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Rap-specific GTPase Activating Protein follows an Alternative Mechanism*

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Rap1 is a small GTPase that is involved in signal transduction cascades. It is highly homologous to Ras but it is down-regulated by its own set of GTPase activating proteins (GAPs). To investigate the mechanism of the GTP-hydrolysis reaction catalyzed by Rap1GAP, a catalytically active fragment was expressed in *Escherichia coli* and characterized by kinetic and mutagenesis studies. The GTPase reaction of Rap1 is stimulated 10^5-fold by Rap1GAP and has a k_{cat} of 6 s^{-1} at 25 °C. The catalytic effect of GAPs from Ras, Rho, and Rabs depends on a crucial arginine which is inserted into the active site. However, all seven highly conserved arginines of Rap1GAP can be mutated without dramatically reducing V_{max} of the GTP-hydrolysis reaction. We found instead two lysines whose mutations reduce catalysis 25- and 100-fold, most likely by an affinity effect. Rap1GAP does also not supply the crucial glutamine that is missing in Rap proteins at position 61. The Rap1(G12V) mutant which in Ras reduces catalysis 10^6-fold is shown to be efficiently down-regulated by Rap1GAP. As an alternative, Rap1(F64A) is shown by kinetic and cell biological studies to be a Rap1GAP-resistant mutant. This study supports the notion of a completely different mechanism of the Rap1GAP-catalyzed GTP-hydrolysis reaction on Rap1.

Rap is a small guanine nucleotide-binding protein implied in signal transduction processes (reviewed in Ref. 1). In humans, four isoforms, Rap1A, Rap1B, Rap2A, and Rap2B, exist. Rap1A and Rap1B share more than 90% sequence identity and in most studies no discrimination between these isoforms has been made. Rap1A/B (Rap1 from now) is the closest relative of the Ras protein with more than 50% sequence identity. Just as Ras, it cycles between a GDP-bound inactive and a GTP-bound active form, and this switching is regulated by specific guanine nucleotide exchange factors (GEFs)[1] and GTPase activating proteins (GAPs). After its original cloning (2) and purification (3), Rap1 was shown to inhibit K-Ras-mediated transformation (4) and growth factor-induced and Ras-mediated MAP kinase activation (5). In contrast to its suggested role as a Ras antagonist, Rap1 becomes activated in parallel to Ras by receptor tyrosine kinase activators and is believed to mediate its own type of intracellular signals (6). It was shown to induce neurite outgrowth in PC12 cells via a pathway involving B-Raf (7) and to act as a mitogenic stimulus in certain cell types (8, 9). Recently it was found to be involved in inside-out regulation of integrin activation (10–12).

A number of guanine-nucleotide exchange factors have been identified which connect Rap1 activation with a variety of upstream signals such as Ca^{2+}/calmodulin, diacylglycerol, cAMP, and receptor tyrosine kinases (13–17). These GEFs are large modular proteins which contain a Ras exchanger motif (18) and a Cdc25 GEF domain. These domains are also found in Ras-specific GEFs like SOS (19). Biochemical experiments have demonstrated that the Cdc25 domain is fully capable for catalyzing nucleotide exchange (20), and the three-dimensional structure of the Ras-SOS complex has shown that the Ras exchanger motif domain is stabilizing the Cdc25 domain (21). Due to the conservation of the Cdc25 domain in both Ras and Rap-specific GEFs it is expected that the basic mechanism of nucleotide exchange will be similar between these, and mutagenesis studies have in fact supported this (22).

In contrast to RapGEFs, Rap-specific GAPs have no sequence homology with RasGAP and other GAPs such as RanGAP or RhoGAP. The first RapGAP, Rap1GAP, was purified as a cytosolic and a membrane-associated higher molecular weight isoform (23, 24). The cytosolic form was cloned and identified as a 663-residue protein which worked on Rap1, somewhat less on Rap2, but had no activity toward Ras and other small GTP-binding proteins (25). Recent results propose an interaction between a splice isoform of Rap1GAP, termed Rap1GAPII, and the $G\alpha_i$ isoform of a heterotrimeric G proteins (26), and similar connections to $G\alpha_z$ and $G\alpha_o$ were also reported (27, 28). The interaction is mediated by the GoLoco domain of Rap1GAPII which is a general $G\alpha$-interacting motif (29).

A catalytic domain sufficient for RapGAP activity was identified in Rap1GAP (30). This domain was later found in Spa1, a 130-kDa protein with high expression in lymphoid tissues (31), and E6TP1, a protein interacting with the E6-oncoprotein of human papilloma viruses (32). Limited homology to Rap1GAP was identified in tuberin which is encoded by a gene disrupted in patients with tuberous sclerosis, a syndrome characterized by the development of benign tumors. Indeed, a weak RapGAP activity was demonstrated for Tuberin (33). GAP^{IP4BP}, which by sequence is closely related to RasGAP, has been reported to stimulate the GTPase activity of both Ras and Rap1 (34). In line with these observations, Bud2 from *Saccharomyces cerevisiae* has sequence homology to the RasGAP domain but acts on Bud1p/Rsr1p, a putative yeast homologue of Rap1 (35).

Nothing is known about the mechanism of GTPase activation

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[1] The abbreviations used here are: GEF, guanine nucleotide exchange factor; GAP, GTPase activating protein; mant, methylanthranoyl; SOS, Son of Sevenless; GST, glutathione *S*-transferase.

of Rap1 by RapGAPs. For RasGAP it has been shown that the catalytic mechanism involves the stabilization of the catalytic Gln^{61} on Ras and the introduction of a catalytic arginine from GAP, the arginine finger, into the active site to complement the catalytic machinery (36, 37), and similar findings have been made for Rho and RhoGAPs (38, 39). By aligning the sequence of GAPs for other small G proteins and identifying invariant arginines (40), it was postulated that they might employ a similar mechanism, as verified in the meantime for the Rab-RabGAP interaction (41, 42). It is, however, anticipated that the mechanism is different for Rap, since Rap is the only small guanine nucleotide-binding protein that does not contain a residue homologous to Gln^{61} in Ras.

By using a bacterial expression system we prepared a catalytically active fragment of Rap1GAP of 341 amino acids (termed here RapGAP-341) to study the mechanism of GTPase activation. We can show that RapGAP-341 accelerates the slow intrinsic hydrolysis by 5 orders of magnitude. However, no arginine is involved in the reaction mechanism. The G12V mutant of Rap1, believed to be insensitive to RapGAPs and thus dominant active, can be efficiently down-regulated by RapGAP-341. Instead, we identify Rap1(F64A) as a mutant whose GTPase activity is not down-regulated by Rap1GAP. For future transfection studies, Rap1(F64A) thus appears to be a suitable dominant active Rap mutant.

EXPERIMENTAL PROCEDURES

Purification of Recombinant Proteins—Truncated forms (amino acids 1–167) of Rap1A, Rap1B, and Rap1 mutants were prepared from *Escherichia coli* strain CK600K using the ptac-expression system, purification on a Q-Sepharose column and subsequent gel filtration as described (for Ras, see Ref. 43).

A 1050-bp fragment of human Rap1GAP coding for amino acid 75–415 was amplified by PCR using primers 5′-CGCGGATCCCCCACAACCAGGTG-3′ and 5′-ATAAGAATGCGGCCGCCTACTACTCGTCGC-3′ (*Bam*HI and *Not*I cleavage sites underlined, codons separated) and cloned in the *Bam*HI-*Not*I cleavage site of pGEX4T1. Mutants were generated using the QuikChange™ Site-directed Mutagenesis Kit (Qiagen).

GST-RapGAP-341 and its mutants were isolated from *E. coli* strain BL21-DE3 using the pGEX-expression system (Amersham Biosciences). Freshly transformed BL21 DE3 were grown in 5 liters of TB medium containing 100 μg/ml ampicillin at 37 °C. 50 μM Isopropyl-D-thiogalactopyranoside was added at an A_{600} ~0.2, and the culture was grown overnight at 18 °C. Bacterial pellets were resuspended in phosphate-buffered saline containing 5 mM $MgCl_2$, 5 mM dithioerythritol, 1 mM ATP, and 0.1 mM phenylmethylsulfonyl fluoride (pH 7.5). After the bacterial lysis by micro-fluidizer (Microfluidics Corp.) and centrifugation (15,000 × *g*, 4 °C, 60 min) the supernatant was applied on a glutathione-Sepharose column which was equilibrated with phosphate-buffered saline containing 2 mM $MgCl_2$, 5 mM dithioerythritol, and 0.1 mM ATP (pH 7.5). After extensive washing, bound proteins were eluted with phosphate-buffered saline containing 5 mM dithioerythritol and 20 mM glutathione (pH 7.5). Cleavage of RapGAP-341 from GST was performed on the glutathione-Sepharose column by incubation with 300 units of thrombin (Serva) overnight at 4 °C under continuous circulation (0.5 ml/min). Proteins were concentrated by centrifugal concentrators (Vivaspin 10-kDa cut off, Viva Science) to 10 mg/ml, 10% glycerol was added, aliquots were snap frozen in liquid nitrogen and stored at −80 °C.

Nucleotide Exchange—Rap1·GTP was prepared by incubating Rap1·GDP in the presence of 12 mM EDTA and 100-fold molar excess of GTP for 1 h at room temperature in 30 mM Tris/HCl and 3 mM dithioerythritol (pH 7.5). Exchange was stopped by adding 25 mM $MgCl_2$. The separation of unbound nucleotides and EDTA from Rap1·GTP was carried out using PD-10 gel filtration column (Amersham Biosciences AB, Uppsala, Sweden). The concentration of Rap1·GTP was determined by high performance liquid chromatography as described (44). [γ-^{32}P]GTP-bound Rap1 was prepared by incubating 1.5 mM Rap1·GTP, 20 μCi of [γ-^{32}P]GTP (800 Ci/mmol, ICN) in the presence of 12 mM EDTA for 30 min on ice. The exchange reaction was stopped by adding 25 mM $MgCl_2$.

GAP Assay—The RapGAP-341-stimulated GTP-hydrolysis of Rap1 was measured according to the charcoal method (45) using increasing amounts of Rap1·[γ-^{32}P]GTP and constant concentration of RapGAP-341 (100 nM) at 25 °C in standard buffer (30 mM Tris (pH 7.5), 2 mM $MgCl_2$, 3 mM dithioerythritol). At different time points, 5-μl aliquots were withdrawn, added to 395 μl of 5% (w/v) Norit in 20 mM H_3PO_4 and vortexed. The charcoal was removed by centrifugation (14,000 rpm for 5 min). The radioactivity of a 200-μl aliquot of supernatant was subjected to scintillation counting. The respective initial GTP-hydrolysis rates were evaluated by linear regression fitting, and K_m and k_{cat} were determined by Michaelis-Menten equation using the program Grafit (Erithacus software).

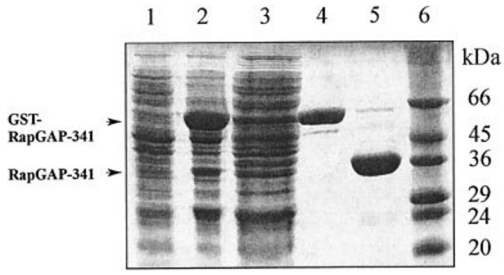

FIG. 1. **Expression and purification of the RapGAP-341.** Residues 75–415 from human Rap1GAP were produced as a GST fusion. *Lanes 1* and *2*, non-induced culture; induced culture. *Lane 3*, soluble extract. *Lane 4,* GST-RapGAP-341 after elution from glutathione-Sepharose. *Lane 5*, RapGAP-341 after thrombin cleavage. *Lane 6*, molecular weight markers.

Rap1·GTP Stability in Cell Culture—COS7 cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal calf serum. The Rap1·GTP pull-down assay was carried out essentially as described in Ref. 46. Shortly, 1.5×10^6 cells were seeded on 10-cm culture dishes and grown overnight. Transfections with the pMT2sm-HA plasmid harboring the respective Rap1A constructs (6) were carried out according to the DEAE-dextran method (47) using 5 μg of plasmid DNA per dish. Cells were grown for 48 h, washed in ice-cold phosphate-buffered saline and lysed for 5 min in ice-cold lysis buffer (50 mM Tris/HCl, pH 7.4, 2 mM $MgCl_2$, 1% Nonidet P-40, 10% glycerol, 100 mM NaCl, 1 mM benzamidine, 1 μg/ml leupeptine, 1 μg/ml pepstatin, 1 μg/ml aprotinin). Insoluble material was removed by centrifugation. The supernatant was split and one-half was incubated with 2 μM RapGAP-341 for 10 min at 20 °C. Each sample was incubated for 30 min at 4 °C with GST-Ral-GDS, produced in BL21 DE3, that was bound to glutathione-coupled Sepharose beads. After extensive washing with lysis buffer, bound proteins were eluted with standard SDS-Laemmli buffer and analyzed for HA-Rap1A by Western blotting using a monoclonal mouse antibody directed against the HA-tag (12CA5, Roche Molecular Biochemicals).

RESULTS AND DISCUSSION

Bacterial Expression of a Catalytically Active Rap1GAP Domain—It has previously been shown by expression of proteins in Sf9 cells that residues 75 to 416 from Rap1GAP form a stable protein whose catalytic activity is indistinguishable from that of the full-length protein (30). We tried to express various fragments of Rap1GAP in *E. coli* as GST fusion proteins, but these fusion proteins were mostly insoluble or could not be purified away from chaperones such as GroEL. The fragment that worked best was the 75–415 fragment (RapGAP-341), and no smaller soluble fragment could be produced in line with earlier observations (30). By expressing the gene fragment at low temperatures and performing protein purification in the presence of ATP to remove bound chaperones, we were able to produce reasonable large amounts of the corresponding highly active GST fusion protein (4 mg/liter culture). This protein could in turn be cleaved by thrombin to obtain the catalytically active RapGAP-341 (Fig. 1).

Stimulation of Intrinsic Hydrolysis of Rap1 by RapGAP-341—Qualitative measurements of the RapGAP-catalyzed GTPase reaction on Rap1 have been reported so far, but the affinity or the maximal rate of the reaction are unknown (23, 25, 48). In the case of Ras, fluorescent mant derivatives of GTP or the tryptophan fluorescence of the Y32W mutant were used

to follow the kinetics of the RasGAP-catalyzed reaction (49, 50). However, we did not find sufficiently large fluorescent changes using either mant-GTP or tryptophan fluorescence of a Y32W mutant of Rap1A (data not shown). This indicates that the interaction between Rap1A and RapGAP-341 is different from the Ras system. We thus employed radioactively labeled [γ-^{32}P]GTP and measured the increase of radioactive inorganic phosphate with the charcoal method (45). Using 100 nM RapGAP-341 (the enzyme), we measured the rate of the reaction with increasing amounts of Rap1A·GTP (the substrate). Plotting the rates against the concentration of substrate and treating the data with the Michaelis-Menten equation, we obtained a k_{cat} of 5.6 s^{-1} and a K_m of 52 μM at 25 °C (Fig. 2). The k_{cat} value is very similar to that of the GAP-stimulated GTPase reactions of Ras (51), Rho (38), Ran (52), and Rab (41) which all have a k_{cat} of ~5–20 s^{-1}. We also tested Rap1B as substrate for RapGAP-341 and obtained identical values for k_{cat} and K_m (data not shown). Because we could prepare Rap1B in much larger amounts than Rap1A, it was used in the following experiments.

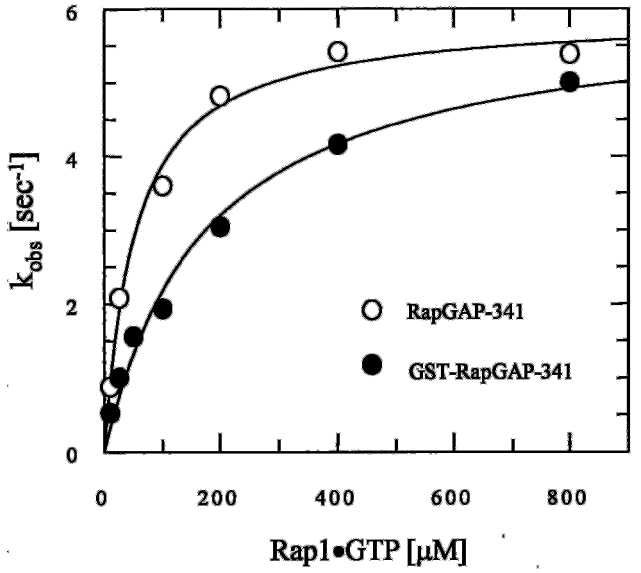

FIG. 2. **Michaelis-Menten kinetic of the RapGAP-341-stimulated reaction.** 100 nM RapGAP-341 or GST-RapGAP-341 were incubated in standard buffer at 25 °C with increasing concentrations of Rap1A·[γ-^{32}P]GTP. GTP hydrolysis was monitored by measuring P_i release. Data evaluation was performed as described under "Experimental Procedures." The obtained rates (as rate constants) were fitted to the Michaelis-Menten equation for calculation of k_{cat} and K_m.

Since we wanted to prepare several RapGAP-341 mutants for the analysis of the reaction mechanism, and since the mutants might be less stable and thus more difficult to prepare, we wondered whether GST-RapGAP-341 would have similar catalytic properties as compared with RapGAP-341. Fig. 2 shows that the K_m for the GST fusion is somewhat lower but k_{cat} is the same (k_{cat} = 5.6 s^{-1}, K_m = 175 μM). Under appropriate conditions, we have thus used wild-type and mutants as GST fusion proteins.

Mutational Analysis of RapGAP-341—Previous studies on the RasGAP reaction have shown that the crucial catalytic arginine cannot be replaced by any other amino acid without drastically reducing GAP activity (36). To identify an analogues residue in RapGAP-341 which participates in the GTP hydrolysis reaction, we aligned various RapGAPs from different species and looked for conserved arginine residues (see Fig. 3). We identified seven totally conserved arginines at positions 91, 128, 132, 284, 286, 388, and 390 and mutated them to alanines. For one of the mutants, R132A, we obtained only very little soluble protein, which was only partially cleavable by thrombin indicating that the amino acid exchange somehow destabilized the protein. The mutant RapGAPs-341 were tested for activity under standard conditions where 100 nM GST-RapGAP-341 was incubated with 200 μM Rap·GTP, and the initial linear rate of the reaction was measured at 25 °C. Under these conditions, the R91A, R128A, R286A, and R390A mutants retained more than 50% activity in comparison to wild-

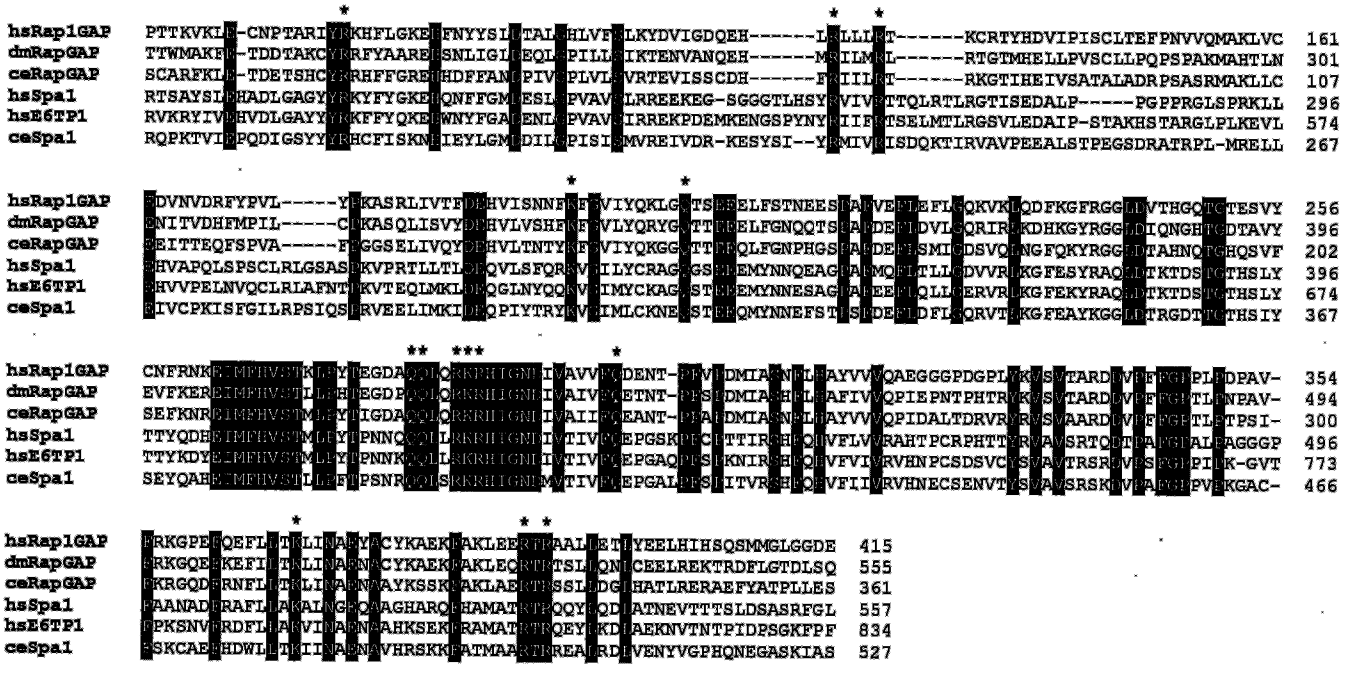

FIG. 3. **Alignment of different RapGAPs.** Sequences are from *Homo sapiens* Rap1GAP (Swiss-Prot number P47736), *Drosophila melanogaster* RapGAP (Swiss-Prot number O44090), *Caenorhabditis elegans* RapGAP (Swiss-Prot number P91315), *H. sapiens* Spa1 (Swiss-Prot number O60618), *H. sapiens* E6TP1 (Swiss-Prot number Q9UNU4), and *Caenorhabditis elegans* Spa1 (Swiss-Prot number Q20016) with the amino acid positions indicated. The alignment was performed using the program Genedoc (70). Invariant residues are *boxed*, and those mutated in the present studies are indicated with "*".

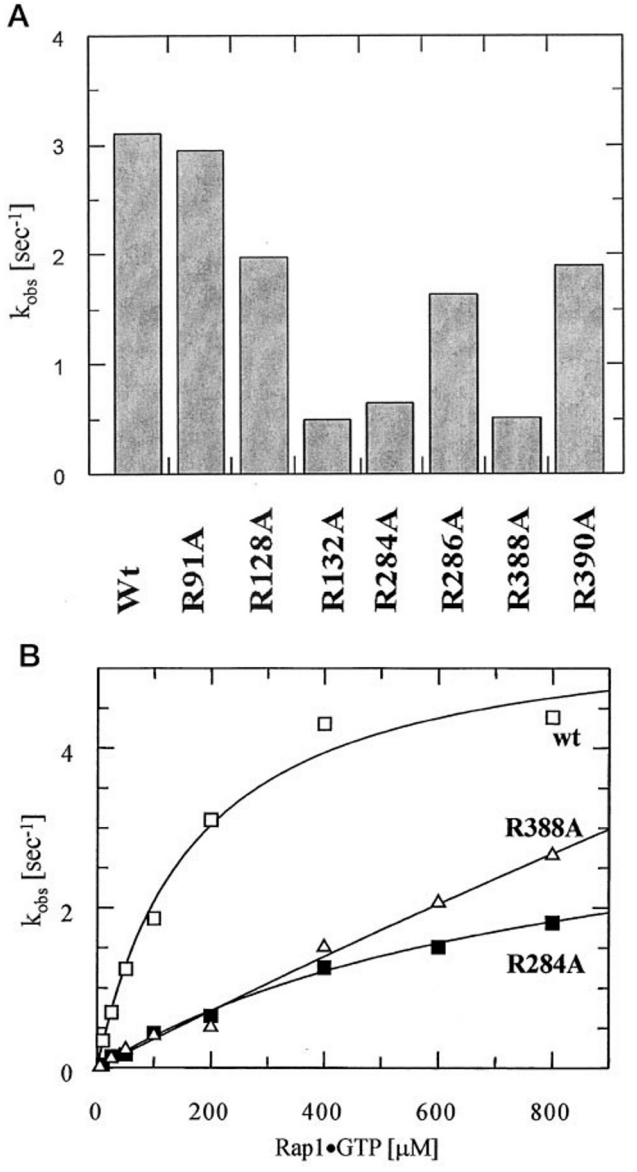

FIG. 4. **Probing the GTPase mechanism by arginine mutations.** *A*, invariant arginine residues (see Fig. 3) were mutated to alanine. The mutants were purified as GST fusion proteins and the GAP activity was measured under one standard set of condition, which are 100 nM GST-RapGAP-341, 200 μM Rap1·[γ-^{32}P]GTP in standard buffer at 25 °C. Initial rates were determined with several time points and plotted in a *bar diagram*. *B*, Michaelis-Menten kinetics of the RapGAP-341 (R284A) and (R388A) mutants compared with wild-type under standard conditions (see Fig. 2).

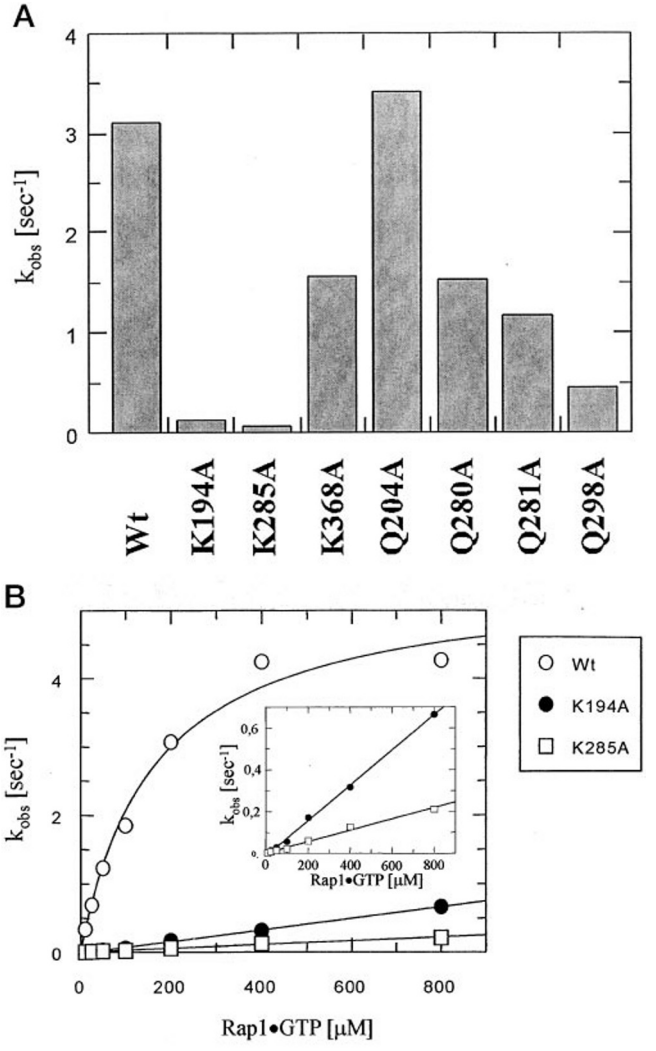

FIG. 5. **Mutational analysis of conserved lysine and glutamine residues.** *A*, the conserved lysines and glutamines of RapGAP-341 were mutated to alanine. The GAP activity of these mutants was analyzed under one set of conditions as described in the legend to Fig. 4*A* and plotted in a *bar diagram*. For mutants K194A and K285A, an enzyme concentration of 2 and 5 μM was used. *B*, for further analysis, Michaelis-Menten kinetics of mutants K194A and K285A were generated under standard conditions (see Fig. 2), shown in comparison to wild-type (*main*). In the *inset*, rates are on a different scale.

type, whereas mutants R284A and R388A exhibited a more than 3-fold decrease in GAP activity (Fig. 4*A*). The mutant R132A showed an activity between 10 and 20% of wild-type, depending on the preparation, which we explain with the instability of this protein. To learn about the specific defects of the stable mutants R284A and R388A, they were further studied by Michaelis-Menten kinetics. Fig. 4*B* shows that GST-RapGAP-341(R284A) has both an increase in K_m to 860 μM, and a slightly reduced k_{cat} of 3.8 s^{-1}. The reaction with the R388A mutant cannot be saturated under these conditions. This indicates that the mutation affects K_m although a slight effect on k_{cat} cannot be dismissed. We conclude that the Rap1GAP-stimulated GTPase reaction does apparently not employ an arginine finger like in Ras, Rho, and Rab. The mutation of the crucial arginine reduces k_{cat} 1800- and 540-fold in Ras and Rho, respectively (36, 38), and completely inactivates the RabGAPs Gyp1p and Gyp7p (41). Previous studies with Arf had already implied that the arginine finger mechanism may not be ubiquitous, although the evidence is conflicting. Mutagenesis studies with the Arf/ArfGAP system indicated an effect of an arginine on the overall reaction (53), whereas the structure of the Arf·GDP·ArfGAP complex shows no arginine in the neighborhood of the active site (54).

Which Residues of RapGAP Are Crucial for GAP Activity?—Since we could not find a major contribution to catalysis from the arginine residues of RapGAP-341 we asked the question whether other conserved residues might be involved in catalysis. Since lysine could in principle be used for stabilization of the transition state, as found for the nitrogenase complex in the ADP·AlF_4^- state (55), we mutated all three invariant lysine residues in RapGAP-341 (Fig. 5*A*). Whereas Lys^{368} does not seem to be important for catalysis we could observe a large drop in catalytic activity for mutants K194A and K285A under the standard condition. Using an enzyme concentration of 2 μM and 5 μM, respectively, we estimate that K194A and K285A are at least 25- and 100-fold less active than wild-type at 200 μM

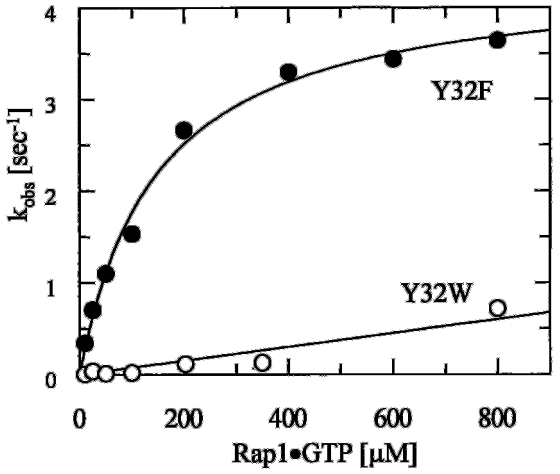

FIG. 6. **The phenolic hydroxyl group of Tyr^{32} in Rap1 is not involved in Rap1GAP mediated catalysis.** Michaelis-Menten kinetics for the GTPase activation of Rap1(Y32W) and (Y32F) mutants by GST-RapGAP-341 under standard conditions (see Fig. 2).

substrate concentration. With the same enzyme concentrations, a Michaelis-Menten kinetic was generated for these mutants (see Fig. 5*B*). In contrast to wild-type, both of the mutants could not be saturated using 800 μM Rap·GTP indicating that they have a decreased affinity for the substrate. However, we cannot exclude that these mutants may also display a reduced V_{max}.

Rap is the only small GTP-binding protein which possesses a threonine instead of a glutamine residue at position 61. Given the crucial importance of Gln for the GTP-hydrolysis reaction of all other small GTPases, we reasoned that RapGAP-341 might supply a catalytic glutamine for the reaction. We thus mutated all four invariant glutamine residues of RapGAP-341 to alanine and analyzed the properties of the mutants. Fig. 5*A* shows that the Q204A mutation had no effect. Q280A and Q281A mutations have a 2-fold and Q298A a 6-fold effect on catalysis. This is, however, very much smaller than expected for a residue replacing Gln^{61} whose mutation in Ras or Ran reduces GAP activity by more than 5 orders of magnitude.

Mutants of Rap1 in the RapGAP-341 Catalyzed Reaction—In the search for a fluorescent Rap1 mutant we had observed that the Y32W mutant shows a reduced GAP-stimulated GTP hydrolysis. In the three-dimensional structure of Rap2·GTP, the hydroxyl group of Tyr^{32} was found to contact the γ-phosphate of GTP similar to what is seen in the Raps-RafRBD structure (56). It was discussed whether such a contact would contribute to catalysis (57). The loss of activity of the Rap1(Y32W) mutant seems to confirm this expectation (Fig. 6). However, the Rap1 mutant (Y32F) which is unable to contact the γ-phosphate is fully active in the GAP reaction arguing that the phenolic hydroxyl group does not participate in RapGAP-341 stimulated catalysis. We conclude that the mutation to tryptophan might sterically interfere with catalysis.

Our data and the fact that Thr^{61} in Rap1 is not required for GTP hydrolysis (58) indicate that Rap1GAP employs a completely different reaction mechanism for the stimulation of the GTP hydrolysis than Ras. We wondered therefore whether the canonical mutation G12V in Rap1, which blocks the GTPase reaction in Ras, affects the GTPase stimulation of RapGAP-341. Maruta *et al.* (58) had previously demonstrated that Rap1(G12V) could not be activated by GAP3, a Rap1GAP fragment purified from bovine brain. Since then, the Rap1(G12V) mutant has been used in numerous transfection experiments as the constitutive active Rap mutant.

When the GTP-hydrolysis reaction of Rap1(G12V) was tested

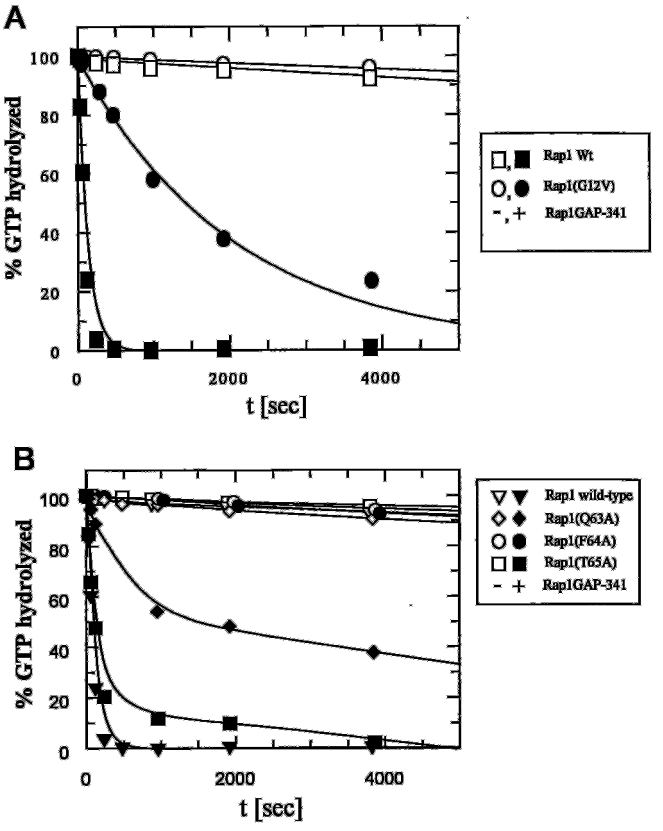

FIG. 7. **Mutational analysis of Rap1 for stimulation by Rap1GAP.** *A*, analysis of the Rap1(G12V) mutant and comparison to Rap1 wild-type under one standard condition as described in the legend to Fig. 4*A*. *Open* and *closed symbols* are in the absence or presence of RapGAP-341, respectively. *B*, several residues from switch II were mutated and the intrinsic and RapGAP-341 stimulated reaction was measured under one standard condition (see Fig. 4*A*).

in the presence of RapGAP-341, we surprisingly found a drastic GAP-catalyzed GTPase activity with a difference to Rap1 wild-type of only ~9-fold (Fig. 7*A*). Again, this result is different from Ras where no down-regulation of the G12V mutant by RasGAP can be detected.

Considering the importance of GTPase negative mutants of Rap for cell transfection experiments, we looked for mutations in Rap1 that might interfere with the GTPase stimulation by RapGAP-341. We created alanine mutants in the switch II region of Rap1 (amino acids 61–67) which had previously been shown to be important for the GAP3-catalyzed reaction (58–60). As seen in Fig. 7*B*, Rap1(T65A) is insensitive to the substitution. The Q63A mutant of Rap1 has a somewhat reduced rate, comparable to G12V. However, the F64A mutation completely blocks RapGAP-341 stimulated GTP hydrolysis. This is in agreement with the finding that the F64E mutation in Rap1 prevents stimulation of GTP hydrolysis by GAP3 (60). Thus, Rap1(F64A) or possibly Rap1(F64E) appear to be reasonable candidates for dominant active Rap1 mutants.

So far, Ras and Rap have been found to share overlapping effectors which have a Ras-binding domain (61–63). These types of Ras-binding domains interact with Ras and Rap only via switch I (amino acids 32–40) (56, 64–66). We wanted to determine whether the F64A mutant is still able to interact with effectors and confirm that the differences in GAP stimulation of Rap1 wild-type, Rap1(G12V) and Rap1(F64A) *in vitro* are also seen *in vivo*. Thus, we transfected COS-7 cells with HA-tagged Rap1 constructs and precipitated GTP bound Rap1 using the immobilized Rap1 effector RalGDS (67). We could detect only a slight increase in GTP bound Rap1(G12V) in

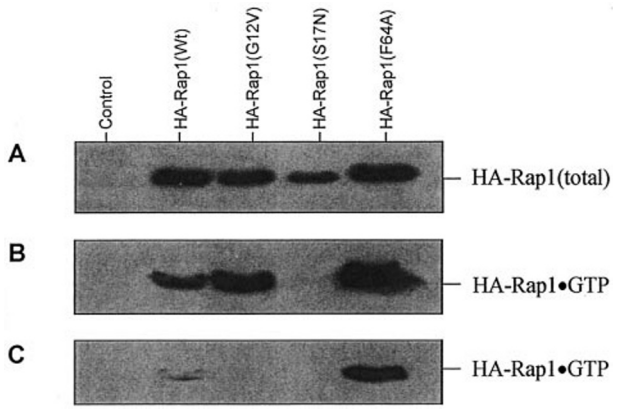

FIG. 8. **Stability of the GTP bound state of Rap1 in cells.** COS-7 cells expressing the indicated HA-tagged Rap1 proteins were lysed and split (lysate control in *A*). One part was incubated with 2 μM RapGAP-341 (*C*), the other left untreated (*B*). GTP-bound Rap1 was precipitated using GST-RalGDS coupled to GSH-agarose beads. After SDS-PAGE and Western blot, HA-Rap1 was detected by a monoclonal antibody directed against the HA-tag.

comparison to Rap1 wild-type (see Fig. 8*B*). However, a markedly increased level of the Rap1(F64A) mutant could be precipitated using RalGDS. As negative control, the Rap1(S17N) mutant was used which has a decreased affinity for nucleotides.

When the lysate was incubated for 10 min in the presence of 2 μM RapGAP-341 before the Rap1 precipitation, only Rap1(F64A) could be detected in the GTP bound form (see Fig. 8*C*). These results demonstrate that in cell culture experiments the Rap1(F64A) mutant shows an increased level of GTP loading as compared with Rap1 wild-type and Rap1(G12V). We also demonstrate that Rap1(F64A) is still able to interact with its putative effector RalGDS and can be used for pull-down experiments. Whether it is indeed able to activate its effectors such as B-Raf (7) and can therefore be considered a true dominant active mutant has to await further studies.

Conclusion—The RapGAP-341 mediated GTPase reaction on Rap1 stimulates the intrinsic reaction by approximately 5 orders of magnitude. It works by a different mechanism than hitherto described for the GAPs for Ras (37), Rho/Cdc42 (39, 68) and postulated for the GAPs of Ran (69). It does not supply an arginine finger or a catalytic glutamine. The mutations affecting most drastically the activity of RapGAP-341 were K194A and K285A which clearly reduce the binding of substrate. However, these lysines may additionally act in a catalytic fashion as observed for a lysine residue in nitrogenase (55). Tyr^{32} which had been suggested from the structure of Rap2 to participate in the GTPase reaction can be replaced by phenylalanine, making it unlikely that the phenolic hydroxyl group is involved in catalysis. In contrast to most other small and large GTP-binding proteins, the G12V mutation of the P-loop glycine interferes only slightly with the GTPase reaction. However, we present Rap1(F64A) as a GAP-insensitive Rap1 mutant *in vitro* and in cell culture experiments. The current failure to find effectors that are specific for Rap might have been due to the use of a mutant that is not as fully GTPase resistant as anticipated from the analogy to oncogenic Ras.

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