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NMR resonance assignments for the active and inactive conformations of the small G protein RalA

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Abstract

The Ral proteins (RalA and RalB) are small G proteins of the Ras family that have been implicated in exocytosis, endocytosis, transcriptional regulation and mitochondrial fission, as well as having a role in tumourigenesis. RalA and RalB are activated downstream of the master regulator, Ras, which causes the nucleotide exchange of GDP for GTP. Here we report the ¹H, ¹⁵ N and ¹³C resonance assignments of RalA in its active form bound to the GTP analogue GMPPNP. We also report the backbone assignments of RalA in its inactive, GDP-bound form. The assignments give insight into the switch regions, which change conformation upon nucleotide exchange. These switch regions are invisible in the spectra of the active, GMPPNP bound form but the residues proximal to the switches can be monitored. RalA is also an important drug target due to its over activation in some cancers and these assignments will be extremely useful for NMR-based screening approaches.

Keywords $Ras \cdot Gtpase \cdot Ral \cdot Signalling \cdot G$ Protein

Biological context

The Ral small G proteins are members of the Ras superfamily and are regulated by Ras, which activates guanine nucleotide exchange factors (GEFs) that act on the Ral proteins. The RalGEFs sanction the removal of GDP from the Ral proteins, thereby permitting its replacement by GTP, which is more abundant in the cell (Bodemann and White 2008; Cooper et al. 2013). Once GTP-bound, the Ral proteins are active and can interact with various downstream effectors, including: components of the exocyst complex, Sec5 (Fukai et al. 2003) and Exo84 (Jin et al. 2005); RalBP1 (also known as RLIP76) (Fenwick et al. 2010); the Y-box transcription factor ZONAB (Frankel et al. 2005); and phospholipases C- δ 1 (Sidhu et al. 2005) and D1 (Jiang et al. 1995). The Ral proteins regulate a variety of cellular processes through these effector molecules including exocytosis, endocytosis, actin cytoskeletal dynamics, transcriptional activation and

mitochondrial fission. In addition, Ral proteins have been implicated in the initiation and regulation of oncogenic transformation of human cells (van Dam and Robinson 2006). The Ral proteins like other small G proteins act as molecular switches, which cycle between GTP bound active and GDP bound inactive states. They contain two dynamic switch regions [switch I (residues 41-51) and switch II (residues 69-81)], which are involved in effector binding and undergo conformational changes upon nucleotide exchange (Milburn et al. 1990). There are two Ral isoforms, RalA and RalB, which have approximately 85% sequence identity and in vitro bind with similar affinities to their effectors (Campbell et al. 2015). In vivo however, RalA and RalB appear to perform distinctive functions in normal cells and to play different roles in tumourigenesis and cancer progression. The sequence differences are mainly within the C-terminal, hypervariable region of RalA and RalB, and indeed all of the residues known to contact effector proteins are conserved (Mott and Owen 2010). There is also a single residue insertion in the loop between helix $\alpha 3$ and strand β 5, which, while not contacting effectors directly, is close to the effector binding regions. These differences are likely to determine differential Ral protein interactions with their effector molecules (Campbell et al. 2015; Shipitsin and Feig 2004). Furthermore, the divergent C-terminal region of Ral

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proteins is also thought to determine their cellular localization (Shipitsin and Feig 2004).

The resonance assignments for RalB·GMPPNP were published in 2007 (Prasannan et al. 2007) but there are no resonance assignments currently available for RalA. Here, we report the backbone and sidechain assignments for RalA bound to the non-hydrolysable GTP analogue, GMPPNP. We also report the backbone amide assignments for the GDP bound, inactive form of RalA.

Methods and experiments

Uniformly ¹⁵ N- and ¹⁵ N, ¹³C-labelled simian RalA G domain (residues 1–184) containing the activating Q72L mutation was expressed and purified as described earlier (Campbell et al. 2015). The bound nucleotide was exchanged for GMPPNP (Sigma) as described previously (Thompson et al. 1998). Uniformly ¹⁵ N and ¹⁵ N-¹³C wild type (wt) RalA G domain (residues 1–184) was also expressed and purified as described above. To prepare a protein sample bound only to GDP, wt RalA was incubated at 25 °C for 16–20 h to allow complete hydrolysis of the bound GTP. HPLC analysis was used to confirm the identity of all bound nucleotides.

NMR samples contained 0.26 mM and 0.45 mM of ¹⁵ N- and ¹⁵ N, ¹³C-labelled Q72L RalA·GMPPNP respectively or 0.75 mM of ¹⁵ N-labelled wt RalA·GDP in NMR buffer (20 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1 mM MgCl₂, 0.05% NaN₃) with an additional 10% D₂O. All NMR spectra were acquired on a Bruker DRX500 spectrometer at 298 K except ¹³C HSQC, HCCH-TOCSY and ¹³C-separated NOESY experiments, which were recorded on a Bruker DRX800 at 298 K. The following experiments were recorded on ¹⁵ N, ¹³C-labelled Q72L RalA·GMPPNP: HNCA, HN(CO)CA, HNCACB, CBCA(CO)NH, HCCH-TOCSY, ¹³C HSQC, and ¹³C-separated NOESY. ¹⁵ N HSQC, ¹⁵ N-separated TOCSY and ¹⁵ N-separated NOESY experiments were recorded on ¹⁵ N-labelled samples of both Q72L RalA·GMPPNP and wt RalA·GDP. A 2D homonuclear ¹H NOESY was recorded on 0.70 mM unlabelled Q72L RalA-GMPPNP. All NOESY experiments were recorded with 100 ms mixing times. NMR data were processed using AZARA (Boucher, https://www.bioc.cam.ac.uk/azara), and analysed using CCPN Analysis (Vranken et al. 2005).

For Q72L RalA·GMPPNP the backbone assignments were achieved using standard triple resonance methodology (Gardner and Kay 1998).The sidechain resonances were assigned using the triple resonance experiments (HNCACB, CBCA(CO)NH), in combination with ¹⁵ N-separated TOCSY and HCCH-TOCSY experiments, as well as ¹⁵ N-separated and ¹³C-separated NOESY. Sidechain resonances of aromatic residues were assigned using the aromatic region of a ¹³C HSQC and the relevant planes in the ¹³C-separated NOESY. Assignments were validated by the presence of return peaks in the related ¹³C β planes. The methyl groups of methionine were identified using their strong peaks at distinct chemical shifts in the ¹³C HSQC and the sign of the crosspeaks in constant time ¹³C HSQC. Sidechain amide resonances for Asn, Gln and Arg were assigned using the ¹⁵ N-separated and ¹³C-separated NOESY spectra. The 2D homonuclear ¹H NOESY was used to identify proton chemical shifts for the bound GMPPNP. The backbone resonances for wt RalA·GDP were assigned with the aid of the assignments of Q72L RalA·GMPPNP, in combination with the ¹⁵ N-separated NOESY and TOCSY and ¹⁵ N HSQC recorded on both proteins (Clore and Gronenborn 1998a, b; Marion et al. 1989).

Assignments and data deposition

Backbone resonances were never observed for the 11 N-terminal residues (1–11) of RalA. Subsequent electrospray ionisation mass spectrometry (ESI–MS) analysis showed that the N-terminus of RalA undergoes proteolysis in solution. This region is not the part of G-domain of Ral proteins (Bourne et al. 1991) and was similarly absent (and therefore unassigned) in RalB (Fenwick et al. 2009; Prasannan et al. 2007).

The complete backbone assignments were obtained for residues 12–184 of Q72L RalA·GMPPNP except for residues 43–49, which are within switch I, and 68–70, which are in switch II. The statistics for the backbone assignments are shown in Table 1. The $^{1}H-^{15}N$ HSQC spectrum showing the spectral quality and the assignments is shown in Fig. 1. The switch I (41–51) and switch II (68–81) regions in small G proteins exhibit internal mobility in the active form, on a timescale that makes their assignment problematic (Feltham et al. 1997; Kraulis et al. 1994; Mott et al. 1999). The missing assignments in these regions were therefore not surprising.

Sidechain assignments were achieved for all residues with observable backbone resonances in Q72L

 Table 1
 Extent of assignments of RalA in the active (GMPPNP) and inactive (GDP) forms

	Atom	Number of expected	Number assigned	Percentage assignment (%)
RalA·GMPPNP (Q72L)	HN ¹⁵ NH	171 173	162 162	94.7 93.6
	¹³ C	173	165	95.4
RalA·GDP	HN ¹⁵ NH	171 173	169 169	98.8 97.7



Fig. 1 ¹H-¹⁵ N HSQC spectrum of RalA·GMPPNP at 298 K and 500 MHz

RalA·GMPPNP. In addition, sidechains were assigned for residues Thr69 Ala70 in switch II. Outside the two switch regions, aliphatic side-chains for residues 11 to 184 were almost fully assigned except for sidechain carbons of Lys27, Arg176 and Lys179. The ¹H and ¹³C of all proton-bearing aromatic sidechains were fully assigned, except Tyr43 in switch I. H ϵ /N ϵ were assigned for Arg108 and Arg135, H η was assigned for Tyr153 and OH were assigned for Thr31, Ser100, Ser129 and Thr160. All Gln and Asn sidechain NH₂ groups were also assigned. The H1, H2 and H8 resonances of the purine ring and H1' of the ribose in GMPPNP were assigned using a 2D ¹H NOESY spectrum. Assignments of RalA·GMPPNP have been deposited in the BMRB database, accession number 28046.

For wt RalA·GDP, backbone amides of all of switch 1 could be assigned and assignments were missing only for Ala70 and Asn81 in switch II. The statistics for the backbone assignments are shown in Table 1. The ${}^{1}\text{H}{-}^{15}\text{N}$ HSQC spectrum of wt RalA·GDP showing the spectral quality and the assignments is shown in Fig. 2. Assignments of wt RalA·GDP have been deposited in the BMRB database, accession number 28047.



Fig. 2 ¹H-¹⁵ N HSQC spectrum of RalA·GDP at 298 K and 500 MHz

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References

- Bodemann BO, White MA (2008) Ral GTPases and cancer: linchpin support of the tumorigenic platform. Nat Rev Cancer 8:133–140
- Bourne HR, Sanders DA, McCormick F (1991) The GTPase superfamily: conserved structure and molecular mechanism. Nature 349:117–127
- Campbell LJ, Peppa M, Crabtree MD, Shafiq A, McGough NF, Mott HR, Owen D (2015) Thermodynamic mapping of effector protein interfaces with RalA and RalB. Biochemistry 54:1380–1389

- Clore GM, Gronenborn AM (1998a) Determining the structures of large proteins and protein complexes by NMR. Trends Biotechnol 16:22–34
- Clore GM, Gronenborn AM (1998b) NMR structure determination of proteins and protein complexes larger than 20 kDa. Curr Opin Chem Biol 2:564–570
- Cooper JM, Bodemann BO, White MA (2013) The RalGEF/Ral pathway: evaluating an intervention opportunity for Ras cancers. Enzymes 34(Pt. B):137–156
- Feltham JL, Dotsch V, Raza S, Manor D, Cerione RA, Sutcliffe MJ, Wagner G, Oswald RE (1997) Definition of the switch surface in the solution structure of Cdc42Hs. Biochemistry 36:8755–8766
- Fenwick RB, Prasannan S, Campbell LJ, Nietlispach D, Evetts KA, Camonis J, Mott HR, Owen D (2009) Solution structure and dynamics of the small GTPase RalB in its active conformation: significance for effector protein binding. Biochemistry 48:2192–2206
- Fenwick RB, Campbell LJ, Rajasekar K, Prasannan S, Nietlispach D, Camonis J, Owen D, Mott HR (2010) The RalB-RLIP76 complex reveals a novel mode of ral-effector interaction. Structure 18:985–995
- Frankel P, Aronheim A, Kavanagh E, Balda MS, Matter K, Bunney TD, Marshall CJ (2005) RalA interacts with ZONAB in a cell density-dependent manner and regulates its transcriptional activity. EMBO J 24:54–62
- Fukai S, Matern HT, Jagath JR, Scheller RH, Brunger AT (2003) Structural basis of the interaction between RalA and Sec5, a subunit of the sec6/8 complex. EMBO J 22:3267–3278
- Gardner KH, Kay LE (1998) The use of 2H, 13C, 15N multidimensional NMR to study the structure and dynamics of proteins. Annu Rev Biophys Biomol Struct 27:357–406
- Jiang H, Luo JQ, Urano T, Frankel P, Lu Z, Foster DA, Feig LA (1995) Involvement of Ral GTPase in v-Src-induced phospholipase D activation. Nature 378:409–412

- Jin R, Junutula JR, Matern HT, Ervin KE, Scheller RH, Brunger AT (2005) Exo84 and Sec5 are competitive regulatory Sec6/8 effectors to the RalA GTPase. EMBO J 24:2064–2074
- Kraulis PJ, Domaille PJ, Campbell-Burk SL, Van Aken T, Laue ED (1994) Solution structure and dynamics of ras p21.GDP determined by heteronuclear three- and four-dimensional NMR spectroscopy. Biochemistry 33:3515–3531
- Marion D, Driscoll PC, Kay LE, Wingfield PT, Bax A, Gronenborn AM, Clore GM (1989) Overcoming the overlap problem in the assignment of 1H NMR spectra of larger proteins by use of threedimensional heteronuclear 1H–15N Hartmann-Hahn-multiple quantum coherence and nuclear Overhauser-multiple quantum coherence spectroscopy: application to interleukin 1 beta. Biochemistry 28:6150–6156
- Milburn MV, Tong L, deVos AM, Brunger A, Yamaizumi Z, Nishimura S, Kim SH (1990) Molecular switch for signal transduction: structural differences between active and inactive forms of protooncogenic ras proteins. Science 247:939–945
- Mott HR, Owen D (2010) RLIP76 (RalBP1): The first piece of the structural puzzle. Small GTPases 1:157–160
- Mott HR, Owen D, Nietlispach D, Lowe PN, Manser E, Lim L, Laue ED (1999) Structure of the small G protein Cdc42 bound to the GTPase-binding domain of ACK. Nature 399:384–388

- Prasannan S, Fenwick RB, Campbell LJ, Evetts KA, Nietlispach D, Owen D, Mott HR (2007) 1H, 13C and 15N resonance assignments for the small G protein RalB in its active conformation. Biomol NMR Assignments 1:147–149
- Shipitsin M, Feig LA (2004) RalA but not RalB enhances polarized delivery of membrane proteins to the basolateral surface of epithelial cells. Mol Cell Biol 24:5746–5756
- Sidhu RS, Clough RR, Bhullar RP (2005) Regulation of phospholipase C-delta1 through direct interactions with the small GTPase Ral and calmodulin. J Biol Chem 280:21933–21941
- van Dam EM, Robinson PJ (2006) Ral: mediator of membrane trafficking. Int J Biochem Cell Biol 38:1841–1847
- Vranken WF, Boucher W, Stevens TJ, Fogh RH, Pajon A, Llinas M, Ulrich EL, Markley JL, Ionides J, Laue ED (2005) The CCPN data model for NMR spectroscopy: development of a software pipeline. Proteins 59:687–696

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