PROTEIN STRUCTURE REPORT

Solution structure of the N-terminal domain of DC-UbP/UBTD2 and its interaction with ubiquitin

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Abstract: DC-UbP/UBTD2 is a ubiquitin (Ub) domain-containing protein first identified from dendritic cells, and is implicated in ubiquitination pathway. The solution structure and backbone dynamics of the C-terminal Ub-like (UbL) domain were elucidated in our previous work. To further understand the biological function of DC-UbP, we then solved the solution structure of the N-terminal domain of DC-UbP (DC-UbP_N) and studied its Ub binding properties by NMR techniques. The results show that DC-UbP_N holds a novel structural fold and acts as a Ub-binding domain (UBD) but with low affinity. This implies that the DC-UbP protein, composing of a combination of both UbL and UBD domains, might play an important role in regulating protein ubiquitination and delivery of ubiquitinated substrates in eukaryotic cells.

Keywords: DC-UbP/UBTD2; solution structure; ubiquitin-binding domain; ubiquitin-like domain; NMR

Introduction

Protein ubiquitination is a very important post-translational modification process that regulates diverse cellular events such as protein degradation, DNA repair, translocation, and immune response.[1,2] Many proteins, domains, and complexes are involved in this particular process.[3–5] Dendritic cell-derived ubiquitin (Ub)-like protein (DC-UbP) was first identified from dendritic cell as a Ub-like (UbL) protein of 106 amino acid residues.[6] Then a 190-residue protein was reported, which includes a C-terminal UbL domain and an N-terminal extension (GenBank AAH19910.1). Since DC-UbP contains an UbL domain, this protein has also been named as UBTD2 (ubiquitin domain containing protein 2). We have elucidated the solution structure and backbone

Additional Supporting Information may be found in the online version of this article.

Ai-Xin Song and Chen-Jie Zhou contributed equally to this work.

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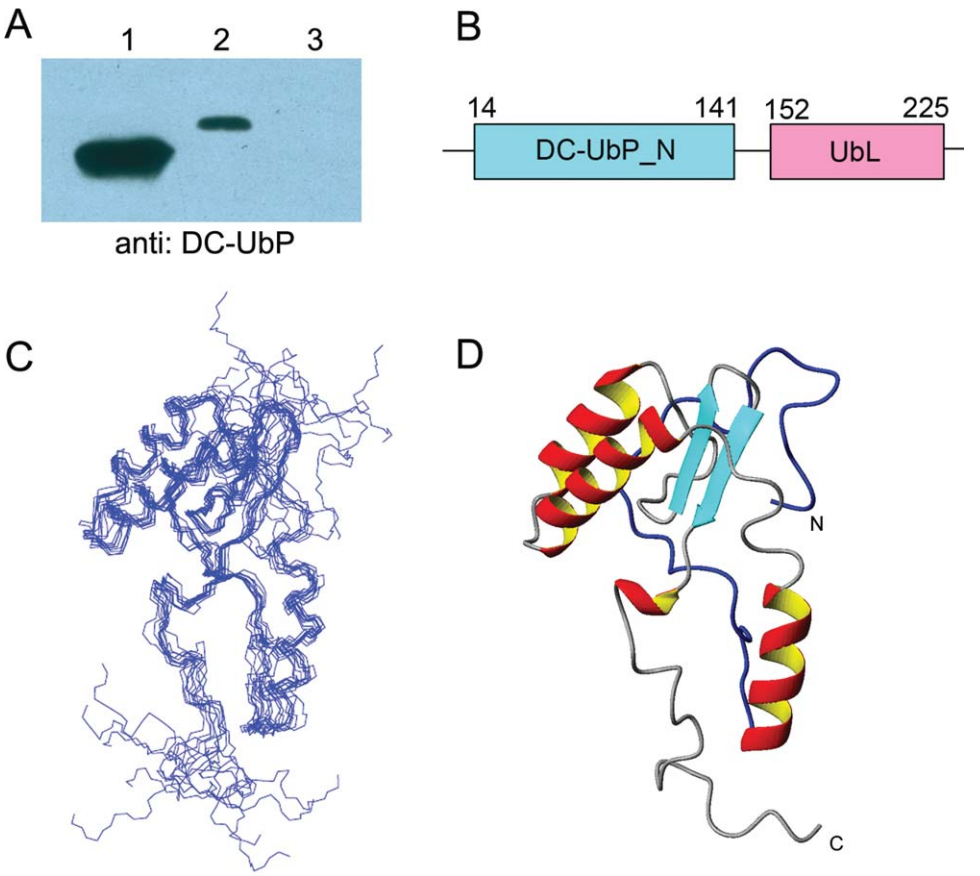

Figure 1. The N-terminal domain of DC-UbP/UBTD2. A: Characterization of eukaryotic expression of the DC-UbP by anti-DC-UbP antibody. 1, Recombinant DC-UbP fragment (residues 45–234) as a control; 2, Detection of DC-UbP expression in A431 cells; 3, Detection of DC-UbP expression in SH-SY5Y cells. B: Domain architecture of DC-UbP protein. DC-UbP_N, the N-terminal domain (residues 14–141); UbL, the C-terminal Ub-like domain. C: Superposition of the backbone traces of 10 representative structures. D: Ribbon diagram of a representative structure with secondary structure elements labeled. The N-terminal region (14–46) is colored in blue.

dynamics of the C-terminal UbL domain of DC-UbP by NMR spectroscopy.[7] Although the UbL domain takes similar fold as Ub, its electrostatic surface is largely different from that of Ub, suggesting that the UbL domain of DC-UbP may have different interacting partners from Ub and other Ub-like proteins or domains but with yet unknown function related to ubiquitination.[7]

To further our understanding of the biological function of DC-UbP, we solved the solution structure of the N-terminal domain of DC-UbP (DC-UbP_N) and determined its Ub binding properties by NMR techniques. DC-UbP_N holds a novel structural fold and exhibits a weak Ub binding ability.

Results and Discussion

DC-UbP is a 234-residue protein with two separate domains

DC-UbP is consisting of a C-terminal UbL domain and an N-terminal putative domain with structure and function unknown. The linker region between the two domains contains many proline residues that cannot be included in the N-terminal domain. We first prepared an expression construct based on the sequence released in GenBank (AAH19910.1) that encoding a protein of 190 residues, and purified the soluble protein. Then, we tested with many clones for the N-terminal domain based on this sequence version. Unfortunately, these fragments are actually not well structured as characterized by circular dichroism and NMR HSQC spectroscopies (data not shown). To our surprise, Western blot experiment on the mammalian cell lysates shows that the exact molecular weight of the endogenous DC-UbP is larger than what we expected from the original sequence [Fig. 1(A)]. We deduced that the endogenous DC-UbP may cover a further extension at its N-terminus. By BLAST searching the protein database, we got an almost identical sequence but with an N-terminal extension (Q8WUN7). The protein is composed of 234 amino acid residues and displays the same gel shift with that of the endogenous protein as detected with an antibody against DC-UbP (data not shown). So,

the full-length DC-UbP may contain up to 234 residues and can be divided into two separate domains [Fig. 1(B)]. We then cloned another construct of the N-terminal domain of DC-UbP (DC-UbP_N, residues 14–141) for structural analysis. The circular dichroism spectrum exhibits typical double negative peaks (data not shown), suggesting that the DC-UbP_N domain is dominantly comprised of α-helical structures.

Solution structure of the DC-UbP_N domain

We labeled uniformly the DC-UbP_N protein with ^{15}N and ^{13}C for acquiring NMR spectra, assigned both backbone and side-chain resonances [see Fig. 2(A)], and then elucidated the solution structure based on the NOE-derived distance restraints together with dihedral angle and hydrogen bond restraints. A summary of the NMR experimental restraints and structural model statistics is presented in Table I. A superposition of the 10 lowest-energy structures is displayed in Figure 1(C), where the root mean square deviations (RMSD) for the backbone atoms and all heavy atoms in secondary structure elements are 0.48 and 0.97 Å, respectively. A ribbon presentation of the domain structure is shown in Figure 1(D). DC-UbP_N shows an αααββ structural pattern with a short helix at the C-terminus. Interestingly, the N-terminal 33-residue region (14–46) is extended, but it is in contact with the secondary structure core and may contribute to the stability of the overall structure. This also explains why the deletion of this region (initial construct composed of residues 45–141) destroys the three-dimensional structure of the domain. We also performed experiment on the backbone dynamics by measuring R1, R2, and NOE (see Supporting Information Figure S1). It corroborates that the N-terminal region of DC-UbP_N is relatively flexible.

Sequence analysis by BLAST server indicates that there is no homologous sequence of known structure in PDB database with that of DC-UbP_N. By using the DALI server,[9] we have not got a similar structure (with $Z > 3.0$) in PDB database with that of DC-UbP_N, but only got the structures (e.g., 2A86, 1N2B) with the largest Z-score of 2.6, which belong to a family of pantothenate synthetases.[10] This implies that the N-terminal domain of DC-UbP may represent a unique domain structure.

DC-UbP_N can bind with Ub

DC-UbP contains two well-structured domains and a proline-rich linker, and the C-terminal UbL domain holds an Ub-like fold. We tested whether the N- and C-terminal domains of DC-UbP interact with each other by GST pulldown and NMR titration experiments. The data show that these two domains do not interact with each other under the experimental conditions (data not shown). However, in the NMR titration experiment, unlabeled Ub causes significant chemical shift changes of some amide resonances of ^{15}N-labled DC-UbP_N [Fig. 2(A)], suggesting that DC-UbP_N can interact with Ub. By analyzing the chemical shift changes of three typical residues with a 1:1 stoichiometry fitting model [Fig. 2(B)], we calculated the dissociation constants (~1 mM) for this weak but significant binding between DC-UbP_N and Ub. The diagram of the chemical shift changes of DC-UbP_N against residue number shows that some residues are significantly perturbed [Fig. 2(C)], based on which, the putative Ub-binding sites are mapped on DC-UbP_N as shown in Figure 2(D) and its opposite surface in Supporting Information Figure S2. Some hydrophobic residues (Ile70, Leu88, Ile92, Ile98, and Leu112) may contribute to the interaction with the hydrophobic surface of Ub, whereas some negatively charged residues (Glu65, Glu69 Asp94, and Glu111) are proposed to interact with the positively charged residues on Ub. On the other hand, when ^{15}N-labeled Ub is titrated with unlabeled DC-UbP_N, it also causes chemical shift changes of the amide resonances of some residues on Ub [Fig. 2(E)]. The binding sites are located close to the well-known binding surface centered at Ile44 of Ub [Fig. 2(F)], as in the cases of the binding with UBA,[11,12] UIM,[13] and PFU.[14] It seems that the C-terminal extension (Leu71, Arg72, and Leu73) of Ub contributes largely to the specific interaction with DC-UbP_N, analogous to the cases of ZnF-UBP domains binding with Ub.[15]

Biological significance of Ub binding

Ub is widely distributed in eukaryotic cells as its name implicated. Up to date, a large number of Ub-binding domains (UBDs), such as UBA,[11,16,17] CUE,[18] UIM,[13] ZnF-UBP,[15] and PFU,[14] have been identified to bind specifically with Ub and its analogs with variable affinities within micromolar to millimolar scale.[4] Interestingly, SH3, a canonical domain binding with proline-rich motifs found in diverse signal transduction proteins, has also been identified to bind with Ub.[19,20] Our current finding that the novel N-terminal domain of DC-UbP can bind with Ub may provide a possibility that DC-UbP protein plays a role in regulating ubiquitination of proteins and delivery of ubiquitinated substrates in eukaryotic cells.

Materials and Methods

Protein expression and purification

The cDNAs encoding various fragments of DC-UbP (residues 45–141, 45–234, 1–141, and 14–141) were cloned into pET-22b$^+$ vector and transformed into *E. coli* BL21 (DE3) strain for overexpression. The proteins were purified by a Ni^{2+}-NTA column (Qiagen), followed by size-exclusion chromatography (Superdex-75, GE Healthcare). ^{15}N- and $^{15}N/^{13}C$-labeled DC-

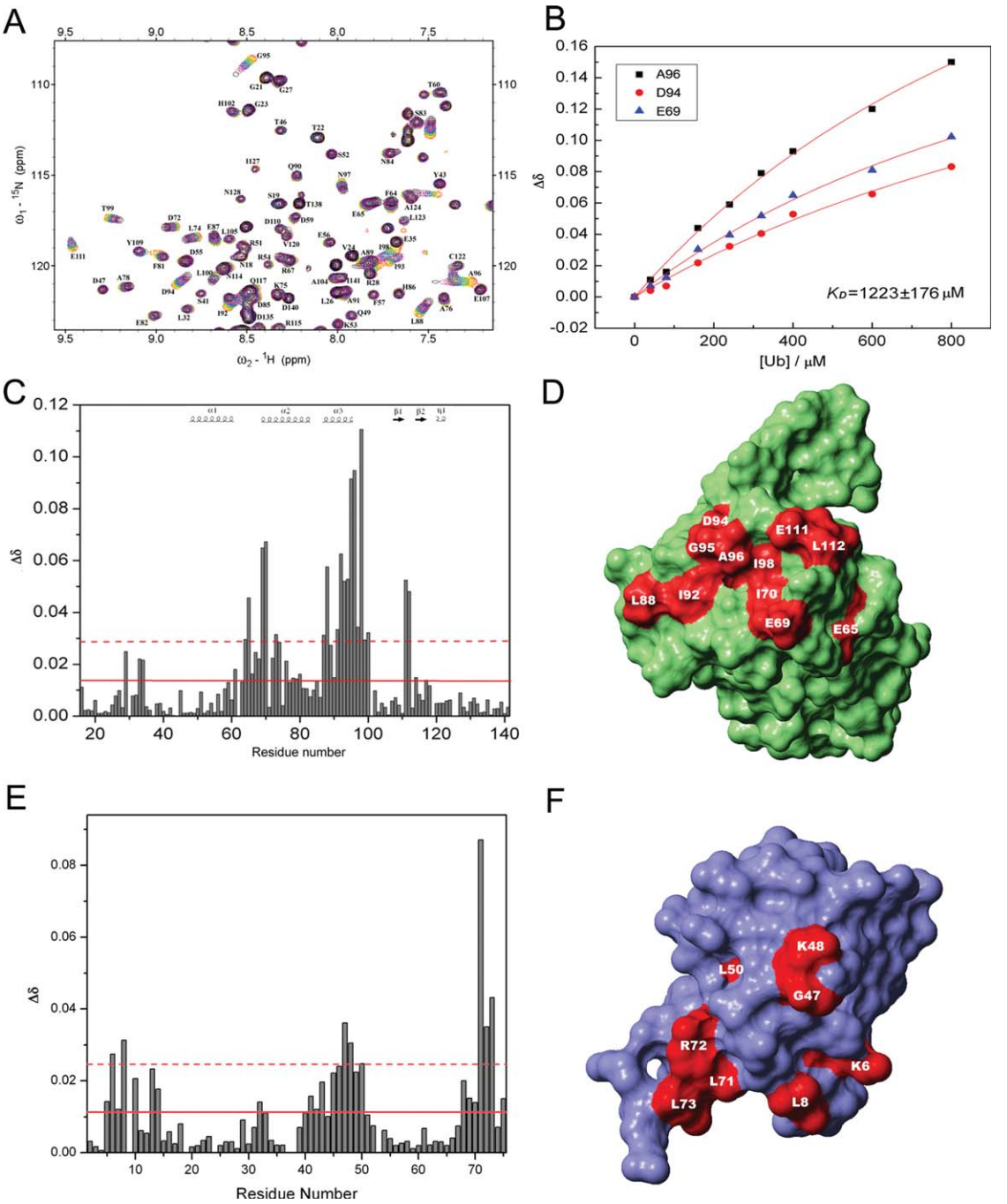

Figure 2. Interaction of DC-UbP_N with Ub. A: Overlay of the ^{1}H-^{15}N HSQC spectra of ^{15}N-labled DC-UbP_N in free form and on titration with Ub. The backbone amide resonances are also labeled in the spectrum of the free-form protein. B: Titration of ^{15}N-labled DC-UbP_N with Ub showing the chemical shift changes ($\Delta\delta$) of the three representative residues (Glu69, Asp94, and Ala96). C: Diagram of the chemical shift changes ($\Delta\delta$) of DC-UbP against residue number at a molar ratio (DC-UbP_N/Ub) of 1:2. The solid line denotes mean value and the dashed line is mean + SD. The secondary structures of DC-UbP_N are indicated at the top. D: Mapping the Ub-binding sites on the surface of DC-UbP_N. The potential Ub-binding sites are highlighted in red. E: Diagram of the chemical shift changes ($\Delta\delta$) of Ub against residue number at a molar ratio (Ub/DC-UbP_N) of 1:2. F: Mapping the DC-UbP_N binding sites on the surface of Ub. The crystal structure of Ub (PDB code: 1UBQ) was used for mapping the molecular surface.[8]

UbP_N (residues 14–141) was overexpressed in M9 minimal media using [^{15}N]-NH_4Cl and/or [^{13}C]-glucose as the sole nitrogen and carbon sources (Cambridge Isotope Laboratories). The purified proteins were dialyzed against water, lyophilized, and stored at –20°C. The DC-UbP_N protein with a His_6-tag at the C-terminus was directly used for the NMR study without further removing the tag.

Table I. *Experimental Restraints and Structural Model Statistics of the DC-UbP_N Domain (Residues 14–141)*

Number of experimental restraints	
Total unambiguous distance restraints	1784
Intra residual	852
Sequential ($\|i-j\| = 1$)	435
Medium range ($2 \leq \|i-j\| \leq 4$)	265
Long range ($\|i-j\| \geq 5$)	232
Hydrogen bond restraints	60
Dihedral angle restraints	
ϕ	68
ψ	68
Structure model statistics	
RMSD from experimental restraints	
NOE distances (Å)	0.020 ± 0.004
Dihedral angles (°)	0.57 ± 0.09
RMSD from idealized geometry	
Bonds (Å)	0.002 ± 0.000
Angles (°)	0.40 ± 0.006
Impropers (°)	0.28 ± 0.01
Ramachandran analysis (residues 27–126)	
Residues in most favored regions (%)	84.9
Residues in additionally allowed regions (%)	12.1
Residues in generously allowed regions (%)	1.3
Residues in disallowed regions (%)	1.7
Average atomic RMSDs	
All residues (27–126)	
Backbone atoms (Å)	1.36
Heavy atoms (Å)	2.10
Secondary structures	
Backbone atoms (Å)	0.48
Heavy atoms (Å)	0.97

NMR data acquisition, titration, and structure analysis

The ^{15}N- or ^{15}N/^{13}C-labeled DC-UbP_N samples (~1 m*M*) were dissolved in a PBS buffer (20 m*M* phosphate, 100 m*M* NaCl, pH 6.5) containing 8 or 100% D_2O. All NMR spectra were acquired at 298 K on a Bruker Avance 600 MHz NMR spectrometer equipped with a TCI cryoprobe. The backbone and side-chain ^{1}H, ^{15}N, and ^{13}C resonances were assigned based on the spectra of 3D HNHA, HNCO, HNCACB, CBCA(CO)NH, CC(CO)NH, and 3D ^{13}C HCCH-COSY, HCCH-TOCSY. NOE distance restraints for structure calculations were obtained from 3D ^{15}N-edited NOESY and ^{13}C-edited NOESY (aliphatic ^{13}C regions). The protein structures were calculated by using the CNS program[21] with the ARIA module,[22] assessed by PROCHECK,[23] and displayed by MOLMOL.[24] Backbone dihedral angle restraints (ϕ and ψ) were derived from the TALOS program.[25] The calculation in combination with iterative NOE peak assignments was performed for nine cycles, and a total of 200 structures were finally obtained. Ten structures with the lowest energies, which exhibit no NOE violation >0.3 Å and no dihedral violation >5 Å, were selected and displayed.

^{15}N-labeled DC-UbP_N (0.2 m*M*) was titrated stepwise with unlabeled Ub. A series of ^{1}H-^{15}N HSQC spectra were obtained and the chemical shift changes were measured at each molar ratio. The combined average chemical shift changes ($\Delta\delta_{ave}$) were calculated as $\Delta\delta_{ave} = [(0.2 \times \Delta\delta_N)^2 + (\Delta\delta_{HN})^2]^{1/2}$, where $\Delta\delta_{HN}$ and $\Delta\delta_N$ are the chemical shift changes in the ^{1}H and ^{15}N dimensions, respectively. The dissociation constant (K_D) for the binding of DC-UbP_N with Ub was calculated by fitting the titration curves.[26]

Western blotting assays

Cells were cultured at 37°C in Dulbecco's modified Eagle's medium (DMEM)/F-12 (Gibco) supplemented with 10% (V/V) fetal bovine serum (Hyclone), penicillin and streptomycin, and under a humidified atmosphere containing 5% CO_2. After cultured for 48 h, the cells (60-mm dish) were harvested and lysed in the buffer (50 m*M* HEPES, 150 m*M* NaCl, 1% Triton-X-100, 1mM EDTA, pH7.6) at 4°C for 10 min. The cell lysates were then subjected to SDS-PAGE, followed by Western blotting with the mouse monoclonal antibody against DC-UbP (Abnova) and ECL detection reagents (Pierce). The purified recombinant DC-UbP fragment (residues 45–234) was used as a control.

Coordinates

Coordinates and structural information have been deposited in the PDB with the accession code 2KSN.

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