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Small GTPases and their GAPs

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Summary

Widespread utilization of small GTPases as major regulatory hubs in many different biological systems derives from a conserved conformational switch mechanism that facilitates cycling between GTP-bound active and GDP-bound inactive states under control of guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs), which accelerate slow intrinsic rates of activation by nucleotide exchange and deactivation by GTP hydrolysis, respectively. Here we review developments leading to current understanding of intrinsic and GAP catalyzed GTP hydrolytic reactions in small GTPases from structural, molecular and chemical mechanistic perspectives. Despite the apparent simplicity of the GTPase cycle, the structural bases underlying the hallmark hydrolytic reaction and catalytic acceleration by GAPs are considerably more diverse than originally anticipated. Even the most fundamental aspects of the reaction mechanism have been challenging to decipher. Through a combination of experimental and *in silico* approaches, the outlines of a consensus view have begun to emerge for the best studied paradigms. Nevertheless, recent observations indicate that there is still much to be learned.

Introduction

Regulated hydrolysis of GTP by small GTPases (also known as small G proteins) is a biologically important example of the more general class of phosphoryl transfer reactions in aqueous solution and structured protein environments¹⁻³. The deceptively simple reaction chemistry involves nucleophilic attack by a water molecule on the terminal γ phosphate of a GTP substrate and cleavage of the phospho-monoester bond to produce GDP (the leaving group) and inorganic phosphate. Unlike classical enzymes that evolved to rapidly convert weakly bound substrates into products with catalytic efficiencies (k_{cat}/K_M) in some cases approaching diffusion controlled limits, the majority of GTPases, including nearly all small GTPases, evolved under very different selective pressures to bind GDP/GTP with very low dissociation constants (K_D typically in the nanomolar-picomolar range) and slowly hydrolyze GTP with turnover numbers rarely exceeding 10^{-1} s^{-1} and more generally in the range of 10^{-3} - 10^{-6} s^{-1} or even lower⁴⁻⁷. With co-evolution of biosynthetic pathways to maintain free GTP at steady state concentrations in excess of free GDP by at least 10 fold⁸, the result is a superfamily of enzymes that slowly exchange GDP for GTP and slowly hydrolyze GTP to GDP in an effectively unidirectional cycle between metastable GDP- and

GTP-bound states characterized by structurally and dynamically distinct conformations (Fig. 1A). Indeed, the timescale for interconversion between nucleotide-bound states is generally much slower than that of the relevant biological processes such that two major classes of regulatory proteins co-evolved to control exchange and hydrolysis. Guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs) accelerate GDP/GTP exchange and GTP hydrolysis by orders of magnitude, with catalytic turnover numbers exceeding the intrinsic rates by as much as 10^5 fold⁹ or even more in cases where the intrinsic rates are particularly low.

The general functional significance of this remarkable switch mechanism becomes apparent with the co-evolution of another major class of proteins known as 'effectors', which selectively bind the GTP-bound conformation to couple the GTPase cycle with the downstream molecular machinery involved in signaling, cytoskeletal dynamics, trafficking, nuclear transport and many other cellular processes¹⁰. Thus, the GTPase cycle emerged as one of the most highly conserved switch mechanisms, in which cell autonomous or external signals coupled through GEFs and GAPs are the primary inputs controlling transitions between GDP-bound 'inactive' and GTP-bound 'active' conformations while selective effector binding to the GTP-bound conformation is the primary output mediating physiological responses. The remarkable versatility of the GTPase cycle as a widespread regulatory mechanism for numerous biological processes reflects the structural diversity of the GTPases, GEFs, GAPs and effectors, which contributes to high functional specificity at the level of molecular interactions. Finally, the co-evolution of additional ancillary proteins, including GDP-dissociation inhibitors (GDIs) and related factors that selectively bind the GDP-bound conformation, is crucial for post-translational modification of GTPases as well as delivery to sub-cellular sites of action.

This review focuses on current understanding of the molecular mechanisms, structural underpinnings and chemical energetics/dynamics related to the tightly regulated GTP hydrolytic reaction in small GTPases. Selected examples are used to illustrate general conceptual frameworks as well as variations and exceptions that have extended, shifted or redefined established paradigms. Given this emphasis, other very important aspects of the GTPase cycle including relevant biology and associated diseases are largely neglected. The interested reader can find further information on related topics in many excellent reviews in this issue and elsewhere^{1,2,10-21}.

Small GTPase architecture

As illustrated in Fig. 1B, small GTPases of the Ras superfamily share a common architecture with a core 'GTPase domain' consisting of five helices ($\alpha 1$ - $\alpha 5$) surrounding a six-stranded beta sheet ($\beta 1$ - $\beta 6$)²². At the primary structural level, five highly conserved 'G motifs' (G1-G5) encode invariant residues that mediate critical contacts with the nucleotide as well as an essential Mg^{2+} ion required for high affinity nucleotide binding and hydrolytic activity⁴. The G1 motif (GxxxxGKS/T) encodes the phosphate binding loop (P-loop) characteristic of nucleotide binding proteins. The cryptic G2 motif (xTx) contains an invariant threonine residue that mediates hydrogen bonding interactions with γ phosphate and is also coordinated by the Mg^{2+} ion in the GTP-bound state. In addition to invariant aspartic acid

and glycine residues involved in stabilization of Mg^{2+} binding and hydrogen bonding with the γ phosphate, respectively, the G3 motif (DxxG) is typically followed by a glutamine residue that is conserved in many families (including Ras, Rho, Rab, Arf, Arl, and G_{α} subunits) and plays a particularly important role in the intrinsic and, with notable exceptions discussed below, GAP-catalyzed hydrolytic reactions. Finally, the G4 (N/TKxD) and G5 (xAx) motifs encode residues important for base contacts and guanine nucleotide binding specificity.

N- and C-terminal extensions to the GTPase core vary considerably between and even within families. Although typically too short to form independent domains, the variable extensions include distinctive sequence motifs or other structural features that confer additional functionality, most notably related to subcellular localization or binding specificity for effectors, GEFs, GAPs and GDIs. Ras, Rho and Rab GTPases possess C-terminal cysteine motifs that specify post-translational modification of the cysteine thiol group to form a stable thioether linkage to either one (CAAX motifs) or two (CC, CXC motifs) non-polar farnesyl (15 carbon) or geranyl geranyl (20 carbon) moieties consisting of three or four isoprenoid units, respectively²³⁻²⁷. The prenyl groups anchor these GTPases at membrane surfaces but confer little if any targeting specificity, which depends on other elements in the variable regions and/or molecular interactions with GDIs, GEFs, GAPs, and effectors^{12,16,28}. Moreover, since many small GTPases are involved in or otherwise influenced by membrane dynamic processes (e.g. vesicle budding/transport/fusion) their steady state distributions can differ from their initial sites of membrane targeting.

Membrane targeting of all Arf GTPases depends on partitioning of an N-terminally myristoylated (14 carbon) amphipathic helix that inserts into the hydrocarbon core²⁹⁻³¹. Some Arl GTPases are also known or predicted to be N-terminally myristoylated (e.g. Arl1, Arl4, Arl5, Arl11, and Arl14)³²⁻³⁴ whereas others either lack the essential glycine at position 2 or are otherwise predicted to be poor substrates for N-myristoyl transferase (Arl2, Arl3, Arl8, Arl9, Arl10, Arl13, Arl15, and Arl16)^{31,35}. Arl2 and Arl3 have the required glycine at position 2; however, there is no evidence of myristoylation even though the N-terminal amphipathic helix contributes to membrane association³⁶. A few non-myristoylated Arl GTPases are instead N-terminally acetylated, a modification that is functionally important and (e.g. yeast Arl3 and fly Arl8)³⁷⁻³⁹. Whether myristoylated or not most Arl GTPases have an N-terminal amphipathic helix that is known or expected to contribute to membrane partitioning^{31,36,40,41}. As with prenylated GTPases, membrane targeting and steady state localizations of Arfs and Arls are dependent on multiple molecular interactions.

Comparisons of GDP- vs. GTP-bound crystal structures of Ras⁴²⁻⁴⁴, and subsequently others from every major family⁴⁵⁻⁴⁹, revealed prominent conformational differences within two localized 'switch regions', referred to as the switch I ($\alpha 1/\beta 2$ loop) and switch II ($\beta 3/\alpha 2$ loop, $\alpha 2$, and $\alpha 2/\beta 4$ loop). To a first approximation, the switch regions are flexible in the GDP-bound state but adopt relatively well ordered conformations in the GTP-bound state due to hydrogen bonding interactions between the γ phosphate and: i) the main chain NH as well as side chain hydroxyl of the invariant threonine in the switch I G2 motif; and ii) the main chain NH of the invariant glycine in the switch II DxxG motif. In addition, waters displaced from two sites in the coordination sphere of the Mg^{2+} ion in the GDP-bound state

are replaced by a non-bridging oxygen from the γ phosphate and the side chain hydroxyl oxygen of the invariant switch I threonine in the GTP-bound state. The nucleotide-dependent conformational changes in Arf GTPases include a distinctive 'myristoyl switch' involving a two residue shift of the β 2 strand known as the 'interswitch toggle' that displaces the myristoylated N-terminal amphipathic helix to facilitate membrane targeting⁴⁶.

The effects of oncogenic mutations in Ras provided insights into the GTPase mechanism and implicated the conserved DxxGQ glutamine as a critical catalytic residue⁵⁰. The Q61L mutation, for example, abolishes intrinsic as well as GAP-accelerated hydrolysis and is constitutively activating. The analogous QL substitutions have been widely used to generate constitutively active forms of small GTPases. Another common oncogenic mutation in the P-loop, G12V, has a similar effect and in fact every Gly 12 substitution except proline is oncogenic⁵¹. The lack of oncogenicity for G12P Ras is likely related to the observation that its intrinsic hydrolytic rate is slightly higher than that of wild type Ras despite being GAP insensitive whereas other mutations typically have lower intrinsic rates^{52,53}. A second important insight came from comparison of Ras with the G_{α} subunits of heterotrimeric G proteins, which have considerably higher intrinsic hydrolytic rates on the seconds rather than minutes timescale. In addition to conserving the catalytic glutamine equivalent to Gln 61 in Ras, G_{α} subunits also possess a catalytic switch I arginine that is absent in Ras and other small GTPase families⁴. It was hypothesized that GAPs for small GTPases including Ras might supply a catalytic arginine in trans.

Ground and transition state mimetics

Metal fluoride coordination complexes (e.g. AlF_3 , AlF_4^- , BeF_3^- and MgF_3^-) have been extensively used in combination with nucleotide diphosphates to mimic the ground or transition states for phosphoryl transfer reactions⁵⁴. BeF_3^- favors tetrahedral coordination geometry with a β phosphate oxygen of GDP as the fourth ligand and should in principle approximate the GTP ground state⁵⁵. Interestingly, the stability of RasGAP complexes with Ras-GDP- BeF_3^- are less sensitive to oncogenic substitutions than complexes with Ras-GppNHp, suggesting that the two ground state GAP complexes though similar are not identical⁵⁶. Aluminum fluoride forms mixed coordination species and trigonal bipyramidal (AlF_3) as well as octahedral (AlF_4^-) geometries have been reported in crystal structures of GDP-bound GTPases alone (G_{α} subunits^{57,58}) and in complexes with GAPs⁵⁹⁻⁶⁶. In both geometries, the equatorial sites are occupied by fluoride ions while the apical sites are occupied by a β phosphate oxygen of GDP and a water. The possibility that aluminum fluoride complexes might be transition state mimetic was suggested on the basis of biochemical evidence^{67,68} and strongly supported by crystal structures of GDP-bound G_{α} subunits activated with aluminum fluoride^{57,58}. MgF_3^- favors trigonal bipyramidal geometry and has a net charge approximating that expected for the transition state and thus may be a better transition state mimetic than octahedral AlF_4^- or charge neutral AlF_3 complexes⁶⁹. Here it is worth noting that the electron density in a number of medium resolution crystal structures has been interpreted as AlF_3 ; however, the atomic scattering factors and expected geometry for AlF_3 are nearly indistinguishable from MgF_3^- and it is possible that MgF_3^- is the actual chemical species since Mg^{2+} is also present in the crystallization buffers^{69,70}.

Although metal fluorides were originally shown to activate heterotrimeric G proteins and stabilize complexes of small GTPases with GAPs^{55,71,72}, Cdc42-GDP binds effectors in the presence of aluminum or beryllium fluorides⁷³ and a GDP-bound Ras variant, in which the switch I Tyr 32 was mutated to tryptophan to monitor activation by intrinsic tryptophan fluorescence, binds BeF_3^- with a high K_d in the millimolar range⁷⁴. It is not known whether the Y32F mutation influences BeF_3^- binding. However, a Rab5 variant capable of binding aluminum fluoride in the absence of a GAP was generated by substituting proline for alanine at a P-loop position proximal to the hydrolytic site⁷⁵. BeF_3^- also binds to the Ran-GDP complex with Ran binding protein in the absence of RanGAP⁷⁶. Thus, the affinity of metal fluorides for GDP-bound GTPases in the presence and absence of GAPs depends on multiple determinants and not just the presence of catalytic arginine/glutamine residues.

Chemical kinetic barriers and reaction pathways

The remarkable stability of phosphomonoester bonds in aqueous solution derives from the net negative charge of the ionizable phosphoryl group at physiological pH, which impedes nucleophilic attack³. Reduction of negative charge on the terminal phosphate through electrostatic interactions with the divalent Mg^{2+} cation, the P-loop lysine and often a cis or trans arginine is a general feature exploited by nucleotide triphosphatases to reduce this barrier; however, other factors are also important, including differences in the stereochemistry of polar interactions, Mg^{2+} coordination and steric strain in the ground vs. transition states^{1,18}. From a theoretical perspective, GTP hydrolytic mechanisms are generally considered with respect to the lowest energy pathway over a potential surface defined by reaction coordinates for phosphomonoester bond breakage and bond formation with the nucleophilic water. Three extreme cases (Fig. 2) illustrate the range of possible trajectories over the high energy transition state/intermediate region: i) a fully dissociative transition state intermediate for which bond breakage precedes bond formation; ii) a fully associative transition state intermediate for which bond formation precedes bond breakage; and iii) a $\text{S}_\text{N}2$ -like transition state for which bond breakage and formation are concerted. In fact, the reaction pathways can be mixed and the possibilities are even more complex when the low lying d orbitals of phosphorous or pre/post-bond breakage/formation steps are considered^{1,77}. The problem has been intensively investigated through experimental as well as theoretical approaches, with considerable debate reflecting the inherent complexity of the underlying chemical energetics and the dependence on the detailed structural and chemical environment of the hydrolytic site.

Structural and other experimental observations have been interpreted to support mainly dissociative or associative mechanisms as well as concerted mechanisms with substantial dissociative or associative character in GTPases with or without GAPs^{14,64,75,78-80}. Computational studies on intrinsic and GAP catalyzed hydrolytic reactions in small GTPases have focused primarily on Ras, for which multiple constitutively-activating oncogenic substitutions have well established effects on hydrolytic activity^{52,81}. Detailed thermodynamic analysis of linear free energy relationships and *ab initio* quantum mechanical(QM)/molecular mechanical(MM) free energy calculations suggested that experimental observations do not distinguish between associative, dissociative or concerted mechanisms⁸²⁻⁸⁴. The calculations further suggested that the lowest energy reaction

pathways for both associative and dissociative mechanisms traverse flat transition regions such that dissociative (metaphosphate) or associative intermediates would be weakly populated and therefore difficult to detect. Consequently, mechanistic details can differ significantly between solution, intrinsic GTPase and GAP-catalyzed reactions, and need to be worked out for each case using theoretical calculations subject to experimental observations.

A related and also much debated mechanistic problem concerns transfer of the proton from the nucleophilic water to an acceptor, which can in principle be an amino acid residue, the γ phosphate or a second water¹⁴. Based on the crystal structure of active Ras-GppNHp, it was noted that the DxxGQ glutamine (Gln 61) was sufficiently close to potentially activate the nucleophilic water⁴³. However, the glutamine carboxamide group is a very weak base and free energy perturbation calculations combined with electrostatic considerations suggested that Gln 61 is unlikely to function as a general base due to the high barrier for protonation⁸⁵. An alternative proposal based on analysis of linear free energy relationships suggested that the γ phosphate, which has a pK_a near 6.5 in solution, might function as a general base in a substrate-assisted mechanism⁸⁶. Subsequent QM/MM calculations suggested that proton transfer to the γ phosphate may be indirectly mediated by Gln 61 in the GAP reaction and involve a second water assisted by Gln 61 in the absence of the GAP^{70,87}. Recent QM/MM calculations including intermediates after bond breakage revealed a seemingly counterintuitive possibility that proton transfer to Gln 61 results in the imide tautomer form of the carboxamide group and that regeneration of the amide form may be the highest energy chemical barrier along the GAP-accelerated hydrolytic pathway⁷⁷.

Regardless of the proton transfer pathway, analyses of QM/MM free energy surfaces suggest that the acceleration provided by the GAP is likely due to a combination of electrostatic stabilization and protein conformational changes influencing the transition region preceding proton transfer^{83,84}. FTIR studies combined with QM/MM simulations indicate that the non-bridging phosphate oxygens of GTP adopt a low energy staggered configuration in solution compared to a high energy eclipsed configuration for the non-bridging oxygens of the β - γ phosphates when bound to Ras and eclipsed configurations for the non-bridging oxygens of all three phosphates in the GAP complex⁸⁸. These staggered-to-eclipsed configurational changes correlate with increases in the length of the scissile (P_γ - $O_{\beta\gamma}$) bond and are predicted to contribute to ground state destabilization.

Finally, another issue relates to the protonation state of the γ phosphate in Ras-GTP, which is thought to be fully deprotonated based on the pH dependence of ^{31}P NMR chemical shifts^{52,86,89}. In a recent neutron scattering structure of ground state Ras-GppNHp exchanged with D_2O , however, a deuteron is observed on one of the γ phosphate oxygens at a calculated pD of 8.4⁹⁰. This apparent discrepancy may be due to differences in the pK_a of the γ phosphate, which is higher for GppNHp than GTP in solution⁵². Given the potential mechanistic implications, the question of whether the γ phosphate is protonated in Ras-GTP with or without RasGAP would benefit from clarification. It might be interesting, for example, to compare the protonation of state of constitutively active Ras mutants loaded with GTP vs. GppNHp.

Non-chemical kinetic barriers and rate-limiting steps

Beyond the chemical steps, there are pre- and post-transition state barriers related to the conformational transitions in the GTPase and GTPase/GAP complex on the pathway from the ground to transition state and from the transition state to products. The ground to transition state conformational changes could in principle be rate limiting, although it is difficult to experimentally distinguish chemical steps from conformational changes if the latter are rate-limiting. In some ground state crystal structures of active GTPases, the switch I region adopts a 'closed' conformation incompatible with the insertion of catalytic residues into the hydrolytic site, suggesting that transition to an open conformation either precedes or accompanies formation of the initial GAP complex. For example, a switch I tyrosine in Rab1 (equivalent to Ras Tyr 32) interacts with the γ phosphate and occludes the hydrolytic site, except when trapped by crystal contacts in an open conformation⁹¹. The equivalent switch I tyrosine in Ran also blocks the active site and detailed studies combining X-ray crystallography, FTIR and MD simulations indicate that the closed conformation contributes substantially to the slow rate of GTP hydrolysis compared to Ras⁷⁶. One role of the GAPs in these instances may be to stabilize an open switch I conformation compatible with insertion of catalytic residues. In addition, allosteric modulation at a remote site by small molecules including calcium acetate in various crystal forms of Ras-GppNHp has been observed to shift the equilibrium between catalytically competent and resistant conformations, suggesting that global conformational changes in the ground state, potentially modulated by physiological interactions, may also play an important role^{92,93}.

On the product side, there are at least three barriers, in particular relaxation to the GDP-bound conformation, release of phosphate after hydrolysis and, for GAP accelerated reactions, dissociation of the GTPase-GAP complex⁹⁴. Using time resolved FTIR to monitor vibrational modes for a number of GTPase-GAP complexes¹⁸, it has been possible in certain cases to distinguish the hydrolytic event from subsequent phosphate release, with concomitant accumulation of a GDP + P_i intermediate state. In the case of Ras/RasGAP, for example, phosphate release is roughly 8 fold slower than hydrolysis^{95,96}. Phosphate release is also rate-limiting for Rap1b with either Rap1GAP or the dual specificity Ras/Rap1GAP⁹⁷ whereas the hydrolytic step is evidently rate limiting for RhoA/RhoGAP, Ran/RanGAP, and Rab1b/TBC1D20^{60,98}. Relaxation of the switch regions to the GDP-bound conformation is presumably the major post-hydrolysis barrier preceding phosphate release. For GAP-catalyzed reactions, dissociation of the GAP-GTPase complex may limit the rate of conformational relaxation and consequently the rate of phosphate release. Whether conformational relaxation and/or phosphate release can precede GAP dissociation is unclear; however, the crystal structure of ArfGAP in complex with Arf1-GDP⁹⁹ suggests that this possibility warrants further investigation.

The arginine finger/cis-glutamine paradigm and variations on the theme

RasGAP

A key insight facilitating atomic resolution crystallographic studies of GAP complexes with small GTPases involved the discovery that aluminum fluoride stabilizes complexes between the GDP-bound forms of several small GTPases including Ras and their cognate GAPs⁷².

The first crystal structure of a small GTPase-GAP complex was determined for Ras-GDP-AlF₃ in complex with the catalytic domain of RasGAP⁶⁴. The structure revealed polar and electrostatic interactions involving Gln 61 and an 'arginine finger' from the GAP that have similar stereochemistry to interactions mediated by the catalytic glutamine and arginine residues in G_α subunits, despite different aluminum fluoride coordination geometries and different arginine rotamer configurations (Fig. 3A and B). Consistent with an important role in catalysis, mutation of the arginine finger reduces GAP activity by three orders of magnitude⁹. The RasGAP-Ras-GDP-AlF₃ structure further clarified how oncogenic substitutions interfere with formation of the transition state. The frequently occurring G12V oncogenic mutation, for example, introduces van der Waals conflicts with both the arginine finger and cis-glutamine (Fig. 3C). The van der Waals conflict with the cis-glutamine involves the C_β and may help to explain why all Gly12 mutations except proline are oncogenic⁵¹. Indeed, as noted above, the intrinsic activity of G12P Ras is slightly higher than that for wild type Ras^{52,53}.

Rho family GAPs

The crystal structure of RhoA-GDP-AlF₃ in complex with the catalytic domain of RhoGAP revealed an arginine finger/cis-glutamine arrangement similar to Ras⁶³ (Fig. 3D) and, unexpectedly, a related overall architecture for the RhoGAP and RasGAP catalytic domains despite limited sequence homology¹⁰⁰. The crystal structure of RhoGAP in complex with Cdc42-GppNHP suggests that the arginine finger and DxxGQ glutamine are not engaged with the γ phosphate in the ground state complex¹⁰¹. From these and subsequent crystallographic studies of RhoGAP in complex with RhoA-GDP-MgF₃⁻⁶⁹, which more closely approximates the expected transition state geometry than the AlF₄⁻ complex, as well as a catalytically compromised Cdc42GAP (arginine finger mutated to alanine) in complex with Cdc42-GDP-AlF₃¹⁰², a coherent paradigm emerged for transition state stabilization involving a cis-glutamine and trans-arginine finger that mediate equivalent polar/electrostatic interactions even though the arginine finger inserts from different directions and adopts different rotamer configurations in RhoGAP vs. RasGAP complexes.

Arf/Arl family GAPs

Since the majority of small GTPase families conserve the DxxGQ glutamine, it was anticipated that their GAP complexes would conform to the cis-glutamine/trans-arginine paradigm. Although this is indeed the case for several GTPase families, there are nevertheless differences in structural detail that have implications for the catalytic mechanism. For example, the retinitis pigmentosa protein 2 (RP2) GAP in complex with Arl3-GDP-AlF₃ revealed an arginine finger/cis-glutamine configuration similar to that observed for Ras/Rho GAPs⁶⁵ (Fig. 3E). However, the cis-glutamine is not optimally positioned to donate a hydrogen bond to an equatorial oxygen in the transition state due to a van der Waals conflict with an aspartic acid residue at the P-loop position equivalent to Ras Gly 12. An arginine finger/cis-glutamine configuration was also observed in the crystal structure of the zinc finger GAP domain-ankyrin repeats of the ArfGAP ASAP3 in a complex with Arf6-GDP-AlF₄⁻ that was stabilized by generating a fusion construct¹⁰³ (Fig. 3F). Although the resolution of the latter structure is too low for detailed stereochemical analysis, the same van der Waals conflict is predicted. Notably, however, both GAPs supply

the arginine finger from an angle that circumvents potential steric conflict with the P-loop aspartic acid.

GAP mechanisms without a catalytic arginine and/or glutamine

RanGAP

The hydrolytic reactions in some small GTPase/GAP systems proceed in the absence of a DxxGQ glutamine and/or arginine finger. When crystal structures were determined for RanGAP in ternary complexes with Ran binding protein (RanBP) and ground state Ran-GppNHp or transition state mimetic Ran-GDP-AlF₃, it became clear that while the DxxGQ glutamine mediates polar interactions similar to its counterpart in the Ras and Rho GAP complexes, RanGAP does not supply an arginine finger¹⁰⁴ (Fig. 4A). Since RanBP binds to a distal site, the catalytic mechanism likely involves allosteric effects, including stabilization of a catalytically competent orientation of the cis-glutamine similar to RGS GAPs for G_α subunits^{105,106}, in addition to proposed solvent shielding of the hydrolytic site by the switch I tyrosine equivalent to Ras Tyr 32. Indeed, the switch I tyrosine in Ran adopts a closed conformation that contrasts with the open conformation required for insertion of the arginine finger in most GTPase/GAP complexes. Interestingly, a conserved arginine from RanGAP packs against the switch I tyrosine and appears to be sufficiently close to potentially insert into the hydrolytic site if the switch I tyrosine was in an open conformation. Alanine substitution of the conserved arginine, or a nearby conserved glutamic acid with which it forms an ion pair, has little effect on GAP activity whereas alanine substitution of another conserved arginine proposed to be a catalytic arginine finger reduces k_{cat} by two orders of magnitude^{107,108}. The proposed arginine finger, however, is located roughly 15 Å from the hydrolytic site in the transition state mimetic complex¹⁰⁴. Time resolved FTIR studies reveal a small conformational change in the hydrolytic site preceding rate limiting bond breakage, and further suggest that the switch I tyrosine inhibits intrinsic hydrolysis since alanine substitution increases the intrinsic rate by roughly two orders of magnitude but has little effect on GAP-accelerated hydrolysis⁹⁸.

Rap GAPs

Although closely related to Ras, the small GTPase Rap has a non-catalytic threonine rather than a glutamine following the DxxG motif. Rap is deactivated by two distinct GAPs, Rap-specific RapGAPs and dual specificity Ras/Rap GAPs. Unlike the dual specificity Ras/Rap GAPs, RapGAPs lack an arginine finger¹⁰⁹. A mutational analysis based on the structure of the RapGAP1 catalytic domain indicated that RapGAPs supply an 'asparagine thumb' in lieu of the cis-glutamine¹¹⁰. The crystal structure of RapGAP1 in complex with Rap-GDP and the ground state mimetic BeF₃⁻ confirmed that the asparagine thumb is appropriately oriented towards BeF₃⁻ to function in catalysis¹¹¹ (Fig. 4B). In addition, the switch I tyrosine equivalent to Ras Tyr 32 adopts a closed conformation similar to that in the Ran/RanGAP complex. The tyrosine phenyl moiety rather than its hydroxyl group appears to play an important role in the GAP reaction, since mutation to alanine reduces catalytic activity 25 fold compared to less than two fold reduction for mutation to phenylalanine. Time resolved FTIR experiments combined with mutational analyses of wild type and mutated dual specificity Ras/Rap GAPs (GAP1 and RASAL) provide compelling evidence that the

GAP domain reorients switch II such that a glutamine three residues after the DxxG motif (i.e. not the typical *cis*-glutamine) assumes a catalytic role analogous to the DxxGQ glutamine in Rho, Ras and G α subunits¹¹². As these examples illustrate, different GAPs can exploit structurally distinct catalytic mechanisms to deactivate common GTPase substrates.

Sar1 GAP

In the Sar1 GTPase involved in vesicle budding from endoplasmic reticulum exit sites, and also in evolutionarily conserved prokaryotic and eukaryotic GTPases involved in translation elongation (EF-Tu, EF1A, EF-G and IF2), the DxxGQ glutamine is replaced by a catalytic histidine. Unlike glutamine, the relatively low pK_a for histidine (typically near 6) would appear to make it a reasonable candidate for general base catalysis through activation of the nucleophilic water. However, simulations for EF-Tu in the context of the ribosome suggest that the pK_a for the catalytic histidine is several units higher due to nearby counter ions such that the histidine is protonated in the ground state and instead functions in electrostatic compensation of the hydroxide ion generated during transfer of the proton from the nucleophilic water to the γ phosphate¹¹³. Although it is not clear if the histidine in Sar1 functions in an analogous substrate-assisted mechanism, the Sar1 GAP (i.e. the Sec23/24 complex) supplies a putative arginine finger that is directed into the hydrolytic site in the crystal structure of the Sar1-GppNHp complex with Sec23/24¹¹⁴.

Cation-dependent GAPs

Finally, it is interesting that several GTPases substitute the DxxGQ glutamine with a non-catalytic hydrophobic amino acid residue and have been termed 'HAS-GTPases'^{115,116}. These include the cation-dependent MnmE and dynamin family GTPases, which utilize either K⁺ (MnmE) or Na⁺/K⁺ in lieu of a catalytic arginine. In the crystal structure of dimeric MnmE with the transition state mimetic GDP-AlF₄⁻, a conserved glutamate residue from switch II compensates for the absence of a *cis*-glutamine through an indirect water-mediated polar interaction with the nucleophilic water¹¹⁷. These and related examples have been reviewed in the context of potentially unifying principles, including steric hindrance arising from P-loop substitutions at the position equivalent to Ras Gly12, as a selective pressure for evolution of diverse GAP-GTPase mechanisms¹¹⁸.

The dual trans-finger mechanism in TBC domain Rab GAPs

Rab proteins comprise the largest GTPase family, with 11 members in yeast, 20-30 in lower metazoan organisms, and 60 members in humans^{119,120}. Multi-genome phylogenetic analyses predict that the last eukaryotic common ancestor (LECA) had approximately 23 different Rab GTPases, indicating that the Rab genes diverged early in eukaryotic evolution to regulate trafficking between different organelles of the endomembrane system^{121,122}. TBC domain proteins also emerged early during eukaryotic evolution with slightly lower numbers than Rab GTPases in diverse genomes, suggestive of a co-evolutionary relationship¹²³. From the initial characterization of TBC proteins in yeast, which are known as GAPs for Ypt (Rab) GTPases or Gyps, it was clear that most yeast TBC domain proteins were GAPs for different though overlapping subsets of Rab GTPases¹²⁴⁻¹²⁷. It is generally thought that most TBC proteins in other organisms are Rab GAPs and that most Rab

GTPases have TBC domain GAPs, although the Rab substrate specificity profiles remain to be established for a number of mammalian TBC domain proteins¹¹.

For the prototypical yeast Rab GAP Gyp1, a putative arginine finger was identified by mutational analysis based on the crystal structure of the TBC domain¹²⁸. Given that Rab GTPases conserve the DxxGQ glutamine (except Rab25, which has a natural leucine substitution), the standard cis-glutamine/trans-arginine configuration was expected. Rab-GDP-aluminum fluoride complexes with Rab GAPs are marginally stable and thus difficult to crystallize; however, the Gyp1 complex with mammalian Rab33 was a fortuitous exception⁶². High resolution phylogenetic analyses indicate that Rab33 first appeared in multicellular metazoan organisms and is absent from the fungi lineage¹²². Nevertheless, Gyp1 has 5 fold higher catalytic efficiency for Rab33 than the best characterized yeast substrate Ypt1, and forms sufficiently stable complexes with Rab33-GDP and aluminum fluoride to support crystallization⁶². Although the crystal structure of Gyp1 in complex with Rab33-GDP-AlF₃ confirmed the predicted arginine finger, it also revealed a major surprise regarding the role of the DxxGQ glutamine (Fig. 4C). Rather than adopting the expected transition state stereochemistry, the DxxGQ glutamine instead mediates bivalent polar interactions with the main chain of the TBC domain, consistent with a role in binding rather than catalysis. Substituting for the DxxGQ glutamine, is a 'glutamine finger' from the TBC domain, which mediates canonical bivalent polar interactions with the transition state mimetic. Notably, the glutamine and arginine fingers are both conserved in nearly all TBC domains, with the exception of the small TBC1D3 subfamily in which both are replaced. The dual finger configuration has also been observed in the crystal structure of the TBC1D20-Rab1-GDP-AlF₃ transition state complex⁶⁰ (Fig. 4D), and mutational analyses of these and other TBC domain/Rab systems suggest that the dual finger modality is a fundamental feature of TBC domain GAPs^{62,129}.

Lessons from pathogenic bacterial GAPs that manipulate host GTPases

Some pathogenic bacteria have evolved GAP proteins that deactivate one or more small GTPases in host cells during infection. Several examples and the underlying evolutionary significance are discussed below.

Rab GAPs from *Shigella flexneri* and enteropathogenic *E. coli*

The structurally similar bacterial proteins VirA from *Shigella flexneri* and EspG from enteropathogenic *E. coli* have high GAP activity for Rab1 and crystal structures of VirA and EspG in transition state mimetic complexes with Rab1-GDP-AlF₃ revealed a dual arginine/glutamine finger configuration analogous to that of TBC domains⁵⁹ (Fig. 4E). Considering that the overall tertiary structures of VirA/EspG and TBC domains are unrelated, the common dual finger mechanism is evidently an example of convergent evolution.

A Rab GAP from *Legionella pneumophila*

Another Rab1 GAP LepB is injected into lung macrophages during infection by the intracellular pathogen *Legionella pneumophila*¹³⁰. The catalytic efficiency of LepB for Rab1 ($k_{\text{cat}}/K_M \approx 10^6 \text{ M}^{-1} \text{ s}^{-1}$) is an order of magnitude higher than that of the host Rab1 GAP

TBC1D20, a difference which likely reflects a very high turnover number for LepB ($k_{\text{cat}} \approx 10^2 \text{ s}^{-1}$) approaching the range of traditional enzymes^{131,132}. Crystal structures of LepB in complex with either the ground state mimetic BeF_3^- or the transition state mimetic AlF_3 revealed a cis-glutamine/trans-arginine configuration rather than the dual trans-finger configuration of TBC domains and VirA/EspG^{66,131,133} (Fig. 4F). Although the low resolution of the transition state mimetic structures limits the certainty of conclusions regarding specific polar interactions, there is nevertheless an elaborate network of polar residues, four from Rab1 and two from LepB, that likely contribute directly to transition state stabilization as suggested by their disposition in the hydrolytic site of the ground/transition state mimetic structures and by the relative magnitude of changes in catalytic efficiency for systematic mutation of polar residues throughout the binding interface¹³¹. In addition to the DxxGQ glutamine and arginine finger, the other putative catalytic residues include a P-loop serine corresponding to Ras Gly 12, a switch I serine preceding the invariant threonine, and a switch II arginine that forms an ion pair with a 'glutamic acid finger' from LepB. The P-loop and switch I serines are appropriately disposed to mediate polar interactions with the γ phosphate in the transition state. The switch I serine may also interact with the cis-glutamine and nucleophilic water. The ionic interaction between the switch II arginine and LepB glutamic acid finger secures the cis-glutamine in a catalytically competent orientation. Alanine substitution of either the DxxGQ glutamine or switch II arginine reduces catalytic efficiency by four orders of magnitude, while individual alanine substitutions of the P-loop/switch I serines or arginine/glutamic acid fingers reduces catalytic efficiency by two orders of magnitude. Alanine substitutions of other polar residues in the binding interface reduce catalytic efficiency by 10 fold or less. Finally, LepB facilitates insertion of its arginine finger by clamping the switch I tyrosine equivalent to Ras Tyr 32 in an open conformation.

Evolutionary significance

The convergent evolution of dual finger mechanisms in TBC domains and VirA/EspG along with independent evolution of LepB to exploit an atypical polar catalytic network, implies a selective pressure that is likely due to the inability of the cis-glutamine to adopt an optimal stereochemistry for stabilization of the transition state in GTPases with residues other than glycine at the P-loop position equivalent to Ras Gly 12¹¹⁸. Indeed, all but a few Rab GTPases including Rab1 have residues other than glycine at the equivalent P-loop position. One solution to this problem is for the GAP to supply a glutamine finger that inserts from a different orientation to avoid van der Waals conflicts as in VirA/EspG and TBC1D20. An alternative solution is to exploit a polar network of catalytic residues to compensate for the sub-optimal stereochemistry of the cis-glutamine as in LepB.

GAP regulatory mechanisms

Given that most small GTPases are membrane localized it is not surprising that a major paradigm for regulation of GAPs involves membrane recruitment, which can be mediated directly by lipid interactions and/or indirectly by interactions with membrane-bound proteins. Another important regulatory paradigm involves post-translation modification of either the GAP or substrate GTPases, which can alter membrane recruitment, interactions

with the GTPase, and potentially the hydrolytic activity. Allosteric and autoregulatory mechanisms have also been identified for some GAPs, although the extent of autoregulation and the underlying structural mechanisms remain poorly characterized in most cases. Cross-talk between small GTPases and non-cognate GAPs has also been reported to influence the GAP activity. Finally, expression levels of GAPs can be regulated, either during development or during progression of physiological or pathological processes. Several selected examples illustrating different mechanisms and functional consequences are highlighted below. GAP regulatory mechanisms and their implications for feedback amplification loops have been recently reviewed¹⁵.

Autoregulation in Rho family GAPs

The RacGAP β 2-chimaerin is activated by diacylglycerol (DAG) binding to its C1 domain. The crystal structure of the inactive form of β 2-chimaerin revealed an autoinhibitory mechanism whereby the N-terminus of the protein intrudes into the Rac binding site in the GAP domain¹³⁴ (Fig. 5A). The autoinhibited conformation is stabilized by multiple intramolecular interactions involving the N-terminus, SH2 domain, RacGAP domain and the SH2-C1 linker, which occlude the DAG/phospholipid membrane binding site in the C1 domain. DAG/phospholipid membrane binding to the C1 domain likely relieves autoinhibition by destabilizing these interactions. Another Rho family GAP, p50RhoGAP, is proposed to be autoregulated, although the structural basis remains unknown¹³⁵. Biochemical and yeast two hybrid analyses of truncation constructs suggest that the GAP activity may be autoinhibited by a mechanism involving the N-terminal region of the protein as well as the Sec14 domain, which has also been implicated in endosome targeting¹³⁶, and relieved by binding of the N-terminal region to the prenylated C-terminus of Rac. The activities of p190B RhoGAP and RA-RhoGAP are modulated by interaction with Rac and Rap1, respectively^{137,138}; however, it is not known if activation involves relief of autoinhibitory constraints or another mechanism.

Arf GAP regulation by cooperative membrane recruitment and curvature sensing

The Arf GAP ASAP1 has several modular domains including a PH domain. The GAP activity of ASAP1 exhibits cooperative dependence on phosphatidylinositol 4,5-bisphosphate (PIP2) density in liposome membranes¹³⁹. Although the cooperative effect requires PIP2 binding to the PH domain, the underlying structural mechanism was enigmatic¹⁴⁰. The recent crystal structure of the PH domain in complex with soluble dibutyl PIP2 offered an unexpected insight¹⁴¹ (Fig. 5B). In contrast to the phosphoinositide binding modalities observed in other PH domains, where the phosphoinositide typically binds to either a 'canonical site' or a proximal 'non-canonical' site, both sites are simultaneously occupied in the ASAP1 PH domain. The cooperative mechanism, which is also thought to involve dynamic interactions between the PH and GAP domains detected by NMR¹⁴⁰, is proposed to confer switch-like sensitivity of ASAP1 GAP activity to PIP2 density. The GAP activity of ArfGAP1, which is required for COPI vesicle budding from Golgi membranes, increases by two orders of magnitude as the membrane curvature of liposomes is increased to approximate that of a budding vesicle¹⁴². Membrane curvature is sensed by the folding of an ArfGAP1 Lipid Packing Sensor (ALPS) motif proximal to the GAP domain¹⁴³. ALPS motifs form amphipathic helices stabilized by partitioning with high curvature membranes.

Unlike typical amphipathic helices, however, the hydrophilic surfaces of ALPS motif helices are rich in serine/threonine rather than basic residues. How curvature sensitive folding/partitioning of the ALPS motif increases GAP activity remains to be established.

Rab cascades and regulation of Rab distributions by Rab GAPs

The membrane recruitment mechanism for the Rab GAP Gyp1, which as noted above deactivates the yeast Rab1 ortholog Ypt1, illustrates a functional ‘Rab cascade’ in which the GAP for Ypt1, a Rab required for fusion of ER-derived vesicles with the cis-Golgi, is recruited to the Golgi through an effector binding interaction with active Ypt32, a downstream Rab involved in intra-Golgi transport^{125,126,144}. This Rab cascade enhances segregation between Ypt1 and Ypt32 through a Gyp1-dependent temporal mechanism in which Ypt1 compartments are converted to Ypt32 compartments.

Kinase regulation of a Rab GAP in insulin-stimulated glucose transporters translocation

The Akt kinase substrate AS160 (TBC1D4) has GAP activity for several Rab substrates including Rab10¹⁴⁵. Under basal conditions in adipocytes, AS160 is recruited to vesicles containing the GLUT4 glucose transporter, where it deactivates Rab10 and possibly other Rab GTPases^{146,147}. In response to insulin stimulation, Akt phosphorylation of AS160 results in its release into cytoplasm and concomitant accumulation of active Rab10, which is important for GLUT4 translocation to the plasma membrane.

Regulation of Rab GAP reactions by substrate AMPylation

During infection of lung macrophages, the intracellular bacterial pathogen *Legionella pneumophila* injects at least 270 distinct ‘effector proteins’ (i.e. bacterial effectors as opposed to GTPase effectors), including a GEF (DrrA/SidM), GAP (LepB) and binding protein (LidA) for Rab1^{130,148,149}. In addition to a P4M domain that selectively binds PI4P to facilitate targeting to the *Legionella*-containing vacuole¹⁵⁰ and a GEF domain that activates Rab1¹⁵¹⁻¹⁵³, DrrA/SidM contains an AMP transferase (ATase) domain that covalently attaches AMP to a conserved tyrosine in the switch II region of Rab1⁹¹. AMPylated Rab1 is inert to deactivation by LepB or the host Rab1 GAP TBC1D20 and must be de-AMPylated by another *Legionella* effector SidD prior to de-activation by GAPs^{132,154}. Notably the AMPylated tyrosine is located at the binding interface in the crystal structures of the LepB (Fig. 5C) and TBC1D20 complexes with Rab1. In contrast, AMPylation does not impair binding to LidA, which has picomolar affinity for Rab1 independent of nucleotide state^{132,155,156}. Thus, AMPylation repurposes Rab1 for selective interactions with LidA, possibly to facilitate tethering of ER-derived vesicles for fusion with the LCV¹⁵⁷.

Regulation of Arl3 subfamily GAPs by interfacial calcium

The crystal structure of Arl3-GDP-AlF₃ in complex with the ASAP3 GAP domain revealed a Ca²⁺ ion at the GTPase-GAP binding interface that is coordinated by residues from both proteins¹⁰³ (Fig. 5D). Ca²⁺ was found to simulate the GAP activity of ASAP1 and ASAP3 (and likely ASAP2) but not ArfGAPs from other subfamilies. Ca²⁺ activation may couple

the GAP activity of ASAP isoforms to release of Ca^{2+} from internal stores and thereby generate crosstalk between Ca^{2+} and Arf signaling pathways.

GAPs, disease and therapeutic targets

Given that small GTPase regulatory networks control many fundamental biological processes, it is not surprising that they are increasingly implicated in disease initiation, progression, severity and/or therapeutic resistance. The examples below illustrate some of the different roles for GAPs in pathological processes as well as some potential targets and strategies for therapeutic intervention. More detailed discussion of disease relationships for small GTPases and their regulatory factors can be found in other in depth reviews^{13,15,20,50,158-161}.

RasGAPs in tumor suppression

Although constitutively activating Ras mutations (including the aforementioned Gly 12 and Gln 61 substitutions) occur in roughly 30% of human tumors, they are curiously absent in some cases where down stream signaling pathways are nevertheless hyperactivated¹⁶⁰. In this context, the 14 human RasGAPs are increasingly appreciated as *bona fide* or potential tumor suppressors. The RasGAP neurofibromin encoded by the NF1 gene is the earliest and best characterized example¹⁶². Deletion or loss of function mutations in NF1 are responsible for peripheral nervous system tumors (neurofibromas) that are the hallmark of neurofibromatosis type 1 and studies in mice provide evidence for a more general role of NF1 in familial as well as sporadic cancers¹⁶⁰.

A RheB GAP with a RapGAP-like domain in tuberous sclerosis

The tuberous sclerosis complex (TSC), consisting of the hamartin and tuberin proteins encoded by the TSC1 and TSC2 tumor suppressor genes respectively, functions as a GAP for the small GTPase RheB in the mechanistic target of Rapamycin (mTOR) signaling pathway¹⁶³. Interestingly, missense mutations that map to the Rap1GAP-like domain of tuberin and disrupt GAP activity for RheB have been identified in patients with tuberous sclerosis. The unconventional TBC domain protein TBC1D7 was shown to be a third component of the TSC¹⁶⁴. Although TBC1D7 knockdown results in increased mTOR Complex 1 (mTORC1) signaling, delayed initiation of autophagy and resistance to glucose or growth factor withdrawal, it does not appear to be associated with tuber sclerosis mutations. Homology modeling based on the Rap1GAP-Rap1 structure described above suggests that at least one patient mutation (R388P) in the TSC2 GAP domain disrupts binding to RheB (or possibly Rap1) rather than hydrolytic activity *per se*¹¹⁰.

RP2 in X-linked retinitis pigmentosa

In the Arl3 GAP RP2 described above, loss of function mutations in the GAP domain including the arginine finger interfere with trafficking between the Golgi and sensory cilium membrane, leading to retinal degeneration, and account for ~15% of X-linked retinitis pigmentosa cases^{165,166}. Several missense mutations involve residues in the GAP domain including the arginine finger⁶⁵.

An unconventional TBC domain protein in DOORS syndrome

Mutations in TBC1D24 are associated with DOORS syndrome^{167,168}, a rare familial disorder characterized by multiple clinical presentations including infantile epileptic seizures and developmental delay, as well as rare autosomal-dominant hearing loss¹⁶⁹⁻¹⁷¹. TBC1D24 interacts with Arf6, and evidently functions as a negative regulator¹⁷². Notably, TBC1D24 lacks the characteristic arginine and glutamine fingers, suggesting that it does not function as a Rab GAP or otherwise deviates from the dual finger paradigm. Whether the TBC domain of TBC1D24 has GAP activity for Arf6 or Rab substrates remains to be determined.

The Rab3GAP complex in neurological disorders

Rab3GAP is a heterodimer consisting of catalytic and regulatory subunits, and deactivates Rab3 isoforms in neurons to regulate synaptic vesicle fusion^{173,174}. Mutations in both subunits have been linked to the autosomal recessive neurological disorders Warburg Micro Syndrome (catalytic subunit mutations) and Martsolf Syndrome (regulatory subunit mutations)^{175,176}. In the case of Warburg Micro Syndrome, the majority of mutations occur in the C-terminal region of the catalytic subunit required for catalytic activity and are proposed to disrupt GAP activity. Since the catalytic subunit lacks homology with other GAP domains and the tertiary structure remains to be determined, the underlying structural bases and etiology are poorly understood.

GAPs from pathogenic bacteria

Several pathogenic prokaryotes supply GAPs that manipulate small GTPases during infection¹⁵. These include VirA/EspG and LepB described above as well as the *Salmonella typhimurium* RhoGAP SptP, which functions in conjunction with the *Salmonella typhimurium* RhoGEF SopE to control Rac-1 and Cdc42 activation and actin dynamics^{177,178}. SptP supplies an arginine finger and utilizes the DxxGQ glutamine but otherwise does not resemble the tertiary structure of host Rho family GAPs, indicative of a convergent evolutionary process that preserves underlying stereochemical functionality¹⁷⁹. Several other pathogenic bacteria have proteins with structurally related RhoGAP domains including *Yersinia pestis* YopE¹⁸⁰, *Pseudomonas aeruginosa* ExoS¹⁸¹ and ExoT¹⁸², and *Aeromonas hydrophila* AexT¹⁸³. As with bacterial RabGAPs, the catalytic activities of the bacterial RhoGAP domains are considerably higher than those of host RhoGAPs¹⁵.

Therapeutic targets, validation and strategies

At least some GTPase regulatory factors, in particular those required for maintenance of pathologic conditions that can also be validated as *in vivo* targets in animal models for disease, are potential candidates for development of new mechanisms-based therapeutic interventions. Inspired in part by natural inhibitors such as the fungal metabolite Brefeldin A, which inhibits Arf GTPase activation by forming an interfacial ternary complex with Arf1-GDP and Golgi Arf GEFs¹⁸⁴, efforts to identify chemical inhibitors of small GTPase GEFs have led the way^{185,186}. However, there is no fundamental reason to exclude GAPs or mutated GTPases as potential targets. Indeed, it has been proposed that chemical compounds might be found that would counter the effects of constitutively activating Ras mutations, possibly by mimicking acceleration of GTP hydrolysis by GAPs, trapping ternary complexes

with Ras GAPs, or modulating protein expression or degradation¹³. A fundamentally different approach that has recently generated considerable excitement as well as concern involves correction of mutations or knockout/inactivation of mutated genes through gene-editing¹⁸⁷. High efficacy gene-editing approaches would require improved specificity to minimize off-target effects, possibly in conjunction with better vehicles for delivery to affected cells in order to increase potency, penetrance and selectivity. Short of therapeutic applications, gene editing tools are already playing an important role in target validation and assessment of potential therapeutic strategies in cell and animal models¹⁸⁸.

Concluding remarks

Since the discovery of small GTPases several decades ago, considerable progress has been made with respect to delineating the structural bases and mechanisms underlying the intrinsic vs. GAP-catalyzed GTP hydrolytic reactions for all of the major GTPase families. Most GAPs provide a catalytic arginine finger, although some GAP/GTPase systems utilize a K^+ or Na^+ ion to achieve analogous electrostatic compensation whereas others lack a catalytic arginine and do not depend on monovalent ions. Simple generalizations regarding the role of the largely conserved DxxGQ glutamine are complicated by structural variations and exceptions. In the known cases where the DxxGQ glutamine is either non-catalytic or replaced by a non-catalytic residue, a distinct though functionally equivalent glutamine/asparagine may be supplied in trans or even in cis. In retrospect, it appears that some of the structural variations and trans vs. cis alternatives may be related to oncogenic mutations in Ras. For example, the trans glutamine alternative avoids van der Waals conflicts imposed by natural substitutions at the P-loop position corresponding to Ras Gly12 that prevent the DxxGQ glutamine from adopting optimal stereochemistry in the transition state. Thus, the chemical functionality is better preserved than individual residues or their functional roles.

The nature of the transition state/intermediates and specific chemical functions of catalytic residues in the hydrolytic site has been the subject of considerable debate, with multiple alternatives proposed on the basis of experimental observations and computational analyses. It is generally accepted that the arginine finger contributes to electrostatic reduction of developing charge in the transition state. The cis/trans-glutamine or asparagine equivalent is thought to orient the nucleophilic water and stabilize the transition state geometry, with some differences in stereochemistry forced by P-loop substitutions as noted above. For Ras/RasGAP, which is by far the best characterized GTPase/GAP system, there appears to be an emerging though still developing consensus supporting a transition state/intermediate reaction pathway with substantial associative character but without a general base for activation of the nucleophilic water. Proton transfer from the nucleophilic water to the γ phosphate may not be direct, but instead mediated either by a second water in the intrinsic reaction or the DxxGQ glutamine in the GAP reaction. Regardless of the proton transfer pathway, the catalytic effect of the GAP appears to derive primarily from electrostatic compensation by the arginine finger in combination with allosteric modulation. The allosteric contribution includes a staggered-to-eclipsed configurational change involving the non-bridging oxygens of the α - β phosphates, which adds to the steric strain in the phosphate-bonding network incurred by an analogous configurational change for the non-bridging oxygens of the β - γ phosphates accompanying GTP binding to Ras.

Beyond well characterized instances, the regulatory mechanisms for most GAPs are either unknown or partially characterized. The dearth of detailed information is due in part to the challenges associated with expressing and purifying full length GAPs proteins and/or complexes in quantities required for structural and biophysical studies. Nevertheless, it is already clear that the mechanisms for regulation of GAP localization/activity are even more diverse than the catalytic mechanisms. Likewise, many diseases including a substantial fraction of cancers are genetically-linked to disruption or impairment of hydrolytic activity as a consequence of mutations in small GTPases as well as their GAPs. Pathogens also supply GAPs to manipulate the hydrolytic activity of host small GTPases.

Despite considerable progress, there are still major questions that require clarification and additional paradigm-shifting observations can be expected. With respect to intrinsic and GAP catalyzed reaction mechanisms, it is not entirely clear whether or how the results from extensive studies of Ras/RasGAP can be extended to other GTPases/GAP systems, especially those with significant structural divergence in the hydrolytic site. Further biophysical and computational investigation of the intrinsic and GAP reactions for other GTPase/GAP families would be useful. Meaningful comparisons of computational analyses with experimental observations will require more detailed kinetic and time-resolved spectroscopic studies in order to distinguish chemical steps from other potentially rate limiting pre/post-transition state barriers including phosphate release. There is also a clear need for exploration of GAP regulatory mechanisms in the context of the full length proteins, complexes and membranes as well as the application of quantitative experimental approaches to extend *in vitro/in silico* observations to cellular environments. Finally, there is a growing awareness of the important role of small GTPases and GAPs with respect to disease onset, progression and severity. The surface has hardly been scratched with respect to development of new mechanism-based therapeutics targeting components of GTPase regulatory networks. A strong case can be made for further exploration of GTPases and GAPs in disease, including the underlying molecular/structural mechanisms, target validation, and testing of therapeutic strategies in disease models.

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Figure 1. Small GTPase cycle and architecture

(A) GTPase cycle with key nucleotide exchange and GTP hydrolysis intermediates. Also indicated are metal fluoride complexes commonly used as ground and transition state mimetics in GAP complexes with GDP-bound small GTPases. (B) Structure of the prototypical small GTPase Ras in the active conformation bound to the non-hydrolyzeable GTP analog GppNHp (PDB ID 5P21). Characteristic G motifs, conformational switch regions and key functional residues are also indicated.

Figure 2. Reaction pathways and transition state intermediates for GTP hydrolysis
 Transition state geometries corresponding to dissociative, associative, and concerted reaction pathways are depicted with approximate interatomic distances for the bond breakage ($P_{\gamma}-O_{\beta\gamma}$) and bond formation ($P_{\gamma}-O_{\text{wat}}$) coordinates.

Figure 3. The arginine finger/cis-glutamine paradigm and variations

Hydrolytic sites in crystal structures of GTPases and GAP/GTPase complexes with ground or transition state mimetics. (A) G_{ta} -GDP- AlF_4^- (PDB ID 1TAD). Note Mg^{2+} is replaced by Ca^{2+} from the crystallization buffer. (B) RasGAP-Ras-GDP- AlF_3 (PDB ID 1WQ1). (C) RasGAP-RasG12V-GDP- AlF_3 with the G12V mutation modeled on the structure in B. (D) RhoGAP-Rho-GDP- MgF_3^- (PDB ID 1OW3). (E) RP2-Arl3-GDP- AlF_4^- (PDB ID 3BH7). (F) ASAP3-Arf6-GDP- AlF_3 (PDB ID 3LVR).

Figure 4. Exceptions to the arginine finger/cis-glutamine paradigm

Hydrolytic sites in crystal structures of GAP/GTPase complexes with ground or transition state mimetics. (A) *RanGAP-Ran-GDP-AlF₃* (PDB ID 1K5G). (B) *RapGAP-Rap1-GDP-BeF₃⁻* (PDB ID 3BRW). (C) *Gyp1-Rab33-GDP-AlF₃* (PDB ID 2G77). (D) *TBC1D20-Rab1-GDP-BeF₃⁻* (PDB ID 4HLQ). (E) *EspG-Rab1-GDP-AlF₃* (PDB ID 4FMC). (F) *LepB-Rab1-GDP-AlF₃* (PDB ID 4IRU).

Figure 5. Examples of GAP regulatory mechanisms

(A) Structure of autoinhibited β 2-Chimaerin (PDB ID 1xA6) with Rho and phorbol ester derived from the structures of the RhoGAP complex with Rho-GDP-AIF₄⁻ (PDB ID 1TX4) and the PKC δ C1 domain bound to a phorbol ester (PDB ID 1PTR). (B) Structure of the ASAP3 PH domain with two molecules of dibutyl PIP2 simultaneously occupying the canonical and alternative phosphoinositide binding sites (PDB ID 5C79). The PH domain is shown in a hypothetical orientation relative to a simulated bilayer membrane¹⁸⁹. (C) Location of the switch II tyrosine AMPylated by Rab1 within the interface with LepB in the crystal structure of LepB-Rab1-GDP-AIF₃ (PDB ID 4IRU). (D) Calcium binding site at the GAP-GTPase interface in the crystal structure of ASAP3-Arf6-GDP-AIF₃ (PDB ID 3LVR).