Biomol NMR Assign
DOI 10.1007/s12104-007-9076-2

ARTICLE

Backbone assignment of osteopontin, a cytokine and cell attachment protein implicated in tumorigenesis

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Received: 14 November 2007 / Accepted: 11 December 2007

Abstract OPN is an RGD-containing protein overexpressed in cells transformed by *v-myc* and *v-mil*(*raf*) oncogenes. Here we report the resonance assignment of recombinant quail OPN and provide NMR evidence that quail OPN is an intrinsically unstructured protein in solution.

Keywords Osteopontin · NMR signal assignment · Intrinsically unfolded protein · *myc* oncogene · Target gene

Biological context

The protein product (c-Myc) of the protooncogene c-*myc* is a transcriptional regulator playing a crucial role in cellular growth, differentiation, and apoptosis. Deregulated *myc* alleles like the retroviral *v-myc* oncogene cause cell transformation. In order to delineate the molecular mechanism of cell transformation, knowledge of structure and function of oncogenic target genes is indispensable. Avian fibroblasts simultaneously transformed by *v-myc* and *v-mil*(*raf*) display significantly elevated levels of the transcription factor complex AP-1 which subsequently leads to upregulation of specific target genes, such as the osteopontin (OPN) encoding gene (*OPN*), *126MRP*, and *rac2* (Hartl et al. 2006). OPN is an arginine-glycine-aspartate (RGD)-containing glycoprotein that was first identified as a major sialoprotein in bones (Denhardt et al. 2001; Rangaswami et al. 2006). It comprises two receptor-recognition sites, a central integrin-binding domain and a C-terminal CD44-binding site, which are separated by a protease-hypersensitive cleavage site. To provide molecular information about the solution structure of this important cancer target we have started the NMR structure determination of quail OPN.

Methods and experiments

Bacteria of the *E. coli* strain BL21(DE3) transformed with the pET11d-OPN220 expression vector described previously (Hartl et al. 2006) were grown in LB medium overnight. The culture was diluted 1:100 in 50 ml of minimal medium (D'Silva et al. 2005), supplemented with 0.5 g of NaCl, 1 g of $^{15}NH_4Cl$, and 2 g of D-[^{13}C]glucose per 1000 ml, and the bacteria were incubated at 37°C for 12–16 h. The 50-ml preculture was added to 1000 ml of minimal medium, and the bacteria were grown at 37°C until the culture reached an absorbance at 600 nm of 0.7. Recombinant protein synthesis was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM, and incubation was continued for 12 h at 28°C. The cells were collected by centrifugation at 5000 rpm for 20 min and resuspended in 20 ml of ice-cold PBS buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 0.05% NaN_3) per liter of the original bacterial culture. Bacteria were lysed by passing through a French press, and the cell lysate was cleared by centrifugation at 15,000 rpm for 30 min. 20-ml portions of

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the supernatant containing the soluble protein fraction were adjusted to a $(NH_4)_2SO_4$ concentration of 50%, and then clarified by centrifugation at 15,000 rpm for 30 min. The precipitate containing most of the OPN recombinant protein was dialyzed against PBS buffer at 4°C, concentrated to 6 ml by centrifugation through an Amicon Ultra-15 centrifugal filter device 10K NMWL (Amicon), and loaded onto an FPLC anion exchange column (GE Healthcare). Protein was eluted with a sodium chloride gradient generated by mixing PBS (140 mM NaCl) and high salt PBS (1 M NaCl). The supernatant containing most of the OPN protein was concentrated to 1.5 ml by centrifugation through an Amicon Ultra-15 centrifugal filter device 10 K NMWL (Amicon), and loaded onto a Superdex 200 HiLoad 16/60 prep grade (GE Healthcare) gel filtration column equilibrated in a phosphate buffer (150 mM NaCl, 100 mM NaH_2PO_4/Na_2HPO_4 pH 6.5). The final yield of homogeneous OPN was approximately 4 mg/l of bacterial culture. For NMR analysis, protein samples were concentrated to 4 mM.

NMR spectra were recorded at 25°C on Varian Inova 800 MHz, 600 MHz, and 500 MHz spectrometers. The NMR sample contained 1.4 mM osteopontin, 100 mM sodium phosphate, 150 mM NaCl, pH 6.5, in 90% H_2O and 10% D_2O. The suite of standard triple-resonance experiments for extracting $^{13}C\alpha$, $^{13}C\beta$ and $^{13}C'$ chemical shifts included 3D HNCA, 3D HN(CO)CA, 3D HNCACB, 3D CBCA(CO)NH, 3D HNCO, and 3D HN(CA)CO (Cavanagh et al. 1996). In addition, 3D HNN and 3D HN(C)N experiments (Panchal et al. 2001) were performed to establish sequential connectivities and to obtain a high extent of unambiguous backbone assignments. NMR spectra were processed and analyzed using the NMRPipe (Delaglio et al. 1995) and SPARKY (Goddard et al. 2002) software packages, respectively.

Extent of assignments and data deposition

Figure 1 shows the 2D ^{15}N–^{1}H HSQC spectrum of quail OPN collected at 800 MHz. In total, 181 of 207 non-proline ^{1}H and ^{15}N backbone chemical shifts were assigned (87.4%). $^{13}C\alpha$, $^{13}C\beta$ and $^{13}C'$ chemical shifts were allocated for 195 residues (89.0%). Complete backbone assignment was prevented by intense signal overlap. The assignments have been deposited in the BioMagResBank (http://www.bmrb.wisc.edu) under BMRB accession number 15519. The secondary structures of quail OPN were indicated by the consensus chemical shift index (CSI) (Wishart et al. 1995) using assigned $^{13}C\alpha$, $^{13}C\beta$ and $^{13}C'$ chemical shiftsafter correction for sequence effects (Schwarzinger et al. 2001). Overall quail OPN exists as an intrinsically unstructured protein in solution. However,

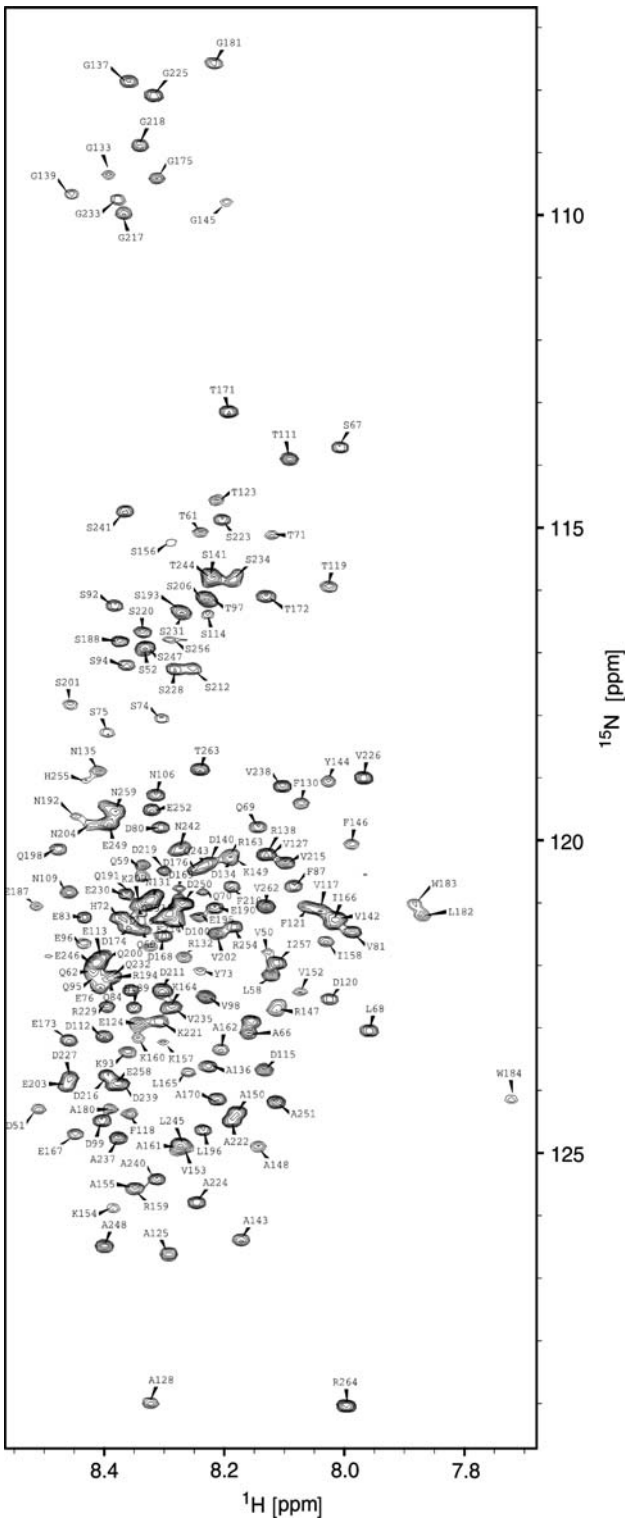

Fig 1 800 MHz 2D ^{1}H–^{15}N HSQC spectrum of quail osteopontin. The residue positions are indicated in the spectrum

significant ^{13}C secondary shifts were observed for several parts of the polypeptide chain. For example, the following secondary structure elements were found: α-helix (Q59-S74; slightly extended conformations for E77–F87

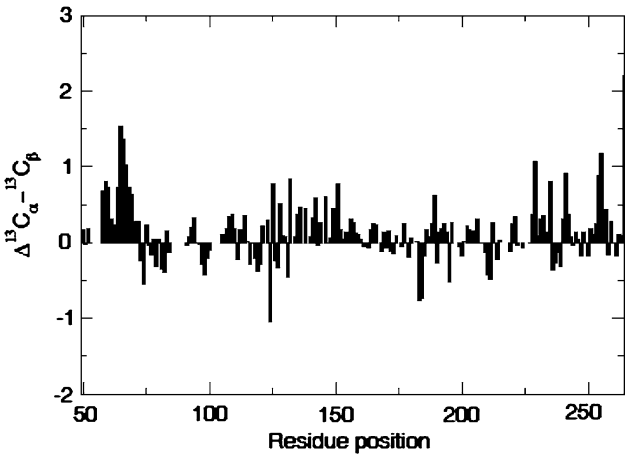

Fig 2 Residue plot of $\Delta^{13}C\alpha - \Delta^{13}C\beta$ secondary shift of quail osteopontin

and V117–R132. The integrin binding site (RGD-motif) is located at residue position 131. The conformational flexibility of quail osteopontin was independently corroborated by a primary sequence analysis based on our recently developed meta-structure approach (Mayer et al. 2007). In this novel sequence analysis tool the local residue-specific compactness value (C_i) is taken as a quantitative parameter describing the structural complexity of an individual residue in the context of the 3D protein fold. A survey of PDB structures revealed an average compactness value of about 300 for a typically folded protein. The obtained average compactness value of quail OPN was found to about 172, and thus indicative of an intrinsic flexibility of quail osteopontin in solution (Fig. 2).

Acknowledgements This work was supported by grants P17041 (to K.B.) and P18148 (to M.H.) from the Austrian Science Foundation (FWF), and WWTF LS612 (to R.K.). R.K. acknowledges general funding by the FWF.

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