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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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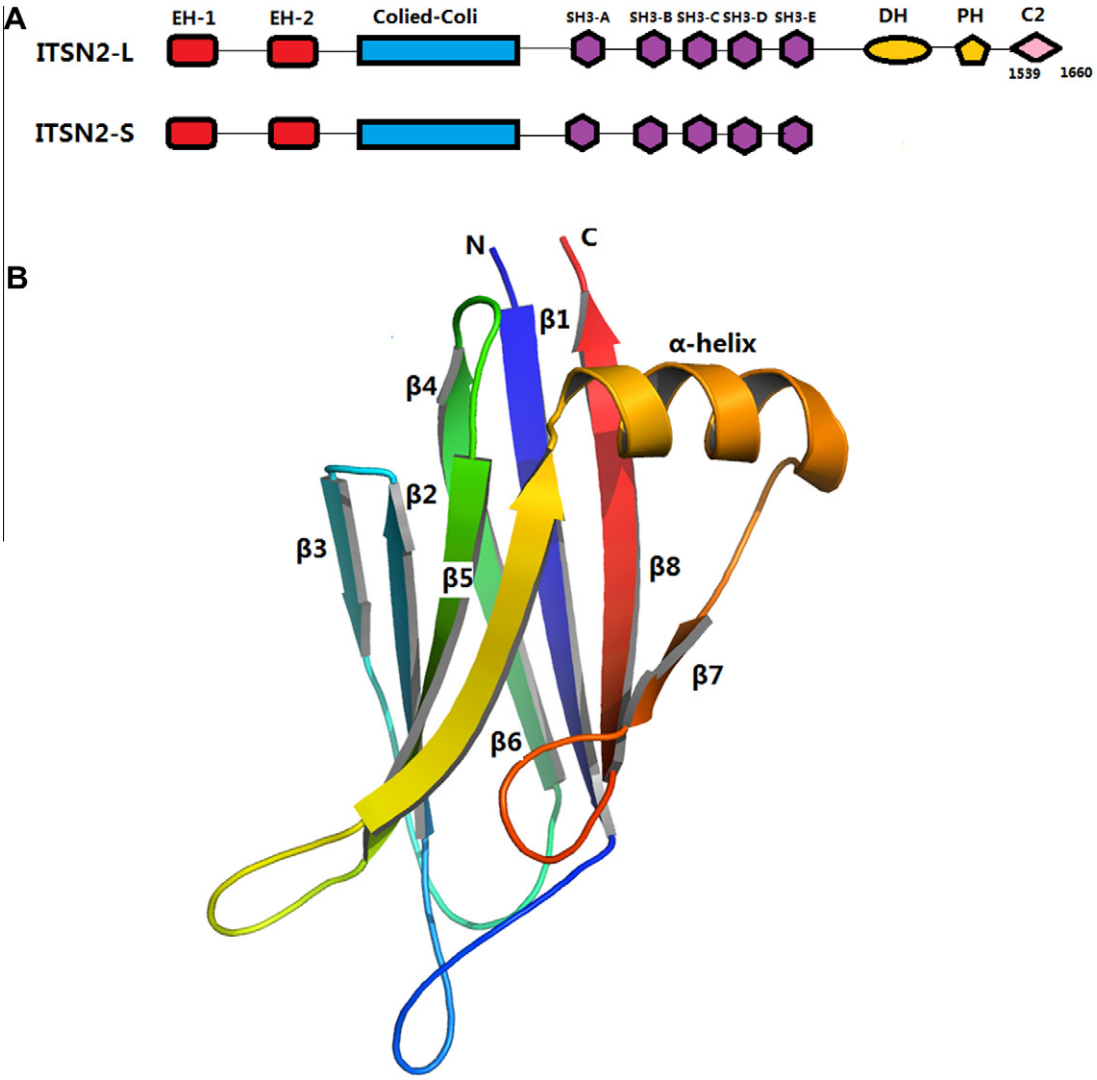

Fig. 1. The domain organization of ITSN-2 and over structure of the human ITSN-2L C2 domain: (A) Schematic representation of ITSN-2 shows the domain organization of ITSN-2S and ITSN-2L. Numbers below indicate amino-acid positions of C2 domain boundaries. (B) The overall structure of C2 domain exhibits 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