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# Common mechanisms of catalysis in small and heterotrimeric GTPases and their respective GAPs

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**Abstract:** GTPases are central switches in cells. Their dysfunctions are involved in severe diseases. The small GTPase Ras regulates cell growth, differentiation and apoptosis by transmitting external signals to the nucleus. In one group of oncogenic mutations, the 'switch-off' reaction is inhibited, leading to persistent activation of the signaling pathway. The switch reaction is regulated by GTPase-activating proteins (GAPs), which catalyze GTP hydrolysis in Ras, and by guanine nucleotide exchange factors, which catalyze the exchange of GDP for GTP. Heterotrimeric G-proteins are activated by G-protein coupled receptors and are inactivated by GTP hydrolysis in the Gα subunit. Their GAPs are called regulators of G-protein signaling. In the same way that Ras serves as a prototype for small GTPases, $G\alpha_{i1}$ is the most well-studied Gα subunit. By utilizing X-ray structural models, time-resolved infrared-difference spectroscopy, and biomolecular simulations, we elucidated the detailed molecular reaction mechanism of the GTP hydrolysis in Ras and $G\alpha_{i1}$. In both proteins, the charge distribution of GTP is driven towards the transition state, and an arginine is precisely positioned to facilitate nucleophilic attack of water. In addition to these mechanistic details of GTP hydrolysis, Ras dimerization as an emerging factor in signal transduction is discussed in this review.

**Keywords:** biomolecular simulations; FTIR spectroscopy; heterotrimeric GTPases; Ras; reaction mechanism; small GTPases.

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## Introduction: small and heterotrimeric GTPases

The overall structure of and common motifs present in small and heterotrimeric GTPases have been previously discussed in several excellent reviews (Hamm, 1998; Vetter and Wittinghofer, 2001; Wennerberg et al., 2005; Wittinghofer and Vetter, 2011; Ligeti et al., 2012; Cherfils and Zeghouf, 2013; Wittinghofer, 2014a,b; Carvalho et al., 2015; Lu et al., 2016; Mishra and Lambright, 2016; Sprang, 2016; Syrovatkina et al., 2016). Here, we will focus mainly on the results obtained from the orchestration of time-resolved Fourier transform infrared (FTIR) difference-spectroscopy experiments and biomolecular simulations. These studies were performed within the collaborative research center 642 of the Deutsche Forschungsgemeinschaft (DFG), and the experimental methods were previously described in detail in Kötting and Gerwert (2015).

GTPases are molecular switches that regulate a plethora of cellular events (Brunsveld et al., 2006; Cherfils and Zeghouf, 2013). The signaling state is encoded into surface alterations of the GTPase that are determined by the presence of the third phosphate group of GTP (Wittinghofer and Vetter, 2011). GTP hydrolysis, which switches the signal off, has been the focus of extensive research spanning dozens of years. When this reaction occurs in water, it is very slow, with a half-life of about 60 days at 37°C (Kötting and Gerwert, 2004). However, a typical small GTPase, such as Ras, catalyzes GTP hydrolysis with an efficiency that exceeds this by five orders of magnitude, to $t_{1/2} = 25$ min at 37°C (Tucker et al., 1986; Neal et al., 1990). GTPase-activating proteins (GAPs) can further accelerate hydrolysis by forming a protein-protein complex with the GTPase; in the case of GAP-Ras, the hydrolysis is catalyzed by another five orders of magnitude, down to $t_{1/2} = 36$ ms at 25°C (Gideon et al., 1992).

In contrast, the Gα subunits of heterotrimeric GTPases can hydrolyze GTP in seconds, which is much faster than the reaction mediated by small GTPases (Linder et al., 1990). Many Gα subunits are also catalyzed by GAPs, known as regulators of G-protein signaling (RGS) proteins,

and these promote reaction rates similar to those of the Ras-GAP complex. Importantly, mutations that interfere with GTP hydrolysis are frequent in various tumors (Pylayeva-Gupta et al., 2011; O'Hayre et al., 2013) and fibrous dysplasia (Dumitrescu and Collins, 2008). Other diseases result from modifications of catalytic residues within the binding pocket, such as ADP-ribosylation of Gα subunits by cholera toxin (Northup et al., 1980).

All GTPases share a G-domain with five α-helices and a six-stranded β-sheet. They form the two large families described above, the heterotrimeric GTPases (Sprang, 2016) and the small GTPases (Mishra and Lambright, 2016), as well as the dynamin superfamily (Daumke and Praefcke, 2016) and translational GTPases (Maracci and Rodnina, 2016). The small GTPases are grouped into five subfamilies: Ras GTPases are mainly involved in cell growth (Cox and Der, 2010), Rho GTPases function in the regulation of the cytoskeleton (Sit and Manser, 2011), Rab (Stenmark, 2009) and Arf (Khan and Ménétrey, 2013) regulate vesicular transport, and Ran (Jamali et al., 2011) controls nuclear transport. In contrast, heterotrimeric G-proteins are involved in mediating vision, smell, or regulation of the cAMP second messenger system, that modulates among others the blood pressure (Walland, 1975). To date, several different α-, β- and γ-subunits, that can form a heterotrimer, are known, most prominently $G\alpha_t$, which mediates vision, and $G\alpha_i$ and $G\alpha_s$, which inhibit and stimulate adenylate cyclase, respectively. The Gα subunit, the actual GTPase, consists of a so-called Ras-like domain with high structural similarity to Ras proteins, and in addition a helical all-α domain (Figure 1A). Several conserved active site elements catalyze GTP hydrolysis in small and heterotrimeric GTPases. A conserved Gln/Asn coordinates the attacking water molecule and the charge distribution of the substrate is shifted, mainly by a bound $Mg^{2+}$-ion, a conserved lysine (GxxxGKS/T motif) and an arginine 'finger' (Figure 1B). In this article, several findings on how this active site elements catalyze GTP hydrolysis are summarized.

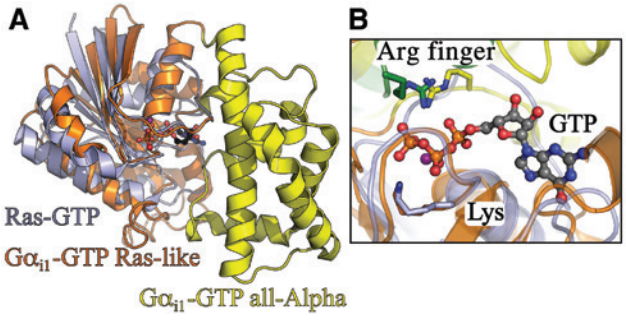


**Figure 1:** Structural similarities between Ras and $G\alpha_{i1}$.
(A) The Gα subunit of heterotrimeric G-proteins consists of a Ras-like domain (orange) and in addition a helical all-Alpha domain (yellow). Ras is shown in blue. (B) The active sites of the Ras · GAP and $G\alpha_{i1}$ · RGS protein complexes are shown. The active sites share several highly conserved structural elements, most prominently a conserved lysine in the P-loop and the arginine 'finger', which is a residue introduced by the GAP in case of Ras but an intrinsic residue from the all-Alpha domain in $G\alpha_{i1}$, allosterically positioned by RGS.

## GTP hydrolysis in Ras

GTP hydrolysis alone is a slow process with a high-energy transition state. The attacking water molecule must cross the negatively charged oxygen atoms for an in-line nucleophilic attack at γ-GTP. The GTPase active site contains various specific amino acids that function to compensate charges on the GTP molecule, coordinate the $Mg^{2+}$ atom between β-GTP and γ-GTP, and position the attacking water molecule. Two conserved residues in particular, the Lys16 in the P-loop and the arginine 'finger', that is supplied by the GAP, alter the charge distribution of β-GTP and γ-GTP (Li and Zhang, 2004).

Ras is involved in many signal transduction processes (Cox and Der, 2010; Wittinghofer and Vetter, 2011) and can be activated by its guanine nucleotide exchange factor (GEF) son of sevenless (SOS), which catalyzes the exchange of GDP for GTP. SOS reduces the affinity of Ras for the nucleotide and the bound $Mg^{2+}$ ion (Gureasko et al., 2010), allowing the exchange of GDP (off) for GTP (on) and thus, turning the switch on (Boriack-Sjodin et al., 1998). In the GTP bound 'on' state, Ras interacts with downstream effector proteins, transducing the signal mostly *via* the MAPK pathway to the nucleus. The switch-off is mediated by hydrolysis of GTP to GDP, and this is accelerated and thereby regulated by GAPs.

The detailed reaction mechanisms for Ras and the Ras-GAP complex have been elucidated by time-resolved-FTIR difference-spectroscopy (Cepus et al., 1998; Kötting et al., 2008). Integration of results from X-ray crystallography structures and FTIR spectroscopy experiments (Pai, 1990; Scheffzek et al., 1997) provides not only a static picture of the ground state, but also a 'movie' of the reaction mechanism at subatomic detail (available at www.bph.rub.de/movies). Specifically, during the hydrolysis reaction, three apparent rate constants were observed in the Ras-GAP complex by time-resolved FTIR difference spectroscopy, as summarized in Figure 2. To reveal the reactions of individual protein and substrate groups in the catalytic center, difference spectra between the initial-state GTP and the

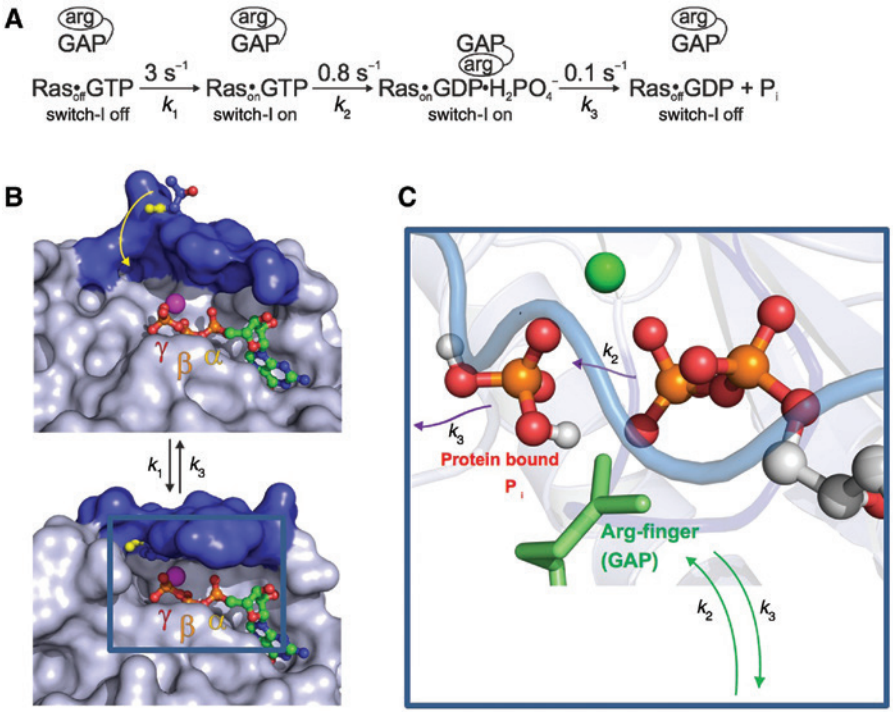


**Figure 2:** The Ras · GAP GTPase reaction.
(A) The reaction mechanism can be described by three apparent rate constants; the shift to the 'on' conformation ($k_1$), the movement of the Arg-finger with immediate hydrolysis of GTP ($k_2$), and the release of $P_i$ into the bulk solvent ($k_3$). Note that the given rate constants were measured at 260 K. (B) With $k_1$, the signal transducing 'on' state is formed by the switch-I movement, closing the binding site. Only with the last apparent rate, $k_3$, does the protein return to the 'off' state. (C) The Arg-finger moves into the binding pocket with $k_2$. With the same rate, $k_2$, bond breakage is observed, and protein-bound $H_2PO_4^-$ is formed. In the rate-limiting step, $k_3$, $H_2PO_4^-$ is released from the protein into the bulk solvent.

reaction intermediates were calculated. In this way, the large background absorbance of all non-contributing protein groups and the solvent is subtracted.

In FTIR experiments, to trigger the reaction *in situ*, caged GTP (*cg*GTP) is bound to Ras and then uncaged to produce GTP by a short laser flash (Cepus et al., 1998). Thereby all Ras proteins start the GTPase reaction in a highly synchronized manner with their natural substrate GTP (Kötting and Gerwert, 2015). In the first step (with apparent rate constant $k_1$), the absorbance change of the switch-I marker band indicates the 'off' to 'on' conformational change after uncaging Ras-*cg*GTP to Ras GTP. In the second step (with apparent rate constant $k_2$), the arginine finger moves into the catalytic site to complete the formation of the active site for catalysis of GTP hydrolysis (Kötting et al., 2008). Concurrently, cleavage of the γ-phosphate bond occurs, and protein-bound $P_i$ is formed as $H_2PO_4^-$ (Figure 2C) (Kötting et al., 2006; Xia et al., 2012). In the last step (with apparent rate constant $k_3$), the protein-bound phosphate is released into the bulk solvent, the switch-I returns to the 'off' state, and the arginine finger leaves the binding pocket again. Thus, time-resolved FTIR spectroscopy demonstrated, under near-physiological conditions, that in contrast to the transition state analog, the arginine finger is not located within the GTP binding pocket in the ground state (Scheffzek et al., 1997). This confirmed the model from X-ray structural models of the small GTPase Rho in complex with RhoGAP, where the transition state analog showed the GAP arginine interacting with the nucleotide, whereas in the ground state, the arginine was not in the active site (Rittinger et al., 1997a,b). The movement of the arginine finger into the catalytic site could be observed directly in $k_2$, immediately followed by bond cleavage. Further, Ras returned to the 'off' state after $P_i$ was released into the bulk solvent, making $P_i$ release the rate-limiting step in Ras-catalyzed GTP hydrolysis.

Structural details and charge distribution of Ras-bound GTP were further revealed by integration of the experimental data with biomolecular simulations (Rudack et al., 2012). Specifically, the detailed molecular information encoded in the FTIR difference spectra of GTP can be decoded using quantum mechanics/molecular mechanics (QM/MM) simulations. As validation of the simulation accuracy, the agreement between the experimental and simulated infrared

(IR)-GTP spectra at the catalytic center was used as very sensitive measure. Based on their excellent agreement, further detailed conclusions were possible. These provide profound and detailed insights, which are summarized in Figure 3. Here, we show the nucleotide GTP in water, GTP bound to Ras, and GTP bound to the Ras-GAP complex. Protein binding induces catalytically important alterations in GTP, and both the charge distribution and geometry are shifted towards the transition state. That is, the charge of the γ-phosphorous atom becomes more positive, facilitating the nucleophilic attack of the water molecule. Additionally, the distance between the β- and γ-phosphorus atoms increases. Most remarkably, a conformational change in the phosphate groups, from a staggered to an eclipsed conformation, induces strain in the GTP molecule. These alterations in the reactant GTP also drive it energetically closer to the transition state and, in turn, lower the activation barrier for GTP hydrolysis (Figure 4) (Kötting and Gerwert, 2004; Kötting et al., 2008). The high intrinsic binding energy of GTP overcompensates for the energy-costly process of inducing these changes (Jencks, 2006).

With this elucidation of the catalytic details for the wild-type protein, it will next be important to investigate the catalytic mechanism in GTPases containing oncogenic mutations and, specifically, how these alterations slow the reaction. In this context, the role of the nucleophilic attacking water molecules in particular will be elucidated in detail.

## GTP hydrolysis in heterotrimeric GTPases

Heterotrimeric G-proteins are involved in numerous physiological processes, such as vision, scent, and blood pressure regulation (Malbon, 2005). They consist of an α-, β- and γ-subunit. The Gα-subunit contains the G-domain, which is conserved among GTPases, as well as a helical all-α domain. The latter also contributes the arginine finger, which in case of Ras, is supplied by the GAP. During the off-switch reaction, Gα is not bound to the βγ subunits, and thus GTP hydrolysis can be investigated with Gα alone (Coleman et al., 1994). The GEFs associated with heterotrimeric G-proteins are usually G-protein coupled receptors (GPCRs), which catalyze the exchange from GDP to GTP at the Gα subunit and are currently the focus of investigations by numerous research groups worldwide. A seminal advancement in this field was the elucidation of the β-adrenergic receptor in complex with the G protein (Rasmussen et al., 2011). GPCR signaling pathways are important targets in pharmacology. However, not only dysfunctions in GPCRs, but also in heterotrimeric G-proteins, are responsible for a number of serious diseases (O'Hayre et al., 2013).

In Gα proteins, the nucleotide exchange is much slower than the hydrolysis reaction, and thus, multiple turnover measurements consisting of numerous nucleotide exchanges with subsequent hydrolysis almost exclusively probe the nucleotide exchange. This prevents a detailed study of the hydrolysis reaction by multiple

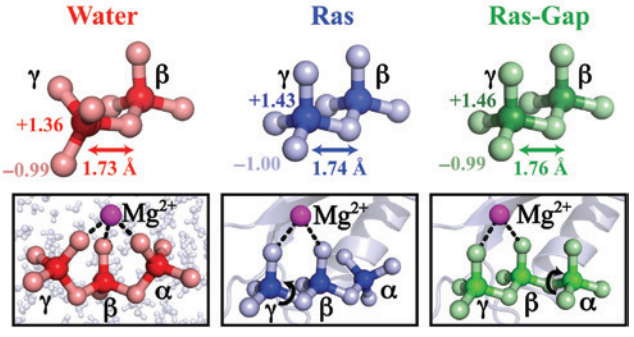


**Figure 3:** Comparison of the detailed structures and charge distributions of GTP free in water, bound to Ras, and in the Ras-GAP complex, as obtained by an integration of experimental FTIR spectroscopy and biomolecular simulations (Rudack et al., 2012).

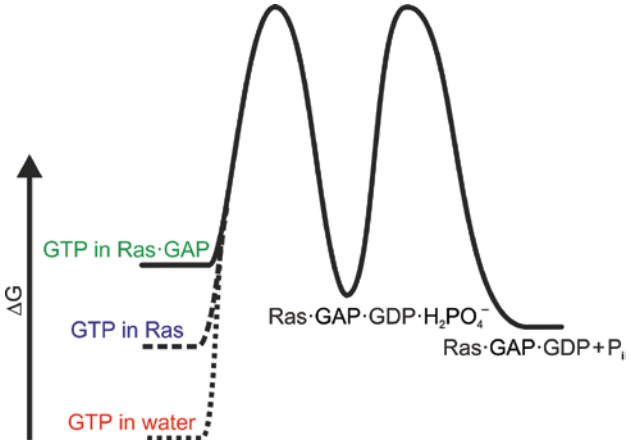


**Figure 4:** The free energy during GTP hydrolysis within the Ras-GAP complex (Kötting et al., 2008).
The structural changes and charge distributions of GTP in water, bound to Ras, and in the Ras-GAP complex are shown in Figure 3. These shift GTP in Ras and Ras-GAP closer to the transition state for bond cleavage, as indicated here. Note that the dotted lines for the smaller systems indicate their positions relative to the first transition state only, neglecting the energy for complex formation. The subsequent reaction profile is only valid for the Ras · GAP system. Formally, this situation corresponds to a preferential binding of the transition state over the ground state within the protein (Roston and Cui, 2016).

turnover assays; for example, the influence of site-directed mutants on the hydrolysis reaction cannot be studied. Consequently, time-resolved FTIR measurements are especially valuable, as they provide unambiguous single turnover reaction rates for the hydrolysis reaction.

We investigated the kinetics of nucleotide exchange and of hydrolysis in $G\alpha_{i1}$ separately, using a fluorescence assay for the exchange reaction and time-resolved FTIR difference spectroscopy for the hydrolysis reaction (Schröter et al., 2015). In Figure 5, we show that a mutation of the intrinsic arginine finger, R178S, does not alter the nucleotide exchange rate, but rather slows hydrolysis by two orders of magnitude (O'Hayre et al., 2013). This could not be deduced from multiple turnover measurements where the reaction is barely slowed. Conversely, the mutant D272N lacks an interaction between the G-domain and the base of the nucleotide, which accelerates the nucleotide exchange kinetics, while the hydrolysis kinetics remain unaltered, as demonstrated by single-turnover FTIR measurements. By integrating time-resolved FTIR difference spectroscopy and molecular dynamics (MD) simulations, new details of the nucleotide exchange reaction can be elucidated. For example, we see that residue D229 positions R242 of the Ras-like G-domain, enabling a stable hydrogen bond to Q147 in the all-α domain. Further, we observe that D231 forms a hydrogen bond to R144. Thereby a detailed picture of the inter-domain coordination, that regulates the nucleotide exchange, emerges (Schröter et al., 2015).

Single turnover measurements of the RGS4-catalyzed GTPase reaction in $G\alpha_{i1}$ using time-resolved FTIR spectroscopy revealed an acceleration of about two orders of magnitude, as compared to the intrinsic reaction. Further integration of these data with biomolecular simulations decoded the IR-spectra and provided an RGS4 catalysis mechanism at subatomic resolution. In particular, the role of the catalytic intrinsic arginine finger was elucidated and found to be in a similar position as the arginine finger provided externally by Ras · GAP. The most striking feature is the different role of the intrinsic arginine finger with, and without, the RGS protein. In the X-ray structural models, the nucleotide is substituted by GTPγS or GDP · AlFx (Coleman et al., 1994; Kaya et al., 2016), and the position of the arginine is artificially altered due to the presence of the γS and $AlF_4^-$ analogs. In contrast to this, QM/MM calculations of $G\alpha_{i1}$ with the natural nucleotide GTP show a direct hydrogen bond to the γ-phosphate (Figure 6A) (Mann et al., 2016). This interaction was further confirmed experimentally by FTIR spectroscopy: A mutation deleting this hydrogen bond leads to a blue-shift of the absorption band of the γ-phosphate only, whereas the α- and β-phosphate bands are unchanged. Unlike Ras · GAP, RGS has no direct

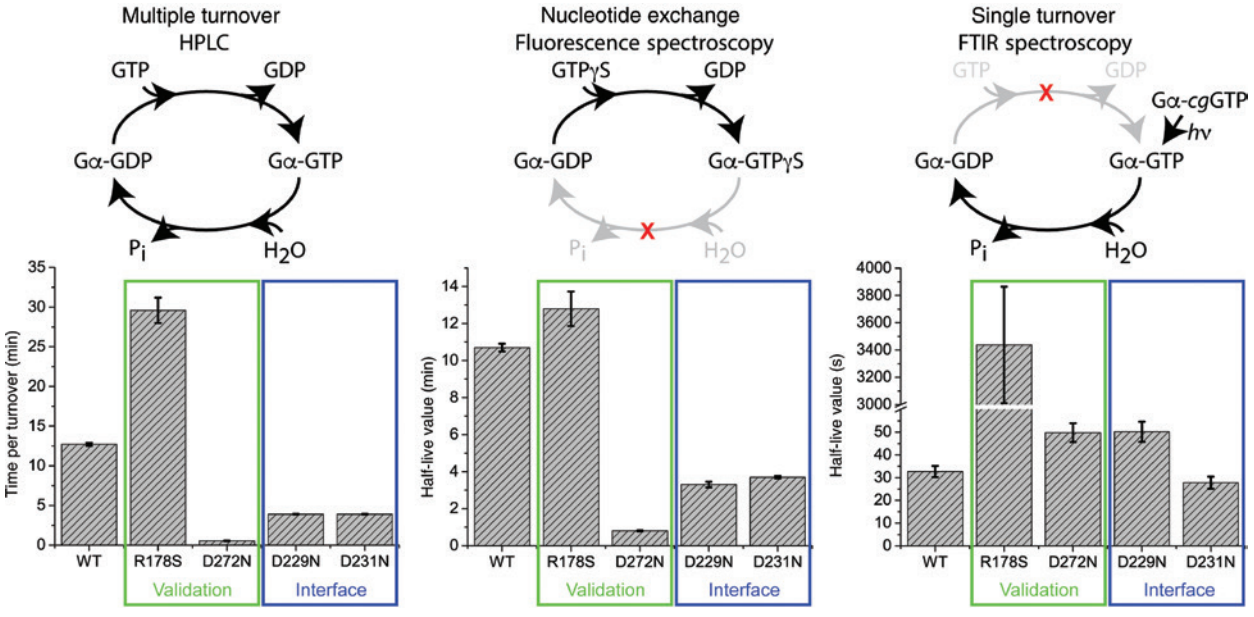


**Figure 5:** Multiple turnover kinetics of GDP to GTP exchange and GTP hydrolysis under steady-state conditions in $G\alpha_{i1}$ (left), and the underlying kinetics of single turnover nucleotide exchange (middle) and single turnover GTP hydrolysis (right).
For validation, the arginine finger which effects hydrolysis only, and Asp272 that coordinates GTP that effects nucleotide exchange were mutated (green box). Furthermore, residues at the Ras-like/all-alpha interface were investigated (blue box). They play a role in nucleotide exchange.

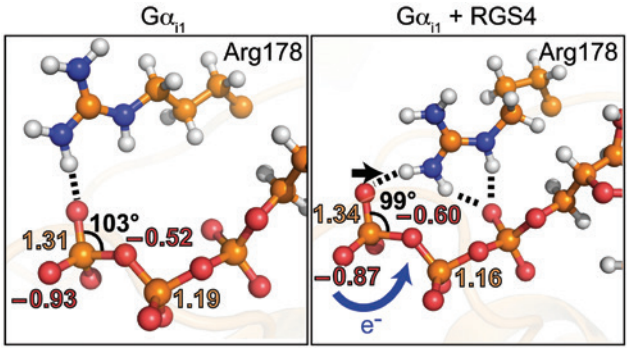


**Figure 6:** The role of the intrinsic arginine finger of $G\alpha_{i1}$ with, and without, RGS4.

contact with the phosphate groups. Integration of QM/MM calculations and FTIR spectroscopy further revealed that RGS catalysis is achieved through the intrinsic arginine finger of $G\alpha_{i1}$. RGS pushes the arginine finger into a bidentate coordination of the GTP with two additional hydrogen bonds to the α-phosphate (Figure 6B). A similar effect of the arginine finger on the α-phosphate is also seen in Ras. The bidentate coordination shifts the terminal $PO_3$ of the γ-phosphate to a more planar conformation, closer to the transition state of the nucleophilic attack of water at the γ-phosphate. Notably, the planar structure of the $G\alpha_{i1}$ transition state is also observed with the analog GDP · AlFx (Tesmer et al., 1997). Further, as in Ras, the negative charge is transferred towards the β-GTP, inducing a more product-like charge distribution. Whereas in GTP the formal charge of the β-GTP is –1, for β-GDP, it is –2. The more dissociative the mechanism, the closer the charge distribution of the transition state is to that of the product (Carvalho et al., 2015). The α-phosphate is turned into an eclipsed conformation relative to the β-GTP *via* close coordination between the arginine finger and Thr48. As similarly noted for the Ras · GAP system, this induces strain that can facilitate bond breakage (Rudack et al., 2012).

An advantage of the simultaneous investigation of small and heterotrimeric GTPases is that the presence of different rate-limiting steps can help to elucidate common mechanisms, which are not resolved in the other system. For example, although it is not possible to measure coupling of the arginine finger to GTP in the Ras-GAP system, this was nicely resolved in the $G\alpha_{i1}$ system (Mann et al., 2016). Similarly, mutation of Lys16, which is found in the conserved GTPase GxxxxGKS/T motif, was not possible in small GTPases due to the fact that mutated proteins were unstable (Du and Sprang, 2009). However, this mutation

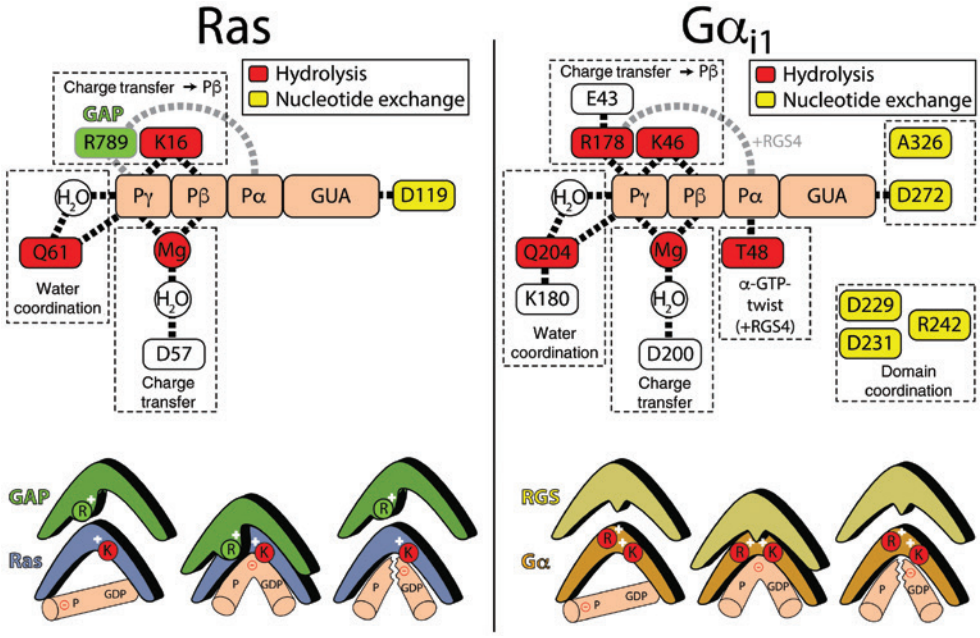


**Figure 7:** Schematic mechanism for GTP hydrolysis in Ras and $G\alpha_{i1}$.
The most important residues for GTP hydrolysis (red or green) and nucleotide exchange (yellow) are given. The conserved lysine and the arginine finger transfer charges toward β-GTP and thereby facilitate GTP hydrolysis. The arginine finger induces a twist at α-GTP, in case of $G\alpha_{i1}$ aided by Thr48. Further charge transfer is caused by $Mg^{2+}$, which is precisely positioned *via* a conserved aspartic acid. Furthermore, the nucleophilic attacking water molecule is held in position by a conserved glutamine. Residues that affect the nucleotide exchange, either by direct binding or allosterically, are highlighted in yellow. Charge shifts and geometry twisting induce strain in GTP, facilitating GTP hydrolysis.

could be generated in $G\alpha_{i1}$, because the binding pocket is more closed to the outside. Measurement of FTIR spectra for this mutant, which were subsequently decoded by QM/MM calculations, elucidated the role of the conserved lysine. Specifically, this residue shifts negative charges from γ-GTP to β-GTP and thereby facilitates GTP hydrolysis similarly to the arginine finger, with the mutant showing similarly slowed hydrolysis kinetics (Mann et al., 2017). In conclusion, these findings reveal novel intrinsic and GAP-catalyzed hydrolysis mechanisms for G-proteins at subatomic resolution (Figure 7).

## Membrane binding of Ras

Ras is a peripheral membrane protein that is attached to the membrane by lipid anchors. Four isoforms (H-Ras, N-Ras, K-Ras-4A, and K-Ras-4B) each contain a distinct anchor, which regulates membrane targeting (Rocks et al., 2005; Brunsveld et al., 2006; Ahearn et al., 2011; Tsai et al., 2015), and this specific Ras localization is also an essential factor for signaling. Similar to the way in which GDP-dissociation inhibitors (GDIs) influence Rho and Rab function, Ras localization can be influenced by the prenyl-binding protein PDEδ (Chandra et al., 2012), which binds to Ras, preventing membrane binding. Localization can also be altered *via* the cleavage of one (N-Ras, K-Ras-4A) or two (H-Ras) lipid anchors or by phosphorylation (K-Ras-4B) (Ahearn et al., 2011; Schmick et al., 2015).

It was shown more than 10 years ago that Ras can form nanoclusters (Hancock and Parton, 2005), and recently, we demonstrated dimerization of N-Ras at a 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) model membrane, suggesting that Ras plays an active role in this nanoclustering (Güldenhaupt et al., 2012). Specifically, through the integration of attenuated total reflection (ATR)-FTIR spectroscopy, MD simulations, and fluorescence resonance energy transfer (FRET) experiments, we showed for the first time that N-Ras dimerizes at the membrane (Figure 8), and several investigations on Ras dimerization have been published since then. For example, the Groves group found that H-Ras forms dimers on membrane surfaces (Lin et al., 2014); however, these dimers may be induced by a photochemical reaction within the confocal fluorescence microscope (Chung et al., 2016). Evidence for dimer formation by K-Ras was also obtained using super-resolution fluorescence microscopy (Nan et al., 2013, 2015), even though the distance between the monomers remains unclear and the dimerization might be influenced by the label, i.e. mCherry alone tethered to the membrane with the K-Ras tail behaved in exactly the same way as did mCherry-KRas. However, these studies showed that Ras dimerization activates the MAPK pathway and therefore likely plays an important role in signal transduction. Similarly, a role for dimerization and nanocluster formation in signaling (Solman et al., 2015; Roob et al., 2016), possibly due to orientation changes of the G-domain (Mazhab-Jafari et al., 2015), was also reported by other groups. Specifically, Muratcioglu et al. (2015) reported that K-Ras dimer formation is nucleotide dependent using dynamic light scattering and nuclear magnetic resonance (NMR) spectroscopy. Of note, this dimer formation by the Ras proteins provides a novel drug target (Santos, 2014;

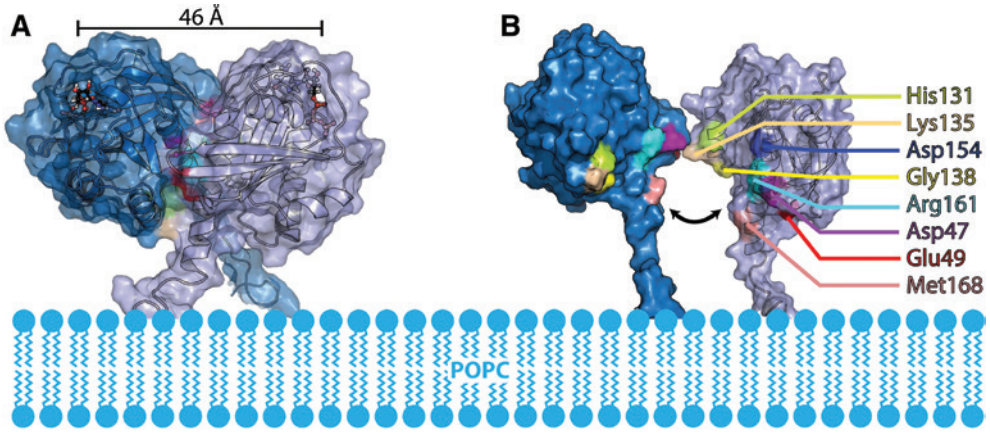


**Figure 8:** N-Ras dimers at a POPC model membrane.
(A) The N-Ras dimer with an interface consisting of residues from the β2, β3 loop, helix 4, and helix 5 as obtained by MD simulations (Güldenhaupt et al., 2012). The distance between the fluorophores in the simulations agrees nicely with the experimental FRET-determined distance. (B) The proposed dimer interface with coordinating residues as obtained by MD simulations. A similar interface was proposed for K-Ras (Spencer-Smith et al., 2016; Prakash et al., 2017).

Stephen et al., 2014; Chen et al., 2016; Lu et al., 2016), and elucidating the dimer interface at atomic detail will facilitate therapeutic approaches that modulate this interaction.

In 2012, we proposed a model for the N-Ras dimer interface based on our experimental findings (Güldenhaupt et al., 2012). Subsequently, several Ras dimer interfaces, both similar and different to ours, were proposed based on MD simulations, mainly for K-Ras (Jang et al., 2016; Prakash et al., 2016; Sayyed-Ahmad et al., 2016), and currently, there is no consensus on the detailed dimer interface. Some proposals are questionable from a physiological point of view; for example, dimerization including the effector domain (Muratcioglu et al., 2015) would not lead to the observed increased signaling (Nan et al., 2013, 2015). While measurements in cells have revealed that Ras forms nanoclusters and/or dimers, and FRET between Ras molecules is in agreement with this (Solman et al., 2015), a detailed investigation of the interaction remains challenging. In addition, proteins like galectin seem to interact with Ras and play a role in nanocluster formation in the cell (Prior et al., 2003). However, it remains unclear whether this is primarily due to the general effects of galectin on the membrane or to a specific galectin-Ras interaction. Notably, a recent report describes the direct interaction of galectin-1 dimers with Raf effectors, indicating an indirect interaction with Ras (Blaževitš et al., 2016). Further, the GEF SOS binds two Ras molecules (Margarit et al., 2003; Sondermann et al., 2004; Gureasko et al., 2008), and the effector BRAF dimerizes and also potentially binds to two Ras molecules (Poulikakos et al., 2010). In both cases, the two Ras molecules are in a distance enabling FRET without any direct Ras-Ras interaction. Consequently, it is difficult to prove direct Ras-Ras interactions in cells. Recently, an NMR study did not detect dimerization using the G-domain without the anchor region and without a bilayer (Kovrigina et al., 2015). This indicates that interactions within the hypervariable region (HVR) and/or membrane attachment anchor are required for dimerization. The latter can possibly promote a preorientation that leads to an entropic benefit for dimerization, similar to a crosslink (Zaman et al., 2002). On the other hand, dynamic light scattering experiments indicate dimerization for the Ras G-domain alone when bound to GTP-γ-S (Muratcioglu et al., 2015). As H-, N- and K-Ras have differences in their helix 4 and helix 5 regions, it is possible that these lead to the variable behaviors observed for the different isoforms (Parker and Mattos, 2015). Thus, the unambiguous characterization of the correct dimerization interface is still challenging.

In summary, integration of FTIR difference spectroscopy and biomolecular simulations provides a detailed insight into Ras and $G\alpha_{i1}$ catalysis by their respective GAPs *via* protein-protein interactions and common features have been elucidated. Using the ATR technique further allows us to study the role of membrane binding of Ras. This opens the door for reconstitution experiments at the membrane in a near native but accurately defined system.

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