Crystal structure of the ARF-GAP domain and ankyrin repeats of PYK2-associated protein β

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ADP ribosylation factors (ARFs), which are members of the Ras superfamily of GTP-binding proteins, are critical components of vesicular trafficking pathways in eukaryotes. Like Ras, ARFs are active in their GTP-bound form, and their duration of activity is controlled by GTPase-activating proteins (GAPs), which assist ARFs in hydrolyzing GTP to GDP. PAP β , a protein that binds to and is phosphorylated by the non-receptor tyrosine kinase PYK2, contains several modular signaling domains including a pleckstrin homology domain, an SH3 domain, ankyrin repeats and an ARF-GAP domain. Sequences of ARF-GAP domains show no recognizable similarity to those of other GAPs, and contain a characteristic Cys-X₂-Cys-X_{16–17}-Cys-X₂-Cys motif. The crystal structure of the PAP β ARF-GAP domain and the C-terminal ankyrin repeats has been determined at 2.1 Å resolution. The ARF-GAP domain comprises a central three-stranded β -sheet flanked by five α -helices, with a Zn²⁺ ion coordinated by the four cysteines of the cysteine-rich motif. Four ankyrin repeats are also present, the first two of which form an extensive interface with the ARF-GAP domain. An invariant arginine and several nearby hydrophobic residues are solvent exposed and are predicted to be the site of interaction with ARFs. Site-directed mutagenesis of these residues confirms their importance in ARF-GAP activity.

Keywords: ankyrin repeats/ARF-GAP domain/PAP β /
X-ray crystallography/zinc-binding module

Introduction

ADP ribosylation factors (ARFs), members of the Ras superfamily of GTP-binding proteins, were identified initially based on their ability to stimulate the ADP-ribosyltransferase activity of the cholera toxin A subunit (Kahn and Gillman, 1986). However, ARFs are now known to play critical roles in vesicular trafficking (Rothman, 1994) and stimulation of phospholipase D (Donaldson and Klausner, 1994). In mammals, the ARF family consists of six proteins and >10 ARF-like (ARL) proteins (Moss and Vaughan, 1998). The six ARFs are grouped into three classes (Moss and Vaughan, 1995):

class 1 (ARF1, ARF2 and ARF3), class 2 (ARF4 and ARF5) and class 3 (ARF6).

The best characterized member of the ARF family, ARF1, cycles between the cytosol and membrane depending on its nucleotide status. Membrane association of ARF1 occurs through conversion of ARF1 to the active, GTP-bound form by guanine nucleotide exchange factors (GEFs). Once membrane-bound, ARF1 participates in recruitment of coatamer proteins which are required for budding and fission of membranes (Rothman and Wieland, 1996; Schekman and Orci, 1996). A coated vesicle must be uncoated before fusion with the acceptor compartment can occur. Uncoating requires hydrolysis of GTP to GDP, a process dependent on the interaction of ARF1 with GTPase-activating proteins (GAPs). Mutation of the ARF1 ortholog in yeast has shown that yeast ARF1 is required for maintenance of both Golgi and endosome structure (Gaynor *et al.*, 1998). ARF6 is postulated to be involved in recycling of transferrin receptors from endosomal compartments to the plasma membrane (Radhakrishna and Donaldson, 1997; D'Souza-Schorey *et al.*, 1998). The functions of other ARFs and ARL proteins are not yet clear.

ARF1 adopts a similar structural fold to that of Ras, though they share only 11% sequence identity (Amor *et al.*, 1994; Greasley *et al.*, 1995; Goldberg, 1998). Apart from the consensus sequences required for GTP binding (Sprang, 1997), ARFs contain two structural elements that have no counterparts in Ras: an extra β -strand (β 2E) and an N-terminal α -helix. The N-terminal amphipathic helix of ARF1 is myristoylated and interacts with membrane lipids in a GTP-dependent manner (Franco *et al.*, 1995; Randazzo *et al.*, 1995). ARFs have extremely low intrinsic GTPase activity (Kahn and Gillman, 1986); hydrolysis of GTP to GDP is dependent on ARF-GAPs.

Although the overall folds of ARF1 and Ras are very similar, their respective GAPs show no sequence similarity. Sequences of ARF-GAP domains contain a characteristic pattern of four cysteine residues which coordinate a Zn²⁺ ion. Overexpression of ARFGAP1, the first mammalian ARF-GAP protein to be characterized (Cukierman *et al.*, 1995), induces the release of COPI from Golgi membranes to the cytosol and the redistribution of the entire Golgi complex to the endoplasmic reticulum (ER) (Aoe *et al.*, 1997; Huber *et al.*, 1998). Gcs1 and Glo3 are two yeast ARF-GAP proteins that function in the ER–Golgi vesicular transport system (Poon *et al.*, 1996, 1999). Yeast lacking both of these ARF-GAPs are not viable, exhibiting defects in protein transport between the ER and Golgi.

PYK2 is a non-receptor tyrosine kinase involved in modulation of ion channel function and activation of MAP kinase pathways in response to various stimuli that elevate intracellular calcium levels (Lev *et al.*, 1995). Recently, PYK2-associated protein β (PAP β) was cloned from a mouse pituitary cDNA library based on its ability to bind

Fig. 1. Experimental electron density map. Stereo view of the solvent-flattened, MAD-phased electron density map (contoured at 1σ) in the interface between the ARF-GAP domain and the ankyrin repeats. Superimposed on the electron density is the refined atomic model. Atoms in the ARF-GAP domain are colored yellow, atoms in the ankyrin repeats are colored green, and water molecules are represented as red spheres. Prepared with SETOR (Evans, 1993).

Table 1. Data collection, phasing and refinement summary

	Native	$\lambda 1$ (0.9879 Å)	$\lambda 2$ (0.9794 Å)	$\lambda 3$ (0.9790 Å)	$\lambda 4$ (0.9686 Å)
Resolution range (Å)	25.0–2.1	25.0–2.1	25.0–2.1	25.0–2.1	25.0–2.1
R_{sym}^a (%)	4.5 (11.9) ^b	3.8 (11.6)	3.9 (12.6)	4.1 (14.6)	3.8 (13.6)
Reflections (total/unique)	78 917/17 055	123 816/32 622	124 781/32 671	124 716/32 664	124 941/32 537
Completeness (%)	99.1 (97.8) ^b	98.4 (97.1)	98.4 (96.5)	98.4 (96.7)	98.4 (97.8)
Signal ($\langle I/\sigma I \rangle$)	24.6	24.6	23.0	21.5	22.8
Phasing power ^c (centric/acentric)	–	–	1.9/1.7	2.2/1.9	1.6/1.4
R_{cullis}^d (%) (centric/acentric)	–	–	0.48/0.64	0.61/0.61	0.93/0.74
Overall figure of merit (centric/acentric)	–	0.72/0.67			
Atomic model: 2073 non-hydrogen protein atoms, 96 water molecules, 1 Zn^{2+} ion					
Resolution range (Å)	25.0–2.1				
Reflections	16 886				
R_{cryst}^e (%)	21.4 (26.5) ^f				
R.m.s.d.					
Bond lengths (Å)	0.009				
Angles (°)	1.4				
B -factors (Å ²) (bonded atoms)	2.0				

^a $R_{\text{sym}} = 100 \times \sum_{\text{hkl}} \sum_i |I_i(\text{hkl}) - \langle I(\text{hkl}) \rangle| / \sum_{\text{hkl}} \sum_i I_i(\text{hkl})$.

^bThe value in parentheses is for the highest resolution shell: 2.18–2.10 Å.

^cPhasing power = r.m.s. heavy atom structure factor/r.m.s. lack of closure.

^d $R_{\text{cullis}} = \text{r.m.s. lack of closure/r.m.s. } \Delta \text{ structure factor}$.

^e $R_{\text{cryst}} = 100 \times \sum_{\text{hkl}} |F_o(\text{hkl}) - |F_c(\text{hkl})|| / \sum_{\text{hkl}} |F_o(\text{hkl})|$, where F_o and F_c are the observed and calculated structure factors, respectively.

^fThe value in parentheses is the free R_{cryst} determined from 10% of the data.

to PYK2 (Andreev *et al.*, 1999). The PAP β sequence predicts an 88 kDa protein with an N-terminal coiled-coil region, a pleckstrin homology (PH) domain, an ARF-GAP domain, ankyrin repeats, a proline-rich region and a C-terminal SH3 domain. A truncated form of PAP β containing the PH domain, the ARF-GAP domain and the ankyrin repeats showed phosphatidylinositol-4,5-bisphosphate (PIP₂)-dependent GAP activity on ARF1 and ARF5 and less activity on ARF6 (Andreev *et al.*, 1999). A similar protein, ASAP1, has been purified from bovine brain (Brown *et al.*, 1998).

The crystal structure of ARFGAP1 in complex with GDP-bound ARF1 has been reported previously (Goldberg, 1999). ARFGAP1 lacks the C-terminal ankyrin repeats present in PAP β and in many other proteins with ARF-GAP activity. To provide insights into the mechanism by which PAP β and related proteins assist in ARF GTP hydrolysis, we have determined the crystal structure of

the ARF-GAP domain and the ankyrin repeats of PAP β . Examination of the PAP β structure reveals that several conserved, solvent-exposed residues cluster near an invariant arginine residue, suggesting that these residues may be important for interaction with ARFs and for ARF-GAP activity. We present site-directed mutagenesis data that are consistent with this hypothesis. The structural and mutagenesis results reported here support an alternative mode of interaction between ARFs and ARF-GAPs to that proposed by Goldberg (1999).

Results and discussion

Structure determination

Limited proteolysis with Glu-C of a 411 residue fragment of murine PAP β (112–522) expressed in *Escherichia coli* resulted in a protease-resistant core of 272 residues (247–518) comprising the ARF-GAP domain and the ankyrin

Fig. 2. Overall architecture of the PAP β ARF-GAP domain and ankyrin repeats. (A) Ribbon diagram of PAP β -GA with the α -helices of the ARF-GAP domain colored red, the α -helices of the ankyrin repeats colored purple, and β -strands colored yellow. The Zn $^{2+}$ ion is colored white and the four Zn $^{2+}$ -coordinating cysteine residues are colored green. The termini are denoted by N and C. The view in the right panel is 90° away from the view in the left panel as indicated by the arrow. Prepared with MOLSCRIPT (Kraulis, 1991) and RASTER3D (Merrit and Bacon, 1997). (B) Topology diagram of PAP β -GA with α -helices shown as cylinders and β -strands as arrows. The coloring scheme is the same as in (A).

repeats. Based on this result, an *E.coli* expression vector was constructed to encode PAP β residues 247–522. The resulting protein product is referred to as PAP β -GA (for PAP β GAP domain and ankyrin repeats). The crystal structure of PAP β -GA was determined by the multi-wavelength anomalous diffraction (MAD) method (Hendrickson and Ogata, 1997) using selenomethionyl-substituted PAP β -GA. The expressed protein contains six methionine residues, four of which are well ordered in the structure. The structure has been refined at 2.1 Å resolution with a crystallographic *R*-value of 21% (free *R*-value of 26%). The atomic model includes PAP β residues 247–522, one Zn $^{2+}$ ion and 96 water molecules. A portion of the experimental electron density map is shown in Figure 1, and data collection, phasing and refinement statistics are given in Table I.

ARF-GAP domain

The structure of PAP β -GA comprises two domains, an ARF-GAP domain and an ankyrin repeat domain, with significant interaction between the two (Figure 2A). The ARF-GAP domain consists of a short, three-stranded β -sheet, which is flanked on three of six sides by α -helices within this domain and on one side by α -helices from the ankyrin repeat domain. The α -helices in the ARF-GAP domain associate pair-wise in a parallel fashion: A with C, B with E, and D with G from the first ankyrin repeat. The overall dimensions of the ARF-GAP domain are $\sim 42 \times 28 \times 26$ Å. The ARF-GAP domain is organized around a metal-binding module in which the metal is coordinated by the cysteines of the Cys-X $_2$ -Cys-X $_{16-17}$ -Cys-X $_2$ -Cys sequence motif present in all proteins with known ARF-GAP activity (Figure 4). A large peak (9.8 σ) in the solvent-flattened electron density map is found approximately equidistant from the sulfhydryl groups of Cys264, Cys267, Cys284 and Cys287. Based on the type (sulfur) and geometry (tetrahedral) of the coordinating

Fig. 3. Superposition of the Zn $^{2+}$ -binding site in PAP β and PKC (Zhang *et al.*, 1995). The C α trace and carbon atoms are colored orange (PAP β) or green (PKC), sulfur atoms are colored yellow, nitrogen atoms are colored blue, and the Zn $^{2+}$ ion is colored violet (PAP β) or black (PKC). The positions of β -strands 1–3 and α -helix B in PAP β are indicated. Prepared with MOLSCRIPT (Kraulis, 1991) and RASTER3D (Merrit and Bacon, 1997).

ligands, the electron density most probably corresponds to a Zn $^{2+}$ ion. The non-restrained Zn $^{2+}$ -S distances range from 2.3 to 2.4 Å.

The precise role of the Zn $^{2+}$ ion in ARF-GAP activity is not clear, but its importance is underscored by mutations of the Zn $^{2+}$ -coordinating cysteines in ARFGAP1 (Cukierman *et al.*, 1995) and Gcs1 (Ireland *et al.*, 1994),

which abrogate ARF-GAP activity. The coordination of the Zn²⁺ ion by four cysteines would indicate that this ion plays a structural rather than a catalytic role. An invariant arginine (Arg292) and a nearly invariant tryptophan (Trp274), which are important residues for ARF-GAP activity (discussed below), are located in the Zn²⁺-binding module. Therefore, the Zn²⁺ ion probably functions to stabilize the region of the protein in which these critical residues reside. Whether the Zn²⁺-binding module is also involved in protein–protein or protein–lipid interactions is not known.

A search of the Brookhaven structural database using DALI (Holm and Sander, 1995) revealed that the Zn²⁺-binding module of PAP β closely resembles one of the Zn²⁺-binding modules in the C1 domain of protein kinase C (PKC) (Hommel *et al.*, 1994; Zhang *et al.*, 1995), even though the Zn²⁺ ion in PKC is coordinated by three cysteines and one histidine rather than four cysteines (Figure 3). At the cores of the PAP β and PKC Zn²⁺-binding modules are two β -strands with either an adjacent α -helix (PAP β) or a 3_{10} helix (PKC). The structural homology extends further to include the third strand of the β -sheet, although the connectivity is different in the two structures: β – β – α – β for PAP β and β – β – β – 3_{10} for PKC. A superposition of 24 C α atoms (versus 1PTQ; Zhang *et al.*, 1995) yields a root-mean-square deviation (r.m.s.d.) of 1.2 Å. A DALI search using either the entire ARF-GAP domain or just the five α -helices in the domain yielded no matches of statistical significance.

Ankyrin repeats

Four ankyrin repeats, each of which consists of a helix–turn–helix–loop (Gorina and Pavletich, 1996), follow the ARF-GAP domain (Figure 2). The first ankyrin repeat is atypical due to the lack of a β -turn in the loop connecting the first and second repeats. This loop is also longer than the other two connecting loops, enabling residues within this loop (Glu411 and Glu414) to contact residues in the third repeat (Ser450 and Tyr455). The β -turn in the loop connecting repeats two and three is stabilized by a hydrogen bond between the side chain of Thr446 and a main chain amide nitrogen across the turn. Similarly, Asn479 stabilizes the β -turn in the loop connecting repeats three and four. Hydrophobic residues C-terminal to the fourth ankyrin repeat (Tyr517, Trp519 and Leu521) interact with residues in helices A and C of the ARF-GAP domain, positioning the C-terminus of the ankyrin repeat domain near the N-terminus of the ARF-GAP domain.

The ARF-GAP domain and the first two ankyrin repeats form an extensive interface with 1835 Å² of total surface area buried. Both hydrophobic and polar interactions are present in the interface. Ile387, Phe388, Leu391, Tyr394 and Phe434 from helices G and I in the first two ankyrin repeats participate in hydrophobic interactions with Leu315, Leu316, Ile320 and Ile328 from helices C and D in the ARF-GAP domain (Figure 1). Gln437 and Asn438 from helix I in the second ankyrin repeat are hydrogen bonded to Thr277 and Asn278 in the loop between β -strands 2 and 3 in the ARF-GAP domain.

Only a subset of proteins with known or putative ARF-GAP activity contain ankyrin repeats C-terminal to the ARF-GAP domain (Figure 4) and, therefore, ankyrin repeats are evidently not essential for ARF-GAP activity

per se. Yet the extensive interface between the ARF-GAP domain and the first two ankyrin repeats in the PAP β -GA structure implies that the ankyrin repeats are important for the stability of the ARF-GAP domain in those proteins that contain ankyrin repeats. Since ankyrin repeats typically mediate protein–protein interactions, the ankyrin repeats of PAP β may also interact, via residues in the connecting loops, with proteins that are involved in subcellular localization or modulation of catalytic activity.

Insights into catalytic mechanism

Biochemical and structural studies of Ras-GAP and Rho-GAP have led to the ‘arginine finger’ model for stimulation of GTP hydrolysis (Scheffzek *et al.*, 1998a). In this model, the GTPase (e.g. Ras or Rho) lacks a critical arginine for catalysis, which is supplied by the corresponding GAP and inserted into the active site of the GTPase. The ‘finger’ arginine hydrogen-bonds to the γ -phosphate of GTP, stabilizing the transition state.

Although there is no discernible sequence or structural homology between ARF-GAP and Ras-GAP or Rho-GAP, one arginine is invariant in ARF-GAPs: Arg292 in PAP β . Arg292 resides in helix B, five residues C-terminal to the last of the four cysteines that coordinate the Zn²⁺ ion. The Arg292 side chain is largely solvent exposed, indicating that it probably does not play a role in stabilization of the ARF-GAP domain. In contrast, the highly conserved His291 which immediately precedes this arginine clearly does function to stabilize the domain, making hydrogen bonds with conserved Asp266 and Ser299 (all three residues are buried in the domain core). Interestingly, both Arg789 in Ras-GAP and Arg282 in Rho-GAP (Arg305 in Cdc42-GAP)—the ‘finger’ arginines—reside in a loop, whereas Arg292 in PAP β resides in an α -helix. Another positively charged residue is highly conserved in the Ras-GAP (Arg903) and Rho-GAP (Lys319) families. The side chain of this residue makes one or more hydrogen bonds with main chain carbonyl oxygens in the loop containing the catalytic arginine, which serves to stabilize the loop conformation (Barrett *et al.*, 1997; Rittinger *et al.*, 1997; Scheffzek *et al.*, 1997, 1998b; Nassar *et al.*, 1998). In the PAP β -GA structure, no positively charged residue corresponding to Arg903/Lys319 is present.

In the crystal structures of the complexes between Ras and Ras-GAP (Scheffzek *et al.*, 1997) and between Rho and Rho-GAP (Rittinger *et al.*, 1997), several conserved hydrophobic residues are positioned near the catalytic arginine, Leu902 and Leu910 of Ras-GAP and Val394 and Ile413 of Rho-GAP, which contact hydrophobic residues in the switch I and II regions of their respective GTPases. In the PAP β -GA structure, there are a number of hydrophobic residues as well as a conserved aspartic acid that are solvent exposed and are situated near Arg292 (Figure 5A). Asp307 is conserved in nearly all ARF-GAP domain sequences (Figure 4). In the PAP β -GA structure, one of the carboxylate oxygens of the Asp307 side chain is hydrogen bonded to the main chain amide nitrogen of Ser303, while the other carboxylate oxygen faces the solvent. Trp274 is highly conserved in ARF-GAP sequences, and hydrophobic residues predominate at the positions corresponding to solvent-exposed Ile285 and Leu306.

To investigate whether invariant Arg292 and nearby

solvent-exposed residues are important in ARF-GAP activity, either catalytically or in ARF binding, site-directed mutagenesis was performed. The ARF-GAP assay, which was established for PIP₂-stimulated ARF-GAPs (Brown *et al.*, 1998), was conducted with wild-type and mutant PAP β proteins that contain the PH domain in addition to the ARF-GAP domain and ankyrin repeats. PAP β -GA

(which lacks the PH domain) has minimal ARF-GAP activity, which is probably attributable to a requirement for co-localization of ARF1 and PAP β on membranes, mediated by PIP₂. ARF1 has been shown to bind to PIP₂-containing Triton X-100 micelles with a K_d of ~50 μ M (Randazzo, 1997), and PH domains from many proteins are known to bind PIP₂ (Lemmon *et al.*, 1997). Based on

A

	hhhhhhhhA	ss1	ss2	hhhhhhhhB	ss3		
	1#4013203628205400003167-021000000000164003005#136#3030300473						
	250	260	270	280	290	300	
PAPbeta	MELTKEIIESEVQRMGTNDV	CD	CGAPD-PTWLS	TNLGILTCE	CSGIHRELGVHYSRM	QSLTLD--	
ASAP1	EDLTKAIIEDVQRLPGNDIC	CD	CGSSE-PTWLS	TNLGILTCE	CSGIHREMVGVIHSRI	QSLTLD--	
PIP3bp	MAKERKKAIVLELLQRPGNAR	CD	CGAPD-PDWAS	YTLGVFICL	SCSGIHRNIP-HVSKVKS	SVRLD--	
CentA	MAGELRRALLELLTRPGNSRC	AD	CGAPD-PDWAS	YTLGVFICL	SCSGIHRNIP-QVSKVKS	SVRLD--	
GIT1	MSRKGPRAEVC	AD	CSAPD-PGWAS	ISRGVLVCE	CCSVHRESLGRHISIVK	HLRHS--	
GCS1	VDPDTRRRLLQLQKIGANKKC	MD	CGAPN-PQWAT	PKFGAFICL	EAGIHRRLGLGVHISFV	RSITMD--	
GLO3	TTQQVFQKLGNSNMENRV	FC	DCGNKN-PTWT	SVPFGVMLCI	QCSAVHRNMGVHITFV	KSSTLD--	
SPS18	MENRKRLLRAKKAAGNNNC	FE	CKSVN-PQFV	SCSFGIFICV	NCANLLRGMGTNIFC	VKSITMD--	
KIAA0041	EKLLKGESALQRVQCIPGNAS	CD	CGLAD-PRWAS	INLGITLCE	CSGIHRESLGVHFSK	VRSSTLD--	
KIAA0050	REPGGVGHVVAQVQSV	DGNAQC	CD	CREPA-PEWAS	INLGVTLICQ	CSGIHRESLGVHFSK	VRSSTLD--
KIAA0167	TDSQSEAVAIQAIRNAKGN	SI	CV	DCGAPN-PTWAS	LNGLALICE	CSGIHRLNGLTHLSR	VRSSTLD--
KIAA0580	AETLSDEYAEKIWFNESNR	SC	ADCKAPD-PDWAS	INLCVVIC	KKAGQHRSLGPKDSK	VRSSTLD--	
KIAA0782	ALSTSEVAERIWAAPNRF	CA	DCGAPQ-PDWAS	INLCVVIC	KRCAGEHRLGAGVSK	VRSSTLD--	
dm2286211	MASPRTRRVLLQELKPDEN	SKCF	ECGTHN-PQWV	SVTYGIWICL	ECSGKHRSLGVHLSF	VRSSTMD--	
ce3947610	AGFSSSTTAFEQVRVP	GVNEVC	AD	CGSPA-PKWVS	INLGVVLCI	ECSGAHRESLGVQTSK	VRSSTMD--
sp1723238	YHASANIFIQMLRKTDVSN	SVCA	DCGSVDVTWCS	INIPVVLCE	CSGIHRESLGLTHISK	TRSLD--	
sc2131544	ASNRDELLSTVIRKIDKSN	LKCC	DCGSTATVEWVS	INLLCILCE	KCSGVHRESLGSISK	IRSLD--	
at1931654	SRQNIQDDVSTILRLGLP	GNAC	ECNAPE-PDWAS	INLGVLLCE	CSGVHRLNGLVHISK	VRSSTLD--	
ARFGAP1	MASPRTRKVLKEVRAQDEN	NNVC	FC	GAFN-PQWV	SVTYGIWICL	ECSGRHRLGLGVHLSF	VRSSTMD--
	hhhhhhhhA	ss3	ss4	hhhhhhhhB	ss5		
	700001000012000410171015523##8330-----54816473056002000452500						
	310	320	330	340	350	360	
PAPbeta	VLGTSELLAKNIGNAGFNEI	MECLPSEDPVKP-----	NPGSDMIARKDYITAKY	MERRYA			
ASAP1	KLGTSELLAKNVGNNSFNDI	MEANLPSPPSP-KP-----	TPSSDMTVRKEYITAKY	VDHRFS			
PIP3bp	TWEDVQVEFMASRGNAIAR	ATFESRVPPFFYY-RP-----	SASDCPLLREQWIRAKY	ERQEFA...			
CentA	AWDETQVEFMSTSHGNEAAR	ATFESKVPFFFFYY-RP-----	TFSDCQLLREQWIRAKY	ERQEFL...			
GIT1	AWPPTLLQMVHTLASNGANS	IWEHSLDDPAQVQS-GRRKAN	PQDKVHPKSEFIRAKY	QMLAFV...			
GCS1	QFKPEELLRMEKGGNEPLET	EWFFK-----	SHNIDLS	PQKVYDNPVAE...			
GLO3	KWTINNLLRRFKLGGNHKAR	DFFL-----	KNNGKQLLNTANVDAK	TKYTSVPAK...			
SPS18	NFEKDVRRVEKSGNNRFGS	FLS-----	KNGLIQNGIPLREKY	DNLFPAK...			
KIAA0041	TWEPPELLKMLCELGN	DVINRVYEANVEKMG	IKKP-----	QPGQRQEKEYIRAKY	VERKFV...		
KIAA0050	SWEPPELLKMLCELGN	VIIINQIYEAREVAMAV	KKP-----	GPSCSRQEKEAWIHAKY	VEKFL...		
KIAA0167	DWPRELTLVLTAIGNDTAN	RVWESDTRGRA--KP-----	SRDSSREERESWIRAKY	EQLLFL			
KIAA0580	IWSNELIELFIIVIGNKRAN	DFWAGNLQKDEELHM-----	DSPVEKKRNFITQKY	KEGKFR			
KIAA0782	VWTETLIELFLQLGNGAGNR	FWAANVPPSEALQP-----	SSSPSTRRCHLEAKY	REGKYR			
dm2286211	KWKDIELEKMKAGGNRNAR	EFLE-----	DQEDWNERAPITQRY	NSKAAA...			
ce3947610	SIDNELRDVLLALGNRQVNEI	FLAHLPPADSI	VPPQIN----	EKSARPAAREAWIKAKY	VERRFA...		
sp1723238	SLSQSKVLLCKIGNAAVN	RVEYKGLSNPSLKPKP-----	EHNAQVKLAFQKKY	VEHAFI			
sc2131544	NFTSLELMHLLQN-NVSN	SNVNAIYESNLRNFP	VKKI----	TANSDDSERSKFI	IDKYQKKFV		
at1931654	VWETLIDFLRNLGNVYCN	SLWEGLLHLLDDDCED	GSALSHASVSKPC	PEDSFVSKEKY	ILGKYL		
ARFGAP1	KWKDIELEKMKAGGNAR	KFRELE-----	AQDDYEPSWSLQDKY	SSRAAALFRDKVAT	LAEGKEWSLESSPAQ	NWTP..	
	hhhhhhhhH	hhhhhhhhD		hhhhhhhhhhH	gggG		

B

	hhhhhhhhhhH	hhhhhhhhH	hhhhhhhhH	hhhhhhhhH	hhhhhhhhH	hhhhhhhhH	hhhhhhhhH	
	#46275662015100#0046340320010100415014#0529#351550000000033044400000000011940							
	370	380	390	400	410	420	430	440
PAPbeta	RKKHADTAALKHSLCEAVK	TRDIFGLLQAYADGV	DLTEKIPLANGHEP	DETALHLAVRSVDR	TSLHIVDFLVQNSGNL			
ASAP1	RKTCASSAKLNELLEAIKSR	DLALQVYAEGLVMEFL	-LEPGQELGETALHLAV	RTADQTSLSLHVD	FLVQNCNL			
KIAA0050	SEDLGSLHPGALLFRASGH	PSLPTMADALAHGADV	NVWNGQDNA----	TPLIQATAA----	NSLLACEFLQNGANV			
KIAA0167	APLSTSEPLGQLWAAVQAQ	DVATVLLLLAHARHG	PLDTSVEDPQL--	RSPLHLAELAHVIT	QLLLWYGADVAAR			
KIAA0580	LLASLTKEELNKALCAAV	KPDVLETMALLFSGAD	VMCATGDPVHS----	TPYLLAKKA----	GQSLQMEFLYHNKFS	...		
KIAA0782	HPLFGNQEELDKALCAAV	TTDLAETQALLGCGAG	INCFSGDPEAP----	TPALALAEQA----	GQTLQMEFLRNRTTE	...		
sp1723238	DFAGVDADATLLEGLEQN	KISKILLGLAAPHFEEN	-----	GVVFLKAVTRDTS	SKLHLEFLFMNGLL	...		
sc2131544	IDSNQGREASLSLIKAIH	LDVSFMMQRAIAQSKY	SLR(14aa)SSIFQY	SLKHYEIVDG-TP	IFFITEFLLCNGIHE	...		
at1931654	-EKATDIDAYHHIDA	AEKAVKKRHDFTVC	QRIKESNEPRS----	CLQGC	SLHVAACHI--	GDSVLLLELLQFGADL		
	hhhhhhhJ	hhhhhhhhK	hhhhhhhhL	hhhhhhhhhhH				
	24404410000000035310100100023802162316#62202300#53#2#403#00421567#2474040503062							
	450	460	470	480	490	500	510	520
PAPbeta	DKQTGKGSTALHYCYCL	TDNAECLKLLLRKAS	IEIANESGETPLDIA	KRLKHEHCEELT	QALSGRFSNVHVEY	EWRL		
ASAP1	DKQTSVGNVTVLHYCS	MYGKPECLKLLLR	SKPTVDIVNQNGE	TALDIKRLKATQ	CEDLSSQAKSGKFN	PNHVEYEWNL		
KIAA0050	NQADSAGRGPLHATIL	GLTGLACFLKRGAD	LGGARDSEGRDPL	TTAMETANADIV	TLRLAKMREA			
KIAA0167	---DAQGR	TALFYARQAGS	QLCADILLQH					
at1931654	NIRDYHGRTPLHHC	ISSGNHFKAKILL	RRGARPSIEDDG	GLSVLERAMEMGA	ITDEELFLLLAECA			

the structural comparison with ARFGAP1 (Goldberg, 1999; discussed below), it is clear that the absence of the PH domain does not alter the fold of the ARF-GAP domain as seen in the PAP β -GA structure.

The results of the site-directed mutagenesis experiments are presented in Figure 5B. All of the mutant proteins behaved like wild type during expression and purification, indicating that the various mutations did not significantly

Fig. 5. Site-directed mutagenesis studies. (A) Full-sphere representation of the PAP β -GA structure highlighting conserved residues in the ARF-GAP domain that are solvent exposed and near Arg292. Selected residues are colored green (hydrophobic), red (acidic) or blue (basic), and the Zn²⁺-coordinating cysteines are colored yellow. All other atoms are colored light gray (ARF-GAP domain) or dark gray (ankyrin repeats). The viewing direction is similar to that in the right panel of Figure 2A. Prepared with MOLSCRIPT (Kraulis, 1991) and RASTER3D (Merrit and Bacon, 1997). (B) ARF-GAP activity of wild-type PAP β and various PAP β mutants. Top panel: autoradiograph of thin layer chromatographic separation of the ARF1 GTP hydrolysis reaction mediated by wild-type PAP β , the Asp307→Ala mutant and the Arg292→Lys mutant. A single round of GTP hydrolysis on non-myristoylated ARF1 (10 μ M), which was pre-loaded with [α -³²P]GTP, was assayed as described (Randazzo and Kahn, 1994). The values above the autoradiograph indicate the concentrations (in nM) of PAP β (wild-type or mutant) used in the assay. Bottom panel: quantitation of ARF1 GTP hydrolysis stimulated by PAP β and various PAP β mutants. The radioactivity in the GTP and GDP spots was quantified by phosphorimager. The percentage values are averages of two determinations.

Fig. 4. Structural alignment of the ARF-GAP domain and ankyrin repeats. (A) Alignment of ARF-GAP domain sequences. (B) Alignment of ankyrin repeat sequences. (Only a subset of proteins with an ARF-GAP domain contain ankyrin repeats.) Highly conserved residues or conserved hydrophobic positions are colored according to residue type: green for hydrophobic, magenta for polar, red (or magenta) for acidic, and blue (or magenta) for basic. Threonine is colored either green (hydrophobic) or magenta (polar) depending on the context. Invariant residues are boxed. An ellipsis (...) at the end of a line of sequence indicates that the sequence that follows cannot be aligned with PAP β . Secondary structure assignments were taken from PROCHECK (Laskowski *et al.*, 1993). Residues in α -helices are denoted by 'h' (or the helix designation letter), residues in β -strands are denoted by 's' (or the strand designation number) and residues in 3_{10} helices are denoted by 'g' (or the helix designation letter). The secondary structure assignments for ARFGAP1 (Goldberg, 1999), performed with PROCHECK, appear at the bottom of the alignment. The strand numbering is according to Goldberg (1999). Strands 1 and 2 of ARFGAP1 are designated β -turn by PROCHECK rather than β -strand; this β -turn is also present in PAP β (residues 263–270). The numbers under the residue numbering represent the fractional solvent accessibility (FSA) of the residue in the PAP β -GA structure. The FSA is the ratio of the solvent-accessible surface area of a residue in a Gly-X-Gly tripeptide to that in the PAP β -GA structure. A value of 0 represents an FSA between 0.00 and 0.09, 1 represents an FSA between 0.10 and 0.19, etc. The higher the FSA, the more solvent exposed the residue. A hash sign (#) in the FSA line indicates that the side chain for that residue is not included in the atomic model due to disorder. The accession Nos for the sequences are: AAC98349 (ASAP1), AAC52337 (ARFGAP1), BAA20132 (PIP3bp), AAC52683 (CentA), AAC83348 (GIT1), P35197 (GCS1), P38682 (GLO3), P32572 (SPS18), Q15057 (KIAA0041), Q15027 (KIAA0050), AAC39522 (KIAA0167), BAA25506 (KIAA0580), BAA34502 (KIAA0782), AAB64300 (dm2286211), CAA19462 (ce3947610), Q10165 (sp1723238), S69580 (sc2131544) and AAB65489 (at1931654).

Fig. 6. Stereo view of a superposition between the ARF-GAP domains of PAP β and ARFGAP1 (Goldberg, 1999). The coloring for PAP β is the same as in Figure 2A. The ARF-GAP domain of ARFGAP1 is colored green (the Zn²⁺ ion, not shown, superimposes with the PAP β Zn²⁺ ion), ARF1 is colored light blue, and the GDP molecule is colored tan. The viewing direction is similar to that in the left panel of Figure 2A. The position of Arg292 in PAP β is indicated. Prepared with MOLSCRIPT (Kraulis, 1991) and RASTER3D (Merrit and Bacon, 1997).

alter the structure/integrity of the proteins (as predicted from the structure). Mutation of Arg292 to lysine (Arg292→Lys) results in a >10 000-fold increase in the concentration of mutant PAP β needed to obtain a level of activity similar to wild-type PAP β , and mutation to alanine results in an additional 5-fold reduction in activity. This establishes the importance of Arg292 in ARF-GAP activity. Substitution of conserved Trp274 with alanine also severely impaired ARF-GAP activity. Single mutations of Ile285 and Leu306 to alanine caused an ~100-fold reduction in activity, and the double mutation of Ile285/Leu306→Ala was as impaired as the Arg292→Lys mutant. These data suggest that Trp274, Ile285 and Leu306 may interact with ARF. The activity of the Asp307→Ala mutant was ~1000-fold less than that of wild type, which suggests that this conserved residue may also be involved in ARF binding.

Comparison with ARFGAP1 structure

The structures of the ARF-GAP domains of PAP β and ARFGAP1 (Goldberg, 1999) are quite similar from helix A through helix D (r.m.s.d. for 82 C α atoms is 1.2 Å), but diverge substantially thereafter (Figure 6). A large insertion is present between helices D and E in PAP β and in other ARF-GAP proteins that contain ankyrin repeats (Figure 4). The structures briefly reconverge at the conserved Lys–Tyr tandem at the end of helix E (PAP β residues 357–358). The latter portion of the ARF-GAP domain of ARFGAP1, containing helices F and G, occupies a spatial position that is generally similar to the helices in the first two ankyrin repeats of PAP β (Figure 6). Thus, residues either in the C-terminal portion of the ARF-GAP domain (for non-ankyrin-containing ARF-GAPs) or in the first two ankyrin repeats buttress the back of the Zn²⁺-binding module.

The most surprising feature in the superposition of the two structures is the position of GDP-bound ARF1. There is considerable overlap between ARF1 and the third and

fourth ankyrin repeats of PAP β (Figure 6). This implies that either the ankyrin repeats are dislodged from the ARF-GAP domain before or upon interaction with ARF1, or the mode of binding of ARF1 to the PAP β ARF-GAP domain (and presumably other ARF-GAP domains) is significantly different from that observed in the structure of the ARF1–ARFGAP1 complex. The extensive interface between the PAP β ARF-GAP domain and ankyrin repeats would argue against the first possibility, raising the issue of the biological relevance of the ARF1–ARFGAP1 interaction observed in the crystal structure (Goldberg, 1999).

That the ARF1–ARFGAP1 complex includes GDP-bound ARF1 is significant, since GDP is the product of the reaction rather than the substrate. Also, there are several major structural differences between the GDP- and GTP-bound forms of ARF1 (Goldberg, 1998). Previous crystal structures revealing the catalytic role of the invariant arginine in Ras/Rho-GAP have been determined in complex with Ras/Rho bound to GDP and aluminum fluoride, which mimics the transition state of the reaction (Rittinger *et al.*, 1997; Scheffzek *et al.*, 1997).

In the ARF1–ARFGAP1 structure, the GDP molecule is ~28 Å away from the invariant arginine. Based on this finding, and the observation that coatamer protein enhances GTP hydrolysis *in vitro*, Goldberg suggests that the invariant arginine is not involved in catalysis but rather serves a structural role, and that coatamer protein may provide the ‘finger’ arginine (Goldberg, 1999). Rather than supplying a missing catalytic residue, coatamer protein may simply increase the local concentration of ARF1 and ARFGAP1; in the absence of coatamer protein, GTP hydrolysis is still clearly detected (Goldberg, 1999).

Our structural and mutagenesis data indicate that the invariant arginine plays more than a structural role. In the PAP β -GA crystal structure, the arginine makes only minor contacts with other residues, which is also true in the ARF1–ARFGAP1 complex. Moreover, a conservative mutation of this arginine to lysine drastically reduced

ARF-GAP activity. In addition, mutation of solvent-exposed hydrophobic residues that are in proximity to the arginine also impairs ARF-GAP activity, suggesting that these hydrophobic residues are involved in ARF binding. Thus, although our data do not establish the precise role of the invariant arginine in ARF-GAP catalytic activity, they do indicate that this arginine and surrounding residues play a central role.

Materials and methods

Expression, purification and crystallization

A region of PAP β corresponding to the PH domain, the ARF-GAP domain and the ankyrin repeats (residues 112–522) was subcloned into the pET-22 vector (Novagen) which contains a His₆ fusion at the C-terminus. The protein was expressed in *E. coli* and purified by Ni affinity (Pharmacia) chromatography followed by gel filtration chromatography and Mono Q (Pharmacia) anion-exchange chromatography. Limited proteolysis with Glu-C (Boehringer) resulted in a protease-resistant fragment comprising the ARF-GAP domain and ankyrin repeats (residues 247–518). PAP β cDNA corresponding to residues 247–522 was subcloned into pET-22, and the protein was purified as above.

Crystals were obtained initially by the hanging drop vapor diffusion method at 20°C by mixing an equal volume of protein solution (10 mg/ml) and reservoir solution (~30% PEG 4000, 0.2 M sodium acetate, 0.1 M Tris-HCl pH 9.5). Single crystals of sufficient size for X-ray data collection were grown at 4°C by streak seeding into pre-equilibrated drops that contained protein, reservoir solution and 15% ethylene glycol. Crystals grew to an average size of $0.6 \times 0.3 \times 0.1$ mm over 1–2 weeks. The crystals belong to orthorhombic space group C222₁ and have unit cell dimensions of $a = 73.94$ Å, $b = 132.22$ Å, $c = 59.82$ Å when frozen. There is one molecule in the asymmetric unit and the solvent content is 48% assuming a partial specific volume of 0.74 cm³/g.

Preparation of selenomethionine-labeled protein

Selenomethionyl-substituted protein was prepared by expressing the protein in a methionine auxotrophic strain (DL41DE3). Cells were grown for 6–8 h in 20 ml of LB medium and the bacteria were recovered by centrifugation. The pellet was gently suspended in M9 medium supplemented with all the amino acids except methionine, for which L-selenomethionine was substituted (Hendrickson *et al.*, 1990). Bacteria were grown and protein expressed as described (Hendrickson *et al.*, 1990). Selenomethionyl-substituted protein was purified using the same protocol as for native protein. Incorporation of six Se was verified by mass spectrometry. Selenomethionyl-containing crystals were grown under the same conditions as used to grow native crystals, using native crystals as seeds.

Data collection, structure determination and analysis

Native and selenomethionyl data sets were collected at beamline X4A at the National Synchrotron Light Source, Brookhaven National Laboratory, using a Rigaku R-Axis IV image plate detector. Crystals were flash-cooled directly in liquid nitrogen and then transferred to the goniostat, which was bathed in a dry nitrogen stream at –170°C. Selenomethionyl data were taken at four X-ray wavelengths about the K absorption edge of Se (Table I). Typically, at a given X-ray wavelength, 10° of data were collected in 2° oscillations (90–120 s per frame) at the + ϕ goniostat setting, followed by 10° at the – ϕ goniostat setting (inverse beam mode). The X-ray wavelength was then changed. A total of 224° of data were collected at each wavelength. For the native data set, 120° of data were collected in 2° oscillations (90–120 s per frame). All data were processed using DENZO and SCALEPACK (Otwinowski and Minor, 1997). Bijvoet pairs were processed separately, and all of the measurements for a given reflection were merged after rejection criteria had been applied.

Four Se sites were found by inspection of Bijvoet difference Patterson maps and heavy atom search and refinement procedures as implemented in CNS (Brünger *et al.*, 1998). Structure factor amplitudes for the four wavelengths were scaled together and put on a quasi-absolute scale using CNS. MAD phasing was performed with CNS using maximum likelihood refinement of anomalous scattering parameters, with the data at λ_1 used as the reference set. The overall figure of merit was 0.67 (25.0–2.1 Å). Electron density maps computed for the two possible enantiomorphs revealed the correct one unambiguously. After solvent flattening and histogram matching were performed with DM (Collaborat-

ive Computational Project Number 4, 1994), the overall figure of merit increased to 0.80. The resulting electron density map was of high quality (Figure 1), and model building in O (Jones *et al.*, 1991) proceeded straightforwardly. Torsion angle simulated annealing and positional/*B*-factor refinement against the native 2.1 Å data set were performed using CNS. A bulk solvent correction was applied. The atomic model includes all residues except the first two N-terminal residues and the last eight C-terminal residues, which includes the His₆ tag. The average *B*-factor is 31.8 Å² for all atoms, 31.7 Å² for protein atoms, 35.1 Å² for water molecules and 26.7 Å² for the Zn²⁺ ion.

Site-directed mutagenesis and ARF-GAP assay

Mutations were performed using the Quick Change kit (Stratagene) and confirmed by DNA sequencing. Mutant proteins were purified as described above. Non-myristoylated ARF1 was prepared as described (Randazzo *et al.*, 1992). ARF-GAP activity was determined using an *in vitro* assay that measures a single round of GTP hydrolysis on recombinant ARF (Randazzo and Kahn, 1994). Crude phosphoinositides [containing PtdIns(4,5)P₂] were obtained from Sigma. Phospholipids were solubilized in 0.1% Triton X-100 and added to the assay as mixed micelles. Thin layer chromatography was carried out on PEI-cellulose (J.T.Baker) plates.

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