INVESTIGATION OF THE FUNCTIONAL MECHANISM OF SMALL G PROTEIN RAN

PhD thesis

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1. Introduction

The small GTPase Ran (**Ra**s-related **N**uclear protein) is a key regulator of nucleocytoplasmic transport, mitotic spindle assembly, and nuclear envelope reformation. By modulating importins and exportins, Ran ensures proper cargo delivery across nuclear pore complexes (NPCs). Disruption of Ran signaling results in spindle defects, mitotic failure, and chromosomal instability. Notably, Ran is frequently overexpressed in many human cancers, while its loss is well tolerated in normal cells, highlighting its prognostic and therapeutic potential. This study, therefore, aims to investigate Ran's structural features and dynamic behavior using simulation-based approaches.

Ran belongs to the Ras superfamily, a group of 20-30 kDa monomeric GTP-binding proteins that function as molecular switches. These proteins cycle between inactive GDP-bound and active GTP-bound states to regulate signaling pathways controlling growth, differentiation, cytoskeletal organization, and intracellular transport. Their activity is regulated by guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs), and other modulators that coordinate spatial and temporal signaling.

Within the Ras superfamily, Ran uniquely governs nucleocytoplasmic transport. Directionality depends on a RanGTP/RanGDP gradient, maintained by RCC1 in the nucleus and RanGAP1 in the cytoplasm.

Structurally, Ran contains a conserved G-domain with a phosphate-binding P-loop, switch I and II regions that undergo conformational changes upon nucleotide exchange, and a distinctive C-terminal helix that regulates interactions depending on its GDP- or GTP-bound state.

2. Objectives

Objective 1: Exploration of Structural Dynamics of RanGDP and RanGTP

We aim to characterize and compare the structural and dynamic properties of Ran in its GDP- and GTP-bound states, with particular emphasis on conformational flexibility, nucleotide-dependent behavior, and determinants of nucleotide specificity, using MD and aMDeNM simulations.

Objective 2: Impact of C-terminal movement of RanGTP on ligand binding

We aim to investigate the functional implications of the highly flexible, frequently unresolved C-terminal region of RanGTP and determine how its conformational mobility influences ligand recognition and binding.

Objective 3: Investigation of switch I opening of RanGTP

We aim to define and characterize the molecular mechanism underlying switch I opening in RanGTP by reproducing the observed conformational transition in simulations and identifying the key interactions and perturbations that trigger this process.

3. Methods

3.1. Structure preparation

The starting structures of RanGDP (PDB ID: 5CIQ) and RanGTP (PDB ID: 5CLL) were obtained from the Protein Data Bank. Because the RanGTP structure lacked C-terminal residues (185–207), this segment was modeled using coordinates from PDB ID 5CLQ, aligned via residues 176–184. The Y39A mutant (5CLQ) was not used as the primary model. The unresolved acidic tail (residues 210–216) was not modeled to avoid bias. The GDP-BeF analogue was converted to GTP. Protonation states were assigned with PROPKA (pH 7.0).

Systems were solvated in TIP3 water using CHARMM-GUI with 0.15 M NaCl. Electrostatics were treated with PME, and vdW interactions were handled with a 12 Å cutoff. After staged minimization, simulations were performed using CHARMM with the C36m force field to investigate RanGTP opening mechanisms.

3.1.1. Reproduction of switch I opening

To reproduce the switch I opening observed in initial simulations, two approaches were used. First, a structure from 103 ns - just before the opening event - was selected, and seven parallel simulations were initiated with different initial velocity distributions. Second, after identifying interactions that affect the stabilization of switch I, harmonic distance constraint was applied between the atoms of the interacting amino acids (Lys152:NZ-Glu186:OE2), gradually reducing their distance to 3.4 Å to match the interaction seen in the RanGDP crystal structure (PDB: 5CIQ).

To examine the role of Mg^{2+} coordination, the 5CLL RanGTP structure was combined with the C-terminal conformation from 20 ns and solvated in an 85 × 85 × 85 Å water box with 0.15 M NaCl. Energy minimization was performed in NAMD (10,000 steps) with harmonic restraints. A 20 ns MD simulation followed, maintaining a 3.4 Å Lys152–Glu186 restraint (20 kcal/mol/Å^2).

3.2. MD and MDeNM simulations

Production runs of 200 ns (extended to 1 μs) were performed for RanGDP and RanGTP. Additional 1 μs parallel simulations included restrained and modified systems targeting Lys152–Glu186 and Thr42–Mg^{2+} interactions. To enhance conformational sampling, aMDeNM simulations were conducted. Normal modes were calculated in CHARMM after thorough minimization. While no suitable low-frequency modes were found for RanGDP, four were selected for RanGTP. All

MD and MDeNM simulations were performed using NAMD with CHARMM C36m force field.

System	Name	Type	Eq. bias	Prod. bias	Time
RanGDP	*md1-3*	MD	∅	∅	3 × 0.2/1 µs
RanGTP	*md1-3*	MD	∅	∅	3 × 0.2/1 µs
	md1'-7'	MD	∅	∅	7 × 1 µs
	md_constr152_1-3	MD	20 ns, Lys152-Glu186	∅	3 × 1 µs
	md_mgoct_152_1-5	MD	20 ns, Lys152-Glu186	Upperwall Lys152-Glu186	5 × 1 µs
	md_152_mg42_1-5	MD	20 ns, Thr42 - Mg^{2+}	∅	5 × 1 µs
	MDeNM	aMDeNM	∅	∅	183 × 0,2 ns

Table 1. Summary of simulations performed for Ran in GDP- and GTP-bound states. *Traditional MD simulations were run from multiple replicas, some systems with initial 200 ns segments followed by extended 1 µs production runs. Additional biased simulations targeted key interactions during equilibration, with either unbiased or restrained production phases and enhanced sampling using aMDeNM to explore collective motions.*

4. Results

4.1. Nucleotide-dependent functional dynamics of full-length Ran

Previous MD studies of the Ras superfamily have shown that key functional regions - particularly switch I and switch II - exhibit nucleotide-dependent flexibility. Consistent with these findings and prior experimental data, we anticipated that the C-terminal region of Ran would be more flexible in the GTP-bound state than in the GDP-bound form. Conventional MD simulations confirmed this expectation: the C-terminus of RanGTP showed markedly higher fluctuations, with minimum fluctuations nearly twice those of RanGDP. Unlike K-Ras, however, switch I in RanGTP also showed slightly increased flexibility relative to RanGDP, likely reflecting a conformational transition from an ordered open structure (α-helix followed by β-turn) to a more disordered, loop-like closed state. In contrast, switch II became more rigid upon GTP binding, as expected. These results underscore distinctive features of Ran's conformational dynamics that may relate to its specialized function in nuclear transport.

To further expand conformational sampling, we applied the aMDeNM method, which enhanced exploration of C-terminal motions in RanGTP. This approach revealed even greater C-terminal flexibility than conventional MD, although the increased fluctuations partly reflect methodological bias from selecting normal modes targeting this region. Consequently, switch I fluctuations were not captured in aMDeNM simulations.

Because no complete experimental structure of free RanGTP exists, this study provides the first detailed computational characterization of its C-terminal dynamics. Three independent RanGTP MD trajectories revealed distinct local minima for C-terminal conformations. Simulations 1 and 2 sampled relatively confined spaces, while simulation 3 explored broader motions. In all cases, conformations clustered on one side of the G-domain near the switch regions, suggesting stabilizing interactions that restrict movement. By contrast, aMDeNM simulations mapped diverse open conformations on multiple sides of the G-domain.

Available crystal structures of RanGTP - resolved only in complexes with Ran-binding proteins - consistently show the C-terminal helix embracing the Ran-binding domain, reflecting constrained conformations. Complementarily, aMDeNM results demonstrates that unbound RanGTP can sample a wide conformational space before stabilizing upon partner binding.

In RanGDP, stabilizing interactions anchor the C-terminus in its inactive conformation. Residues 171–180 interact with the α5/β2 interface; residues 181–186 contact the α1 helix and β6–α5 loop; and the N-terminal end of the C-terminal helix forms three strong ionic interactions with the G-domain. These hydrogen bonds and electrostatic contacts maintain structural stability.

A common feature across all three RanGTP MD simulations is the persistence of the interaction between the N-terminal segment of the C-terminus and the interface between the α_5 helix and β_2 sheet of the G-domain. This consistent intercalation suggests that the N-terminal portion of the C-terminus remains anchored in this region regardless of the distal conformational variability. Distinct interaction patterns emerged: in simulation 1, the C-terminal loop engaged the β6–α5 loop; in simulation 2, the helix shifted toward β2–β3; and in simulation 3, the C-terminus formed ionic contacts with switch I, reducing its flexibility.

Overall, by using MD and aMDeNM method in the active GTP-bound form, we generated an extensive conformational landscape of the C-terminal region, which allowed us to envisage how RanGTP interacts with its macromolecular partners.

4.2. The mechanism of induced switch I opening in RanGTP

During one RanGTP MD simulation, switch I transitioned from its closed, active conformation to an open, GDP-like state. To understand this shift, we analyzed interactions correlating with switch I “openness.” Two key contacts were identified: π–π interaction between Phe35 and the guanine base of GTP, and hydrogen bonds between Glu36 and the GTP phosphates.

In the closed RanGTP state, the GTP aromatic ring also engages in hydrophobic contacts with the aliphatic portion of Lys152. Together these residues create a stabilizing Phe35–GTP–Lys152 triad that anchors switch I to the G-domain. Hydrogen bonds between Glu36 and GTP phosphates further reinforce this network. Disruption of all these interactions appears necessary for full opening.

The trigger for triad breakdown involves Glu186. When Glu186 approaches Lys152, a salt bridge forms between them, pulling Lys152 away from the triad. This displacement destabilizes Phe35’s interaction with GTP and disrupts Glu36's hydrogen bonds, collectively weakening the attachment of switch I. Structural analysis reveals a stepwise transition: closed (intact triad and hydrogen bonds), intermediate (Lys152–Glu186 salt bridge forms, triad destabilizes), and fully open (switch I detached, resembling RanGDP).

Interestingly, in simulations in which the switch remained closed, transient “ring-flipping” motions of Phe35 were observed. Despite temporary displacement from GTP, the Glu36-GTP hydrogen bonds persisted, and Lys152 maintained contact with GTP, enabling rapid reformation of the triad. This dynamic resilience explains the stability of the active conformation. Only when Phe35 flipping occurred simultaneously with the loss of the Glu36 hydrogen bond did switch I fully open, without requiring Lys152 displacement.

Elevated RMSF values near switch I prompted investigation of Mg^{2+} coordination. After energy minimization, Asp65 unexpectedly entered the Mg^{2+} coordination sphere, displacing the γ-phosphate and a coordinating water molecule. A hydrogen bond formed between the γ-phosphate and Thr42, while Mg^{2+} adopted a transient hepta-coordinated state, deviating from its preferred octahedral geometry. Over time, Thr42 dissociated from Mg^{2+}, partially relieving distortion and promoting partial switch I opening.

To test whether triad disruption alone was sufficient, simulations were performed with preserved Mg^{2+} octahedral coordination and enforced proximity between Lys152 and Glu186. Although the triad consistently broke and Phe35 moved away from GTP, Mg^{2+} coordination remained intact and switch I adopted only a partially open state. Therefore, triad disruption alone was insufficient for full transition.

Finally, to mimic GAP-like effects, the Mg^{2+}–Thr42 distance was gradually increased to 10 Å, and five unrestrained simulations were run. Both Phe35–GTP and Mg^{2+}–Thr42 distances increased, leading to a fully open switch I conformation resembling RanGDP.

Overall, results indicate that switch I opening requires coordinated disruption of the Phe35–GTP–Lys152 triad and alteration of Mg^{2+} coordination. The Lys152–Glu186 salt bridge destabilizes the triad, while the loss of the Thr42–Mg^{2+} interaction facilitates the complete transition to the open state.

5. Conclusion

By employing different simulation approaches (MD and aMDeNM), we were able to characterize the nucleotide-dependent dynamical behavior of the C-terminal end of Ran. This biological role has been extensively reported in experimental studies.

In the inactive GDP-bound form, the C-terminus end stays rigidly attached to the G-domain, auto-inhibiting the effector binding site. In addition to previously reported contacts, our analysis revealed three strong ionic interactions that strongly stabilize the association of the C-terminal helix with the G-domain: Asp190–Lys127, Glu198–Lys159, and Asp200–Lys134.

In the active, GTP-bound form, we achieved extensive mapping of C-terminal conformations, enabling visualization of how RanGTP interacts with its macromolecular partners.

We defined the mechanism underlying switch I opening in RanGTP, demonstrating that full transition to the open switch I conformation requires two perturbations: disruption of the Phe35-GTP-Lys152 triad, initiated by the reorientation of Lys152 through interaction with Glu186, and the displacement of Thr42 from the Mg^{2+} coordination sphere. These changes, in turn, also destabilize the hydrogen bonds between Glu36 and GTP, enabling switch I to transition into an open conformation that resembles the inactive state of RanGDP.

Together, our findings define a new mechanistic basis for the switch I 'on' - to 'off' transition and point to potential therapeutic strategies for modulating small GTPases in cases where pathogenic mutations stabilize the active state.

6. Bibliography of the candidate's publications

Publications related to the thesis:

J. Czigleczki, P.T. de Resende Lara, B. Dudas, H. Jang, D. Perahia, R. Nussinov, E. Balog. "Small GTPase Ran: Depicting the nucleotide-specific conformational landscape of the

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Submitted:

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J. Czigleczki, P.T. de Resende Lara, B. Dudas, H. Jang, D. Perahia, R. Nussinov, E. Balog. “Functionally important C-terminus of small GTPase Ran: exploring its nucleotide-specific conformational surface.” PhD Scientific Days 2023, Semmelweis University, 22-23 June 2023, Budapest

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