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4-1-2012

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Accepted version. *Biomolecular NMR Assignments*, Vol. 6, No. 1 (April 2012) 91-93. DOI: [10.1007/s12104-011-9332-3](https://doi.org/10.1007/s12104-011-9332-3). © 2012 Springer Nature Switzerland AG. Part of Springer Nature. Used with permission.

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# Assignments of backbone $^1\text{H}$ , $^{13}\text{C}$ and $^{15}\text{N}$ resonances in H-Ras (1–166) complexed with GppNHp at physiological pH

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## Abstract

The small GTPase Ras is an important signaling molecule acting as a molecular switch in eukaryotic cells. Recent findings of global conformational exchange and a putative allosteric binding site in the G domain of Ras opened an avenue to understanding novel aspects of Ras function. To facilitate detailed NMR studies of Ras in physiological solution conditions, we performed backbone resonance assignments of Ras bound to slowly hydrolysable GTP mimic, guanosine 5'-[ $\beta$ ,  $\gamma$ -imido]triphosphate at pH 7.2. Out of 163 non-proline residues of the G domain, signals from backbone amide proton, nitrogen and carbon

spins of 127 residues were confidently assigned with the remaining unassigned residues mostly located at the exchange-broadened effectors interface.

Keywords

NMR; Ras; Assignments

## Biological context

Ras is the founding member of a superfamily of small monomeric GTPases ubiquitous in cellular signaling cascades within the cell (Malumbres and Barbacid 2003). These proteins bind guanine nucleotides and catalyze the hydrolysis of GTP. When bound to GTP, Ras associates with a variety of effectors driving cellular proliferation, gene activation, and apoptosis. The Ras subfamily proteins (H, N and K-Ras) contain a soluble G domain responsible for binding guanine nucleotides and a lipid-modified C-terminal tail mediating membrane association. The G domains of Ras isoforms (roughly 170 amino-acid residues) are highly conserved while C-terminal tails (of 20–23 residues) are variable. Mutations in the G domain that prevent GTP hydrolysis are intimately linked to human disease with nearly 30% of all human cancers harboring such mutations in Ras genes. In 2008, we discovered a global conformational exchange process in the G domain of H-Ras occurring on a millisecond timescale (O'Connor and Kovrigin 2008). More recently, X-ray diffraction studies located a novel allosteric site in the G domain that was suggested to control GTPase activity (Buhrman et al. 2010). Global conformational heterogeneity and a novel allosteric site may indicate a new pathway for cellular regulation of Ras as well as provide an approach to controlling Ras function by small molecules.

To further investigate the relationship between structure, dynamics and function in Ras, one needs an accurate knowledge of NMR signal assignments at physiological pH. Previously, assignments of H-Ras-GppNHP were obtained at acidic pH 5.5 to minimize amide proton exchange with water (Ito et al. 1997). In our 2008 paper we reported amide proton and nitrogen assignments at pH 7.4 for 117 out of total 163 non-proline spin systems obtained by titrating NMR samples from 5.5 to 7.4 and acquiring the 3D  $^1\text{H}$ - $^{15}\text{N}$ -NOESY-HSQC and 3D  $^1\text{H}$ - $^{15}\text{N}$  TOCSY HSQC data sets. In this work, in order to expand the available set of assignments in Ras, we performed de novo backbone triple-resonance assignment of the GTPase domain of H-Ras bound to GppNHP at pH 7.2 corresponding to the native cytosolic pH of a eukaryotic cell.

## Methods and experiments

The G domain of H-Ras residues 1–166 was expressed in *E. coli* as inclusion bodies, isolated, refolded and purified as described in our earlier publication. After ion-exchange and size-exclusion chromatographic separation, protein purity was >95% (judged by SDS-PAGE).

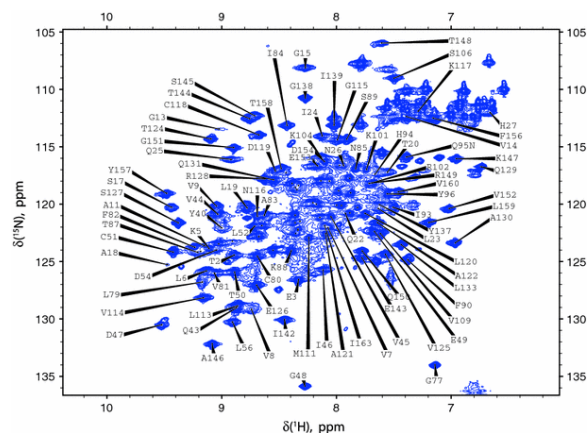
For triple-resonance experiments, 1 mM  $^{13}\text{C}$ - $^{15}\text{N}$  protein samples were prepared in buffer containing 10 mM TRIS, 10 mM NaCl, 5 mM  $\text{MgCl}_2$ , 1 mM DTT, 0.1 mM EDTA, 1 mM GppNHP, 10%  $\text{D}_2\text{O}$ , 0.01%  $\text{NaN}_3$ , and Roche protease inhibitors (Cat. #S8830) in a thin-walled Shigemi tube with a final volume of 330  $\mu\text{l}$ . Triple resonance experiments were performed at 293.15 K and 14.1 T using a Bruker Avance II spectrometer equipped with a cryoprobe. Backbone experiments included HNC(O), HN(CA)CO, HN(CO)CA, HN(CO)CACB, HNCA, HNCACB with small modifications (Markley et al. 2009). The 3D  $^1\text{H}$ - $^{15}\text{N}$ - $^1\text{H}$  NOESY-HSQC experiments were performed in order to support sequential assignments and cross-validate the data against NOE values calculated using the crystal structure (PDB ID: 5P21).

Spectra were processed with NMRPipe and viewed with Sparky (Delaglio et al. 1995; Goddard and Kneller 2004). Because GppNHp is a slowly hydrolysable GTP mimic, care was taken to exclude from analysis the resonances originating from Ras-GDP, which appeared over the course of multi-day acquisition.

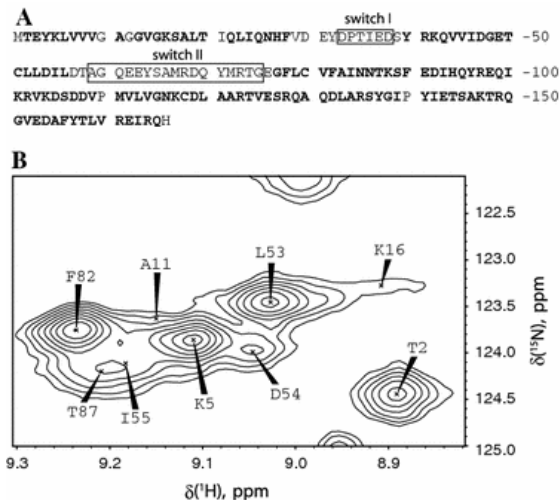
## Assignments and data deposition

Initial peak lists for triple-resonance experiments were generated via restricted peak picking in Sparky based on resonances observed in the 2D  $^1\text{H}$ - $^{15}\text{N}$  HSQC. The Probabilistic Interaction Network of Evidence (PINE, <http://www.pine.nmr.fam.wisc.edu/>) algorithm was employed to perform the first round of assignment guesses. The PINE-SPARKY software was used to facilitate import of PINE results into Sparky and the final assignment of resonances has been performed manually as described (Bahrami et al. 2009; Lee et al. 2009).

Out of 163 non-proline residues 127 were confidently assigned. Backbone  $^1\text{H}$ ,  $^{13}\text{C}$ ,  $^{15}\text{N}$  chemical shifts have been deposited to the Biological Magnetic Resonance Data Bank (Ulrich et al. 2008; <http://www.bmrb.wisc.edu>) under accession number 17678. Figure 1 displays a  $^1\text{H}$ - $^{15}\text{N}$  HSQC spectrum demonstrating overall spectral quality and peak dispersion. A summary of residue assignments mapped on the protein sequence is given the Fig. 2, panel a. The 14 new  $^1\text{H}$ - $^{15}\text{N}$  correlations were added to the ones reported in 2008: A11, G13, F28, Y40, V45, T87, Q99, I100, K101, R102, E162, I163, R164, and Q165. There were considerably overlapped regions in the 2D  $^{15}\text{N}$ - $^1\text{H}$  HSQC that were also successfully assigned (for example, Fig. 2, panel b). A number of residues residing at the effector interface of the G domain have not been confidently assigned due to strong exchange broadening and, therefore, excluded from our BMRB deposition.



**Fig. 1** An overall view of  $^1\text{H}$ - $^{15}\text{N}$  HSQC of H-Ras (1–166) complexed with GppNHp. Some peak labels in most crowded regions were removed for clarity



**Fig. 2 a** Primary amino acid sequence of H-Ras showing residues with assigned  $^1\text{H}$ – $^{15}\text{N}$  correlations in a *bold* typeface. Residues of the effector interface regions “switch I” and “II” are indicated by *boxes*. **b** Selected region from  $^1\text{H}$ – $^{15}\text{N}$  HSQC of H-Ras(1–166) complexed with GppNHp demonstrating successful assignment of an overlapped group of peaks

A few of low-intensity and poorly resolved  $^1\text{H}$ – $^{15}\text{N}$  HSQC peaks, which we tentatively assigned previously utilizing pH titrations and NOESY/TOCSY correlations, were corrected using information from triple-resonance experiments. Identities of resonances originally assigned to residues M72, T74, G75, and E76 located at the C-terminal end of switch II were not confirmed by triple-resonance correlations. The residues T2, A18, R41, V44, L52, F78, H94, E98, R102, and D108 were re-assigned to other (mostly—neighboring) resonances. It is important to note that the above corrections do not affect the main conclusion from 2008 that the G domain of H-Ras exhibits global correlated motion occurring on a millisecond time scale.

A comparison of resonance frequencies of amide  $^{15}\text{N}$  and  $^1\text{H}$  spins in H-Ras observed at a neutral pH with those reported at acidic conditions (Ito et al. 1997) reveals a significant pH-dependence for more than 25 residues. A few of these residues (I93, H94, Q95, Y96, V109, V112, K147, L159, and E162) exhibit very dramatic peak shifts exceeding 1 ppm in  $^{15}\text{N}$  or/and 0.1 ppm in  $^1\text{H}$  dimensions. It is remarkable that the residues I93, Q95, Y96, V112, and K147 out of this group were also found to be involved in global conformational exchange in Ras, thus suggesting the global dynamics might involve reorganization of Coulombic interactions in Ras structure. In summary, the extended set of confident assignments described in the current report enables further investigation of the mechanistic details of global conformational exchange in Ras and its functional implications.

## Notes

### Acknowledgments

The authors acknowledge Dr. Elizaveta Kovrigina for help with NMR sample preparation and Dr. Francis Peterson for assistance with setting up the triple-resonance NMR experiments. The funding for this work came from the Medical College of Wisconsin as Program Development funds to Evgenii L. Kovrigin.

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