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Eukaryotic Rab5s are highly conserved small GTPase-family proteins that are involved in the regulation of early endocytosis. Leishmania donovani Rab5a regulates the sorting of early endosomes that are involved in the uptake of essential nutrients through fluid-phase endocytosis. Here, the 1.80 Å resolution crystal structure of the N-terminal GTPase domain of L. donovani Rab5a in complex with GDP is presented. The crystal structure determination was enabled by the design of specific single-site mutations and two deletions that were made to stabilize the protein for previous NMR studies. The structure of LdRab5a shows the canonical GTPase fold, with a six-stranded central mixed β/C12-sheet surrounded by five α11-helices. The positions of the Switch I and Switch II loops confirm an open conformation, as expected in the absence of the γ-phosphate. However, in comparison to other GTP-bound and GDP-bound homologous proteins, the Switch I region traces a unique disposition in LdRab5a. One magnesium ion is bound to the protein at the GTP-binding site. Molecular-dynamics simulations indicate that the GDP-bound structure exhibits higher stability than the apo structure. The GDP-bound LdRab5a structure presented here will aid in efforts to unravel its interactions with its regulators, including the guanine nucleotide-exchange factor, and will lay the foundation for a structure-based search for specific inhibitors.

1. Introduction

Leishmania donovani, a causative agent of the disease visceral leishmaniasis, lives a digenetic life between phlebotomine sand flies and human beings. While the parasites adopt a procyclic promastigote form in the insects, in human hosts they reside in the highly acidic environment of macrophages within the parasitophorous vacuole as the ovoid amastigote form (Real & Mortara, 2012; Young & Kima, 2019). In both of the forms, essential nutrients and other molecules are accessed through various processes including endocytosis. The transport of endocytic cargoes is carried out through a series of coordinated and specific vesicle-fusion events (Doherty & McMahon, 2009). This specific targeting and transport of internalized cargoes is highly regulated by small GTP-binding members of the Rab family (Markgraf et al., 2007). Leishmania has a robust endocytosis system for nutrient uptake and defense from the humoral arm of the host immune system. One essential key pathway that is absent in Leishmania is the biosynthesis of heme, as it lacks most of the required enzymes (Korény et al., 2010; Orrego et al., 2019). An essential process for the acquisition of heme involves the transport of the...
hemoglobin receptor to the flagellar pocket region, the rapid
decay of hemoglobin and its transport to the lysosomes
for degradation. These steps are regulated by various Rab
proteins (Sengupta et al., 1999; Rastogi et al., 2016).

Rab proteins are members of the largest subfamily of small
GTPases. They are highly conserved and share about 30–75% 
370
homology from yeast to mammals (Zahraoui et al., 1989).
More than 70 Rabs have been identified in humans to date. All 
383
Rabs have conserved N- and C-terminal regions which have
distinct functions. The N-terminal domain has a canonical
GTPase fold, while the long C-terminal unstructured region,
which terminates preferentially in a CC or CXC sequence, is
the site of geranylgeranylation and consequently of anchorage
to the membrane (Leung et al., 2006). The GTPase fold
is made up of a central six-stranded β-sheet, which is surrounded
by five α-helices. Rab-family members contain five amino-acid
segments that contain highly conserved functional residues.
These are labeled G1–G5 and connect some of the α-helices
and β-strands. Of these, the G1, G2 and G3 loops, which are
commonly known as the P-loop, Switch I and Switch II,
respectively, are important for binding to the phosphate
moiety of the GTP and for the GTPase activity of the protein
(Stenmark & Olkkonen, 2001).

Rab proteins interchange between a cytosolic inactive form
and a membrane-bound active form, depending on their
nucleotide-binding status (Vetter & Wittinghofer, 2001). All
newly synthesized Rabs are recognized by Rab escort protein
(REP) and are presented to Rab geranylgeranyltransferase
(Rab GGTase), which geranylgeranylates the Rab at one or
two C-terminal cysteine residues (Anant et al., 1998; Goody et
al., 2017). REP exhibits a higher affinity for GDP-bound Rab
in comparison to GTP-bound Rab, but a lower affinity for
prenylated Rab in comparison to free Rab. Prenylated Rab is
recruited by specific membranes through membrane-localized
guanine-exchange factors (GEFs) and other factors (Lange-
meier et al., 2018; Blümer et al., 2013). GEF also stimulates
the release of GDP from membrane-anchored prenylated Rab
protein, with concomitant binding of GTP, which is present
in the cytosol at a tenfold higher concentration than GDP
(Ullrich et al., 1994). Once in the GTP-bound form,
membrane-anchored Rab proteins adopt their active confor-
mation and begin to recruit and interact with specific effector
proteins and consequently affect further downstream events.
Distinct Rabs function as different components of cellular
trafficking by targeting specific membranes, even though they
share an overall similar fold (Guo et al., 2013). Targeting to
specific membranes is related to specificity towards regulatory
and effector proteins. A small number of subfamily-specific
nonconserved residues provide the specificity for particular
partners (Delprato & Lambright, 2007; Zhu et al., 2004).
However, all Rab proteins go through nucleotide-exchange
cycles in order to change their conformation between the
active and inactive forms. Restructuring of the effector-
binding site is coupled to nucleotide exchange. A GTPase-
activating protein (GAP) converts Rab back to its inactive
GDP-bound form. The extraction of inactive Rab from the
membrane and its diffusion back to membrane compartments
for another round of activation is enabled by GDP dissocia-
tion inhibitor (GDI), an evolutionarily conserved REP
catalyst which has a higher affinity for GDP-bound and
prenylated Rab (Gavrilyuk et al., 2013; Pylypenko et al., 2018).

In Leishmania, more than 11 Rabs have been identified,
including Rab1, Rab4, Rab5, Rab6, Rab7 and Rab11
(Chauhan et al., 2015). Higher eukaryotes possess three
distinct functions of the Rab5 protein, namely Rab5a, Rab5b and
Rab5c, while in Leishmania only two isoforms, Rab5a and
Rab5b, have been identified. Rab5a and Rab5b in Leishmania
share ~62% similarity to each other and are reported to
function at an early endocytic stage. In L. donovani, Rab5b
is involved in the regulation of hemoglobin uptake via receptor-
mediated endocytosis, while Rab5a has been shown to
mediate HRP uptake through fluid-phase endocytosis. Null
mutants of both Rab5a and Rab5b have been reported to be
lethal to the parasite (Rastogi et al., 2016). Therefore, there is
clearly potential for these Rab proteins to become novel drug
targets. It is intriguing that a number of Rab proteins are
targets for cancer and other diseases (Hutagalung & Novick,
2011; Qin et al., 2017). However, so far, there is no drug
targeting any protozoan Rab5 or Rab protein.

Here, we present the crystal structure of the GTPase
domain of Rab5a from L. donovani (LdRab5a) in the
presence of GDP. The structure has been compared with
GDP-bound and GTP-bound structures of homologous Rab5
proteins, which shows that the Switch I region adopts a unique
disposition in LdRab5a. Molecular-dynamics (MD) simulations
show that GDP imparts conformational stability to the apo
LdRab5a structure. The LdRab5a structure presented here
will be helpful in understanding the basis of interaction with
effectors which only bind to the GDP-bound form of Rab.

2. Materials and methods

2.1. Purification of LdRab5a

The cloning and expression of stabilized LdRab5a has been
described previously (Maheshwari et al., 2018). For stabilization,
Q93L, P58D, P59G and C107S mutations and Δ60–79 and
Δ196–235 deletions were incorporated sequentially, with
monitoring of the linewidths and dispersion in the
15N-1H-HSQC spectrum of the protein over a period of nine or more
days. The residues of the stabilized LdRab5a mutant are
numbered consecutively from 1 to 175. However, in the crystal
structure only the residues from Ala11 to Leu175 are visible.
LdRab5a was recombiantly expressed in Escherichia coli as a
GST-fusion protein with a thrombin protease cleavage site
between the two proteins, as described previously (Mahesh-
wari et al., 2018). Purification of the protein was performed as
follows. Briefly, E. coli cells expressing GST-LdRab5a were
lysed and the supernatant from the lysed cells was incubated
overnight at 4°C with Glutathione Agarose 4B Beads
(Macharya Nagel) that had been pre-equilibrated with buffer A
(50 mM Tris–HCl pH 8.0, 100 mM NaCl, 5 mM MgCl2, 1 mM
GTP). After binding, the beads were packed into a small
column and washed sequentially with buffer A and buffer B
indexed in the trigonal space group P321. The cleaved LdRab5a protein was collected in the flow-through. The protein was dialyzed against 20 mM Tris–HCl buffer pH 8.3 containing 50 mM NaCl, 5 mM MgCl₂, 1 mM DTT and 1 mM GTP and was concentrated to 8 mg ml⁻¹ using a 3 kDa molecular-weight cutoff centrifugal concentrator units (Millipore India). The concentrated protein was further purified by size-exclusion chromatography on a Superdex 75 10/300 GL column (GE Healthcare, Chicago, Illinois, USA) at 22 °C for 12 h. The cleaved LdRab5a protein was collected in the flow-through.

2.2. Crystallization

The purified LdRab5a protein was extensively dialyzed against buffer C (20 mM Tris–HCl pH 8.3, 50 mM NaCl, 5 mM MgCl₂, 1 mM DTT, 1 mM GTP) and was concentrated to 10 mg ml⁻¹ using 3 kDa molecular-weight cutoff centrifugal concentrator units (Millipore India). Protein crystallization was optimized with the Hampton Research Crystal Screen and Crystal Screen 2 kits using the hanging-drop vapor-diffusion method (Jancarik & Kim, 1991) in 24-well plates (Corning, USA). Crystallization drops were set up by mixing equal volumes of protein solution and mother liquor (2 μl each) and were equilibrated against 500 μl reservoir solution. The crystallization plates were incubated at 277 and 295 K. After obtaining initial hits, protein crystals with high diffraction quality were grown using a reservoir solution consisting of 0.1 M Tris–HCl pH 8.5, 0.2 M ammonium acetate, 15% polyethylene glycol 3350 at 277 K in about 20 days. Prior to setting up crystallization, the protein was incubated with an additional 1 mM GTP (in buffer C) for 20 min.

2.3. X-ray diffraction and structure determination

For X-ray data collection, crystals of LdRab5a were soaked in a cryoprotectant consisting of 20% glycerol in the reservoir solution and were picked from the drops using CryoLoops (Hampton Research). The crystals were flash-cooled in a nitrogen stream at 100 K. X-ray diffraction data were collected to a resolution of 1.8 Å using a Rigaku FR-E+ SuperBright X-ray data-collection system with an R-AXIS IV++ detector at the X-ray Diffraction Facility, National Institute of Immunology, New Delhi, India. The crystal-to-detector distance was kept at 125 mm during the collection of diffraction data for each frame. A total of 403 frames were collected and the oscillation steps were kept at 0.75°, with an exposure time of 120 s per frame. The reflections were indexed, integrated and scaled in HKL-2000. The crystal was indexed in the trigonal space group P321, with unit-cell parameters a = b = 58.02, c = 103.42 Å, α = β = 90, γ = 120°. Data-collection statistics are summarized in Table 1. The LdRab5a protein structure was determined by the molecular-replacement method with Phaser (McCoy et al., 2007) using the structure of human Rab5b (HsRab5b; PDB entry 2hei; Structural Genomics Consortium, unpublished work) as the search model. The amino-acid sequence of LdRab5a shares 52% identity with that of HsRab5b. The structure was solved with one molecule in the asymmetric unit, a Matthews coefficient of 2.56 Å³ Da⁻¹ (Matthews, 1968) and a solvent content of 52.05%.

The initial model obtained from Phaser was first refined as a rigid body, followed by restrained and individual isotropic temperature refinement using REFMAC5 (Murshudov et al., 2011). Model building was carried out using Coot (Emsley & Cowtan, 2004; Emsley et al., 2010). The refined model was further subjected to simulated-annealing refinement using Phenix (Liebschner et al., 2019), and a composite OMIT map (2|Fo| - |Fc|) was calculated and checked at the end of the refinement. Iterative rounds of structure refinement and model building were carried out in REFMAC5 and Coot, respectively, until the model was completely built. In the final rounds of refinement, TLS and anisotropic B-factor restraints
were applied for the entire chain. Poor electron density was observed for the side chains of three residues, Asn41, Ile57 and Lys174, but the atoms of these side chains were added in order to better trace the main chain. The final model of LdRab5a consists of 165 residues, one magnesium ion, one glycerol molecule, one acetate molecule and 124 solvent molecules, with an \( R \) factor of 16.5\% and an \( R_{\text{free}} \) of 20.3\% (Bru\ënger, 1993). Ramachandran plot analysis of the final model using PROCHECK (Laskowski et al., 1993) shows that 92.7\% of the residues lie in the most favored region, while 7.3\% of the residues lie in the allowed region. Structure-related figures were generated using PyMOL (Schrodinger).

The final refined atomic coordinates have been deposited in the Protein Data Bank (PDB) as PDB entry 6l6o. Details of the crystal structure determination are summarized in Table 1.

2.4. MD simulations

MD simulations were performed using GROMACS (version 5.1.2). The GROMOS96 54a7 force field was applied to parameterize the protein both in the absence and the presence of GDP (Schmid et al., 2011). For simulation in the presence of GDP, topology and other parameters for GDP were generated using the PRODRG online server (http://prodrg1.dyndns.org/). For solvation of the system, a cubic SPC water box was employed followed by charge neutralization. Prior to adding the positional restraints for GDP, energy minimization was performed using the steepest-descent algorithm. System equilibration was carried out at 300 K for 100 ps using the NVT ensemble and for 100 ps using the NPT ensemble. Production molecular-dynamics simulations were performed for 100 ns for both the GDP-bound and the apo structure. After simulation, the root-mean-square deviation (r.m.s.d.) and root-mean-square fluctuation (r.m.s.f.) per residue were calculated and compared.

3. Results

3.1. Multiple sequence alignment of LdRab5a with other Rab5 proteins

LdRab5a was stabilized through Q93L, P58D, P59G and C107S mutations and /\(\text{C1}\)60–79 and /\(\text{C1}\)196–235 deletions. After these modifications, the residues of the stabilized LdRab5a mutant were numbered consecutively from 1 to 175. Multiple sequence alignment of the stabilized LdRab5a sequence with...
sequences from the crystal structures of HsRab5a (PDB entry 1n6h; Zhu et al., 2003), yeast Ypt51 (PDB entry 1ek0; Esters et al., 2000) and PfRab5a (PDB entry 3clv; Structural Genomics Consortium, unpublished work) was performed using ClustalW (http://www.ebi.ac.uk/clustalomega/) and is shown in Fig. 1. The conserved residues, which include the nucleotide-binding P-loop (RabF1) and switch regions (RabF2 and RabF3), along with Rab5 family-specific sequences (RabF4 and RabF5), are highlighted under the sequence with green ribbons. PfRab5a has an extra stretch of 37 residues (Ile67–Leu103) similar to LdRab5a (the deleted 20-residue stretch Pro60–Met79) at the same position between β2 and β3. HsRab5a and yeast Ypt51 do not possess this loop.

3.2. Crystal structure of the GDP-bound GTPase domain of LdRab5a

The structure of LdRab5a (PDB entry 6l6o) was solved at 1.80 Å resolution (Table 1). Although the protein was crystallized in the presence of GTP, we found one molecule of GDP and one magnesium ion bound to the protein. Additionally, one glycerol molecule and one acetate molecule were also found in the LdRab5a crystal structure. In the crystal structure, no electron density was found for the initial ten residues (Met1–Glu10) at the N-terminus, which were omitted from the structure.

The crystal structure of LdRab5a, as shown in Fig. 2, displays the canonical Rab topology in which a six-stranded central mixed β-sheet is surrounded by five α-helices. The various secondary-structure elements in topological order are as follows: β1 (12–21), α1 (25–36), β2 (49–57), β3 (60–68), α2 (71–84), β4 (87–95), α3 (97–115), β5 (120–126), α4 (137–148), β6 (152–156), α5 (163–175). With the exception of strands β2 and β3, all β-strands run parallel to each other. Some of the β-strands and α-helices are connected by functionally important loops, which are highly conserved in Rab-GTPases and are conventionally labeled G1–G5, as per the Ras-superfamily nomenclature. The G1 loop, which is also called the P-loop, interacts with the α- and β-phosphates of GDP and has the consensus sequence 20GESGAGKS27. The G2 or Switch I loop and the G3 or Switch II loop contain the conserved sequences 44TTI46 and 68DTAGLE73, respectively. Together, these loops adopt an open conformation because of the absence of the γ-phosphate. Along with the phosphate-binding loop (P-loop), these two loops share important interactions with the nucleotide γ-phosphate for the GTPase activity of the protein.

Furthermore, Switch I, Switch II and the inter-switch regions are primarily involved in the interaction with effector proteins, as observed in the crystal structures of various Rab proteins bound to their effectors (Zhu et al., 2004; Eathiraj et al., 2005; Mishra et al., 2010). The crystal structure contains GDP in the pocket formed by the G1–G3 loops, which is stabilized by hydrogen bonds to various residues contributed by these loops and water molecules.

Additionally, one Mg²⁺ ion was also found in the crystal structure. This Mg²⁺ (Mg1A) is located in the GTP-binding pocket and is hexacoordinated by Ser27, the β-phosphoryl group of GDP and water molecules, as shown in Fig. 3. However, the segment conventionally designated helix α2 was partially unfolded and was divided into two α-helical regions, 71–76 and 79–86, which were connected by an elongated stretch. Such a break in helix α2 is also observed in all other GDP-bound Rab GTPases. This helix undergoes a partial deformation, which is coupled to nucleotide exchange, and

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Figure 2
Crystal structure of LdRab5a in the presence of GDP. Color scheme: LdRab5a, gray; phosphate-binding loop, warm pink; Switch I, green; Switch II, marine; GTP, acetate, glycerol, yellow; Mg, olive.

Figure 3
Interaction stabilizing GDP and magnesium in the LdRab5a crystal structure. GDP (yellow) in LdRab5a with neighboring residues (C atoms in gray) that interact through hydrogen bonds that help to stabilize both GDP and the magnesium ion. Mg1A (olive) is hexacoordinated by water molecules (teal), Ser27 and the O atoms of the terminal β-phosphate group of GDP.
alternates between open and closed conformations depending upon the binding of GDP or GTP, respectively.

3.3. Comparison of the LdRab5a crystal structure with GDP-bound and GTP-bound human Rab5a structures

When superimposed on the GDP-bound human Rab5a form A structure (PDB entry 1tu4; Zhu et al., 2004), the crystal structure of LdRab5a overlaps well in most of the regions apart from the Switch I region (Thr34–Phe50; r.m.s.d. of 0.62 Å over 118 Cα atoms). In the GDP-bound HsRab5a structure, helix α1 displays an extra quarter turn, and Switch I and its flanking residues extend from the C-terminus of helix α1 towards the N-terminus of strands β1 and β3, and then curve towards the N-terminus of the antiparallel strand β2. This conformation of Switch I completely exposes the nucleotide-binding site in the HsRab5a structure, as shown in Fig. 4(a) (violet trace). In contrast, in the LdRab5a structure the protein chain turns upwards parallel to helix α1 and then extends towards the C-terminus of strands β1 and β3, before curving towards the N-terminus of strand β2, as highlighted in Fig. 4(a) (green trace). There is, however, no significant difference in the positioning of the Switch II region between LdRab5a and GDP-bound HsRab5a, as shown in Fig. 4(c).

A superimposition of the LdRab5a structure presented here with the GppNHp-bound HsRab5a structure is shown in Fig. 4(b). The average r.m.s.d. over 122 Cα atoms is 0.65 Å. In GppNHp-bound HsRab5a, Switch I and Switch II are together in the closed conformation, which is also referred to as the loaded-spring state. In this conformation, the imidophosphate group is coordinated by Ser29 and Lys33 of the P-loop, Ser51 and Thr52 of Switch I, Gly78 of Switch II and an Mg2+ ion. In comparison, Thr45 (equivalent to Thr52 in HsRab5a) of the Switch I region of LdRab5a is displaced 2.6 Å from the closed conformation of HsRab5a. There is also a significant difference in the orientation of Switch II regions between these two structures. Switch II shows significant changes in arrangement of the 70AGLE73 region, with a largest displacement of 5 Å for Gly71 and Leu72, followed by Glu73, which is displaced by 4.3 Å. This rearrangement makes Gly71 and Leu72 move away from the nucleotide. In contrast, Glu73 reorients its side chain to move closer to the nucleotide-binding site and

Figure 4
Comparison of the Switch I and II regions of GDP-bound LdRab5a and GDP-bound or GppNHp-bound HsRab5a. (a) Overlap of GDP-bound LdRab5a with GDP-bound form A of HsRab5a, showing the G2 loops containing the Switch I regions as tubes. The side chains of residues that correspond between the two superimposed chains are linked by dashed lines. (b) GDP-bound LdRab5a superimposed with GppNHp-bound HsRab5a, showing the G2 loop regions as tubes. (c) GDP-bound LdRab5a superimposed with GDP-bound HsRab5a, showing the Switch II and helix α2 regions as tubes. (d) GDP-bound LdRab5a superimposed with GppNHp-bound HsRab5a, showing the Switch II region and helix α2 as tubes. Color scheme: green, LdRab5a G2 loop; violet, HsRab5a G2 loop; marine, Switch II and helix α2 of LdRab5a; salmon, Switch II and helix α2 of HsRab5a; yellow, GDP of LdRab5a; orange, GDP/GppNHp of HsRab5a.
interacts with Lys26 of the P-loop and water 128 through its side-chain OE2 (hydrogen-bond distance of 2.7 Å).

3.4. Comparison of the LdRab5a crystal structure with the GDP-bound PfRab5a and GppNHp-bound yeast Rab5a (Ypt51) structures

We further superimposed LdRab5a on GDP-bound PfRab5a and GppNHp-bound yeast Rab5a (Ypt51). The r.m.s.d.s for these overlaps are 1.2 Å over 137 Cα atoms and 0.82 Å over 132 Cα atoms, respectively. Superimposition of LdRab5a on PfRab5a shows good overlap for the Switch I region up to the γ-phosphate-interacting residue Thr45. The backbone traces of these two protein chains bifurcate at the Rab5-specific region 46IGAA49, which follows Thr45, as shown in Fig. 5(a). This region shows the largest difference between the GDP-bound LdRab5a and PfRab5a conformations. However, the backbone traces overlap again at Phe50, which incidentally is part of the invariant hydrophobic triad, in combination with Trp67 and Tyr82. This hydrophobic triad acts as an important recognition interface for effector binding.

Besides this, the Switch II residues overlap well in the region 67WDTAGLE73, but the overlap is poorer for the following 74RFRSLA79 region, which corresponds to the elongated helix α2. However, in the elongated region, the orientation of the side chains remains similar in the LdRab5a and PfRab5a structures, as shown in Fig. 5(c).

Upon comparison of the LdRab5a structure with the GppNHp-bound Ypt51 structure from yeast, it was observed that the C-terminal end of the G2 loop is shifted towards helix α2 in Ypt51, as shown in Fig. 5(b). The Switch I threonine (Thr44 in Ypt51) is involved in a hydrogen bond to O1G of GppNHp through its backbone amide NH. In addition, Switch II differs in a similar way at the 70AGL72 segment as seen in the case of HsRab5a–GppNHp. Also, helix α2 in this active conformation is well formed and consequently shifted inwards, as shown in Fig. 5(d).

In order to highlight the differences in the Switch I and II regions of LdRab5a, human Rab5a, PfRab5a and yeast Rab5a, a superimposition of all of these structures is shown in Fig. 6. As can clearly be seen, the Switch I regions of LdRab5a and PfRab5a are shifted away from the vicinity of Switch II and

**Figure 5**

Comparison of the Switch I and II regions of GDP-bound LdRab5a and GDP-bound PfRab5a or GppNHp-bound Ypt51. (a) Overlap of GDP-bound LdRab5a with GDP-bound PfRab5a, showing the G2 loops containing the Switch I regions as tubes. (b) GDP-bound LdRab5a superimposed with GppNHp-bound Ypt51, showing the G2 loop regions as tubes. (c) GDP-bound LdRab5a superimposed with GDP-bound PfRab5a, showing the Switch II and helix α2 regions as tubes. (d) GDP-bound LdRab5a superimposed with GppNHp-bound Ypt51, showing the Switch II region and helix α2 as tubes. Color scheme: green, LdRab5a G2 loop; violet, PfRab5a–GDP or yeast Ypt51–GppNHp G2 loop; marine, Switch II and helix α2 of LdRab5a; salmon, Switch II and helix α2 of PfRab5a–GDP or yeast Ypt51–GppNHp; yellow, GDP in LdRab5a; orange, GDP/GppNHp in PfRab5a/yeast Ypt51.
towards the N-terminus of strand β2, thus forming a crevice between the two switches, in comparison to the HsRab5a and yeast Rab5a structures (Fig. 6a). However, the Switch I region of LdRab5a is shifted further towards helix α1 than that in PfRab5a. On the other hand, overlap of the Switch II region, as shown in Fig. 6(b), displays differences in the nucleotide-binding region of Switch II and the unstructured part of helix α2 in comparison to GppNHp-bound human and yeast Rab5a.

For the various GDP-bound and GTP-bound structures that have been compared above, only weak hydrogen-bond or hydrophobic contacts have been found with the adjacent symmetry-related molecules. In LdRab5a–GDP, Ile46 in the Switch I region forms a hydrogen bond through its backbone carbonyl to the backbone NH2 group of Gly59 of the adjacent symmetry-mate chain. In GDP-bound PfRab5a, Ile40, Gly41 and Ala42 interact with the side-chain NH2 group of Asn177 of the adjacent symmetry-mate chain through hydrogen bonds, while in GppNHp-bound yeast Rab5a the equivalent Ile40 and Ala42 form hydrogen bonds to the side-chain NH2 group of Lys170 of the symmetry mate. In HsRab5a, Ile53 and Ala55 forms hydrogen bonds to Gly15 of the symmetry mate, while in the LdRab5a Switch II region Ser77 is involved in forming hydrogen bonds to the adjacent symmetry mate in the crystal structure. Further, in LdRab5a, Phe75 is also involved in hydrogen-bond formation to a neighboring symmetry mate. The Switch II residues equivalent to Ser77 and Phe75 of LdRab5a also display similar weak interactions with symmetry-related molecules in the other compared Rab5

Figure 6
Superimposition of GDP-bound and GppNHp-bound Rab structures. Superimposition and enlarged views of the LdRab5a–GDP (green), PfRab5a–GDP (magenta), Ypt51–GppNHp (blue) and HsRab5a–GppNHp (cyan) structures. (a) Superimposition with expansion of the G2 loop showing the Switch I (SwI) and family-specific (TTIGAAF) regions of the GDP-bound LdRab5a and PfRab5a structures and the GppNHp-bound Ypt51 and HsRab5a structures. (b) Superimposition with expansion of the Switch II (SwII) and helix α2 regions of GDP-bound LdRab5a and PfRab5a and of GppNHp-bound Ypt51 and HsRab5a.
structures. For example, Arg68 in yeast Ypt51, which is at a position equivalent to that of Phe75 in LdRab5a, is involved in hydrogen-bond interactions with its symmetry mate.

3.5. Comparison of the invariant hydrophobic triad

Along with the switch and inter-switch regions, the invariant hydrophobic triad has also been identified as an important effector-binding interface in Rab proteins. This region is comprised of residues Phe50, Tyr82 and Trp67 (numbering as per the stabilized LdRab5a sequence). As described above, Phe50 follows the Rab5-specific 46IGAA49 region, and the protein chain of Rab5a once again superimposes with those of PfRab5a and yeast Rab5a at this residue after bifurcating at Thr45 in Switch I. However, the invariant phenylalanine is seen to rotate by around 109.7° along the CG—CB—CG bond axis. A similar rotation is observed for Trp67 and Tyr82 between the GDP-bound and GTP-bound Rab5a structures discussed above. The overlap of GDP-bound LdRab5a and PfRab5a, highlighting the invariant hydrophobic triad, is shown in Fig. 7(a), while a superimposition of GDP-bound LdRab5a and GppNHp-bound yeast Rab5a is shown in Fig. 7(b). The degree of rotation with reference to the GDP-bound LdRab5a for these three residues is given in Table 2.

From the above results, it can be seen that nucleotide exchange from GDP to GTP induces allosteric conformational changes through the switch and inter-switch regions, which are the primary site of effector recognition and binding. Surface representations of various Rab proteins with highlighted Switch I, inter-switch, Switch II and helix α2 regions are shown in Fig. 8. As can be seen from the surface representation, these regions remain separated from each other in the GDP-bound conformation (Fig. 8a). On the other hand, these regions come closer to one another on the binding of a nucleotide triphosphate. In the GppNHp-bound form they combine to form an interacting interface or epitope, which displays a high degree of structural similarity, as shown in Fig. 8(b).

3.6. Molecular-dynamics simulations

An interesting aspect of Rab proteins in the GDP-bound state is their interaction with GEF proteins. GEF proteins stimulate the release of GDP, and structures of GEF bound to nucleotide-free Rab proteins have been determined (Uejima et al., 2010; Delprato & Lambright, 2007). GTP competes with GEF for the nucleotide-free Rab. We performed MD simulations of LdRab5a–GDP and nucleotide-free apo LdRab5a, using the crystal structure presented here as a starting point, in order to map the possible conformational changes on going from the GDP-bound to the nucleotide-free state, which is an intermediate between the inactive GDP-bound form and the activated GTP-bound form. Plots of trajectories during 100 ns simulations show that the r.m.s.d. values of the protein in the GDP-bound conformation are lower than those of the nucleotide-free protein in the GDP-bound conformation, as shown in Fig. 9(a). This depicts the higher stability of the GDP-bound form of LdRab5a in comparison to the protein alone. An analysis of r.m.s.f. values plotted against residue

Table 2

<table>
<thead>
<tr>
<th>Residue</th>
<th>Angle of rotation (°)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ypt51–GppNHp</td>
</tr>
<tr>
<td>Phe50</td>
<td>CG—CB—CG</td>
</tr>
<tr>
<td>Trp67</td>
<td>CE3—CG—CE3</td>
</tr>
<tr>
<td>Tyr82</td>
<td>CG—CB—CG</td>
</tr>
</tbody>
</table>

Figure 7

Rotameric orientation comparison of conserved hydrophobic triad residues. (a) Superimposed cartoon and stick model views showing the orientation of the conserved hydrophobic triad residues (Phe50, Trp67 and Tyr82) in the LdRab5a (gray) and PfRab5a (red) structures. (b) Superimposed cartoon and stick model views showing the orientation of the conserved hydrophobic triad residues (Phe50, Trp67 and Tyr82) in the LdRab5a (gray) and Ypt51–GppNHp (blue) structures.
number shows that the residues in the switch regions, and also in other loop regions, exhibit higher fluctuations in nucleotide-free LdRab5a. This might allow GTP to access the active site. Moreover, the GDP-interacting residues were stable and showed an r.m.s.f. around the baseline in the simulation run of GDP-bound LdRab5a, as shown in Fig. 9(b). Additionally, upon comparing the simulated structure in the presence of GDP with the input crystal structure after every 10 ns interval, we found gradual change in flexibility over the 100 ns simulation.

4. Discussion

In the endocytic pathway, Rab5 is present in sorting endosomes, whereas Rab4 and Rab11 are localized in recycling endosomes. Rab7, Rab9 and Rab24 are associated with the late endosomal compartment (Wandinger-Ness & Zerial, 2014). Rab5 controls endosome biogenesis, maturation and fusion through multiple effectors. The Leishmania parasite possess two Rab5 isoforms, which have been reported to function in early endosome formation and targeting. LdRab5a and LdRab5b are essential proteins. LdRab5a induces FPE, but also enhances the kinetics of lysosomal transport (Rastogi et al., 2016). Leishmania endocytoses hemoglobin (Hb) through a specific Hb receptor located in the flagellar pocket. Rapid receptor-mediated endocytosis is regulated by LdRab5b. Endocytosed Hb is degraded in the lysosomes via Rab7-dependent processes to generate intracellular heme, which is essential for the parasite (Patel et al., 2008). It is imperative that the two LdRab5 proteins execute their roles by binding to their specific effectors. Mass-spectrometric analysis of the interactome of LdRab5a and LdRab5b indicated 32 and 26 exclusive binding partners, respectively, for these proteins (Rastogi et al., 2016). Dynein, kinesin and tubulin were identified as some common LdRab5 interactors, and these proteins have also been shown to regulate endocytosis and vesicular trafficking. Although the interactome analysis did not reveal homologs of any of the

Figure 8
Surface view of the GDP-bound and GppNHp-bound conformations showing changes in the effector-binding interface. (a) Conformational organization of the Switch I (pink), inter-switch (green), Switch II (gold) and helix α2 (orange) regions in GDP-bound LdRab5a and PfRab5a. (b) Conformational organization of the Switch I (pink), inter-switch (green), Switch II (gold) and helix α2 (orange) regions in GppNHp-bound Ypt51 and HsRab5a.
conventional mammalian Rab5 effectors such as EEA1, rabaptin-5 or rabenosyn-5, it is nevertheless interesting to look at the binding modes of these effectors to Rab5. Structures of all of these effectors have been determined in complex with human Rab5 (Rastogi et al., 2016). Early endosomal autoantigen 1 (EEA1) enhances endosome fusion. EEA1 is a long coiled-coil homodimer with an N-terminal C2H2 zinc finger (ZF) and a C-terminal FYVE domain. A contiguous surface of the EEA1 C2H2 ZF formed by residues from the β1–β2 strands, α1 helix and a short N-terminal extension binds to the switch and inter-switch regions of Rab5 through a predominantly nonpolar interface augmented by polar interactions (Mishra et al., 2010). Rabaptin-5 is an essential and rate-limiting component of early endosome fusion. It is recruited to early endosome and endocytic vesicle membranes by Rab5 in a GTP-dependent manner and is involved in both heterotypic and homotypic early endosome fusion. Rabaptin-5 binds specifically to Rab5 with its C-terminal region, which consists of a helix of ~36 residues followed by a tight loop and a short helix. Rab5 mainly uses its Switch II and inter-switch (that is β2 and β3) regions to contact rabaptin-5 (Zhu et al., 2004). Rabenosyn-5 binds to Rab22, which is a member of the Rab5 phylogenetic group, through a helical hairpin. The interaction site on Rab22 is again the switch and inter-switch regions (Eathiraj et al., 2005). In fact, the binding sites of human Rab5 for various effectors exhibit a large degree of overlap, irrespective of the topology of the effector. At the same time, the Rab5 interaction sites on these effectors also exhibit a high degree of physicochemical similarity, indicating convergent evolution. The main feature of the Rab5–effector interface is the shape complementarity of primarily nonpolar surfaces, especially to engage Phe50 in the Switch I region away from the nucleotide moiety in the GDP-bound inactive conformation. It can be anticipated that upon GTP binding this residue will move closer to the γ-phosphate group and its backbone amide NH will interact with an O atom of the γ-phosphate. It can also be anticipated that the segment following Thr45, 46IGAA49, will adopt a conformation similar to that seen for GppNHp-bound HsRab5a and yeast Ypt51 (Zhu et al., 2003; Esters et al., 2000). Concomitantly, the Switch II residues 71GLE73 would shift by ~5 Å and rearrange upon GTP binding, enabling interaction between the NH group of Gly71 and an O atom of the γ-phosphate group. This movement of the 71GLE73 segment is facilitated by the reformation of helix α2, which is split and elongated in the GDP-bound state. Upon reformation, helix α2 will move closer to the C-terminus of the G2 loop. Overall, upon GTP binding, the Switch I and Switch II regions would together adopt the closed loaded-spring conformation, along with a reorientation of the invariant hydrophobic triad residues in LdRab5a, Gln72 has been replaced by leucine to decrease the intrinsic GTPase activity. The conserved Gln72 catalyzes the hydrolysis of GTP by assisting the nucleophilic attack by a water molecule on the
bond between the terminal β-phosphoryl and γ-phosphoryl groups (Pai et al., 1990). Further, upon GTP hydrolysis, Glu72 is oriented in such a way so as to enable interaction between the conserved Lys26 of the P-loop and an aspartate residue of GEF, which leads to the exchange of GDP for GTP (Lange-meyer et al., 2014; Delprato & Lambright, 2007; Delprato et al., 2004).

We are currently trying to crystallize LdRab5a bound to GppNHp in order to map the allosteric changes associated with nucleotide exchange. As mentioned above and in previous reports, effectors of Leishmania Rab5 isomers have not been identified to date. We have searched the genome sequence of *L. donovani* for effector proteins with Rab5-specific binding domains. However, determinants of specificity can only be established through experimental characterization.

5. Conclusion

We have determined the crystal structure of the stabilized GTPase domain of the *L. donovani* Rab5a protein in the GDP-bound state at 1.80 Å resolution. LdRab5a displays a canonical Rab fold, with the P-loop, Switch I and Switch II being regions of high functional relevance. In the structure, the conserved Thr45 residue of the G2 loop or Switch I region moves away from the nucleotide and the following residues trace a unique path up to Phe50. The residues Asp/GLQ of the Switch II region are shifted by up to 5 Å from the anticipated loaded-spring conformation, with helix α2 being split and elongated in the RFRSL segment. The invariant hydrophobic triad residues were found to have rotated away from their anticipated conformation in the GTP-bound state. The structural characterization of LdRab5a will be helpful in unraveling the allosteric changes upon nucleotide exchange and may help in understanding the specificity of LdRab5a towards its effectors.

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