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Plasmodium falciparum Rab6 GTPase: expression, purification, crystallization and preliminary crystallographic studies

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The *Plasmodium falciparum rab6* gene encodes a 208 amino-acid polypeptide. Two recombinant versions of *P. falciparum* Rab6 protein were expressed in *Escherichia coli*: the full-length protein and a truncated form containing residues 1–175. Both forms were purified from the soluble fraction of bacterial extract and were purified by ion-exchange chromatography and size-exclusion chromatography. Purified proteins were crystallized at pH 6.5 using the hanging-drop vapor-diffusion technique at room temperature. The full-length protein diffracted to 2.4 Å and belongs to the tetragonal space group $P4_32_12$ or $P4_12_12$, with unit-cell parameters $a = b = 80.6$, $c = 90.4$ Å. The crystals of the truncated protein were isomorphous with those of the full-length construct and diffracted X-rays to 2.2 Å resolution.

1. Introduction

The G-protein family is composed of a large number of molecules that control a wide range of biological processes but have a structurally homologous GTP-binding domain in common. The biological functions of G-proteins include cell proliferation, signal transduction, protein synthesis, protein targeting, membrane trafficking and secretion and cell cytoskeletal organization and movement. These proteins act as molecular switches that cycle between a GTP-bound active form and a GDP-bound inactive form (reviewed by Nuoffer & Balch, 1994). A common structural core for nucleotide binding is present in all GTP-binding protein structures. This core includes common consensus-sequence elements involved in nucleotide binding (Kjeldgaard *et al.*, 1996). Nevertheless, subtle changes in the common sequences reflect functional differences. Therefore, it becomes increasingly important to focus on how these differences are reflected in the structures and how these structural differences are related to function.

P. falciparum is an obligate intracellular protozoan parasite that causes malaria in humans. The malaria parasite spends much of its life cycle inside erythrocytes. Within the erythrocyte, the parasite is surrounded by its own plasma membrane, a parasitophorous vacuole membrane and the cytoplasm and plasma membrane of the erythrocyte. Mechanisms by which proteins are trafficked within and beyond the plasma membrane are not clear. Several components of the standard eukaryotic trafficking machinery are known to be present in this parasite (Langsley & Chakrabarti, 1996; de Castro *et al.*, 1996). On the other hand, the trafficking machinery of *Plasmodium* also possesses distinctive features (Foley & Tilley, 1998; Ward *et al.*, 1997).

Rab GTPases represent a family of proteins that are localized on the surfaces of distinct membrane-bound organelles. The cytoplasmic surface of each compartment along the secretory pathway appears to have its own unique Rab protein (Schimmöller *et al.*, 1998; Pfeffer, 1994). The Rabs alternate between a GTP-bound form associated with membrane and a GDP-bound cytosolic form. Cycling of Rab proteins is regulated, at least in part, by a GDP dissociation inhibitor (GDI) and a GDP/GTP exchange protein (GEP). Current models suggest that Rab proteins catalyze membrane fusion in their GTP-bound conformations (Schimmöller *et al.*, 1998). They are then acted upon by Rab-specific GTPase activating proteins (GAPs), converting them to GDP-bound forms. GDI retrieves Rab proteins from their fusion targets and recycles them back to their membrane of origin (Schimmöller *et al.*, 1998).

Rab6 is a ubiquitous protein involved in intra-Golgi transport in eukaryotic cells (Martinez & Goud, 1998). De Castro *et al.* (1996) described the homologue of mammalian Rab6 in *P. falciparum* (*Pf*Rab6). The full-length *Pf*Rab6 consists of 208 amino-acid residues, with the C-terminal three residues being CLC, which is the site for geranylgeranylation. The C-terminal region of monomeric GTPases is known to have a flexible structure (de Vos *et al.*, 1988). Therefore, we also expressed a C-terminally truncated version of the protein containing residues

Table 1
Data-collection statistics.

	Full length	Truncated
Resolution (Å)	50–2.4	50–2.2
Total number of reflections	414504	483835
Number of unique reflections	12197	15345
Highest resolution shell (Å)	2.49–2.40	2.28–2.20
Overall completeness (%)	99.7	97.8
Completeness (highest resolution shell) (%)	99.9	97.2
R_{merge} (%)	7.7	4.4
R_{merge} (highest resolution shell) (%)	45.0	20.5
Overall $I/\sigma(I)$	11.2	19.2
Reflections with $I/\sigma(I) > 3$ in the highest resolution shell (%)	55.4	78.4
Unit-cell parameters (Å)		
$a = b$	80.86	80.17
c	90.48	90.19

1–175. This represents the catalytic domain of *Pf*Rab6. Here, we describe the bacterial expression, purification and crystallization and preliminary crystallographic analysis of the full-length *Pf*Rab6 and its catalytic domain (*Pf*Rab6t).

2. Materials and methods

2.1. Expression of *Pf*Rab6 and *Pf*Rab6t

Coding sequences for residues 1–208 and 1–175 were separately amplified using PCR. The amplified DNAs were engineered into the *Nde*I and *Hin*dIII sites of the *E. coli* expression vector pET21 (Novagen, Inc.). The resulting plasmids were analyzed by automated DNA sequencing.

The recombinant proteins were expressed in *E. coli* strain BL21(DE3)plysS cells. For protein expression, bacterial cells harboring the recombinant plasmid were grown at 310 K in LB medium containing ampicillin (50 µg ml^{-1}), chloramphenicol (34 µg ml^{-1}) and 0.2% glucose. When the absorbance of the culture reached 0.6 (at 600 nm), isopropyl-thio-β-D-galacoside (IPTG) was addded to the culture to a final concentration of 1 m*M*. The cultures were grown for 4 h post-induction.

2.2. Purification of *Pf*Rab6 and *Pf*Rab6t

Full-length and truncated versions of recombinant *Pf*Rab6 were purified in a similar manner. Cell pellets were extracted in buffer *A* [25 m*M* Tris acetate, 10 m*M* $MgCl_2$, 0.1 m*M* phenylmethylsulfonyl fluoride (PMSF) pH 8.2]. The extract was treated with 1 µg ml^{-1} DNAase I for 30 min at 277 K and then subjected to centrifugation at 30 000 rev min^{-1} for 30 min. The soluble supernatant was mixed with 20 µ*M* guanosine diphosphate (GDP) and applied to a Q-Sepharose FF column (Pharmacia) equilibrated with buffer *A*. Following the application of the sample, the column was washed with buffer *A*. Recombinant protein was recovered in the column flow-through and wash, which were pooled together. To the resulting pool, solid ammonium sulfate was added with constant stirring first to attain 40% saturation and then 70% saturation. At each step, the suspension was allowed to stir for an additional 30 min following which the precipitate was separated by centrifugation. The pellet obtained between 40 and 70% saturation containing recombinant Rab6 protein was suspended in buffer *B* (20 m*M* sodium borate, 10 m*M* $MgCl_2$ pH 9.25) and dialyzed overnight against the same buffer. The dialyzate was applied to a Mono Q 10/10 (Pharmacia) column equilibrated with buffer *B*. After the column was washed thoroughly with the same buffer, the bound protein was eluted from the column by applying a linear gradient of NaCl (0–0.4 *M*) in ten column volumes of buffer *B*. Nearly homogeneous protein was eluted at about 100 m*M* NaCl. Selected fractions were pooled and concentrated by ultrafiltration and further purified by size-exclusion chromatography on a Superdex 75 (Pharmacia) column.

2.3. Crystallization

Crystallization conditions for the full-length protein and the truncated form were similar. The protein (10 mg ml^{-1}) was crystallized at room temperature using the hanging-drop vapor-diffusion technique. Crystal Screen reagents (Hampton Research, CA, USA) were used for screening crystallization conditions. Several reagents (Nos. 16, 17 and 30 of Crystal Screen I) containing either lithium sulfate, a combination of lithium sulfate and polyethylene glycol or a mixture of polyethylene glycol and ammonium sulfate yielded crystals in the preliminary screen.

For crystallization, 4 µl of the full-length protein was mixed with 4 µl of a reservoir solution containing 24% polyethylene glycol 8000, 0.25 *M* ammonium sulfate, 0.1 *M* sodium cacodylate pH 6.5 and the mixture was equilibrated against 1 ml of reservoir solution at 295 K. The crystals of the truncated version were grown by mixing 4 µl of a protein solution with an equal volume of a reservoir solution containing 0.8–1.5 *M* sodium sulfate, 0.1 *M* sodium cacolylate pH 6.5 and equilibrating the mixture against 1 ml of reservoir solution at 295 K.

2.4. Data collection and processing

X-ray diffraction data for crystals of the full-length and truncated form were collected at 103 K using an R-AXIS IV image plate. Data-collection statistics are shown in Table 1. The crystals were dipped into a cryoprotectant solution containing 1.0 *M* sodium sulfate and 25% glycerol before being placed in the Cryostream. Intensity data were processed and scaled using the programs *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997).

3. Results and discussion

Rab6 is a ubiquitous Rab protein found in plants, yeast and mammals. In mammalian cells, Rab6 is thought to be involved in retrograde vesicle transport between trans-Golgi and medial Golgi cisternae (Martinez & Goud, 1998). In *P. falciparum* the Golgi is apparently less developed, lacking distinct stacks of cisternae. Nevertheless, presence of Golgi markers such as *Pf*Rab6 and *Pf*ERD2 is consistent with the existence of transport mechanisms similar to those in higher eukaryotic cells (Li *et al.*, 1996; Wye *et al.*, 1996).

The three-dimensional structure of several small monomeric GTPases are known (de Vos *et al.*, 1988; Cherfils *et al.*, 1997; Hirshberg *et al.*, 1997; Kjeldgaard *et al.*, 1996). Of these, human oncogene Ras-p21 GTPase has been extensively studied and it is apparent that the C-terminal regions of these proteins are highly flexible. Importantly, truncation of the C-terminal 23 residues of Ras-p21 did not affect the GTP binding or GTPase activity (John *et al.*, 1989). Crystal structure analysis of the Ras-p21 truncated 1–166 catalytic domain defined the nucleotide-binding residues (de Vos *et al.*, 1988; Pai *et al.*, 1989). Based on sequence homology between Rab6 and p21 ras proteins, two different constructs of *Pf*Rab6 were expressed in *E. coli*. The full-length protein was composed of 208 amino acids, while the C-terminally truncated version contained 175 amino acids. Both forms were purified from the soluble fraction of the *E. coli* extract. Since Rab proteins are known to bind GDP tightly, we added 20 µ*M* GDP to the protein early in the purification. In addition, 10 m*M* $MgCl_2$ was present throughout the purification. The protein fractions which eluted from the MonoQ column at approximately 100 m*M* NaCl, appeared to be nearly homogeneous as judged by SDS–polyacrylamide gel electrophoresis on 14% gel. On size-exclusion chromatography, the recombinant proteins

were found to exist as monomers. Fig. 1 shows an SDS–polyacrylamide gel electrophoretic pattern of purified *Pf*Rab6 and truncated *Pf*Rab6t proteins.

Crystals of both forms of the protein grew within 2–3 d at room temperature. Diffraction-quality crystals of the full-length protein were obtained from a mixture of polyethylene glycol 8000 and ammonium sulfate. These crystals grew within oily zones in the drop. Crystals of *Pf*Rab6t suitable for X-ray diffraction were grown from sodium sulfate. These crystals grew to maximum dimensions of 0.4 × 0.4 × 0.2 mm and were sometimes unstable. Crystals of the full-length *Pf*Rab6 were relatively smaller in size, with maximum dimensions of 0.2 × 0.2 × 0.2 mm.

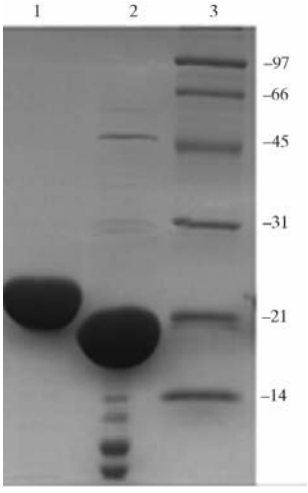

Figure 1
SDS–PAGE showing purified protein samples. 50 µg of protein was boiled with SDS sample-denaturing buffer and subjected to electrophoresis on 14% polyacrylamide gel containing 1% SDS. The gel was fixed in a solution containing 50% ethanol and 15% acetic acid and stained using Coomassie Brilliant Blue (G250). Lane 1, Rab6 full length (1–208); lane 2, Rab6t construct (1–175); lane 3, molecular-weight markers with molecular masses shown.

For data collection, crystals were flash-frozen in cryoprotectant solutions composed of the respective reservoir solutions plus 25%(*v*/*v*) glycerol. Details of the data-collection statistics are provided in Table 1. Both crystals belong to the tetragonal space group $P4_32_12$ or its enantiomer. The diffraction data were 99.7 and 97.8% complete for the full-length and the truncated forms, respectively. Considering one molecule of *Pf*Rab6 (M_r = 23 597) and *Pf*Rab6t (M_r = 20 033) in the asymmetric unit, the solvent contents of these crystals were calculated to be 59.3 and 67%, respectively (Matthews, 1968). The calculated Matthews coefficients, V_m, for these crystal forms were 3.02 and 3.64 Å^3 Da^{-1}, respectively.

Recently, the crystal structures of mammalian Rab3A and a Rab3A–Rabphillin 3A complex have been determined (Ostermeier & Brunger, 1999; Dumas *et al.*, 1999). In both cases, Rab3A exists in complex with guanosine triphosphate (GTP). The structure determination of *Pf*Rab6 in its GDP-bound form will therefore provide a model for a molecular switch in Rab proteins. Moreover, the three-dimensional structure of *Pf*Rab6 will be helpful in understanding the molecular interactions with *Pf*Rab6 effector partners, since these interactions underpin *Pf*Rab6 function inside the parasite.

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