Catalytic mechanism of a mammalian Rab·RabGAP complex in atomic detail

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Rab GTases, key regulators of vesicular transport, hydrolyze GTP very slowly unless assisted by Rab GTPase-activating proteins (RabGAPs). Dysfunction of RabGAPs is involved in many diseases. By combining X-ray structure analysis and time-resolved FTIR spectroscopy we reveal here the detailed molecular reaction mechanism of a complex between human Rab and RabGAP at the highest possible spatiotemporal resolution and in atomic detail. A glutamine residue of Rab proteins (cis-glutamine) that is essential for intrinsic activity is less important in the GAP-activated reaction. During generation of the RabGAP-Rab-GTP complex, there is a rapid conformational change in which the cis-glutamine is replaced by a glutamine from RabGAP (trans-glutamine); this differs from the RasGAP mechanism, where the cis-glutamine is also important for GAP catalysis. However, as in the case of Ras, a trans-arginine is also recruited to complete the active center during this conformational change. In contrast to the RasGAP mechanism, an accumulation of a state in which phosphate is bound is not observed, and bond breakage is the rate-limiting step. The movement of trans-glutamine and trans-arginine into the catalytic site and bond breakage during hydrolysis are monitored in real time. The combination of X-ray structure analysis and time-resolved FTIR spectroscopy provides detailed insight into the catalysis of human Rab GTases.

GTP hydrolysis | dual-finger | isotopic labeling | enzyme catalysis

Rab GTases are regulators of vesicular trafficking processes in eukaryotic cells and constitute the largest family of small GTPases (1). Interaction with effectors takes place in the GTP-bound “on” state, and the inactivation occurs via GTP hydrolysis to GDP; this is catalyzed by GTPase activating proteins (GAPs), thereby downregulating the GTPase. Most RabGAPs contain Trc2-Bub2-Cdc16 (TBC) domains, and accelerate the intrinsic GTP hydrolysis rate up to 104-fold (2, 3). As regulators of Rab-dependent pathways, RabGAPs are involved in a number of diseases, contributing to viral and bacterial infection, cancer development, predisposition to obesity, and others (1, 2). Therefore, detailed insight into the RabGAP-mediated catalysis with highest possible spatiotemporal resolution is of great interest, and is an important prerequisite for the design of small molecules for molecular therapy.

The stabilization of the nucleophilic-attacking water molecule by the carboxamide moiety of a glutamine or asparagine side chain is crucial for GTP hydrolysis in all small GTPases (4). In Ras (5), Ran (6), and Rho (7), the carboxamide moiety is provided by the conserved glutamine of the so-called “switch II.” In Rap, which has a threonine at the homologous position (acidic amino 61), the amide is either not supplied by Rap itself, but by the asparagine thumb of RapGAP (8, 9), or supplied by Gln63 of Rap in the case of catalysis by dual-specificity GAPs (10). In addition, the active site of many small GTPases is completed by a catalytic “arginine finger” that is used, e.g., by Ras-, Rho-, and RabGAPs, but is not needed for catalysis in Ran and Rap. Conventional TBC domains possess conserved arginine and glutamine residues (2, 11). In the case of activation of Rab33 (mouse) by the yeast GAP Gyp1, it was shown that a glutamine residue of Gyp1 (termed trans-glutamine) positions the water molecule rather than the conserved glutamine in Rab33 (termed cis-glutamine) (11). Whether this mechanism applies to bona fide Rab-RabGAP pairs from mammalia has not yet been established.

Ras is the most intensively studied small GTPase. It has been investigated by X-ray crystallography (5, 12–14); biomolecular simulations (15–17); electron paramagnetic resonance and NMR (18); and FTIR (19–22). Time-resolved (tr) FTIR spectroscopy allows monitoring of protein reactions at atomic detail in real time and provides information about charge shifts, protonation states, and protein side-chain movements (19, 21). Based on trFTIR experiments and biomolecular simulations, it has been proposed for RasGAP that the arginine finger movement into the binding pocket displaces water molecules and thereby changes the dielectric in the active site. This process induces a charge shift from the γ- toward the β-phosphate, drives GTP toward the transition state, and contributes to a reduction of the free activation energy (15, 21). Furthermore, Ras and RasGAP drive GTP into a strained conformation and further reduce the free activation energy (15). In Ras, bond breakage is rapid once the arginine finger enters the active site. The released γ-phosphate and the attacking water form a protein bound H2PO4− in a reversible reaction, and the following Pγ-release is the rate-limiting step (19, 21).

Here, we investigated the RabGAP mechanism of a human RabGAP-Rab pair, TBC1D20 and Rab1b. TBC1D20 is a Rab1- and Rab2-specific GAP that is involved in hepatitis C virus (HCV) replication and is an efficient GAP in vitro (23, 24). Though the kinetic parameters of most known mammalian RabGAPs either have not been determined or demonstrate low catalytic efficiency (2), we found more than 104-fold acceleration. Rab1 mediates endoplasmatic reticulum–Golgi trafficking and plays a role in Legionella pneumophila pathogenesis (1, 25–28). Here, we describe the structure of the Rab1b-GDP-BeF3−·TBC1D20 and define the catalytic mechanism using complementary FTIR difference spectroscopy with the help of isotopic labeling of Rab, GAP, and the nucleotide. This approach provides a detailed understanding of the catalytic mechanism at the highest spatiotemporal resolution.

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Data deposition: Coordinates and structure factors have been deposited in the Protein Data Bank, www.pdb.org (PDB ID codes: 4HLQ for TBC1D20, 14-305 and 4HLDQ for Rab1b: TBC1D20, 1-305).

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Results

Structure of the Isolated TBC1D20, α-Subdomain. The crystal structure of the isolated TBC1D20, α-subdomain was determined at 2.2 Å resolution (see Table S1 for data collection and refinement statistics), and was used as a search model in the structure determination of the Rab1-TBC1D20 complex by molecular replacement. Fig. S1 depicts a comparison between the isolated and the Rab1-bound state. The arginine-finger residue R105 of the isolated structure has an alternative conformation to that found in the complexed form. The rest of the structure does not undergo other significant structural rearrangement upon binding to Rab1.

Structure of the Rab1-TBC1D20 Complex. To provide a basis for understanding the mechanism of GTP hydrolysis of Rab1 stimulated by TBC1D20, we determined the crystal structure of Rab1b-TBC1D20 in complex with TBC1D20, α-subdomain, GDP, and BeF₃⁻ at 3.3 Å (Table S1). There are five complex molecules in the asymmetric unit that are highly similar to each other, with rmsd values of 0.394–0.716 Å for 2,255 Cα residues. Similar to other TBC domain-containing GAPs, the structure of TBC1D20 can be subdivided into two subdomains. The amino-terminal subdomain comprises helices α1–α5, and the carboxyl-terminal subdomain contains helices α6–α11 (Fig. A). In accordance with the previously reported Rab33-Gyp1 complex crystal structure, the IxxDxxR and YxQ signature motifs are critical for GAP activity, and they directly contact the nucleotide, whereas the RxxxW motif appears to be important for the structural integrity of the TBC domain (11). In comparison with other TBC domain-containing GAPs, the isoecucine in the IxxDxxR motif is replaced by valine. Helices α5/α7 of the N-terminal subdomain and α8/α9 and α11/α14 of the C-terminal subdomain of TBC1D20 form a groove that is large enough to bind the switch I, switch II, and the P-loop of Rab1.

Additional electron density was found in the interface between Rab1b and TBC1D20 in which GDP, Mg²⁺, and BeF₃⁻ with a trigonal pyramidal conformation could be reliably modeled. In contrast to several other GTPase-GAP structures, no water molecule was found in the active site that was positioned to attack the γ-phosphate group of GTP. However, the structures showing such a water molecule were all with aluminum fluoride (or tetrafluoroaluminate), a transition-state analog, with one exception, the Rap1-RapGAP complex, which was also with beryllium fluoride, a ground-state analog (9). In the latter case, there was also no observable attacking water molecule, but the resolution of the solved structure was relatively low (3.3 Å), as in the structure presented here. Therefore, it is possible that a water molecule in this position is not well-defined enough to be seen at this resolution. In analogy to the results seen in aluminum fluoride complexes with other GTPase-GAP pairs (5, 7, 11), the GDP/BeF₃⁻ complex is stabilized via polar contacts with the arginine and glutamine fingers, with the glutamine finger coming not from Rab but from the GAP molecule, as seen in the Rab33-Gyp1 structure (11). Residue R105 is located in the α4 helix and mediates bidentate interactions with the α- and β-phosphates of GDP, whereas Q144γ is located in loop α5/α7 and forms interactions via its carboxamide side chain with one of the fluoride atoms of BeF₃⁻ and with the backbone amide of Q67α of Rab1b (Fig. B). Residue Q67α, or its equivalent in the switch II region is involved in the intrinsic hydrolysis of GTP by Rab GTPases, but in the structure presented here, Q67α has moved away from the nucleotide and displays hydrogen bond interactions with the backbone carbonyl of Y142 and the amide of Q144 in the YxQ motif of TBC1D20.

The structure of the complex between Rab1b, TBC1D20, GDP, and beryllium fluoride agrees with the results obtained earlier on a hybrid Rab (mouse)/GAP (yeast) system (11). The superposition of the protein complex structures of Rab1b-TBC1D20 and Rab33-Gyp1 reveals analogies in the GTP hydrolytic mechanism (Fig. C). In both complexes the significance of the arginine

Fig. 1. Structure of Rab1b in complex with TBC1D20. (A) Ribbon representation of Rab1b-GDP-BeF₃⁻ complex, colored as indicated by labeling. (B) Overlay of the crystal structures of TBC1D20-Rab1b (white) and Gyp1-Rab33 (cyan, PDB ID 2G77). The electron density map contoured at 1.0 σ of the R105α side chain is highlighted in black. The indices G and T are used for Gyp1 and TBC1D20 amino acid legends, respectively.
and glutamine fingers for catalysis is a striking feature. R105$_T$ in TBC1D20 is the equivalent of R343$_G$ in Gyp1 and Q144$_T$ of Q378$_G$. The glutamine residues of Rab1b and Rab33 (Q67$_R$ and Q92$_R$, respectively) point away from the GDP·BeF$_3$· and GDP·AlF$_3$ moieties. These residues are oriented toward the α5/α6 and α6/α7 ordered loops, respectively, and are involved in polar interactions with the backbone Y142$_Y$/Y376$_G$ carbonyl and Q144$_Y$/ Q378$_G$ amino group. Though the GDP moieties and Mg$^{2+}$ possess nearly identical coordinates, the positions of BeF$_3$ and AlF$_3$ are different. AlF$_3$ shares an axial water with Q378$_G$ in the Rab33 Gyp1 structure, whereas there is no observable electron density for an axial water of BeF$_3$ in the Rab1b-TBC1D20 crystal structure. As a consequence, Q144$_Y$ of TBC1D20 makes polar contacts just to one fluoride atom of BeF$_3$ but not to a water molecule. Residue Q378$_G$ of Gyp1 instead contributes two interactions to AlF$_3$, one to the equatorial fluoride and a second indirectly through the axial water molecule.

The conformations of the arginine and glutamine fingers in the Rab1b-TBC1D20 complex are essentially identical to those in the Rab33-Gyp1 complex structure. The side chain of R343$_G$ of Gyp1 forms polar contacts to the α- and β-phosphate, as does R105$_T$ of TBC1D20. As shown in Fig. 1C, the arginine conformer of TBC1D20 is supported by the crystallographic data.

**Kinetics of GTP Hydrolysis.** The rate constants of GTP hydrolysis were determined by time-resolved FTIR in single-turnover experiments; this was done for the intrinsic reaction of Rab1b and for the GAP-catalyzed reaction of wild-type and two mutant proteins, TBC1D20Q144L and Rab1bQ67L (Fig. 2). The intrinsic Rab1b GTPase activity is slow (1.5 × 10$^{-3}$ s$^{-1}$ at 393 K), but GAP catalyzes the reaction by more than five orders of magnitude (0.9 s$^{-1}$ at 368 K). Mutation of the catalytic fingers (Q144$_Y$ or R105$_T$ of TBC1D20) results in a 1,000-fold decrease in GAP activity, whereas mutation of the cis-glutamine (Q67$_R$ of Rab1b) only reduces the hydrolysis rate by one order of magnitude. This result is in agreement with multiple turnover assays conducted at low GAP concentrations using hybrid Rab-RabGAP pairs from yeast/mouse (11, 29), and shows that the effect is not due to reduced affinity toward Rab1b, but rather due to a distortion of the active site. The result also confirms the idea that the cis-glutamine is not directly involved in GAP-catalyzed GTP hydrolysis. The intrinsic GTPase activity of Rab1bQ67L is at least 100-fold reduced, which underscores the importance of Q67$_R$ for the intrinsic reaction in contrast to GAP-catalyzed hydrolysis.

Detailed absorbance changes of the GAP-catalyzed reaction obtained by global fit analysis according to Eq. S1 are shown in Fig. 3. At 1,651 cm$^{-1}$, an intermediate appears with an apparent rate constant $k_1$ = 7 (± 0.5) s$^{-1}$ and disappears with $k_2$ = 0.9 (± 0.06) s$^{-1}$ at 368 K (SDs from 15 independent measurements).

In the GAP-catalyzed Ras reaction, protein-bound H$_3$PO$_4$ was identified as an intermediate (19). To examine the bond-breaking step, measurements are performed in H$_2$O. The incorporation of the H$_2$O-labeled attacking water molecule into the phosphate leads to band shifts of the phosphate. The protein-bound phosphate in the GAP-catalyzed Ras reaction was identified in this manner (19). The amplitude spectrum of the process occurring with rate constant $k_1$ shows no frequency shifts, and only the free P$_i$ bands at 1,078 cm$^{-1}$ and 996 cm$^{-1}$ of the product state are shifted in the spectrum of $k_2$ (Fig. S2). Therefore, we can conclude that in the Rab1b-TBC1D20 reaction, bond breakage and $P_i$ release occur essentially simultaneously with the rate constant $k_2$, whereas the first rate ($k_1$) corresponds to a conformational change. The release of the $P_i$ and simultaneous disappearance of GTP can be monitored at 1,078 cm$^{-1}$ and 1,256 cm$^{-1}$, respectively (Fig. 3).

The corresponding amplitude spectra reflect the molecular changes within the protein. In intrinsic hydrolysis, bond breakage and $P_i$ release occur as they do for the RabGAP complex in $k_2$.
but the amplitude spectrum of $k_2$ differs from that of intrinsic hydrolysis. However, the sum of the amplitude spectra of $k_1$ and $k_2$ of the GAP catalyzed reaction resembles the amplitude spectrum of intrinsic hydrolysis (Fig. S2B), indicating that there are transient TBC1D20 interactions present in the intermediate. In the Ras-RasGAP reaction (19, 21), three reaction rates are resolved, a conformational change from RasGDP to RasGTP, formation of a protein-bound phosphate intermediate, and $P_i$ release as the rate-limiting step. In the RapGAP-catalyzed reaction of Rap, there is also a detectable protein-bound phosphate intermediate (30, 31). In case of Ran (32), there are only two rates, and the observed intermediate still contains GTP and is characterized by a conformational change of both Ran and RanGAP. Therefore, the Rab1b-TBC1D20 reaction is similar to Ran and the accumulating intermediate is the complex of Rab1b-TBC1D20 forming with rate constant $k_1$, and dissociating together with $P_i$ release ($k_2$).

Mechanism of GTP Hydrolysis. The $\alpha$- and $\beta$-phosphate bands were assigned for the intrinsic and GAP-catalyzed GTPase reaction (Figs. S3 and S4). To further characterize the accumulating reaction intermediate and assign the reaction mechanism of the protein, TBC1D20 was labeled with [5-13C]glutamine. Q144F provides the carboxamide involved in catalysis via a glutamine finger, as seen in the X-ray structure (Fig. 1B). Part of the band at 1,648/1,651 cm$^{-1}$ is shifted due to labeling, as clearly observed in the double-difference spectra (Fig. 4); it is assigned to the $\nu$(CO) vibration of a glutamine side chain, most likely of Q144, because only this group is crucial for catalysis and no other glutamine band is observed in the amplitude spectra. A clear-cut band assignment can be made in $k_2$, where the reaction is reversed. The extinction coefficient of the glutamine side-chain increases in a hydrophobic environment (33). Therefore, the band indicates the movement of the glutamine finger of TBC1D20 into a more hydrophobic environment toward the active center at rate constant $k_1$. The glutamine finger is relocated back with $k_2$ into the hydrophilic environment, simultaneously with $P_i$ release.

In the next step, we labeled Rab1b with [5-13C]glutamine to identify the movement of the cis-glutamine (Fig. S5A). This band is also assigned but shows vibrational coupling to the backbone of Y142f and Q144f of TBC1D20, as expected for interacting amide groups (34) (Fig. S5B). Therefore, a pattern with several band-shifts instead of a single marker band is observed. Because Q67f is the only glutamine in the switch regions of Rab1b, the band-shifts indicate the movement of the cis-glutamine that forms the interaction with the YxQ motif in k1 and is released in k2.

In further experiments, TBC1D20 was labeled with [13C,15N] tyrosine (Fig. S64). There are a total of nine tyrosines in TBC1D20 with three tyrosines in the vicinity of the glutamine finger (Y142f, 143f as seen in Fig. 1B, and 146f). As expected, they also cause multiple band-shifts of amide and ring vibrations. The tyrosine bands that appear in k1 are inverted in k2. They probably indicate conformational changes of Tyr142f, 143f, and 146f in the glutamine finger loop. The large band at 1,650 cm$^{-1}$ also belongs to a tyrosine backbone vibration and overlaps with the band of the glutamine finger. Thus, the band at 1,650 cm$^{-1}$ presents a convenient marker band for formation and disappearance of the intermediate (Fig. 3).

To elucidate the conformational change between the ground state and the intermediate in more detail, we used complex formation with beryllium fluoride. For Ras, BeF$_2$ induces the same “on” conformation in Ras-GDP as does GTP, as shown by FTIR (35). BeF$_2$ represents an analog of the $\gamma$-phosphate (36). The amplitude spectrum of complex formation of Rab1b-GDP with beryllium fluoride and TBC1D20 is very similar to the amplitude spectrum of $k_1$. Therefore, the BeF$_2$ complex clearly mimics the accumulating reaction intermediate. Also, [5-13C]glutamine labeling of Rab1b leads to the same marker pattern as in k1 (Fig. S6B). Therefore, the cis-glutamine still interacts with the YxQ motif in the BeF$_2$ state. The Q144A mutant of TBC1D20 exhibited the same marker pattern on BeF$_2$–complex formation due to isotopic labeling of Rab1b as wild-type TBC1D20 (Fig. S6B), which suggests that the cis-glutamine still interacts with the YxQ motif even if the space in the active site is not occupied by the glutamine finger. Thus, the glutamine finger does not sterically push out the cis-glutamine. The TBC domain of RabGAPs is tailored to pull the cis-glutamine out of the active site so that its own glutamine finger can be inserted.

The glutamine of the DxxGQ motif is crucial for positioning of a water molecule for nucleophilic attack in the intrinsic reaction of most small GTPases. The intrinsic hydrolysis amplitude spectrum of [5-13C]glutamine-labeled Rab1b shows no band-shifts (Fig. S6C), indicating that the cis-glutamine does not change at all. Therefore, we propose that Q67f is rather flexible in the GTP and GDP states of Rab1b and is not able to stabilize the position of the attacking water in the GTP state. Though there is no available crystal structure of Rab1b with a GTP analog, the structure of the slowest Rab GTPase, Rab6a, in the GTP state revealed higher B-factors for the switch II region than for faster Rab GTPases (37), indicating high flexibility of the switch II and, consequently, of the intrinsic glutamine. High flexibility of Q67f could therefore result in low intrinsic GTPase activity of Rab1b.

In contrast, the interaction with GAP leads to conformational changes resulting in a well-defined active site, the glutamine finger taking the position of the cis-glutamine and stabilizing the attacking water molecule. Strongly hydrogen-bonded water molecules lead to continuum absorbances in infrared spectra (38). Indeed, such a continuum band arises in the amplitude spectrum of k1 and disappears in k2, which is a possible marker band for the attacking water molecule stabilized by the glutamine finger (Fig. S7).

In addition to carboxamide side chains, “arginine fingers” complete the catalytic centers of many small GTPases. To determine the contribution of R105f, TBC1D20 was labeled with [15N]arginine. The arginine band was identified as described for RasGAP (21). The arginine band shifts from an aqueous environment to

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Fig. 4. Band assignment of the glutamine finger of TBC1D20. Amplitude spectra of the GTPase reaction using unlabeled (cyan) and [5-13C]glutamine-labeled (red) TBC1D20 in $H_2$O. In $k_1$, negative bands belong to the GTP state, positive bands to the intermediate state. In $k_2$, negative bands belong to the intermediate state, positive bands to the GDP $+$ P$_i$ state. The double difference (labeled – unlabeled) is shown in green. The band at 1,648/1,651 cm$^{-1}$ can be assigned to a glutamine side-chain and most likely represents the catalytic glutamine finger moving into the active site with $k_1$ and in the reverse direction with $k_2$.
a hydrophobic environment with $k_1$ (Fig. S8A). The band also overlaps with tyrosine bands, as discussed above. The same bands can also be assigned in the amplitude spectrum of AlF$_3$ complex formation (Fig. S8A), a transition-state analog. Therefore, we can conclude that the arginine finger enters the active site with $k_1$. In contrast to RasGAP, bond-breaking does not occur with the same rate constant as arginine moves into the catalytic center, but in the next reaction step described by $k_2$. Notably, the spectra of AlF$_3$ and BeF$_3^{-}$ complex formation differ between 1,580 and 1,540 cm$^{-1}$, indicating a slight conformational difference, whereas AlF$_3$ is more similar to the spectrum of $k_1$ than is BeF$_3^{-}$. Because several tyrosine vibrations were found in this region (Fig. S6A), the spectra may represent a change in the $\alpha_5$$\alpha_\bar{F}$ glutamine finger loop. Furthermore, the $\beta$-phosphate band in the AlF$_3$ complex is shifted to lower wave numbers relative to the BeF$_3^{-}$ complex (Fig. S8B), which indicates a different charge distribution, in line with a more product state-like charge distribution. However, the charge shift might be of artificial nature due to the analogs used.

The catalytic mechanism for the mammalian Rab-RabGAP pair elucidated here agrees well with the proposed mechanism based on the hybrid Rab-RabGAP complex with yeast Gyp1p and mammalian Rab33. Gyp1p also employs an arginine finger, and its glutamine finger takes the role played by the cis-glutamine in most other GTPases (11).

Discussion

Our results are summarized in Fig. 5 and show the dynamics of the active center in RabGAP. With the rate constant $k_1$, Q67$_R$ of Rab is pulled out of the active site by the YxQ motif of GAP by interacting with the YxQ backbone, leaving the active site accessible for the catalytic GAP residues; this allows Q144$_T$ of GAP to move into the active site. In contrast to the intrinsic case, the attacking water molecule can now be stabilized in the correct position for nucleophilic attack, which is probably the key contribution of RabGAP to catalysis. Furthermore, R105$_T$ of GAP is pulled into the active site, probably by coulomb interactions with the negatively charged GTP with $k_2$ as well; in contrast to RasGAP, this does not immediately induce bond breakage, which occurs with the final rate $k_2$. Simultaneously, the conformational changes of Q67$_R$, Q144$_T$, and R105$_T$ are reversed as P$_i$ is released. There is no observable accumulation of a protein-bound phosphate intermediate as in the RasGAP catalysis, because bond breakage is the rate-limiting step.

We have demonstrated here a bona fide human Rab-RabGAP pair, that RabGAP proteins with a TBC domain catalyze GTP hydrolysis using a dual-finger mechanism, as first proposed for a mouse/yeast hybrid pair (11). It is therefore highly likely that all TBC domain-containing RabGAPs use this basic mechanism. We have provided detailed insights into the reaction mechanism using time-resolved FTIR, which revealed a coordinated removal of the cis-glutamine from the region of the $\gamma$-phosphoryl group of GTP and the recruitment of a trans-glutamine from the GAP molecule. The cis-glutamine is essential for the intrinsic reaction but not for GAP activity, in which case the mutant is almost as strongly accelerated as wild type. Importantly, mutation of the cis-glutamine is still widely used to generate GTP-locked Rab GTPases for in vivo studies. However, it needs to be recognized that these mutants can be activated by GAPS present in vivo.

There are now several different mechanisms of GAP activation of GTPases that have been recognized, some (but not all) of which involve an arginine from the GAP molecule. However, there appear to be no exceptions to the observation that a carboxamide function from a glutamine or an asparagine residue is essential for catalysis. This carboxamide function is contributed either by the GTPase itself or by the GAP molecule, as in the case described here. The significance of these differences in the physiological context is not clear, and it is also not yet clear whether restrictions imposed by the GTPase structure itself determine the need for a different carboxamide, or specific type of GAP activation mechanism, except in the case of Rap, where the lack of a correctly positioned cis-glutamine necessitates this. Interestingly, in the case of dual-specificity GAPS, there is indeed an involvement of a cis-glutamine of Rap, which occurs two residues removed from the glutamate in other small GTPases. This finding suggests that different GAP mechanisms can operate on a single type of GTPase, and it will be of interest to search for other examples of this nature. The characterization of the different mechanisms at the level described here will help to define the significance of the differences and lead to approaches for manipulating or emulating GAP activity in specific cases.

Materials and Methods

Protein Isolation. Rab1bs-$\Delta$77 and TBC1D20-$\Delta$305/1-362/1-305 were expressed and purified as described previously (27, 39) and in more detail in SI Materials and Methods. Semet labeling of TBC2D2+$\Delta$305 was achieved during protein overexpression in M9 media by inhibition of methionine biosynthesis (40). For isotopic labeling, we used optimized M9 media (composition as described in ref. 41; amino acids raised to 1.2 mM, NH$_4$Cl to 46 mM) in which the amino acid of interest was replaced by its isotopically labeled form (166 mg/L in all cases). For glutamine labeling of proteins, the E. coli strain TH1644 (42) was used, and concentrations of glutamate, asparagine, and aspartate in the media were raised to 2.4 mM, $\eta$-[1$^{15}$N]Arginine, [5$^{13}$C$^{15}$N]glutamine, and [13$^{15}$C]$^{15}$N]tyrosine were purchased from Cambridge Isotope Laboratories. The degree of incorporation and possible spreading were checked by mass spectrometry (41) (Table S2).

Structure Determination. Details of crystallization, structure determination, and synthesis of caged nucleotides are provided in SI Materials and Methods.

FTIR Measurements. For FTIR measurements, Rab1b was loaded with the nonhydrolysable, photoactivatable caged nucleotide (43), and the buffer was exchanged to 1 mM Hepes (pH 7.0), 2 mM NaCl, 0.05 mM DTT, and 0.05 mM MgCl$_2$. TBC1D20-$\Delta$305 used for FTIR measurements was kept in a higher concentrated buffer to avoid precipitation: 5 mM Hepes (pH 7.0), 20 mM NaCl, 1 mM DTT. The sample was prepared between two CaF$_2$ windows as described (44). The final sample composition was 5.6 mM Rab1b, 6.1 mM TBC1D20, 20 mM MgCl$_2$, 20 mM DTT, and 200 mM Hepes (pH 7.0) for the 1:1 complex measurements. Photolysis of the caged compounds was
performed by an LPX 240 XeCl excimer laser (308 nm, Lambda Physics) by 12 flashes within 24 ms. A modified Bruker IFS 66/sV spectrometer in the fast-scan mode was used for the measurement (45). The data were analyzed between 1,800 and 950 cm⁻¹ with a global fit method (46). Further details of sample composition, measurement conditions, and fit equations are given in SI Materials and Methods.


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