberous sclerosis,[7] where it functions as a GAP for the cognate GNBP RheB.[8] The mechanism of down-regulation by these GAPs is of considerable interest because, to our knowledge, it is different from the canonical Ras-RasGAP model that involves a catalytic glutamine in the GNBP and an arginine finger from the GAP.[9] Rap1 does not possess a catalytic glutamine at position 61, which is essential for the intrinsic and GAP-mediated catalysis in nearly all other GNBPs examined so far, but possesses a threonine residue, which is required for the intrinsic reaction[10,11] but not for the GAP-stimulated GTP hydrolysis reaction.[12] It was demonstrated recently that the intrinsic glutamine in Rab proteins is not required for catalysis but is functionally replaced by an extrinsic glutamine from the TBC domain of RabGAPs.[13] Rap1GAP, the GTPase-activating protein for Rap1, has another unique feature, in that it can down-regulate the G12V mutation of Rap1,[14] in contrast to the corresponding oncogenic mutations in Ras or Rho, which cannot be down-regulated by their respective GAPs. Understanding the mechanism of Rap1GAP-mediated hydrolysis is therefore of prime importance for designing molecules that might be able to stimulate GTP hydrolysis of the Gln61 and Gly12 mutants of Ras.

Recently, the structure of Rap1GAP was solved by X-Ray crystallography.[15] It was shown to consist of a catalytic domain and a dimerization domain. Our initial analysis suggested that the major determinant of catalysis is the presentation of an Asn residue, the "asparagine thumb", into the active site of Rap1. To further explore the catalysis of the Rap1GAP-mediated GTP hydrolysis, we have employed mutants of Rap1 and Rap1GAP, and analyzed the reaction biochemically using fluorescence-based rapid kinetics and time-resolved Fourier transform infrared spectroscopy (FTIR). We demonstrate that these two techniques give complementary information concerning the reaction mechanism.

Results

Replacement of threonine 61 in Rap1

To study the role of Thr61 in the GTPase reaction of Rap1, we analyzed the effect of several Thr61 substitutions (T61A, T61L, and T61Q) on the intrinsic and GAP-stimulated GTP hydrolysis. Glutamine is the residue found in all other Ras-like proteins, and has been shown to be essential for catalysis, most likely for positioning the catalytic water molecule relative to the γ-phosphate group.[16] Gln61 is an oncogenic Ras mutation found frequently in human tumors, and this mutation abolishes both the intrinsic and the GAP-stimulated reaction.[17–20]

The intrinsic GTPase reaction of the wild-type, T61A, T61Q and T61L was followed by HPLC and analyzed using an exponential fit, as described (Figure 1(a); Table 1).[14] Wild-type Rap1 has an

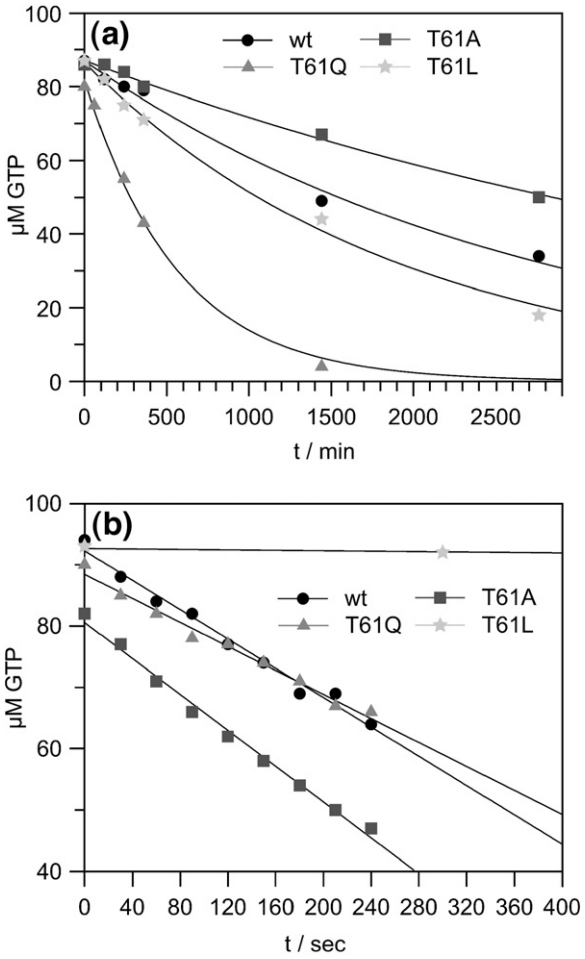

Figure 1. (a) Intrinsic and (b) GAP-catalyzed hydrolysis reaction using 100 µM wild-type and mutant Rap1 in the presence or in the absence of 50 nM Rap1GAP in standard buffer (50 mM Hepes (pH 7.6), 100 mM NaCl, 5 mM dithioerythritol, 5 mM $MgCl_2$). The course of reaction was monitored by HPLC as described.[14] In (b), only two data points are shown for Rap1(T61L), the other data points are outside the indicated time-range.

Table 1. Intrinsic and Rap1GAP-catalyzed reaction of Rap1wild-type and mutants at 293 K

Construct	Intrinsic rate (min^{-1})	Ratio k(mut)/k(wt)	GAP-catalyzed rate (min^{-1})	Ratio k(mut)/k(wt)	Fold activation
Rap1B C′(wt)	0.00035	1	7.2	1	2.0×10^4
T61A	0.00020	0.6	8.8	1.2	4.4×10^4
T61Q	0.0018	5	5.9	0.8	3.3×10^3
T61L	0.00052	1.5	0.11	0.015	28.8

For the Rap1GAP reaction, 50 nM Rap1GAP and 100 μM Rap1•GTP or the corresponding Rap1 mutants were used.

eightfold lower intrinsic reaction rate than Ras, which is increased to the Ras level by the T61Q mutation, similar to what has been described,[10] arguing that threonine is inefficient in positioning the attacking water molecule. The Rap1-T61A mutant hydrolyses GTP only slightly slower than wild-type Rap1, indicating that the hydroxyl group of the threonine is not important for the intrinsic reaction.[12] While the intrinsic rate of Ras is reduced at least tenfold by the Q61L mutation, the GTPase reaction by Rap1-T61L is even slightly faster than that of wild-type, again demonstrating that Thr61 does not have a significant role in the intrinsic catalysis.

The Rap1GAP-stimulated reaction was studied first by multiple turnover analysis with substrate Rap1•GTP in excess over Rap1GAP using HPLC analysis. Wild-type or mutant Rap1•GTP at a concentration of 100 μM was incubated with 50 nM Rap1GAP, and the hydrolysis reaction at 293 K was monitored up to 40% GTP hydrolysis and fit to a linear equation (Figure 1(b); Table 1). In these experiments, the T61A and the T61Q mutant showed a GAP-stimulated reaction similar to that of the wild-type. This indicates that the hydroxyl group of threonine has no essential role in the GAP-stimulated hydrolysis. While glutamine stimulates the intrinsic GTPase, it has, under these conditions, no effect on the GAP-stimulated reaction. The GAP-mediated reaction of the T61L mutant is reduced drastically, possibly because the bulky leucine interferes sterically with Rap1•GAP interaction.

Single turnover measurements with Thr61 mutants

In order to investigate the reaction steps under non-steady state conditions, we used a fluorescent single-turnover, stopped-flow assay as described.[21] Typically, 2 μM wild-type or mutant Rap1 labeled with Aedans on Cys86 was used in the GTP-bound form and mixed with 50 μM Rap1GAP at 283 K, and the time-dependent fluorescence change was monitored (Figure 2(a)). The fluorescence increase in the first part of the transient is due to formation of the complex between Rap1•GTP and Rap1GAP, and the subsequent decrease is due to hydrolysis of GTP and the concomitant dissociation of the proteins.

Strikingly, the fluorescence increase of the T61A, T61L and T61Q mutants is significantly smaller than that of wild-type Rap1, as seen in Figure 2(a). A small increase is observed for the T61A, and an even smaller increase for the T61Q mutant, but no signal is observed for the T61L mutant. These results point to a reduced affinity between the Rap1 mutants and Rap1GAP, which is only 13 μM even for the wild-type.[14,21] A reduced affinity would lead to more pronounced differences in the single-turnover assay than in the multiple-turnover assay (see Table 1) where Rap1GAP is more saturated by excess of substrate. Nevertheless, when we normalize the fluorescence traces to the amplitude of the wild-type reaction (Figure 2(b)), we can define an order of reactivity under those conditions where wild-type Rap1 is faster than Rap1T61A > Rap1T61Q, while Rap1T61L does not show a measurable reaction under these conditions. This lack of saturation prompted us to employ another technique, Fourier transform infrared spectroscopy (FTIR), for an additional analysis of these mutants (see below).

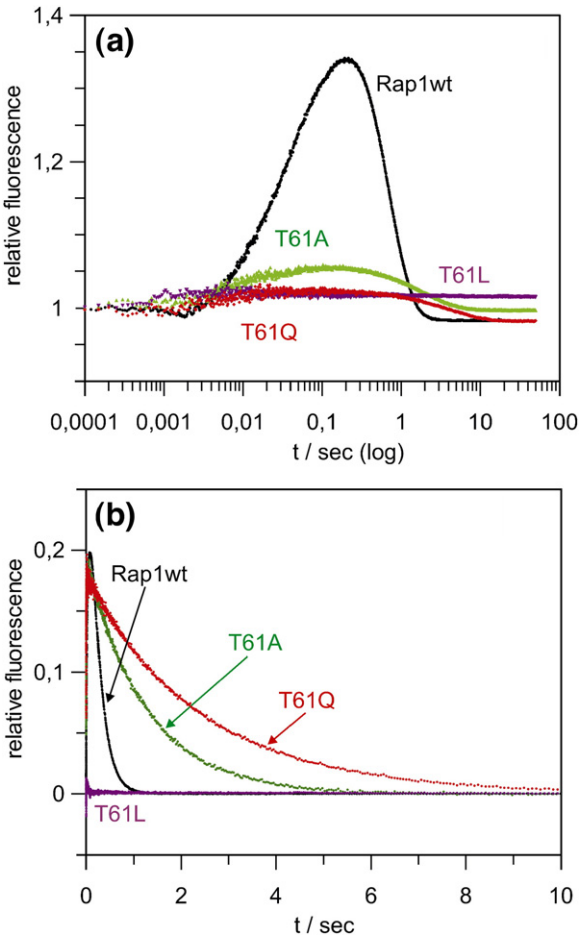

Figure 2. Single turnover analysis of the Rap1-Rap1GAP reaction. (a) Fluorescence transients obtained by reacting 50 μM wild-type Rap1GAP with 2 μM fluorescently labeled wild-type (wt) or mutant Rap1 as indicated by the labels. Relative fluorescence is plotted against the logarithm of time. (b) The same experiment as in (a) but plotted against a linear time axis, with normalization of the fluorescence amplitude.

Metal ion complexes as γ-phosphate analogues

BeF_3^-,[22,23] AlF_x,[24] MgF_3^-,[25–27] and vanadate[28,29] are commonly used mimics for the γ-phosphate group of GTP-binding and ATP-binding proteins. Such studies have been done on myosin,[28,29] F-actin,[30,31] sarcoplasmic ATPases,[32] nitrogenase,[33] Gα subunits of heterotrimeric G-proteins,[22,34] and tubulin.[35] It has been shown that some members of the Ras superfamily bind BeF_x and AlF_x with reasonable affinity only in the presence of the respective GAPs.[19,36] In accordance with previous findings,[15] Rap1GAP forms a complex with Rap1•GDP in the presence of AlF_x (Figure 3(a)). The association process is slow and takes 200–300 s to complete. Magnesium fluoride complexes have been suggested as transition-state analogues for certain phosphoryl transfer reactions.[26,27,37] In our system, MgF_3^- does not induce complex formation under the experimental conditions (Figure 3(a)). Likewise, addition of vanadate does not lead to any increase in fluorescence, indicating that vanadate is not a suitable transition-state mimic for this system, unlike myosin ATPase, which has an asparagine residue in the active site.[28,29,38]

BeF_3^- is a widely accepted ground-state analogue. In the presence of beryllium fluoride, Rap1•GDP and Rap1GAP shows a distinct biphasic association reaction with time constants of 19.6 s^{-1} and 0.12 s^{-1} (Figure 3(a)). The two-phase binding of BeF_x most likely indicates the presence of two species. In a previous study with the transducin Tα subunit, such an effect was attributed to the presence of two species, $BeF_3^-•H_2O$ and $BeF_2(OH)_3^-•H_2O$ forming the complex.[34] In analogy to those studies, the first phase of fluorescence increase represents concurrent binding of both species and the second phase represents slower but higher-affinity binding of the second complex. Once formed, the Rap1•GDP•BeF_x• Rap1GAP complex is stable and can be purified *via* gel-filtration, unlike the corresponding complex with AlF_x (data not shown).

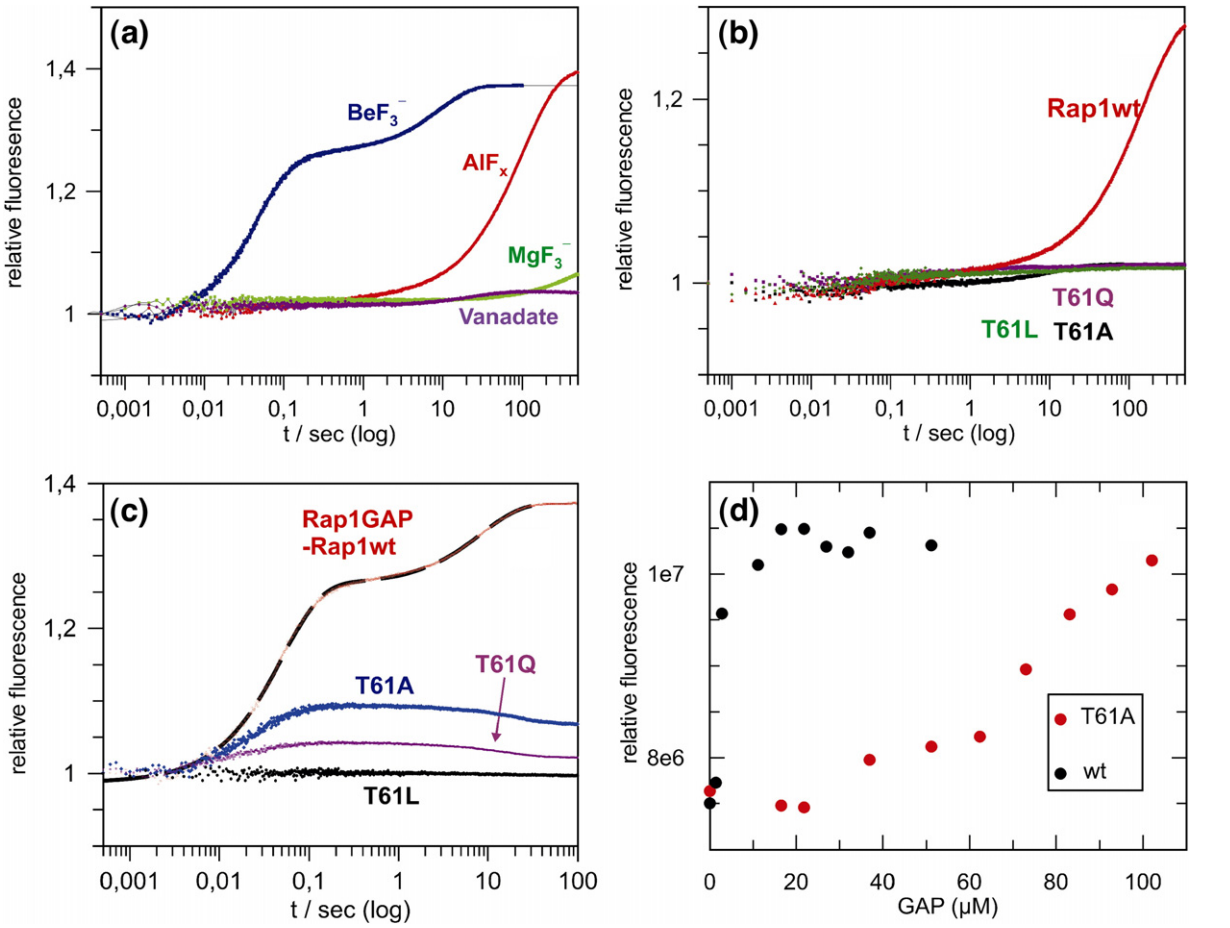

Figure 3. (a) Fluoride complexes in the reaction analysis: 2 μM Rap1•GDP in the presence of 500 μM BeF_3^-, and 500 μM AlF_x, vanadate and MgF_3^- (5 mM $MgCl_2$ and 5 mM NaF) was mixed rapidly with 50 μM Rap1GAP, and the fluorescence emission (λ > 408 nm) was monitored as a function of time. (b) The conditions used in (a) were used for AlF_x association to wild-type and mutants of Rap1 (T61L, T61Q and T61A) in the presence of Rap1GAP. (c) BeF_3^- association to wild-type and mutants of Rap1 in the presence of Rap1GAP, as described in (a). (d) Difference in affinity shown by titration of 0.2 μM wild-type and mutant Rap1(T61A) with increasing concentrations of Rap1GAP, as indicated. Fluorescence emission at 480 nm reaches a similar plateau at saturation for Rap1(T61A), but requires a nearly 100-fold higher concentration of Rap1GAP.

As deduced from structural studies of corresponding complexes, aluminum fluoride binding to GNBPs in the presence of GDP and GAP is believed to mimic the formation of a GTPase transition state. Since mutants of Thr61 do not behave like wild-type in the single-turnover stopped-flow experiments, we wondered how these mutants would behave in metal fluoride-binding studies. AlF_x cannot induce complex formation of Rap1GAP with the T61A, T61Q and T61L mutants. This is in contrast to the multiple-turnover hydrolysis experiments, which show that at least the T61A and T61Q mutants are able to hydrolyze GTP, albeit at reduced rates (Table 1), and suggests that the threonine mutants of Rap1 have a lower affinity towards Rap1GAP which does not allow complex formation under the conditions used, in accordance with the stopped-flow experiments.

While only wild-type Rap1•GDP can associate with Rap1GAP and AlF_x, BeF_3^-, association is apparently less stringently controlled. Both Rap1(T61A)•GDP and Rap1(T61Q)•GDP show a fluorescence increase with Rap1GAP in the presence of beryllium fluoride, although the amplitudes of the fluorescence increases are reduced three- to fivefold as compared to that of the wild-type (Figure 3(c)). Again, Rap1-T61L shows negligible association. Using equilibrium titration with higher concentrations of components, it can be shown that the apparent lower amplitude is indeed caused by reduced affinity. In the presence of BeF_x, wild-type Rap1 has an affinity towards Rap1GAP of ~1 μM, while Rap1(T61A) has an estimated 80–100-fold reduced affinity but shows a very similar amplitude of fluorescence change at saturation (Figure 3(d)).

The catalytic asparagine of Rap1GAP

Previously, we showed that mutation of the catalytic Asn290 of Rap1GAP to Ala eliminates catalysis.[15] To investigate the role of the catalytic asparagine in more detail, it was mutated to Asp, Arg and Lys to see if the carboxyamide side-chain can be substituted by a carboxylate group, or whether a positive charge could, in analogy to the catalytic lysine of nitrogenase[33] and the arginine finger of RasGAP[9] and RhoGAP,[39] function as a stabilizer of the transition state. We also mutated it to glutamine to find out whether the side-chain of Asn can be elongated by one extra carbon atom.

The N290D mutant has a drastically reduced GAP activity (>1000-fold) in multiple-turnover assays (data not shown). In contrast to the N290A mutant, which binds Rap1•GTP but does not stimulate GTP hydrolysis,[15] N290D does not bind Rap1 under these conditions (Figure 4(a)). N290R and N290K are also completely inactive, as measured by HPLC (data not shown) and the fluorescence assay. In contrast to the N290D mutant, they bind to Rap1•GTP, as seen from the amplitude of the stopped-flow experiment (Figure 4(a)). Finally, the N290Q mutant binds and induces GTP hydrolysis, but binding and hydrolysis are reduced, as seen from the smaller amplitude of the fluorescence change and the much slower

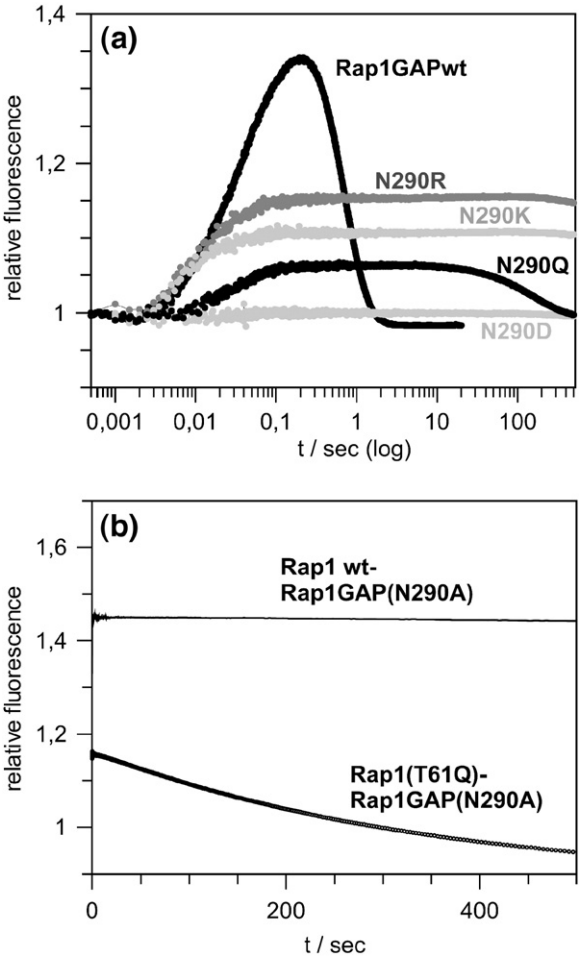

Figure 4. (a) Reaction of wild-type Rap1 and mutants of Rap1GAPs, under the conditions described for Figure 2, with mutants as indicated. (b) Reaction of Rap1GAP (N290A) with wild-type or mutant T61Q Rap1, under the conditions described above, plotted on a linear time-scale.

decrease of fluorescence (Figure 4(a)). Thus, while the carboxyamide side-chain appears to be essential for catalysis, and the longer side-chain is partially tolerated in the Rap1-Rap1GAP system, the geometric requirements seem less stringent than in the Ras-RasGAP system, where replacement of Gln61 is apparently not tolerated.[10]

The Rap1(T61Q) mutant was also tested in the fluorescence reporter assay against the N290R mutant of Rap1GAP to find out if a Ras-RasGAP-like glutamine-arginine combination can rescue both binding and catalysis. Rap1GAP(N290R) can bind to Rap1(T61Q) but it does not stimulate GTP hydrolysis (data not shown).

Earlier, we postulated that the role of the catalytic asparagine in Rap1GAP is similar to that of the catalytic glutamine of Ras in positioning the attacking water molecule.[9,15] We reasoned that the removal of the asparagine might be compensated for by the introduction of a catalytic glutamine in Rap1, and that such a Rap1 mutant should be able to react with the otherwise inactive Rap1GAP(N290A). This "cross activation" reaction was monitored using

the stopped-flow assay under standard conditions. Indeed, association and a clear stimulation of the GTPase reaction are observed, with an initial observed rate of 0.21 min^{-1} (Figure 4(b)). In comparison to the intrinsic reaction of Rap1(T61Q) (0.00052 min^{-1} at 283 K; data not shown), this is an approximately 400-fold stimulation of GTPase activity. We conclude that Gln61 in Rap1 can partially replace the function of the catalytic asparagine residues of Rap1GAP. This also supports our original assumption, that Asn290 is likely involved in positioning the attacking water molecule.[15]

Fluoride complexes for Rap1GAP mutants

We have shown that formation of a complex between Rap1•GDP, Rap1GAP(N290A) and AlF_x is not possible.[15] This was an indication that the asparagine is the crucial residue for catalysis, and demonstrated that aluminum fluoride binding might be a litmus test for judging the importance of residues involved in phosphoryl transfer, at least for GTP-hydrolyzing systems. In line with the observation that activity can be partially restored to the completely inactive Rap1GAP(N290A) mutant by using Rap1(T61Q), the combination of these two proteins is capable of forming an AlF_x complex (Figure 5(a)), although the lower amplitude indicates that complex formation is different from the wild-type situation. That wild-type Rap1GAP does not form a stable complex with Rap1(T61Q) in the presence of AlF_x under standard conditions (Figure 5(a)) is most likely due to the reduced affinity of T61 mutants, as demonstrated above.

Since beryllium fluoride complexes are considered to mimic the ground state rather than the transition state of the GTPase reaction, and since Rap1GAP (N290A) binds Rap1•GTP with an affinity comparable to that of wild-type, it should bind Rap1•GDP in the presence of beryllium fluoride, which is indeed borne out by the experiment (Figure 5(b)), where we observe biphasic kinetics of association. This mutant forms a complex with Rap1(T61Q) also, in line with expectation, although the amplitude of the fluorescence change and the kinetics of binding are different, probably due to the lower affinity. The first phase of the association reaction has a similar rate, while the second is at least threefold slower. The slower rate of the second association step would be expected if the carboxyamide group of Asn290 contacts directly and stabilizes the beryllium species in the ground state (or the γ-phosphate group in the case of GTP). In line with the low affinity of Rap1 (T61L) and the complete absence of a GTPase reaction, this mutant does not show any complex formation with either wild-type or N290A.

Time-resolved FTIR measurements of Rap1 and Rap1GAP mutants

We have used FTIR as the method of choice to analyze GTPase reactions in the Ras-RasGAP and the Rap1•RapGAP systems[40–42] which provided

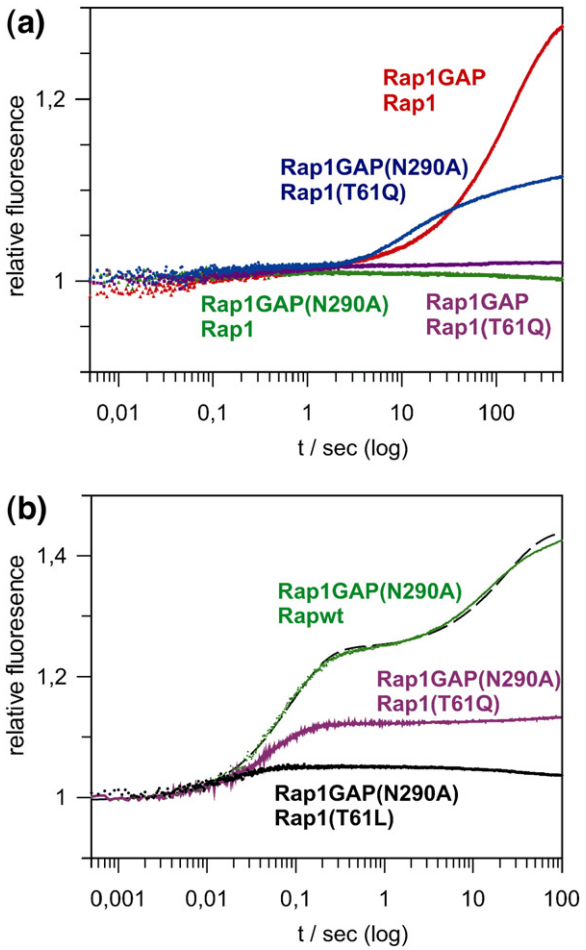

Figure 5. Fluoride complexes in the reaction analysis, with wild-type and mutants of Rap1 and Rap1GAPs as indicated, under conditions similar to those described for Figure 3, using AlF_x (a) or BeF_x (b).

atomic-resolution details of active site interactions on the millisecond time-scale. This method provides direct signals of the participating functional groups and all the phosphate band assignments are available.[40,43] For both RasGAP and Rap1GAP-catalyzed reactions, we have shown the accumulation of an intermediate (rate constant k_2) with protein-bound P_i-like properties whose decay (rate constant k_3) is rate limiting. For Ras1•RasGAP, the intermediate was identified recently as protein-bound $H_2PO_4^-$.[42] For Rap1•Rap1GAP, the band positions and isotopic shifts of the intermediate deviate, and its accumulation and its reverse reaction to produce GTP are more pronounced than those for Ras•RasGAP.[40,43] A further advantage of FTIR is that the reaction is typically performed at 5–6 mM GNBP and GAP and, thus, is likely to be saturated and less sensitive to changes in affinity between the proteins, particularly for the mutants under investigation (see below).

A representative experiment showing time-dependent amplitude changes at different individual IR frequencies for the reaction between Rap1•GTP and Rap1GAP is presented in Figure 6(a), where the

major IR absorption bands (as indicated) have been assigned.[43] At 1271 cm^{-1}, the disappearance of the GTP absorbance and at 1172 cm^{-1} the concomitant appearance of the protein-bound P_i is seen. The reaction of the T61A mutant (not shown; see Table 2) under standard FTIR conditions is similar to the wild-type reaction, in that the appearance and decay of an intermediate at 1172 cm^{-1} can be observed. The major difference is that less intermediate accumulates and consequently the intermediate peak height is much lower. This is reflected by the different half-lives for the second and third rates obtained by global fit analysis, corresponding to the chemical cleavage of the γ-phosphate group of GTP and release of the phosphate intermediate (P_i) to bulk solvent, which are 1.4 s and 3–5 s ($1/k$) for Rap1(T61A), as compared to 1 s and 10–20 s for wild-type Rap1, respectively. Less accumulation of the intermediate peak for Rap1(T61A) can thus be explained by the smaller difference between these two rates, which is 15-fold and threefold for the wild-type and T61A, respectively.

Interestingly, an intermediate is observed for Rap1 (T61Q), as it is for Rap1 wild-type, but with different kinetics. Under the same conditions the release of P_i is two to three times faster for this mutant than for the wild-type, and the intermediate is less resolved. When the temperature was reduced further, the intermediate could be resolved as in the wild-type

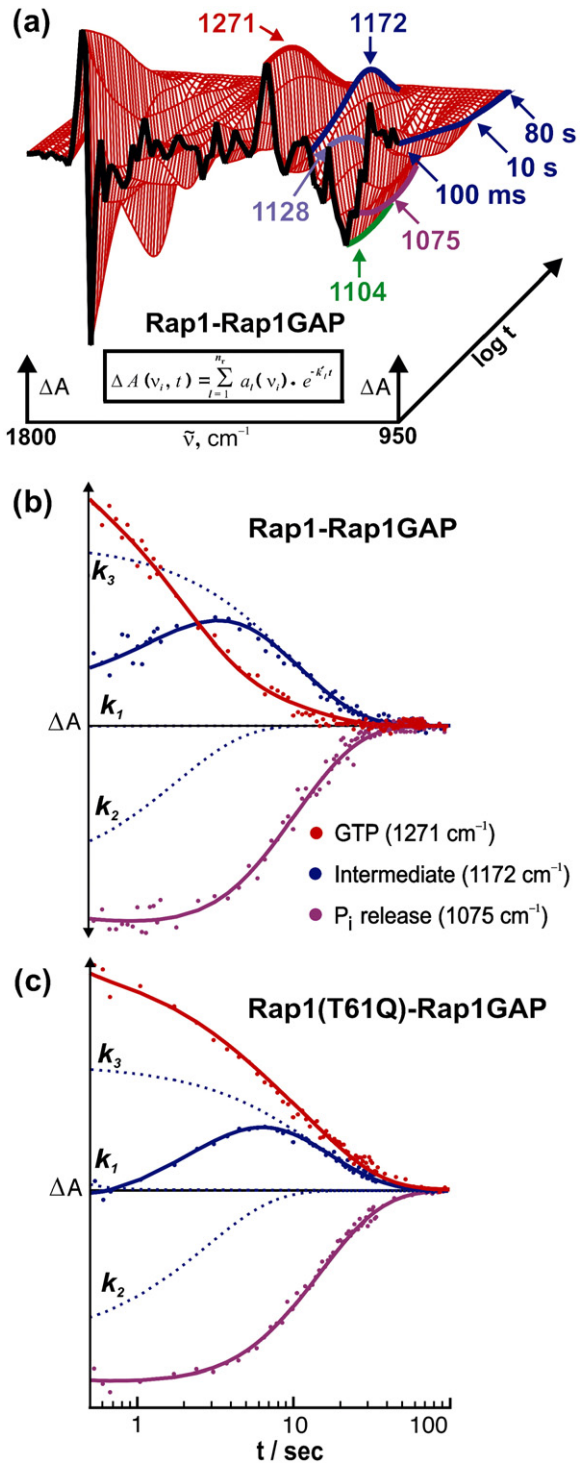

Figure 6. FTIR on the Rap1-Rap1GAP reaction. (a) Absorbance changes in the infrared between 1800 cm^{-1} and 950 cm^{-1} for the Rap1GAP-catalyzed reaction of wild-type Rap at 258 K are shown as a function of wave number and time (logarithmic). In the fitted curves of the global multi-exponential kinetic analysis for wild-type Rap,[43] the disappearance of GTP-α at 1271 cm^{-1} and GTP-β at 1128 cm^{-1}, the accumulation of an intermediate at 1172 cm^{-1}, and the appearance of the products GDP and P_i at 1101/1104 and 1075 cm^{-1}, respectively, are highlighted. (b) Single-frequency kinetics are extracted from the 3D plot of time-dependent amplitude changes at different individual IR frequencies for the wild-type. Shown are the actual data (points) and the fitted curves (continuous curve) for IR bands corresponding to GTP (1271 cm^{-1}), intermediate (1172 cm^{-1}) and phosphate release (1075 cm^{-1}), and the contributions from the apparent rate constants (k_i) of the multi-exponential fit of the intermediate (dotted colored lines). (c) An analogous extraction was done with measurements at lower temperature at 251 K using Rap1(T61Q), showing very similar behavior.

Table 2. FTIR with wild-type and mutant Rap1 and Rap1GAP

Rap1	Rap1GAP	T (K)	$1/k_2$ (s)	$1/k_3$ (s)
Wild-type[a]	Wild-type	258	1.3–1.8	10–20
Wild-type	Wild-type	258	0.4–0.6	15–20
T61Q	Wild-type	258	2–5	
T61A	Wild-type	258	1.2–1.4	3–5
T61L	Wild-type	258	400–500	
T61Q	N290A	273	–	–
		288	600–1200	
Wild-type	N290A	258	–	–
		268	–	–
Wild-type	N290K	268	–	–
Wild-type	N290D	268	1500–1700	
		278	350–850	
Wild-type	N290Q	273	750–900	
Wild-type	R286A	258	1–1.5	60–65
Wild-type	R388P	258	0.7–1.5	10–16

[a] Old measurements using NPE-caged GTP.[43] Here, production of GTP is slow, which affects k_2. Further, due to instability of Rap1GAP at low concentrations of buffer, we had to use the Rap1GAP directly from stock. As a result, the pH of the measurements varies between 6.8 and 7.5, which gives a higher than usual uncertainty of the reaction rate.

reaction (Figure 6(b) and (c)). This shows that the residue at position 61 in Rap1 is not critically important for the catalytic pathway.

In line with the results obtained with the HPLC or fluorescence assays, FTIR did show the cleavage step to be at least 30 times slower for Rap1(T61L) in the presence of Rap1GAP as compared to wild-type Rap1, with no accumulation of an intermediate (summarized in Table 2).

To confirm the results obtained with HPLC and fluorescence, Rap1GAP mutants were tested with FTIR. Rap1GAP(N290A) shows no detectable hydrolysis within 90 min at the standard temperature range of 258–268 K reinforcing the role of Asn290 as a residue crucial for catalysis. While the N290K mutant has no activity between 258 K and 268 K within 90 min, the N290Q and N290D mutants have very weak GAP activity, with rates ($1/k$) of 850 s and 1600 s at 273 K and 268 K, respectively, confirming that the exact positioning and the chemical nature of the carboxamide side-chain of Asn for catalysis cannot be substituted by Gln or Asp.

As expected from the results with multiple-turnover measurements and the fluorescence titration with fluoride complexes, the Rap1GAP(N290A) is partially active on Rap1(T61Q). Raising the temperature to 288 K, the rate ($1/k$) of the reaction is 1000 s and shows no accumulation of an intermediate. This is, however, still 80 times faster than the intrinsic reaction as measured by HPLC. It should be noted that different temperatures have been used for the two mutants compared to wild-type in order to accelerate the reaction and facilitate the data collection despite the mutation of the prime catalytic residue. This helps to avoid baseline instability in the FTIR measurements. We estimate that the temperature difference of 30 K accelerates the reaction by a factor of 10–30, so that the catalysis is less effective than in wild-type by a factor of about 10^3.

Arg388P, analysis of a weak binding mutant by FTIR

Rap1GAP has a two-domain structure[15] where the catalytic domain is highly conserved between the RapGAP family and Tuberin. Structural and mutational analysis indicated that the residues most important for catalysis are located in a conserved helix α7 (Figure 7(a)) that harbors the catalytic asparagine.[15] Interestingly, there are regions away from the catalytic core that appear to be important for catalysis. One such residue is Arg388 in the C-terminal helix α9 of the catalytic domain, which is conserved between RapGAPs and Tuberin. Mutation of this residue to proline is analogous to a tuberous sclerosis mutation in Tuberin/TSC2. (R1743P). This mutation renders Rap1GAP nearly inactive in multiple or single-turnover biochemical assays.[15] Considering that the FTIR method requires high concentrations of components, which in turn assist saturation and gives individual signals for participating functionalities, we asked if Arg388 is important for Rap1 binding or catalysis.

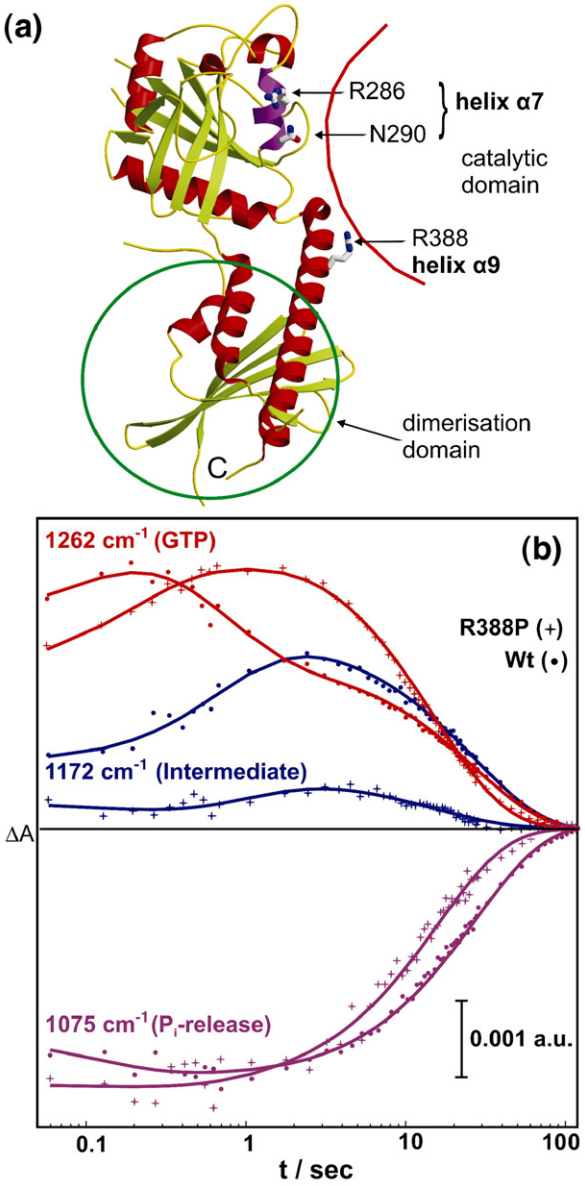

Figure 7. Location of Arg286, Asn290 and Arg388 in the structure of RapGAP. (a) A structural overview of Rap1GAP, highlighting the two sub-domains, the catalytic helix α7 (in purple) with Arg286 and Asn290, and helix α9, which has extensive contacts with the dimerization domain (green circle) bearing Arg388. The putative interface with Rap1 is indicated by the red semicircle. (b) Comparison of single-frequency kinetics of the wild-type and R388P Rap1GAP-catalyzed reactions. Accumulation of the intermediate (blue lines, 1172 cm^{-1}) is reduced significantly for the mutant, when compared to the wild-type. For this mutant, the second α-GTP band at 1262 cm^{-1} (red line) is more intensive than the band at 1271 cm^{-1}.

The FTIR measurements show that hydrolysis with the R388P mutant is almost as fast as with wild-type Rap1GAP, as seen from the kinetics of the GTP band at 1262 cm^{-1} (Figure 7(b), red line) or the (free) P_i band at 1075 cm^{-1} (purple line). The major difference between wild-type and mutant is less accumulation of intermediate as seen by the single-frequency kinetics at 1172 cm^{-1} (blue line). If,

nevertheless, the hydrolysis reaction is fitted by two exponentials, the rates ($1/k$) are 1 s for the cleavage and 12 s for P_i release, which are both similar to what is found for wild-type.[43] This shows that, while helix α9 is part of the binding site for Rap1 (or RheB in the case of tuberin from analogy), the conserved arginine is not required for catalysis, but rather for recognition and affinity towards Rap1 or the cognate GNBP RheB. Loss of this recognition site might be the reason why this mutation in tuberin leads to the formation of tuberous sclerosis.

We have demonstrated that Arg286 on the catalytic helix α7 is not important for catalysis,[14] and single-turnover FTIR analysis confirms the data obtained by HPLC under multiple-turnover conditions (Table 2).

Discussion

There are three aspects where the Rap•Rap1GAP reaction is fundamentally different from the Ras-RasGAP-catalyzed reaction. The GNBP does not have an intrinsic Gln, the GAP does not employ an arginine finger to stabilize the transition state, and the system tolerates mutations of Gly12 in the P-loop. Oncogenic versions of Ras have a mutation of either the catalytically important Gln,[10] or sterically restricted Gly12,[9] where any replacement except by Pro makes Ras an oncogene.[44] For our ongoing effort to develop small molecules that stimulate GTP hydrolysis of oncogenic Ras as potential anti-cancer lead compounds, it is of great importance to understand the mechanism of the Rap1•Rap1GAP reaction in great detail.

Rap1(G12V) behaves in a way similar to that of some of the Rap1 mutants of position 61 (T61A and T61Q). It has a rate of hydrolysis similar to that of T61A in the intrinsic situation and that of T61Q under multiple-turnover condition, as measured by HPLC. Under the standard stopped-flow conditions, Rap1(G12V) is slightly slower than T61Q, and the increase of fluorescence amplitude is comparable to that of T61Q and T61A (data not shown). Considering the analogy between tuberin and RapGAPs, it is thus not surprising that RheB, the target of tuberin, has a bulky arginine residue in the Gly12 position of the P-loop.

We show here that fluoride complexes of aluminum and beryllium can be used to carefully analyze the role of particular residues in phosphoryl transfer. For reasons not understood, vanadate, which has been used successfully in the analysis of many phosphoryl transfer enzymes,[45] does not mimic the transition in the Rap1•Rap1GAP system (Figure 3(a)) or in other GTPase reactions analyzed previously (A.W., unpublished results). BeF_3^- is well suited to mimic the ground state of phosphoryl transfer reactions and indeed it is found here to form complexes between proteins carrying mutations in either GNBP or GAP, although the analysis is partially hampered by the fact that the already low affinity is further reduced by many of the mutations. Complex formation with AlF_x seems to be more selective, since Rap1 mutants such as T61A and T61Q, which are efficiently down-regulated by Rap1GAP, show no sign of complex formation under standard conditions. Considering that the main difference between AlF_x and BeF_3^- is the number of possible coordination bonds, it is an additional hint that the transition state for this reaction is significantly more dissociative. This is as suggested for the Ran•RanGAP system, which retains an intrinsic Gln but also lacks an arginine finger in the active site,[46] in contrast to the Ras-RasGAP system, where the transition state mimic is stabilized by a positively charged arginine finger, which can contribute to catalysis only in the case of an associative mechanism.[9,47] This is in line with previous spectroscopic results,[43] where it has been shown that the main effect of Rap1GAP mediated catalysis is withdrawal of electron density towards the non-bridging β-oxygen atom of GTP, thus weakening the GTP β–γ bond, in the ground state.

The combined analysis by stopped-flow fluorescence and FTIR experiments show how well the two approaches complement each other. Fluorescence measurements of Ras proteins with their cognate GAPs are well suited to study kinetics and equilibria of protein–protein interactions, which may be correlated to elementary steps in the reaction pathway,[48–52] and are reasonably performed at a concentration of 0.1–10 μM. In the case of the Rap1•Rap1GAP reaction, stopped-flow fluorescence studies have suggested the presence of an intermediate in the reaction pathway,[21] which is much more clearly visible in FITR measurements as a post-hydrolysis state with inorganic phosphate in fast equilibrium with activated GTP.[43] Mutagenesis studies now show that mutants of Rap1 or Rap1•GAP can be analyzed as a function of a two-step hydrolysis reaction. The data show that an intermediate does not accumulate in most of the mutants. This, we believe is either (i) due to a less efficient cleavage reaction that reduces the difference in rates between cleavage and P_i release and thus does not allow accumulation of the product or, more likely, (ii) due to a reduced affinity between Rap1 and Rap1GAP, as seen from fluorescence titrations in the presence of BeF_x, which cannot be saturated under the chosen conditions. Thus, the already low affinity between Rap1GAP and the product Rap1•GDP is further reduced by the mutation, 80–100 fold in the case of Rap(T61A), and leads to a faster dissociation after hydrolysis. The most drastic example for the complementarity between the methods is the R388P mutation, which seemed to be totally inactive in the fluorescence assay,[15] while FTIR shows that the cleavage step is unperturbed, although less intermediate accumulates in the reaction.

Together with the results of previous experiments, our data clearly show the crucial importance of Asn290, which is located on the highly conserved helix α7[15] and is itself totally conserved between RapGAPs and tuberin. It cannot be replaced by a loss of function mutation, such as Ala, or by Gln with a longer side-chain, or charged residues such as

Asp and Lys. Asp or Glu residues are frequently found in the active site of phosphoryl transfer enzymes, such as proteins kinases[53] and F1-ATPase[54] and in GNBPs such as MnmE,[55] where they act as general bases for activation of nucleophilic water. Considering the intrinsic Gln of Ras, that positions the nucleophilic water molecule relative to the γ-phosphate group,[16] we postulate an analogous role for Asn (the Asn thumb) supplied *in trans* by Rap1GAP. Similar to the situation of the intrinsic Gln in Ras, no replacement of N290 of Rap1GAP seems to be tolerated in the Rap1•Rap1-GAP reaction. Substitution of the Asn thumb is tolerated only when Thr61 of Rap1 is mutated to Gln, where now the presence of an intrinsic carboxamide group leads to a partial stimulation of hydrolysis even with Rap1GAP(N290A). Surprisingly, an extrinsic Gln with exactly the same orientation and a role similar to that of the intrinsic Gln of Ras and Rho has been found for the Rab-RabGAP system of the TBC family.[13] Since Asn is found in the active site of ATPses such as myosin,[38] it has been postulated to be involved in phosphoryl transfer reactions as well. The true function of Asn290 in Rap1GAP or of Asn1643 in Tuberin will need to be evaluated by crystallographic analysis of a complex between the GNBP and its cognate GAP.

Materials and Methods

Protein purification

Recombinant Rap1B (1-167) and the mutants were expressed in *Escherichia coli* CK600K using the ptac expression system and were purified on a Q-Sepharose column and subsequent gel-filtration as described.[56] For most fluorescence and FTIR measurements, Rap1 proteins carry an additional A86C mutation for fluorescent labeling, which has been shown not to interfere with the GAP-mediated GTPase reaction.[21]

The catalytic domain of Rap1GAP (75–415) and the mutants were expresses in *E. coli* strain BL21DE3 using the pGEX4T1 expression system and purified as described.[14,57] Mutants were prepared using the Quick-Change protocol (Stratagene).

Fluorescence labeling, nucleotide exchange and –nucleotide detection

For coupling of a fluorophore to wild-type or mutants of Rap1, the buffer was exchanged to 50 mM Hepes (pH 7.6), 5 mM $MgCl_2$, 2 mM ascorbate by repeated dilution and ultrafiltration steps as described.[21] Protein was then incubated overnight with a tenfold excess of Iaedans (Molecular Probes, Leiden, NL). The reaction was stopped by adding an excess of dithioerythritol. Unbound fluorophore was removed by sequential dilution and ultrafiltration steps. Efficiency of the labeling reaction was analyzed by mass spectrometry. In the context of fluorescence spectroscopy in this study, Rap1 or any mutant of Rap1 designates a protein labeled with the Aedans fluorophore at Cys86.

Nucleotide exchange was performed as described.[56] Exchange to (P^3-1-(2-nitro)phenylethyl guanosine 5′-*O*-triphosphate (caged-GTP) was done according to a modified protocol as described.[58] Rap1•GDP was incubated with a fivefold excess of caged-GTP and a catalytic amount of alkaline phosphatase in 50 mM Tris–HCl (pH 8.5), 5 mM DTT, 10 μM $ZnSO_4$, 200 mM ammonium sulfate. After incubation at room temperature for 3 h, the protein was re-buffered and excess caged nucleotide was removed by gel-filtration. Nucleotide detection by HPLC was carried out as described.[59]

Rapid kinetics using stopped-flow and FTIR

Transient kinetic experiments were carried out as described,[21] using a SX18MV-stopped-flow-apparatus (Applied Photophysics, Leatherhead, U.K.). Aedans-labeled protein was excited with monochromatic light (λ = 350 nm, band-width = 6.4 nm) and emission was followed using a cut-off filter (λ > 408 nm). A total of 1000 data points were measured for every time course.

Initial reaction assays

The reaction buffer was 50 mM Hepes (pH 7.5), 100 mM NaCl, 5 mM $MgCl_2$. 2 μM Rap1-Aedans•GTP (or mutant) was mixed with 50 μM Rap1GAP in the stopped-flow apparatus and the fluorescence was followed. All traces shown are the average of three to five individual measurements.

Transition state experiments (AlF_x, BeF_3^-, MgF_3^- and vanadate)

Aedans-Rap1 (or mutants) was incubated with a catalytic amount of Rap1GAP at 293 K for 2 h to convert it into the GDP-bound form. For stopped-flow measurements, 2 μM Aedans-Rap1•GDP in 50 mM Hepes (pH 7,5), 100 mM NaCl, 5 mM $MgCl_2$, 500 μM $AlCl_3$ (or BeF_2) and 5 mM NaF was mixed rapidly with the corresponding Rap1GAP construct (50 μM) and the fluorescence monitored over 500 s. The MgF. experiments were done using 5 mM NaF and 5 mM $MgCl_2$. Similarly; the vanadate experiments were done with 500 μM vanadate prepared in the standard buffer as described.[60]

Analysis of biochemical data

The fluorescence were normalized and analyzed with the program Grafit 5.0.6 (Erithacus Software Limited) or Scientist 4.0 (Micromath Scientific, Microsoft Corp.).

FTIR spectroscopy

The sample solutions were prepared essentially as described for GAP-catalyzed reaction of Rap1.[43] The exchange to caged-GTP was ~65% complete, 35% of Rap1 remaining in the GDP-bound state. The caged group was either P^3-*para*-hydroxyphenacyl (pHP) or P^3-1-(2-nitrophenyl)ethyl (NPE).[61,62] Rap1GAP was used in 1.5 molar excess over Rap1•caged nucleotide, which was 6 mM. The GAP-catalyzed reactions were done at 258 K if not stated otherwise. The FTIR measurements were performed by the fast scan technique,[63] where the GTPase reaction was initiated by UV laser flashes as described.[40,41,43] This converted caged-GTP to GTP, and subsequently the IR absorbance change was monitored. The data of the time-resolved fast scan measurements for the GAP-catalyzed reactions were analyzed between

1800 cm^{-1} and 950 cm^{-1} with a global fit method that yields amplitude spectra and apparent rate constants.[64]

Acknowledgements

We thank the DFG SFB642 to K.G., Y.S. and C.K. (grant number A1), O.D. thanks the Boehringer Ingelheim Fonds and P.P.C. the International Max Planck Research School for Chemical Biology for support. We thank Rita Schebaum for secretarial assistance.

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Edited by D. E. Draper

(Received 28 September 2006; received in revised form 27 October 2006; accepted 6 November 2006)
Available online 10 November 2006