Short communication

Structure of the extremely slow GTPase Rab6A in the GTP bound form at 1.8 Å resolution

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Abstract

Rab/Ypt GTPases represent a > 60 member large family of membrane traffic regulators in eukaryotic cells. Members of this group display intrinsic GTPase activity varying over two orders of magnitude. Here, we show that Rab6A represents the RabGTPase with the slowest spontaneous GTPase activity yet measured (5 × 10⁻⁶ s⁻¹). Due to the very low intrinsic hydrolysis rate we were able to crystallise and solve the structure of the Rab6A-GTP complex to 1.82 Å resolution. Analysis of the structure suggests that low catalytic activity of the Rab6A might be due to high flexibility of the Switch II region and a low degree of constraint of critically important for catalysis Gln 72.

Keywords: RabGTPases; Vesicular transport; Crystal structure; Hydrolysis rate

1. Introduction

Small monomeric Rab proteins are members of the Ras-like superfamily of guanine nucleotide-binding proteins (GNBRs) that act as indispensable regulators of intracellular trafficking between subcellular compartments of the eukaryotic cell (Stenmark and Olkkonen, 2001; Zerial and McBride, 2001). Like all G proteins, Rabs share a structurally homologous GTP-binding domain allowing them to function as molecular switches that cycle between a GTP bound active conformation and a GDP-bound inactive conformation. A guanine exchange factor (GEF) catalyses the transition from the GDP-bound state to the GTP bound state. As a consequence, two otherwise mobile regions (Bourne et al., 1991) of the GTPase called Switch I and Switch II change their conformation to form a well ordered, hydrophobic interface, to which downstream effectors bind (Stroupe and Brunger, 2000). Rabs return to the GDP-bound state and effectors dissociate upon hydrolysis of GTP to GDP, which occurs due to intrinsic GTPase activity. This, usually low, GTPase activity is enhanced by the action of a Rab-specific GTPase-activating protein (GAP), lowering the transition state barrier of the reaction (Schimmoller et al., 1998). Earlier studies showed that whereas Rab5 and Rab3 display similar hydrolytic activity the hydrolysis rates of Rab7 is slower by a factor of ~20 (Dumas et al., 1999; Simon et al., 1996a). Such variations are presumed to reflect the important functional differences of these biological timers.

While structural data has been accumulating for Rab GTPases, the available structures of the GDP- or GppNHp bound forms have failed to identify structural determinants responsible for the large differences in the intrinsic rates of GTP hydrolysis. To address this issue, we set out to determine and evaluate the structure of Rab6, the Rab GTPase with the lowest GTP hydrolysis rate measured so far (Yang et al., 1993). Rab6 was originally identified as a key regulator of microtubule-dependent retrograde traffic, controlling vesicular trafficking from early endosomes to the trans-Golgi network, through the Golgi apparatus and from the

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Golgi to the endoplasmatic reticulum and appears to be involved in a complex network of protein–protein interactions (Dachen and Goud, 2000).

1.1. Determination of the intrinsic GTP hydrolysis rate of Rab6A

To analyse the enzymatic activity of human Rab6A we expressed the protein in Escherichia coli and purified it to homogeneity as described in “Supplementary materials.” HPLC analysis of the purified recombinant Rab6A demonstrated that more than 90% of the protein was in the GTP bound form (not shown). This suggests that the intrinsic rate of hydrolysis of this GTPase is unusually low since most of RabGTPases are purified recombinantly. To measure the intrinsic GTPase activity we incubated the recombinant Rab6A protein at 25 °C for 6 days while continuously analysing spontaneous hydrolysis of GTP under the same conditions is shown as empty squares (□). The hydrolysis reaction was started by addition of 10 mM MgCl₂. Concentrations of GTP and GDP were determined by HPLC (see Supplementary materials). The data of the wild-type protein was fitted to the single-exponential curve giving a rate constant of $5 \times 10^{-6}$ s⁻¹.

We determined a rate constant for the decrease in GTP concentration upon triggering the reaction by addition of Mg²⁺ of $5 \times 10^{-6}$ s⁻¹. While this is in good accordance to the GTPase rate of the yeast homologue Ypt6 (3 $\times 10^{-6}$ s⁻¹) found at 30°C (Will and Gallwitz, 2001) it is much slower than that observed for other Rab proteins under comparable conditions. To confirm that the observed GTP → GDP conversion was indeed a consequence of enzymatic activity we measured the GTPase activity of Rab6A(Q72L) mutant that is expected to lack enzymatic activity (closed circles on Fig. 1). The previously known Rab GTPase with the slowest observed catalytic activity Rab7 displays a rate of GTP hydrolysis of $4.5 \times 10^{-5}$ s⁻¹. The RabGTPase with the fastest rate of catalysis is Rab5, which displays a rate of $5.5 \times 10^{-4}$ s⁻¹ (Simon et al., 1996b). The observed, extremely low hydrolysis rate of Rab6A could potentially enable determination of its structure in GTP bound form, which so far was not possible for other Rab GTPases. Generally the GTP bound conformation of Rab and other GTPases is simulated by complexing them with the non-hydrolysable analogues GTPγS or GPPNP or by using mutants with impaired GTPase activity (Zhu et al., 2003). The structure of the Rab6A:GTP complex could provide an insight into the structural basis of extreme differences in catalytic activity within the Rab family and also validate the use of non-hydrolysable GTP analogues for structural research on RabGTPases.

1.2. Structure solution and analysis of Rab6A:GTP complex

We expressed an N- and C-terminally truncated version of Rab6A and crystallised it as described in Table 1 and Supplementary materials section. The diffraction data were collected to 1.82 Å resolution and structure was solved.

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Table 1

Statistics of diffraction data and refinement

<table>
<thead>
<tr>
<th>Data collection</th>
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<tr>
<td>Spacegroup</td>
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<tr>
<td>Unit cell (a,b,c) [Å]</td>
<td>β = 107.57°</td>
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<td>X-ray source</td>
<td>ESRF ID14-4</td>
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<td>Wavelength [Å]</td>
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<tr>
<td>Detector</td>
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<tr>
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</tr>
<tr>
<td>$R_{	ext{sym}}^{\text{obs}}$</td>
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<tr>
<td>Observations total/unique</td>
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<tr>
<td>Completeness [%]</td>
<td>87.7(52.2)</td>
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<tr>
<td>$&lt;I&gt;/σ(I)$</td>
<td>17.7(4.3)</td>
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<table>
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<th>Refinement</th>
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<tr>
<td>$R_{\text{work}}/R_{\text{free}}$</td>
<td>20.1/26.5</td>
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<tr>
<td>Protein atoms</td>
<td>5202</td>
</tr>
<tr>
<td>GDP:GTP-Mg atoms</td>
<td>132</td>
</tr>
<tr>
<td>Water molecules</td>
<td>868</td>
</tr>
<tr>
<td>Rmsd bonds/angles (Å/deg)</td>
<td>0.012/1.35</td>
</tr>
<tr>
<td>Method</td>
<td>Vapour diffusion, hanging drop geometry, at 20 °C. Reservoir volume 0.5 ml, drops volume 1 µl of protein was mixed with 1 µl of reservoir solution</td>
</tr>
<tr>
<td>Conditions</td>
<td>100 mM Tris–HCl, pH 8.0, 1 M LiCl, 32% (w/v) polyethylene glycol 1000, and 3.3 mM Nonyl-β-D-glucoside</td>
</tr>
</tbody>
</table>

$^a$ Completeness, $R_{\text{sym}}$ and $(<I>/σ(I))$ are given for all data and for the highest resolution shell: 19.0–18.2 Å.

$^b$ $R_{\text{work}} = \Sigma F_{\text{calc}} - k F_{\text{obs}}/\Sigma F_{\text{calc}}$, 5% of randomly chosen reflections were used for the calculation of $R_{\text{free}}$.

$^c$ $R_{\text{sym}} = \Sigma |F| - <|F|>/|F|$, where $<|F|$ is the average intensity of reflection $j$ for its symmetry equivalents.
using the coordinates of the *Plasmodium falciparum* homologue of Rab6 (PDB code 1D5C) (Chattopadhyay et al., 2000). The asymmetric unit contained four molecules of Rab6 arranged into two dimers. The molecules in a dimer adopt somewhat different conformations with $\alpha$ RMSD between molecules of 1.24 Å.

The overall structure of Rab6A displays a typical Ras-like GTPase fold (Pai, 1998). Fig. 2 shows a ribbon representation of the residues 13–174 that were visible in the electron density map. The structure comprises five $\alpha$-helices surrounding five parallel and one antiparallel $\beta$-strands, a fold common to most small GTPases. The position of GTP and magnesium ion overlap with those found in GppNHp complexes with Ras, Rab5A, and Rab3A confirming that non-hydrolysable analogues represent a good approximation of the native GTP ligand. Crystal contacts to symmetry-related molecules of the discussed structure were found to contribute to constraining the Switch II regions of molecules B and D of the asymmetric cell unit. In molecules A and C, the area surrounding the nucleotide-binding site is free of contacts with other molecules. Hence, we assume that structure of this region represents an unperturbed view on Rab6A in the GTP bound state.

The observed electron density map at the Switch II region of molecules A and C was sufficient to define region’s conformation, although the poor quality of the map reflected high flexibility of the region and led to high B-factors. Flexibility of Switch II region provides possible clue to the molecular basis of the low catalytic activity. As can be seen in Fig. 3A and S2, the Switch II region of Rab6A appears to be the most flexible region of the molecule. One of the important features of the Switch II region is the presence of Gln 72 (equivalent to the catalytic Gln 61 of Ras) that is required for efficient catalysis. Recent biochemical and structural studies on the small GTPase RHEB suggested that displacement of this residue from the active site may account for the extremely low intrinsic catalytic activity of this protein (Yu et al., 2005a). Comparison of B-factors of the structure of Rab6A:GTP with the structures of Rab7:GPPNHP and Rab5:GPPNHP led us to the conclusion that Switch II is most constrained in the Rab5A molecule, while Rab7 represents an intermediate situation (not shown). Therefore it appears likely that flexibility of Switch II and as a consequence of Gln 72 plays an important role in determining the intrinsic catalytic activity in Rab and possibly other GTPases.

Further comparison of the Rab6A:GTP structure with the structure of Rab5A:GPPNHP complex indicates some additional differences. The main difference to the structure of Rab5 is the presence of Tyr 32 in the Switch I region that covers the phosphate groups of GTP, with its OH group forming a hydrogen bond with the oxygen of the $\gamma$-phosphate (Fig. 3A). This residue forms a lid burying the phosphate groups of the nucleotide. Comparison of Rab6A solvent-exposed surfaces with that of Rab5A which possesses an active site with well exposed
nucleotide and Mg\textsuperscript{2+} ion shows that the presence of Tyr 32 reduces the exposed surface of the nucleotide by nearly two thirds (Figs. 3B and C). To determine whether the occluded positioning of the phosphates could correlate with the observed rates of spontaneous hydrolyses we analysed the structure of Rab7:GPPHNp, which is the second catalytically slowest RabGTPase. Interestingly, Rab7 also pos sesses a bulky lid-forming tyrosine (Tyr 37) forming a hydrogen bond with the oxygen of the \(\gamma\)-phosphate (Fig. 3E). In contrast, another rapidly hydrolysing GTPase Rab3A has Phe51 residue at this position that cannot from a hydrogen bond with the nucleotide and does not shield the phosphates (data not shown). Although, it is not currently clear what the consequences of Tyr 37 in Switch I and its hydrogen bonding with the \(\gamma\)-phosphate of GTP are, the observed feature is not restricted to Rab6A and Rab7. Tyrosine occurs at an analogous position in a number of RabGTPases, including another catalytically inefficient Rab GTPase Rab24 (Colicelli, 2004; Erdman et al., 2000). Moreover, in the already mentioned structure of the RHEB:GTP complex, Tyr 35 is also present in Switch I and forms a hydrogen bond to the \(\gamma\)-phosphate of GTP, leading to closure of the nucleotide-binding site (Yu et al., 2005a). It is possible that shielding of the nucleotide-binding site does not itself influence the catalytic activity but that it is an accompanying feature of slowly hydrolysing RabGTPases GTPases that increases the fidelity of Rab:GAP interactions. RabGAPs are known to be promiscuous and shielding of phosphates by Tyr residue may provide an additionally occluding feature of slowly hydrolysing RabGTPases GTPases that increases the fidelity of Rab:GAP interactions. RabGAPs are known to be promiscuous and shielding of phosphates by Tyr residue may provide an additionally occluding feature of slowly hydrolysing RabGTPases GTPases that increases the fidelity of Rab:GAP interactions. Rab24 is an atypical member of the Rab GTPase family. Deficient GTPase activity, GDP dissociation inhibitor interaction, and prenylation of Rab24 expressed in cultured cells. J. Biol. Chem. 275 (6), 3848–3856.


References


