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¹H, ¹³C and ¹⁵N backbone resonance assignment of the human BRCA2 N-terminal region

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Abstract

The Breast Cancer susceptibility protein 2 (BRCA2) is involved in mechanisms that maintain genome stability, including DNA repair, replication and cell division. These functions are ensured by the folded C-terminal DNA binding domain of BRCA2 but also by its large regions predicted to be disordered. Several studies have shown that disordered regions of BRCA2 are subjected to phosphorylation, thus regulating BRCA2 interactions through the cell cycle. The N-terminal region of BRCA2 contains two highly conserved clusters of phosphorylation sites between amino acids 75 and 210. Upon phosphorylation by CDK, the cluster 1 is known to become a docking site for the kinase PLK1. The cluster 2 is phosphorylated by PLK1 at least at 2 positions. Both of these phosphorylation clusters are important for mitosis progression, in particular for chromosome segregation and cytokinesis. In order to identify the phosphorylated residues and to characterize the phosphorylation sites preferences and their functional consequences within BRCA2 N-terminus, we have produced and analyzed the BRCA2 fragment from amino acid 48 to amino acid 284 (BRCA2₄₈₋₂₈₄). Here, we report the assignment of ¹H, ¹⁵N, ¹³CO, ¹³C α and ¹³C β NMR chemical shifts of this region. Analysis of these chemical shifts confirmed that BRCA2₄₈₋₂₈₄ shows no stable fold : it is intrinsically disordered, with only short, transient α -helices.

Keywords. BRCA2, mitosis, breast cancer, intrinsically disordered protein, phosphorylation, NMR.

Biological context

BReast Cancer susceptibility 2 (BRCA2) is a tumor suppressor gene identified in 1994, commonly mutated in hereditary breast and ovarian cancers (Wooster et al. 1994). BRCA2 was initially characterized as a DNA repair protein involved in the recruitment and loading of the recombinase RAD51, which, in turn, drives the homologous recombination process at DNA double-strand breaks (DSB) (Thorslund et al. 2007, Moynahan et al. 2010, Jensen et al. 2010). Two decades later, BRCA2 is mainly described as a key platform protein for genome stability contributing to DNA repair, telomere maintenance, stressed replication fork stabilization (Fradet-Turcotte et al. 2016) and mitosis (Daniels et al. 2004, Mondal et al. 2012, Choi et al. 2012, Venkitaraman et al. 2014,). These functions are ensured by the folded C-terminal DNA binding domain of BRCA2 (Yang et al. 2002) and the large regions predicted to be disordered that contain several protein binding motifs. Among them, two short fragments were already structurally characterized in complex with their partner: one of the repeated BRC motifs BRC4 bound to RAD51 (Pellegrini et al. 2002) and BRCA2 fragment between amino acid 21 and amino acid 39 interacting with PALB2 (Oliver et al. 2009). Both of these BRCA2 segments form α -helices upon binding to their partner. Here, we focus on the N-terminal region of BRCA2, which contains 1000 amino acids with a structure that remain elusive. It is predicted to be disordered (**Figure 1.B**) and contains two clusters of phosphorylation (**Figure 1.A**) identified by mass spectrometry (<https://www.phosphosite.org/>) between amino acid 75 and amino acid 80 for cluster 1, and amino acid 190 and aa 210 for cluster 2. These two clusters are very well conserved from mammals to fishes and their biological relevance is still unclear. The cluster 1 was reported to be phosphorylated by Cyclin-Dependant Kinase (CDK) at position T77 during late S-phase to mitosis (Yata, et al. 2014). This phosphorylation generates a genuine Polo-Like Kinase 1 (PLK1) docking site (Takaoka et al. 2014, Yata et al. 2014) suggested to promote further phosphorylation by PLK1 both on BRCA2 itself (Lin et al. 2003, Takaoka et al. 2014) and on BRCA2 partners such as RAD51 (Yata et al. 2014). However, biochemical data are missing to describe to which extent this BRCA2 phosphorylation by CDK influences later phosphorylation kinetics by PLK1. The cluster 2 is phosphorylated by PLK1 during mitosis (Lin et al. 2003). This regulates BRCA2 interaction with P300/CBP-associated factor (P/CAF) (Lin et al. 2003). It also creates a supplementary PLK1 docking site that ensures chromosome segregation (Ehlen et al. BioRxiv). Furthermore, phosphorylation of S193 by PLK1 leads to the recruitment of BRCA2 at the Flemming body, an important step that warrants a complete cytokinesis (Daniels et al. 2004, Takaoka et al. 2014). While these phosphorylation events have been observed to dynamically regulate these BRCA2 functions throughout the cell cycle, little is known about the structure of the BRCA2 N-terminal region and the phosphorylated residues are only partially identified. Here, we report the assignment of the ^1H , ^{15}N , $^{13}\text{C}\alpha$ $^{13}\text{C}\beta$ and ^{13}CO NMR chemical shifts of the WT BRCA2₄₈₋₂₈₄ fragment.

Methods and experiments

Protein expression and purification

Five fragments spanning the human WT BRCA2 N-terminal phosphorylation clusters were designed: a fragment from amino acid 53 to amino acid 131 (BRCA2₅₃₋₁₃₁), a fragment from amino acid 53 to amino acid 228 (BRCA2₅₃₋₂₂₈), a fragment from amino acid 48 to amino acid 218 in which all cysteines are mutated into alanines (BRCA2_{48-218(C4A)}) a fragment from amino acid 190 to amino acid 284 (BRCA2₁₉₀₋₂₈₄) and a fragment from amino acid 48 to amino acid 284 with the first four cysteines mutated into alanines (BRCA2_{48-284(C4A)}) (**Figure 2.A**). All fragments were expressed in *Escherichia coli* BL21 (DE3) Star using a pETM13 vector (BRCA2₅₃₋₂₂₈, BRCA2_{48-218(C4A)} and BRCA2_{48-284(C4A)}), a pGEX-6P-1 vector (GE Healthcare, BRCA2₁₉₀₋₂₈₄) or a pET-41b vector (BRCA2₅₃₋₁₃₁). cDNA of BRCA2₅₃₋₁₃₁, BRCA2₅₃₋₂₂₈, BRCA2_{48-218(C4A)} and BRCA2_{48-284(C4A)} were optimized for expression in *E. coli* (Genscript).

Bacteria were grown in M9 medium supplemented with $^{15}\text{NH}_4\text{Cl}$ (0.5 g/L) and ^{13}C -glucose (2 g/L) as sole sources of nitrogen and carbon. Recombinant expression was induced at an optical density of 0.6-0.8 using 1 mM isopropyl β -D-1-thiogalactopyranoside during 4 hours at 37°C. Cells were harvested by centrifugation, resuspended in lysis buffer (20 mM Tris, 150 mM NaCl, 1 mM EDTA, 5 mM DTT, 1 mM PMSF, pH 8) and lysed by sonication. The soluble fraction was obtained upon centrifugation of the whole cell lysate at 20,000 g during 15 min at 4°C. BRCA2₅₃₋₁₃₁, BRCA2₅₃₋₂₂₈ and BRCA2₁₉₀₋₂₈₄ were produced with a N-terminal GST tag followed by either a TEV cleavage site (ENLYFQG) or a PreScission cleavage site (LEVLFQGP) and purified by Gluthatione Sepharose affinity chromatography. The tag was cleaved by the TEV protease (BRCA2₅₃₋₁₃₁ and BRCA2₅₃₋₂₂₈) or the PreScission protease (BRCA2₁₉₀₋₂₈₄). The BRCA2₅₃₋₁₃₁ and BRCA2₁₉₀₋₂₈₄ samples were then boiled at 95°C during 10 min to remove the GST tag, centrifuged 10 minutes at 16,000g, and the supernatant was later injected on a gel filtration column (Highload 16/60 Superdex 75pg; GE Healthcare) equilibrated with buffer A (50 mM HEPES, 1 mM EDTA, 2 mM dithiothreitol, pH 7.0). The influence of boiling on structure was verified by ^1H - ^{15}N HSQC NMR (data not shown). BRCA2₅₃₋₂₂₈ precipitates after cleavage, and therefore was pelleted by centrifugation (10 minutes, 16,000g), then resuspended into buffer A supplemented with 8 M urea and finally diluted 10 times with buffer A containing 10 mM β -mercaptoethanol before injection on a gel filtration column (Highload 16/60 Superdex 75pg; GE Healthcare) equilibrated with 50 mM HEPES, 1 mM EDTA, 5 mM tris(2-carboxyethyl)phosphine (TCEP), pH 7.0. BRCA2_{48-218(C4A)} and BRCA2_{48-284(C4A)} were expressed with a N-terminal octa-histidine tag and purified by Ni-NTA affinity chromatography. The tag was cleaved using the TEV protease and the sample was injected on a gel filtration column (Highload 16/60 Superdex 75 pg; GE Healthcare) equilibrated with buffer A at pH 7.0 or buffer A at pH 6.4 for assignment of BRCA2_{48-218(C4A)}. Samples were concentrated to 200-400 μM for assignment experiments (BRCA2₅₃₋₁₃₁, BRCA2_{48-218(C4A)} and BRCA2₁₉₀₋₂₈₄) and to 50 μM for BRCA2₅₃₋₂₂₈ and BRCA2_{48-284(C4A)} characterization.

NMR Spectroscopy

NMR experiments were performed on uniformly ^{15}N and ^{13}C labelled fragments in buffer A at pH 7.0 for BRCA2₅₃₋₁₃₁, BRCA2_{48-218(C4A)}, BRCA2₁₉₀₋₂₈₄ and BRCA2_{48-284(C4A)} ($\text{H}_2\text{O}:\text{D}_2\text{O}$ ratio 90:10), in buffer A at pH 6.4 ($\text{H}_2\text{O}:\text{D}_2\text{O}$ ratio 95:5) for BRCA2_{48-218(C4A)} and in 50 mM HEPES, 1 mM EDTA, 5 mM TCEP, pH 7.0 ($\text{H}_2\text{O}:\text{D}_2\text{O}$ ratio 95:5) for BRCA2₅₃₋₂₂₈. All samples were supplemented with 50 μM Sodium trimethylsilylpropanesulfonate (DSS). NMR experiments were recorded at 283 K on a 600 MHz Bruker Advance II spectrometer and a 700 MHz Bruker Advance Neo spectrometer, both equipped with a triple resonance cryogenically cooled probe. Spectra were referenced using DSS ^1H chemical shifts (Wishart et al. 1995) and ^1H , ^{13}C and ^{15}N resonance frequencies were assigned using 2D ^1H - ^{15}N SOFAST-HMQC, 3D BEST-HNCACB, CBCA(CO)NH, BEST-HNCO, BEST-HN(CA)CO and HN(CO)(CA)NH experiments. The data were processed using Topspin 3.6 (Bruker) and analyzed with CCPNMR Analysis (Vranken et al. 2005). The assignments were deposited in the BioMagResBank (<http://www.bmrb.wisc.edu/>) under the following codes: 50077 for BRCA2_{48-218(C4A)}, 50078 for BRCA2₅₃₋₁₃₁ and 50079 for BRCA2₁₉₀₋₂₈₄.

Assignments and data deposition

To facilitate the assignment of the human BRCA2 region from amino acid 48 to amino acid 284, we designed and produced three overlapping fragments: BRCA2₅₃₋₁₃₁ centered on the phosphorylation cluster 1, BRCA2₅₃₋₂₂₈ containing the two phosphorylation clusters and BRCA2₁₉₀₋₂₈₄ that contains only the cluster 2 (**Figure 2.A**). BRCA2₅₃₋₁₃₁ and BRCA2₁₉₀₋₂₈₄ were purified in sufficient amounts and were stable enough for NMR analysis. However,

BRCA2₅₃₋₂₂₈ was prone to aggregation due to the oxidation of its four solvent-exposed cysteines. These cysteines are not conserved within BRCA2 from fishes to human. Therefore, we designed the construct BRCA2_{48-218(C4A)}, which corresponds to the fragment from amino acid 48 to amino acid 218 with all cysteines mutated into alanines (C132A, C138A, C148A and C161A; see **Figure 2A**). The ¹H-¹⁵N HSQC spectra of BRCA2₅₃₋₂₂₈ and BRCA2_{48-218(C4A)} overlap to a large extent: we observed differences only in the vicinity of the mutated residues and N-ter or C-ter ends (Figure 2.B). This shows that the alanine mutations did not modify the average structural ensemble of the peptide. Then, we assigned the ¹H, ¹⁵N, ¹³C α , ¹³C β and ¹³CO chemical shifts of the fragments BRCA2₅₃₋₁₃₁, BRCA2_{48-218(C4A)} and BRCA2₁₉₀₋₂₈₄ using a series of 3D heteronuclear NMR experiments. We obtained high assignment coverages along the sequences of fragments BRCA2₅₃₋₁₃₁, BRCA2_{48-218(C4A)} and BRCA2₁₉₀₋₂₈₄: 94 %, 97% and 96% of ¹H-¹⁵N pairs, 87 %, 98 % and 95% of ¹³C α , 99%, 96 % and 97 % of ¹³C β and 96 %, 98 %, 95 % of ¹³CO resonances were assigned, respectively. **Figure 3** shows a very good crosspeak superimposition between the ¹H-¹⁵N spectra of every fragment and that of BRCA2_{48-284(C4A)}. The narrow range of backbone amide ¹H chemical shifts (between 7.5 and 8.5 ppm) for all BRCA2 fragments reveals their disordered behavior. Furthermore, only the crosspeaks corresponding to the N-terminal or C-terminal residues of each fragment differ from those of the largest construct of BRCA2, i.e. BRCA2_{48-284(C4A)}. The secondary structure analysis, based on ¹³C α and ¹³C β chemical shifts and the neighbor corrected structural propensity method (Tamiola et al. 2010, Tamiola et al. 2012), confirms the absence of a stable fold for BRCA2₅₃₋₁₃₁, BRCA2_{48-218(C4A)} and BRCA2₁₉₀₋₂₈₄ (**Figure 4**). We observed α -helical propensities of about 25 % around residues 100-110 and 255-260. We concluded that the fragment of BRCA2 from amino acid 48 to amino acid 284 is disordered and that its shorter segments BRCA2₅₃₋₁₃₁, BRCA2_{48-218(C4A)} and BRCA2₁₉₀₋₂₈₄ have the same structural properties when isolated or within BRCA2_{48-284(C4A)}. From this set of data, it is now possible to monitor phosphorylation reactions within the two conserved BRCA2 clusters of phosphorylation using ¹H-¹⁵N and ¹H-¹³C NMR spectroscopy.

Acknowledgments

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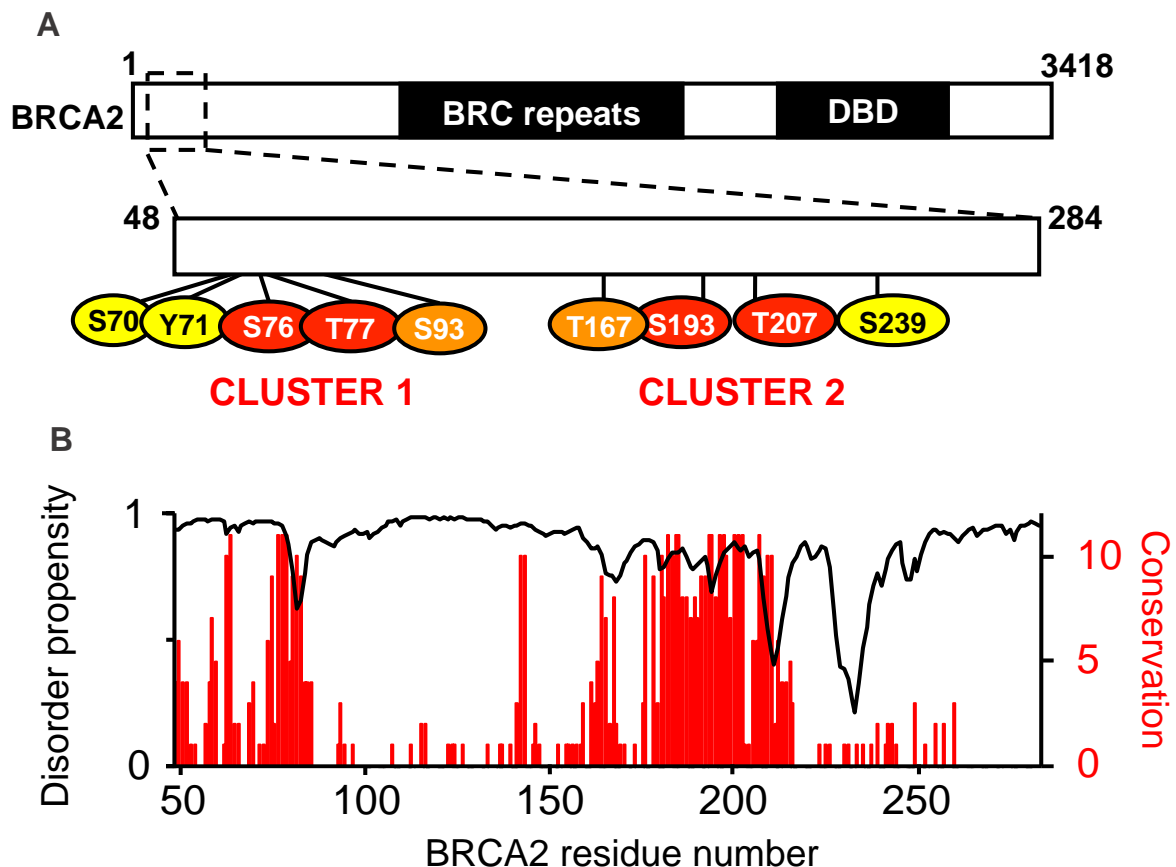


Figure 1. The N-terminal BRCA2 fragment includes 2 phosphoclusters well-conserved from mammals to fishes. (A) BRCA2 is composed of i) a 1000 residues long N-terminal region containing the PALB2 binding site (from amino acid 21 to amino acid 39) and a DNA binding domain (DBD) (from amino acid 250 to amino acid 500) (Von Nicolai et al. 2016), ii) an intermediate region made of 8 successive BRC repeats (from amino acid 1002 to amino acid 2085 and iii) a folded C-terminal DBD (from amino acid 2500 to amino acid 3200). The N-terminal region of the protein contains several phosphorylation sites (<https://www.phosphosite.org/>) organized in two phosphorylation clusters. Alignment of 30 BRCA2 sequences from fishes to mammals revealed that 4 of these positions are 100% conserved: Ser76, Thr77, Ser193 and Thr207. We focused on the BRCA2 region from amino acid 48 to amino acid 284, which includes these 4 phosphosites. In this region, residues identified as phosphorylated in more than one mass spectrometry study are indicated in red if conserved in all 30 sequences, in orange if conserved in more than 80% and yellow if conserved in less than 80% of the sequences. (B) The disorder propensity and conservation of the BRCA2 region from amino acid 48 to amino acid 284 are displayed as a function of the sequence. The disorder propensity was calculated using SPOT-Disorder (Hanson et al. 2016). A score of 1 corresponds to a predicted disorder propensity of 100%. The conservation score was calculated using Jalview 1.0 (Clamp et al. 2004). A score of 11 corresponds to a position identical in 100% of the sequences, while a score of 1 indicates that only one chemical criteria (size, hydrophobicity, global charge) is common to all the variants.

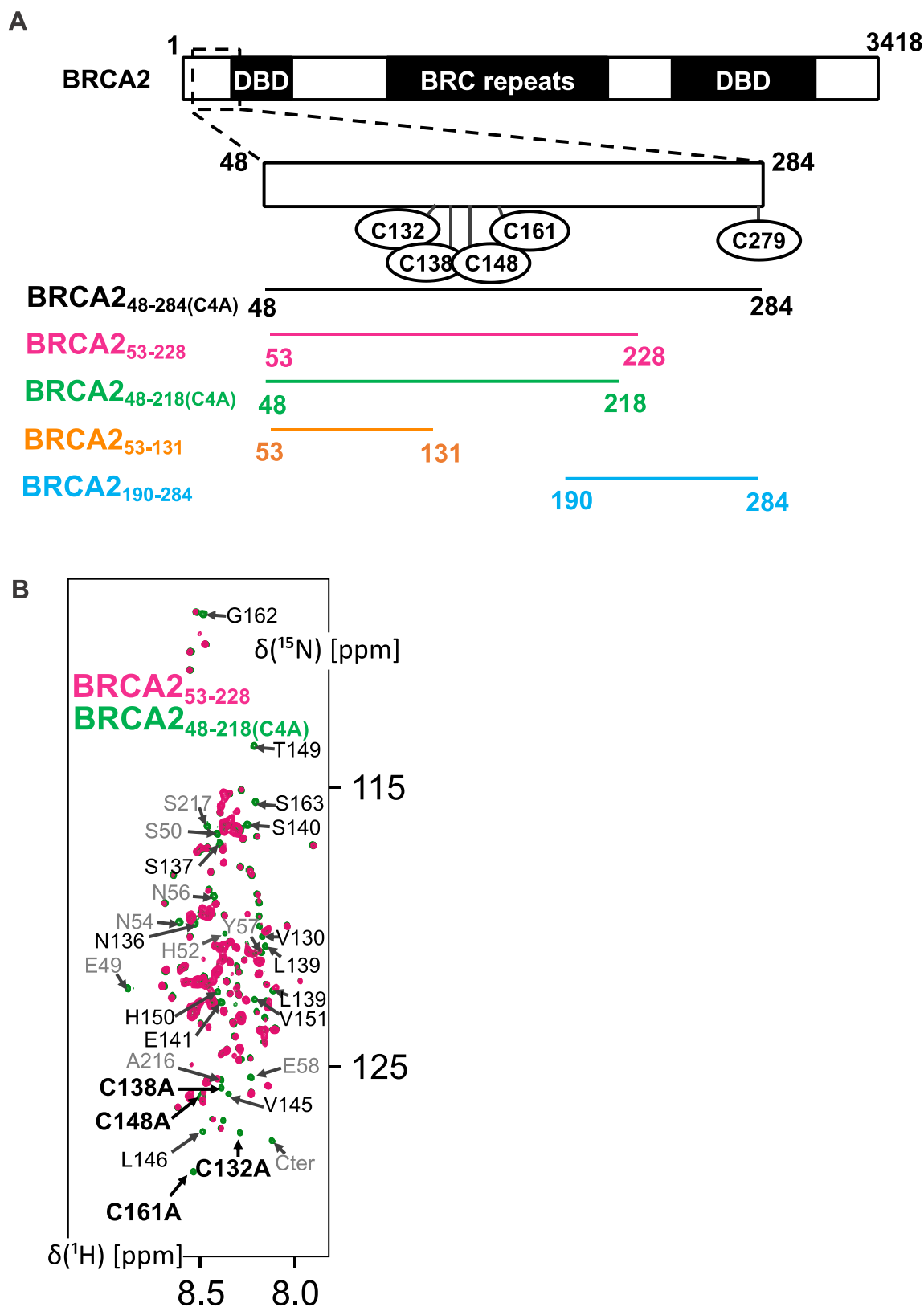


Figure 2. Overlapping BRCA2 constructs were used to analyze the two phosphorylation clusters. (A) Five constructs were designed for this study, containing either phosphorylation cluster 1 (BRCA2₅₃₋₁₃₁) or cluster 2 (BRCA2₁₉₀₋₂₈₃) or both (BRCA2₅₃₋₂₂₈, BRCA2_{48-218(C4A)} and BRCA2_{48-284(C4A)}). BRCA2₅₃₋₂₂₈ comprises 4 cysteines (C132, C138, C148, C161). In construct BRCA2_{48-218(C4A)} and BRCA2_{48-284(C4A)}, the four cysteines are mutated into alanines.

(B) The ^1H - ^{15}N SOFAST-HMQC spectra of BRCA2₅₃₋₂₂₈ (pink) at 50 μM in buffer A containing TCEP and BRCA2₄₈₋₂₁₈ C4A (green) at 50 μM in buffer A containing DTT are superimposable, peaks overlap for residues spanning the whole sequence except around the mutated positions (labelled in black) and close to the N-terminal or C-terminal ends (labelled in grey). These spectra were recorded at 283 K and pH 7.0 on a Bruker 700 MHz spectrometer.

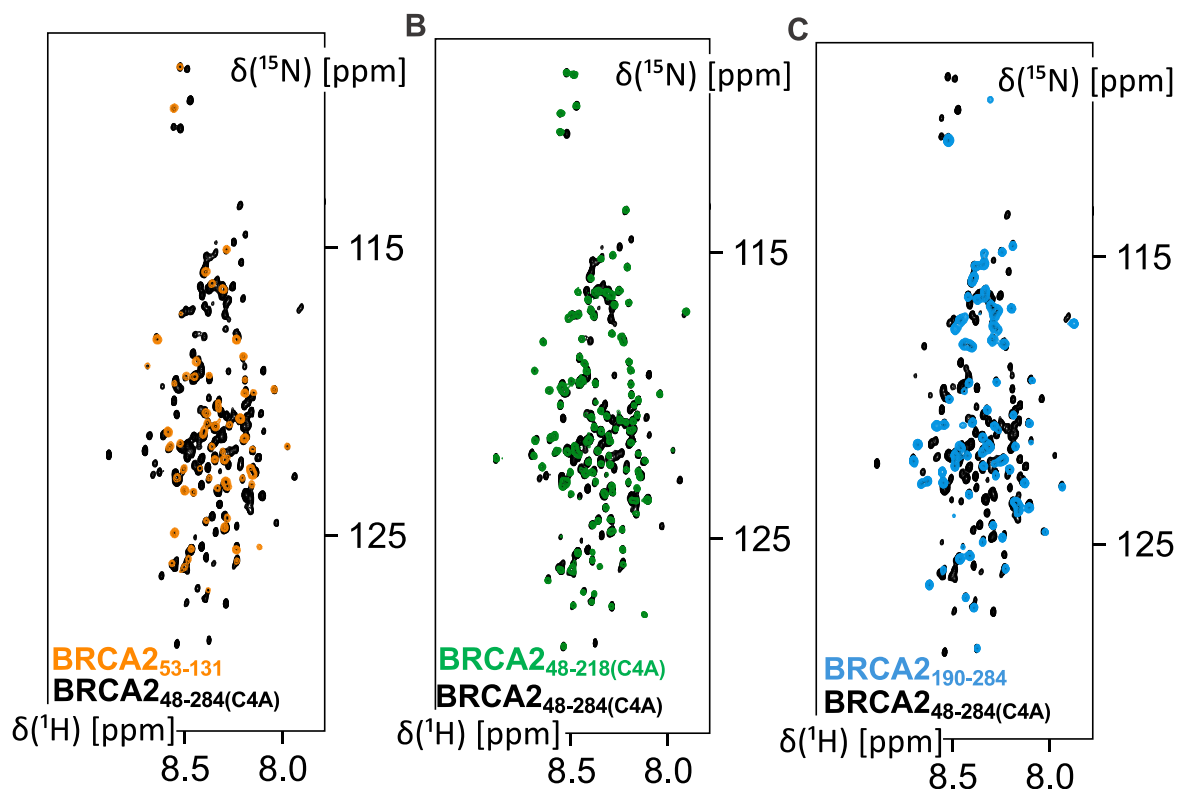


Figure 3. Superimposition of the constructs spanning the BRCA2 region from amino acid 48 to amino acid 284 confirms the intrinsically disordered behavior of this region. The ^1H - ^{15}N SOFAST-HMQC spectra of (A) BRCA2₅₃₋₁₃₁ (orange), (B) BRCA2_{48-218(C4A)} (green) and (C) BRCA2₁₉₀₋₂₈₄ (blue) are superimposed over BRCA2_{48-284(C4A)} in the different panels. All peptides are at 50 μM in buffer A and spectra were recorded at 283 K and pH 7.0 on a Bruker 700 MHz spectrometer. Only chemical shifts of residues close to the extremities vary between shorter constructs and BRCA2_{48-284(C4A)}. In order to simplify the figure, peaks corresponding to these residues are not labelled, however, they can easily be highlighted by comparison of chemical shift values available on the BMRB.

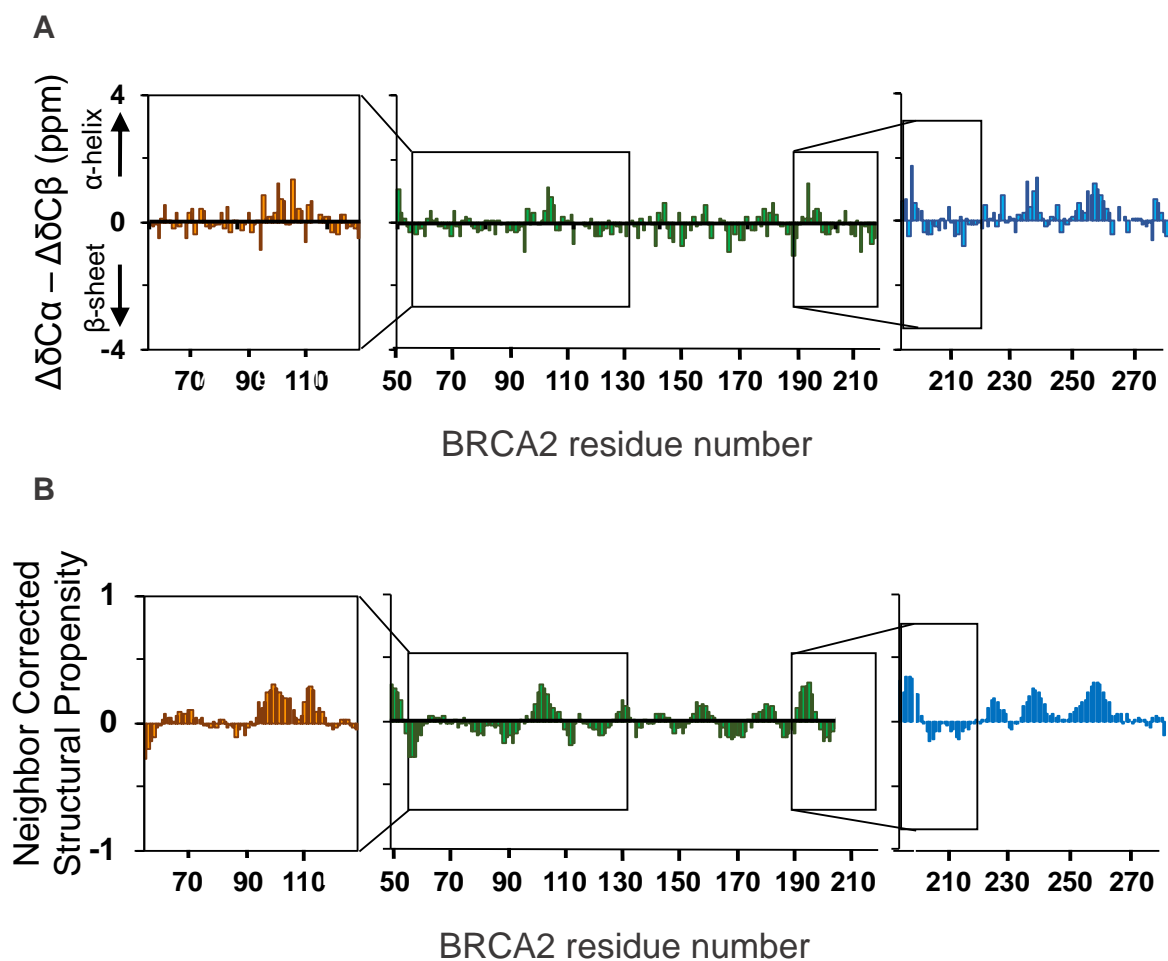


Figure 4. Analysis of the secondary propensity BRCA2₅₃₋₁₃₁ (orange), BRCA2_{48-218(C4A)} (green) and BRCA2₁₉₀₋₂₈₄ (blue) based on the C α and C β chemical shifts.

(A) Analysis of experimental C α and C β chemical shifts compared to predicted values for a disordered region (<http://nmr.chem.rug.nl/ncIDP>) (Tamiola et al. 2010). (B) Analysis based on the calculator available on the website <http://linuxnmr02.chem.rug.nl/ncSPC/> (Tamiola, 2012) and the library of Tamiola, Acar and Mulder (2010), with an average window of 5 residues.

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