GTPase-activating proteins: helping hands to complement an active site

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Stimulation of the intrinsic GTPase activity of GTP-binding proteins by GTPase-activating proteins (GAPs) is a basic principle of GTP-binding-protein downregulation. Recently, the molecular mechanism behind this reaction has been elucidated by studies on Ras and Rho, and their respective GAPs. The basic features involve stabilizing the existing catalytic machinery and supplementing it by an external arginine residue. This represents a novel mechanism for enzyme active-site formation.

GTP HYDROLYSIS IS a key process in intracellular signal transduction. Numerous vital processes, including protein synthesis, visual perception, vesicular and nucleocytoplasmic transport, protein targeting, growth control and differentiation, are controlled enzymatically by the conversion of GTP to GDP and inorganic phosphate (P_i)[1]. GTP-binding proteins are the molecular machines that catalyse this reaction. As essential factors in protein biosynthesis, heterotrimeric G proteins or small Ras-related GTP-binding proteins function as molecular switches that cycle between GTP-bound 'ON' and GDP-bound 'OFF' states. Exchange of the bound GDP is facilitated by guanine-nucleotide-exchange factors (GEFs), which increase the dissociation rate of nucleotides. This promotes binding of GTP, which allows the GTP-binding proteins to interact with effector molecules. Hydrolysis of bound GTP is the timing mechanism that returns these proteins to their GDP-bound OFF state and thereby completes what is called the GTPase cycle[1,2].

GTP-hydrolysis by GTP-binding proteins is intrinsically very slow but can be accelerated by orders of magnitude upon interaction with GTPase-activating proteins (GAPs)[3], which are specific for their respective GTP-binding proteins[4]. GAPs are primarily downregulators of the GTP-bound form, but some are also active signal transduction molecules. For example, the Ras-specific p120GAP contains signalling domains that have a dramatic impact on the reorganization of the cytoskeleton[5]. The importance of GTPase regulation is evident from diseases associated with mutations in either GTP-binding proteins themselves or GAPs: certain GTP-binding-protein mutants are oncoproteins[6]; and loss of GAP function (as a consequence of disruption or mutation of the presumed tumour-suppressor gene) is responsible for the disease phenotype in type 1 neurofibromatosis patients[7].

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GTPase activation – ten years after

During microinjection studies of Ras (originally termed p21ras) function, Trahey and McCormick noticed that, contrary to observations *in vitro, in vivo* 'Gly12p21 was predominantly guanosine diphosphate (GDP)-bound because of a dramatic stimulation of Gly12p21-associated guanosine triphosphatase (GTPase) activity'[8]. The cytosolic protein responsible for this increased activity, now known as p120GAP, stimulated GTP-hydrolysis by normal Ras *in vitro*, but had no effect on oncogenic Ras mutants[8,9]. Since the discovery of this first GTPase-activating protein, many studies have shown GTPase activation to be a general regulatory principle within systems that involve GTP-binding proteins and have provided considerable insight into the mechanism of GAP action.

The use of activation of nucleoside triphosphate hydrolysis as a regulatory mechanism is not confined to GTPases; ATP-converting enzymes are also regulated in this way. Actin stimulates ATP turnover by myosin, thereby acting as an ATPase-activating protein ('AAP'). Similarly, the ATPase activity of the bacterial chaperone DnaK is stimulated by DnaJ.

GAPs that are specific for the Ras, Rho, Rab, Ran and Arf subfamilies of Ras-related GTP-binding proteins have been described (see Box 1, p. 260). Although members of any one subfamily share sequence homology, GAPs from different subfamilies do not[4]. Accordingly, they are termed RasGAPs, RhoGAPs, etc. Modular architecture is commonly used to combine the downregulatory activity of GAPs with various other functions, including signalling. Originally detected genetically in yeast, as negative regulators of G-protein signalling (RGSs), a large number of GAPs that target heterotrimeric-G-protein α subunits (Gαs) have been described recently[10].

The variability of GAP function is demonstrated by elongation factor Tu (EF-Tu). EF-Tu possesses an almost unmeasurable, intrinsic GTPase activity, which is stimulated dramatically by the large subunit of the mRNA-primed ribosome. It has been proposed that the L7/L12 protein C-terminal domain acts as an EF-TuGAP (Ref. 11). FtsY and Ffh, the *Escherichia coli* homologues of the signal-recognition particle (SRP) and its receptor, respectively, catalyse the cotranslational targeting of proteins into membranes. By a mechanism that remains to be defined, they stimulate each other's GTPase activity[12].

Most of the concepts underlying the structure, function and biochemical mechanism of small GTP-binding proteins have been derived from studies on Ras[1,2,6]. During the past two years, Ras has again come into focus: studies on the Ras–RasGAP system have elucidated the mechanism behind GAP catalysis, both biochemically[13,14] and in terms of structural biology[15,16]. This mechanism has been confirmed, independently, by biochemical and structural studies of the Rho–RhoGAP system[17–21]. These two systems are therefore the focus of this review.

Hypotheses on the GTPase-activating protein mechanism

Investigation of GTPase acceleration focused on two questions: which steps of the GTPase reaction are controlled by

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GAP; and does a universal mechanism exist? In the acto-myosin system, release of P_i is rate limiting and is stimulated by actin. By contrast, in both intrinsic and GAP-stimulated reactions, the release of P_i by GTP-binding proteins is not rate limiting[22,23]. Two models for the mechanism of GAP action have been discussed. The first postulates that the GTP-binding protein is itself an efficient GTPase and that GAP acts catalytically to drive the GTP-binding protein into an enzymatically competent conformation. Experiments using fluorescently labelled GTP analogues designed to test such a model yielded conflicting results[24–26]. The second hypothesis proposes that GAP participates actively in the process of GTP hydrolysis, possibly by contributing a catalytic residue to the active site; in this case, stoichiometric amounts of GAP would be needed for catalysis[3]. The latter model is based on the structure of very efficient phosphoryl-transfer enzymes, such as adenylate kinase, where a number of positively charged residues are involved in catalysis[27].

Experiments with aluminium fluoride

A major breakthrough in the elucidation of the nature of GTPase acceleration came from studies using fluorescently labelled guanine nucleotides and aluminium fluoride (AlF_x). AlF_x was originally found to activate heterotrimeric G proteins in their inactive GDP-bound state[28]. The hypothesis that AlF_x was trapped in the γ-phosphate-binding site, thereby mimicking at least some aspects of the GTP-bound form, was confirmed by the crystal structures of Gα–AlF_x complexes. In addition, these structures showed AlF_4^- to be a square-planar entity; together with biochemical data, this observation supported the idea that GDP–AlF_4^- does not simply act as a GTP analogue but, rather, mimics the transition state in the GTPase reaction[29,30]. On the basis of numerous studies on GTP- and ATP-converting enzymes, aluminium fluoride is now considered to be a general mimic of the phosphoryl group transferred in GTP/ATP hydrolysis and other phosphotransfer reactions[31].

The observations that Ras–GDP does not bind AlF_x (Ref. 32) and that the helical domain in the effector region (as defined in Ras) of Gα subunits acts as an internal GAP (Ref. 2) prompted studies of the effect of RasGAPs on the AlF_4^-–Ras interaction. Indeed, Ras–GDP forms a stoichiometric complex with AlF_x in the presence of NF1-333 or GAP-334, the catalytic domains of neurofibromin and p120GAP, respectively[13]. Complex formation does not occur if the invariant arginine residue, Arg1391 (from the RasGAP identifier motif $FLRX_3PAX_3P$) in NF1-333, is mutated to methionine, or if an oncogenic mutant of Ras is used. The experiments using transition-state analogues favoured the hypothesis that GAPs actively participate in the process of Ras-mediated GTP hydrolysis. The general implications of the AlF_x experiments, for the interaction between GAPs and their respective GTP-binding proteins, are obvious from studies of the Rho system. Stable complexes between Cdc42–GDP and RhoGAP can form in the presence of AlF_4^-, and experiments with Ran and Rap have yielded similar results[17].

Gα proteins bind AlF_4^-, but are also targets of specific GAPs, the RGS proteins. Some of these (such as RGS4) bind much more tightly to the transition-state complex (as represented by Gαi–GDP–AlF_4^-) than to the ground-state complex (as represented by Gαi–GTPγS)[10]. Thus, stabilizing AlF_4^- binding is not the only indication of GTPase activation.

Structures of GTPase-activating proteins

Numerous GAPs that are specific for Rho/Rac/Cdc42 have been identified, including p190 and p50. The RhoGAP-like domain of the p85 subunit of phosphoinositide 3-kinase comprises a 200-residue helical protein[18] that is highly similar in structure to the corresponding domain of p50RhoGAP (Ref. 19) but has no GAP activity[33]. Its core contains a four-helix bundle, one face of which contains most of the conserved residues and has been proposed to be the G-protein-binding site (Fig. 1a)[18,19].

Of the five mammalian RasGAPs described to date, p120GAP and neurofibromin are the best studied[34]. The catalytic fragment of p120GAP, GAP-334, is a helical, elongated protein (Fig 1b)[15]. The structure defined a central domain of 218 amino acid residues that contains all the residues conserved among RasGAPs and corresponds to a minimal catalytic domain of neurofibromin that retains full GAP activity (Fig. 1b)[35]. On the basis of a large number of

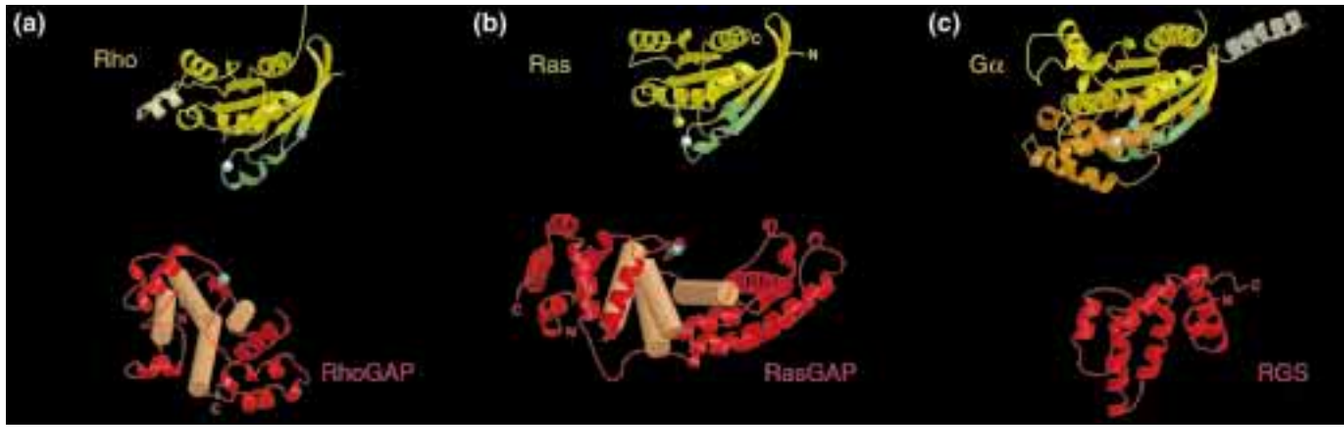

Figure 1

Structures of GTP-binding proteins and their GTPase-activating proteins (GAPs; drawn using MOLSCRIPT[48] and Raster3D[49]) shown in the orientations found for their respective complexes. In each case the nucleotide has been omitted for clarity. The G domains are shown in yellow; additional elements not present in Ras and not associated with GAP activity are shown in pale yellow; the helical domain of Gα is shown in orange; the common switch I/II regions and the P-loop of the GTP-binding proteins are shown in green. GAPs are shown in red; the positions of the catalytic arginines and the critical glutamines are indicated by cyan and white dots, respectively. Helices belonging to the proposed evolutionary module in RasGAP and RhoGAP are shown as solid, pink cylinders. Gly12 in Ras is shown in yellow. **(a)** Structures of Rac1 (PDB accession code 1MH1), representative of Rho proteins[50], and p50RhoGAP (PDB accession code 1RGP)[19]. **(b)** H-Ras (PDB accession code 5P21)[44] and GAP-334 (PDB accession code 1WER)[15]. **(c)** Gαi and regulator of G-protein signalling 4 (RGS4; PDB accession code 1AGR)[39].

biochemical studies of Ras–RasGAP interaction[9,34], a docking model has been proposed, in which two invariant arginine residues (Arg789, Arg903) – candidates for residues involved in GAP catalysis – are brought within reach of the nucleotide[15].

The catalytic domains of p120GAP and p50RhoGAP have been reported to share no detectable tertiary structural similarity[20,21]. However, alignment of the models (on the basis of the way in which the GAPs communicate with their GTP-binding protein partners[16,21]) reveals at least distant structural relationships (Fig. 1a,b). An additional region (present in GAP-334) that includes the C-terminal part of the central domain is missing in RhoGAP, and an α-helical hairpin corresponding to α3c and α4c in GAP-334 is considerably shorter and adopts an orientation that is different from that of the equivalent region in GAP-334. A structural overlay suggests that the helical core described for the RhoGAP domain[18,19] is a possible evolutionary module (Fig. 1a,b).

No apparent similarity between the Rho/RasGAPs and RGS4 (Fig. 1c) has yet been detected. From the structural studies, it appears that the GTP-binding proteins represent a *tema con variazioni* (variations on a theme). In contrast, their GAPs share far less structural similarity, although they are not completely unrelated (Fig. 1a–c).

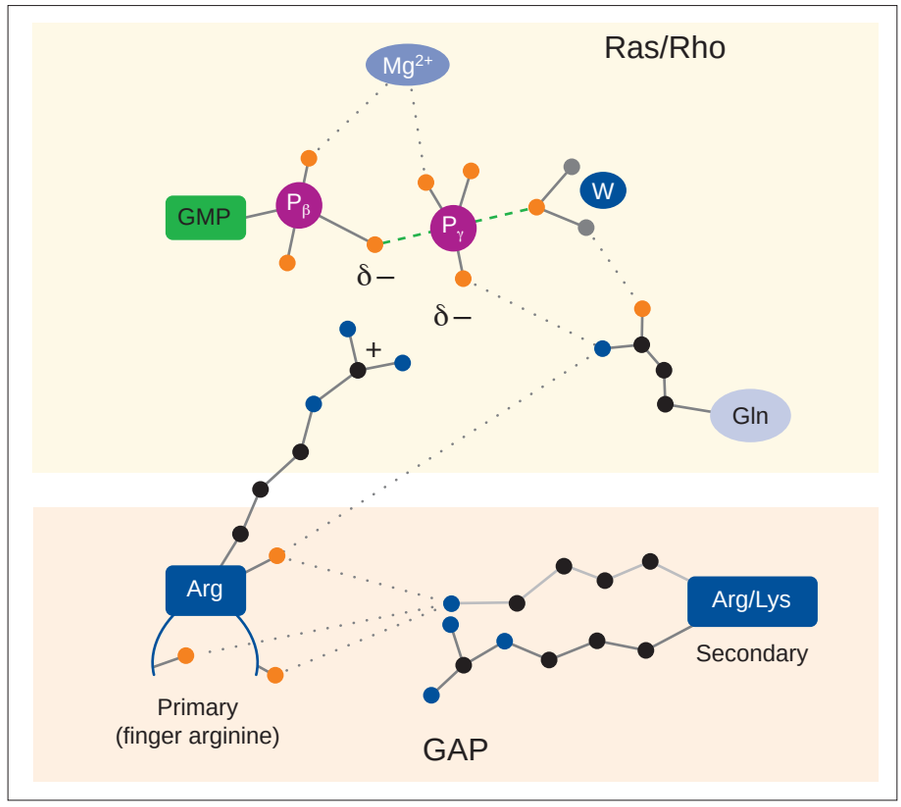

Figure 2

Complementation of the active site of the small GTP-binding proteins Ras and Rho by their respective GTPase-activating proteins (GAPs). A 'primary', finger-arginine residue, together with the finger loop, crosses the 'gap' between the proteins in order to neutralize developing charges in the transition state of the reaction and stabilize the critical glutamine residue. A 'secondary', positively charged residue, (Arg in RasGAP and Lys in RhoGAP) stabilizes the finger loop. The transition state is shown as having a pentacoordinate phosphate group, in which the degree of bond making and bond breaking between the transferred phosphate, and the leaving group and nucleophilic oxygen (broken green lines), respectively, determines its associative/dissociative character. GMP, guanosine monophosphate.

GTPase-activating-protein communication in three dimensions

Within the Ras–RasGAP complex formed by Ras–GDP and GAP-334 in the presence of AlF_3, GAP-334 interacts predominantly with the switch regions and the P-loop of Ras. This interaction is similar to that proposed in the docking model[15], and the complex is stabilized by hydrophobic and hydrophilic contacts (Fig. 1b)[16]. An exposed loop in RasGAP is placed close to the nucleotide, the guanidinium group of Arg789 interacting with the β phosphate of GDP and AlF_3. In addition, the main-chain carbonyl oxygen of Arg789 forms a hydrogen bond with the side-chain amide group of the catalytically important Gln61 in Ras. Because Arg789 and the loop point into the active site, they have been called the 'arginine finger' and the 'finger loop', respectively[16]. Gln61 also contacts AlF_3 and a water molecule that corresponds to the attacking nucleophile. As in unligated GAP-334 (Ref. 15), Arg903 of the FLR motif stabilizes the finger loop by side-chain–main-chain interactions. This situation is depicted schematically in Fig. 2.

Mutations of Gly12 and Gln61 in Ras that are commonly found in human tumours lock the GTP-binding protein in its active conformation, thereby activating its oncogenic potential[36,37]; homologous mutations in other GTP-binding proteins show a similar, constitutively activated, phenotype. The structure provides a simple explanation for why these mutants are insensitive to GAP: Gly12 lies sufficiently close to the finger loop that even the smallest possible amino acid change (to alanine) would sterically interfere with the geometry of the transition state. Because Gly12 mutants bind to GAP with almost wild-type affinity, it appears that larger side chains at position 12 can be tolerated in the Ras–RasGAP ground-state complex but not in the transition state. The apparent involvement of Gln61 in stabilization of the transition state, together with biochemical data[13], confirms the notion that Gln61 has a vital role in catalysis.

The situation observed in the active site of the Ras–GDP–AlF_3–GAP-334 complex was confirmed by the structure of the corresponding complex between the catalytic domain of p50RhoGAP and RhoA, which revealed the details of communication between the two proteins[21]. In this structure, the invariant arginine (Arg85, which corresponds to Arg282 of the full-length protein) contacts the nucleotide and a fluoride ligand of the square-planar AlF_4^-. As in the Ras–RasGAP complex, the carbonyl oxygen of this arginine forms a hydrogen bond with the amide group of the critical glutamine (Gln63), which also contacts AlF_4^- and the nucleophilic water molecule. The loop carrying Arg85 is stabilized by an invariant lysine (Lys122), which appears to play a similar role to that of Arg903 of RasGAP (Fig. 2).

Comparison of the AlF_4^--bound complex with the ground-state complex, as represented by p50RhoGAP(242-residue fragment)–Cdc42–GppNHp (Ref. 20), revealed that major structural changes

RapGAPs	Acc. No.	Motif 1	Motif 2	Motif 3
*hs*RapGAP	M64788	KGFRGGL	LQRKRHI	EERTRAA
*dm*RapGAP	AF023478	KGYRGGL	LQRKRHI	EQRTRTS
*ce*Rap1GAP	P91315	QKYRGGL	LQRKRHI	AERTRSS
*mm*Spa1	P46062	EQYRAQL	LLRKRHI	ATRTRQQ
*mm*Rap1GAP	P70204	ESYRAQL	LLRKRHI	ATRTRQQ
*hs*Rap1GAP	D1023058	ESYRAQL	LLRKRHI	ATRTRQQ
*rn*Spa1	G2555183	EKYRAQL	LLRKRHI	ATRTRQE
Consensus		**cxaRsxL**	**LxRKRHI**	**xpRTRxx**
RasGAPs				
*hs*NF1	M89914	TLFRGNS	MFLRFIN	
*rn*NF1	M89914	TLFRGNS	MFLRFIN	
*ss*GAP^{ip4BP}	Q14644	TIFRGNS	IFLRFFG	
*bt*R-GAP	Q28013	TIFRGNS	IFLRFFG	
*hs*RasGAP	Q15283	TIFRGNS	VFLRFFA	
*rn*GAP1^{m}	Q29594	AIFRGNS	VFLRFFA	
*mm*GAP3	D30734	TIFRGNS	IFLRFFA	
*dm*GAP1	U202238	TIFRGNT	IFLRFFA	
*ce*GAP	M86655	LMFRGNT	IFLRFLC	
*btp*120	P09851	TLFRATT	VFLRLIC	
*rnp*120	P50904	TLFRATT	VFLRLIC	
*hsp*120	M23379	TLFRATT	VFLRLIC	
*sc*IRA1	M24378	DILRRNS	VFLRFIG	
*sc*IRA2	M33779	DILRRNS	VFLRFFC	
*sp*Sar1	S37449	SLLRANT	FFLRFVN	
Consensus		**xhhRsNo**	**hFLRFhx**	
RhoGAPs				
*hsp*50GAP	Z23024	GIFRRSA		
*mm*Rip1	Q62172	GVYRVSG		
*rn*Cytocentrin	G2697022	GIYRVSG		
*hs*Rlip76	Q15311	GIYRVSG		
*rn*RalBP1	Q62796	GIYRVSG		
*hs*ABR	Q13693	GIYRISG		
*hs*GRP	Q12979	GIYRISG		
*hs*Bcr	P11274	GIYRVSG		
*hs*N-Chimerin	P15882	GLYRVSG		
*hs*ß-Chimerin	P52757	GLYRVSG		
*mm*p190-B	P97393	GLYRVSG		
*hs*RGC1	P98171	GIFRVSG		
*rnp*190GAP	P81128	GIYRVSG		
*sc*Rga1/Dbm1	P39083	GIYRKSG		
*mm*3BP-1	X87671	GLFRLAA		
consensus		**GhaRhSG**		
YptGAPs				
*sc*Gyp1	Ref. 4	FAFRWMN	LLMREFQ	
*ce*Gyp6	P32806	WLIRWTR	LFLRELP	
*sc*Gyp7	P48365	FCFRMLL	WFHREFE	
Gyp7-like	P09379	FFFRMLL	LFRRELS	
*yl*Gyp7	E339717	FFFRMLL	WFHRELL	
Consensus		**FhFRhhx**	**xFhREhx**	
RanGAPs				
*xl*RanGAP1	G2062659	FTGRLRP	AGRNRLE	
*stp*RanGAP1	G2623618	FTGRLRS	AGRNRLE	
*mm*RGP1	P46061	FTGRLRS	AGRNRLE	
*hs*RanGAP1	P46060	FTGRLRT	AGRNRLE	
*mm*RNA1	Q60801	FTGRLRS	AGRNRLE	
*sp*RNA1	P41391	FTGRVKD	CGRNRLE	
*sc*RNA1	P11745	YTSRLVD	CGRNRLE	
Consensus		**FTGRh+x**	**AGRNRLE**	
ArfGAPs				
*hs*ARD1	P36406	AKHRRVP		
*rn*ArfGAP	Q62848	GRHRGLG		
*dm*ArfGAP	G2286211	GKHRSLG		
*sc*Gcs1	P35197	GIHRGLG		
*sc*Sps18	P32572	NLLRGMG		
*sc*Glo3	P38682	AVHRNMG		
*btIn*3PBP	O02753	GIHRNIP		
*rn*Centaurin	Q63629	GIHRNIP		
*ssp*42IP4	O02780	GIHRNIP		
Consensus		**shHRxhx**		
L7/L12				
E. coli	V00339	KAVRGAT		
S. typhimur.	P18081	KAVRGAT		
P. putida	P31855	KAVRELT		
M. luteus	P02395	KVVREIT		
B. stearoth.	P05392	KVVREIT		
Th. maritima	P29396	KVVREIT		
H. pylori	P55834	KVVREIT		
Consensus		**KxVRxhT**		

The invariant arginine residues (highlighted in yellow) are aligned. Invariant residues are shown in red; conserved residues are green; homologous residues are shown in blue. Acc. No., accession number; a, aromatic; c, charged; h, hydrophobic; o, Ser or Thr; s, Gly or Ala; +, positive; x, any residue. *at*, *Arabidopsis thaliana*; *bt*, *Bos taurus*; *sec*, *Serinus canaria*; *ce*, *Caenorhabditis elegans*; *dm*, *Drosophila melanogaster*; *hs*, *Homo sapiens*; *mm*, *Mus musculus*; *rn*, *Rattus norvegicus*; *sc*, *Saccharomyces cerevisiae*; *sp*, *Schizosaccharomyces pombe*; *ss*, *Sus scrofa*; *stp*, *Strongylocentrotus purpuratus*; *xl*, *Xenopus laevis*; *yl*, *Yarrowia lipolytica*.

Box 1. Are all GTP-binding proteins switched off by arginine fingers?

The increasing number of new GTPase activating proteins (GAPs) identified for members of subfamilies of GTP-binding proteins such as Rap, Ran, Arf, Ypt and elongation factors prompted us to analyse the sequence relationships between the various GAP subfamilies in order to identify possible catalytic arginine residues. GAPs within a subfamily share high sequence similarity, but the degree of sequence similarity between subfamilies is low[a–g]. Nevertheless, we were able to identify sequence motifs that contain invariant arginine residues within the GAPs described for the Rap, Ypt, Ran and Arf proteins [motifs 1, 2 and 3 (see figure)]. The ribosomal L7/L12-proteins, which are proposed to be GAPs for elongation factors[h], contain one invariant arginine conserved in 38 protein sequences analysed from different bacteria and chloroplasts.

The arginine-finger hypothesis is strongly supported by biochemical and structural data on RasGAPs and RhoGAPs showing that the arginine finger (motif 1) is the primary element required for GTPase-rate enhancement. The second invariant arginine residue in RasGAPs (motif 2) stabilizes the finger loop, a function that appears to be dependent on a conserved lysine residue at the equivalent position in RhoGAPs (Fig. 2 in review). The Rho and Ras systems reveal characteristics that might be common to other arginine fingers. The catalytic residues in RasGAPs and RhoGAPs are located in the N-terminal portion of the catalytic domains (motif 1). Although RasGAPs and RhoGAPs do not share obvious sequence homology, their structures are related. It is therefore likely that the putative arginine fingers in members of other GAP subfamilies are also localized within the N-terminal half of the protein's catalytic domain (motif 1). Interestingly, the motif-1-arginine residues are preceded by an aromatic amino acid residue (phenylalanine or tyrosine) in RasGAPs, RhoGAPs, RapGAPs and YPTGAPs. In RasGAPs and RhoGAPs, this aromatic amino acid residue stabilizes the adjacent hydrophobic core and balances the orientation of the arginine finger. This hydrophobic stabilization, however, is not realized in all GAPs. In RanGAP and ArfGAP, and in the presumed GAP for the elongation factors L7/L12, the invariant arginine residues are not preceded by an aromatic residue. Furthermore, ARD1 is an Arf protein that has an ArfGAP domain in the N-terminal region[i]. The invariant arginine residue (Arg164) in the ArfGAP domain of ARD1 has recently been shown to be critical for the ARD1 GAP activity. Its replacement by a glycine residue almost completely abolishes GTP-hydrolysis[i], which supports the proposal that it is in fact an arginine finger.

Although the evidence is far from conclusive, and the structural data for the other GAPs are not available, our analysis of GAPs has identified a limited number of arginine residues that are good candidates for arginine fingers. Arginine fingers must meet the following requirements: (1) they are invariant within a subfamily of GAPs; (2) they cannot be replaced, not even by a lysine residue; (3) a mutation in the residue drastically impairs GAP activity without changing binding affinity. In other words, the critical arginine finger should show up if it is broken by site-directed mutagenesis.

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occur upon formation of the transition state. These involve a 20°, rigid, body rotation of the two proteins relative to each other[21]. In the ground-state complex, Arg85 contacts the P-loop of Cdc42 and is not in a position that would support catalysis. Phosphorus-NMR experiments using Ras have shown that the presence of RasGAP does not induce a chemical-shift change in any of the Ras–GppNHp-complex phosphate resonances, which would be expected if they were contacted by a positively charged arginine side chain[38]. This, together with biochemical studies, suggests that, as with RhoGAP, the RasGAP finger arginine is not in a position that would accelerate GTP-hydrolysis, in the ground state of the RasGAP–Ras complex.

As in the Ras–RasGAP complex[16], larger side chains at the critical Gly12 position in Cdc42/RhoA can be accommodated in the ground state but would cause steric hindrance upon transition-state formation (K. Rittinger and S. Smerdon, pers. commun.). This explains why these mutants bind, but are not sensitive to, GAP, although the conformational changes observed in these studies did not involve complexes containing identical GTP-binding species.

Gα proteins differ from small GTP-binding proteins in that they contain an additional helical domain that is apparently important for positioning a catalytically important arginine residue such that it contacts the nucleotide and a fluoride ligand in the complex with GDP–AlF_4^- (Refs 29, 30). The crystal structure of the complex with RGS4 revealed that RGS4 has a helical architecture (Fig. 1c)[39] and that it predominantly contacts the switch regions of Gα. RGS4 is therefore believed to stabilize the transition state by reducing the mobility of these regions, and thus acts similarly to RasGAPs and RhoGAPs.

The mechanism of GTPase activation

Arginine and lysine residues play critical roles in phosphoryl-transfer reactions. Positively charged under physiological conditions, these residues are able to neutralize negative charges that develop on the transferred phosphoryl group or the leaving group oxygen, depending on whether the mechanism is associative or dissociative[40]. In addition, their side chains are comparatively long, which allows them to bridge larger distances at the protein–protein-complex interfaces. In nucleoside monophosphate kinases, arginine residues are essential for catalysis[27].

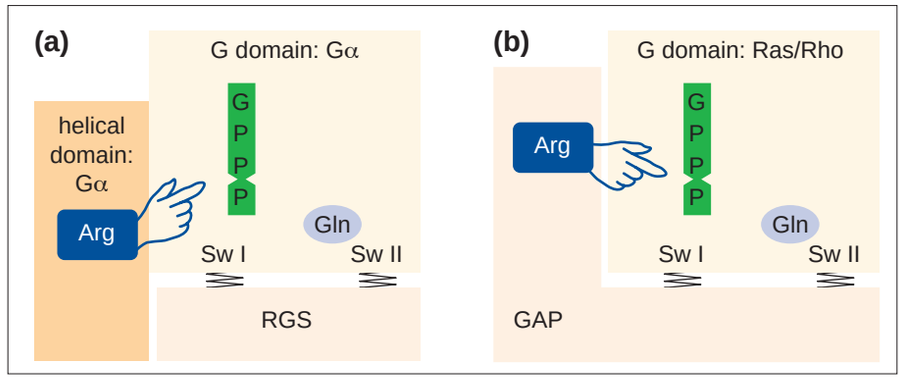

Figure 3

Common principles in the requirements for efficient GTP-hydrolysis. The universal G domain (shown in yellow) contains a number of functionally important residues, especially a critical glutamine, that provide the scaffold for the active site. **(a)** In heterotrimeric-G-protein α subunits (Gαs; shown in yellow), a catalytically essential arginine is supplied *in cis* and positioned by an inserted helical domain (shown in orange), which is supplemented by a regulator of G-protein signalling (RGS; shown in pink) that stabilizes the switch regions. **(b)** In the case of the small GTP-binding proteins Ras and Rho (shown in yellow), the arginine residue is supplied *in trans*, by GAPs (shown in pink). The GAP also provides components that stabilize the switch regions. Sw, Switch.

In RhoGAPs and RasGAPs, invariant arginines are critical for interaction with the GTP-binding protein[15,19]. In GAP-334, Arg789 (Arg1276 in neurofibromin) is extremely important for GTPase acceleration; even conservative mutation of this residue, to lysine, has dramatic effects. Arg903 (Arg1301) is less critical, but double mutants such as Arg789→Lys Arg903→Ala are unable to accelerate the GTPase activity beyond the intrinsic rate of Ras-mediated GTP hydrolysis[14]. These observations are in very good agreement with the structure of the Ras–GAP-334 complex. Arg789 points into the active site and neutralizes negative charges, and, by means of the finger loop, anchors Gln61. This stabilizes the transition state (as represented by Ras–GDP–AlF_3). As one would expect, mutation of the invariant 'finger' arginine in p190 has a detrimental effect on catalysis in Rho proteins[41]. The finger loop is stabilized by Arg903. Interestingly, an invariant lysine residue (Lys122) seems to play the role of this residue in the RhoGAP system (Fig. 2). Gln61 apparently positions the water molecule for nucleophilic attack and stabilizes the transferred phosphoryl group. The structures of Rho and Ras in complexes with their respective GAPs did not reveal a general base for the activation of the nucleophilic water molecule, which is consistent with the notion that the proposed mechanism of substrate-assisted catalysis[42] also applies to the GAP-catalysed reaction[43].

By comparing the roles of GAPs with those of RGSs, we can conclude that nature has developed at least two themes in order to realize efficient GTP hydrolysis: (1) use of arginine residues for stabilizing the transition state; (2) stabilization of the switch regions in order to optimize the orientation of the catalytic machinery in the GTP-binding protein, the most important element of which is a glutamine residue. In heterotrimeric G proteins the critical arginine residue is part of the GTP-binding protein itself and is supplied *in cis* with an extra domain necessary for orientation and an extra protein (RGS) required for proper alignment of the entire machinery (Fig. 3a). For Ras-related GTP-binding proteins, this residue is supplied as an arginine finger (i.e. *in trans*) by the respective GAP (Fig. 3b). Stabilization of the switch region is best documented in Ras, where the Gln61 region is highly mobile in the isolated protein[44–46].

Although the basic features of GAP-catalysed GTPase reactions have been worked out, many questions remain. For example, why do most known phosphoryl-transfer enzymes prefer arginine residues for catalysis? Why do we find differently coordinated AlF_x (AlF_4^- or AlF_3) in the active sites? We also do not know how the transition-state mimic containing AlF_x, which is kinetically and thermodynamically very stable, is related to the high-energy state of the real transition state. In addition, do AAPs such as DnaJ work in a similar manner? What is the mechanism of phosphoryl transfer in myosin, which has a non-actin-stimulated single-turnover ATPase of about 100 s^{-1}, but does not have a unique positively

charged amino acid residue in the active site[47]? Clever studies have to be designed in order to answer these questions.

A heterodimeric enzyme

Transition-state stabilization is the basic principle of enzyme catalysis. In GTP-binding proteins, a substrate-binding site is formed by amino acid residues derived from fingerprint sequence motifs, and the catalytic machinery is in principle able to perform GTP cleavage at a rate significantly greater than that of spontaneous hydrolysis in water. However, this rate is increased even more upon interaction with GAPs, in a way that represents a novel biological principle.

What is special about this enzyme? Nature has developed numerous strategies for optimizing metabolism and for regulating enzyme activities, such as allosteric control, proteolytic activation, reversible covalent modification and activation by control proteins. Enzymes are also commonly composed of two or more subunits, and the active site can be shared between subunits – a strategy that optimizes regulation and formation of the induced-fit conformation required for catalysis. In the Ras–RasGAP/Rho–RhoGAP system, the active site is shared by two completely different proteins. They come together as a transient heterodimeric enzyme, in order to catalyse GTP-hydrolysis, and separate after the job is done.

Separation of components of the enzymatic machinery is of major physiological importance. p120GAP is a cytosolic protein and becomes localized to the plasma membrane by binding (through its SH2 domain) to activated receptor tyrosine kinases, such as the platelet-derived-growth-factor receptor. Considering the low affinity of p120GAP for Ras–GTP, which is in the micromolar range, and the low concentrations of the reaction partners, it seems reasonable to assume that GAP acts on Ras only when both are localized to the plasma membrane (where the local concentration in the two-dimensional space is very high). Neurofibromin is predominantly cytoplasmic and has been found attached to microtubules, where it might not be available for Ras downregulation under normal conditions. The affinity of neurofibromin for Ras–GTP is about 50-fold higher than that of p120GAP, which indicates a differential requirement for the GAP reaction. Other GAP isoforms might also exhibit differential behaviour; however, detailed knowledge is limited.

Another reason for the physical separation of the GTPase active centre could be a requirement for efficient and inefficient GTPase machineries under different physiological conditions. In order to avoid unnecessary GTP turnover because of nucleotide exchange and GTP-hydrolysis, both reactions are very slow in the absence of GEFs and GAPs, respectively. Only when the signalling mechanisms of the cell require fast Ras activation or deactivation do external factors increase the rates of these reactions. It appears that the newly discovered principle that an inefficient GTPase centre is complemented, under certain physiological conditions, by a GAP is a universal principle in the regulation of many if not all GTP-binding proteins.

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