

Resonance assignment and secondary structure of the middle MA-3 domain and complete tandem MA-3 region of the tumour suppressor protein Pdc4

Lorna C. Waters · Ojore Oka · Frederick W. Muskett · Sarah L. Strong · Thore Schmedt · Karl-Heinz Klemm · Mark D. Carr

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Abstract Pdc4 (Programmed Cell Death Protein 4) is a novel eukaryotic tumour suppressor protein, which is involved in the regulation of both transcription and translation (reviewed in Lankat-Buttgereit and Göke 2009). The protein contains two interacting MA-3 domains (MA-3_M and MA-3_C), which are linked by a short semi-flexible linker region (Waters et al. 2007; Suzuki et al. 2008). The MA-3 domains are involved in mediating specific protein–protein interactions with functional partners such as eIF4A (Yang et al. 2003). Here we report essentially complete backbone and side chain ¹⁵N, ¹³C and ¹H assignments for a construct composed of the middle MA-3 domain and subsequent linker region (MA-3_M) and backbone assignments for the entire tandem MA-3 region of Pdc4 (Pdc4 MA-3_{M-C}). Analysis of the backbone chemical shift data obtained indicates that Pdc4 MA-3_M contains eight helical regions corresponding to over 74% of the protein backbone and that Pdc4 MA-3_{M-C} contains fifteen helical regions (72%). Comparison of the position of these helical regions with those observed in the crystal structures suggests that the solution and crystal structures of both proteins are very similar.

Keywords Pdc4 · MA-3 domain · NMR resonance assignments · Secondary structure

Biological context

The eukaryotic regulatory protein Pdc4 (Programmed Cell Death Protein 4) was initially discovered in a screen for genes activated during apoptosis and later identified as a tumour suppressor in studies of a mouse keratinocyte model of tumour promotion. A number of subsequent studies have also highlighted Pdc4's role as a tumour suppressor, for example, Pdc4 has been shown to suppress tumour development in a mouse model of skin carcinogenesis (reviewed in Lankat-Buttgereit and Göke 2009). Pdc4 has been shown to play critical roles in the regulation of both transcription and translation, mediated via specific protein–protein and perhaps protein–RNA interactions (Bitomsky et al. 2004; Yang et al. 2003).

The 469 residue mouse Pdc4 protein contains at least three domains, an N-terminal RNA-binding region (residues 1–157) and two interacting MA3 domains (residues 157–275 and 319–449), which are linked by a short semi flexible region (Waters et al. 2007; Suzuki et al. 2008). To date the majority of the molecular and structural studies of Pdc4 have focused on the interaction between the two MA-3 domains of Pdc4 and the eukaryotic translation initiation factor eIF4A, with complex formation resulting in the inhibition of cap-dependent translation (Yang et al. 2003). The structures of both the isolated C-terminal (MA-3_C) MA-3 domain, and a construct composed of the middle MA-3 domain and subsequent linker region (MA-3_M) of Pdc4 have been solved and were shown to be composed of three or four layers of atypical HEAT repeats respectively (Waters et al. 2007; Suzuki et al. 2008). More recently, the

Lorna C. Waters and Ojore Oka are equal first authors.

L. C. Waters (✉) · O. Oka · F. W. Muskett · S. L. Strong · M. D. Carr (✉)
Department of Biochemistry, Henry Wellcome Building,
University of Leicester, Lancaster Road,
Leicester LE1 9HN, UK
e-mail: lw83@le.ac.uk

M. D. Carr
e-mail: mdc12@le.ac.uk

T. Schmedt · K.-H. Klemm
Institut für Biochemie, Westfälische-Wilhelms-Universität
Münster, Wilhelm-Klemm-Str. 2, 48149 Münster, Germany

crystal structure of the entire tandem MA-3 region (MA-3_{M-C}) has also been determined (Loh et al. 2009). To date only the assignments for the Pdc4 MA-3_C have been deposited (BMRB accession number 6900).

Here we report the determination of essentially complete sequence-specific backbone and side chain assignments for Pdc4 MA-3_M and backbone assignments for Pdc4 MA-3_{M-C}, as well as the positions of elements of regular secondary structure.

Methods and experiments

Unlabelled and uniformly ¹⁵N and ¹⁵N/¹³C labelled samples of mouse Pdc4 MA-3_M (residues 157–318) and Pdc4 MA-3_{M-C} (residues 157–449) were prepared from pGex-6P-2-based *E. coli* expression vectors essentially as described previously for Pdc4 MA-3_C (Waters et al. 2006a), however, for Pdc4 MA-3_{M-C} transformed cells protein expression was induced for 20 h at 15°C. In addition, the cleaved Pdc4 constructs and GST were separated by a second round of glutathione based affinity chromatography prior to gel filtration. A ¹³C labelled Pdc4 MA-3_M sample which was used for the ¹³C/¹H NOESY-HSQC experiment was prepared as described above, but with the addition of non-isotopically labelled aromatic amino acids to the minimal media (His, Phe, Trp and Tyr at 50 mg/l).

NMR spectra of Pdc4 MA-3_M were acquired from 0.35 ml samples of 0.5–1.5 mM Pdc4 MA-3_M in a 25 mM sodium phosphate, 100 mM sodium chloride, 0.5 mM DTT, 10 μM EDTA, 200 μM AEBF and 0.02% (w/v) sodium azide buffer at pH 6.5, containing 10% D₂O/90% H₂O or 100% D₂O as appropriate. All NMR data were acquired at 25°C on either 600 MHz Bruker Avance/DRX systems or an 800 MHz Bruker Avance II spectrometer. The 2D and 3D spectra recorded to obtain sequence specific assignments for Pdc4 MA-3_M were: ¹H-¹H TOCSY with mixing times of 35 and 55 ms, ¹H-¹H NOESY with an NOE mixing time of 100 ms; ¹⁵N/¹H HSQC; TOCSY-HSQC with a mixing time of 55 ms; NOESY-HSQC with an NOE mixing time of 100 ms; ¹³C/¹H HCCH-TOCSY with a mixing time 19.6 ms, ¹³C/¹H HSQC-NOESY with an NOE mixing time of 100 ms; ¹⁵N/¹³C/¹H HNCACB, CBCA (CO)NH and HNCO (reviewed in Cavanagh et al. 2006). Typical acquisition times in F₁ and F₂ for the 3D experiments were 12–25 ms for ¹⁵N, 6.7–9.0 ms for ¹³C (35 ms for HNCO experiments) and 15–22.5 ms for ¹H, and with an acquisition time of 55–70 ms in F₃ (¹H). Typical acquisition times in F₁ for 2D experiments were 50 ms for ¹⁵N and 35 ms for ¹H, with 80–100 ms in F₂.

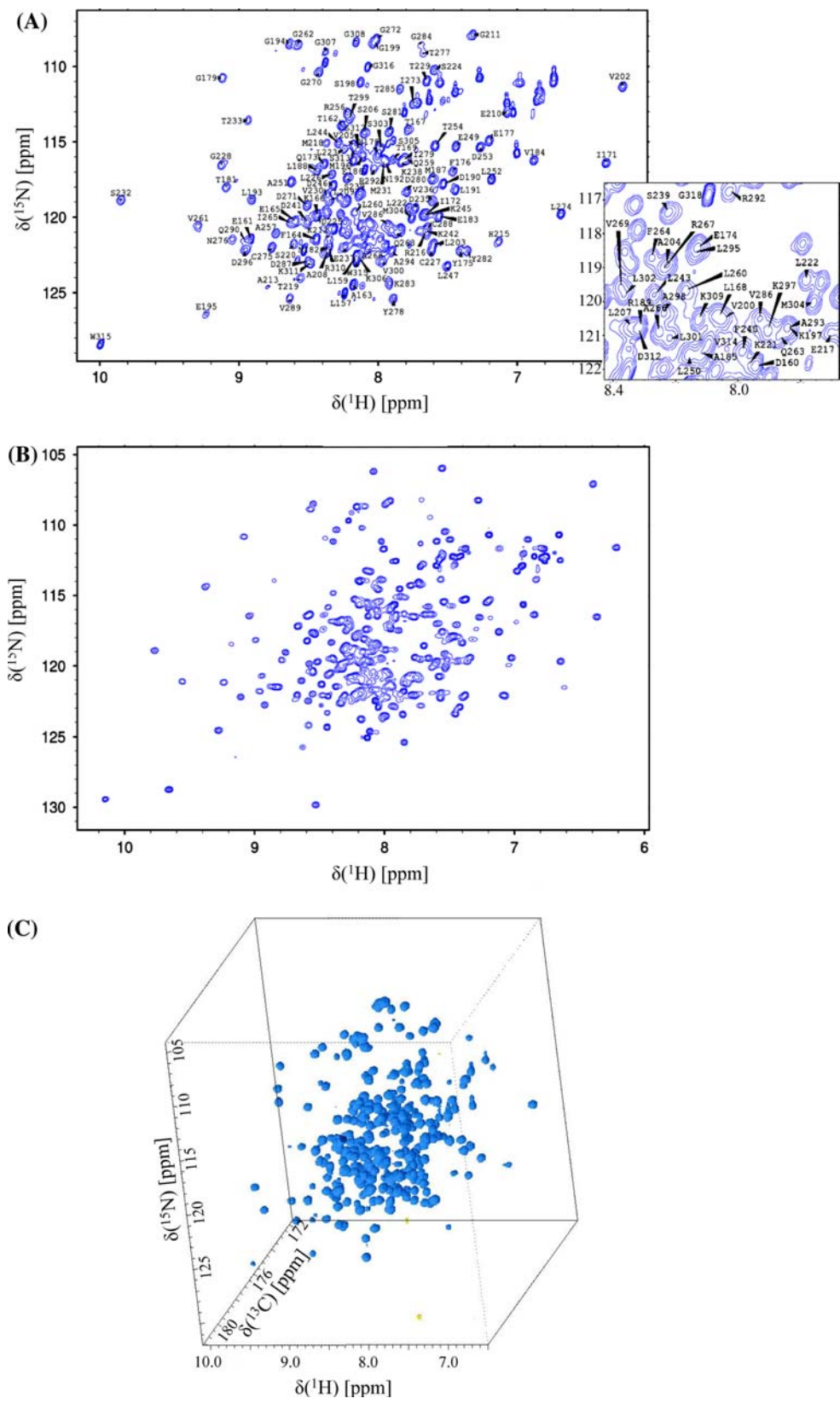
NMR spectra of Pdc4 MA-3_{M-C} were acquired from 0.35 ml samples of 0.15–0.50 mM Pdc4 MA-3_{M-C} in a 25 mM sodium phosphate, 100 mM sodium chloride,

Fig. 1 Quality of the NMR spectra obtained for Pdc4 MA-3_M and Pdc4 MA-3_{M-C}. *Panel A* shows an ¹⁵N/¹H HSQC spectrum of Pdc4 MA-3_M. The assignments of the signals from backbone amide groups are indicated by residue type and number. The region between 6.7 and 7.8 ppm in ¹H and 110.0 and 113.3 ppm in ¹⁵N contains, with the exception of E210, S224, T229 and I273, signals from the sidechain amide groups of the asparagine and glutamine residues. The signal located at 10.0 ppm in ¹H and 128.4 ppm in ¹⁵N ppm is from the indolic-NH group of W315. The assignments obtained for overlapped signals in the region between 7.7 and 8.4 ppm in ¹H and 116.6 and 122.3 ppm in ¹⁵N are indicated in the *enlarged region*. *Panel B* shows an ¹³N/¹H HSQC spectrum of Pdc4 MA-3_{M-C} and panel C a three dimensional view of a ¹⁵N/¹³C/¹H HNCO spectrum of Pdc4 MA-3_{M-C}

2.5 mM DTT, 2.5 mM TCEP, 50 μM EDTA, 200 μM AEBF, and 0.02% (w/v) sodium azide buffer at pH 6.5, containing 5% D₂O/95% H₂O. NMR data were acquired at 35°C on an 800 MHz Bruker Avance II spectrometer. The 3D spectra recorded to obtain backbone assignments for Pdc4 MA-3_{M-C} were: ¹⁵N/¹H NOESY-HSQC with an NOE mixing time of 75 ms and ¹⁵N/¹³C/¹H HNCA, HNCO, TROSY-HN(CO)CA and CBCA(CO)NH. Typical acquisition times in F₁ and F₂ for the 3D experiments were 10–25 ms for ¹⁵N, 6.7–9.0 ms for ¹³C (22.5 ms for HNCO experiments) and 16 ms for ¹H, and with an acquisition time of 60 ms in F₃ (¹H).

The majority of the 3D spectra were collected over approximately 72 h. The WATERGATE method was used to suppress the water signal when required. The 3D NMR data were processed using Topspin (Bruker Biospin Ltd) with linear prediction used to extend the effective acquisition times by up to twofold in F₁ and F₂. The spectra were analysed using the Sparky package (T. D. Goddard and D. G. Kneller, SPARKY 3, University of California, San Francisco).

Sequence-specific backbone and side chain resonance assignments were obtained for Pdc4 MA-3_M using the approach described previously (Waters et al. 2006b). Initially, tentative sequence-specific backbone resonance assignments (N, NH, Cα and CO) for Pdc4 MA-3_{M-C} were obtained by transposing the assignments from the isolated MA-3 domains (acquired at 25°C) onto the triple resonance spectra obtained for Pdc4 MA-3_{M-C} (acquired at 35°C). Ambiguities between the assignments obtained for the individual MA-3 domains and Pdc4 MA-3_{M-C}, arising from differences in the temperature at which the spectra were recorded and/or changes in the chemical environment of residues located at the interface between the two MA-3 domains, were resolved by comparing the amide nitrogen/proton and carbonyl chemical shifts of residues in HNCO spectra recorded at a range of temperatures (25 and 35°C for the individual MA-3 domains and 25, 30 and 35°C for Pdc4 MA-3_{M-C}). Finally, the inter-residue amide nitrogen/proton to Cα connectivities observed in triple resonance spectra and the NH to NH NOEs identified in NOESY-HSQC spectra were used to confirm all the assignments made.



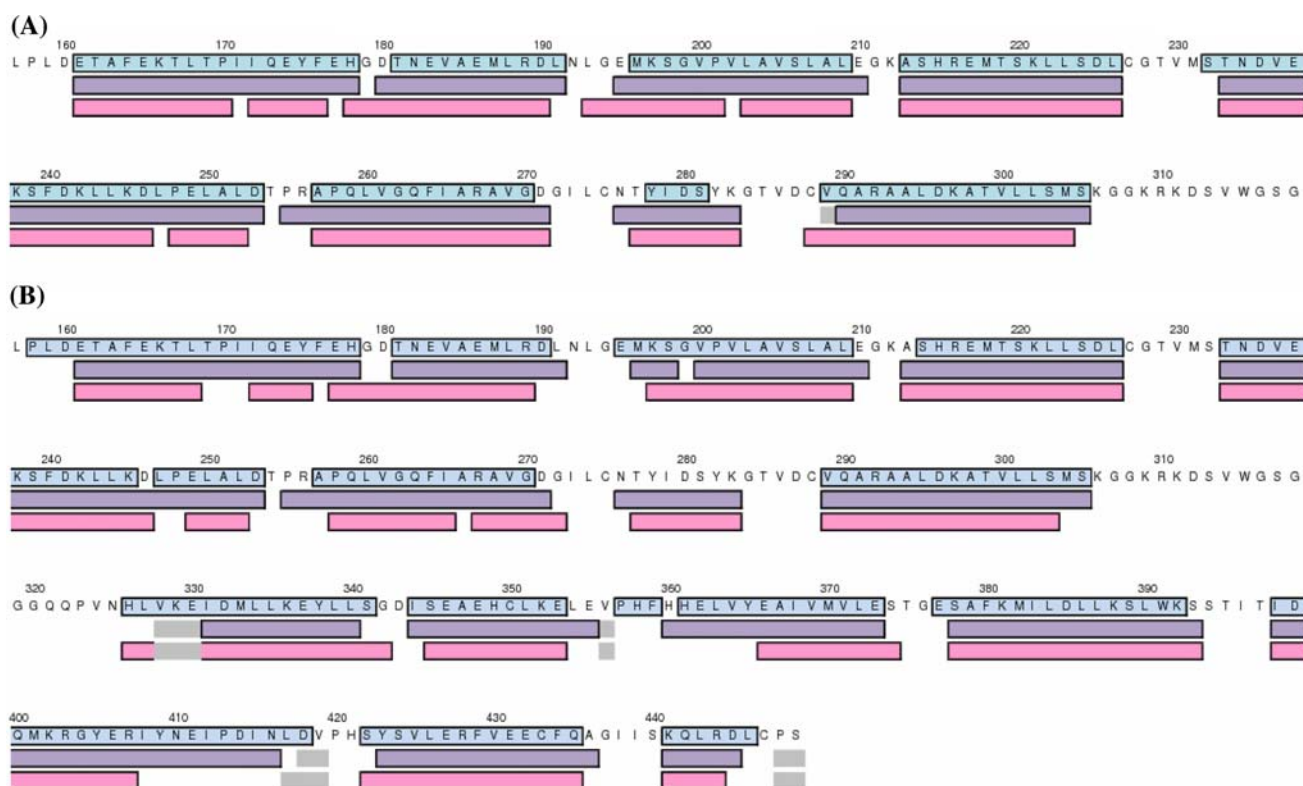


Fig. 2 Location of the secondary structure elements in Pdc4 MA-3_M and Pdc4 MA-3_{M-C}. *Panel A* shows the amino acid sequence of Pdc4 MA-3_M. Highlighted in blue on the sequence are the positions of the helical regions observed in the crystal structure of Pdc4 MA-3_M (Suzuki et al. 2008; PDB code 2RG8). Indicated underneath the sequence in purple and pink are the regions suggested to be helical by the programmes TALOS (shown immediately below) and CSI

respectively. Residues for which no data could be obtained, due to a lack of input chemical shifts, are highlighted in grey. *Panel B* shows the amino acid sequence for the entire Pdc4 MA-3_{M-C} region. Helical regions are indicated as in *panel A*. In this case, residues highlighted in blue show the positions of the helical regions observed in the crystal structure of Pdc4 MA-3_{M-C} (Loh et al. 2009; PDB code 3EIJ)

Extent of assignments and data deposition

Pdc4 MA-3_M gives rise to well-resolved spectra, as illustrated by the ¹⁵N/¹H HSQC spectrum shown in Fig. 1a. This allowed essentially complete backbone resonance assignments to be made. Despite the relatively large size of Pdc4 MA-3_{M-C} (293 residues) and consequent overlap of signals in 2D ¹⁵N/¹H HSQC spectra, we were also able to obtain comprehensive sequence-specific backbone resonance assignments. The quality of the NMR spectra obtained is illustrated by the HSQC and HNC0 spectra shown in Fig. 1b, c.

Backbone amide assignments were obtained for all residues in Pdc4 MA-3_M except: D180, S214 and A291 (98.1%), for all C α and C β signals apart from M218 (C β only), C288 and Q290 (98.5%) and for all CO signals, except for residues followed by a proline, and G179, A213, T233, Q290 and G318 (93.2%). In the case of H α and H β signals, assignments were obtained for all residues except: P201, L207 (H β 3 only), R216, E217 (H β 2, H β 3), M218 (H β 3 only), K221 (H β 3 only), P258, L260, R267, C288,

Q290 and S317 (H β 3 only) (94.1%). For the remaining non-exchangeable side chain signals (¹³C and ¹H) complete assignments were obtained apart from: L188 ((H δ 2)₃), L193 (C γ), K197 (H ϵ 3), P201 (C γ , C δ , H γ 2, H γ 3, H δ 2, H δ 3), L207 (C γ , H γ), R216 (C γ , C δ , H γ 2, H γ 3, H δ 2, H δ 3), P248 (C γ), P258 (C γ , C δ , H γ 2, H γ 3, H δ 2, H δ 3), L260 (C γ , C δ 1, C δ 2, H γ , (H δ 1)₃, (H δ 2)₃), R267 (C γ , C δ , H γ 2, H γ 3, H δ 2, H δ 3), V269 (C γ 1), Q290 (C γ , H γ 2, H γ 3) and W315 (H ζ 2, H η 2) (93.5%).

Backbone amide assignments were obtained for all non-proline residues in Pdc4 MA-3_{M-C} except: D180, S214, A291, G319, H326, V328, K329, E330, I331, V356, H360, T397, D418, V419, S422 and S449 (94.0%), for all C α signals except: H326, V328, K329, E330, V356, I415, L417, D418, V419, S422, P448 and S449 (95.9%), and for all CO signals except residues followed by a proline, and G179, A213, T233, Q290, G318, N325, L327, V328, K329, E330, E355, F359, I396, D414, L417, D418, H421, P448 and S449 (89.8%). The comprehensive ¹⁵N, ¹³C and ¹H resonance assignments obtained for Pdc4 MA-3_M and Pdc4 MA-3_{M-C} have been deposited

at the BioMagResBank database (accession numbers 16538 and 16542 respectively).

Analysis of the backbone chemical shifts of Pdc4 MA-3_M, using the programme TALOS (Shen et al. 2009), shows that the domain contains eight helical regions (residues E161-H178, D180-L191, E195-E210, A213-L226, T233-D253, P255-D271, N276-K283 and Q290-S305). These regions are in close agreement with the position of the secondary structure elements observed in the crystal structure of Pdc4 MA-3_M, as shown in Fig. 2a (Suzuki et al. 2008). Similarly, analysis of the backbone chemical shifts of Pdc4 MA-3_{M-C} showed that there were 15 helical regions (residues E161-H178, T181-L191, M196-E210, A213-L226, T233-D253, P255-D271, N276-K283, V289-S305, I331-L340, I344-E355, H360-E373, S378-S393, I398-N416, Y423-A436 and K441-D445). These results are also in close agreement with the position of the secondary structure elements observed in the crystal structure of Pdc4 MA-3_{M-C}, as shown in Fig. 2b (Loh et al. 2009). The one exception is the absence of the N276-K283 helix in the crystal structure, where this region is instead composed of a series of bends and turns. However, it should be noted that B-factors for this region are somewhat higher than average. The NMR results reported here clearly indicate that the solution and crystal structures of both proteins are very similar.

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