Allostery and dynamics in small G proteins

Helen R. Mott* & Darerca Owen

Department of Biochemistry
80, Tennis Court Road
Cambridge
CB2 1GA

*Correspondence to hrm28@cam.ac.uk

Abstract

The Ras family of small guanine-nucleotide binding proteins behave as molecular switches: they are switched off and inactive when bound to GDP but can be activated by GTP binding in response to signal transduction pathways. Early structural analysis showed that two regions of the protein, which change conformation depending on the nucleotide present, mediate this switch. A large number of X-ray, NMR and simulation studies have shown that this is an over-simplification. The switch regions themselves are highly dynamic and can exist in distinct sub-states in the GTP-bound form that have different affinities for other proteins. Furthermore, regions outside the switches have been found to be sensitive to the nucleotide state of the protein, indicating that allosteric change is more widespread than previously thought. Taken together, the accrued knowledge about small G protein structures, allostery and dynamics will be essential for design and testing of the next generation of inhibitors, both orthosteric and allosteric, as well as for understanding their mode of action.

Introduction

Small G proteins of the Ras superfamily are essential components in almost all aspects of cell biology and since they were discovered their structures and functions have been intensively studied. One of their most important features is their ability to exchange GDP for GTP, often mediated by exchange factors (GEFs) that are activated by upstream signals. In their GTP-bound form, the G proteins selectively bind to downstream effector proteins that execute the signalling outcomes, and to GTPase activating proteins (GAPs) that switch off the signal by stimulating the intrinsic GTPase activity.

The structure of Ras was solved almost 30 years ago[1,2] and this has been followed by structures of representatives of all five families within this large superfamily. All of these proteins have the same canonical architecture, comprising a Rossmann-fold like structure with a parallel β-sheet surrounded by α-helices and an extra, anti-parallel two-stranded β-sheet on one side (Figure 1). Some of the families are decorated with extra structural features: the Rho family includes an insertion, which forms extra pair of helices within the G domain itself, (Figure 1C, D) while Arf has an N-terminal helix and Ran has a C-terminal helix. The Arf and Ran helices are both packed against the G domain in the inactive, GDP-bound form and are released when GTP binds, allowing them to be involved in interaction with other molecules.

Most of the biochemical and biophysical studies have concentrated on just a handful of the large number of Ras superfamily members. As more proteins have been identified and characterised it has become clear that some Rho family members are atypical: they do not undergo cycling and they are likely to be regulated by other mechanisms (reviewed in [3]). Here, we will concentrate on the Ras and typical Rho families, summarising the findings of a large number of studies investigating dynamics and allostery within the G domain. For brevity, we will not discuss dynamics of the C-terminal extensions in these proteins.

Dynamics in the Switch Regions

The Ras family

Early on it became clear that, as the small G proteins are sensitive to the state of the bound nucleotide, there must be some conformational changes when GTP replaces GDP. Comparison of the Ras structures in the GDP-bound and GTP-bound forms gave some insight into the nucleotide sensitivity, disclosing two regions, known as switch 1 and switch 2, whose position shifted (Figure 1A, B). The backbone amides of two conserved residues, Thr35 (switch 1) and Gly60 (switch 2), form hydrogen bonds with the γ-phosphate, which is only present in the GTP-bound form. These contacts are sufficient to pull the rest of the switch residues into new positions, which are competent to interact with effectors and GAPs. This simple picture of the active form of the G protein has been described as a 'loaded spring' to reflect the fact that this form is of a higher energy and is presumed to be relatively rigid[4].

The propensity for allostery in the Ras switch regions was therefore obvious from the earliest X-ray structures. It was also clear that the switch regions often exhibited low electron density, indicating that they were dynamic in the crystals even in a single nucleotide state. Indeed, some parts of the switches were missing density altogether so that the switches were often only fully visible when they were involved in crystal contacts.

Direct evidence for the different conformations in the Ras switch regions came from ^{31}P NMR spectra recorded at low temperatures, which revealed the presence of two conformational states for the three phosphate resonances in H-Ras bound to GMPPNP[5], a GTP analogue, indicated by the presence of two peaks corresponding to each phosphorus atom. These were arbitrarily named state 1 (the left peak) and state 2 (the right peak).

Titrations with Raf showed that state 2 was stabilised in the presence of the effector, suggesting that this is the active conformation, while GAP interacted preferentially with state 1. It was suggested that the origin of these two states resides in the orientation of Tyr32, a conserved residue in switch 1, with respect to the phosphates. As discussed below, this simple explanation was shown to be incorrect by a number of experimental structures that have subsequently been determined.

A solution structure of H-Ras·GDP[6] showed that residues 30-38 and 58-66 were poorly defined by the NMR restraints. NMR uniquely yields information on dynamics of individual residues in proteins, and analysis of of H-Ras·GDP revealed that 27-32 and 58-66 were indeed moving rapidly on the nsec timescale. Motion on this timescale meant that the cross peaks for the backbone amides of these residues were very sharp in the ^{15}N HSQC NMR spectrum of Ras·GDP. Interestingly, experiments recorded on the GMPPNP-bound form of H-Ras showed that a large number of cross peaks were missing in the ^{15}N HSQC spectrum[7]. These missing peaks corresponded to residues in the P-loop (or phosphate-binding loop), switch 1 and switch 2, which suggested that these regions were undergoing conformational exchange between two (or more) states whose exchange rates and frequency differences made them invisible in the NMR spectra. This was supported by subsequent NMR experiments designed to probe the existence of invisible states[8] and the observation that peaks for residues around the missing regions were selectively more intense in spectra recorded at high temperature. This indicates that they are also subject to exchange but the chemical shift differences between the two states are smaller so that only one species is visible. Analysis of the dynamics showed that some of the residues close to switches 1 and 2 are involved in rapid internal motion (psec-nsec timescales) while others close to the P-loop and switch 2 exhibit slower motion in the msec timescale range[7]. The authors suggested that the Tyr32 side-chain orientation is only a subprocess of a more substantial 'regional polysterism' in the Ras protein. This has been shown to be the case as more structures have been solved (Figure 2).

Interestingly, NMR spectra recorded with H-Ras bound to GTPγS or to GTP showed that the rate of exchange between the two forms increased as the nucleotide became more native *i.e.* more peaks were observed when GMPPNP was replaced by GTPγS and more again when the latter was replaced by GTP. This was supported by the ^{31}P NMR, which showed only one

species for the GTPγS and GTP forms[5], which are dominated by state 2[9]. It is thought that the multiple species exist in all the active forms but that the exchange between them varies with the nucleotide analogue used. Nevertheless, the affinities of GMPPNP and GTPγS-bound Ras for the effector Raf are the same[9]. These were, however, measured by an equilibrium method (isothermal titration calorimetry) that would not uncover any differences in the *kinetics* of binding, which is more likely to be affected by the exchange rates between states. It is clear however, that the different nucleotide analogues do affect the interaction of Ras with Raf, since the enthalpic and entropic changes upon binding vary when different analogues are used[9].

A study of M-Ras showed that, while the M-Ras·GDP structure looked just like H-Ras, in M-Ras·GMPPNP switch 1 adopts an unusual conformation: the Thr at position 45 (equivalent to Thr35 in H-Ras) does not coordinate the Mg^{2+} in the structure and instead there is a water molecule present[10]. This results in all of switch 1 being pulled away from the nucleotide and a change in the orientation of the switch 2 helix. Low temperature ^{31}P NMR was performed on the M-Ras·GMPPNP form and it was found that there was only one state present at low temperatures. This was assumed to be state 1, because the addition of Raf caused the appearance of another species that, for the γ phosphate at least, was the only one visible in the 1:1 complex. This suggested that in the state 1, inactive form, Thr35 does not contact the Mg^{2+} and the nucleotide (Figure 2A). Interestingly, M-Ras binds more weakly to Raf than H-Ras does, so the higher population of state 1 could be responsible for the lower affinity.

The same group also investigated switch dynamics in Rap1A, Rap2A and RalA[11]. Rap1A/2A switch 1 sequences are identical to those in Ras but RalA is different. In the RalA·GMPPNP structure there is no Thr35-γ phosphate interaction and ^{31}P NMR showed it was mostly in state 1, although addition of the effector Sec5 caused interconversion to state 2. In contrast, Rap1A/2A·GMPPNP were mostly in state 2. The on- and off-rates for GTPγS were measured and it was found that M-Ras and RalA, which are in state 1, had higher k_{on} and k_{off} for nucleotide compared to H-Ras, which is mostly in state 2. This suggested that state 1 binds GTP and that state 2 is then formed by a conformational change. So far it has been assumed that effectors bind to and stabilise state 2. We found however that RalB·GMPPNP has a single set of resonances for the phosphates, which corresponds to state 1[12]. When RalB·GTP was tested there were two sets of peaks as state 2 became populated so, like H-Ras, there is some dependence on the nucleotide/analogue itself. However when Sec5 was added to the

GTP form there were still 2 peaks *i.e.* more than one species was observed even in the presence of effector.

Mutations have been found that affect the relative populations of the two states, for example Ras mutations T35S or T35A in switch 1 stabilize state 1[13] and both mutations lead to dramatic reductions in affinity for the effector Raf. Two structures of T35S H-Ras·GMPPNP[14] gave more credence to the idea that Thr35 and its contacts with the Mg^{2+} ion, either direct or indirect, are central to the transition between state 1 and state 2. The changes in orientation of Thr35 lead to a shift in the position of Y32 position, which causes the small changes in the phosphate positions due to ring current effects. Furthermore, the change in switch 1 seemed to affect switch 2 as well: in one of the two crystal forms Gly60 failed to form hydrogen bonds with the γ-phosphate, while in the other form although the Gly60 interactions were restored, much of the electron density of switch 2 was missing. The dynamics of the switch regions in the T35S mutant was further probed by an NMR study comparing wild-type and T35S H-Ras[15]. T35S H-Ras·GMPPNP NMR peaks were all visible because it is predominantly in state 1, while as previously observed, conformational exchange meant that, in the wild-type protein, the P-loop and the switch regions were partially missing. An analysis of the dynamics of the T35S protein showed that it resembled the GDP form of the wild-type H-Ras rather than the GMPPNP or GTP form: the switch regions were very dynamic on a fast (psec-nsec) timescale. Lowering the temperature to 5 ˚C led to the appearance of 20 new peaks in the wild-type H-Ras·GMPPNP spectra, many of which could be assigned by comparison with spectra of T35S (state 1) or wild-type Ras in complex with Raf (state 2). Interestingly, for some residues in the switch regions there were two distinct species visible in the spectra at 5 ˚C, corresponding to state 1 and state 2, indicating that there was slow exchange between them at this temperature.

Mutations were made in M-Ras that increased the state 2 population and the structure of one of them, P40D/D41E/L51R, showed that state 2 was achieved, with Thr45 interacting with the γ-phosphate[14]. The M-Ras single mutant D41E was solved in two crystal forms, one of which was in state 1 and one in state 2[16]. In the state 1 structure, neither Thr45 nor Gly70 (equivalent to Gly60) interacted with the γ-phosphate. This suggested that there is some interdependence between the switch regions when the conformation moves between state 1 and state 2. Similarly, structural analysis of K-Ras bound to GMPPCP (another GTP analogue) showed that switch 1 is in an open state 1 conformation[17] that is most similar to

T35S H-Ras·GMPPNP. In this structure Thr35 does not contact the γ-phosphate and switch 2 is disordered.

Replica-exchange molecular dynamics performed on H-Ras starting from state 2[18] resulted in two low energy states corresponding to state 1 and state 2. Closer analysis showed that state 2 was actually composed of two sub-states: the major one had Tyr32 pointing towards the GTP (like the starting structure), while in the other Tyr32 was pointing away from the nucleotide. The authors analysed crystal structures with Thr35 contacting the Mg^{2+} ion (*i.e.* they were in state 2) and found that Tyr32 was not always in the same orientation. When Tyr32 was flipped out it is found in a similar position to that in the GAP complex, where Tyr32 has to move out of the way to allow a catalytic Arg residues from the GAP to enter the active site and stimulate GTP hydrolysis.

It is clear therefore that the position of Tyr32, rather than being the origin of state 1 and state 2 as originally thought[5], actually represents different sub-states of state 2. State 1 is instead defined by the lack of contacts between Thr35 and Mg^{2+}.

The Rho family

The dynamics of the switch regions and the presence of at least two states in the active, GTP-analogue bound form are well established for the Ras family. What of the other families within the Ras superfamily? These have been less well studied, mainly because Ras itself is such an important target for disease and most effort has gone into understanding the three Ras isoforms.

Among the Rho family members, the dynamics of Cdc42 is the best studied and the switches seem to have different dynamics in Cdc42 than in Ras[19]. In the GDP form, parts of the switch regions could not be assigned, suggesting that there was conformational change on a msec timescale, unlike in Ras, where only fast motion (psec-nsec) was observed in the GDP form. As in Ras, in the GMPPCP form, there was also msec timescale motion for the switch regions. The dynamics of Cdc42 were also investigated in complex with the binding region of the effector protein, PAK. Overall, switch 1 was disordered in the GDP and GMPPCP forms and even in the PAK complex, although it was reduced in the latter. For those residues that could be assigned the dynamics was fast, on a nsec timescale. Switch 2 was also flexible in all of

the forms but the longer timescale (msec) suggested that it was undergoing conformational exchange. The dynamics in the Cdc42 switch regions in the PAK complex was also apparent when the same group published the solution structure, which showed that the switch regions were poorly defined in the structure[20]. Our own structure of a slightly different fragment of PAK in complex with Cdc42 showed however, that both proteins were more ordered, with only three (non-interacting) residues in switch 2 being poorly defined[21]. This indicates that the switches in Cdc42 can, as in Ras, form a single, rigid conformation when they interact with their binding partners. The NMR studies of Cdc42 with ACK[22] and WASP[23] also showed that the switches rigidified on binding.

The similarity in the dynamics of the GDP and GMPPCP forms observed by NMR was reinforced when the Cdc42·GMPPCP structure was solved[24] and it was found to be identical to the GDP structure. This raises the question as to how effectors such as PAK can recognise the active form of Cdc42. The authors suggested that effectors cause conformational rearrangement of switch 1, which is secured by interactions between Tyr32 and the γ-phosphate and is therefore less stable in the GDP-bound Cdc42. To probe the differences in the two forms, Y32W mutants were generated in H-Ras and Cdc42 and intrinsic tryptophan fluorescence used to probe conformational rearrangements in switch 1. When Ras was tested there were significant differences in the fluorescence between the GDP and GMPPCP forms but in Cdc42 the fluorescence of both nucleotide forms was the same. In the Cdc42·GMPPNP structure Thr35 did not coordinate the Mg^{2+} and switch 1 was highly mobile. Low temperature ^{31}P NMR showed only one peak for each phosphate and this shifted when effector was added, indicating that Cdc42 was in state 1. Similarly, in the T35A mutant (which should be in state 1) the phosphate peaks were in a similar position to those in the wild-type protein. The T35A Cdc42·GMPPNP was, however, less dynamic than wild-type as judged by appearance of peaks for switch 1 in the NMR spectra and analysis of the backbone dynamics by NMR[25]. Hence even though there was only one species observable by ^{31}P NMR this is likely to represent only the most populated of a number of conformations.

There have been few detailed studies on the other Rho family proteins. It is likely that the closely related protein Rac1 is similar to Cdc42: Thr35 does not coordinate Mg^{2+} in either the GDP or the GMPPNP-bound structures with Arfaptin, where the switches were well-defined[26]. Structures of RhoC bound to GDP, GMPPNP and GTPγS (Figure 2C) showed that

the GMPPNP form resembled the GDP form in switch 1, while the GTPγS-bound RhoC protein, had the hallmarks of an active, state 2 structure[27]. The two GTP analogue structures were, however, more similar in switch 2 (Figure 2C).

Allostery beyond the switch regions

Given their amenable size, a large number of small G proteins of the Ras superfamily have been studied by NMR, in both their GDP- and GTP analogue-bound forms. NMR chemical shifts are exquisitely sensitive to the local environment and it is apparent that there are numerous changes and widespread changes in the NMR spectra when the active and inactive forms are compared. These changes cannot be explained on the conformational variation of the two switch regions alone, suggesting that there are longer-range allosteric effects of nucleotide exchange.

The insert region of the Rho family

The Rho family are defined by presence of an extra region within their sequence known as the insert region, which forms an additional α-helix together with a short, less stable second helix (Figure 1). The insert helix is not closely connected to the rest of the structure and is relatively mobile. The role of the insert remains elusive: it interacts with formin proteins[28,29] but not with other regulators or effectors [30]. In Cdc42·GDP the edges of the insert region are in conformational exchange while the central residues are undergoing faster timescale small motions[19]. This suggests that the insert helix moves in a block in the GDP form. In the GMPPCP form of the protein the entire insert undergoes smaller, fast movement. There are no chemical shift changes in the insert region when the nucleotide is changed, suggesting that any nucleotide dependence of this region is only in the dynamics.

Biophysical and NMR analysis of a Rac1 protein lacking an insert showed that it behaved similarly to the protein with the insert present, implying that the insert does not impinge on the G domain[31]. Like Cdc42 however, the residues on either side of the insert helix had low order parameters, suggesting that they formed a hinge[32]. Interestingly, when a domain from Plexin-B1 was added these hinges became less mobile, implying a change in dynamics. The structure of the complex of Rac1 with Plexin-B1 however, showed that the insert was not involved in the interaction[33], suggesting that the change in the insert dynamics is relayed across the Rac1 protein and is not a direct effect.

The dynamics of the methyl groups in Cdc42 and how they change upon PAK binding have been studied[34]. Some of the residues on either side of the insert have low order parameters: *e.g.* Val84 and Ile137. Methyl group dynamics are a powerful probe of conformational entropy and analysis showed that although the insert region does not change in structure (based on chemical shifts) when PAK binds, its dynamics do change[35]. It was suggested that this is part of an allosteric change where residues that are not directly involved in binding to PAK become less rigid to counteract the unfavourable entropic cost of the reduced flexibility in those regions that bind to PAK. Long-range contacts between the switch regions and the insert loop were also implied by a study of the F28L mutant of Cdc42, which has a lower affinity for nucleotides and is therefore 'fast cycling'[36]. Overall this mutant had a more flexible structure than the wild-type protein, with Leu28 and residues in the P-loop being particularly dynamic, as expected. Interestingly the insert region is more rigid in this mutant as judged by NMR backbone dynamics and slower hydrogen/deuterium exchange rates.

The structure of RhoC was solved in the GDP, GMPPNP and GTPγS forms, as described above[27]. Interestingly, in RhoC·GTPγS, the only form with switch 1 in state 2, the insert helix and following loop are at a slightly different orientation than in the other two forms, where they are almost identical (Figure 2C). This implies that the insert region is also sensitive to the state of switch 1, raising the intriguing possibility that it could be involved in nucleotide-sensitive interactions with other molecules.

The two-lobe theory

The insert region is specific to the Rho family but there is also evidence for longer-range allosteric effects being a general feature of the small G proteins. A number of studies have suggested that there is an allosteric change transmitted from the 'effector lobe' (essentially the N-terminal ~85 amino acids) to the 'allosteric lobe' (the remainder of the protein up to the start of the hypervariable C-terminal region). The effector lobe is highly conserved and includes the switch regions and the P-loop, while the allosteric lobe is more variable between the different proteins. Communication between the two lobes offers a relatively straightforward explanation for differences in binding affinities despite almost identical effector regions in related G proteins.

One region in the effector lobe that transmits conformational change in the Rho family proteins is the anti-parallel β-sheet (β2-β3) that connects the switch regions and the final α-helix of the G domain (α5). Shifts in this sheet can therefore relay the nucleotide state to α5, which is particularly important for the Rho proteins because α5 is involved in binding to the CRIB effectors (reviewed in [30]). High mobility in the β2-β3 region was observed in Rac1[32], which was reduced in complex with Plexin-B1, even though the effector does not contact this region[33]. The same region is also flexible in Cdc42 and is rigidified in the PAK complex[34,35] but this region contacts PAK directly so the changes can be explained by induced fit. In the RhoC·GTPγS structure (switch 1 in state 2), the β2-β3 hairpin is in a different orientation than the same region in the GMPPNP and GDP-bound structures, where switch 1 is in the inactive conformation (Figure 2C), implying that this region is also sensitive to the activity state of switch 1. The β2-β3 region has also been implicated in transmitting the nucleotide state to the C-terminal hypervariable region (HVR) in H-Ras[37], which is close to the membrane. Molecular dynamics simulations suggested that H-Ras·GDP and H-Ras·GTP interact with the lipid bilayer in different ways[38]: in the GDP form residues at the end of helix α5 contact the lipid headgroups, while in the GTP form residues in helix α4 contact the membrane. The driving force for this switch is the reorientation of salt bridges that are formed between acidic residues in the β2-β2 loop and basic residues in helix α5. The change in nucleotide loading causes a reorientation, which can then be transmitted to the HVR.

Another interesting region in the allosteric lobe was found by a thorough analysis of all of the available H-Ras structures[39]. A continuum of different conformations was observed, compatible with the results from ^{31}P NMR that first highlighted the different states of the active protein. As well as the switch regions and the β2-β2 region already discussed, principal component analysis showed that the region around residues 99-108, comprising helix α3 and loop L7 (the α3-β5 loop), was also variable in the different structures. Accelerated molecular dynamics (MD) showed that, in the GTP form, there was correlated motion between helix α2 in switch 2 and this region, indicating that switch 2 is responsible for the interlobe communication to α3 and loop L7. This correlation between movement in the switch regions and helix α3 was also suggested by NMR experiments that probed the dynamics of H-Ras·GMPPNP on the msec timescale[40]. These timescales are consistent with the interconversion between state 1 and state 2, and suggested that the protein is 'breathing'. Most of the residues observed to move were in the effector region as expected but there was a

notable cluster of mobile residues within helix α3. NMR spectra of H-Ras recorded at 5 °C[15] showed that two distinct species were visible for the switch regions. However, there were also two species for one residue in helix α3, indicating that this helix is undergoing conformational exchange when state 1 and state 2 interconvert.

The hypothesis that the region around helix α3 represents an allosteric site has been reinforced by a number of crystal structures (Figure 2B). A structure of H-Ras was solved in the presence of calcium acetate[41]. Acetate binds to Arg97 in helix α3 and this leads to a shift in the end of this helix (98-103) and loop 7 (104-108) as well as in residues 69-75 at the end of switch 2. This results in the ordering of switch 2 and the placement of the catalytic Gln61, mimicking the effect of GAP binding. Water-mediated hydrogen bonds between switch 2 and helix α3 are observed in the active, R state only (Figure 2C), which are presumably responsible for the stabilization of switch 2. Multiple solvent crystal structures were also solved to find binding site hot spots[42] and one was located between helix α3 and switch 2. Again, binding ligand here leads to ordering of switch 2, as does binding of other small molecules[43].

Allosteric communication between helix α3/loop L7 and switch 2 has been observed in other Ras family members. Ras isoform sequence differences cluster around helix α3, loop 7 and helix α4. An NMR analysis of K-Ras to look at dynamics on a μsec-msec timescale suggested that, like H-Ras, there was a global conformational rearrangement[42]. N-Ras binds Raf more weakly than K and H-Ras and has a slower hydrolysis rate than H-Ras[44]. A crystal structure of N-Ras in its GMPPNP-bound state[44] showed that switch 1 is ordered but switch 2 is more open towards the C-terminus. This results in helix α3 being shifted towards the T state (the inactive state) *i.e.* it is closer to switch 2. A number of residues unique to N-Ras seem to be responsible for these interactions that drive the equilibrium towards the T state.

The Ras-like protein Rheb enhances apoptosis and although it binds to Raf it has a very low affinity that is unlikely to be physiologically relevant. Rheb has a similar sequence to Ras, with just a few changes in switch 1 being responsible for the different effector affinities. In the X-ray structures of rat Rheb·GDP and Rheb·GMPPNP, switch 2 looks identical (reviewed in [45]). NMR analysis showed that in the GDP form the switch regions were flexible

on a psec-nsec timescales while in the GMPPNP form the switch regions were missing and therefore moving on a slower, msec timescale[46]. Hence, as in Ras, switch 2 is involved in conformational selection. Work on the human Rheb protein also showed that residues in loop 7, 109-112, are dynamic on the sub nsec timescale and there was evidence for conformational exchange in helix $\alpha 3$[47].

RalA and RalB are 85% identical, with 100% identity in the effector binding regions but they have different functions *in vivo*. One of the differences between the two proteins is an extra residue in loop L7 in RalB and our work has shown that this loop is highly dynamic[12], undergoing motion on a psec-nsec timescale. This suggests that loop L7 is central to the allosteric differences between RalA and RalB that underlie their diversity.

Work on two Rho family proteins indicates that allostery in helix 3 and loop L7 exists in the Rho family as well. In Cdc42 switch 2 was flexible in both the GDP and GMPPCP forms, with motion on a msec timescale indicating conformational exchange[19]. Chemical exchange was also observed for the face of helix 3 that borders switch 2, suggesting that the movement of switch 2 causes changes in this helix as well. Insight into a role for this region of Rac1 emerged with our structure of this G protein in complex with the HR1b domain from its effector PRK1[48]. This structure was of full-length Rac1, which is unusual: the C-terminus of the protein, which is hypervariable between even highly related G proteins, is usually removed for structural analysis. In this case the C-terminus was necessary for the interaction. In the Rac1-HR1b complex, the C-terminus loops back and contacts residues at the end of switch 2 and in the L7 loop, as well as contacting the effector protein. The allostery of the switch 2-L7-$\alpha 3$ patch may therefore facilitate effector binding in this case.

Druggability of allosteric sites

One of the major goals of Ras research has been to generate inhibitors that could begin to address the over-activation of mutant Ras in some cancers. These efforts have so far met with limited success, but there is hope that the improved knowledge of dynamics and allostery in Ras will open the door to new target sites. The discovery of small molecules that bind to an allosteric site that exists in a common lung cancer mutant K-Ras has excited new interest in the possibility of exploiting the allostery of Ras to find new therapeutics[49].

The existence of the two states in switch 1 suggested that there might be some differences in their surfaces that could be exploited. In the structures of H-Ras·GMPPNP (T35A mutant) and M-Ras·GMPPNP, both of which are in state 1, a pocket was observed between the two switch regions[14]. This pocket between the switches was also observed in a wild-type H-Ras·GMPPNP crystal structure that was in state 1[50] but was not present in state 2 structures, suggesting that state 1 might be more druggable than state 2. Molecules that bind this pocket could stabilise state 1 (and hence the inactive state). The same group also used NMR and hydrogen/deuterium exchange to compare T35S (state 1) and wild-type H-Ras (state 1 and state 2 mix) and found that helix α3 residues were less protected in the wild-type protein. This reinforces the view that allostery at helix α3 is related to the state transition and that a search for allosteric regulators at this site might also bear fruit.

A fragment-based screen was performed using full-length, farnesylated K-Ras·GTP in the presence of phosphatidylserine[51]. G12V Ras bound to one of the fragments was analysed by ^{31}P NMR and it was found that the compound trapped K-Ras in the inactive, state 1 form. The binding site for the fragment was mapped and it was shown to bind to a similar pocket as inhibitors of full-length K-Ras·GDP[52]. This suggests that inhibitors that selectively bind to and stabilise state 1 are possible.

The importance of targeting the allosteric lobe of Ras, rather than the switch regions, was demonstrated recently using a synthetic protein monobody (NS1) that inhibits both H-Ras and K-Ras signalling. Surprisingly, when the structure of the NS1-H-Ras complex was solved it was apparent that NS1 does not bind the switch regions, and instead interacts with the opposite face around helices α4 and α5 and the intervening strand β6[53]. A careful analysis of all the X-ray structures of H-Ras that have been solved showed that there is a preferential dimerization interface encompassing this region of Ras, suggesting that NS1 actually disrupts Ras dimerization. The NS1 monobody was then shown to block Ras nanoclustering in cell-based assays and therefore Raf activation.

Conclusions

After almost three decades of research into the structures and dynamics of Ras superfamily proteins, a picture is emerging of the role of allostery in these important proteins. The idea that even in the active proteins there are two states holds for the Ras and Rho family

members that have been studied, as well as for Ran[54]. The G domain in these proteins, far from being a rigid scaffold with two nucleotide-sensitive switch regions is actually rather allosteric. There are two regions that can transfer information between the switches and the rest of the G domain: the β2 and β3 strands and the intervening loops transmit the nucleotide state to helix α5, and this may be particularly important for the Rho family proteins where the CRIB effectors bind to helix α5; an allosteric site around helix α3 and loop 7 relays to and from switch 2. The highly dynamic state of the small G proteins is not surprising, given that such a small domain is able to direct specific interactions with a large number of modulator and effector proteins. With each binding partner induced fit must be necessary for specificity and it seems that conformational selection is at work in these proteins. Discovery of allosteric transitions in these proteins has also led to the identification of temporary binding pockets, which offer new hope for targeting Ras directly.

References

1. Pai, E. F. *et al.* Refined crystal structure of the triphosphate conformation of H-ras p21 at 1.35 A resolution: implications for the mechanism of GTP hydrolysis. *EMBO J.* **9,** 2351–9 (1990).
2. Milburn, M. V *et al.* Molecular switch for signal transduction: Strutural differences between active and inactive forms of protooncogenic ras proteins. *Science* **247,** 939–945 (1990).
3. Aspenström, P., Ruusala, A. & Pacholsky, D. Taking Rho GTPases to the next level: The cellular functions of atypical Rho GTPases. *Exp. Cell Res.* **313,** 3673–3679 (2007).
4. Vetter, I. R. & Wittinghofer, A. Signal transduction - The guanine nucleotide-binding switch in three dimensions. *Science* **294,** 1299–1304 (2001).
5. Geyer, M. *et al.* Conformational transitions in p21(ras) and in its complexes with the effector protein Raf-RBD and the GTPase activating protein GAP. *Biochemistry* **35,** 10308–10320 (1996).
6. Kraulis, P. J., Laue, E. D., Domaille, P. J., Campbell-Burk, S. L. & Van Aken, T. Solution Structure and Dynamics of Ras p21·GDP Determined by Heteronuclear Three- and Four-Dimensional NMR Spectroscopy. *Biochemistry* **33,** 3515–3531 (1994).
7. Ito, Y. *et al.* Regional polysterism in the GTP-bound form of the human c-Ha-Ras protein. *Biochemistry* **36,** 9109–9119 (1997).
8. Long, D. *et al.* A comparative CEST NMR study of slow conformational dynamics of small GTPases complexed with GTP and GTP analogues. *Angew. Chemie - Int. Ed.* **52,** 10771–10774 (2013).
9. Spoerner, M., Nuehs, A., Herrmann, C., Steiner, G. & Kalbitzer, H. R. Slow conformational dynamics of the guanine nucleotide-binding protein Ras complexed with the GTP analogue GTPγS. *FEBS J.* **274,** 1419–1433 (2007).
10. Ye, M. *et al.* Crystal structure of M-Ras reveals a GTP-bound 'off' state conformation of Ras family small GTPases. *J. Biol. Chem.* **280,** 31267–31275 (2005).
11. Liao, J. *et al.* Two conformational states of Ras GTPase exhibit differential GTP-binding kinetics. *Biochem. Biophys. Res. Commun.* **369,** 327–332 (2008).

12. Fenwick, R. B. *et al.* Solution Structure and Dynamics of the Small GTPase RalB in Its Active Conformation: Significance for Effector Protein Binding. *Biochemistry* **48,** 2192–2206 (2009).
13. Spoerner, M., Herrmann, C., Vetter, I. R., Kalbitzer, H. R. & Wittinghofer, A. Dynamic properties of the Ras switch I region and its importance for binding to effectors. *Proc. Natl. Acad. Sci. U. S. A.* **98,** 4944–9 (2001).
14. Shima, F. *et al.* Structural basis for conformational dynamics of GTP-bound ras protein. *J. Biol. Chem.* **285,** 22696–22705 (2010).
15. Araki, M. *et al.* Solution structure of the state 1 conformer of GTP-bound H-ras protein and distinct dynamic properties between the state 1 and state 2 conformers. *J. Biol. Chem.* **286,** 39644–39653 (2011).
16. Matsumoto, K. *et al.* Critical roles of interactions among switch I-preceding residues and between switch II and its neighboring alpha-helix in conformational dynamics of the GTP-bound Ras family small GTPases. *J. Biol. Chem.* **286,** 15403–15412 (2011).
17. Parker, J. A., Volmar, A. Y., Pavlopoulos, S. & Mattos, C. K-Ras Populates Conformational States Differently from Its Isoform H-Ras and Oncogenic Mutant K-RasG12D. *Structure* **26,** 810–820 (2018).
18. Li, Y. *et al.* Specific Substates of Ras to Interact with GAPs and Effectors: Revealed by Theoretical Simulations and FTIR Experiments. *J. Phys. Chem. Lett.* **9,** 1312–1317 (2018).
19. Loh, A. P., Guo, W., Nicholson, L. K. & Oswald, R. E. Backbone dynamics of inactive, active, and effector-bound Cdc42Hs from measurements of (15)N relaxation parameters at multiple field strengths. *Biochemistry* **38,** 12547–12557 (1999).
20. Gizachew, D., Guo, W., Chohan, K. K., Sutcliffe, M. J. & Oswald, R. E. Structure of the complex of Cdc42Hs with a peptide derived from P-21 activated kinase. *Biochemistry* **39,** 3963–3971 (2000).
21. Morreale, A. *et al.* Structure of Cdc42 bound to the GTPase binding domain of PAK. *Nat. Struct. Biol.* **7,** 384–388 (2000).
22. Mott, H. R. *et al.* Structure of the small G protein Cdc42 bound to the GTpasebinding domain of ACK. *Nature* **399,** 384–388 (1999).
23. Abdul-Manan, N. *et al.* Structure of Cdc42 in complex with the GTPase-binding domain of the 'Wiskott-Aldrich syndrome' protein. *Nature* **399,** 379–383 (1999).
24. Phillips, M. J., Calero, G., Chan, B., Ramachandran, S. & Cerione, R. A. Effector proteins exert an important influence on the signaling-active state of the small GTPase Cdc42. *J. Biol. Chem.* **283,** 14153–14164 (2008).
25. Chandrashekar, R., Salem, O., Krizova, H., McFeeters, R. & Adams, P. D. A switch I mutant of Cdc42 exhibits less conformational freedom. *Biochemistry* **50,** 6196–6207 (2011).
26. Tarricone, C., Xiao, B., Justin, N., Walker, P. A. & Rittinger, K. The structural basis of Arfaptin- mediated cross-talk between Rac and Arf signalling pathways. *Nature* **411,** 215–219 (2001).
27. Dias, S. M. G. & Cerione, R. A. X-ray crystal structures reveal two activated states for RhOC. *Biochemistry* **46,** 6547–6558 (2007).
28. Lammers, M., Meyer, S., Kuhlmann, D. & Wittinghofer, A. Specificity of Interactions between mDia Isoforms and Rho Proteins. *J. Biol. Chem.* **283,** 35236–35246 (2008).
29. Kühn, S. *et al.* The structure of FMNL2-Cdc42 yields insights into the mechanism of lamellipodia and filopodia formation. *Nat. Commun.* **6,** 7088 (2015).
30. Mott, H. R. & Owen, D. Structures of Ras superfamily effector complexes: What have we learnt in two decades? *Crit. Rev. Biochem. Mol. Biol.* **50,** 85–133 (2015).
31. Thapar, R., Karnoub, A. E. & Campbell, S. L. Structural and biophysical insights into

the role of the insert region in Rac1 function. *Biochemistry* **41,** 3875–3883 (2002).
32. Bouguet-Bonnet, S. & Buck, M. Compensatory and Long-Range Changes in Picosecond-Nanosecond Main-Chain Dynamics upon Complex Formation: 15N Relaxation Analysis of the Free and Bound States of the Ubiquitin-like Domain of Human Plexin-B1 and the Small GTPase Rac1. *J. Mol. Biol.* **377,** 1474–1487 (2008).
33. Bell, C. H., Aricescu, A. R., Jones, E. Y. & Siebold, C. A dual binding mode for RhoGTPases in plexin signalling. *PLoS Biol.* **9,** e1001134 (2011).
34. Loh, A. P., Pawley, N., Nicholson, L. K. & Oswald, R. E. An increase in side chain entropy facilitates effector binding: NMR characterization of the side chain methyl group dynamics in Cdc42Hs. *Biochemistry* **40,** 4590–4600 (2001).
35. Moorman, V. R. *et al.* Dynamic and thermodynamic response of the Ras protein Cdc42Hs upon association with the effector domain of PAK3. *J. Mol. Biol.* **426,** 3520–3538 (2014).
36. Adams, P. D., Loh, A. P. & Oswald, R. E. Backbone dynamics of an oncogenic mutant of Cdc42Hs shows increased flexibility at the nucleotide-binding site. *Biochemistry* **43,** 9968–9977 (2004).
37. Abankwa, D. *et al.* A novel switch region regulates H-ras membrane orientation and signal output. *EMBO J.* **27,** 727–735 (2008).
38. Gorfe, A. A., Hanzal-Bayer, M., Abankwa, D., Hancock, J. F. & McCammon, J. A. Structure and dynamics of the full-length lipid-modified H-Ras protein in a 1,2-dimyristoylglycero-3-phosphocholine bilayer. *J. Med. Chem.* **50,** 674–684 (2007).
39. Gorfe, A. A., Grant, B. J. & McCammon, J. A. Mapping the Nucleotide and Isoform-Dependent Structural and Dynamical Features of Ras Proteins. *Structure* **16,** 885–896 (2008).
40. O'Connor, C. & Kovrigin, E. L. Global conformational dynamics in Ras. *Biochemistry* **47,** 10244–10246 (2008).
41. Buhrman, G., Holzapfel, G., Fetics, S. & Mattos, C. Allosteric modulation of Ras positions Q61 for a direct role in catalysis. *Proc. Natl. Acad. Sci.* **107,** 4931–4936 (2010).
42. Buhrman, G. *et al.* Analysis of binding site hot spots on the surface of Ras GTPase. *J. Mol. Biol.* **413,** 773–789 (2011).
43. Holzapfel, G., Buhrman, G. & Mattos, C. Shift in the equilibrium between on and off states of the allosteric switch in ras-GppNHp affected by small molecules and bulk solvent composition. *Biochemistry* **51,** 6114–6126 (2012).
44. Johnson, C. W. *et al.* The small GTPases K-Ras, N-Ras, and H-Ras have distinct biochemical properties determined by allosteric effects. *J. Biol. Chem.* **292,** 12981–12993 (2017).
45. Schöpel, M. *et al.* The small GTPases Ras and Rheb studied by multidimensional NMR spectroscopy: Structure and function. *Biol. Chem.* **398,** 577–588 (2017).
46. Karassek, S. *et al.* Ras homolog enriched in brain (Rheb) enhances apoptotic signaling. *J. Biol. Chem.* **285,** 33979–33991 (2010).
47. Cicco, M. De, Kiss, L. & Dames, S. A. NMR analysis of the backbone dynamics of the small GTPase Rheb and its interaction with the regulatory protein FKBP38. *FEBS Lett.* **592,** 130–146 (2018).
48. Modha, R. *et al.* The Rac1 polybasic region is required for interaction with its effector PRK1. *J. Biol. Chem.* **283,** 1492–1500 (2008).
49. Ostrem, J. M., Peters, U., Sos, M. L., Wells, J. A. & Shokat, K. M. K-Ras(G12C) inhibitors allosterically control GTP affinity and effector interactions. *Nature* **503,** 548–551 (2013).
50. Matsumoto, S. *et al.* Molecular Mechanism for Conformational Dynamics of Ras·GTP

Elucidated from In-Situ Structural Transition in Crystal. *Sci. Rep.* **6,** 1–12 (2016).
51. Jansen, J. M. *et al.* Inhibition of prenylated KRAS in a lipid environment. *PLoS One* **12,** 1–21 (2017).
52. Maurer, T. *et al.* Small-molecule ligands bind to a distinct pocket in Ras and inhibit SOS-mediated nucleotide exchange activity. *Proc. Natl. Acad. Sci.* **109,** 5299–5304 (2012).
53. Spencer-Smith, R. *et al.* Inhibition of RAS function through targeting an allosteric regulatory site. *Nat. Chem. Biol.* **13,** 62–68 (2016).
54. Geyer, M. *et al.* Conformational states of the nuclear GTP-binding protein ran and its complexes with the exchange factor RCC1 and the effector protein RanBP1. *Biochemistry* **38,** 11250–11260 (1999).

Figure Legends

Figure 1. Representative structures of Ras and Rho family proteins in their inactive and active forms. The active proteins are coloured yellow, with the switch regions in pink. The inactive proteins are coloured pale blue with the switch regions in dark blue. The nucleotide is shown as sticks with carbons yellow (active) or cyan (inactive), nitrogen blue, oxygen red and phosphate orange, and the Mg^{2+} ion is a green sphere. The insert helices in the Rho family are coloured brown (active form) or pink (inactive form). Secondary structural elements discussed in the text are labelled.

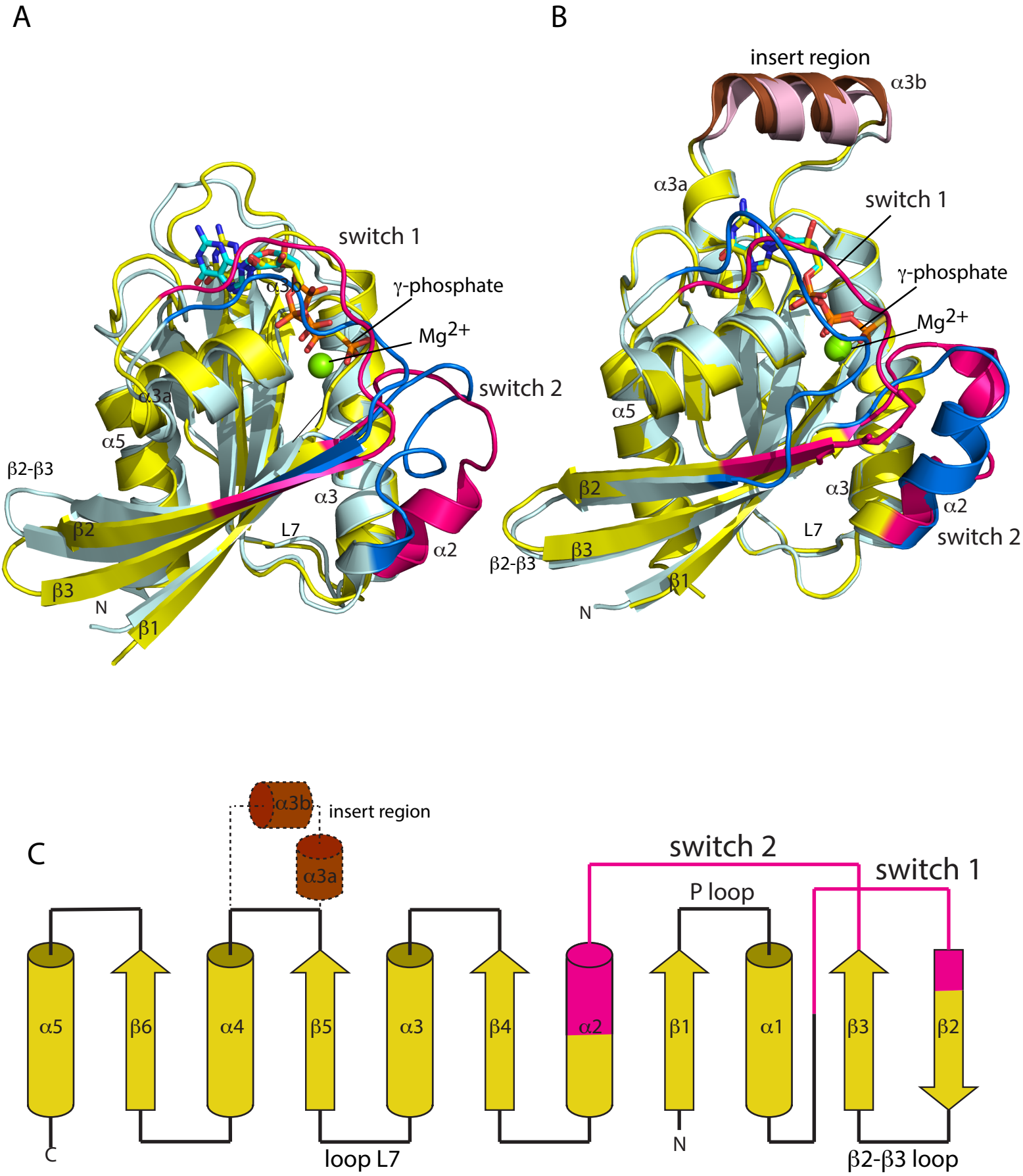
A. The structure of H-Ras·GDP (pdb 1AA9) and H-Ras with a GTP analogue, GMPPCP (pdb 121P)
B. The structure of RhoC·GDP (pdb 2GCN) and the structure of RhoC·GTPγS (pdb 2GCP)
C. Schematic secondary structure of the Ras and Rho family proteins. The colour scheme the same as that of the active forms in panels A and B. The locations of the regions discussed in the text are indicated, as is the P loop, which is responsible for binding the phosphate groups in the guanine nucleotide.

Figure 2. Allostery in the Ras and Rho family.

A. The structures of H-Ras·GMPPNP with the switch 1 regions in state 1 (pdb 4EF1) and state 2 (pdb 3K8Y). The switch regions are coloured pink, with Thr35 and Tyr32 shown as yellow (state 1) and cyan (state 2) sticks. The nucleotide is shown as sticks with carbon green, nitrogen blue, oxygen red and phosphate orange. The Mg^{2+} ion is a grey sphere. Water molecules that coordinate Mg^{2+} are shown as yellow spheres. The various features are denoted by the subscripts 1 and 2 for state 1 and state 2 respectively. In state 2, Thr35 coordinates the Mg^{2+} ion, along with two water oxygens, and tyrosine 32 points towards the nucleotide. In state 2, Thr35 and Tyr32 are flipped out and a third water oxygen coordinates the Mg^{2+} instead.
B. The allosteric site in H-Ras involving helix α3 and the L7 loop. The structures and their colours are as follows: switch 1 state 2, allosteric site T – yellow (pdb 2RGE); switch 1 state 2, allosteric site R – slate grey (pdb 3K8Y); switch 1 state 1, allosteric site R (pdb 4EF1). The T and R states are labelled.
C. Network of water-mediated hydrogen bonds that connect helix α3 and switch 2 in H-Ras, linking the allosteric site to the nucleotide-sensitive switches. The T state (pdb 2RGE) is yellow and the R state (pdb 3K8Y) is slate grey. Much of switch 2 is missing in the T state structure and a yellow dotted line connects Gly60 to Asp69. Water interactions are shown for the R state only, with water oxygens represented by

a green sphere and hydrogen bonds by black dotted lines. Residues in the allosteric site, those involved in hydrogen bonds and the nucleotide are shown for the R state in a stick representation with carbon slate grey, nitrogen blue, oxygen red and phosphate orange. The same residues in the T state (if they are visible in the structure) are shown with the same colour scheme except that carbon is yellow.

D. The structures of RhoC in three nucleotide forms: GDP (yellow, pdb 2GCN), GMPPNP (cyan, pdb 2GCO) and GTPγS (blue, pdb 2GCP). The nucleotide is shown as sticks with carbon green, nitrogen blue, oxygen red and phosphate orange. On the left the two switches are shown, in the middle is the β2-β3 hairpin discussed in the text and on the right is the Rho-family specific insert region. It is apparent that the GDP and GMPPNP forms are more similar, with switch 1, β2-β3 and the insert in the same orientations. In contrast, GMPPNP and GTPγS have a similar switch 2 conformation, which is distinct from that of GDP.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switch 2
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β2
L7
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β3
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β1
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switch 1
γ-phosphate
Mg2+
α5
β2
α3
α2
L7
switch 2
β3
β2-β3
β1
N
C
α3b
insert region
α3a
switch 2
switch 1
P loop
α5
β6
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α2
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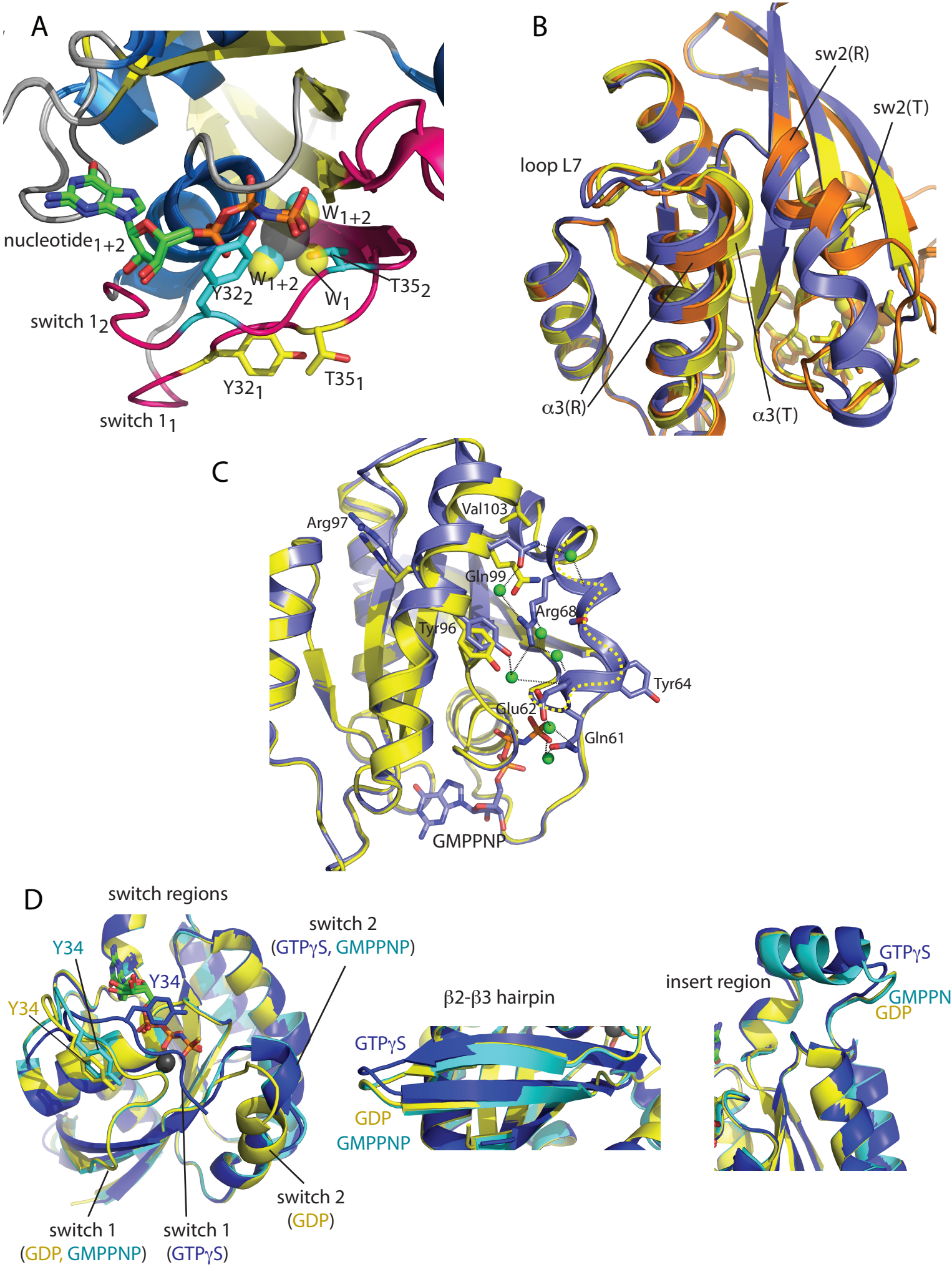
α1
β3
β2
C
loop L7
N
β2-β3 loop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W1+2
Y322
W1+2
W1
T352
switch 12
Y321
T351
switch 11
B
sw2(R)
sw2(T)
loop L7
α3(R)
α3(T)
C
Arg97
Val103
Gln99
Arg68
Tyr96
Tyr64
Glu62
Gln61
GMPPNP
D
switch regions
Y34
Y34
Y34
switch 2
(GTPγS, GMPPNP)
switch 1
(GDP, GMPPNP)
switch 1
(GTPγS)
switch 2
(GDP)
β2-β3 hairpin
GTPγS
GDP
GMPPNP
insert region
GTPγS
GMPPNP
GDP