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ORIGINAL PAPER

# Comparative modeling of Rab6 proteins: identification of key residues and their interactions with guanine nucleotides

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**Abstract** The cytoplasm of a eukaryotic cell consists of a wide variety of membrane bound cell organelles and continuous flow of proteins amongst these organelles is a major challenge and must be stringently maintained in order to continue the correct biochemical functioning inside a cell. The transportation of various proteins amongst these organelles is facilitated by a vast Tubulo-vesicular network mediated by carrier proteins. The Rabs belong to small G proteins super family involved in the regulation and vesicle transport in between the organelles by shuttling between the active GTP and inactive GDP bound states. In this paper we put forth the homology modeling and docking studies of Rab6A proteins (*Mus musculus, Gallus gallus* and *Caenorhabditis elegans*) with GTP, GMP-PNP and GDP molecules and a comparative study between these proteins is done to identify key residues out of which serine of the phosphate binding loop (P – loop) and aspartic acid showed prominent interactions with the GTP, GDP and GMP-PNP nucleotides and cogitate that aspartic acid might also help in the stabilization of the switch I region of the Rab proteins besides serine.

**Keywords** GDP · GMP-PNP · GTP · FlexX · Modeller9.10 · Rab6 · Sybyl · Vesicle trafficking

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## Introduction

The high complexity of the cell structure and its components is a characteristic feature of any eukaryotic cell. The cytoplasm of a eukaryotic cell consists of wide variety of membrane bound cell organelles, each carrying out specific functions inside the cell. The continuous flow of proteins amongst these organelles is a major challenge that all organelles must stringently maintain in order to continue the correct biochemical composition and functioning inside the cell. The transportation of various proteins amongst these organelles is facilitated by a vast tubulovesicular network mediated by carrier proteins as established by different research laboratories and till date continues to be one of the key areas of research. There are two different kinds of transport mechanisms namely the exocytosis pathway – transport of synthesized proteins from the endoplasmic reticulum (ER)/ Golgi apparatus to the lysosome or plasma membrane and the endocytic pathway- transport of nutrient material from the exterior of the cell into early endosome where the nutrients are processed accordingly and the proteins required for further processing are subsequently transported to the late endosome and then to the lysosome/ vacuole [1]. The transport of cargo materials takes place in four steps, firstly the assortment of cargo material and subsequent packing into target specific vesicles followed by the transport of these vesicles via molecular motors on the cytoskeletal components, i.e., either on microtubules or actin filaments, thirdly careful tethering by tethering complexes facilitates the vesicles and the target membrane approximation followed by the final step of fusion with the target membrane via the action of soluble NSF attachment

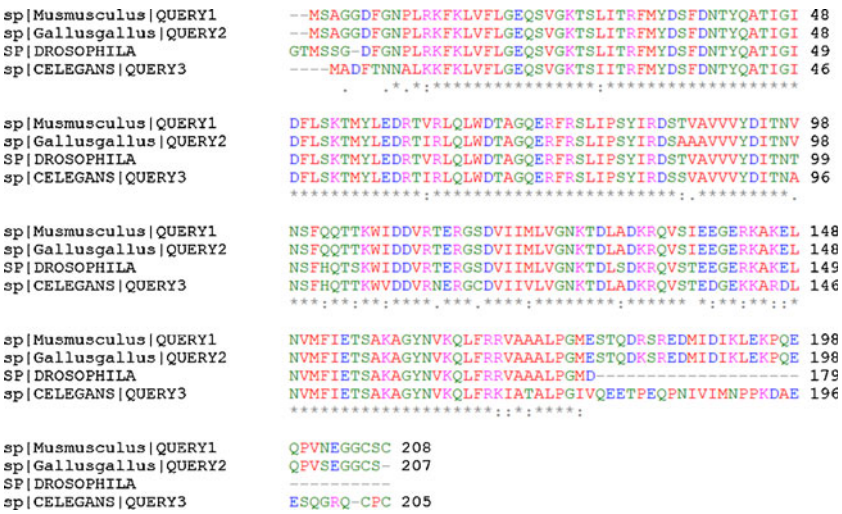


**Fig. 1** sequence alignment of *Drosophila, Mus musculus, Gallus gallus* and *Caenorhabditis elegans* Rab6A sequences using Clustal W

protein receptors (SNAREs) along with its respective associated machinery [2]. Although it is generally accepted that the transportation of cargo material is based on vesicle trafficking between the organelles, relatively very little is known about the actual mechanism of how the cargo is specifically identified? How these vesicles are transported on the cytoskeletal network? How tethering, fusion and docking of the cargo material takes place via SNARE complexes? It has been observed that in all these steps the Rab GTPases family of proteins plays a key role by acting as "molecular switches" to control formation, transfer, tethering and fusion of vesicles between the organelles [2].

*The Rab GTPases* Rabs are ubiquitous form of proteins belonging to small G proteins super family [1] associated with the Golgi complex [3, 4], evidence of the Rab proteins

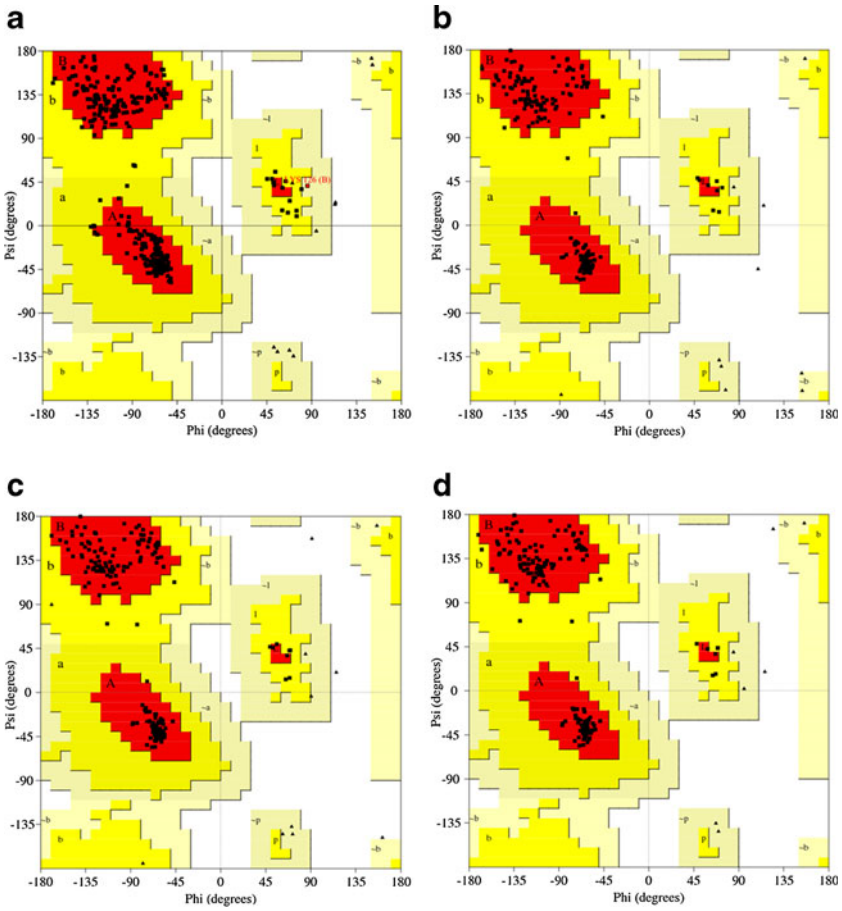


**Fig. 2** Ramachandran plots of **a** *Drosophila melanogaster*, **b** *Mus musculus* **c** *Gallus gallus* and **d** *Caenorhabditis elegans* obtained from PROCHECK software showing Psi and Phi bond angles

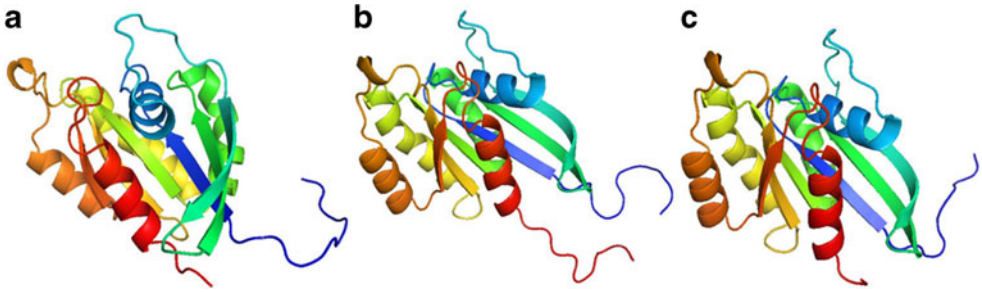


**Fig. 3** Modeled structures of **a** *Mus musculus*, **b** *Gallus gallus* and **c** *Caenorhabditis elegans* by Modeller 9.10 based on the crystal structure of *Drosophila melanogaster*, all these proteins show typical 6 stranded β sheet and 5 α-helices structure as observed in other Rab and Ras family of proteins

involvement in regulation and vesicle transportation in between the organelles was first obtained from the yeast - *S. cerevisiae* [5–7] and subsequently in many other systems as well. To date 11 forms of Rabs are identified in yeast and over 60 have been indentified in humans and about 16 different crystal structures Rab proteins exist either with inactive GDP or active GTP bound states [1, 8]. These monomeric Ras like proteins of 20–29 KDa size [9] shuttle between active GTP and inactive GDP bound states controlled with the help of guanine nucleotide exchange factors (GEFs), triggering the fastening of GTP and GTPases activating proteins (GAPs) and subsequent accelerated hydrolysis of GTP into GDP [10].

Although many Rab crystal structures are determined by various research groups, we primarily focused on the Rab6 family which are linked with the Golgi and trans Golgi network (TGN) membranes, wherein well established evidence suggests that they are involved with the regulation of COPI – independent Golgi to ER trafficking [11, 12]. In this paper we put forth the homology modeling and docking studies of Rab6A proteins (*Mus musculus, Gallus gallus* and *Caenorhabditis elegans*) with GTP, GNP-PNP and GDP molecules and also we have observed that not only the serine residue of the phosphate binding loop (P – loop) but also aspartic acid showed prominent interactions with the GTP, GDP and GMP-PNP nucleotides and might help in the stabilization of the switch I region of the Rab proteins.

## Materials and methods

### Sequence alignment, comparative protein modeling and model validation

The primary Rab6 protein sequences of mouse (*Mus musculus* - accession ID: P35279**)**, Chicken (*Gallus gallus* - accession ID: Q1KME6), and *Caenorhabditis elegans* (accession ID: P34213**)** were obtained from the SIB ExPASy Bioformatics Resources Portal [13]. To find related protein templates to build models for these primary sequences, a sequence similarity search was done individually by using Protein BLAST tool against solved protein structures deposited in Protein Data Bank (PDB). Although the search yielded many highly similar sequences the *Drosophila melanogaster* Rab6A chain; PDB entry: 2Y8E [14] was chosen

**Table 1** Ligand binding pockets as detected by the SiteID module of Tripos Sybyl 6.7 software

| *Caenorhabditis elegans* | | | *Gallus gallus* | | | *Mus musculus* | | | |
|---|---|---|---|---|---|---|---|---|---|
| Pocket 1: | Pocket 2: | Pocket 3: | Pocket 1: | Pocket 2: | Pocket 3: | Pocket 1: | Pocket 2: | Pocket 3: | Pocket 4: |
| Ser 21 | Phe 12 | Ile 28 | Thr 27 | Ile 30 | Phe 14 | Gly 25 | Phe 33 | Ile 63 | Ile 30 |
| Gly 23 | Phe 31 | Leu 49 | Ser 28 | Tyr 35 | Phe 167 | Lys 26 | Met 34 | Ile 79 | Thr 31 |
| Lys 24 | Leu 55 | | Thr 31 | Leu 51 | | Thr 27 | Leu 57 | Trp 107 | Leu 51 |
| Thr 25 | Leu 62 | | Phe 38 | | | Ser 28 | Val 62 | Asp 110 | |
| Ser 26 | Phe 165 | | Asp 39 | | | Thr 31 | Leu 64 | | |
| Phe 36 | Ala 169 | | Tyr 42 | | | Phe 38 | Phe 167 | | |
| Asp 37 | | | Gln 43 | | | Asp 39 | Arg 168 | | |
| Tyr 40 | | | Thr 45 | | | Tyr 42 | | | |
| Gln 41 | | | Lys 158 | | | Gln 43 | | | |
| Thr 43 | | | | | | Thr 45 | | | |
| Gly 69 | | | | | | Asp 68 | | | |
| Lys 156 | | | | | | Asp 129 | | | |
| | | | | | | Ala 157 | | | |
| | | | | | | Lys 158 | | | |

to build models for the above sequences since the *Drosophila* sequence has the best known crystal resolution of 1.4 Å besides good sequence similarity. A comparative sequence alignment was then done with *Drosophila* template using Clustal X tool and online Clustal W tools [15].

MODELLER 9.10 was then used to gain satisfactory models; an automated approach to comparative modeling by satisfaction of spatial restrains [16, 17]. After manually modifying the alignment input file in MODELLER 9.10 to match the template and query sequence, 20 models were generated and were then minimized using the molecular dynamics and simulation procedure CHARMM in MODELLER for each of the primary sequences [17] out of which the models with least Modeller objective function were then chosen. These models were then checked in detail for the protein structure stereochemistry by using PROCHECK [18], which generates Ramachandran plots and comprehensive residue by residue listing facilitates the in depth assessment of Psi/Phi angles and the backbone conformation of the models.

### Docking studies

The GTP, GMP-PNP and GDP molecules were manually drawn using Silicon Graphics 02 – Tripos SYBYL 6.7 software and were minimized after adding Hydrogens to their most appropriate conformation using the Powell method and Gasteiger - Huckel charges. These molecules were then added to a database for FlexX docking in SYBYL 6.7. Hydrogen's were added to the modeled proteins and possible active sites were predicted using SiteID of SYBYL software which predicts and identifies the potential binding sites by mixing and matching aspects such as depth, exposure, temperature factor, hydrophobicity, solvent accessible surface and hydrogen-bonding capability, the molecules were then docked into the predicted active site of the proteins using FlexX docking studies which docks the ligands based on the incremental construction algorithm that combines a strategy of efficient methods for overlap detection and for the search of new interactions [19]. Default parameters were used in FlexX along with some minor changes which will be discussed later in the results and discussions section.

## Results and discussion

### Sequence alignment and homology modeling of Rab6

The Rab family of proteins shows highly conserved amino acids and only a small heterogeneity can be seen in the switch domains of the active form of Rab proteins [2]. After protein BLAST of the primary sequences of mouse, chicken

**Table 2** Mouse, chicken and *C. elegans* Rab6 protein amino acids interactions with GTP, GDP and GMP-PNP and their respective docking scores. (Representations in the table: Amino acids showing interactions with only: a) GDP and GTP (represented in **bold**); b) GTP and GMP-PNP (represented in *italics*); c) GDP and GMP-PNP (represented in ***bold italics***)

| Organism and docking scores | Interactions with GTP | Interactions with GDP | Interactions with GMP-PNP |
|---|---|---|---|
| | *Ser28* | ***Gly25*** | ***Gly25*** |
| *Mus musculus* | Thr31 | ***Lys26*** | ***Lys26*** |
| GTP: -17.2 | Asp39 | ***Thr27*** | ***Thr27*** |
| GDP: -23.5 | **Gln43** | Asp39 | *Ser28* |
| GMP-PNP: -16.4 | *Asp129* | **Gln43** | Asp39 |
| | Lys158 | | Tyr42 |
| | | | *Asp129* |
| | Thr25 | Thr25 | Thr25 |
| *Caenorhabditis elegans* | Ser26 | Ser26 | Ser26 |
| GTP: -17.7 | Asp37 | Asp37 | Asp37 |
| GDP: -14.3 | Gln41 | Gln41 | Gln41 |
| GMP-PNP: -13.8 | | Thr43 | |
| | *Thr27* | Ser28 | *Thr27* |
| *Gallus gallus* | Ser28 | Thr31 | Ser28 |
| GTP : -26.3 | Thr31 | Asp39 | Thr31 |
| GDP: -19.9 | Tyr35 | Gln43 | Asp39 |
| GMP-PNP: -26.2 | Asp39 | | Tyr42 |
| | Gln43 | | Gln43 |
| | | | Thr45 |

**Table 3** *Drosophila melanogaster* Rab6 protein amino acids interactions with GTP and GDP molecules (represented in **bold**) in comparison to the interactions as observed in the *Drosophila* crystal structure (PDB ID: 2Y8E)

| Organism and docking scores | Interactions with GTP | Interactions with GDP | Interactions with GMP-PNP according to the PDB - 2Y8E crystal structure |
|---|---|---|---|
| | Lys25 | | Lys25 |
| *Drosophila melanogaster* | Thr26 | **Ser27** | Thr26 |
| GTP: -37.0 | **Ser27** | **Asp38** | Ser27 |
| GDP: -28.9 | **Asp38** | Asn39 | Asp38 |
| | Tyr41 | **Asn125** | Asn39 |
| | Thr44 | **Asp128** | Tyr41 |
| | Gly70 | **Ala156** | Thr44 |
| | **Asn125** | **Lys157** | Gly70 |
| | **Asp128** | | Asn125 |
| | **Ala156** | | Asp128 |
| | **Lys157** | | Ala156 |
| | | | Lys157 |

and *C. elegans* with the predetermined structures deposited in PDB bank, the *Drosophila* sequence (PDB entry: 2Y8E [14]) showed greatest similarity (see Fig. 1 for sequences alignment), and the primary reason for selecting the crystal structure sequence of *Drosophila* is its low resolution of 1.4 Å which no other determined Rab6 crystal structures possess, hence the models built for all the three primary sequences based on the predetermined crystallographic structure of *Drosophila* can be considered to be much closer to their native configuration and as well as the models generated will be of high quality. Twenty models were generated using the MODELLER 9.10 program based on the sequence alignment files generated by ClustalX program. The alignment file was tweaked manually to best fit the sequences. Out of the generated models for all the primary sequences, the model with the Least object function were selected for further evaluation for protein stereochemistry (phi and psi angles) with PROCHECK software.

The PROCHECK software generates a number of files which list complete residue by residue data and the assessment of the overall quality of the generated structure as compared to well refined structures of the same resolution

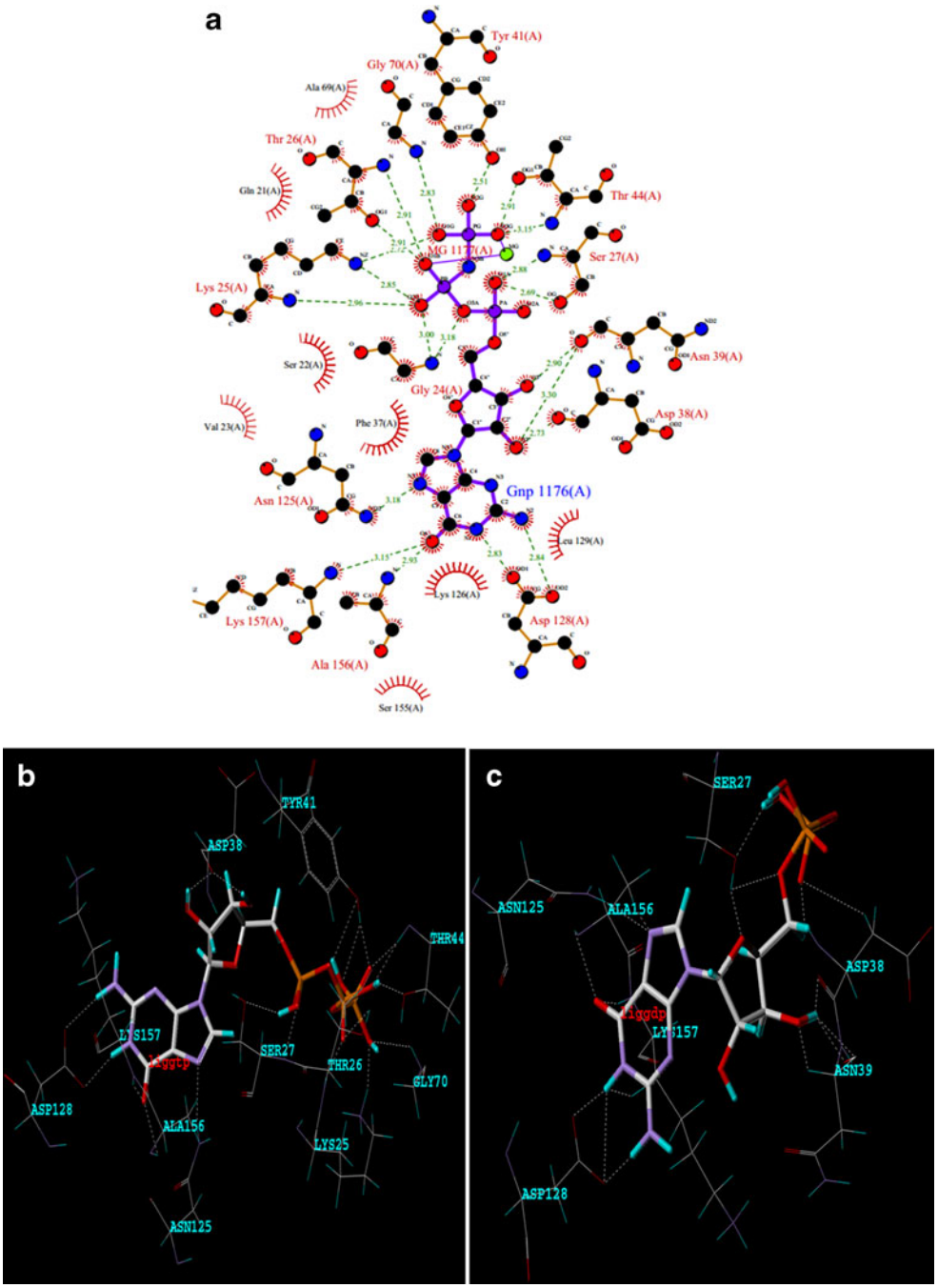


**Fig. 4** **a** Amino acid interactions as shown in the *Drosophila melanogaster* crystal structure [14]; **b** Predicted amino acid interactions between GTP and Rab6 protein of *Drosophila* using FlexX dock; **c** Predicted amino acid interactions between GDP and Rab6 protein of *Drosophila* using FlexX dock. *Note*: The following representations were used for representing ligands – liggtp - GTP ligand molecule; liggdp - GDP ligand molecule; liggmppnp - GMP-PNP ligand molecule

[18]. The Ramachandran plot of the *Drosophila* template shows 289 amino acid residues (93.5 %) in most favorable regions with 19 amino acid residues (6.1 %) falling into additionally allowed regions and with one amino acid residue (0.3 %) falling into the generously allowed regions but whereas for the modeled structures, 178 amino acid residues (95.2 %) in mouse, 178 amino acid residues (95.2 %) in chicken and 175 amino acid residues (94.1 %) in *C. elegans* were observed to fall into most favored regions with nine amino acid residues (4.8 %) in mouse, nine amino acid residues (4.8 %) in chicken and 11 amino acid residues (4.8 %) in *C. elegans* are in additionally allowed regions and with no amino acids falling into either generously allowed regions or disallowed region, these results clearly indicate that the generated models are much more sophisticated and more conformationally better than the template *Drosophila* structure. (See Fig. 2 for Ramachandran plots of *Drosophila*, mouse, chicken and *C. elegans)*.

The striking feature of all Ras and Rab super family proteins is its GTPase fold, which is made up of six stranded β-sheet with five α-helices present on its sides. The GTPase region -COOH terminal is a hyper variable region followed by CAAX boxes consisting of two cysteine residues that are covalently bonded to the Geranylgeranyl moieties and help in the membrane insertion of Rabs [2]. It can be said that due to the highly conserved structure, the specific functions of each Rab can be determined by its active and inactive states. The nucleotide dependant Rab function is based on the

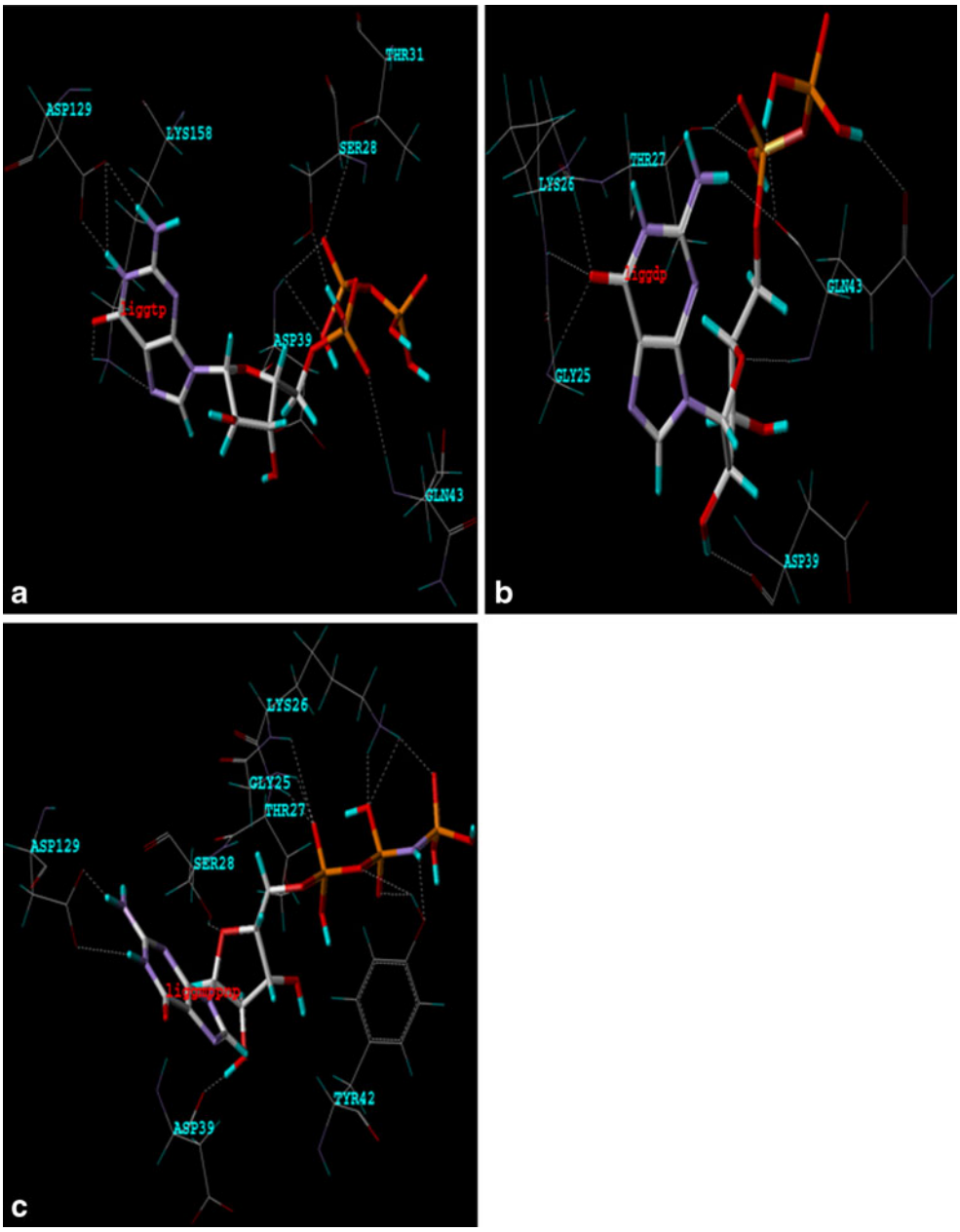


**Fig. 5** Mouse Rab6 protein amino acids showing interactions with **a** GTP, **b** GDP and **c** GMP-PNP

switch regions I and II that make contact with the γ-phosphate of GTP. The switch regions tend to organize based on the GDP/GTP moiety it binds to, i.e., these regions are well ordered structurally when GTP binds to it and vice versa when GDP binds to it and also upon juxtaposing of different GTP bound active Rab structures the greatest structural diversity can be seen in these switch domains especially in the α3/β5 loop lying next to switch II region [2, 8] and these differences can explain the definite recruitment of effectors and regulation of their relevant pathways[8, 20, 21].

The models that we generated for mouse, chicken and *C. elegans* also showed typical 5 α-helices and 6 stranded β sheets with one anti parallel strand, and is consistent with observed experimental data further validating the generated models [2, 14, 20, 22–24] (See Fig. 3).

## Docking studies of modeled Rab6 proteins

The active site for docking was predicted using Tripos Sybyl SiteID module after adding hydrogen atoms to the modeled proteins. The SiteID module predicts and identifies the potential binding sites by combination and correlating key criterion such as depth, exposure, temperature factor, hydrophobicity, solvent accessible surface and hydrogen-bonding capability. Table 1 shows the ligand binding pockets as predicted by SiteID module using the above said factors.

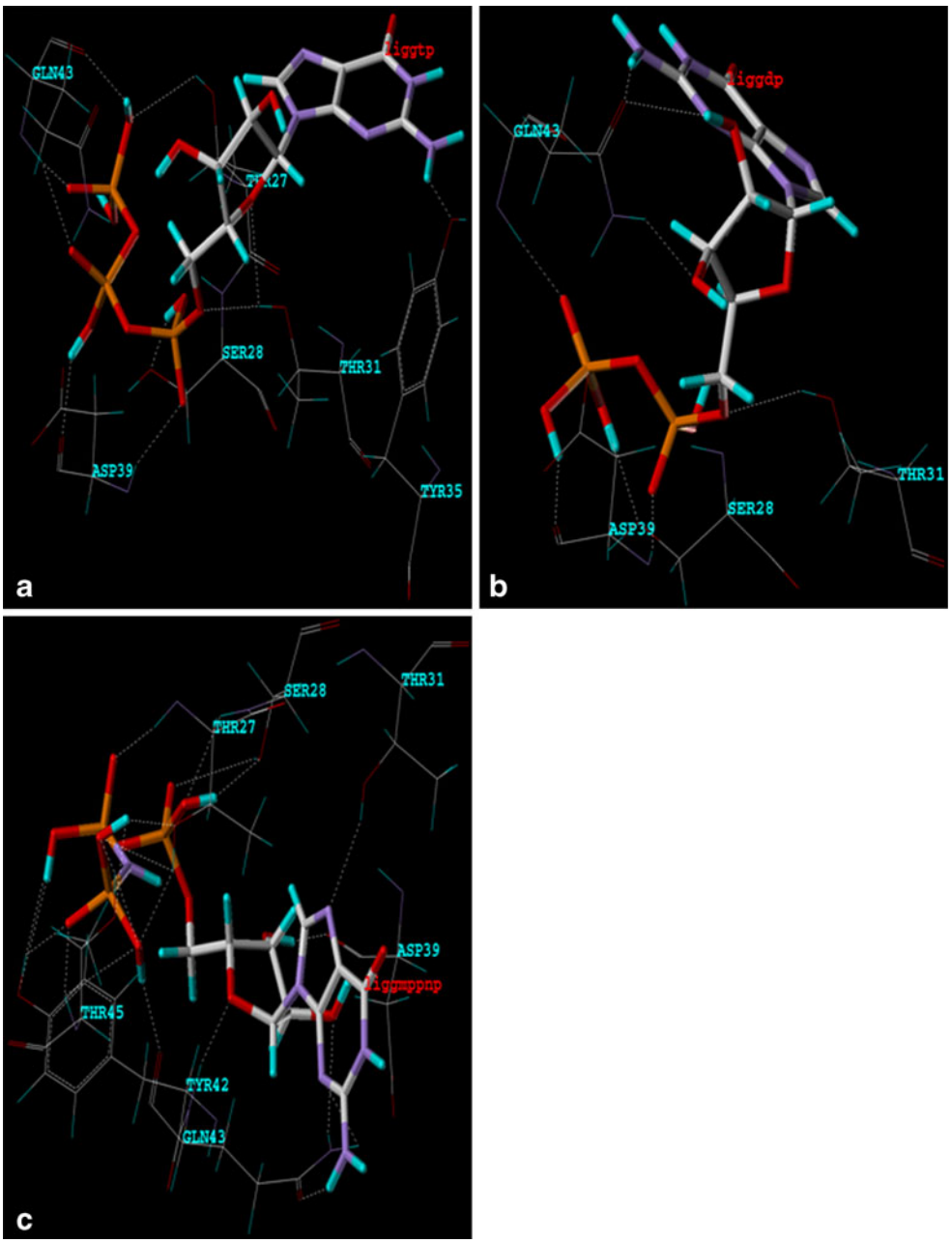


**Fig. 6** Chicken Rab6 protein amino acids showing interactions with **a** GTP, **b** GDP and **c** GMP-PNP

While detecting the potential binding sites the SiteID module generates necessary files required for performing FlexX docking studies. For the FlexX docking module the receptor descriptor files (RDF file) generated by the SiteID module plays a very important role as it contains information related to the protein's amino acids and their torsion angles besides active site parameters. The FlexX module works on formal charges rather than the partial atomic charges hence during the docking procedure formal charges were assigned to the proteins and their respective active site pockets as predicted by the SiteID module were selected within 3 Å radius of the ligand. By taking these parameters into account and other default parameters in the FlexX module the GTP, GDP and GMP-PNP ligand molecules were docked into the three Rab6 proteins. The FlexX module during the docking procedure selects only the best fit active site pocket with respect to the ligands in order to dock them. Table 2 below shows Rab6 protein amino acids interacting with GTP, GDP and GMP-PNP.

It can be seen from Table 2 that in chicken, amino acids Ser28, Thr31, Asp39 and Gln43 show interactions with all the

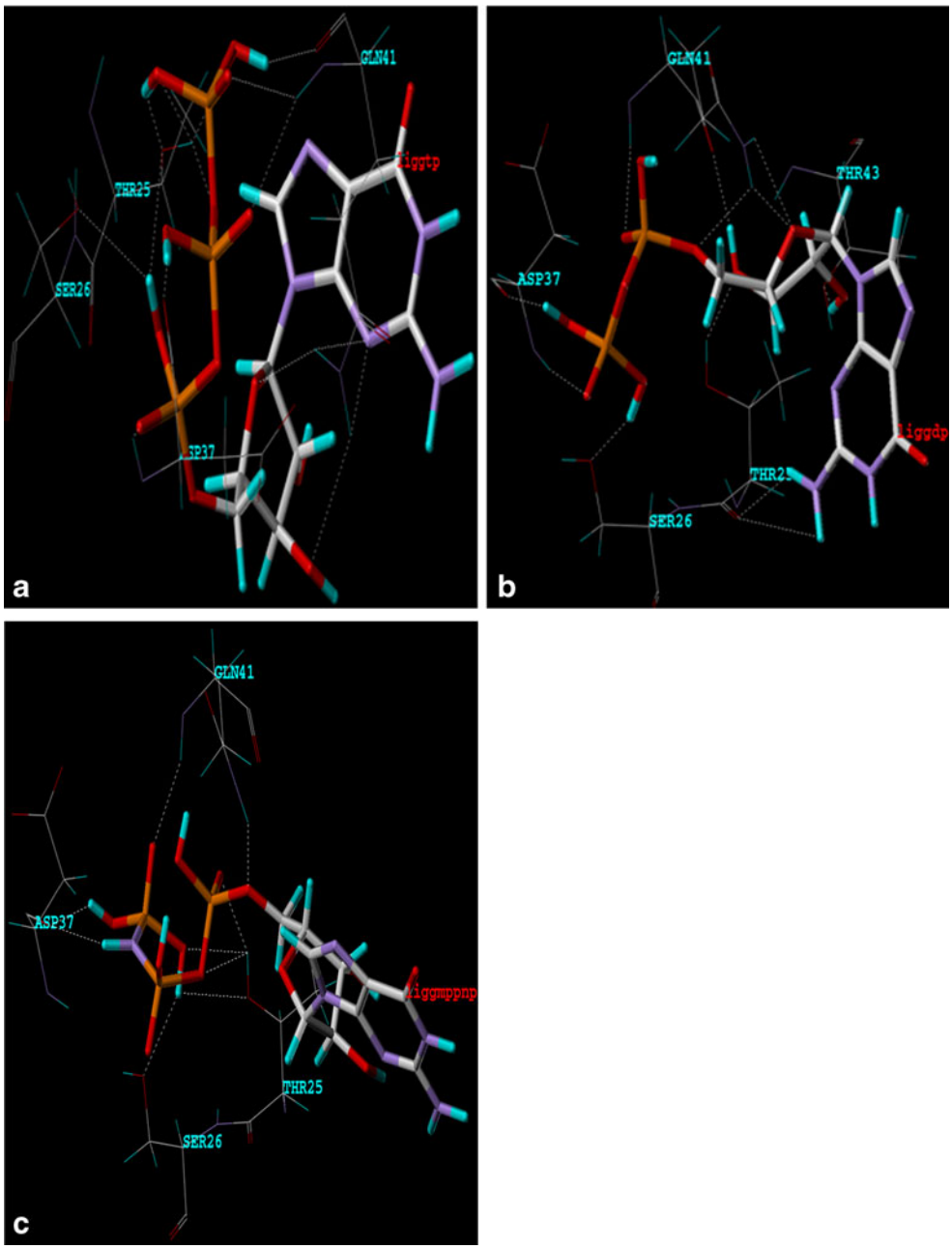


**Fig. 7** C. elegans Rab6 protein amino acids showing interactions with **a** GTP, **b** GDP and **c** GMP-PNP. In all these pictures it can be seen that Serine residue of the P-loop and aspartic acid shows consistent binding with the ligands

ligands except for Thr27 which shows interactions only with GTP and GMP-PNP molecules. In the case of *C. elegans* residues Thr25, Ser26, Asp37 and Gln41 seem to show interactions with all three ligands with no specific amino acids showing interactions with only GTP or GMP-PNP but unlike the above two cases in mouse amino acids Ser28, Asp129 shows interactions with GTP and GMP-PNP, Gln43 with GTP and GDP and amino acids Gly25, Lys26, Thr27 show interactions with GDP and GMP-PNP molecules only, which might suggest that cofactors like $Mg^{2+}$ ion might not only be required for specific binding to GTP or GMP-PNP but could also help the nucleotides to bind much more efficiently with the protein. Also from Table 2 it can be clearly seen that Asp39/ Asp37 (underlined in Table) is the only common amino acid that shows consistent interactions with all three ligands GTP, GDP and GMP-PNP in mouse, chicken and *C. elegans* which could suggest that the Asp38/37 rather than helping in GTPase activity, it might help in stabilizing the switch I region.

We have also docked GTP and GDP molecules into the A chain of the *Drosophila* Rab6 protein to find out the possible interactions. We can see from Table 3 that almost all the interactions with GTP moiety are the same except for Asn39 which bonds with the ribose group of GMP-PNP moiety [14] (see Fig. 4) and also amino acids Ser27, Asp38, Asn39, Asn125, Asp128, Ala156 and Lys157 are the only ones showing interactions with the GDP moiety thereby confirming the *in silco* methods that we used for docking the ligands in all the Ra6 proteins showed reliable results and are analogous with the crystallographic experimental data [14, 22, 24].

### Conserved residues in the P-binding loop

It is observed in Rab3A and in many Rab GTPases involved in the regulation of exocytic vesicles trafficking pathways that the serine residue of P - loop is highly conserved and plays a key role in the stabilization of the switch I region, although it is variable among the Rabs involved in endocytic pathways and is not present in the P-loop [23], and mutation of Ser31 with glycine increased the GTPase activity several fold [23, 25] while simple substitution of Gly12 to Ser in Ras protein disrupted its basic function implying that serine residue would rather help in stabilizing the active conformation of the switch I region than help in GTPase activity.

This data correlates with the data we generated and we observed the same Ser26 (*C. elegans*), Ser27 (*Drosophila*) and Ser28 (mouse and chicken) residues binding to the gamma phosphate of the GTP and GMP-PNP moieties when bound to Rab6, and not only this we observed Asp37 (*C. elegans*), Asp38 (*Drosophila*) and Asp39 (mouse and chicken) also showed consistent binding with GTP, GDP and GMP-PNP moieties (see Fig. 5, 6, and 7) and believe Asp might as well help in stabilizing the switch I region in Rab proteins, although to date no experimental evidence exists on whether or not aspartic acid is involved in stabilization, further investigation and crystallographic evidence is required to address it and as well as to further elucidate the mechanism of vesicle trafficking inside the cell.

## Conclusions

In conclusion 3D molecular models for Rab6 GTPases of *Mus Musculus, Gallus gallus* and *Caenorhabditis elegans* that are involved with Golgi and trans Golgi network (TGN) membranes protein trafficking are built and refined. The valuation results by PROCHECK program indicated that the generated models are reliable and are analogous with the already established structures of other Rab proteins.

The docking results with GTP, GDP and GMP-PNP with Rab6 proteins of *Mus Musculus, Gallus gallus* and *Caenorhabditis elegans* and docking results with GTP and GDP with Rab6 protein of *Drosophila melanogaster* showed many similar residues binding with the ligands, especially the residue serine which is one of the highly conserved residues in Rab GTPase family that helps in stabilizing the active conformation of the switch I region, serine showed prominent interactions with the γ–phosphate of GTP and GMP-PNP moieties and we think that aspartate is another residue that might also help in stabilizing the conformation the switch I region due to it showing consistent interactions with GTP, GDP and GMP-PNP molecules. The detailed 3D structure, interaction information and the key residues identified are helpful for guiding the site-directed mutagenesis investigation and understanding the vesicle trafficking mechanism inside the cell.

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