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

Valsan Mandiyan, Julian Andreev, Joseph Schlessinger1 and Stevan R. Hubbard1

Department of Pharmacology and Skirball Institute of Biomolecular Medicine, New York University Medical School, New York, NY 10016, USA

1Corresponding authors
E-mail: hubbard@tallis.med.nyu.edu

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. PAPβ, 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-X16–17-Cys-X2-Cys motif. The crystal structure of the PAPβ 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/PAPβ/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 coatomer 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). 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 (PAPβ) was cloned from a mouse pituitary cDNA library based on its ability to bind...
Structure of PAP\(\beta\) ARF-GAP domain and ankyrin repeats

Fig. 1. Experimental electron density map. Stereo view of the solvent-flattened, MAD-phased electron density map (contoured at 1\(\sigma\)) 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 I. Data collection, phasing and refinement summary

<table>
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<tr>
<th></th>
<th>Native</th>
<th>(\lambda_1) (0.9879 Å)</th>
<th>(\lambda_2) (0.9794 Å)</th>
<th>(\lambda_3) (0.9790 Å)</th>
<th>(\lambda_4) (0.9686 Å)</th>
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<tbody>
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<td>25.0–2.1</td>
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<td>(R_{crys}) (%)</td>
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<td>3.8 (11.6)</td>
<td>3.9 (12.6)</td>
<td>4.1 (14.6)</td>
<td>3.8 (13.6)</td>
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<tr>
<td>Reflections (total/unique)</td>
<td>78 917/17 055</td>
<td>123 816/32 622</td>
<td>124 781/32 671</td>
<td>124 716/32 664</td>
<td>124 941/32 537</td>
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<tr>
<td>Completeness (%)</td>
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<td>98.4 (97.1)</td>
<td>98.4 (96.5)</td>
<td>98.4 (96.7)</td>
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<tr>
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<td>24.6</td>
<td>23.0</td>
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<td>22.8</td>
</tr>
<tr>
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<td>–</td>
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<td>2.2/1.9</td>
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Atomic model: 2073 non-hydrogen protein atoms, 96 water molecules, 1 Zn\(^{2+}\) ion

<table>
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</tr>
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<td>Angles (°)</td>
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<tr>
<td>B-factors (Å(^2)) (bonded atoms)</td>
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\(^a\)\(R_{crys} = 100 \times \Sigma \left| F_{o}(hkl) \right| - \left| F_{c}(hkl) \right| / \Sigma \left| F_{c}(hkl) \right|,\) where \(F_{o}\) and \(F_{c}\) are the observed and calculated structure factors, respectively.

\(^b\)The value in parentheses is for the highest resolution shell: 2.18–2.10 Å.

\(^c\)Phasing power = r.m.s. heavy atom structure factor/r.m.s. lack of closure.

\(^d\)\(R_{crys} = \sqrt{\sum_{hkl} \left( F_{o}(hkl) - F_{c}(hkl) \right)^2 / \sum_{hkl} F_{o}^2(hkl)},\) where \(F_{o}\) and \(F_{c}\) are the observed and calculated structure factors, respectively.

\(^e\)The value in parentheses is the free \(R_{crys}\) determined from 10% of the data.

to PYK2 (Andreev \textit{et al.}, 1999). The PAP\(\beta\) 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\(\beta\) containing the PH domain, the ARF-GAP domain and the ankyrin repeats showed phosphatidylinositol-4,5-bisphosphate (PIP\(_2\))-dependent GAP activity on ARF1 and ARF5 and less activity on ARF6 (Andreev \textit{et al.}, 1999). A similar protein, ASAP1, has been purified from bovine brain (Brown \textit{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\(\beta\) and in many other proteins with ARF-GAP activity. To provide insights into the mechanism by which PAP\(\beta\) 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\(\beta\). Examination of the PAP\(\beta\) 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\(\beta\) (112–522) expressed in \textit{Escherichia coli} resulted in a protease-resistant core of 272 residues (247–518) comprising the ARF-GAP domain and the ankyrin
repeats. Based on this result, an *E.coli* expression vector was constructed to encode PAPβ residues 247–522. The resulting protein product is referred to as PAPβ-GA (for PAPβ GAP domain and ankyrin repeats). The crystal structure of PAPβ-GA was determined by the multi-wavelength anomalous diffraction (MAD) method (Hendrickson and Ogata, 1997) using selenomethionyl-substituted PAPβ-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 PAPβ residues 247–522, one Zn2+ 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 PAPβ-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 ~42 × 28 × 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-X2-Cys-X16-17-Cys-X2-Cys sequence motif present in all proteins with known ARF-GAP activity (Figure 4). A large peak (9.8σ) 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 ligands, the electron density most probably corresponds to a Zn2+ ion. The non-restrained Zn2+-S distances range from 2.3 to 2.4 Å.

The precise role of the Zn2+ ion in ARF-GAP activity is not clear, but its importance is underscored by mutations of the Zn2+-coordinating cysteines in ARFGAP1 (Cukierman *et al*., 1995) and Gcs1 (Ireland *et al*., 1994),
which abrogate ARF-GAP activity. The coordination of the Zn$^{2+}$ 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 Zn$^{2+}$-binding module. Therefore, the Zn$^{2+}$ ion probably functions to stabilize the region of the protein in which these critical residues reside. Whether the Zn$^{2+}$-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 Zn$^{2+}$-binding module of PAP$\beta$ closely resembles one of the Zn$^{2+}$-binding modules in the Cl domain of protein kinase C (PKC) (Hommel et al., 1994; Zhang et al., 1995), even though the Zn$^{2+}$ ion in PKC is coordinated by three cysteines and one histidine rather than four cysteines (Figure 3). At the cores of the PAP$\beta$ and PKC Zn$^{2+}$-binding modules are two $\beta$-strands with either an adjacent $\alpha$-helix (PAP$\beta$) or a $3_10$ helix (PKC). The structural homology extends further to include the third strand of the $\beta$-sheet, although the connectivity is different in the two structures: $\beta$–$\beta$–$\alpha$–$\beta$ for PAP$\beta$ and $\beta$–$\beta$–$3_10$ for PKC. A superposition of 24 C$\alpha$ 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 $\alpha$-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 $\beta$-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 $\beta$-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 $\beta$-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 Å$^2$ 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). Glu437 and Asn438 from helix I in the second ankyrin repeat are hydrogen bonded to Thr277 and Asn278 in the loop between $\beta$-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$\beta$-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$\beta$ 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 (Schefzek 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 $\gamma$-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$\beta$. Arg292 resides in helix B, five residues C-terminal to the last of the four cysteines that coordinate the Zn$^{2+}$ 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$\beta$ resides in an $\alpha$-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; Schefzek et al., 1997, 1998b; Nassar et al., 1998). In the PAP$\beta$-GA structure, no positively charged residue corresponding to Arg903/Lys319 is present.

In the crystal structures of the complexes between Ras and Ras-GAP (Schefzek 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$\beta$-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$\beta$-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

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6893
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 PAPβ proteins that contain the PH domain in addition to the ARF-GAP domain and ankyrin repeats. PAPβ-GA (which lacks the PH domain) has minimal ARF-GAP activity, which is probably attributable to a requirement for co-localization of ARF1 and PAPβ on membranes, mediated by PIP2. ARF1 has been shown to bind to PIP2-containing Triton X-100 micelles with a Kd of ~50 µM (Randazzo, 1997), and PH domains from many proteins are known to bind PIP2 (Lemmon et al., 1997). Based on
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 PAPβ-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 PAPβ-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 PAPβ and various PAPβ mutants. Top panel: autoradiograph of thin layer chromatographic separation of the ARF1 GTP hydrolysis reaction mediated by wild-type PAPβ, the Asp307→Ala mutant and the Arg292→Lys mutant. A single round of GTP hydrolysis on non-myristoylated ARF1 (10 µM), 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 PAPβ (wild-type or mutant) used in the assay. Bottom panel: quantitation of ARF1 GTP hydrolysis stimulated by PAPβ and various PAPβ mutants. The radioactivity in the GTP and GDP spots was quantified by phosphoimager. 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 PAPβ. 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 PAPβ (residues 263–270). The numbers under the residue numbering represent the fractional solvent accessibility (FSA) of the residue in the PAPβ-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 PAPβ-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), AAC53348 (GTT1), P55197 (GCS1), P38682 (GLO3), P32572 (SPS18), Q15057 (KIAA0041), Q15027 (KIAA0050), AAC39522 (KIAA0167), BAA25506 (KIAA0580), BAA34502 (KIAA0782), AAB64300 (dm2286211), CAA44022 (cP32860861), Q10165 (sp1723238), S69580 (sc2131544) and AAB65489 (at1931654).
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 PAPβ needed to obtain a level of activity similar to wild-type PAPβ, 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 impairs 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 PAPβ and ARFGAP1 (Goldberg, 1999) are quite similar from helix A through helix D (r.m.s.d. for 82 Cα atoms is 1.2 Å), but diverge substantially thereafter (Figure 6). A large insertion is present between helices D and E in PAPβ 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 (PAPβ 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 PAPβ (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 PAPβ (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 PAPβ 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 PAPβ 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 coatomer 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 coatomer protein may provide the ‘finger’ arginine (Goldberg, 1999). Rather than supplying a missing catalytic residue, coatomer protein may simply increase the local concentration of ARF1 and ARFGAP1; in the absence of coatomer 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 PAPβ-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 PAPβ 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). PAPβ 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 × 0.3 × 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 goniotat, 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 1). Typically, at a given X-ray wavelength, 10° of data were collected in 2° oscillations (90–120 s per frame) at the +φ goniotat setting, followed by 10° at the −φ goniotat 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 (Otwinski 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 1.0 Å 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 (Collaborative 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 purified 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 phosphoanisidases [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 PEl-cellulose (J.T.Baker) plates.

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References


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