Journal of Biomolecular NMR, **16:** 355–356, 2000.
KLUWER/ESCOM

Letter to the Editor: Sequential NMR assignment of the RAS-binding domain of Byr2

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Received 8 December 1999; Accepted 7 February 2000

Key words: Byr2, Ras-binding domain, sequential assignment

Biological context

Ras-related small GTPases, e.g. Ras, Rho, Rac, are essential components of signal transduction pathways in the cell. It was found that 30% of all human tumours lead back on mutations in the Ras gene (Barbacid, 1987). Effectors of Ras-related GTP-binding proteins are defined as proteins interacting mainly with the active GTP-bound form of a Ras molecule. Known effectors are for example the serine/threonine kinase c-Raf1, the Ral guanine-nucleotide exchange factors RalGEF, Rgl, and Rlf and the protein kinase Byr2 from *Schizosaccharomyces pombe*. From partial loss of function mutations it could be shown that different Ras effectors recognise similar but not identical structural regions of the Ras molecule. To learn more about the structural basis of the different Ras-effector interactions, it is important to have a broad knowledge about their structures. So far solution structures are known for the Ras-binding domains of Raf-1 (Terada et al., 1999), RalGEF (Geyer et al., 1997), Rgl (Kigawa et al., 1998) and Rlf (Esser et al., 1998). Since Byr2 is unrelated in primary sequence to the other Ras effectors it was suggested that it might be characterized by a different tertiary fold as well as by a different mode of protein-binding interaction. Using deletion cloning, the regulatory Ras-binding domain was defined as a fragment of 116 amino acids in the N-terminal part of Byr2. We are using heteronuclear high resolution NMR to investigate the solution structure of the regulatory domain of Byr2. As the first stage of the study, we report here the ^{1}H, ^{15}N and ^{13}C sequence-specific resonance assignment and the secondary structure elements of the protein.

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Methods and results

Expression and isotopic enrichment. Byr2 amino acid residues 65–180 were synthesized in *Escherichia coli* strain Bl21 DE3 using the plasmid pGEX4T3 (Pharmacia). After the cells were grown in standard I medium (Merck, Darmstadt) to $OD_{600} = 0.8$, they were incubated overnight at 30 °C in the presence of 0.1 mM isopropyl-β-D-thiogalactoside. Cells were harvested by centrifugation, resuspended in phosphate buffered saline and lysed by ultrasonication. The cell lysate was cleared by centrifugation at 30000 × g and run over a column containing glutathione sepharose (Pharmacia). The GST-Byr2 fusion protein immobilized on this material was cleaved by incubation with 10 U/ml thrombin (Serva) overnight at 4 °C. Finally, Byr2 was purified by size exclusion chromatography and thereby transferred into 20 mM potassium phosphate, 0.5 mM EDTA, 0.5 mM NaN_3 and 3 mM DTE at pH 6.9. Only the monomer fractions were used for further experiments, which were more than 95% pure as judged by SDS polyacrylamide gel electrophoresis.

For isotopic labeling minimal medium was used instead of standard I in order to grow the cells.

NMR spectroscopy. All NMR samples contained 25 mM DTE, 200 mM deuterated glycine, 20 mM phosphate buffer, 0.5 mM EDTA, 0.5 mM NaN_3 and 0.1 mM DSS for referencing. The DTE content was

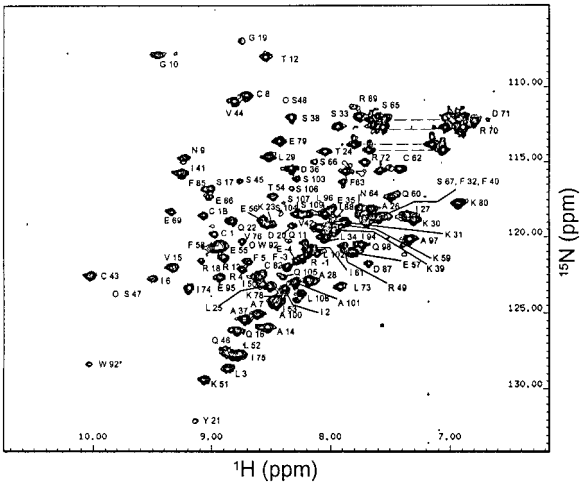

Figure 1. ^{1}H-^{15}N HSQC spectrum of the Ras-binding domain of Byr2 (pH 6.9; 25 °C), measured at a ^{1}H resonance frequency of 800 MHz. The side chain of Trp-92 is indicated by an asterisk while the resonances originating from the Asn and Gln side chains are marked with dotted lines. For reasons of clarity, not all resonances have been marked.

raised from 3 mM to 25 mM to increase long-term stability. The pH was adjusted to 6.9 for all samples. Protein concentration varied between 0.7 and 1.2 mM. One major problem in the assignment process was the poor long-term stability of the molecule, which was probably caused by the formation of disulphide bridges. NMR experiments were recorded at 25 °C on either a Bruker DRX600 or DRX800 spectrometer equipped with triple resonance probes and pulse field gradient capabilities.

The sequence-specific backbone assignment was performed using CBCA(CO)NH, CBCANH, HBHA-(CO)NH, HNCA, HN(CO)CA, TOCSY-HSQC and NOESY-HSQC spectra (for a review see Sattler et al., 1999). Figure 1 shows a 2D ^{1}H-^{15}N HSQC spectrum of Byr2 obtained at a ^{1}H resonance frequency of 800 MHz. Side chain assignments are based on the HCCH-TOCSY spectra with some additional information derived from the HBHA(CO)NH experiment. Aromatic ^{1}H resonances were identified from the 2D NOESY and TOCSY spectra measured in D_2O. Amide proton exchange rates were determined from a series of ^{1}H-^{15}N HSQC spectra acquired in D_2O. NOE contacts were identified from ^{13}C and ^{15}N edited NOESY-HSQC spectra. Processing and analysis of the data was accomplished using the Bruker programs XWINNMR and AURELIA (Neidig et al., 1995). Analysis of the patterns of NOE connectivities, chemical shifts (Wishart et al., 1991) and hydrogen exchange rates suggests the presence of 5 β-strands and 2 α-helices in the first 86 amino acids, while the following 30 residues remain relatively unstructured (data not shown). The helices are located between strands 2 and 3 and strands 4 and 5, indicating a ββαββαβ-topology for the molecule. Since a similar topology is found for the ubiquitin fold, we suggest that Byr2 adopts a comparable overall fold.

Extent of assignments and data deposition

Sequential assignments were obtained for all amino acids with the exception of P83, S84, R90 and P110. Please note that our amino acid numbering starts with R-5, which corresponds to R 65 in the native protein. Side chain assignments were obtained for 99% of the assigned residues, not counting G10 and G19. However, in the region P83–E93 signals in all spectra were generally very weak and only rudimentary side chain assignments could be obtained. 100% of the Cα and 96% of the Cβ resonances could be determined for the assigned residues. A list of the ^{1}H, ^{15}N, and ^{13}C chemical shifts has been deposited with the BioMagResBank under accession number 4463.

Acknowledgements

We thank T. Maurer, G. Horn and W. Kremer for helpful discussions. This work was supported by the Deutsche Forschungsgemeinschaft.

References

Barbacid, M. (1987) *Annu. Rev. Biochem.*, **56**, 779–827.
Esser, D., Bauer, B., Wolthuis, R.M.F., Wittinghofer, A., Cool, R.H. and Bayer, P. (1998) *Biochemistry*, **37**, 13453–13462.
Geyer, M., Herrmann, C., Wohlgemuth, S., Wittinghofer, A. and Kalbitzer, H.R. (1997) *Nat. Struct. Biol.*, **4**, 694–699.
Huang, L., Hofer, F., Martin, G.S. and Kim, S.H. (1998) *Nat. Struct. Biol.*, **5**, 422–426.
Kigawa, T., Ito, Y., Shirouzu, M., Kikuchi, A. and Yokoyama, S. (1998) *FEBS Lett.*, **441**, 413–418.
Neidig, K.-P., Geyer, M., Görler, A., Antz, C., Saffrich, R., Beneicke, W. and Kalbitzer, H.R. (1995) *J. Biomol. NMR*, **6**, 255–270.
Sattler, M., Schleucher, J. and Griesinger, C. (1999) *Prog. NMR Spectrosc.*, **34**, 93–158.
Terada, T., Ito, Y., Shirouzu, M., Tateno, M., Hasimoto, K., Kigawa, T., Ebisuzaki, T., Takio, K., Shibata, T., Yokoyama, S., Smith, B.O., Laue, E.D. and Cooper, J.A. (1999) *J. Mol. Biol.*, **286**, 219–232.
Wishart, D.S., Sykes, B.D. and Richards, F.M. (1991) *J. Mol. Biol.*, **222**, 311–333.