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Letter to the Editor: Sequence-specific resonance assignment of the Ras-binding domain of AF6

G. Steiner[a], W. Kremer[a], T. Linnemann[b], C. Herrmann[b], M. Geyer[a] & H.R. Kalbitzer[a]
[a]*Institut für Biophysik und Physikalische Biochemie, Universität Regensburg, Postfach, D-93040 Regensburg, Germany*
[b]*Max-Planck-Institut für molekulare Physiologie, Otto-Hahn-Straße 11, D-44227 Dortmund, Germany*

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Biological context

AF6, a protein with high sequence similarity to *Drosophila* Canoe, was originally identified as a fusion partner of ALL-1 in acute myeloid leucaemia (Prasad et al., 1993). Further interest arose when it was found that AF6 binds to Ras and thus might be a putative effector in a Ras-driven signalling cascade (Kuriyama et al., 1996). Binding was also observed to ZO-1, a protein involved in the formation of tight junctions in epithelial cells (Yamamoto et al., 1997). As a result of recent studies, a picture emerged where AF6 could play a role in the regulation of cell–cell contacts through a Ras-modulated interaction with ZO-1 (Taya et al., 1998).

It has been shown that the amino terminus of AF6 (residues 1–141) adopts a stable fold and constitutes the minimal Ras-binding domain (RBD) (Linnemann et al., 1999). AF6 RBD exhibits Ras binding characteristics very similar to other effectors such as Raf and Ral GDS, but is significantly larger and shares hardly any sequence homology to these. So it will be interesting to determine the structure of AF6 RBD and compare it with the fold of other RBDs. As a first step, we report here the sequence specific assignment of the NMR resonances of AF6 RBD.

Methods and results

Protein expression and isotopic labeling. All NMR experiments were carried out with AF6 RBD from *rattus norwegicus* (rnAF6) which shares ∼99% sequence homology with human AF6. Expression was done with the pGEX plasmid encoding the glutathione S-transferase/AF6 RBD fusion protein. The RBD of AF6 (residues 1–141) was synthesized in *E. coli* strain BL21(DE3), grown in standard I medium (Merck) to OD = 0.6. The culture was incubated at 30 °C for 4 h in the presence of 0.1 mM isopropyl-β-D-thiogalactoside and then centrifuged. The resuspended cell pellet was sonicated, and the lysate was cleared by centrifugation at 32000 *g*. The GST/AF6 RBD fusion was run over a glutathione-sepharose column (Pharmacia). The protein was cleaved by incubation with thrombin (10 U/ml, overnight, 4 °C). AF6 RBD was eluted from the column and further purified by size exclusion chromatography (Superdex 75, Pharmacia), thereby transferred into 20 mM potassium phosphate, 0.5 mM EDTA, 0.5 mM NaN_3 and 3 mM DTE at

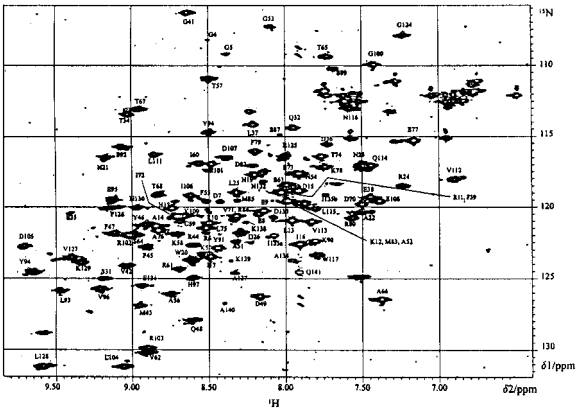

Figure 1. ^{1}H-^{15}N HSQC spectrum of the RBD of AF6 (residues 1–141) at a ^{1}H resonance frequency of 800 MHz (pH 6.9, 303 K).

*To whom correspondence should be addressed. E-mail hans-robert.kalbitzer@biologie.uni-regensburg.de

pH 6.9. The protein fractions were analyzed for purity by SDS-polyacrylamide gel electrophoresis and concentrated to 20 mg/ml by ultrafiltration (Vivaspin 15, Vivascience).

For isotopic labeling, standard I was replaced with minimal medium for cell growth. For single ^{15}N labeling the incubation procedure yielded 5 mg pure protein per liter of culture. For double labeling (^{15}N and ^{13}C), typically 3 mg AF6 were obtained from 1 liter of culture.

NMR spectroscopy. NMR samples contained 2 mM DTE, 0.2 mM EDTA, 1 mM NaN_3, 0.1 mM DSS in 20 mM potassium phosphate buffer (pH 6.9). The protein concentration varied between 0.9 and 1.3 mM. Measurements were done with 0.6 ml samples in a 92% H_2O, 8% D_2O solvent mixture. The spectra were recorded on Bruker DRX600 and DRX800 NMR spectrometers at 303 K. ^1H chemical shifts were referenced to internal DSS, while IUPAC recommendations were used to calculate the corresponding calibration for ^{15}N and ^{13}C nuclei (Markley et al., 1998).

Sequence specific assignments were obtained by combining the data from HNCA, HNCO, HN(CO)CA, CBCA(CO)NH, TOCSY-HSQC and NOESY-HSQC experiments (for a review see e.g. Sattler et al., 1999). Information about side chain chemical shifts was taken from a HCCH-TOCSY spectrum. Data processing and analysis was carried out on a Silicon Graphics Indy workstation with the software packages XWINNMR and AURELIA from Bruker (Neidig et al., 1995).

Extent of assignments and data deposition

With the combined information from all heteronuclear experiments we were able to assign 93% of the backbone ^{15}N and amide proton resonances, not counting the proline residues. Most of the unassigned signals belong to one of two bigger gaps (residues 27–30 and 119–123). From secondary structure analysis we know that these regions belong to less structured loops in the protein. It is therefore reasonable to assume that the corresponding NMR signals are weakened by fast exchange with the bulk water and/or broadened by conformational exchange.

Of the assigned signals, we were able to obtain 100% of the C^α and C' and 95% of the C^β and H^α chemical shift values. Figure 1 shows an assigned 2D ^1H-^{15}N-HSQC spectrum of AF6 RBD, recorded at 800 MHz. A detailed examination of the NOE contacts found in the ^{15}N edited NOESY-HSQC spectrum suggests that the Ras-binding domain of AF6 is made up of three α-helices and five-β strands, which also agrees with the chemical shifts calculated according to Wishart et al. (1994).

A full list of ^1H, ^{15}N and ^{13}C chemical shifts has been deposited in the BioMagResBank under accession number 4719.

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