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Crystal structure of human Intersectin-2L C2 domain

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ABSTRACT

Intersectin-2L (ITSN-2L) is a long isoform of ITSN family, which is a multimodule scaffolding protein functioning in membrane-associated molecular trafficking and signal transduction pathways. ITSN-2L possesses a carboxy-terminal extension encoding a Dbl homology domain (DH), a pleckstrin homology domain (PH) and a C2 domain, suggesting that it could act as a guanine nucleotide exchange factor for Rho-like GTPases. But the role of C2 domain is obscure in this process. Here we report the crystal structure of human ITSN-2L C2 domain at 1.56 Å resolution. The sequence and structural alignment of ITSN-2L C2 domain with other members of C2 domain protein family indicate its vital cellular roles in membrane trafficking, the generation of lipid-second messengers and activation of GTPases. Moreover, our data show the possible roles of ITSN-2L C2 domain in regulating the activity of Cdc42.

1. Introduction

ITSN (also known as Ese-1, EHSH-1, Dap-160) is an evolutionarily conserved scaffolding protein found in diverse metazoan organisms ranging from nematodes to mammals [1–3]. There are two ITSN genes in mammals, *ITSN-1* and *ITSN-2*, which have high identity in sequence. There are two splice variants in human *ITSN-2*, each encoding a short isoform (ITSN-2S) and a long isoform (ITSN-2L) [4,5]. Both the long and short ITSN-2 isoforms process two Eps15 homology domains (EH1 and EH2), a coiled-coil region and five Src homology 3 domains (SH3A–E), while ITSN-2L contains a carboxy-terminal extension encoding a DH, a PH and a C2 domain (Fig. 1A). The role of ITSN-2L has been shown in regulating actin remodeling and mitotic spindle orientation as well as its interaction with the K15 protein of human *herpesvirus* 8 [6–8]. As a guanine nucleotide exchange factor (GEF) for the small GTPase Cdc42 [9], the tandem DH and PH domains of ITSN-2L regulate cell polarity and the actin cytoskeleton in the development of embryonic through regulating formation of finger-like actin projections [10,11].

The DH domain is a conserved domain in GEF proteins and sufficient for the nucleotide exchange activity of Dbl family proteins [12,13]. In human ITSN-L, the adjacent domain of DH may function in regulating Cdc42 exchange activity of DH. The SH3 (E) of ITSN-1L has been shown to be sufficient for the autoinhibition of DH activity [14]. These data propose a model in which the intramolecular interaction may block or distort the GTPase binding region of the DH domain. The PH domain of INST-L is present in many intracellular signaling proteins and its invariant positioning immediately C-terminal to the catalytic DH domain suggests an important role. DH–PH fragments, both *in vivo* and *in vitro*, have greater nucleotide exchange activity than the respective DH domains alone [15]. But in some instances, the PH domain has an inhibitory effect on DH domain mediated nucleotide exchange [16,17]. The C2 domain of ITSN-2L is Ca^{2+}-dependent or Ca^{2+}-independent intracellular protein module, which functions in signal transduction and membrane traffic. Such domains have been shown to bind calcium and phospholipid in a large number of membrane transport proteins [18,19], but the role of C2 domain of ITSN-2L in regulating Cdc42 exchange activity of DH is obscure. To shed light on biological function of ITSN-2L C2 domain, we solved the high-resolution crystal structure of ITSN-2L C2 domain and provided a structural basis of C2 domain for controlling the activity of DH domain.

2. Materials and methods

2.1. Protein expression and purification

The cDNA template of human ITSN-2L (amino acid residues D1174-E1665) encoding a DH domain, a PH domain and a C2 domain was subcloned into a pET-28a-MHL vector via ligase-independent cloning. The cDNA template contains a lys_{1525} mutation compared with reference sequence NP_062541.2. The recombinant protein was over expressed in *Escherichia coli* BL21 (DE3) with the pRARE plasmid for codon-biased expression. Cells were grown in minimal medium (Terrific Broth) at 37 °C with 50 μg/mL Kanamycin and

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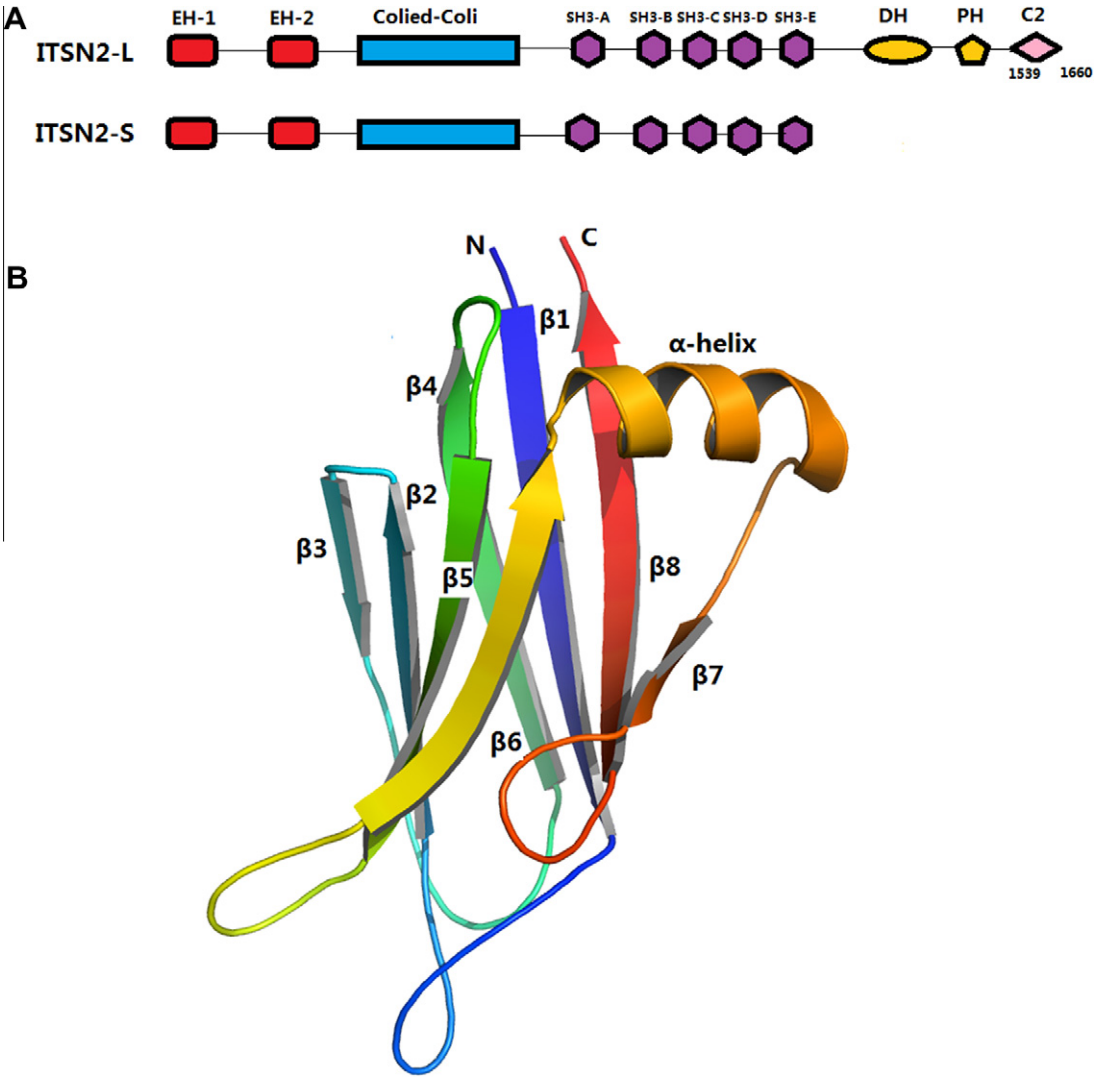

Fig. 1. The domain organization of ITSN-2 and over structure of the human ITSN-2L C2 domain: (A) Schematic representation of ITSN-2 shows the domain organization of ITSN-2S and ITSN-2L. Numbers below indicate amino-acid positions of C2 domain boundaries. (B) The overall structure of C2 domain exhibits a compact region composed of eight β-strands forming two β-sheets. There are two loops, a α-helix at the top of the domain and four at the bottom connect the eight β-strands.

25 μg/mL Chloramphenicol to an optical density of approximately 3.0. Protein expression was induced with 0.5 mM isopropyl-1-thio-D-galactopuranoside (IPTG) in the 1.8 L medium and the cell cultures were grown at 15 °C after induction. The cells were allowed to grow overnight before they were harvested and flash frozen in liquid nitrogen and stored at −80 °C. Frozen cells from 6 L culture were thawed and resuspended in 400 mL extraction buffer with freshly added final concentration of 1 mM PMSF/Benzamidine, 0.5% CHAPS and 5 U/mL Benzonase (Sigma, 250 U/μL), and supplemented with 1 mL protease inhibitor cocktail (Sigma), and lysed by sonication at 10 s 50% duty cycle for 5 min at 120 W.

The lysate was centrifuged for 60 min at 16,000 rpm. The supernatant was incubated with 4 mL Ni-NTA beads (Qiagen Mississauga) for 1 h at 4 °C. The supernatant was then passed through a gravity column (Poly-Prep, Bio-Rad) and the beads were washed with 50 mL binding buffer (1× PBS pH7.3, 500 mM NaCl, 5 mM Imidazole, 5 mM BME, 5% Glycerol) followed by 50 mL washing buffer (1× PBS pH7.3, 500 mM NaCl, 10 mM Imidazole, 5 mM BME, 5% Glycerol). The protein bound to beads was then eluted with 15 mL elution buffer (1× PBS pH7.3, 500 mM NaCl, 300 mM Imidazole, 5 mM BME, 5% Glycerol). The flow-through was collected and loaded onto Supderdex-75 26/60 gel filtration column (GE Healthcare). Eluted fractions were pooled and concentrated with amicon centrifugal filter (m.w. cut-off 10,000). The purified proteins were verified by SDS–PAGE analysis. Protein concentration (23.43 mg/mL) was assayed by the Bradford method using bovine serum albumin as the standard.

2.2. Crystallization, X-ray data collection and structure determination

The protein of original construct includes a DH, a PH and a C2 domain, but no crystals were observed. The DH and PH domains were cleaved off by *E. coli* protease and C2 domain was crystallized. Stock protein solution was added with a final concentration of 2 mM $CaCl_2$. Crystals suitable for X-ray diffraction analysis were obtained by the sitting-drop vapor diffusion method at 291 K by mixing equal volumes of the protein solution and the reservoir solution (2 M $(NH_4)_2SO_4$, 0.2 M NaAc, 0.1 M HEPES, 5% MPD, pH 7.5).

Diffraction data from the protein crystals were collected at beamline 19ID of the Advanced Photon Source (Argonne, Illinois, USA) and reduced with the HKL software suite [20]. Further experimental details are listed in Table 1. The structure was solved by

Table 1
Data collection and refinement statistics.

Data collection	
Space group	$I4_122$
Cell dimensions	
a, *b*, *c* (Å)	54.12, 54.12, 196.71
α, β, γ (°)	90, 90, 90
Wavelength (Å)	0.810
Resolution (Å)	40.0–1.56 (2.12–2.21)
R_{merge} (%)	9.5 (7.9)
$I/\sigma I$	8.4 (3.5)
Completeness (%)	100.0 (99.9)
Redundancy	18.6 (17.7)
Refinement	
Resolution (Å)	40.0–1.56
No. of reflections	21,410
R_{work}/R_{free}	22.2/23.5
No. of atoms	
Protein	991
Heterogen atoms	5
Solvent atoms	68
R.m.s. deviations	
Bond lengths (Å)	0.016
Bond angles (°)	1.607

Values in parentheses correspond to the highest resolution shells.

the multiple wavelength anomalous diffraction [21] method with high energy remote and peak wavelength data sets SHELX [22] software. The protein model was traced automatically with ARP/WARP [23]. Further refinement was performed with the programs COOT [24], PHENIX [25], and REFMAC [26]. The model's geometry was checked with MOLPROBITY server [27].

3. Results and discussion

3.1. Overall structure

The original protein construct included a DH, a PH and a C2 domain of human ITSN-2L (Fig. 1A). During crystallization, the DH and PH domains were cleaved off by a residual *E. coli* protease, and only the crystal structure of C2 domain (residues D1174-E1665) was well defined. C2 domain of human ITSN-2L has a typical C2 domain fold consisting of a stable β-sandwich topology with flexible loops, and a single α-helix (residues 1626–1636). The overall structure of human ITSN-2L C2 domain is high similar to the C2 domain in other members of C2 domain proteins, which forms an eight-stranded β-sandwich consisting of a pair of four-stranded β-sheets (Fig. 1B). There are two loops, a α-helix at the top of the domain and four at the bottom connect the eight β-strands (Fig. 1B). Ca^{2+}, which stabilizes the structure of overall structure C2 domain binding, occurs exclusively at the top two loops and α-helix. But it does not cause a major conformational shift of the domain [28,29].

3.2. Function of C2 domain in vesicular transport

Single and multiple copies of C2 domains have been identified in a growing number of membrane transport proteins, such as

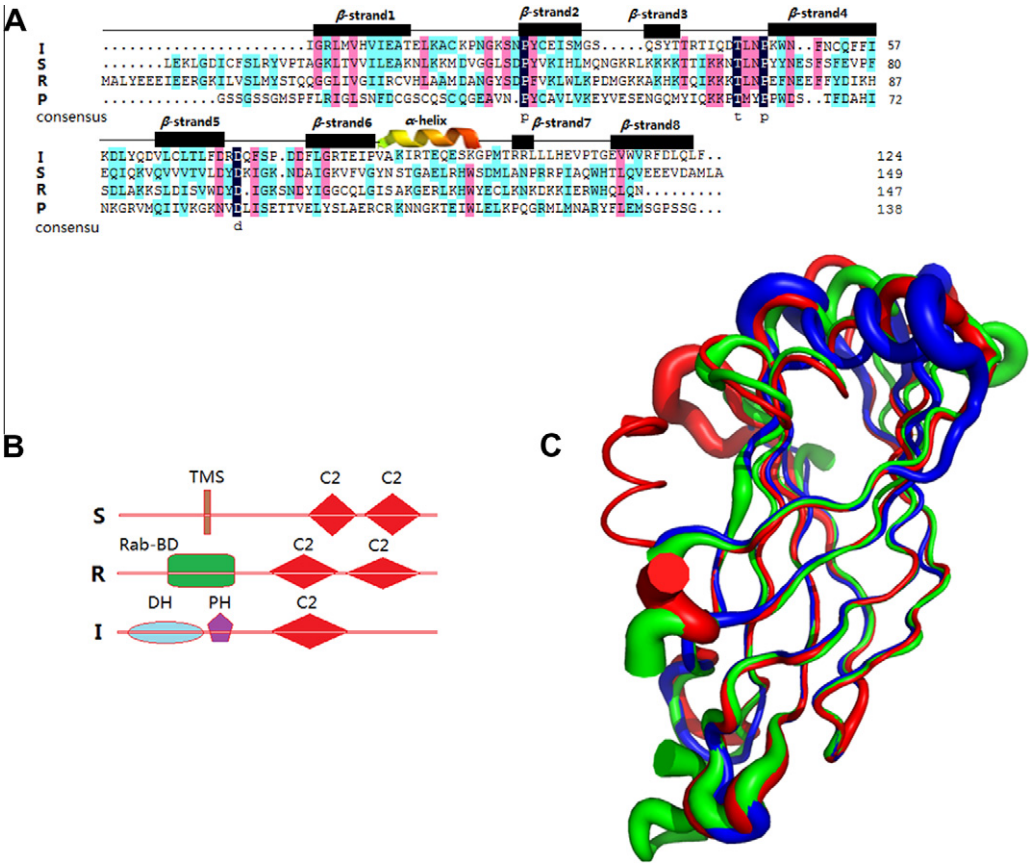

Fig. 2. Structure-based comparison of C2 domain protein subfamily: (A) Structure-guided sequence alignment of C2 domains in four C2 domain protein family members (I: ITSN-2L, S: synaptotagminI, R: rabphilin, P: PKCδ). The dashed lines indicate gaps introduced to optimize alignments. (B) Modular representation of functional domains of C2 domain protein family members in vesicular transport (TMS: transmembrane segment, Rab-BD: Rab-binding domain, I, S, R represent the same protein as in the Fig. 2A). (C) Structural comparison of C2 domain in vesicular transport members (ITSN-2L: blue, PDB code 3JZY; synaptotagminI: green, PDB code 1UOV; rabphilin: red, PDB code 2CM5). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

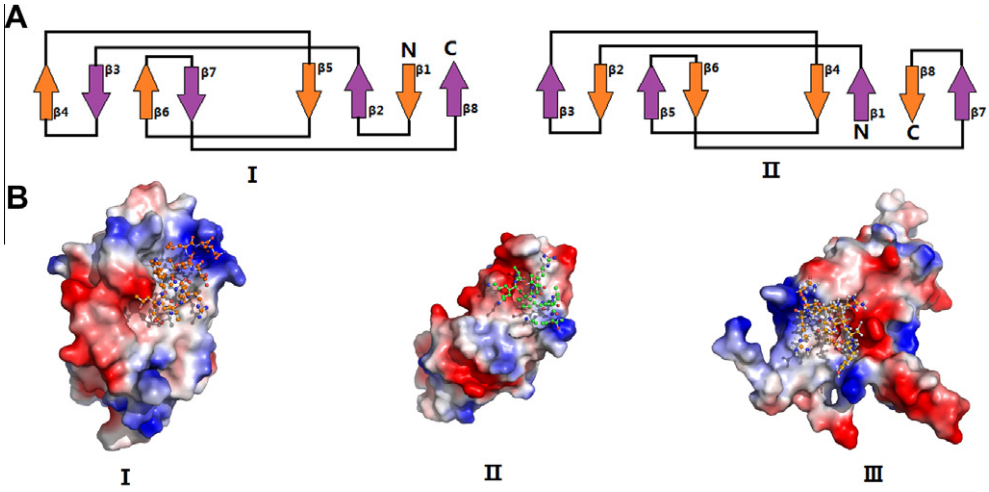

Fig. 3. Schematic representation of the two C2 domain topologies and comparison of the solvent accessible surface of C2 domain from synaptotagminI, ITSN-2L and PKCδ: (A) Schematic representation of the two prototypical C2 domain topologies illustrated by synaptotagmin I (I) and PKCδ (II). (B) Three orthogonal views of solvent accessible grasp surface with charge distribution (blue for positive charge, and red for negative charge). The ball and stick mode of α-helix of C2 domain locate in low charge groove of the protein (I: synaptotagmin I, II: ITSN-2L, III: PKCδ). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

synaptotagmins, rabphilin, DOC2, perforin and so on [30]. In these proteins, C2 domain display the remarkable property of binding a variety of different ligands and substrates, including Ca^{2+}, phospholipids, inositol polyphosphates, and intracellular proteins. Similar to these membrane transport proteins, the human ITSN-2L has a conserved C2 domain at C-terminus of the protein (Fig. 2B). The multiple crystal structure alignments of human ITSN-2L C2 domain with synaptotagmin I and rabphilin show that there is a high degree of structural homology between C2 domains in the core β-sandwich and less similarity in the top and bottom loops (Fig. 2C). The high degree of structural identity between the core β-sandwiches of C2 domains suggests that the β-sandwich represents a scaffold in vesicular transport. The reports showed that the short isoform of ITSN (ITSN-S) is expressed in all tissue whereas the ITSN-L is expressed only in neurons or other specific tissues [4,31]. It suggests that the β-sandwich of ITSN-2L C2 domain may serves as scaffolds in the assembly of endocytic vesicles. ITSN-L in specific cellular membrane may distribute through the interaction of the β-sandwich of C2 domain with phospholipids. The different distributions of ITSN-1L and ITSN-2L [1,6,32] indicate that the loops or α-helix along the β-sheets may determine the functional specificity of a C2 domain.

3.3. Function of C2 domain in cellular signaling pathways

There are two types of topology referred to as topology I (such as synaptotagmin I C2A domain) and topology II (such as PLC δ1C2 domain, PKCδ C2 domain), differing slightly in their β-strand connectivity [33,34]. From the crystal structure of human ITSN-2L C2 domain, we find that it belongs to topology I. In the two types of topology, the first strand of topology I occupies the same structural position as the eighth β-strand of topology II, which shifts the order of homologous strands in the primary structure (Fig. 3A). The multiple Ca^{2+} ions bind in a cluster exclusively at the top loops or α-helix in the most C2 domain, where these loops are widely separated in the primary sequences (Fig. 2A). The multiple sequence alignments of C2 domain family indicate that there is a certain similarity among them, and the conserved residues locate flexible loops which are involved in the Ca^{2+} binding (Fig. 2A).

The comparison of the solvent accessible surface of two types of C2 domain shows that the top α-helix in all C2 domains located in low charge groove of the protein (Fig. 3B). Ca^{2+} binding does not induce a major conformational change in the C2 domains but causes a major change in electrostatic potential of the C2 domain proteins that may be important for regulating protein interactions [10,28,29,35]. In human ITSN-2L, C2 domain may act as Ca^{2+}-activated, molecular switches to turn on DH activity. It provides a possible mechanism that human ITSN-2L C2 domain affects the DH activity through the Ca^{2+} influx, while the precise mechanism is unknown.

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