Structures of *Drosophila melanogaster* Rab2 and Rab3 bound to GMPPNP

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Rab GTPases belong to the large family of Ras proteins. They act as key regulators of membrane organization and intracellular trafficking. Functionally, they act as switches. In the active GTP-bound form they can bind to effector proteins to facilitate the delivery of transport vesicles. Upon stimulation, the GTP is hydrolyzed and the Rab proteins undergo conformational changes in their switch regions. This study focuses on Rab2 and Rab3 from *Drosophila melanogaster*. Whereas Rab2 is involved in vesicle transport between the Golgi and the endoplasmic reticulum, Rab3 is a key player in exocytosis, and in the synapse it is involved in the assembly of the presynaptic active zone. Here, high-resolution crystal structures of Rab2 and Rab3 in complex with GMPPNP and Mg²⁺ are presented. In the structure of Rab3 a modified cysteine residue is observed with an enigmatic electron density attached to its thiol function.

1. Introduction

Rab proteins are small monomeric GTP-binding proteins (GTPases) which constitute the largest branch of the Ras superfamily (Pereira-Leal & Seabra, 2000). They are evolutionarily conserved, with 55–75% identity between orthologues from yeast to mammals. More than 70 different Rab proteins are encoded in the *Homo sapiens* genome (Zerial & McBride, 2001; Bhuin & Roy, 2014), 11 in *Saccharomyces cerevisiae* (Lazar *et al.*, 1997), 29 in *Caenorhabditis elegans* (Pereira-Leal & Seabra, 2000), 57 in *Arabidopsis thaliana* (Vernoud *et al.*, 2003) and about 33 in *Drosophila melanogaster* (Chan *et al.*, 2011). Rab GTPases act as key regulators of membrane organization and intracellular trafficking in all eukaryotic cells (Pfeffer, 1994; Zerial & McBride, 2001; Stenmark, 2009; Bhuin & Roy, 2014), and as such take part in vesicle formation, motility, tethering and fusion of the vesicles with their target membrane (Zerial & McBride, 2001; Pfeffer, 2007). These functions are carried out by a diverse collection of effector molecules, which are recruited by specific Rab proteins, owing to their role as molecular switches. Thus, Rab proteins regulate their particular pathways by interacting with various effector proteins.

In their function as molecular switches, Rab proteins undergo two alternate conformational transitions upon binding to either GDP or GTP. Firstly, the protein is activated by a guanine-exchange factor (GEF), which exchanges GDP for GTP. In the GTP-bound active form, each Rab can interact with a different set of proteins (effectors) to facilitate the delivery of transport vesicles to different acceptor membranes (Molendijk *et al.*, 2004). While in this conformation, Rabs can associate with their target membrane and interact with their effectors to recruit them to specific subcellular compartments or to activate them. Upon stimulation by GTPase-activating proteins (GAPs) the GTP is hydrolyzed, releasing an inorganic phosphate group, and the now inactive Rab returns to the cytosol. Owing to the identical mechanisms of effector binding and nucleotide exchange and hydrolysis, Rabs share a conserved and well characterized fold with most of the small GTPase family members.

The tertiary structure is composed of a six-stranded β -sheet surrounded by α -helices. Extensive analyses of other GTPases have defined two regions, termed switches I and II, located near the phosphate region of the bound guanine nucleotide (Dumas *et al.*, 1999; Ostermeier & Brunger, 1999). These regions undergo dramatic

Table 1

Macromolecule-production information.

	<i>dmRab2</i> ^{O65L}	<i>dmRab3</i> ^{O80L}
Source organism	<i>D. melanogaster</i>	<i>D. melanogaster</i>
DNA source	cDNA	cDNA
Forward primer	ACCATGGGATGCTCTACGGTACTTG	TATACCATGGGATGCGGAGTGGCG
Reverse primer	TATAGTCGACTCACTGGATCTTCTGTAATC	TATAGTCGACTCACTGGACATCTTATCG
Expression vector	pET-MBP	pET-MBP
Expression host	<i>E. coli</i>	<i>E. coli</i>
Complete amino-acid sequence of the construct produced†	GAMSYAYLFKYIIIGDTGVGKSCLLQLQFDRFQPVHDLTIGVEFGARMITIDGKQ- IKLQIWDTAGLEAFRSITRSYYRGAAGALLVYDITRRETFNHLTTWLEDARQHS- NSNMVIMLIGNKSDLSRREVKKKEGEAFAREHGLVFMETSARTANVEEAFIN- TAKEIYEKIQ	GAMASGGDPKWQKDAADQNFDMFKLLIIGNSSVGKTSFLFRYADDSFTSAFVSTV- GIDFKVKTVFRHDKRVKLQIWDTAGLERYRTITTAAYRGAMGFILMYDVTNEDS- FNSVQDWVTQIKTYSWDNAQVILVGNKCDMEDQRVISFERGRQLADQLGVEFFE- TSAKENVNVKAVFERLVDIICDKMSE

† The remaining tag sequence after TEV cleavage is underlined.

Table 2

Crystallization.

	<i>dmRab2</i> ^{O65L}	<i>dmRab3</i> ^{O80L}
Method	Sitting-drop vapour diffusion	Sitting-drop vapour diffusion
Plate type	Crychem plate	Crychem plate
Temperature (K)	291	291
Protein concentration (mg ml ⁻¹)	50	40
Buffer composition of protein solution	200 mM NaCl, 20 mM HEPES pH 7.5, 5 mM magnesium acetate, 2 mM DTT, 2% (v/v) glycerol	200 mM NaCl, 20 mM HEPES pH 7.5, 5 mM magnesium acetate, 2 mM DTT, 2% (v/v) glycerol
Composition of reservoir solution	34% (v/v) polyethylene glycol (PEG) 400, 200 mM sodium acetate pH 4.6	28% (v/v) PEG 200, 5% (w/v) PEG 3000, 100 mM MES buffer pH 6.0
Volume and ratio of drop	1:1	1:1
Volume of reservoir (μl)	600	600

conformational changes on nucleotide exchange and are involved in protein–protein interactions; hence, they account for the nucleotide-dependency of most GTPase interactions (Bhuin & Roy, 2014; Sprang, 1997). In the GDP-bound form these regions are highly disordered and thus inactive. They only become ordered upon GTP binding and then expose a triad of hydrophobic amino acids to the surface of the protein. This triad, together with other residues of the switch I and switch II regions, is crucial for the interaction of the Rabs with their respective effector proteins, and this is thought to define the specificity of Rabs for their different effector partners (Merithew *et al.*, 2001; Eathiraj *et al.*, 2005; Burguete *et al.*, 2008).

Rab2 has been identified as a specific regulator of vesicle transport between the Golgi and the endoplasmic reticulum (Liu & Storrie, 2012; Stenmark, 2009) with several known effector proteins, including GM130 and golgin-45 (Short *et al.*, 2001). For instance, Rab2 is able to promote the recruitment of COP I vesicles by binding to its effector PKC 1/λ (Tisdale, 2000). Furthermore, Tisdale and Balch showed that the amino-terminus of Rab2 might be involved in the maturation of pre-Golgi intermediates (Tisdale & Balch, 1996).

Rab3 is one of the most investigated Rab GTPases in the context of neuronal functions. It has been identified as a specific regulator in the exocytosis of secretory granules, including synaptic vesicles and vesicles, from the trans-Golgi-network to apico-lateral membranes (Stenmark, 2009; Bhuin & Roy, 2014). There are several known effector proteins of Rab3, including RIM, which plays a role in synaptic vesicle trafficking (Wang *et al.*, 1997). In *Drosophila* Rab3 seems to have a different function at the synapse: not synaptic vesicle trafficking but rather the trafficking of membrane/cargo for assembly of the presynaptic active-zone cytomatrix (Graf *et al.*, 2009).

2. Materials and methods

2.1. Macromolecule production

The cDNAs for full-length *D. melanogaster* Rab2 (*dmRab2*) and Rab3 (*dmRab3*) were purchased from the *Drosophila* Genomics

Resource Center. The Rab2 and Rab3 genes were amplified by polymerase chain reaction and cloned into the pET-MBP vector using *NcoI* and *SalI* restriction sites (Table 1). The resulting constructs comprise an N-terminal MBP tag followed by a *Tobacco etch virus* (TEV) protease cleavage site followed by the N-terminal GTPase domain of *dmRab2* (amino acids 1–172) and *dmRab3* (amino acids 1–188), respectively. These construct boundaries were chosen based on bioinformatic analysis of deposited GTPase structures in the Protein Data Bank. Furthermore, the conserved glutamine (*dmRab2* Gln65 and *dmRab3* Gln80) located in the switch II region and involved in transition-state stabilization (Privé *et al.*, 1992; Der *et al.*, 1986) was mutated to a leucine. Rab mutants were prepared by site-directed mutagenesis according to the manufacturer's protocol (EURx 'Site-directed mutagenesis'). The correctness of the DNA sequences was confirmed by DNA sequencing.

2.2. Protein expression and purification

Protein expression was conducted using chemically competent *Escherichia coli* Rosetta cells. The cells were grown in autoinduction ZY medium (Studier, 2005) with kanamycin and chloramphenicol for 4 h at 37°C. The temperature was then decreased to 18°C and the cells were grown overnight. The cells were harvested by centrifugation and the cell pellet was resuspended in resuspension buffer [200 mM NaCl, 20 mM HEPES pH 7.5, 5 mM magnesium acetate, 2 mM DTT, 2% (v/v) glycerol, 10 mg l⁻¹ lysozyme, 5 mg l⁻¹ DNase I] and subsequently lysed by sonication for 15 min. The lysate was centrifuged at 56 000g for 45 min to pellet the cell debris. The supernatant was applied to affinity chromatography using a column packed with 20 ml amylose resin (NEB). The average incubation time was 1 h. Two washing steps were then performed using 50 ml washing buffer [200 mM NaCl, 20 mM HEPES pH 7.5, 5 mM magnesium acetate, 2 mM DTT, 2% (v/v) glycerol] for each step. For elution, the amylose resin was incubated with 20 ml washing buffer supplemented with 20 mM maltose for 15 min. The MBP tag of the truncated *dmRab* constructs was cleaved off using TEV protease (1 mg ml⁻¹)

Table 3

Data collection and processing.

Values in parentheses are for the highest resolution shell.

	<i>dmRab2</i> ^{Q65L} –GMPPNP	<i>dmRab3</i> ^{Q80L} –GMPPNP
Diffraction source	BESSY 14.3	BESSY 14.3
Wavelength (Å)	0.895	0.895
Temperature (K)	100	100
Detector	Rayonix MX-225	Rayonix MX-225
Crystal-to-detector distance (mm)	230	145
Rotation range per image (°)	1.0	0.5
Total rotation range (°)	100	110
Exposure time per image (s)	5.2	12
Space group	<i>P</i> ₃ 2 ₁	<i>P</i> ₂ 1 ₂ 1
<i>a</i> , <i>b</i> , <i>c</i> (Å)	81.4, 81.4, 53.1	37.2, 80.5, 123.9
α , β , γ (°)	90.0, 90.0, 120.0	90.0, 90.0, 90.0
Mosaicity (°)	0.2	0.1
Resolution (Å)	50.00–2.00 (2.12–2.00)	50.00–1.50 (1.54–1.50)
Total No. of reflections	70633	222056
No. of unique reflections	13981 (2209)	60482 (4425)
Completeness (%)	99.8 (99.2)	99.9 (100.0)
Multiplicity	5.0 (5.0)	3.7 (3.7)
$\langle I/\sigma(I) \rangle$	7.9 (1.8)	12.9 (2.6)
$R_{\text{meas}}^{\dagger}$	0.216 (0.989)	0.082 (0.616)
$CC_{1/2}^{\ddagger}$	99.0 (72.4)	99.8 (75.0)
Wilson <i>B</i> (Å ²)	25.7	19.7

$\dagger R_{\text{meas}} = \sum_{hkl} \{N(hkl)/[N(hkl) - 1]\}^{1/2} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $\langle I(hkl) \rangle$ is the mean intensity of symmetry-equivalent reflections and $N(hkl)$ is the redundancy. $\ddagger$ The high-resolution cutoff was estimated using $CC_{1/2}$.

in the presence of 100 μM guanosine 5'-(β,γ -imido)triphosphate (GMPPNP; Jena Bioscience). The protease was added to the eluted protein at a molar ratio of 1:25 and the reaction was incubated at 4°C overnight. TEV-cleaved constructs were purified using a Superdex 75 26/60 column (GE Healthcare). The protein-containing fractions were pooled and concentrated using a 10 kDa molecular-weight cutoff concentrator (Millipore). The progress of protein purification was monitored by SDS–PAGE. Protein concentrations were determined by UV absorption with extinction coefficients $\epsilon(\text{Rab2}) = 21\,430\,\text{l mol}^{-1}\,\text{cm}^{-1}$ and $\epsilon(\text{Rab3}) = 32\,430\,\text{l mol}^{-1}\,\text{cm}^{-1}$, respectively.

2.3. Crystallization

For crystallization experiments, *dmRab2*^{Q65L} was concentrated to 50 mg ml^{−1} and *dmRab3*^{Q80L} to 40 mg ml^{−1} and they were incubated with equimolar concentrations of GMPPNP (Jena Bioscience) prior to crystallization. Crystals were obtained by the sitting-drop vapour-diffusion method at 291 K with drops consisting of 1 μl reservoir solution and 1 μl protein solution (Table 2). No additional cryoprotection was necessary for flash-cooling the crystals in liquid nitrogen.

2.4. Data collection and indexing, structure determination and refinement

Synchrotron diffraction data were collected on beamline 14.3 of the MX Joint Berlin laboratory at BESSY, Berlin, Germany. X-ray data collection was performed at 100 K. Diffraction data were indexed and processed with *XDS* (Kabsch, 2010; Table 3).

2.5. Structure solution and refinement

The structure of *dmRab3*^{Q80L} was solved by molecular replacement using *Phaser* (McCoy *et al.*, 2007) with the known structure of Rab3A from *Rattus norvegicus* (*rmRab3A*; PDB entry 3rab) as a search model (Dumas *et al.*, 1999). The structure of *dmRab2*^{Q65L} was solved by molecular replacement using our initially solved structure of *dmRab3*^{Q80L}. For the calculation of the free *R* factor, a randomly generated set of 5% of the reflections from the diffraction data sets was used and was excluded from the refinement. The structures were

Table 4

Structure solution and refinement.

Values in parentheses are for the highest resolution shell.

	<i>dmRab2</i> ^{Q65L} –GMPPNP	<i>dmRab3</i> ^{Q80L} –GMPPNP
Resolution range (Å)	42.4–2.0	38.3–1.5
Completeness (%)	99.8	99.7
No. of reflections, working set	132267	57442
No. of reflections, test set	699	3025
Final $R_{\text{work}}^{\dagger}$	0.167 (0.237)	0.157 (0.206)
Final $R_{\text{free}}^{\ddagger}$	0.224 (0.313)	0.194 (0.237)
No. of non H-atoms		
Protein	1412	2964
Mg ²⁺	1	2
GMPPNP	32	64
PEG	21	43
Water	113	378
Total	1579	3451
R.m.s. deviation		
Bond lengths (Å)	0.008	0.010
Bond angles (°)	1.10	1.26
Average <i>B</i> factors (Å ²)		
Protein	22.5	16.5
Mg ²⁺	19.6	9.1
GMPPNP	19.7	9.1
PEG	28.3	26.2
Water	28.4	28.7
Ramachandran plot§		
Outliers (%)	0	0
Favoured (%)	97.0	98.4

$\dagger R_{\text{work}} = \sum_{hkl} ||F_{\text{obs}}| - |F_{\text{calc}}|| / \sum_{hkl} |F_{\text{obs}}|$. $\ddagger R_{\text{free}}$ is the same as R_{cryst} but calculated using 5% of the data, which were excluded from refinement. $\S$ As calculated by *MolProbity*.

initially refined by applying a simulated-annealing protocol and in later refinement cycles by maximum-likelihood restrained refinement using *PHENIX* (Adams *et al.*, 2010; Afonine *et al.*, 2012). Model building and water picking was performed with *Coot* (Emsley *et al.*, 2010). The model quality was evaluated with *MolProbity* (Chen *et al.*, 2010) and *PROCHECK* (Laskowski *et al.*, 1993). Secondary-structure elements were assigned with *DSSP* (Kabsch & Sander, 1983). Final refinement statistics are given in Table 4. Figures were prepared using *PyMOL* (DeLano, 2002). The atomic coordinates and structure-factor amplitudes have been deposited in the Protein Data Bank under accession codes 4rke (*dmRab2*^{Q65L}) and 4rkf (*dmRab3*^{Q80L}).

2.6. Mass spectrometry

Protein masses were analyzed by matrix-assisted laser desorption/ionization–time of flight mass spectrometry (MALDI-TOF MS) using an Ultraflex-II TOF/TOF instrument (Bruker Daltonics, Bremen, Germany) equipped with a 200 Hz solid-state Smart beam laser. The mass spectrometer was operated in the positive linear mode. MS spectra were acquired over an *m/z* range of 5000–25 000 and data were analyzed using the *FlexAnalysis* 2.4 software provided with the instrument. The mass accuracy was estimated to be $\pm 1\%$ in the relevant mass range. Sinapinic acid was used as the matrix and samples were spotted using the dried-droplet technique undiluted and in a 1:5 dilution with 33% acetonitrile/0.1% trifluoroacetic acid.

3. Results

3.1. Expression, crystallization and structure determination

We prepared expression constructs of Rab2 and Rab3 from *D. melanogaster* comprising only the GTPase domain. In addition, we mutated the catalytically important glutamine to leucine, locking both *dmRab2* and *dmRab3* into the activated GTP-bound state (Der *et al.*, 1986; Privé *et al.*, 1992). To unravel the architecture of

dmRab2^{Q65L} and *dmRab3*^{Q80L}, we overexpressed both proteins in *E. coli* and subsequently purified and crystallized them. We could collect high-resolution data sets to 2.0 Å resolution for *dmRab2*^{Q65L} and to 1.5 Å resolution for *dmRab3*^{Q80L}. Whereas the *dmRab3*^{Q80L} crystals were indexed in space group *P*₂₁₂₁₂₁, the *dmRab2*^{Q65L} crystals belonged to space group *P*₃₁₂₁ (Tables 3 and 4). Both structures were solved by molecular replacement, locating two *dmRab3*^{Q80L} molecules and one *dmRab2*^{Q65L} molecule in the asymmetric unit. The structure of *dmRab2*^{Q65L} was refined to *R*_{work} = 0.167 and *R*_{free} = 0.224 and that of *dmRab3*^{Q80L} to *R*_{work} = 0.157 and *R*_{free} = 0.194 with excellent geometry. Data collection and refinement statistics are given in Tables 3 and 4. The electron density maps for both proteins were well defined; therefore, the model of *dmRab3*^{Q80L} could be built except for the 15 N-terminal and the two C-terminal amino acids of the construct. In addition, the structure of *dmRab2*^{Q65L} could also be completely modelled, including four amino acids of the N-terminal linker region that remained after TEV cleavage. The initial 2*mF*_o − *DF*_c and *mF*_o − *DF*_c electron density maps clearly revealed the localization of GMPPNP and Mg²⁺ (Fig. 2). Moreover, we

Table 5

Hydrogen-bonding interactions of GMPPNP in complex with *dmRab2*^{Q65L} and *dmRab3*^{Q80L}.

Distances ≤3.2 Å are given. Canonical interactions of the α- and β-phosphates of GMPPNP with the protein backbone of the P-loops are not listed.

GMPPNP		<i>dmRab2</i> ^{Q65L}	Distance (Å)	<i>dmRab3</i> ^{Q80L}	Distance (Å)
Guanine base	O6	Ala150 N	2.9	Ala165 N	2.9
	N1	Asp122 OD1	2.8	Asp137 OD1	2.8
	N2	Asp122 OD2	2.8	Asp137 OD2	2.9
	N7	Asn119 ND2	3.1	Asn134 ND2	3.2
Ribose	O2'	Gln32 O	2.8	Thr47 O	2.9
	O2'	Pro33 O	2.8		
	O3'	Pro33 O	3.2	Ser48 O	2.7
γ-Phosphate	O1G	Gly64 N	2.8	Gly79 N	2.8
	O2G	Thr38 N	3.0	Thr53 N	2.8
	O2G	Thr38 OG2	2.7	Thr53 OG2	2.9
	O3G	Thr15 OG2	2.6	Ser30 OG	2.7
	O3G	His35 NE2	2.9	Ser52 OG	2.6

observed electron density in a horseshoe shape that we could interpret as PEG fragments originating from the crystallization cocktail.

3.2. Overall structure

Both proteins are monomeric based on an interface analysis with the PISA server (Krissinel & Henrick, 2007) and in agreement with our experimental size-exclusion chromatography. *dmRab2*^{Q65L} and *dmRab3*^{Q80L} adopt the classical fold of the Rab family of GTP-binding proteins, with one β-sheet that is composed of six β-strands surrounded by five α-helices (Fig. 1). *dmRab2* and *dmRab3* share 33% sequence identity and 52% similarity. The overall folds are related to the Ras superfamily (Tong *et al.*, 1989; Pai *et al.*, 1989). *dmRab2*^{Q65L} and *dmRab3*^{Q80L} are practically indistinguishable, with a root-mean-square deviation (r.m.s.d.) of 0.8 Å for 157 pairs of C^α atoms. *dmRab2* shares 65% sequence identity and *dmRab3* shares 73% sequence identity with *mRab3A*. The reported structure of *mRab3A* in a GMPPNP-bound form (PDB entry 3rab) superimposes with *dmRab2*^{Q65L} and *dmRab3*^{Q80L} with an r.m.s.d. of 0.6 Å for 169 pairs of C^α atoms. In both structures the switch I and II regions are very well defined (Sprang, 1997; Kjeldgaard *et al.*, 1996) and are involved in a hydrogen-bonding network to stabilize the bound nucleotide and coordinate the Mg²⁺ cation (Fig. 1).

3.3. Nucleotide-binding site of *dmRab2*^{Q65L}

The guanine function of GMPPNP bound to *dmRab2*^{Q65L} is involved in hydrogen bonding to Ala150, Asp122 and Asn119. The hydroxyl functions are in contact with Gln32 and Pro33 (Table 5). The α- and β-phosphate are stabilized by interactions with the Walker A motif or P-loop (Saraste *et al.*, 1990; Walker *et al.*, 1982). This motif in *dmRab2*^{Q65L} is ¹³GDTDVDKS²⁰, with the catalytic Walker A lysine at position Lys19. The switch II region of exocytic Rab GTPase is highly conserved and is found within the region ⁶⁰WDTAGLE-AFRSITRSYYRGA⁷⁹ in *dmRab2*^{Q65L} (Fig. 1a). The γ-phosphate interacts with Thr15, His35 and Thr38 as well as Gly64 (Table 5). The Mg²⁺ ion is octahedrally coordinated by the hydroxyl functions of Ser20 and Thr38 of *dmRab2*^{Q65L}, the β- and γ-phosphate groups of GMPPNP and two water molecules (Fig. 1a and Table 5). The latter two water molecules are embedded in a dense hydrogen-bonding network including Asp36, Thr38, Asp61, Thr62 and the phosphate functions of GMPPNP.

3.4. Nucleotide-binding site of *dmRab3*^{Q80L}

The GMPPNP bound to *dmRab3*^{Q80L} establishes similar interactions with the protein as described above for *dmRab2*^{Q65L}. The

Figure 1

Structures of *dmRab2*^{Q65L} and *dmRab3*^{Q80L} drawn in cartoon representation. α-Helices are coloured blue, β-strands salmon and connecting loop regions brown. The bound GMPPNP is shown in stick representation, as are the Mg²⁺-coordinating residues. The octahedrally coordinated Mg²⁺ is depicted as a black sphere and coordinating water molecules as red spheres. Grey dashed lines indicate the coordination sphere of Mg²⁺. (a) Structure of *dmRab2*^{Q65L}; (b) structure of *dmRab3*^{Q80L}.

guanine base is hydrogen-bonded to Ala165, Asp137 and Asn134, and the hydroxyls of the ribose moiety are hydrogen-bonded to Ser48 and Thr47 (Table 5). The Walker A motif is established by the motif ²⁸GNSSVGKT³⁵, with the conserved Walker A lysine being Lys34. The side chains of *dmRab2* Lys19 and *dmRab3* Lys34 point to and interact with the O atoms of the β - and γ -phosphates of the bound GMPPNP and hence adopt the so-called 'conventional' conformation (Dikfidan *et al.*, 2014). The switch II region of *dmRab3*^{Q80L} comprises the sequence ⁷⁵WDTAGLERTITTAYYRGA⁹⁴ (Fig. 1b) and is shorter by two amino-acid residues compared with *dmRab2*^{Q65L}. This difference is reflected in a α -helix within the switch II region in the structure of *dmRab3*^{Q80L}. Switches I and II as well as the inter-switch regions are important in effector protein binding (Ostermeier & Brunger, 1999; Dumas *et al.*, 1999). In the GTP-bound state these regions are well ordered and expose a hydrophobic aromatic triad of residues to the protein surface. In concert with other residues, these residues are believed to define the specificity of different Rabs for different effector molecules (Merithew *et al.*, 2001). The triad is conserved in both *dmRab2*^{Q65L} and *dmRab3*^{Q80L}: Phe43, Trp60 and Tyr75 in *dmRab2*^{Q65L} and Phe58, Trp75 and Tyr90 in *dmRab3*^{Q80L}. All residues are solvent-exposed, with one exception, *dmRab2*^{Q65L} Tyr75, the side chain of which points towards the protein. The γ -phosphate of GMPPNP is hydrogen-bonded to Gly79, Thr53, Ser30 and Ser32 (Table 5). In the *dmRab3*^{Q80L} structure two threonine residues, Thr35 and Thr53, are involved in Mg²⁺ coordination as well as the γ -phosphate groups of GMPPNP and two water molecules (Figs. 1b and 2). The latter two water molecules are hydrogen-bonded to Thr35, Asp76, Thr77, Val51 and Thr53.

3.5. Modified cysteine in *dmRab3*^{Q80L}

The free thiol function of cysteine allows a large variety of modifications. Many post-translational modifications such as phosphorylation, S-nitrosylation, S-glutathionylation, sulphydration, sulfenylation, sulfinic acids, sulfonic acid polyprenylation and sulfenyl-amides are known (Walsh *et al.*, 2005; Chung *et al.*, 2013). On

the other hand, the thiol function is extensively exploited in the context of *in vitro* protein modifications such as fluorescent tagging, paramagnetic spin labelling and many more diverse applications. In the initially calculated electron-density maps we could observe additional electron density attached to Cys183 that resides on the very C-terminal α -helix of *dmRab3*^{Q80L}. The electron density has an approximately twofold rotational symmetry and has a planar shape (Fig. 3). The volume of the electron density is large enough to accommodate six atoms. The unknown electron density is located in a hydrophobic pocket on the surface of the protein formed by the side chains of Tyr20, Phe22, His66, Lys68, Val70 and Met186. The plane of the electron density is parallel to the phenolic ring of Tyr20 (Fig. 3), tentatively establishing a π -interaction with the unknown cysteine modification. Even though DTT was present in our purification buffers, the planarity and symmetry of the electron density rules out a mixed disulfide with DTT. To shed light on the modification, we performed MALDI-TOF MS of the *dmRab3*^{Q80L} protein prior to crystallization. The experimental molecular weight of 21 925 Da is in reasonable agreement with the calculated theoretical mass of

Figure 2
Nucleotide-binding site of *dmRab3*^{Q80L}. $2mF_o - DF_c$ simulated-annealing OMIT map contoured at 1σ shown as a blue mesh for the omitted GMPPNP and in violet for the Mg²⁺ ion. The GMPPNP is shown in stick representation and the Mg²⁺ ion is shown as black sphere.

Figure 3
Protein surroundings of Cys183 in *dmRab3*^{Q80L}. (a) $mF_o - DF_c$ electron-density map contoured at 3σ shown as a green mesh. Difference electron density with a planar shape is attached to the S atom of Cys183. (b) The view in (a) rotated by 45° .

21 939 Da. This raised the question of the point in time at which the modification is made. Hence, we washed and dissolved *dmRab3*^{Q80L} crystals in water and subjected them to MALDI-TOF MS. We could now see a mass difference of 135 Da compared with the theoretical mass of the protein. Consequently, the thiol modification must take place during the crystallization process. Next, we intended to identify the atom establishing the thiol linkage. We therefore collected a highly redundant, anomalous diffraction data set at 1.7 Å wavelength. We could detect the positions of most of the sulfur atoms of cysteine and methionine amino-acid side chains in the anomalous difference electron density, but no anomalous difference electron density for the atom covalently attached to the thiol of Cys183. It is tempting to speculate about the origin of the modification. Since the modification seems to be nearly complete for Cys183, the only source could be the crystallization cocktail. We can merely speculate that a degradation product of the precipitant PEG 200 might have caused the modification.

4. Discussion

We have determined the crystal structures of constitutively active *dmRab2*^{Q65L} and *dmRab3*^{Q80L} variants with bound Mg²⁺ and the nonhydrolysable GTP analogue GMPPNP to atomic resolution. Our structures provide information on the residues involved in Mg²⁺ coordination and interaction with GMPPNP. In the crystal structure of *dmRab3*^{Q80L} we detected a covalently attached modification at Cys183. The latter modification remains enigmatic and hence has not been modelled in the crystal structure of *dmRab3*^{Q80L}.

Our structure of *dmRab2*^{Q65L} represents the first crystal structure of a Rab2 protein in the 'GTP'-bound active state and allows comparison with the structure of *mRab2A* (PDB entry 1z0a; Eathiraj *et al.*, 2005) in the GDP-bound state. In the structure of *mRab2A*-GDP the switch I region adopts different conformations in the four copies within the asymmetric unit. In three copies (chains A, B and D) the switch I region is not defined in the electron-density maps and hence is lacking from the model, whereas in one copy (chain C) the entire switch I region could be modelled but with truncated side chains, indicating increased flexibility. *dmRab2*^{Q65L} and *mRab2A* share 94% identity and the structures superimpose with an r.m.s.d. of 1.7 Å for 165 pairs of C α atoms of chain C, whereas for chain A, which lacks residues Pro33–His35, the r.m.s.d. is 1.4 Å for 166 pairs of C α atoms. The different conformations are likely to be influenced by crystal packing. Major structural differences between *dmRab2*^{Q65L} and *mRab2A* are observed in the switch I and II regions (Fig. 4a). In the structure of *dmRab2*^{Q65L} presented here the switch I region is well defined in the electron density and establishes the expected interactions with the hydroxyl functions of the ribose and the γ -phosphate of the GMPPNP nucleotide (Table 5). Even though the switch I region of *dmRab2*^{Q65L} is involved in crystal packing, our structure strongly suggests that the switch I region adopts the conformation of the active state of *dmRab2*. The switch II region undergoes a more drastic conformational change (Fig. 4a). By this conformational change it establishes contact with the γ -phosphate of the GMPPNP (Table 5). Again, the observed conformation of the switch II region could be potentially involved in crystal contacts.

dmRab2 and *dmRab3* share 33% sequence identity and the structures of the proteins are nearly identical, with an r.m.s.d. of 0.8 Å for 157 pairs of C α atoms (Fig. 4b). Whereas the conformation of the switch I region of both proteins is very similar, the conformation of the switch II region is altered. In *dmRab3*^{Q80L} we observe a α -helix of six residues in length from Arg84 to Ala89 (Fig. 1b). In the structure

of *dmRab2*^{Q65L} the switch II region adopts a random-coil conformation. Since the switch region is involved in crystal contacts in both structures, we cannot fully exclude a possible influence on their conformation. These observed alterations, especially within the switch II regions, hint at the capability of *dmRab2* and *dmRab3* to bind to different effector proteins.

A potential interaction partner of Rab3A is Bruchpilot, since upon loss of Rab3 Bruchpilot is dramatically reduced (Graf *et al.*, 2009). Bruchpilot acts as one of the main scaffolding proteins that decorate the intracellular face of the active zone in *Drosophila* where synaptic vesicles fuse with the membrane (Haucke *et al.*, 2011; Kittel *et al.*, 2006; Liu *et al.*, 2011). Bruchpilot is critical for the structural integrity and functionality of the active zone. Graf and coworkers showed that the Rab3 GTPase is essential for correct assembly of the active zone in *Drosophila* (Graf *et al.*, 2009). Owing to their described properties and function in vesicular transport, the question arose of whether Rab GTPases might interact with active zone proteins in synaptic vesicle tethering. In future experiments, we would like to shed light

Figure 4
Superposition of different Rab structures shown in ribbon presentation. (a) Superposition of *mRab2A* (PDB entry 1z0a, chain A) bound to GDP and *dmRab2* bound to GMPPNP. The switch I and switch II regions of *mRab2A*-GDP are coloured purple and orange, respectively, whereas the switch I and switch II regions of *dmRab2A*-GMPPNP are coloured light blue and red, respectively. (b) Superposition of *dmRab2A* (grey ribbon) and *dmRab3A* (black ribbon) both in the GMPPNP-bound state. The switch regions of *dmRab2A* are coloured as in (a). The switch I and switch II regions of *dmRab3A* are coloured green and pink, respectively.

on these possible interactions by using our Rab GTPase constructs in pull-down experiments.

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