Journal of the Korean Magnetic Resonance Society **2017**, *21*, 72-77
DOI 10.6564/JKMRS.2017.21.2.072

Cloning, Purification and NMR Studies on β-catenin C-terminal Domain

Jeongmin Oh[1†], Sooho Choi[1†], Ji-Hye Yun[1], Yoon-Joo Ko[2], Kang-Yell Choi[3], and Weontae Lee[1,*]

[1]Department of Biochemistry, College of Life Science & Biotechnology, Yonsei University, Seoul, Korea 120-749
[2]National Center for Inter-University Research Facilities, Seoul National University, Seoul, Korea 151-742
[3]Department of Biotechnology, College of Life Science & Biotechnology, Yonsei University, Seoul, Korea 120-749

Received May 09, 2017; Revised May 20, 2017; Accepted May 31, 2017

Abstract β-catenin is a key signaling protein which regulates cell signaling and gene transcription. Abnormal activation of β-catenin is linked to many cancers, particularly with colorectal cancers. Although many genetic and biological studies on Wnt/β-catenin have been reported and structures of the complex between β-catenin and its diverse binding partners have been published, many of them have focused on armadillo repeat domain of β-catenin. Both N- and C-terminal domains have been suggested to regulate interactions of β-catenin with other molecules, but still little is known about the C-terminal unstructured domain. To investigate the structure of this domain, construct of C-terminus was designed and structural studies were performed using size exclusion chromatography (SEC), circular dichroism (CD), fluorescence and nuclear magnetic resonance (NMR) spectroscopy. We observed that not only the purified full-length construct but the purified C-terminal construct also dimerizes in solution by SEC, suggesting that this domain involves in dimerization of β-catenin. CD and fluorescence data indicate its flexibility and structural formation in the presence of membrane environments.

Keywords Wnt/β-catenin pathway, β-catenin, CD, C-terminus, structure, NMR

Introduction

β-catenin is a pivotal component of canonical Wnt/β-catenin pathways and is related to cell signaling and cell-cell adhesions. In the absence of Wnt ligands, β-catenin is recognized by cytosolic destruction complexes and degraded by proteasomes. With Wnt ligands, β-catenin is stably accumulated to enter nucleus and function as a transcription factor. Dysregulations of this Wnt/β-catenin signaling are detected from various cancers and familial adenomatous polyposis.[1] β-catenin is a 85 kDa protein consisting of a central domain as armadillo repeat (arm) domain with flanking N- and C-terminal regions. The structure of arm domain from murine β-catenin has been already known[2] and various biological studies using that domain have been conducted, but still little is known about both terminal domains.[3-7] The structure of arm domain and C-terminal region (138-781) of human β-catenin was also solved, but C-terminus was not observed in the crystal structure due to its flexibility. However, these terminal domains were suggested to regulate bindings of β-catenin and its binding partners.[8,9] To understand this unstructured domain in structural and biochemical perspectives, C-terminus (β-catenin 661-781) and the full-length (β-catenin 1-781) constructs of human β-catenin were designed and

† These two authors contributed equally to this work.
* Correspondence to: **Weontae Lee,** Department of Biochemistry, College of Life Science & Biotechnology, Yonsei University, Seoul, Korea 120-749, Tel: 82-2-2123-2706; E-mail: wlee@spin.yonsei.ac.kr

size exclusion chromatography (SEC), circular dichroism (CD), fluorescence and nuclear magnetic resonance (NMR) spectroscopy were performed. It was observed that not only the purified full-length construct but the purified C-terminal construct also dimerizes in solution by SEC, suggesting that this domain involves in dimerization of β-catenin. In addition, the domain which has a feature of intrinsically disordered region is related to lipid environments. This study of its flexibility and dynamic structure will provide an insight to understand and design the specific drug as a β-catenin regulator.

Experimental Methods

Gene Cloning- Full-length human β-catenin cDNA was obtained kindly from Translational Research Center for Protein Function Control (TRCP) and used as a template to clone constructs. β-catenin C-terminal domain construct$^{661\text{-}781}$ was inserted into pET32a vector (Novagen) digested using restriction enzymes with N-terminal Trx-hexa-histidine tag followed by TEV cleavage site.[10] These recombinant vectors were used to transform the *E.coli* BL21(DE3) for protein expression.

Expression and purification of β-catenin C-terminal construct- Protein was overexpressed in *E.coli* BL21(DE3). Cells were grown in LB media at 37°C until optical density at 600 nm (OD_{600}) reached 0.6. The cells were induced with 0.2 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG), incubated for 20 hours at 18°C and harvested by centrifugation at 6000 rpm, 4°C.

Cell pellets of C-terminus$^{661\text{-}781}$ construct were suspended in lysis buffer (25 mM HEPES, 300 mM NaCl, pH 7.5) with protease inhibitor cocktail (Roche) and lysed with sonication. After centrifugation at 14000 rpm, 4°C, supernatant was loaded onto Ni-NTA column (Qiagen). The column was washed by buffer with 40 mM imidazole and Trx His-tag fusion protein was eluted using buffer with 500 mM imidazole. Eluted protein solution was buffer-exchanged using PD10 desalting column to dilute imidazole and Trx-His-tag was cleaved with 0.2X TEV protease treatment at 25°C for 3 hours. Then target proteins were further purified by Ni-NTA column and SEC using Superdex75 column.[11,12]

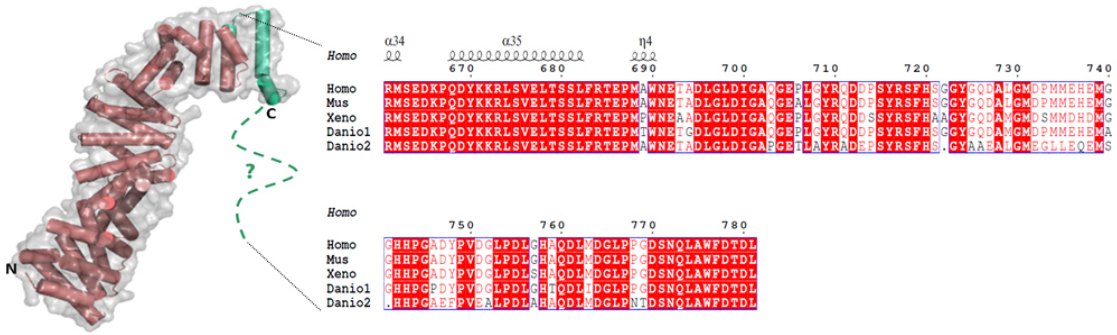

Figure 1. Multiple sequence alignment of β-catenin$^{661\text{-}781}$. Homo, *Homo sapiens*(NP_001091679.1); Mus, *Mus musculus*(NP_001159374.1); Xeno, *Xenopus laevis*(P26233.1); Danio, *Danio rerio*(1, NP_571134.2; 2, NP_001001889.1). Sequence numbering was based on human β-catenin and sequence alignment was generated by T-coffee and Espript. The crystal structure of human β-catenin was depicted (PDB 2z6h).

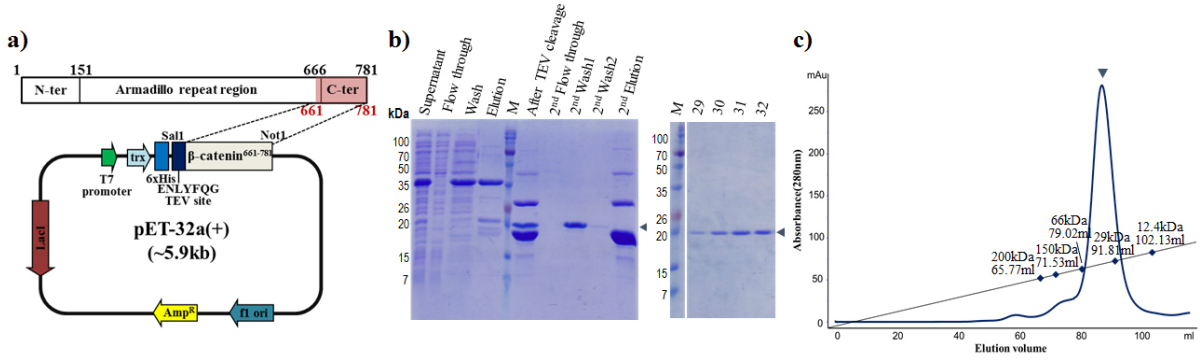

Figure 2. Construct Design and Purification Profile. (a) Recombinant pET-32a(+) vector map for the expression of C-terminal domain$^{661\text{-}781}$ of human β-catenin. The map of *E.coli* expression vector containing TrxA, His-tag, TEV protease cleavage site and recombinant β-catenin was presented. (b,c) The SDS-PAGE and size exclusion chromatography (SEC) results show the solubility, purity and molecular weight of β-catenin. Calculated size of β-catenin by SEC is shown as a dimer in solution.

To prepare uniformly ^{15}N or $^{13}C/^{15}N$-labeled proteins, cells were grown in M9 media containing $^{15}NH_4Cl$ (Cambridge Isotope Lab.) as the source of nitrogen and ^{13}C-D-glucose was used as the source of carbon for $^{13}C/^{15}N$-labeled proteins. Thiamine was added in the media to support optimal growth. The cells were induced with 0.2 mM IPTG at OD_{600} 0.6 and grown for 28 hours at 18°C. Proteins were purified as described above. Purified proteins were finally dissolved in NMR buffer (50 mM HEPES, 50 mM NaCl, 2 mM $MgCl_2$, 2 mM dithiothreitol (DTT), pH 7.4 in 90%H_2O/10%D_2O).

Circular dichroism spectroscopy- CD spectra were recorded on JASCO J-815 spectropolarimeter (Jasco, Tokyo, Japan) calibrated with ammonium D-10-camphorsulfonate at 290 nm and equipped with a thermostatically controlled cell holder attached to water bath with an accuracy ±0.1°C. The parameters for far-ultraviolet (UV) CD measurements were cell of path length 0.1 cm for scanning between 250 nm - 200 nm, 1 nm bandwidth, and a scan speed of 50 nm/min, signal-averaged over at least 6 scans, and baseline corrected by subtracting a buffer spectrum. Secondary structure was calculated using K2D3 method or Yang's reference.[13,14]

NMR spectroscopy- NMR experiments were performed at 298K on a Bruker AVANCE 600MHz equipped with 5 mm TXI cryoprobe. 2D ^{1}H-^{15}N HSQC experiments were performed to test different buffer conditions and effects of detergents. The obtained FIDs were processed using NMRPipe / NMRDraw software and spectrum was analyzed using Sparky program.[15-17]

Results

Protein expression and purification- β-catenin has a high sequence homology of C-terminal domain in various species, as shown in Fig.1. The roles of C-terminal domain to regulate interactions with binding partners have been suggested in some researches corresponding to high homology of that region. Although structures of zebrafish full-length β-catenin and human β-catenin armadillo repeat domain are already known, C-terminal domain have not been able to be detected in crystal structures due to its flexibility. C-terminus of β-catenin is anticipated to have a large propensity of disorder based on sequence database. In accordance with the previous structural studies, this C-terminal domain is thought to be intrinsically disordered region unveiled recently to have crucial roles in cell signaling.

To analyze C-terminal domain in structural view, construct of β-catenin$^{661\text{-}781}$ was used (Fig. 2a) and over-expressed protein was purified. To confirm the

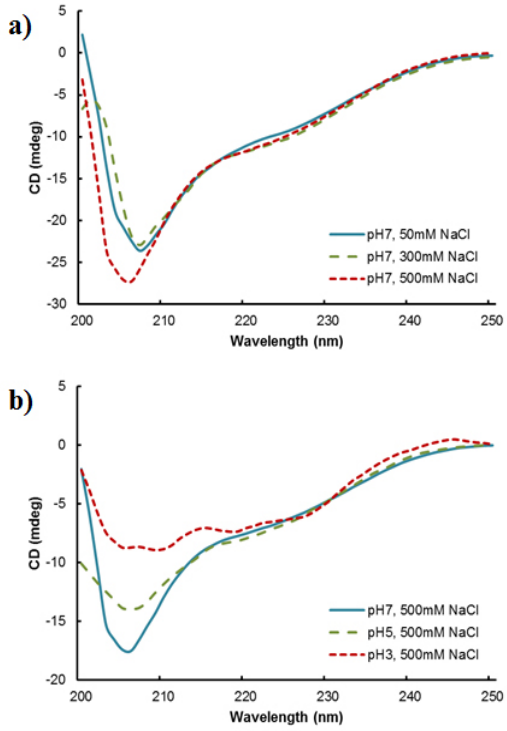

Figure 3. Secondary structure analysis of β-catenin C-terminal domain using circular dichroism (CD) spectroscopy in different pH, salt condition. The CD spectrum of β-catenin$^{661-781}$ was analyzed (a) in different NaCl concentration from 50 mM to 300 mM in pH 7 and (b) in different pH from 3 to 7 in 500 mM NaCl.

purity of target protein, samples of each purification step were loaded on 15% SDS-PAGE (Fig. 2b) and size exclusion chromatography (SEC) results show a single peak indicating that target protein was purely purified (Fig. 2c). Calculated size of β-catenin$^{661-781}$ by SEC is shown as a dimer in solution, suggesting that C-terminus probably involves in dimerization of β-catenin. Considering that disordered proteins often appear to have higher molecular weight on SEC, it can be confirmed using multi-angle light scattering for a dimeric state.

Circular dichroism spectroscopy- To identify pH and salt dependence of β-catenin$^{661-781}$ and optimize buffer conditions, secondary structure analysis was done based on circular dichroism (CD) spectrum.

NaCl concentration affected the spectral shape slightly (Fig. 3a), but low pH conditions made more differences in spectrum (Fig. 3b). It was estimated that C-terminus is highly affected by proton environments. Despite this result, following experiments were carried out around pH 7 to analyze in the physiological pH.

While β-catenin$^{661-781}$ has a little secondary structure in the absence of SDS, α-helical structures were induced with titration of SDS. Over 1 mM concentration of SDS induced a 222 nm negative peak as like in the presence of 2,2,2-trifluoroethanol

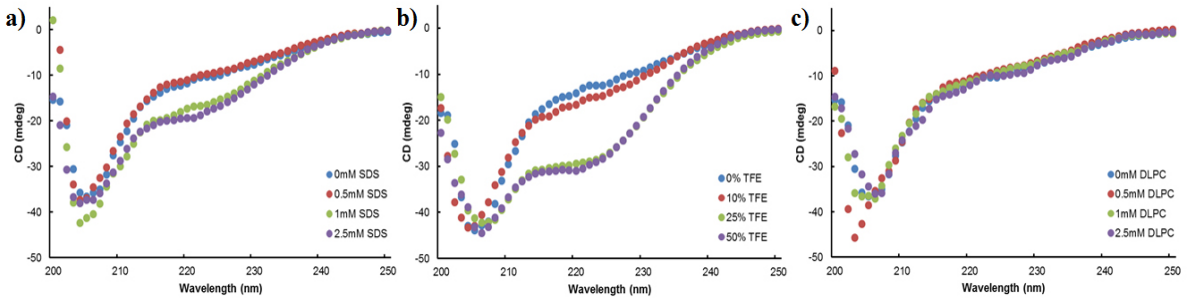

Figure 4. Secondary structure analysis of β-catenin C-terminal domain using CD spectroscopy. The CD spectrum of β-catenin$^{661-781}$ was analyzed (a) in different SDS concentration from 0 mM to 2.5 mM (b) in different TFE percentages from 0 % to 50 % and (c) DLPC concentrations from 0 mM to 2.5 mM.

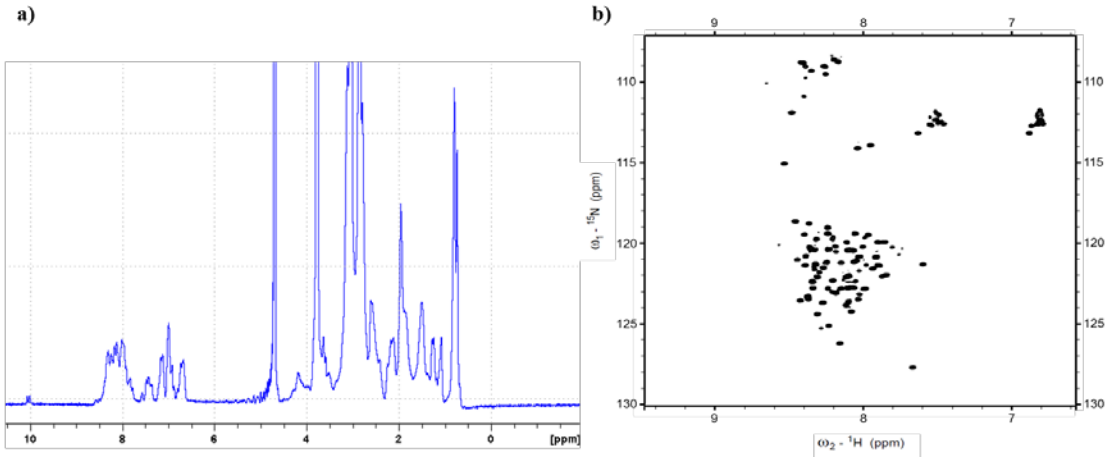

Figure 5. NMR spectroscopy to analyze β-catenin$^{661\text{-}781}$. The NMR spectrum of β-catenin$^{661\text{-}781}$ was analyzed, (a) 1D ^{1}H NMR spectrum of β-catenin$^{661\text{-}781}$ and (b) 2D ^{1}H-^{15}N HSQC spectrum of β-catenin$^{661\text{-}781}$.

(TFE), an inducer of α-helix (Fig. 4a, b) used as positive control. However, 1,2-Dilauroyl-sn-glycerol -3-phosphocholine (DLPC), one of phospholipids, didn't make any change in the shape of CD spectrum (Fig. 4c). According to these results, it is proposed that SDS-like lipid conditions can induce secondary structures and contribute to have probably more stable conformation in membrane-dependent manner.

NMR spectroscopy- 1D ^{1}H spectrum was analyzed to confirm the purified β-catenin$^{661\text{-}781}$ (Fig. 5a). The high peak at 4.7 ppm is originated from water, and peaks at 3.90, 3.26, 3.00 ppm are from buffer component HEPES. Peaks observed around 9~7 ppm are mostly from NH protons of peptide backbones. Little peaks are found around 5~4 ppm where protons in α-helix and β-sheet. To further analyze β-catenin$^{661\text{-}781}$, 2D ^{1}H-^{15}N HSQC experiment was also performed (Fig. 5b). The spectrum has a low dispersion of peaks around 8 ppm, indicating that β-catenin$^{661\text{-}781}$ does not have well-defined structures.

Discussion

In our study, we characterized C-terminal domain of β-catenin using biochemical approaches. The NMR spectrum has low dispersions indicating that this region is not well-structured in accordance with previous data. Intrinsically disordered regions have been emphasized for their significant roles in diverse cellular responses. Considering β-catenin is a key signaling molecule which regulates cell signaling and gene transcription, structural studies and dynamics studies on the C-terminal domain of β-catenin will provide insights on its various functions and regulation mechanisms.

Acknowledgements

This work was supported by Translational Research Center for Protein Function Control (2009-0083522) and Mid-career Researcher Program (NRF-2017R1A2B2008483 to WL).

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