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Coexpression and protein-protein complexing of DIX domains of human Dvl1 and Axin1 protein

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The Dvl and Axin proteins, which are involved in the Wnt signaling pathway, each contain a conserved DIX domain in their sequences. The DIX domain mediates interaction between Dvl and Axin, which together play an important role in signal transduction. However, the extremely low production of DIX domain fragments in *E. coli* has prevented more widespread functional and structural studies. In this study, we demonstrate that the DIX domains of Dvl and Axin are expressed noticeably in a multi-cistronic system but not in a mono-cistronic system. Formation of the DIX_{Dvl1}-DIX_{Axin1} complex was investigated by affinity chromatography, SEC and crystallization studies. Unstable DIX domains were stabilized by complexing with counterpart DIX domains. The results of the preliminary crystallization and diffraction of the DIX_{Dvl1}-DIX_{Axin1} complex may prove useful for further crystallographic studies. [BMB reports 2010; 43(9): 609-613]

INTRODUCTION

Wnt signaling is a key regulatory pathway in animal development and is critical for the homeostasis of adult tissues (1, 2). In the canonical Wnt pathway, Dvl proteins are cytoplasmic and are recruited to the plasma membrane by binding to Fz receptors. Upon activation, Dvl proteins suppress the three component proteins (GSK3β, APC and Axin) of the Axin complex, resulting in regulation of the phosphorylation and stability of β-catenin (1, 2). Three Dvl genes (Dvl1, 2 and 3) along with two Axin genes (Axin1 and 2) have been isolated from mammals. β-catenin acts as a transcriptional coactivator in the Wnt signaling pathway, and its inappropriate activation causes tumor-related diseases such as colorectal cancer (3-5). Therefore, studies on the interaction between Dvl and Axin protein are important to understanding signal transduction in the Wnt pathway. However, the interaction mechanism between the two proteins remains unclear.

Dvl proteins contain three highly conserved domains: an N-terminal DIX domain, a central PDZ domain and a DEP domain (6-9) (Fig. 1). Disruption of the PDZ domain abolishes Dvl activity during Wnt signaling (10, 11), and the DEP domain is critical for the upregulation of β-catenin activity and stimulation of LEF-1, which mediates transcription in mammalian cells (12). Interestingly, the C-terminal region of Axin is homologous to the DIX domain of Dvl. Moveover, the activity of Axin protein is inhibited upon the interaction of its DIX domain with that of Dvl (6-8, 13, 14). However, the 3D structure of the Dvl DIX domain and DIX_{Dvl}-DIX_{Axin} complex remain undetermined. In addition to their heterogeneous interaction, the DIX domains of Dvl and Axin have been reported to mediate self-association both *in vitro* and *in vivo* (6, 15, 16). According to X-ray crystallography, the Axin DIX domain can form head-to-tail filaments by self-association (17).

In this study, we demonstrated that the DIX domains of human Dvl and Axin were not expressed in *E. coli* when using a mono-cistronic vector and highly expressed when using a multi-cistronic vector. Interaction of the DIX domains mediated the formation of a DIX_{Dvl1}-DIX_{Axin1} complex in solution. Moreover, we reported the initial crystallization conditions of the protein-protein complex for x-ray crystallography.

RESULTS AND DISCUSSION

Coexpression of Each DIX domain gene from hDvl1 and hAxin1

To express the DIX domains of Dvl1 and Axin1 with high yield in *E. coli*, we inserted the DIX domains of hAxin1and hDvl1 into multiple cloning sites (MCS) 1 and 2 of the multi-cistronic vector pACYCDuet-1, respectively (Fig. 1). The inserted Axin1 DIX domain was designed to be expressed with a N-terminal hexahisitidine tag while the Dvl1 DIX domain expressed no tag. Dvl1 DIX and Axin1 DIX recombinant proteins had predicted sizes of 230 and 308 amino acid residues, respectively, with theoretical molecular masses of 25.5 and 34.1 kDa. *E. coli* BL21 (DE3) host cells harboring pACYCDuet-1 were induced by IPTG for the production of recombinant proteins, and a distinct band of ~25 kDa and ~35 kDa was observed by SDS-PAGE (Fig. 2A). However, there was no detectable expression of the DIX domains of Dvl1 or Axin1 using mono-cis-

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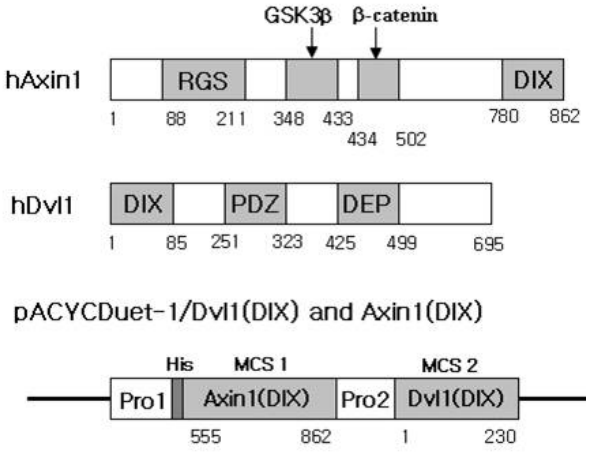

Fig. 1. Domain structures of human Dvl1 and Axin1 proteins, and a schematic diagram of the coexpression vector. Human Axin1 is schematically represented with its ligand proteins, which bind to known structural domains. Pro, promoter; MCS, multiple cloning site; His, histidine tag.

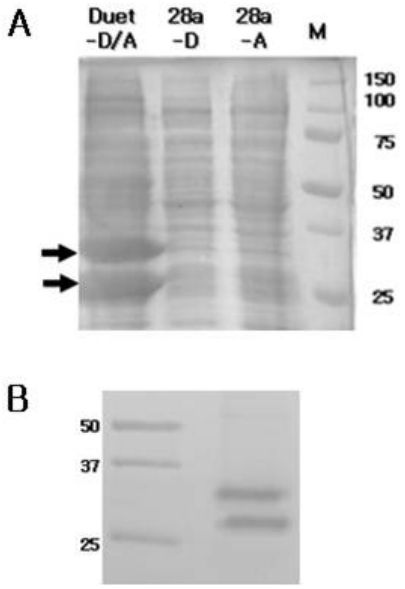

Fig. 2. (A) SDS-PAGE analysis of DIX domains expressed in *E. coli*. Lanes: Duet-D/A, total fraction of both Dvl1 and Axin1 DIX domains expressed in pACYCDuet-1; 28a-D, total fraction of Dvl1 DIX domain expressed in pET28a; 28a-A, total fraction of Axin1 DIX domain expressed in pET28a; M, molecular weight standards (BioRad). Two arrows indicate expressed protein bands, respectively. (B) SDS-PAGE of purified proteins after affinity chromatography.

tronic pET28a vector (Fig. 2A), which implies that the separately-expressed protein in *E. coli* was unstable. Due to this noticeable in expression difference between the multi- and mono-cistronic vectors, instability was avoided by complexing the DIX domains with other proteins.

Affinity purification of recombinant proteins

After the harvested cells were resuspended in ice-cold lysis buffer, homogenization was carried out with an ultrasonic processor. The crude lysate was centrifuged and only the supernatant fraction was loaded onto a Ni-NTA affinity column. Nonspecific proteins were washed away with buffer A containing 50 mM Tris-HCl (8.0) and 100 mM NaCl. Elution of bound proteins was performed in a linear salt gradient, which was facilitated by mixing with buffer B containing 500 mM imidazole.

Both Dvl1 and Axin1 DIX were eluted simultaneously in the range of 100-300 mM imidazole and confirmed as homogeneous by SDS-PAGE (Fig. 2B). Since only Axin1 DIX protein contained a hexahistidine tag, co-elution of Dvl1 DIX protein at an increased imidazole concentration indicated interaction with Axin1 DIX protein. After affinity purification, dialysis was performed against buffer A to remove imidazole salt from the pooled sample.

Formation of DIX_{Dvl1}-DIX_{Axin1} complex

To investigate the formation of the DIX_{Dvl1}-DIX_{Axin1} complex, we analyzed the molecular weight of purified protein using size-exclusion chromatography. Based on the molecular weight of each protein, the DIX_{Dvl1}-DIX_{Axin1} complex was expected to be about 60.4 kDa. A standard curve was prepared as described above. As shown in the SEC profile, the highest peak, which was eluted at ~12-14 ml, corresponded to a molecular weight ranging from 67 kDa to 440 kDa (Fig. 3A). This reveals that the eluted protein particles were definitely larger than Dvl1DIX (25.5 kDa) and Axin1 DIX (34.1 kDa). SDS-PAGE showed that the two proteins were simultaneously eluted (Fig. 3B), although both differed considerably in molecular weight. Together with co-elution by affinity column chromatography, these results reveal that the DIX domains formed a heterogeneous protein-protein complex with another DIX domain.

In addition to heterogeneous interaction, another research group reported the homo-oligomerization of Dvl1 DIX and Axin1 DIX by self-association, with homo-oligomers resembling a trimer or tetramer (6). Interestingly, the band widths of Axin1 DIX and Dvl1 DIX did not show 1 : 1 stoichiometry in SDS-PAGE. Thus, the co-eluted fraction in the SEC profile was a mixture of several species containing a homo-oligomer and hetero-dimer of Dvl1 DIX and Axin1 DIX. However, it was not possible to determine the molecular weight of the hetero-dimer due to the presence of a trimer or tetramer of he homo-oligomer.

Crystallization screening of DIX_{Dvl1}-DIX_{Axin1} complex

Among the three domains involved in Dvl protein folding, the structures of PDZ and DEP have been determined. However, the structure of DIX domain remains unsolved. The structural

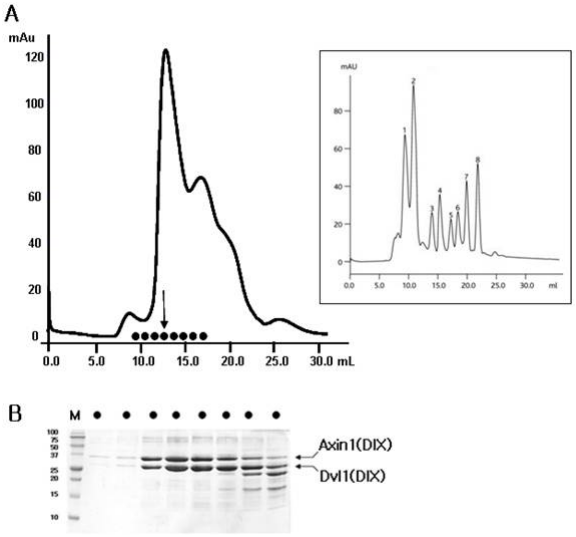

Fig. 3. (A) Profile of size-exclusion chromatography showing shifted elution of each DIX protein due to DIX_{Dvl1}-DIX_{Axin1} complexing. Standard marker curve was prepared in the box (right): 1, thyroglobulin (Mr 669 kDa); 2, ferritin (Mr 440 kDa); 3, BSA (Mr 67 kDa); 4, B-lactoglobulin (Mr 35 kDa); 5, ribonuclease A (Mr 13.7 kDa); 6, cytochrome C (Mr 13.6 kDa); 7, aprotinin (Mr 6.5 kDa); 8, vitamin B12 (Mr 1.4 kDa). Arrow indicates elution volume (~13 ml) of the highest peak, and closed circles denote the collected fractions in the peak. (B) SDS-PAGE analysis of the collected fractions in figure (A).

determination of DIX_{Dvl1}-DIX_{Axin1} complex will explain how the interaction between Dvl1 and Axin1 mediates signal transduction in the Wnt signaling pathway.

Therefore, we tried to crystallize the DIX_{Dvl1}-DIX_{Axin1} complex by X-ray crystallography. The initial screen was performed using commercial screen kits as described above. The best crystals were obtained using a reservoir solution comprising 100 mM ammonium phosphate dibasic, 100 mM Tris-HCl (pH 8.5) and 12-15% PEG 6000 (Fig. 4). The crystals grew to maximum dimensions of 0.1 × 0.1 × 0.1 mm within about one week after 2 μl of protein at a concentration of 5 mg/ml was mixed with 2 μl of reservoir solution. To examine whether the crystals contained the DIX_{Dvl1}-DIX_{Axin1} complex or only a single protein, we performed SDS-PAGE electrophoresis after dissolving the crystals. In order to separate the crystals from the protein sample remaining in the drop, several crystals were washed with reservoir solution and subsequently resuspended in 5x sampling buffer. Two protein bands in the SDS-PAGE showed that the DIX_{Dvl1}-DIX_{Axin1} complex had grown into crystals (Fig. 4), revealing that protein-protein complexing was maintained. As mentioned above, the SEC profile and SDS- PAGE implied that the protein samples were mixtures of several species including homo-oligomers and a hetero-dimer; however, 1 : 1 stoichiometry of the two proteins in Fig. 4 shows that only the hetero-dimer was crystallized under our crystallization conditions.

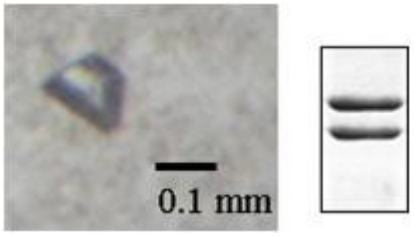

Fig. 4. Crystal of DIX_{Dvl1}-DIX_{Axin1} complex (left) and SDS-PAGE analysis showing protein-protein complexing in the crystals (right).

When mounted with an in-house rotating anode X-ray source, the best crystal diffracted to about 8Å along one axis but worse along other axes (data not shown). Therefore, it seems these crystals were heavily anisotropic, with one direction packed nicely and the other not.

In summary, we reported the successful coexpression of human Dvl1 and Axin1 DIX domains using a multi-cistronic expression vector. Formation of the DIX_{Dvl1}-DIX_{Axin1} complex was examined by affinity chromatography, SEC and crystallization studies. Unstable DIX domains were stabilized by complexing with counterpart DIX domains. Preliminary crystallization and diffraction of DIX_{Dvl1}-DIX_{Axin1} complex will provide clues for further crystallographic studies.

MATERIALS AND METHODS

Materials

The human *Dvl1* and *Axin1* genes were gifted from the 21C Human Gene Bank (Genome Research Center, KRIBB, Korea). Plasmid pACYCDuet-1 was purchased from Novagen (Madison, WI, USA). All enzymes, restriction endonucleases and DNA markers were from Fermentas (Ont., Canada). Plasmid purification kit and gel extraction kit were from Sigma-Aldrich (St. Louis, MO, USA). Sequencing primers were synthesized by Bioneer (Korea). BL21 (DE3) *E. coli* cells for host cells were from Novagen (Madison, WI, USA). Agarose and all other chemicals were of analytical grade.

Construction of multi-cistronic and mono-cistronic vector

The human Dvl1 sequence reported in GenBank (accession no. AAB65242) was used as a template for gene amplification of its N-terminal fragment, which corresponded to amino acids 1-230. The human Axin1 sequence (GenBank accession no. AAC51624) was also amplified by PCR so that the resulting PCR product contained the C-terminal fragment corresponding to amino acids 555-862. After 1% agarose gel electrophoresis, the purified PCR product of the Axin1 fragment was cleaved with *BamHI* and *NotI*, followed by insertion into multiple cloning sites 1 (MCS 1) of pACYCDuet-1 vector using T4 DNA ligase. Transformation into DH5α (DE3) competent cells and colony PCR was carried out to select the positive clones of the

Axin1 fragment. Dvl1 fragment gene was inserted into the MCS 2 sites (*NdeI* and *XhoI*) of pACYCDuet-1 in the presence of the *Axin1* gene at MCS 1. Positive clones were also selected through transformation and colony PCR. Finally, a hexahistidine tag was added to the N-terminus of the Axin1 fragment. The whole coding regions in the pACYCDuet-1 expression vector were sequenced to check for possible mutations during the PCR reaction.

Expression of recombinant fusion protein

The selected clones were inoculated into 5 ml of LB media in the presence of 50 μg/ml of chloramphenicol, followed by culture overnight at 37°C with shaking. Exactly 0.5 ml of the culture was added to 50 ml of LB media containing 50 μg/ml of chloramphenicol, followed by shaking at 37°C at 200 rpm. After an OD_{600nm} of 0.5, 1 mM IPTG (isopropyl-beta-D-thiogalactopyranoside) was added for protein induction. After growing at 37°C for 6 hours, cells were harvested by centrifugation at 4,200 g (6000 rev min^{-1}; Sorvall GSA rotor) at 4°C for 10 min, after which the cell pellet was resuspended in ice-cold lysis buffer (50 mM Tris-HCl pH 8.0, 100 mM NaCl, 1 mM phenylmethylsulfonyl fluoride) and homogenized with an ultrasonic processor. The crude lysate was centrifuged at 70,400 g (30000 rev min^{-1}; Beckman 45Ti rotor) for 1 h at 4°C, after which the recombinant proteins in the supernatant fraction were analyzed by SDS-PAGE and purified by liquid chromatography. We also examined protein induction at 18°C by lowering the temperature shortly before IPTG induction; however, induction temperature did not affect the soluble expression of either protein.

Analysis of DIX_{Dvl1}-DIX_{Axin1} complexing using SEC

After purification, protein samples were analyzed by size-exclusion chromatography (Superdex 200 10/300 GL column; GE Healthcare) in order to determine molecular size. Protein size was judged based on its elution volume (Ve) and band size in SDS-PAGE. The pooled fractions after Ni-NTA column chromatography were concentrated using an YM10 ultrafiltration membrane (Amicon). The injection volume during SEC did not exceed 0.5 ml in order to correspond to the standard curve. The standard protein curve was prepared such that thyroglobulin (Mr 669 kDa), ferritin (Mr 440 kDa), BSA (Mr 67 kDa), B-lactoglobulin (Mr 35 kDa), ribonuclease A (Mr 13.7 kDa), cytochrome C (Mr 13.6 kDa), aprotinin (Mr 6.5 kDa) and vitamin B12 (Mr 1.4 kDa) were eluted at Ve=10 ml, 11 ml, 14 ml, 15 ml, 17 ml, 19 ml, 20 ml and 22 ml, respectively. Protein elution profiles during SEC were monitored by measuring the absorbances at 280 nm.

Crystallization trials

Crystallization was performed at 22°C by the hanging-drop vapor-diffusion method using 24-well tissue-culture plates. Each hanging drop was prepared on a siliconized cover slip by mixing equal volumes (2 μl each) of the protein solution with the corresponding well solutions. Each hanging drop was placed over 1.0 ml of reservoir solution. Initial cystrallization conditions were established using screening kits from Hamton Research (Crystal Screens I, II, and MembFac) and decode Biostructures Group (Wizard I and II).

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