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Letter to the Editor: Sequence-specific assignment of the PAH2 domain of Sin3B free and bound to Mad1

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Biological context

The evolutionary conserved protein Sin3 mediates the assembly of a large multi-protein complex involved in modification of the local chromatin structure. Sin3 has been shown to interact with a number of proteins, such as HDAC, N-CoR, Sap30 and Mad1. Sin3 comprises four repeats of approximately 80 amino acids, called Paired Amphipathic-Helix (PAH) domains, that mediate the interactions of Sin3 with its partners. The second PAH domain of Sin3A and -B has been shown to specifically interact with the N-terminus of Mad1 (Mad1-SID, Sin3 Interaction Domain) (Ayer et al., 1995; Eilers et al., 1999; Xu et al., 1999), a DNA-binding protein that antagonizes the transcriptional activation, proliferation-promoting and transformation functions of the onco-protein Myc (Schreiber-Agus et al., 1998; Foley et al., 1999).

Methods and experiments

The fragment containing the PAH2 domain (residues 148–252) of mouse Sin3B was obtained by PCR and cloned into pGEX2T. Using site-directed mutagenesis, the C241A mutation was introduced in the PAH2 domain to prevent disulphide-bridge mediated dimer formation in solution. The resulting GST-PAH2(C241A) fusion protein was expressed in *E. coli* strain pBL21 using 2×YT medium supplemented with 0.5% glucose. Minimal medium was supplemented with $^{13}C_6$-glucose and/or ^{15}N-nitrate where appropriate. Soluble GST-PAH2 fusion protein was obtained after sonication in lysis buffer (1× PBS, 10 mM EDTA, 0.1 mg/ml lysozyme, 1× complete protease inhibitor cocktail (Boehringer), 1 mM PMSF). DTT and Triton-X-100 were added after sonication to a final concentration of 5 mM and 1%, respectively. After clearance of the extract by centrifugation, GST-PAH2 was bound to a glutathione-sepharose column and eluted with 10 mM glutathione in 50 mM Tris-HCl pH 8.0, reduced as recommended by the manufacturer (Pharmacia). The eluate was diluted fivefold with thrombin cleavage buffer (20 mM Tris-HCl pH 8.5, 100 mM NaCl) and incubated at 25 °C with thrombin (Sigma). Subsequently, the PAH2 polypeptide was separated from the GST moiety by repeating the gluthathione-sepharose column, and the flow-through fraction containing PAH2 was purified over a gel-filtration column (Pharmacia S100) equilibrated with 50 mM NaP_i-buffer pH 6.3, dialyzed against water pH 6.3 and freeze-dried in aliquots.

NMR samples of PAH2 complexed to the chemically synthesised unlabelled 13-amino acid hMad1-SID (denoted as PAH2-Mad1) contained 1–2 mM of complex (1:1 stoichiometry) in 50 mM KP_i-buffer at pH 6.3. NMR samples of free PAH2 (denoted as PAH2) contained 1 mM protein, 50 mM KP_i-buffer at pH 6.5 and 100 mM KCl. All NMR samples were prepared in a H_2O/D_2O (95%/5%) mixture and contained trace amounts of NaN_3 as a preservative.

All NMR spectra were acquired at 20 °C on Varian *Inova* 500, 750 MHz and Bruker DRX600 spectrometers. To obtain assignments 3D HNHA, HNCA, HNCO, HN(CO)CA, HNCACB, CBCA(CO)NNH, (H)CCH-TOCSY and HC(C)H-TOCSY experiments were performed. Stereospecific assignments for prochiral methyl groups of PAH2-Mad1 were obtained using a sample containing 10% ^{13}C-labelled PAH2

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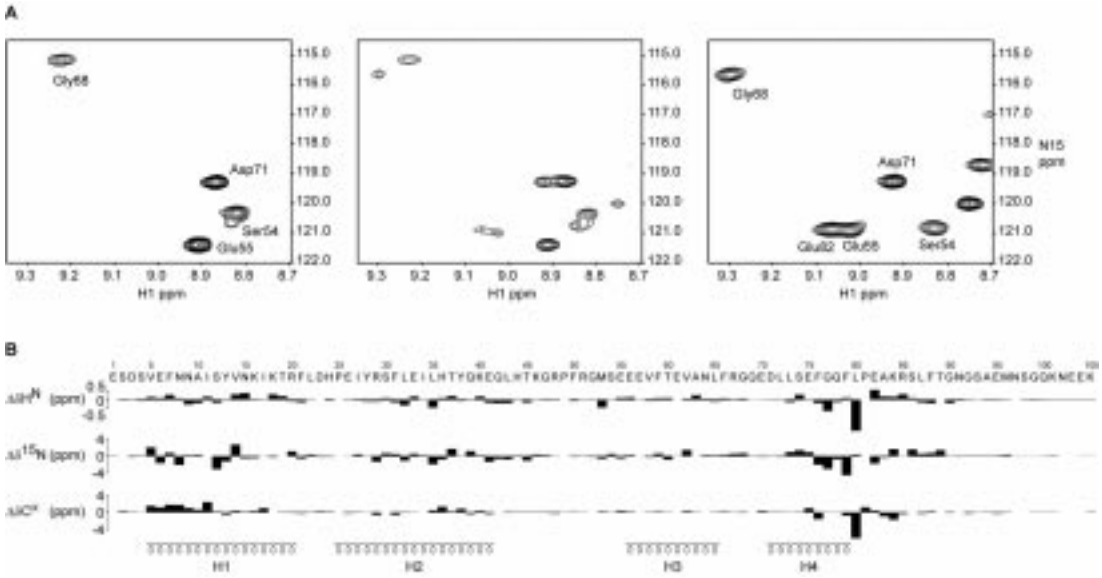

Figure 1. (A) Small regions of the ^{1}H-^{15}N spectrum of PAH2 at 0 (left), 0.5 (middle), and 1 (right) equiv of hMad-SID peptide. (B) Chemical shift differences between the free PAH2 and the PAH2-hMad1-SID complex. Shown are the values of $\delta_{complex} - \delta_{free}$ for H^N, ^{15}N and $^{13}C^{\alpha}$ resonances as a function of residue number.

(Neri et al., 1989). Assignment of the hMad1-SID was accomplished using 2D (^{13}C/^{15}N-filtered)-NOESY and (^{13}C/^{15}N-filtered)-TOCSY spectra. Data processing and analysis was done using NMRPipe (Delaglio et al., 1995) and XEASY (Bartels et al., 1995), respectively.

Figure 1A shows a small region of the ^{15}N-^{1}H HSQC spectra of the PAH2 domain at 0, 0.5, and 1 equiv of hMad1-SID peptide, respectively. The data clearly show the disappearance of the cross peaks of Ser^{54}, Glu^{55}, Gly^{68} and Asp^{71} in conjunction with their appearance at new resonance positions, indicative of a slow-exchange regime. Surface Plasmon Resonance measurements also indicated tight binding of hMad1-SID to PAH2 (K_d of $< 3 * 10^{-7}$). Comparison of the chemical shifts of $^{1}H^{N}$, ^{15}N, and $^{13}C^{\alpha}$ in the free and complexed forms allows for an indication of the regions involved in binding of hMad1-SID. The differences are largest for residues in the first, second and fourth helices of PAH2 (Figure 1B), indicating that these helices form the binding pocket for hMad1-SID. Further details on the structure and interactions in the complex are discussed elsewhere (Spronk et al., 2000).

Extent of assignments and data deposition

The backbone of PAH2-Mad1 was assigned completely, except for the N- and C′-nuclei of the 3 prolines of PAH2. For the unlabelled hMad1-SID we were able to perform a complete sequential assignment of the H^N and H^{α} resonances. Further, >85% of the side chains of the PAH2 protein and the hMad-SID peptide were assigned. For the free PAH2 complete backbone and nearly complete (92%) C^{β} assignments were obtained. Data deposition numbers for assignments of the complex and free PAH2 are BMRB-4841 and BMRB-4874, respectively.

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