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Backbone NMR Assignments of WW2 domain from human AIP4

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Abstract WW domains are small protein modules consisting of three-stranded antiparallel β-sheet, and involved in the protein-protein interaction for various biological systems. We overexpressed and purified WW2 domain from human AIP4/Itch (a member of Nedd4 family) using a pH/temperature dependent cleavage system. The backbone assignments of WW2 domain were completed, and secondary structure was predicted. Furthermore, backbone flexibility of WW2 domain was determined by ^{1}H-^{15}N heteronuclear NOE and amide hydrogen exchange experiments. The structural information would contribute to the structural determination of WW2 domain as well as the interaction study of WW2 domain with various binding partners.

Keywords WW2 domain, backbone NMR assignments, secondary structure, backbone flexibility

Introduction

AIP4/Itch is a HECT type E3 ubiquitin ligase, and a member of Nedd4 (neural precursor cell expressed developmentally downregulated protein 4) family.[1] The proteins of Nedd4 family share the similar domain structure, an amino terminal C2 domain, three or four WW domains, and a carboxyl terminal HECT domain.[2,3]

WW domains are small protein modules that are found in many eukaryotes.[4] The two conserved tryptophan residues of WW domain are spaced 20-22 amino acids apart and play a crucial role in its structure and function.[5] WW domains are typically 35 to 40 amino acids in length and consist of three β-strands forming an antiparallel β-sheet.[4,6,7] WW domains have been classified into four groups based on their binding to ligands.[8-10] Group I WW domains recognize Pro-Pro-X-Tyr (PPXY or PY motifs), and Nedd4 family proteins contain group I WW domains. Group II WW domains bind Pro-Pro-Leu-Pro (PPLP) motifs. Group III and group IV WW domains recognize PR motifs (polyproline motifs flanked by Arg or Lys) and p(S/T)P motifs (phosphorylated serine/threonine-proline sites), respectively .

The WW domain is one of the smallest protein modules that is stable as a monomer, and the WW domain has been a major protein-protein interaction module widely distributed in all biological systems.[11] Here, we report the backbone resonance assignments of WW2 domain from human AIP4/Itch. The secondary structure prediction and backbone dynamics of WW2 domain are also determined.

Experimental Methods

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Gene cloning - The gene encoding the WW2 domain of human AIP4 was purchased from GenScript Corporation (120 Centennial Ave, Piscataway, NJ 08854, USA). The gene encoding WW2 domain was amplified by polymerase chain reaction (PCR), and the amplified products were inserted between the *NcoI* and *PstI* sites in pTWIN1 for N-terminal *Ssp DnaB* intein fusion. The sequence of WW2 domain contained the N-terminal GRA tag (G1, R2, and A3) derived from pTWIN1 vector and additional glycine residue (G5) inserted during the cloning.

Expression and purification - The recombinant plasmid was transformed into *Escherichia coli* strain BL21(DE3) codon plus competent cells. Protein expression was induced by addition of 0.5 mM IPTG when the OD_{600} reached 0.6. After 4 h induction at 37°C, cells were harvested by centrifugation. The cell pellets were resuspended in the lysis buffer (20 mM Tris-HCl, 500 mM NaCl, 1 mM EDTA, pH 8.5), and lysed by sonication. The supernatant was applied to a chitin column equilibrated with buffer containing 20 mM Tris-HCl, 500 mM NaCl, 1 mM EDTA, pH 8.5. Bound intien-WW2 domain was washed and performed on-column cleavage with elution buffer (20 mM Tris-HCl, 500 mM NaCl, 1 mM EDTA, pH 7.0) at room temperature for 12 h. Cleaved WW2 domain was eluted with elution buffer. Protein samples were collected and analyzed by SDS-PAGE. The fraction of the protein was concentrated and further purified using size exclusion chromatography (Superdex 75 10/300; Amersham Biosciences). Uniformly [^{15}N]- or [^{15}N, ^{13}C]- labeled WW2 domain was prepared by growing the cells in M9 minimal medium. NMR samples were prepared as described above, and labeled protein was dissolved in 90% H_2O/10% D_2O, containing 100 mM NaCl, 50 mM sodium phosphate, 1 mM EDTA, pH 6.0.

NMR experiments and backbone assignment- All NMR spectra for backbone assignment were recorded at 303K on a Bruker AVANCE 600 spectrometer equipped with a cryoprobe. Backbone assignments were performed with the HNCA, HNCACB, and HNCO, and confirmed with HNCACO. Chemical shifts were referenced to DSS externally. The ^{1}H-^{15}N heteronuclear NOE experiment was recorded under the same conditions. The heteronuclear NOE values were determined from the ratio of the average intensities of the with (NOE experiment) and without (NONOE experiment) a proton presaturation.[12,13] All NMR spectra were processed using NMRPipe/NMRDraw software[14] and analyzed with NMRView program.[15]

Amide hydrogen exchange experiments – For amide hydrogen exchange experiment, ^{15}N-labeled WW2 was lyophilized from buffer. The NMR measurement was started immediately after the addition of D_2O to a lyophilized sample. ^{1}H-^{15}N HSQC spectra of WW2 domain dissolved in D_2O were obtained at 288K on a Bruker DRX 500 spectrometer.

Results and Discussion

Protein purification – To facilitate the purification of WW2 domain, we employed pH/temperature

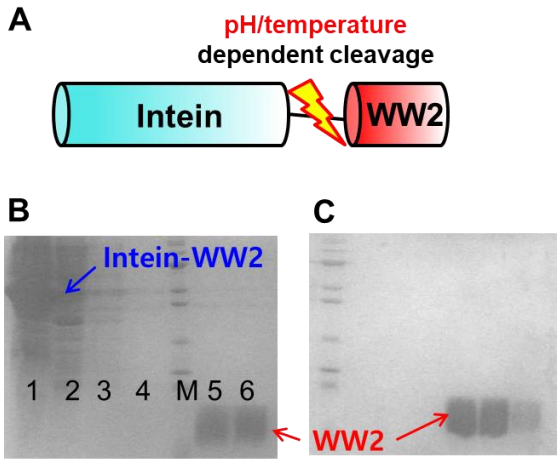

Figure 1. Purification of WW2 domain. (A) Schematic representation of pH/temperature dependent cleavage. (B) On-column cleavage results of WW2 domain. Lanes: 1, cell-lysate of intein-WW2 domain fusion protein; 2, flow-through; 3, washing; 4, flow-through during buffer change; M, molecular weight marker; 5-6, elution fractions after on-column cleavage. (C) Fractions of WW2 domain from a Superdex 75 column.

dependent cleavage method using *Ssp DnaB* intein fusion system (Figure 1A). The intein-fused WW2 domain was well-expressed as soluble form. The fusion protein (intein-WW2 domain) was efficiently cleaved by decreasing pH (from 8.5 to 7.0) and increasing temperature (from 4℃ to 24℃). After on-column cleavage, intein proteins were still bound to the column and unfused WW2 domain could be obtained (Figure 1B). The eluted WW2 domains were further purified by size exclusion chromatography (Figure 1C).

Backbone assignment of WW2 domain - The backbone amide ($^{1}H_{N}$ and ^{15}N) resonances of WW2 domain were completely assigned except 4 prolines and N-terminal glycine and arginine (Figure 2). Carbon resonances (Cα, Cβ, CO) were also assigned. The finally assigned chemical shifts of $^{1}H^{N}$, ^{15}N, $^{13}C\alpha$, $^{13}C\beta$ and ^{13}CO are summarized in table 1.

Secondary structure of WW2 domain – The secondary structure of WW2 domain was predicted on the basis of chemical shifts (Figure 3). Delta values of backbone carbon to random coil chemical shift (δCα-δCβ) were used.[16] Plot of delta values indicates the presence of three potential β-strand regions. We also employed CSI and TALOS+ programs to predict the secondary structure of WW2 domain.[17,18] Although CSI results predicted two potential β-strands, TALOS+ and delta value plot

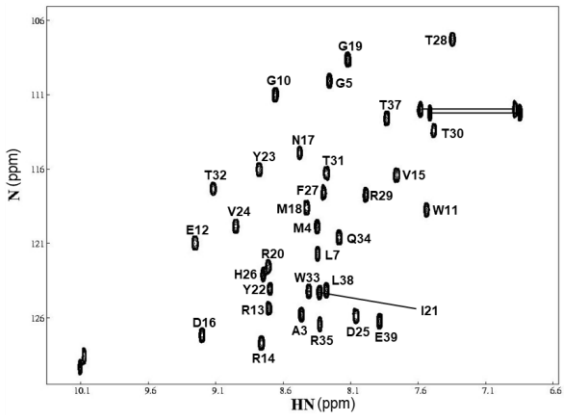

Figure 2. 2D-[^{1}H-^{15}N] HSQC spectrum of WW2 domain. Each resonance in the spectrum is labeled with assigned amino acid residues.

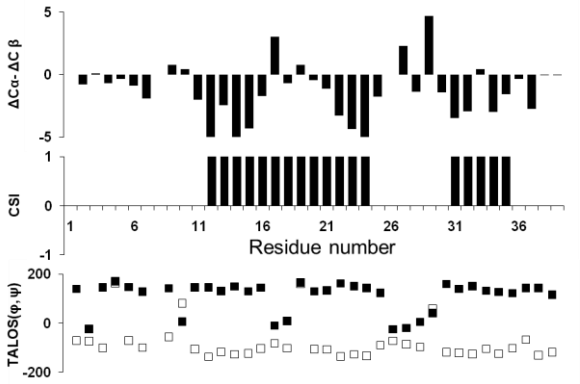

Figure 3. Secondary structure prediction of WW2 domain. Delta values (δCα-δCβ) of backbone carbon to random coil chemical shift were plotted. In the CSI, the values '1' represents the β-strand tendency. Backbone dihedral angles were calculated using TALOS+ server.

implied that there would be a short linker between the first and second β-strands. This is also supported by ^{1}H-^{15}N heteronuclear NOE results (Figure 4A). Taken together, WW2 has three β-strands, and the β-strands correspond to residues 11-16 (β1), 21-24 (β2), and 31-32 (β3) (Figure 3).

Backbone dynamics of WW2 domain – To characterize the backbone dynamics of WW2 domain, ^{1}H-^{15}N heteronuclear NOE and amide hydrogen

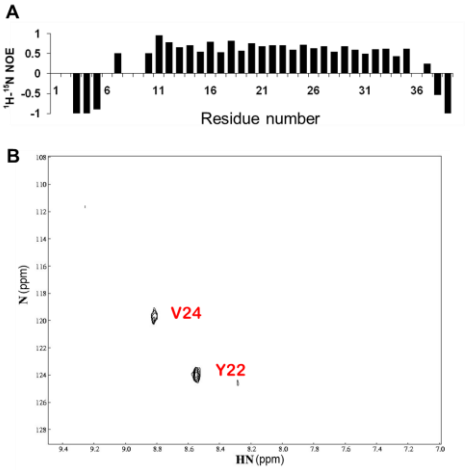

Figure 4. Backbone flexibility of the WW2 domain. (A) ^{1}H-^{15}N heteronuclear NOE values were plotted as a function of residue number. (B) Hydrogen/deuterium exchange experiment of WW2 domain. The detected residues were marked with red color.

exchange experiments were performed. In the result of H/D exchange experiment, only two peaks (Y22 and V24) in the second β-strand appeared in the ^{1}H-^{15}N HSQC spectrum of WW2 in 100% D_2O, which clearly show that overall regions of WW2 domain were exposed to solvent water (Figure 4B). Although WW2 domain may have hydrogen bonds between β-strands, the residues forming hydrogen bonds were exposed and replaced by the deuterium immediately. Because of the low molecular weight of WW2 domain, hydrogen bonding networks could not be protected, and this result is in accordance with the heteronuclear NOE data (Figure 4A)

Table 1. Chemical shifts (ppm) of $^{1}H^{N}$, ^{15}N, $^{13}C\alpha$, $^{13}C\beta$ and ^{13}CO of WW2 domain

a.a.	$^{1}H^{N}$	^{15}N	$^{13}C\alpha$	$^{13}C\beta$	^{13}CO
G1					
R2			56.384	31.372	
A3	8.521	126.101	52.734	19.32	
M4	8.41	120.258	55.545	33.516	
G5	8.32	110.534	44.746		
P6			63.053	32.546	173.906
L7	8.415	121.991	52.934	42.037	171.461
P8					
P9			64.273	32.111	174.218
G10	8.706	111.463	45.55		170.589
W11	7.55	119.019	57.319	31.941	173.658
E12	9.292	121.341	54.997	34.568	171.161
R13	8.765	125.81	55.708	32.339	172.03
R14	8.808	128.174	54.186	34.157	169.701
V15	7.806	116.717	60.528	34.625	174.261
D16	9.248	127.499	52.979	41.496	174.918
N17	8.531	115.284	55.59	37.639	173.102
M18	8.457	118.959	54.703	32.675	173.704
G19	8.157	109.016	45.901		171.294
R20	8.746	122.945	56.372	31.025	172.574
I21	8.395	124.715	60.935	38.751	172.633
Y22	8.742	124.42	55.59	39.573	167.868
Y23	8.823	116.271	56.638	41.667	171.968
V24	9.001	120.136	60.025	35.712	170.782
D25	8.099	126.198	51.78	40.322	174.663
H26	8.79	123.436	58.425	29.454	172.982
F27	8.326	117.869	59.714	38.419	174.406
T28	7.344	107.484	62.252	70.734	171.972
R29	8.012	118.094	57.55	27.056	172.698
T30	7.507	113.707	62.498	71.024	170.281
T31	8.334	116.589	60.936	71.524	171.06
T32	9.166	117.656	60.195	70.244	169.315
W33	8.452	124.528	58.251	30.392	173.216
N34	8.231	120.929	54.999	30.295	171.223
R35	8.395	126.962	54.589	30.372	171.212
P36			62.686	31.648	172.598
T37	7.875	112.934	60.968	70.818	170.892
L38	8.341	124.416	55.544	42.791	173.198
E39	7.935	126.591	58.136	31.423	177.882

Conclusion

In this study, backbone resonance assignments of WW2 domain from hAIP4 are completed, and the secondary structure and backbone dynamics of WW2 domain are determined. The present results provide not only fundamental structural information of WW2 domain at the atomic level but also structural basis for the interaction study of WW2 domain with various binding proteins.

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