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RGK Family G-Domain:GTP Analog Complex Structures and Nucleotide-Binding Properties

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The RGK family of small G-proteins, including Rad, Gem, Rem1, and Rem2, is inducibly expressed in various mammalian tissues and interacts with voltage-dependent calcium channels and Rho kinase. Many questions remain regarding their physiological roles and molecular mechanism. Previous crystallographic studies reported RGK G-domain:guanosine di-phosphate structures. To test whether RGK proteins undergo a nucleotide-induced conformational change, we determined the crystallographic structures of Rad:GppNHp and Rem2:GppNHp to 1.7 and 1.8 Å resolutions, respectively. Also, we characterized the nucleotide-binding properties and conformations for Gem, Rad, and several structure-based mutants using fluorescence spectroscopy. The results suggest that RGK G-proteins may not behave as Ras-like canonical nucleotide-induced molecular switches. Further, the RGK proteins have differing structures and nucleotide-binding properties, which may have implications for their varied action on effectors.

Introduction

The RGK family of small G-proteins was initially isolated by screens for G-proteins that have inducible expression, an uncommon characteristic of the superfamily.[1,2] The two founding members, Gem/Kir and Rad, gave rise to the name of this family (RGK). Subsequently, two additional members were added, Rem1 and Rem2.[3,4] A common structure for the family comprises a variable N-terminal segment of between 70 and 90 residues, a G-domain, and a C-terminal segment that contains a calmodulin binding site[5] and a poly-basic membrane targeting sequence.[6] There have been no reports on any lipid posttranslational modification nor are there any sequences predictive of such. The G-domain exhibits conserved sequence features unique to the family. Upon their identification, the RGK proteins were shown to bind guanosine nucleotides,[1–4] but the effect of the nucleotide on their action is not clear cut, as might be the case for classic small G-proteins such as Ras.

Physiological function for the various family members remains an unanswered question. Our closest estimates derive from transgenic mice studies of Rad, where knock-out[7] and knock-in[8] mutant Rad appeared to cause cardiac pathology,

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Abbreviations used: GNP, guanosine 5′-β,γ-imidophosphate; GDP, guanosine di-phosphate; GTP, guanosine tri-phosphate; mant, 2′(3′)-*O*-(*N*-methylanthraniloyl); FRET, fluorescence resonance energy transfer; PEG, polyethylene glycol; PDB, Protein Data Bank; WT, wild type; TEV, tobacco etch virus; ESRF, European Synchrotron Radiation Facility; RT, room temperature; TCSPC, time-correlated single-photon counting.

Table 1. Crystallographic parameters

	Rad:GppNHp	Rad:GppNHp	Q148A:GppNHp	Rem2:GppNHp
Data statistics				
Wavelength (Å)	0.934	0.934	0.934	0.934
Space group	$P3_1$	$P3_1$	$P3_1$	$P2_1$
Unit cell parameters (Å)	$a=b=38.6$, $c=153.5$	$a=b=39.1$, $c=154.7$	$a=b=38.4$, $c=154.1$	$a=52.5$, $b=59.1$, $c=56.1$
Total reflections	42,495	96,700	42,711	31,389
Unique reflections	8487	30,044	11,324	11,210
Completeness (%)	95.1	94.6	99.2	93.2
R_{merge} (%)	0.080	0.037	0.062	0.088
I/σ	17.6	31.8	20.7	10.9
Resolution range (Å)	50–2.5	50–1.65	50–2.3	50–1.76
X-ray source	ESRF beamline ID14-1	ESRF beamline ID14-2	ESRF beamline ID14-2	ESRF beamline ID14-2
Refinement statistics				
No. of reflections (working/test)	8043/444	29,628/1996	11,052/1112	28,191/1438
d_{min} (Å)	2.5	1.65	2.3	1.76
R_{work}/R_{free} (%)	21.9/26.3	17.59/22.15	16.17/20.52	20.77/23.53
Twinning information				
Fraction		0.495	0.415	
Operator		$h,-h-k,-l$	$h,-h-k,-l$	
RMSD from ideality				
Bond lengths	0.011	0.005	0.008	0.005
Bond angles	1.524	0.954	1.095	1.02
B-factor (Å^2) (RMSD of bonded atoms—main/side chain)	0.69/2.0	4.3/4.9	5.1/6.9	3.5/4.3
Averaged *B*-factor (Å^2)	40.5	31.8	50.7	20.816
No. of protein atoms/solvent	2236/31	2300/129	2233/55	2430/436

implying a role in regulation of $I_{Ca^{2+}}$. In an effort to discover the *bona fide* function for these G-proteins, earlier studies performed yeast two-hybrid screens for protein interactors, providing important leads. Two of these screens demonstrated that Gem interacted with CaV β and Rho kinase β.[9,10] Interactions with CaV β were found also with the other three RGK members.[3,11] The consequence of RGK co-expression with CaV channels in heterologous and endogenous expressing cell lines was strong inhibition of the calcium current.[8,9,11–17] Likewise, RGK proteins had inhibitory action on Rho kinase, thereby affecting cytoskeleton states, and specifically in neurons, generating retraction of neurites.[9] Rad was observed to associate with Rho kinase α, while to date, there is no evidence that Rem1 or Rem2 interacts with any Rho kinase isoform.[10] Another interactor from *in vitro* work was shown to be calmodulin.[5] The binding of calmodulin and its relevance to RGK proteins' physiological action remain unclear.

To date, the near-atomic- or atomic-resolution three-dimensional structures available for the RGK G-domains are limited to guanosine di-phosphate (GDP)-ligated complexes. These include Gem,[18,19] Rad,[20] Rem1 [Protein Data Bank (PDB) ID: 2NZJ], and Rem2 (3CBQ). These structures showed that RGK proteins' switch I did not have any nucleotide interactions. Either it was disordered or it pointed away from the nucleotide pocket. As such, we wondered whether these proteins behave as canonical Ras-like molecular switches. Hence, we sought structural information on the guanosine tri-phosphate (GTP)-ligated state so as to glean any hints of such a nucleotide-dependent conformational switch, as found in most members of the Ras superfamily.[21] Guided by structural information, we measured the thermodynamics and kinetics of nucleotide binding for Gem and Rad in the context of the wild-type (WT) and mutant G-domains. This type of data has been only available for the WT Gem G-domain.[19] In general, the molecular mechanism for the RGK proteins' inhibitory action on their defined interactors remains as yet undefined. Thus, knowledge of these proteins' nucleotide-specific binding properties and structural conformations is necessary information for sorting out this mechanism, particularly given their G-domain architecture.

Results

Crystal structures of Rad and Rem2 guanosine 5′-β,γ-imidophosphate-bound G-domains

In order to obtain three-dimensional structural information of the RGK GTP state, we crystallized the Rad G-domain ligated to guanosine 5′-β,γ-imidophosphate (GNP), a non-hydrolyzable GTP analog, and Mg^{2+}. Prior to crystallization, protein was loaded with GNP, and its nucleotide state was