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Review

GTPase activating proteins: structural and functional insights 18 years after discovery

K. Scheffzek[a,*] and M. R. Ahmadian[b]

[a] European Molecular Biology Laboratory, Structural and Computational Biology Programme and Developmental Biology Programme, Meyerhofstrasse 1, 69117 Heidelberg (Germany), Fax: +49 6221 387 519, e-mail: scheffzek@embl.de
[b] Max-Planck-Institute of Molecular Physiology, Department of Structural Biology, Otto-Hahn-Strasse 1, 44227 Dortmund (Germany)

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Abstract. The conversion of guanosine triphosphate (GTP) to guanosine diphosphate (GDP) and inorganic phosphate (P_i) by guanine nucleotide binding proteins (GNBPs) is a fundamental process in living cells and represents an important timer in intracellular signalling and transport processes. While the rate of GNBP-mediated GTP hydrolysis is intrinsically slow, direct interaction with GTPase activating proteins (GAPs) accelerates the reaction by up to five orders of magnitude *in vitro*. Eighteen years after the discovery of the first GAP, biochemical and structural research has been accumulating evidence that GAPs employ a much wider spectrum of chemical mechanisms than had originally been assumed, in order to regulate the chemical players on the catalytic protein-protein interaction stage.

Key words. Signal transduction; cancer; GTPase cycle; arginine finger; switch region; cytoskeleton; nuclear transport; protein targeting.

Introduction

GTP hydrolysis is a fundamental reaction in biology, controlling numerous vital processes, including protein biosynthesis, growth control and differentiation, sensory perception, various transport processes and cytoskeletal reorganization. Guanine nucleotide-binding proteins (GNBPs) provide the catalytic machinery to perform the chemical reaction converting GTP to GDP and inorganic phosphate (P_i). Like molecular switches these proteins cycle between active forms with GTP and inactive forms with GDP bound (fig. 1A) [1]. To become activated, GDP has to be exchanged for GTP. This reaction requires guanine nucleotide exchange factors (GEFs) [2] to catalyse the release of the usually tightly bound GDP, which is subsequently replaced by abundant cellular GTP. Only in the activated state do GNBPs interact with and activate downstream targets, called effectors, which in turn trigger cellular responses. GTP hydrolysis returns GNBPs to their inactive state, thereby terminating downstream signalling. This intrinsically slow reaction with rate constants of 10^{-1} to $3{\cdot}10^{-3}$ min^{-1} can be accelerated by up to five orders of magnitude through interaction with GTPase activating proteins (GAPs) [3–5]. Thus, GEFs and GAPs are essential modulators of the biological activity of GNBPs (fig. 1A).

The G-domain is the primary structural target of GAPs, frequently exemplified for Ras, one of the best-investigated representatives of the GNBP superfamily [6–9]. The primary sequence folds into an α/β structure with residues from the conserved G-motifs [10] forming a

* Corresponding author.

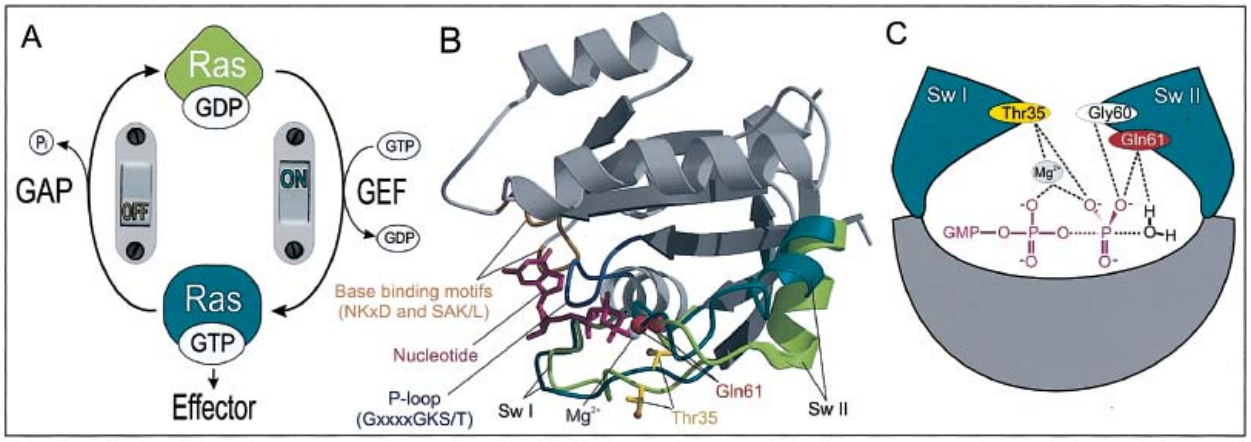


Figure 1. Basic features of GNBPs. (*A*) Conformational switch as the basis functions for the GNBP cycle. GEFs switch 'ON' the GNBPs by binding to the GDP-bound inactive GNBP (green) and catalyse the GDP/GTP exchange reaction. In their GTP-bound active state GNBPs (cyan) either specifically interact with and activate the downstream effectors or are downregulated by GAPs, which switch 'OFF' the signal transduction by stimulating the GTPase reaction of the GNBPs. (*B*) Common characteristics of the G-domain (ribbon). Conserved guanine nucleotide-binding motifs (base and phosphate binding motifs in brown and blue, respectively), the nucleotide (magenta), the Mg^{2+} ion (gray sphere), the invariant threonine (as ball-and-stick in yellow) and the highly conserved catalytic glutamine (red sphere) are highlighted on the G-domain of Ras in both conformations, the GDP-bound (green) [6] and in the GTP-bound state (cyan) [6, 7]. Sw I and Sw II indicate switch I and switch II. (*C*) Schematic view of the intrinsic GTPase reaction mechanism. In the GTP-bound state of the GNBPs, the switch regions (Sw I and Sw II) provide critical residues that fix both the γ-phosphate directly but also *via* the Mg^{2+} ion and the catalytic water molecule. Release of the γ-phosphate after GTP hydrolysis induces a conformational change of switch regions that relax into the GDP-bound state.

shallow surface pocket that accommodates the guanine nucleotides GTP or GDP (fig. 1B). Active and inactive forms differ in the presence or absence of the γ-phosphate on the nucleotide, reflected in considerable conformational differences in regions that contact this terminal phosphate in the GTP-bound form [6, 9]. These regions have been consistently termed switch I and switch II (fig. 1B, C) and are frequently involved in the interactions between the GNBPs and their effectors or/and regulatory proteins [11, 12]. The G-domain can be considered as a structural *tema con variazioni* with the α/β core highly conserved throughout functionally different species but modulated by the insertion or rearrangement of certain structural elements.

Corresponding to their wide variety of functions, GNBPs have been classified into groups that share common sequence motifs implemented into the G-domain (fig. 1B) [1, 11, 13]. Heterotrimeric G-proteins (41–45 kDa), the first class to be discovered, with the GTP/GDP-binding α-subunit, and the regulatory β,γ-subunits, mediate signal transduction from agonist-bound G-protein-coupled receptors to a variety of intracellular effector molecules and ion channels. The multidomain factors, which are important in protein synthesis (e.g. EF-Tu; EF-G) and protein targeting via the signal recognition particle (SRP54 and its receptor SRα) also share a G-domain. The group of interferon γ-inducible GNBPs involve human guanylate binding protein 1 (hGBP1) and MxA proteins (MW 47–65 kDa). Especially interesting is the capability of these proteins to induce GTP hydrolysis down to the level of GMP [14, 15]. In contrast to the large GNBPs, the small GNBPs (also known as 'small GTPases') are globular proteins (MW ~21 kDa) that are commonly counted into the superfamily of Ras-related GNBPs [16], named after its founding member Ras [17, 18]. Among a variety of other processes they control cellular growth and differentiation (Ras family [19]), cytoskeletal dynamics and transcription (Rho/Rac/Cdc42 family [20]), vesicular transport (Rab family [21]), membrane trafficking (Arf family [22]), nucleocytoplasmic transport and mitotic spindle assembly (Ran family [23, 24]). Apart from these major groups and with increasing evaluation of (structural) genomes, one can distinguish other families with growing functional and structural diversity [25].

The GTPase mechanism has attracted considerable attention, largely due to the observation that characteristic G-protein mutants that are not able to hydrolyse GTP contribute to pathological developments [26–28], with cancer leading a growing list of partly life-threatening diseases. In these cases and with Ras in particular, the role of GAPs has been investigated in specific detail since GTPase-deficient mutants are not responsive to these regulators [29–32].

The picture of the general GTPase cycle (fig. 1A) as depicted now in biochemistry textbooks emerged originally from investigations of heterotrimeric G-proteins and EF-Tu. The present conceptual view, that every GNBP is regulated by a GEF, a GAP and in some cases (Rho and Rab families) a guanine nucleotide dissociation inhibitor (GDI), started out with the discovery of the first GAP by Trahey and McCormick 18 years ago [29]. A recent sur-

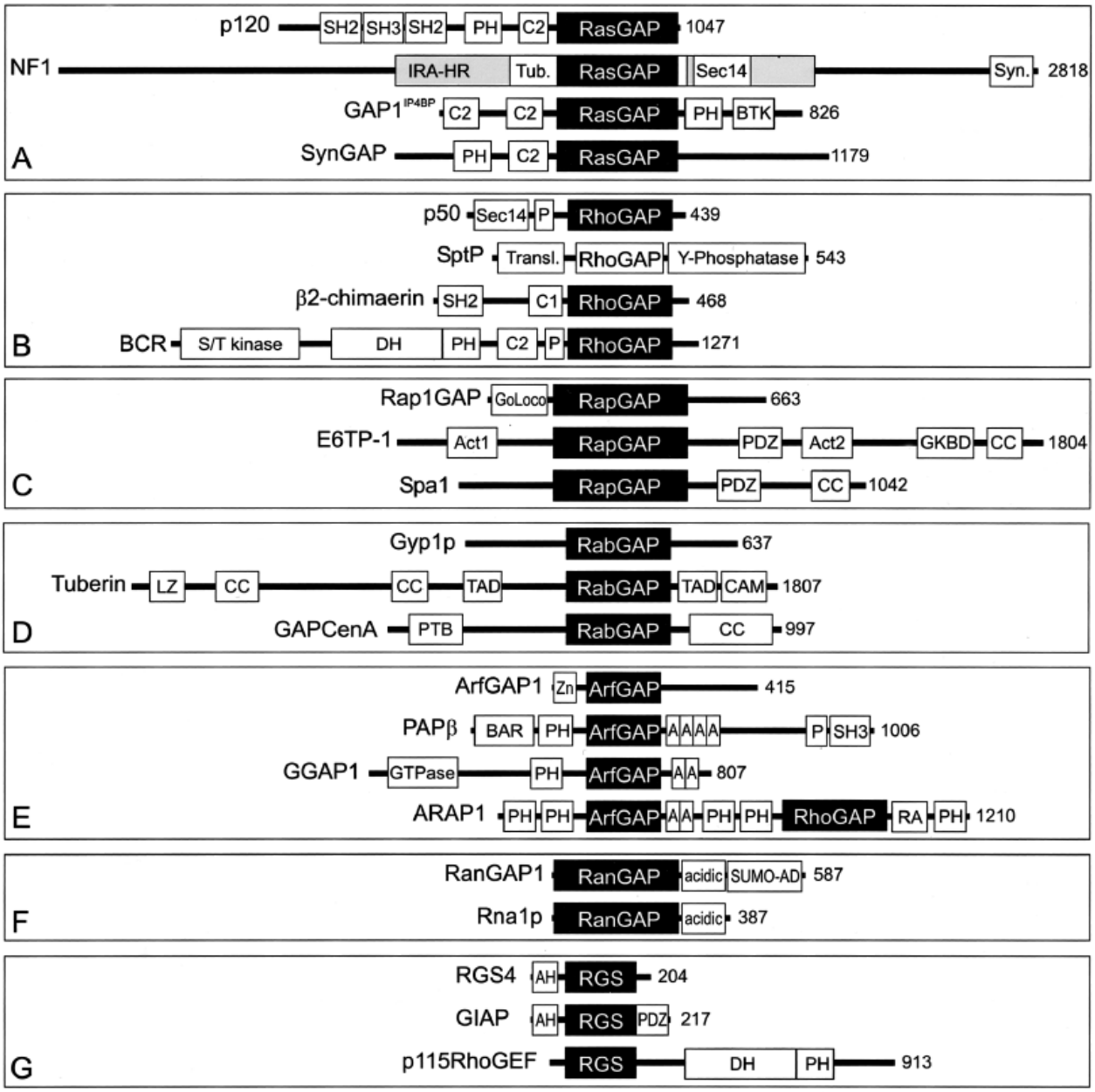


Figure 2. Modular architecture of different GAP families. Multifunctional domain features of different GAP families are illustrated with eukargotic catalytic GAP domains in black. (*A*) RasGAPs, (*B*) RhoGAPs, (*C*) RapGAPs, (*D*) RabGAPs, (*E*) ArfGAPs, (*F*) RanGAPs (*G*) RGS proteins (GαGAPs). The total number of amino acids is shown at the C-terminal end. Abbreviations: Act1/2, actin regulatory; Ank, ankyrin repeat; BAR, Bin-Amphiphysin-Rvs; BTK, Bruton's tyrosine kinase; C2, Ca^{2+}-dependent lipid binding; CAM, calmodulin binding; CC, coiled-coil; DH, Dbl homology; GoLoco, Gα interacting motif; GKBD, guanylate kinase-like binding domain; IRA-HR, IRA homology region; LZ, leucine zipper; P, proline-rich; PBS, paxillin binding subdomain; PDZ, *Drosophila* septate junction protein/Discs-large/ZO-1; PH, pleckstrin homology; PTB, phosphotyrosine binding; RA, Ras association; Sec14, phosphatidylinositol binding; SHD1, Spa2 homology 1 domain; S/T kinase, serine/threonine kinase; Syn., syndecan binding; SH2, Src homology 2; SH3, Src homology 3; SUMO-AD, SUMO association domain; acidic, highly acidic region; TAD, transcription activation domain; Transl., translocation domain; Tub., tubulin-binding; Y-phosphatase, tyrosine phosphatase domain; Zn, zinc finger domain.

vey explored the *Drosophila* and human genomes with respect to GAPs, arriving at the conclusion that about 0.5% of the genes (173 in human and 64 in *Drosophila*) may encode GAPs for Ras superfamily members, many of which still await investigation [4]. Typically and with only few exceptions, GAP activity is contained in a segment of 100–350 residues in a module accompanied by other domains frequently involved in signalling or localization (see fig. 2).

A number of diseases have been found to be associated with genetic alterations of GAPs. The RasGAP encoding neurofibromin, responsible for the pathogenesis of

neurofibromatosis type 1 [33], is one of the best explored in a list of examples that also includes p120GAP [34], N-GAP [35] and the RapGAP homolog tuberous sclerosis protein tuberin [36]. In addition, bacterial pathogens have evolved strategies that interfere with the GTPase cycle of Rho family members and particularly with the GTPase reaction in the absence and presence of functional GAPs [37, 38].

In this article, we review the current knowledge about GAPs with an emphasis on structure-function aspects. We try to focus on concepts where possible and supply more detail where necessary, hoping that both students and advanced researchers will benefit. We do not consider regulatory mechanisms that control GAP activities, which is an important issue most recently reviewed by Bernards and Settleman [39, 40].

Exploring GAP mechanisms

Several approaches have been used to measure the GAP-stimulated GTP-hydrolysis reaction:

1) The most frequently used assay is nitrocellulose filter binding of the (γ-^{32}P)GTP-bound GNBPs, which is inexpensive and rather useful for multiple turnover experiments using catalytic amounts of the GAP.
2) A spectrophotometric method has been developed for measuring the release of P_i during GTP hydrolysis by coupling 2-amino-6-mercapto-7-methylpurine ribonucleoside and purine-nucleoside phosphorylase [41]. The assay was shown to quantitate P_i in solution at concentrations of down to 2 μM.
3) The most accurate technique to date is provided by high-performance liquid chromatography (HPLC), which allows separation of the GTP and GDP peaks and determination of the nucleotide-content [42]. This method, however, consumes high protein quantities.
4) Stopped-flow measurements allow monitoring of the kinetics of the stimulated GTPase reaction under single turnover conditions and determining individual rate constants for the GAP-GNBP interaction using fluorescently labelled GTP analogs [43]. Fluorescence measurements also allow easy observation of the formation of an aluminium fluoride bound state (see below), which is believed to mimic the transition state of the GTPase reaction [44, 45].

Intrinsic GTP hydrolysis

As a hallmark of GNBP function the rate of GTP hydrolysis limits the lifetime of the activated GTP-bound state. While GNBPs have a conserved recognition site for guanine nucleotides, the mechanisms of GTP hydrolysis differ in detail. Small GNBPs are inefficient enzymes and exhibit a large variation in the GTP hydrolysis reaction rates, e.g. Rac1 (0.11 min^{-1}) [46], Ras (0.028 min^{-1}) [47, 48], Rap (0.0031 min^{-1}) [49]. Arf, on the other hand, lacks intrinsic GTPase activity [50, 51]. Isotopic labelling has shown that Ras hydrolyses GTP *via* direct in-line transfer of the γ-phosphate from GTP to water [52]. The activating group was originally proposed to be provided by the critical glutamine (Gln61 in Ras) [7, 53], but studies substituting Gln61 in Ras by the non-natural NGln [54] and linear free energy perturbation calculations [55] disfavoured this mechanism. A detailed investigation using linear free energy relationships (LFERs) suggested that GTP itself acts as a general base in the substrate-assisted catalysis of GNBPs. In the proposed mechanism Mg^{2+}-polarized GTP abstracts a proton from the catalytic water molecule, which then acts as the attacking nucleophile [48]. The role of a second water molecule has been discussed on the basis of a GTP-bound Ras structure, determined at 1.26 Å resolution, proposing a proton-shuffling mechanism between two attacking water molecules and one oxygen of the γ-phosphate [56].

The GTPase reaction catalyzed by GNBPs involves a rather complex free energy surface [57] and several reaction pathways, which can be grouped into two extreme classes: (i) an associative mechanism that involves a phosphorane intermediate, with no bond cleavage to the leaving group, complete bond formation with the incoming nucleophile and a decrease in bond order of the non-bridging oxygens [48]; and (ii) a dissociative mechanism that involves a metaphosphate intermediate characterized by complete bond cleavage on the leaving group, absence of bond formation to the incoming nucleophile and an increase in bond order of the non-bridging oxygens [58]. Typically, a phosphoryl transfer reaction is concerted, with a transition state that is intermediate in structure between metaphosphate and phosphorane extremes [59]. The vast majority of studies have aimed at elucidating the transition state for the phosphoryl transfer reaction of Ras, which is described by a structure somewhere between dissociative and associative extremes [48, 58–61]. Replacement of the catalytic glutamine (Gln61) by virtually any other amino acid significantly reduces the intrinsic hydrolysis rate up to 50-fold [62, 63], prevents GAP-mediated inactivation and thus induces oncogenic transformation by constitutive activation of the GTPase [32, 63–66]. This is because a glutamine has enough polarity and the correct size to be stabilized by the arginine finger of the GAP in order to orient the catalytic water molecule for optimal nucleophilic attack [67, 68]

A second amino acid that is critical for GTP hydrolysis is a glycine in the P-loop (Gly12 in Ras), which appeared to be involved in conformational stabilization of the Gln61 side chain [7]. Glycine 12 mutations in Ras reduce GTPase activity about 20-fold [62, 63, 69, 70] and are oncogenic due to their insensitivity towards RasGAP [71]. Arf

proteins contain an aspartate residue in the P-loop, in the position equivalent to Gly12 in Ras, which may be one of the reasons for the lack of intrinsic GTP hydrolysis [72]. Moreover, two GNBP subgroups, the Rnd proteins of the Rho family [73–75] and DexRas proteins of the Ras family [76, 77], contain amino acid deviations at both positions equivalent to 12 and 61 in Ras. They may thus exist predominantly in the GTP-bound conformation [78, 79] since they are unable to hydrolyse GTP and are insensitive to GAPs [73–75].

GAP-stimulated GTP hydrolysis

Essentially, two models of how GAPs could accelerate the hydrolysis of GTP-bound GNBPs have been proposed. In the first model the GNBP would provide the whole catalytic machinery, and the role of GAPs would be to stabilize the catalytically competent conformation [80–82]. The second model proposed that GAPs supply a catalytic residue into the active site of the GNBP [44, 45, 83]. Arginine would be a good candidate since the long side chain could bridge larger inter-protein distances and the positively charged guanidinium group could contribute to transition state stabilization, as has been derived from structures of adenylate kinase-like enzymes [84].

A breakthrough in the biochemical investigation of the GAP mechanism came from studies employing aluminium fluoride (AlFx). These compounds have been identified as transition state mimics with heterotrimeric G-proteins [85]. In complexes with GDP-bound α-subunits of heterotrimeric G-proteins (Gα) AlF_4 was found in a planar conformation in the position normally occupied by the γ-phosphate of the bound GTP [86, 87]. This was interpreted to represent an approximate mimic of the transferred phosphate moiety, with an oxygen of the GDP β-phosphate and a water molecule serving as the transaxial ligands to complete a tetragonal bipyramid [86, 87]. In contrast to Gα-proteins, GDP-bound Ras alone does not bind AlFx. Addition of RasGAP promotes the formation of a stable ternary complex between the GDP-bound Ras, AlFx and the GAP component. In addition, oncogenic Ras mutants did not form such a complex nor did inactive GAP mutants, in keeping with the impaired GTPase activity and GAP insensitivity [44]. These observations appeared to be the beginning of a catalytic concept unifying heterotrimeric G-proteins and small GNBPs. Formation of a GDP-AlFx bound ternary complex is now seen as indicative of a transition state stabilizing mechanism. Those residues playing a critical role in this process can frequently be identified by mutational analysis in which formation of an AlFx bound complex should be impaired if the functional group is not available.

GAPs for different GNBP families

In the following paragraphs, we review the current knowledge about GNBP-GAP interactions with a focus on the structural and biochemical aspects. Table 1 summarizes current knowledge about the most important features of GAPs, which are discussed below. These features are schematically visualized in figure 4.

RasGAPs

p120GAP [29, 88, 89] and the neurofibromatosis type 1 gene product neurofibromin [90–94] represent the first-identified and best-characterized GAPs. Neurofibromin is the protein responsible for the pathogenesis of neurofibromatosis type 1 [33], a common genetic disorder which predisposes to the formation of tumours and is associated with numerous clinical complications, including learning disabilities. In addition to homologs in *Drosophila melanogaster*, yeast, *Dictyostelium discoideum* and *Caenorhabditis elegans,* 14 RasGAP-related genes are known in mammalian species [4].

The modular structure frequently comprises pleckstrin homology (PH) and C2 [called conserved domain 2 in protein kinase C (PKC)] domains in addition to the catalytic RasGAP module. P120GAP also contains Src homology 2 (SH2) and 3 (SH3) domains and a proline-rich region at the N-terminus (fig. 2A). It appears that at least in some cases these domains are involved in membrane recruitment. Most biochemical studies have been performed with fragments comprising the catalytic domains of neurofibromin and p120GAP. The commonly used segment (330–450 residues) [91, 95] could be reduced to a minimal domain of only 230 residues [96].

Several biochemical studies suggested residues important for the GAP-mediated acceleration of GTP hydrolysis on the Ras as well as on the GAP side. Apart from Gly12, mutation of which to most other residues except proline, unfolds the oncogenic potential of the protein [71], and Gln61 [64], the switch regions are important for the interactions [30, 97, 98]. In RasGAP the primary candidates were derived primarily from the conserved sequence blocks (block1, 2, 3A, 3B), including a fingerprint FLR motif.

The crystal structure of the catalytic domain of p120GAP presented the first three-dimensional model of an active GTPase activating protein [99]. The bowl-shaped helical protein (see fig. 3A) is composed of a central domain that contains all residues important for the interaction with Ras. It basically represents the minimal catalytic domain (GAPc) reported on the basis of biochemical studies [96]. The function of a less conserved extra-domain (GAPex) composed of similar portions from the N- and C-terminus, also present in the GAP-related domain (GRD) of neurofibromin NF1GRD [100], is so far unclear. The conserved residues line up around a shallow groove that

has been proposed to be the docking site for Ras [99] and was confirmed by the crystal structure of the Ras-RasGAP complex (fig. 3A) [101].

In this complex, composed of GDP-AlF_3 bound Ras and the catalytic domain of p120GAP, an invariant arginine (Arg789, the 'arginine finger'), contacts the β,γ portion of the nucleotide in the transition state mimic of the hydrolysis reaction (fig. 3A) [101]. This suggested a catalytic contribution to compensate developing negative charges accumulating during phosphoryl transfer. The arginine finger is presented by the finger loop that also stabilizes the orientation of the catalytic Gln61, whose proposed role is the positioning of the catalytic water molecule for optimal nucleophilic attack, as suggested for Gα-proteins [87] (fig. 4A, B). Mutation of the arginine finger to lysine or alanine reduces GAP activity 2000-fold but does not affect binding, thus representing a major contribution to catalysis. The mutation of the arginine finger in neurofibromin to proline, as found in NF1-patients with malignant peripheral nerve sheath tumors, slightly reduced the binding affinity to Ras, but resulted in an 8000-fold reduction of GAP-stimulated GTP hydrolysis [102]. The conformation of the finger loop is stabilized by the arginine from the FLR fingerprint motif. Its mutation to alanine or lysine decreases GAP activity 50-fold without affecting binding, suggesting a secondary role in catalysis [103, 104].

A number of additional interactions involve an exposed loop segment, termed variable loop, opposite to the finger loop. This segment provides additional basic and acidic residues contributing to the network of polar interactions found in the complex interface [101]. The complex structure explains why oncogenic Ras mutants, particularly those in position 12 [71], are not GAP sensitive: these mutants would interfere with the geometry of the transition state of the GAP assisted GTPase reaction [101]. The observation that they still bind to GAP [105] suggests that the ground state adopts a conformation different from that seen in the transition state. Indeed, nuclear magnetic resonance (NMR) studies have demonstrated that in the ground state GAP does not interact directly with the nucleotide [106], as observed similarly in the Rho-RhoGAP system [107] (see below).

Extensive mutagenesis of NF1-GRD and of GAP-334 has identified three structural fingerprints governing the GAP reaction. These include the catalytic arginine (Arg789)-presenting finger loop, the so-called FLR-region containing the RasGAP fingerprint FLR motif and the α7/variable loop region, which has been identified on the basis of a structure-based sequence alignment [101, 108]. Of particular interest is the finger loop that represents an attractive target to interfere with the Ras-RasGAP interaction in oncogenic Ras mutants. The FLR region and α7/variable loop determine the specificity of the Ras-RasGAP interaction [103, 108].

Fourier transform infrared (FTIR) difference spectroscopy has become an increasingly important tool in the study of the complete intrinsic [109] and GAP-stimulated GTPase reaction pathway of GNBPs in atomic detail and on a millisecond time resolution [110]. FTIR studies have shown [61, 110] that coupled vibrations of the phosphate groups of GTP become largely uncoupled upon Ras binding, and negative charge is shifted towards the non-bridging β-oxygen of GTP [109]. Thereafter, (i) GAP binding increases the negative charge shift towards the non-bridging oxygen of GTP presumably due to the positively charged guanidinium group of the arginine finger, and (ii) a GDP•P_i intermediate accumulates during the reaction. The rate-limiting step is P_i release or dissociation of the GAP from the Ras•GDP•Pi•GAP intermediate, which is in line with conclusions drawn from stopped-flow experiments [82, 111, 112]. The FTIR data also showed that the GTPase reaction is reversible, similar to what is known for other phosphoryl transfer reactions.

Understanding why oncogenic mutants of Ras cannot be switched off by GAP has invoked the concept of restoring GTPase activity of oncogenic Ras mutants by small molecules as a therapeutic approach for Ras-directed cancer therapy [42, 113]. The latter notion has gained impetus by recent reports showing for the first time that oncogenic Ras mutants can be chemically inactivated and are not irreversibly damaged in their capability to act as molecular switches. The defective GTPase reaction of different oncogenic mutants of Ras could be increased by up to three orders of magnitude by using a modified GTP analog, 3,4-diaminobenzophenone-phosphoramidate-GTP (DABP-GTP [114, 115]), instead of GTP [63]. The structures of DABP-GppNHp bound to Pro12 and Val12 mutants of Ras have shown that the DABP moiety is accommodated close to a hydrophobic patch of Pro12 or Val12 in the P-loop. DABP-GTP provides an aromatic amino group that is critical for the mechanism of DABP-GTP cleavage, which differs substantially from the intrinsic and GAP-stimulated GTP hydrolysis by Ras [63, 116]. Catalytic drugs that target the GTPase reaction may be able to complement the insensitive GAP activities in Ras-transformed cancer cells and restore the defective GTPase reaction of oncogenic Ras proteins.

Rho/Cdc42/Rac-specific GAPs

Members of the Rho family of small GNBPs control signal transduction pathways that link cell surface receptors to a variety of intracellular responses. They are well known as regulators of the actin cytoskeleton, but they also control cell polarity, gene expression, microtubule dynamics and vesicular trafficking [20, 117, 118]. The first RhoGAP activity was found by biochemical analysis of cell extracts testing with recombinant RhoA to identify a protein subsequently termed p50rhoGAP [119]. Up to now, at

least 68 RhoGAPs have been identified from the human genome analysis from which about 26 proteins have been analysed [4, 120, 121]. The reason for this vast amount of RhoGAPs reflects the number of different regulatory circuits Rho-like proteins are involved in, requiring coordinated compartmentalized control of Rho activity [121].

RhoGAPs are characterized by the presence of a conserved catalytic module, spanning approximately 150 residues [120], which is sufficient for productive interaction with Rho targets. They are frequently accompanied by domains implicated in regulation, membrane targeting and localization (fig. 2B). Among the well-studied p190RhoGAP and the breakpoint cluster region (BCR) of the BCR-Abl fusion oncogene, the catalytic domain of p50rhoGAP (residues 198–439) has been studied extensively on a structural and biochemical level (fig. 2B). *In vitro*, p50rhoGAP, also referred to as Cdc42GAP or RhoGAP, has been shown originally to stimulate the intrinsic GTP hydrolysis of both Cdc42 [122] and RhoA [123], and to support the formation of an AlFx-bound transition state mimic [45, 124].

The catalytic domain of RhoGAPs folds into a helical topology whose core appears to be arranged as a four-helix bundle as established by crystallographic analysis of the corresponding modules of p50rhoGAP (fig. 3B) [125] and the BCR-homology (BH) domain of Graf [126] (fig. 3B). The structures closely resemble that of the previously solved BH domain from the phosphoinositide 3-kinase p85α-subunit [127], which binds to Cdc42 and Rac but does not show GAP activity [128, 129]. Comparative structural analysis revealed topological similarity with RasGAPs, suggesting that these GAPs are evolutionarily related [5, 130–132]. Crystallographic studies of RhoGAP domains in complex with the Rho targets Cdc42 bound to GppNHp [107] and RhoA/Cdc42 bound to GDP•AlFx [133, 134] gave insight into the catalytic mechanism of GTPase stimulation (figs. 3B, 4A).

As in RasGAP, the GAP domain interacts primarily with the P-loop, the switch I/II regions and the nucleotide itself. The GAP- and the G-domain appear to undergo a rigid body rotation of about 20° relative to each other, when proceeding from the ground (Cdc42•GppNHp•RhoGAP) [107] to the transition state complex (RhoA•GDP•AlF_4^-•RhoGAP) [133], resulting in additional contacts with the G-domain [135]. As with the Ras-RasGAP system, in the transition state complex but not in the ground state, the RhoGAP domain supplies an arginine finger directly into the active site of the Rho target to stabilize the transition state. In addition, the catalytic glutamine (Gln63 in Rho, Gln61 in Rac/Cdc42) of the switch II region is oriented to position the nucleophilic water molecule, similarly to Gln61 in the Ras-RasGAP complex [101]. Comparison of the ground state and the transition state complex reveals that the GTPase-impaired Gly12 mutants, as in the Ras-RasGAP system, would sterically interfere with the transition state geometry but are compatible with the ground state complex [107, 133]. Taken together, binding of GTP-bound Rho appears to establish the complex interface that in a subsequent step adjusts to position the catalytic arginine in the GTPase-competent conformation within the active site of the GNBP component.

The role of the arginine (Arg282 in p50rhoGAP, Arg305 in Cdc42GAP) has been investigated in detail using site-directed mutagenesis along with structural studies. In contrast to the Ras-RasGAP system [103, 104], its mutation to alanine still results in a significant GTPase stimulation and in formation of the GDP•AlFx bound transition state mimic [136, 137], as visualized in the crystal structure of the Cdc42-Cdc42GAP(R305A) complex. A likely explanation of how the residual GAP activity can be maintained lies in the stabilization of the switch regions, particularly switch II for (sub)optimal catalytic adjustment [134].

It has been pointed out that a catalytic arginine is necessary but may not be sufficient for mediating GAP activity, since the regulatory subunit of phosphoinositide-3-kinase (p85), which is homologous to Cdc42GAP (21% identity, 47% similarity), does have the corresponding arginine, but does not display GAP activity [138]. The authors present mutational analyses of GAP residues in the switch interface and suggest that residues from GAP may be critical for stabilization of the switch regions, a function distinct from the contribution of a catalytic residue. The reverse experiment, however, in which corresponding residues of p85 are mutated to their Cdc42GAP counterparts, did not impart GAP activity on p85, suggesting that other interactions or additional protein factors may be important [138].

Of particular importance for the GAP catalysis in Rho/Cdc42 appears to be a conserved tyrosine (Tyr32) in the switch I region, which in the complex with Cdc42 interacts with the guanidinium group of the arginine finger. As has been proposed for Rap proteins [139], Tyr32 appears to be important for GTP hydrolysis through its terminal hydroxyl group, whose precise role is still unclear [138].

Most recently, the crystal structure of full-length β2-chimaerin has been determined, which has been suggested to be in its inactive conformation [140]. β2-Chimaerin contains three conserved domains, an N-terminal SH2, a central C1 and a C-terminal Rac-specific GAP domain (fig. 2B). The latter shows the typical RhoGAP topology and in the crystal structure is sterically blocked in Rac binding through direct interaction with N-terminal regions of β2-chimaerin. Phospholipid binding to the C1 domain, which is buried by extensive intramolecular contacts, has been implicated in triggering the cooperative dissociation of these interactions, resulting in the release of the GAP domain [140].

RhoGAPs as bacterial toxins

Rho family GNBPs and their regulatory circuits play important roles in host invasion by bacterial pathogens. Bacteria have evolved strategies to interfere with host cell Rho signalling at various stages of the GTPase cycle [141]. They encode toxins that reversibly or irreversibly modify the activity of Rho proteins [142]. Employing a multi-protein molecular syringe termed type III secretion system [143], these toxins are injected directly into the cytoplasm of the host cell. Here they act reversibly as mimics of GEFs or GAPs or covalently, i.e. irreversibly, modify critical residues of Rho proteins, with the catalytic glutamine (Gln63 in RhoA) being an important target [144]. It has been proposed that Rho activation by bacterial GEF mimics induces cytoskeletal rearrangements required for pathogen uptake, while inactivation by GAP mimics contributes to restoration of the resting state [145]. Bacterial toxins acting on Rho proteins have been reported from *Salmonella* (SptP [146]), *Pseudomonas* (ExoS [147], ExoT [148]) and *Yersinia* (YopE [147]) (fig. 2B). They encode small proteins with a catalytic portion (~140 residues) that has two highly conserved sequence patterns characteristic for these proteins [149]. In three-dimensional structures derived from the crystalline proteins [150, 151], these sequence motifs fold into exposed loop structures, termed bulges, that carry the functionally most important residues of the helical protein. Most important, they have implemented an invariant arginine, which acts like the arginine finger in the cellular RhoGAPs [135]: it stabilizes the transition state of the GTPase reaction as visualized in complexes with AlFx and GDP-bound Cdc42 or Rac [152, 153]. It is interesting to compare the positions of the guanidinium groups of the catalytic arginine in the various models (fig. 3B). In the transition state, it is consistently in the same position relative to the β/γ-phosphate region, but is presented from different topological areas in the GAP component. Transition state stabilization appears to require the presence of Gly12 in the Rho component, since any larger amino acid in this position would lead to steric hindrance with the arginine and the transferred phosphate, as mimicked by AlF_3.

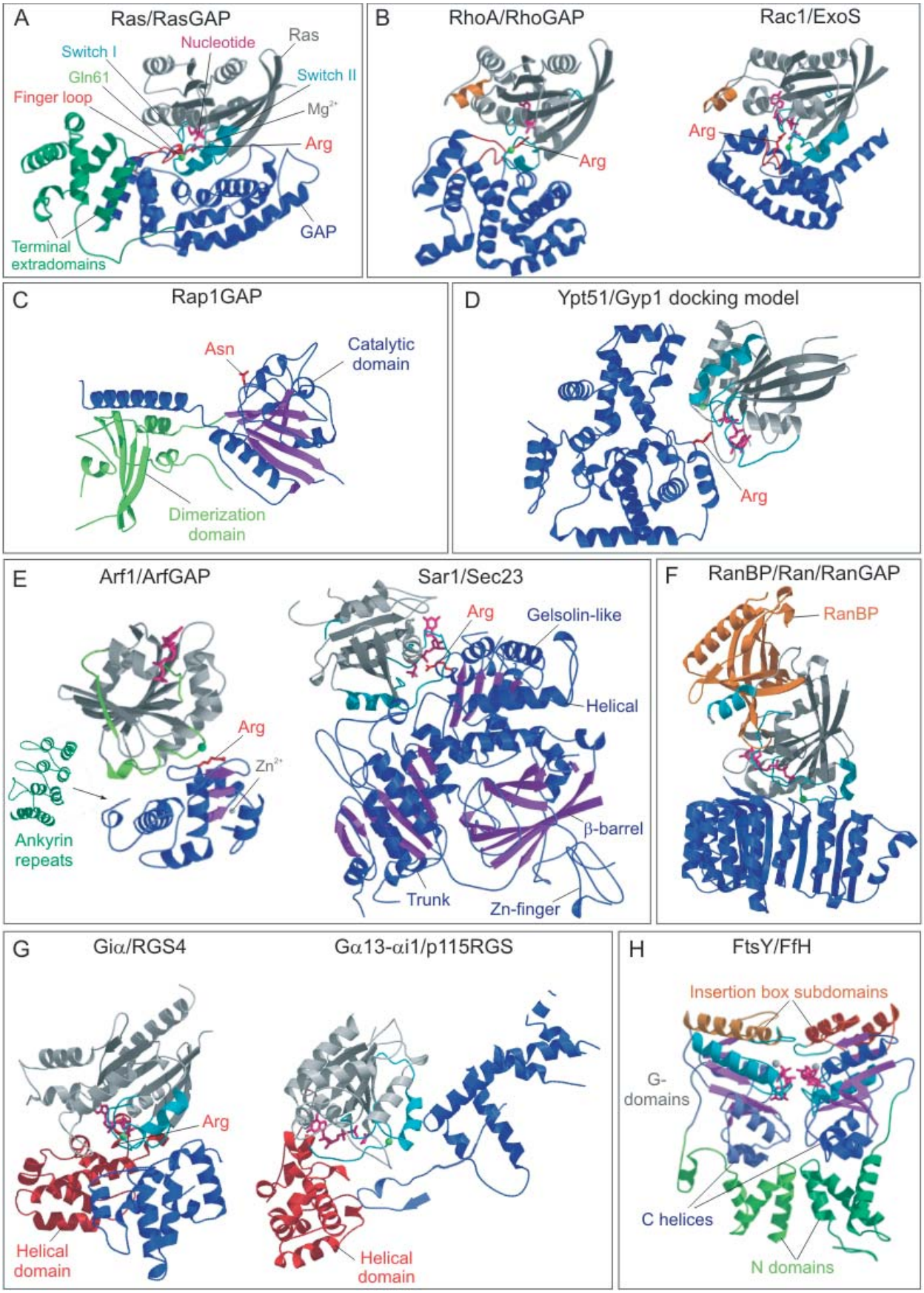
RapGAPs

The small GNBPs of the Rap family (Rap1a, Rap1b, Rap2a, Rap2b), the closest relatives of Ras, are involved in several biological processes, ranging from modulation of growth and differentiation to secretion, integrin-mediated cell adhesion and morphogenesis [154–157]. Rap1 was initially discovered as a protein reverting the phenotypes of Ras transformed cell lines [158].

Several RapGAPs have been found to downregulate Rap specifically, with Rap1GAP (MW 73 kDa) being the first identified [159]. Its GAP activity resides in a 36-kDa segment encompassing 341 amino acids (residues 74–415) [160], which has been detected in the presumed tumour suppressor Spa1 [161] and the papilloma oncoprotein target E6TP1 [162] (fig. 2C). RapGAP activity has been detected also in proteins not containing the typical sequence pattern but containing a RasGAP-domain associated with C2-domains [163, 164] (fig. 2A) (see below). In line with these observations, Bud2 from *Saccharomyces cerevisiae* has sequence homology to the RasGAP domain but acts on Bud1p/Rsr1p, a putative yeast homolog of Rap1 [165]. Such a protein, which exhibits high homology to RasGAP, is GAP^{IP4BP} with multiple GAP activities towards both Ras and Rap proteins [166] (fig. 2A).

RapGAP-mediated GTP hydrolysis displays a number of features distinct from its close relative Ras and from other families of small GNBPs. A threonine (Thr61 in Rap1) corresponds to the position of the catalytic glutamine in Ras (and other GNBPs). This results in a 10-fold decrease of the intrinsic rate of GTP hydrolysis, which can be reconstituted by replacing Thr61 by glutamine [167]. In addition, Thr61 has been shown to be dispensable for the intrinsic and RapGAP-stimulated GTPase reaction of Rap1 [168]. Contrary to the constitutive active mutants of most other small GNBPs, the G12V mutant of Rap1 is efficiently inactivated by Rap1GAP [169]. Instead, replacement of a phenylalanine (Phe64) in the switch II region by alanine in Rap1 results in a drastic reduction of the intrinsic and Rap1GAP-stimulated GTPase activity [169]. The catalytic domain of Rap1GAP, which accelerates the GTPase reaction of Rap by five orders of magnitude, contains several conserved arginines and lysines, but none of them is involved in the reaction mechanism

Figure 3. Representative structures of isolated and GNBP-complexed GAPs. All structures are illustrated with GAPs in blue, arginine finger (Arg) and finger loop in red, GNBPs in grey, switch I and II in cyan, the nucleotide in magenta and the critical glutamine in light green. (*A*) Ras-RasGAP complex in the GDP-AlF_x transition state [101]. (*B*) Rho-RhoGAP complexes in the GDP-AlF_x transition state with RhoA/Cdc42 and the eukaryotic RhoGAP (left panel) [133, 134] and Rac1 and the catalytic domain of the bacterial GAP ExoS/SptP (right panel) [152, 153]. The insert helix of RhoA and Rac is shown in yellow (*C*) RapGAP structure [170] with the proposed catalytic asparagines (Asn). (*D*) The structure of the yeast RabGAP Gyp1 [190] modelled in the complex with yeast Rab protein Ypt51. (*E*) ArfGAPs in the complex with Arf1 (left panel) [207] or alone with the C-terminal ankyrin repeats (green, middle panel) [211] and in the structure of the multidomain Sec23 complexed with the Arf-like GNBP Sar1 (right panel) [216]. (*F*) The ternary Ran-RanGAP-RanBP complex [229]. (*G*) The α-subunit of the heterotrimeric G-proteins with a built in arginine finger positioned by the helical domain in the complex with its GAP RGS4 (left panel) [243] or with p115RGS (right panel) [269]. (*H*) Heterodimeric complex of the GTP-binding multidomain FtsY-FfH proteins [279, 280].


A
Ras/RasGAP
Ras
Nucleotide
Switch I
Switch II
Gln61
Finger loop
Mg2+
Arg
GAP
Terminal extradomains
B
RhoA/RhoGAP
Arg
Rac1/ExoS
Arg
C
Rap1GAP
Asn
Catalytic domain
Dimerization domain
D
Ypt51/Gyp1 docking model
Arg
E
Arf1/ArfGAP
Arg
Zn2+
Ankyrin repeats
Sar1/Sec23
Arg
Gelsolin-like
Helical
β-barrel
Trunk
Zn-finger
F
RanBP/Ran/RanGAP
RanBP
G
Giα/RGS4
Arg
Helical domain
Gα13-αi1/p115RGS
Helical domain
H
FtsY/FfH
Insertion box subdomains
G-domains
C helices
N domains

[169]. These data suggest that the GAP-accelerated GTPase reaction of Rap proteins proceeds via a mechanism completely different from that of other small GNBPs. Interestingly, Rap1GAP supports the binding of AlFx to Rap•GDP even though it does not contain a catalytic arginine [111].

The crystal structure analysis of the catalytic domain of Rap1GAP reveals a two-domain arrangement (fig. 3C), of which the C-terminal part represents the catalytic portion. The N-terminal α/β fold domain appears to mediate dimer stabilization, which is not critical for GAP activity [170]. However, RapGAP activity appears to require the presence of the dimerization domain, possibly to support binding of the Rap target, as concluded by mutational analysis of residues in the contact area with the catalytic domain [170]. In particular, two invariant lysine residues have been demonstrated to interfere with RapGAP activity primarily by reducing target affinity [169]. Intriguingly, the catalytic domain has the G-domain fold known from the GNBPs [11]. In a region corresponding to the switch II regions of G-domain proteins, a highly conserved asparagine (Asn290) was identified to be crucial for the GAP activity, as concluded from its mutation to alanine, which failed to promote the formation of a ternary complex with AlFx that the wild-type protein forms [170]. These data are supported by a recent FTIR spectroscopic study suggesting that RapGAP compensates the absence of both the Gln61 and possibly the catalytic arginine, when compared with the catalytic machinery of the Ras-RasGAP system [61, 110]. In a potential mechanism RapGAP supplies an asparagine into the active site and contributes to the adjustment of the GTP-bound conformation of Rap for catalysis [171] (fig. 4D). The proposal that the catalytic asparagine plays the role of the water-positioning glutamine in Ras and other GNBPs is supported by the observation that the corresponding residue in Rap proteins is a threonine, but we will have to await the structure of a Rap-RapGAP complex for confirmation. Exploring the catalytic properties of the homologous protein tuberin, the above-mentioned asparagine is found mutated during genetic analyses of tuberous sclerosis patients [172]; indeed, the engineered mutant has dramatically decreased activity. This indicates that impaired RapGAP activity plays a role in the pathogenesis of the disease. A similar observation was made in an NF1 patient with severe tumour growth, where the catalytic arginine of neurofibromin is mutated to abolish RasGAP activity [102].

Rab/YptGAPs

Small GNBPs of the Ypt/Rab family have emerged as essential regulators of all stages of membrane traffic [21, 173–175]. These GNBPs have been implicated in diverse aspects of vesicular transport, including vesicle formation, motility, docking and fusion. The Ypt/Rab-proteins constitute the largest family within the Ras superfamily. There are 11 Ypt (yeast protein transport) proteins and 52 mammalian Rab (Ras genes from brain) proteins known so far [176]; in addition 57 genes encoding Rab-like proteins have been identified in the *Arabidopsis* genome [177].

On the basis of sequence similarity with two Ypt-directed GAPs known, Gyp6 [178] and Gyp1 [179], four other Gyp proteins (Gyp2, 3,4,7) and a mammalian RabGAP (called GAPCenA) have been identified [180–183]. Other RabGAPs include the ubiquitously expressed Rab3GAP [184] and RN-tre, which has been implicated to play a role in the pathogenesis of prostate cancer [185] (fig. 2D). RabGAPs share common sequence patterns but vary considerably in their target specificity within the Rab/Ypt-family of small GNBPs.

RabGAPs seem to be inefficient GTPase binders but rather efficient catalysts. For example, Gyp6p has a very low affinity for Ypt6p but is able to accelerate the extremely low intrinsic GTPase activity of Ypt6p by a factor of 5 x 10^6 [186, 187]. Similarly, Rab3GAP affinity for Rab3 is rather low (75 μM); it is nevertheless able to form the transition state mimic with Rab3•GDP•AlF_x, presumably by providing a critical arginine residue (Arg728 in Rab3GAP) [188]. Mutational analysis of Gyp6p has shown that replacement of the presumed arginine finger (Arg155) by either alanine or lysine abolished its GAP activity without impairing Gyp6p/Ypt6p binding [187].

Detailed structural information is so far only available for Gyp1p, whose major target is Ypt51p [179, 180]. The catalytic domain (residues 239–638, termed Gyp1-46p) [189] has been described as a helical molecule that folds the six characteristic sequence motifs in a distinct topological arrangement unrelated to other GAPs [190] (fig. 3D). Sequence heterogeneity in loop regions of RabGAPs has been attributed to determinants of selectivity and specificity for the interaction with other proteins, particularly with substrate GNBPs [190].

Site-directed mutagenesis studies have identified an exposed highly conserved arginine (Arg343) being critical for GAP activity [189]. Together with other conserved residues (including Tyr376, Gln378, Asp340) it is located in a cleft that has been proposed to be the binding site for activated Ypt proteins [190]. A docking model based on the location of conserved residues and on biochemical data with Ypt51 [191] as representative Rab target suggests a GAP mechanism very similar to that of the Ras-RasGAP [101] and Rho-RhoGAP [133, 134] systems (fig. 4A).

ArfGAPs

ADP-ribosylation factors (Arfs) are 21-kDa GNBPs, initially identified as stimulators of cholera toxin-catalysed ADP-ribosylation of Gsα [192]. We now know that Arfs

play a critical role in many vesicular trafficking events in eukaryotic cells, mediated by protein-coated carrier vesicles [193–197]. Activation of Arf1, mediated by Arf-GEFs, triggers association of Arf1•GTP in the heptameric coat protein complex I (COPI) and consequently the recruitment of COPI to the Golgi membrane [198–202]. Hydrolysis of Arf-bound GTP leads to destabilization of the COPI-Arf1 complex. Different from many other GNBPs, this process ultimately requires the participation of ArfGAPs, since Arf itself does not show measurable GTPase activity [50, 51, 72].

The first ArfGAP identified, ArfGAP1, has been reported as a 49-kDa protein which requires additional N-terminal residues including a zinc finger domain for its full GAP activity *in vitro* [203] (fig. 2E). Most recently, it has been demonstrated that ArfGAP1 activity is sensitive to membrane curvature that controls the timing of Arf1-mediated GTP hydrolysis in a COPI bud [204]. At least 15 ArfGAPs exist in humans [205] from which four representative examples are highlighted in figure 2E. The Arf specificity, using predominantly Arf1/5/6 targets, has been investigated for most ArfGAPs with ArfGAP1 being the best-characterized member. Typical K_d values are in the low micromolar range [205, 206].

A catalytic fragment of ArfGAP1 (residues 1–146) has been co-crystallized with N-terminally truncated Arf1 [207]. The structure of this complex was interpreted to be a mimic of the product state, as obtained after incubation of the protein with Arf1•GTP, with GDP bound to the nucleotide-binding pocket [207]. An invariant arginine (Arg50) of ArfGAP1, which has been proposed a candidate arginine finger [5], is distant from the active site of Arf1 and makes extensive contacts with the zinc finger residues (fig. 3E). A unique feature of this structure is that the switch I of Arf1 does not participate in GAP binding. Instead, the vesicle coat protein, coatomer, which binds to the switch I region of the Arf1-GAP complex [208, 209], has been shown to dramatically stimulate GTP hydrolysis on N-terminally truncated Arf1 [207]. Thus, it has been suggested that coatomer rather than GAP may contribute a catalytic residue, e.g. arginine finger, for the GTPase reaction (fig. 3E). Using myristoylated full-length Arf1 bound to phospholipid vesicles, on the other hand, it has been suggested that coatomer indirectly facilitates GTP hydrolysis by bringing GAP into proximity with its substrate Arf1 [210] (fig. 4C).

The crystal structure of a fragment of Pyk2-associated-protein-β (PAPβ), another Arf-specific GAP, that encompasses the GAP domain and the C-terminal ankyrin repeats [211] reveals extensive intramolecular interaction between the ankyrin repeats and a region of the GAP domain considerably overlapping the Arf binding area identified in the Arf1-ArfGAP1 complex [207] (figs. 2E, 3E). Thus, formation of an Arf1-PAPβ complex would require extensive structural rearrangements to allow binding in a way similar to the complex with the Arf1GAP-fragment. With respect to the ArfGAP mechanism [207, 211], a series of mutational and biochemical analyses suggest a catalytic role of an invariant arginine in PAPβ (Arg292) [211], and in the PAPβ-like ArfGAPs ACAP1 (Arg448) and ACAP2 (Arg442) [212]. In addition, ASAP-like proteins have been shown to interact with the switch I region of Arf1 and thus compete with effector binding [213].

The N-terminal Helix (aa 2–17) of Arf1 (missing in the Arf1-ArfGAP1 structure) seems to be crucial not only for anchorage of myristoylated Arf1 to the membrane but also for the interaction with ArfGAPs [214]. In support of a catalytic residue contributed by the ArfGAP component, the crystal structure of a homologous complex composed of the GppNHp-bound Sar1 and the GAP component Sec23 in yeast [215] reveals a conserved arginine (Arg722 in Sec23) protruding into the active site [216] (fig. 3E). In contrast to other GAP complexes with small GNBPs, adjustment of switch II with the catalytic histidine (corresponding to Gln71 in Arf1) to a catalytically competent conformation does not appear to be the major feature of GTPase stimulation. Clearly, more biochemical and structural studies are needed for a comprehensive understanding of this complex system.

RanGAP

Ran plays a central role in nucleocytoplasmic transport, mitotic spindle assembly and nuclear envelope assembly in eukaryotic cells [24, 217, 218]. Consistent with its function in shuttling between intra- and extranucleocytoplasmic compartments, it lacks posttranslational targeting signals. The asymmetric distribution of GDP/GTP nucleotide exchange and GTP hydrolysis causes nuclear Ran to be predominantly GTP-bound, and cytosolic Ran to be predominantly GDP-bound [219]. This is because during interphase, the RanGEF RCC1 is nuclear, while RanGAP is cytosolic [220, 221]. Both proteins accelerate the slow intrinsic reaction rates by several orders of magnitude [222–224].

RanGAP has been identified as Rna1p in the yeast *Saccharomyces cerevisiae* [222, 225, 226] with acceleration rates similar to those of other GAPs [224]. All known RanGAPs show a common modular architecture with a leucine-rich repeat domain comprising approximately 350 residues, followed by a highly acidic region (50 residues) (fig. 2F). In higher eukaryotes, RanGAP1 contains an additional domain at the carboxy terminus, which localizes RanGAP to the nuclear pore complex *via* modification by the ubiquitin-like protein SUMO-1 [220, 221].

RanGAP is a crescent-like molecule [227] made up of 11 leucine-rich repeats similar to the horseshoe structure of ribonuclease inhibitors [228] (fig. 3F). In the ternary complex of RanGAP with activated

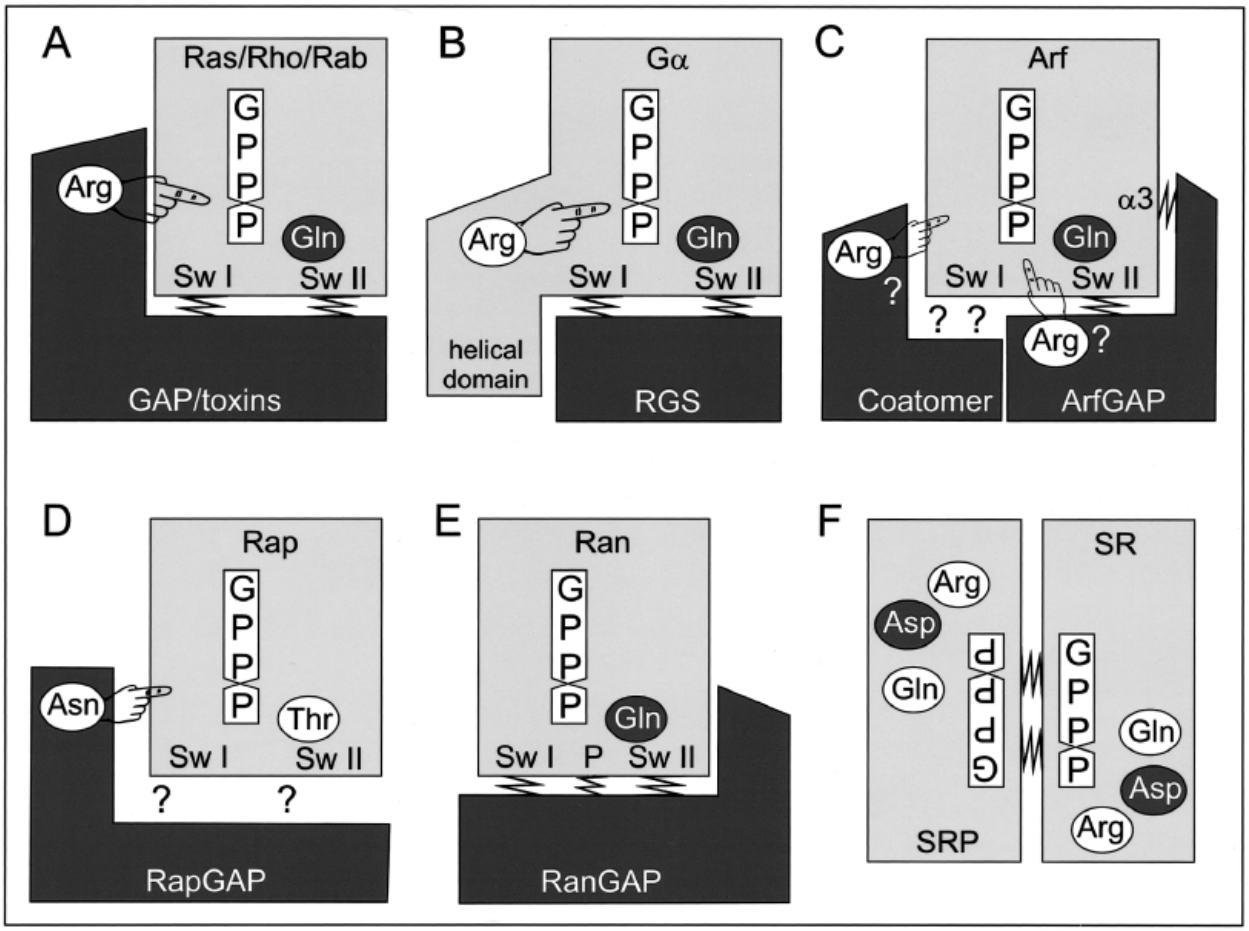


Figure 4. Requirements for efficient GTP-hydrolysis. G-domains of the respective GNBPs are shown in grey with a number of functionally important residues, especially a critical glutamine. GAP is shown in dark grey with the integral elements that are required to bind and fix the switch I and II regions (SwI/SwII) but also in most cases to supply a catalytic residue into the active site. (*A*) GAPs of Ras/Rho/Rab supply a catalytic arginine *in trans*. (*B*) Gα-RGS with catalytically essential arginine supplied *in cis* by the inserted helical domain of the G-domain. (*C*) ArfGAP activity is assisted by coatomers one of which may provide a catalytic arginine. (*D*) RapGAP supplies an asparagine as a catalytic residue. The role of Thr61 instead of a Gln is unclear. (*E*) Ran-RanGAP requires the catalytic glutamine and stabilization of the switch regions; no catalytic residue provided *in trans* has been identified. (*F*) SRP/SR asymmetrically dimerize as GTP-binding proteins and provide several residues to accelerate the GTPase reaction.

Ran (bound to either GppNHp or GDP-AlFx) and the GTPase co-activating Ran binding domain (RBD) of RanBP1, no arginine was found in the vicinity of the active site [229] (figs. 3F, 4E). Candidate residues (Arg74, Arg170) are not involved in nucleotide contacts, neither in the ground nor in the transition state of the GTPase reaction, nor did they show an appreciable effect on the GTPase reaction [227, 229]. A conserved tyrosine (Tyr39) derived from the switch I region interacts with the nucleotide similarly to the homologous Tyr32 in GTP-bound Rap2 [139]. This tyrosine has been implicated to be important in catalysis, also because it is in a position equivalent to the catalytic arginine in heterotrimeric G proteins [86, 87, 230] (figs. 3G, 4B). However, its contribution to catalysis is only minor, as the authors concluded from mutational analysis [229]. In the ternary complex, the RBD component interacts with Ran in a similar molecular embrace, as in the complex with GTP-bound Ran alone [231]. In that complex, the C-terminal extension, which is important for binding to RanGAP [232], is seen in tight interaction with the RBD component that structurally resembles the well-known PH domains [233, 234]. It was proposed that RanBP1 acts as a GTPase coactivator [235] through binding to the C-terminal extension of Ran, which otherwise would inhibit Ran-RanGAP interaction [229].

From the structure of the Ran-RanGAP complex, the major contribution of RanGAP-assisted catalysis appears to derive from stabilization of the switch regions, particularly switch II, with the catalytic glutamine (Gln69 in Ran). Similar to other small GNBPs such as Ras, its mutation to alanine or leucine reduces GAP activity by several orders of magnitude [222, 224].

Table1. Representative GAPs and their characteristics.

GAP-type	Representative example	Rate enhancement	Catalytic feature	Structural models: components, resolution, PDB code, reference
RasGAP	P120GAP [29] neurofibromin [90–92]	30,000–70,000 [43, 62, 313]	arginine [103, 104]	p120GAP(714–1047), 1.6 Å, 1WER, [99]; p120GAP(714–1047).H-Ras(1–166).GDP.AlF_3, 2.5 Å, 1WQ1, [101]; neurofibromin(1198–1530), 2.5 Å, 1NF1, [100]
RhoGAP	P50rhoGAP [123] Cdc42GAP [122]	38,000 [136]	arginine [136]	p85(BH) of PI3K(105–319), 2 Å, 1PBW, [127]; p50rhoGAP(198–439), 2 Å, 1RGP, [125]; p50rhoGAP(198–439).Cdc42(1–184).GppNHp, 2.7 Å, 1AM4, [107]; p50rhoGAP(198–439).RhoA(F25N).GDP.AlF_4, 1.65 Å, 1TX4, [133]; Cdc42GAP(239–443).Cdc42.GDP.AlF_3, 2.1 Å, 1GRN, [134]; Cdc42GAP(239–443,R305A).Cdc42.GDP.AlF_3, 1.9 Å, 1GRN, [134]; GrafGAP-BH(161–391), 2.4 Å, 1F7C, [126]; β2-Chimaerin, 3.2 Å, 1XA6, [140]
RhoGAP	SptP [146], ExoS [147], YopE [314]	n.d.	arginine [152, 153]	SptP(70–543).Rac1(1–184).GDP.AlF_3, 2.3 Å, [152]; ExoS(96–234).Rac1(2–284).GDP.AlF_3, 2 Å, 1HE1, [153]; ExoS(96–234), 2.4 Å, 1HE9, [151]
RapGAP	Rap1GAP [159, 315]	100,000 [169]	asparagine [170]	Rap1GAP(75–415), 2.9 Å, 1SRQ, [170]
RabGAP	Gyp1 [178]	45,000 [180]	arginine [180, 190]	Gyp1(249–637), 1.9 Å, 1FKM, [190]
ArfGAP	Arf1GAP [203, 316]	~1000 in the presence of coatomer [207]	potentially arginine [211]	Arf1GAP(1–146).Arf1(18–181).GDP, 1.95 Å, no entry found [207]; PAPβ(112–522), 2.1 Å, 1DCQ, [211]
Sec23	Sec23 [215]	10–15 in the absence of the membrane fractions [215]	arginine [216]	Sec23(1–768).Sar1(24–190).GppNHp, 2.5 Å, 1M2O, [216]
RanGAP	RanGAP1 [222] Rna1p [225]	40,000 [224]	switch stabilization/ coactivation [317]	Rna1p, 2.66 Å, 1YRG, [227]; Rna1p.RanBP1.Ran.GppNHp, 2.7 Å, 1K5D, [229]; Rna1p.RanBP1.Ran.GDP.AlFx, 3.1 Å, 1K5G, [229]
RGS	RGS4 [242] RGS9 [318]	1000–2000 [244–246]	switch stabilization [243]	RGS4.Giα1.GDP.AlF_4, 2.8 Å, 1FQK, [243]; RGS4, NMR, 1EZY/1EZT, [260]; RGS9(276–422), 1.94 Å, 1FQI, [263]; RGS9(276–422).Gαt/i1.GDP.AlF_4, 2.3 Å, 1FQK, [263]; RGS9(276–422).Gαt/i1.GDP.AlF_3.PDEγ 46–87), 2.02 Å, 1FQJ, [263]; p115RhoGEF(1–239).Gα13/i-5.GDP.AlF_4 , 2.8 Å, 1SHZ, [269]
SRP/SR	FtsY/Ffh [274] SSRP/SR [319]	40 [320, 321]	arginine [322]	FtsY(1/2–304).GppCHp.Ffh(1–300/297).GppCHp, 1.9 Å/2.05, 1RJ9/1OKK [279]/[280]

Regulators of G-protein signalling: GαGAPs

The family of heterotrimeric GNBPs (designated as G-proteins) serves an essential role in transducing receptor-generated signals across the plasma membrane [236]. The GDP/GTP-binding α-subunit (Gα) hydrolyzes GTP much more rapidly (3 min^{-1}) [47] than other GNBPs, which has been ascribed to the GAP-like activity of a helical domain within the G-domain, not found in other GNBPs [237]. This inserted domain positions an arginine *in cis* into the active site of the G-domain, thereby stabilizing the developing negative charge during the phosphotransfer reaction [86, 87]. Consistent with its importance in catalysis, mutations or cholera toxin-catalysed ADP ribosylation of this arginine abolish GTPase activity of the Gα-protein [87, 238–241].

Despite the high intrinsic GTPase rates, the processes of

G-protein-mediated cell responses require rapid down-regulation typically by GAPs specific for Gα-subunits, which are called regulators of G-protein signalling (RGSs) [242]. RGS proteins bind with high affinity to the GDP•AlFx-bound Gα-proteins, as demonstrated for RGS4 and its Gα target [242, 243] and to accelerate the GTPase reaction rate of the α-subunits up to 2000-fold [244, 245, 246]. RGS proteins form a superfamily of about 37 proteins which are highly diverse in structure, expression patterns and specificity [246–248]. They share the conserved RGS domain consisting of 120 amino acids, which exhibits GTPase accelerating activity for Gα-subunits, and thus are negative RGSs [247–250].

Besides the classic RGS proteins, there is another group of proteins that link G-protein signalling to the small GTPases of the Rho family [251]. In addition to an N-terminal RGS domain – which binds to and inactivates the $G\alpha_{12}$ and $G\alpha_{13}$ subunits [252] – these proteins contain a central Dbl homology (DH) domain that exhibits GEF activity for Rho proteins (fig. 2G) [253]. Association of p115RhoGEF with $G\alpha_{12}$ and $G\alpha_{13}$ results in stimulation of its GEF activity towards Rho [253–255]. Similar data have been reported for the other RGS-containing GEFs, such as PDZ-RhoGEF/GTRAP48 and LARG [256–258]. Another protein that accelerates GTP hydrolysis of $G_q\alpha$ by three orders of magnitude is phospholipase C-β, the principal G_q-regulated effector, which has no structural relationship to the RGS proteins [245, 259].

The three-dimensional structure of an RGS-domain was first determined in the GDP•AlF_4^--bound transition state complex of RGS4 with its $G_i\alpha$ target [243], followed by solution structures of GAIP and RGS4 determined by NMR [260, 261], by the crystal structures of Axin-RGS [262], and by the isolated and transition state complex-bound RGS9-domain with $G_t\alpha$ and the inhibitory domain of phosphodiesterase-γ (PDEγ) [263]. The RGS domain (residues 51–178 in RGS4, 276–422 in RGS9) folds into a helical module that consists of two subdomains: a central classical right-handed antiparallel four-helix bundle and an extra domain intertwining helical elements derived from the N- and C-terminus [243] (fig. 3G). Structural variations adding a helical layer to the C-terminal region have been observed in the RGS portion of p115RhoGEF [264] and PDZ-RhoGEF [265]. The RGS box interacts primarily with the switch regions of Gα with no RGS residue of catalytic potential in the immediate vicinity of the nucleotide (figs. 3G, 4B). The structural arrangement suggests stabilization of the switch regions as the major determinant of GAP activity, in contrast to Ras- and RhoGAP [101, 133], but consistent with the view that Gα-proteins have the above-mentioned built-in arginine that is important for catalytic activity and whose guanidinium group is in the functionally equivalent position of the arginine finger in the Ras/RhoGAP complexes (figs. 3A, 4A) [101, 133, 266]. A conserved asparagine (Asn128) found within potential hydrogen bonding distance to the hydrolytic water molecule or the nucleophile derived from it does not contribute significantly to catalysis but rather appears to be important for binding to Gα, as concluded from extensive mutational analyses, suggesting an indirect role in the GAP-assisted reaction [244, 267]. On the basis of the crystal structure of the $G_i\alpha$•GppNHp complex and its comparison with the transition state-stabilized RGS complex, Asn128 was proposed to displace the side chain of the catalytic glutamine (Gln204 of $G_i\alpha$) from its 'anti-catalytic' position observed in the GppNHp (but not in the GTPγS)-bound ground state [268]. The proposal that RGS-domains stimulate Gα-mediated GTP hydrolysis by a transition state mechanism is consistent with the observation that these domains bind much more tightly to the GDP•AlF_x-bound than to the GTP-bound form [242]. Recently, the structure of the RGS domain of p115RhoGEF was determined in complex with a Gαsubscript/subscript chimera, which functionally resembles a Gαsubscript domain and was generated to facilitate overexpression in *Escherichia coli* [269]. In contrast to RGS4/RGS9, productive complex formation requires an N-terminal 42-residue extension [270], which in the structure interacts with the catalytic machinery (fig. 3G), with a direct stabilization of the catalytic arginine not seen in other Gα-RGS complexes. It turned out that the N-terminal extension is in fact sufficient for GAP activity, raising the question for the role of the RGS core domain. It was proposed that this portion might have a role in stimulating the RhoGEF activity in the full-length protein, an interpretation corroborated by effector-like interactions with the Gα-component [269]. Taken together it appears that also in the Gα-system the mechanism of GTPase stimulation may act *via* different structural scaffolds that adjust different portions of the catalytic machinery.

Signal-recognition particle and its receptor

A somewhat unusual case of GTPase stimulation is found in the cotranslational targeting of nascent proteins to membrane compartments, which requires the GTP-dependent interaction of the two structurally homologous GNBPs Ffh and FtsY [271]. These are core components of the signal-recognition particle (SRP) and its receptor (SR), a ubiquitous protein-targeting machinery found in all kingdoms of life, but whose complexity has considerably increased throughout evolution [272, 273]. In a series of complex events SRP binds to signal sequences on nascent proteins, thereby forming a ternary SRP nascent chain ribosome complex, which is targeted to membrane compartments in a GTP-dependent interaction with SR. Resumption of translation through the translocation channel is associated with the release of SRP from the complex [273]. This requires direct interaction between

GTP-bound Ffh (SRP54 in mammals) in the SRP with GTP-bound FtsY (SRα in mammals) in the SR, which is associated with mutual GTPase stimulation on Ffh and FtsY. Thus, FtsY acts as an Ffh-GAP and vice versa [274]. Structures of the so-called NG-domain of SRP-GNBPs, containing an N-terminal helical segment followed by a G-domain module, have been determined in the nucleotide-free state [275, 276] for Ffh and FtsY, and in complex with GDP•Mg^{2+} [277] or GppNHp [278] for Ffh. The SRPs are not related to other families and thus represent a distinct family of GNBPs [1], with an unusual domain, termed insertion box domain (IBD), inserted between the switch regions and the N-terminal helical segment, which is tightly packed against the G-domain core (fig. 3H).

The structure of the complex between FtsY and Ffh, each bound to a nonhydrolysable GTP-analog, reveals a highly symmetric heterodimer stabilized by extensive interaction between the two proteins involving large parts of the G- and to a smaller extent the N- and IBD-domains. The nucleotide binding sites interact to form an 'active site chamber' or 'composite GTP binding cavity', with the P-loop structures interacting with each other, and the bound nucleotides aligned in an unprecedented head-to-tail fashion, described as substrate twinning [279, 280] (fig. 3H). In the complex the IBD domain contributes critical residues to the active site. Acidic residues ($Asp135_{Ffh}$ and $Asp138_{FtsY}$) position water molecules close to the γ-phosphate of the bound nucleotide. The arrangement of conserved arginine ($Arg138_{Ffh}$, $Arg142_{FtsY}$) and glutamine residues ($Gln144_{Ffh}$ and $Gln148_{FtsY}$), as supplied by major conformational rearrangements of the IBD-domain, have been interpreted to support an arginine finger mechanism, i.e. *in trans* transition state stabilization [280], or *in cis* catalysis involving interaction with the β,γ-phosphates [279] (figs. 3H, 4F). The presence of the 3'-OH group on the sugar moiety, which forms a hydrogen bond with the γ-phosphate of the neighbouring nucleotide, appears to be critical, as concluded from the observation that 3'-deoxyGTP is not a substrate [279, 280]. Extensive mutagenesis of residues in the interface area has identified at least 25 amino acids important for the interaction [279], underscoring the requirement of all interactions for successful complex formation. The comparison with the isolated structures of Ffh and FtsY in different states reveals considerable conformational changes upon assembly of the composite active site, with major rearrangements seen in the N-domain, the NG-domain interface and in the switch regions (see above). While the mechanistic details of the GTPase acceleration need further experimental investigation, it seems obvious that the development of negative charges upon GTP hydrolysis would destabilize the composite active site and thus the heterodimeric complex.

Elongation factors

One of the best-characterized roles of GNBPs is in protein biosynthesis, where the elongation factor-Tu (EF-Tu) is responsible for loading amino-acyl transfer RNAs (aa-tRNAs) onto the ribosome during protein biosynthesis [281–283]. EF-Tu in the GTP-bound conformation forms a high-affinity ternary complex with aa-tRNA that binds to the ribosomal A site. Codon recognition by a ternary complex presenting a cognate anticodon induces conformational changes of the ribosome, which in turn stabilize tRNA binding, and triggers the GTPase activity of EF-Tu (10–20 s^{-1}) [284]. Non-cognate ternary complexes are rejected at the initial selection stage prior to GTP hydrolysis, which is due to both the lack of significant codon interaction (no base pair possible), and, most important, a very low rate of GTPase stimulation (~10^{-4} min^{-1}) for such tRNAs [285]. Among the ribosomal proteins that were thought to be responsible for GTPase stimulation of EF-Tu was the L7/L12 protein. It turned out that isolated L7/L12 strongly stimulates GTP hydrolysis of elongation factor G (EF-G) but not that of EF-Tu [286], although a direct interaction between EF-Tu and L7/L12 was recently demonstrated [287]. GTPase stimulation of EF-Tu appears to be controlled by a rotation of the ribosomal 30S subunit upon cognate codon-anticodon duplex recognition that directly stabilizes the position and conformation of EF-Tu [281].

Substrate specificity: promiscuous GAPs

GAPs are typically seen as rather specific catalysts, being unrelated in sequence or structure and acting only on members of a certain family of GNBPs. RasGAPs and RhoGAPs follow this view in that they are GNBP-specific but make an exception in sharing common topological features [5, 130–132]. A curiosity came with the discovery of a GAP^{IP4BP}, which acts on Rap and Ras proteins. Its domain scheme places a RasGAP-domain between an N-terminal C2- and a C-terminal PH-domain [166]. While the structural basis for this bifunctionality is unknown, it is interesting to note that SynGAP has been shown to activate the GTPase of Rap1/2 much more potently than that of Ras [164] and can also act as Rab5-GAP *in vitro* [288]. Whether these common features reside in the neighbouring domains or have a different origin will have to be investigated. Another GAP protein is the product of a familial tuberous sclerosis gene, tuberin, that has been reported to weakly stimulate GTP hydrolysis of Rab5 [289] and Rap1a [36] *in vitro*, and to function as a RhoGAP *in vivo* [290]. More recently, an increasing body of evidence supports the notion that tuberin acts as a GAP towards the small GTPase Rheb (Ras homolog enriched in brain) [291].

Moreover, *in vitro* experiments have shown that isolated catalytic domains of the GAPs are active on several members within a GNBP family, e.g. p50GAP on Rac1, Cdc42 and RhoA, p120RasGAP [292] and neurofibromin on H-, K-, N-, R-Ras proteins [43, 293] or Gyp3p on Sec4p, Ypt6p, Ypt51p, Ypt31/Ypt32p and Ypt1p [180]. However, the specificity of the GAPs *in vivo* is not only controlled by the spatial and temporal expression pattern but also by second messengers [294, 295], phospholipids [166, 212, 296, 297] and adapter proteins [298–300]. Phospholipids have been shown to be involved in determining the substrate specificity of the GAPs. For example, the specificity of p190 for Rho can be switched to Rac in the presence of phosphatidylserine [297].

The catalytic domains of GAPs are generally conserved in sequence. However, the yeast homologs of neurofibromin, IRA1 and IRA2 [301], are exceptions. These GAPs are highly specific for the yeast RAS1 and RAS2, but are completely inactive on human Ras [302]. A residue adjacent to the catalytic arginine (Gly1277 in neurofibromin and Arg1743 in IRA2) has been suggested to determine the substrate specificity [42]. Substitution of Arg1743 in IRA2 to glycine alters the specificity of IRA2 towards human Ras [303], and G1277R mutation of neurofibromin affects both binding to Ras•GTP and Ras•GDP•AlFx, and thus the GAP-catalysed reaction [42].

RasGAP-like domains

There are two groups of signalling proteins containing portions related to RasGAP domains. The proteins belong to the IQGAP and plexin families. IQGAPs have been proposed in mammalian cells as potential regulators of cadherin-based cell adhesion downstream of Cdc42 and Rac1 [304, 305]. All three IQGAPs (called IQGAP1, 2 and 3) found in humans have, among other signalling modules, a RasGAP-related domain at the C-terminus [306]. This domain does not show any GAP activity but specifically binds GTP-bound Rac1 and Cdc42 and inhibits their intrinsic GTP-hydrolysis activity [307–309]. It has been shown that IQGAP1 and α-catenin compete for binding to β-catenin and IQGAP1, thereby dissociating β-catenin from the β-catenin/α-catenin complex [304]. This results in weakening of cell-cell adhesion that can be re-established by activated Cdc42 and Rac1, which specifically bind to the GAP-like domain of IQGAP and inhibit its β-catenin-binding activity of IQGAP1.

Plexins are widely expressed transmembrane proteins that transduce an extracellular semaphorin signal towards axonal guidance through direct interaction with specific Rho-like GNBPs [310]. The cytoplasmic part of plexins shows sequence homology to the catalytic domains of RasGAPs which is, however, interrupted by a central segment of 200 amino acids. This segment has been shown to be responsible for binding to Rho family members [310]. Based on sequence homology between plexins and Ras-GAPs, two conserved arginines, corresponding to the arginine finger and the FLR arginine of RasGAPs, have been shown to be essential for plexin A1 signal transduction [311]. It has been suggested that plexins may be integral membrane proteins with an intrinsic GAP activity that is essential for their ability to induce growth cone collapse. GAP activity has been recently shown for the semaphorin 4D (Sema4D) receptor plexin-B1, which increases the GTPase reaction rate of R-Ras (a member of the Ras family) only in complex with Rnd1, a GTPase-deficient member of the Rho family and Sema4D [312]. It will not be surprising if, with an increasing number of GNBP-systems being discovered and characterized, the situation turns out to be much more complex than originally anticipated.

Concluding remarks

It is well established that GNBPs hydrolyse GTP inefficiently by providing relatively weak catalytic groups from the P-loop and from the mobile switch regions, e.g. a glutamine residue (a threonine in Rap) with an unfourable geometry. Accumulating structural and biochemical information about GAP proteins of GNBP subfamilies supports the view that there is no general mechanism of GTPase acceleration in the sense of the contribution of a catalytic residue (fig. 4, table 1). The GAPs for Ras, Rho and most likely Rab GTPases stimulate the slow reaction rate both by inserting a strong catalytic group (an arginine finger) into the nucleotide binding pocket and by stabilizing the switch regions (fig. 4A). The Gln side chain positions the nucleophilic water molecule for attacking the γ-phosphate of GTP. In contrast, the mechanism of GTPase stimulation of Arf GNBPs by ArfGAPs or coatomer – possibly both together – is still a matter of debate (fig. 4C). The formation of Arf1-ArfGAP1-coatomer complexes suggests that the coatomer serves to localize ArfGAP1 spatially on membranes so that ArfGAP1 catalytic activity is confined to Arf1 molecules in association with the COPI coat. A different function of these complexes could be to allow the coatomer to exert a stimulatory effect on GTPase activity; for example, by increasing the affinity of ArfGAP1 for Arf1, by assisting ArfGAP1 in orienting the Arf1 catalytic machinery or by supplying an arginine finger residue to the Arf1 active site. The GAPs for Ran and G_α-subunits, on the other hand, enhance the reaction rate primarily by stabilizing the switch regions at the transition state, suggesting that correct positioning of the nucleophilic water molecule relative to the γ-phosphate of the GTP substrate is a more conserved mechanism of GTP hydrolysis (figs. 4B, E). In this regard, the SRP-SR complex also provides a com-

posite active site where the nucleophilic water molecule is stabilized by a catalytic aspartate residue for GTP hydrolysis, while RapGAP most likely uses a catalytic asparagine to position the nucleophilic water molecule. However, stabilization of the switch regions containing catalytically important residues appears to be a general feature observed in all known cases.

A remarkable feature of GAP-assisted GTP hydrolysis consists of the transient formation of an efficient catalytic site by two entirely different proteins, thus forming a heterodimeric enzyme that normally only exists for the time of the catalytic reaction. It appears that the physical separation of the GTPase active centre facilitates differential control of GTP hydrolysis depending on the physiological conditions, i.e. efficient GTP hydrolysis when the activation level of the respective GNBP is high and low GTPase activity otherwise. While a large number of structural, biochemical and biophysical studies of individual GNBP/GAP systems have contributed greatly to our current understanding of GAP-stimulated GTPase reaction mechanisms, more detailed studies of the underlying protein-protein interactions will undoubtedly have an important role to play in the future. Finally, the upstream signalling processes regulating the GAPs themselves are a very important and yet open issue for future research.

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