ISSN: 1813-4890

Refolding of the large extracellular loop of human TSPAN12 in 0.5 mM sodium dodecyl sulfate (SDS)

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Abstract

Tetraspanin-12, a crucial member in the Norrin/β-catenin signaling pathway, participates in interaction with FZD4 and NDP via its large extracellular loop (LEL). Furthermore, many of the mutations that cause familial exudative vitreoretinopathy (FEVR) are found on the LEL of TSPAN12. However, the three-dimensional structure of TSPAN12, especially its LEL, remains unknown. In this study, we selected the LEL of TSPAN12 for structural characterization studies. TSPAN12-LEL was successfully produced as inclusion bodies in Escherichia coli and prepared through oxidative refolding and size exclusion chromatography (SEC) purification in 0.5 mM sodium dodecyl sulfate (SDS). The circular dichroism (CD) spectroscopy results revealed that the secondary structure of TSPAN12-LEL refolding in 0.5 mM SDS was mainly composed of α-helix. This work provides a valuable foundation for further investigations into the structural biology of TSPAN12.

Keywords

TSPAN12, Large Extracellular Loop, Refold, Sodium Dodecyl Sulfate, Circular Dichroism.

1. Introduction

The Wnt signaling pathway regulates cell survival, differentiation, proliferation, migration, and other embryogenesis-related processes. It also affects cell proliferation and control in adult tissues[1,2]. As a unique variant of the Wnt signaling pathway, the Norrin/β-catenin signaling pathway plays an important role in retinal vascular growth and development[3,4]. Tetraspanin-12 (TSPAN12), as a crucial member in the Norrin/β-catenin signaling pathway, interacts with the FZD4 and NDP[5]. In the absence of Norrin, both FZD4 and TSPAN12 form homodimers or oligomers. The interaction of FZD4 with TSPAN12 brings FZD4 into tetraspanin-enriched microdomains on the cell membrane. A Norrin dimer binds to an FZD4 dimer with high affinity. Simultaneously, Norrin also binds to Lrp5[6]. This complex together with TSPAN12 activates β-catenin signaling[7]. As a coreceptor, TSPAN12 interacts with FZD4 and NDP through its LEL, promotes ligand selectivity of FZD4, stabilizes the NDP / FZD4 complex, and enhances signaling to physiologically required levels. Moreover, the LEL of TSPAN12 can repair the weakening of the Norrin/β-catenin signaling pathway due to mutations in the NDP and FZD4 genes[8].

Mutations in TSPAN12 are linked to a human retinal disease known as familial exudative vitreoretinopathy (FEVR)[9,10]. FEVR is a rare genetic disease that primarily impairs retinal angiogenesis, resulting in inadequate peripheral retinal blood vessel development and poor vascular differentiation[11]. Mutations in the FZD4, LRP5, TSPAN12, and ZNF408 genes are the most common pathogenic mutations of FEVR, accounting for over half of FEVR cases[5,11-14]. The Norrin/β-catenin signaling pathway relies on the ligand-receptor complex, which is made up of proteins produced by the above genes (excluding ZNF408)[3]. This suggests that this

ISSN: 1813-4890

pathway plays a crucial part in retinal angiogenesis. Currently, many of the mutations that cause familial exudative vitreoretinopathy (FEVR) are found on the LEL of TSPAN12[15].

The LEL of TSPAN12 is particularly important for its function, but its three-dimensional structure, especially the structure of the LEL fragment, remains unknown. In this study, we selected the LEL of TSPAN12 for structural characterization studies. TSPAN12-LEL was successfully produced through oxidative refolding and size exclusion chromatography (SEC) purification in 0.5 mM sodium dodecyl sulfate (SDS). The circular dichroism (CD) spectroscopy results revealed that the secondary structure of TSPAN12-LEL refolding in 0.5 mM SDS was mainly composed of α-helix. This study provides a useful foundation for further research on the structural biology of TSPAN12.

2. Methods

2.1. Construction of the expression vector

The LEL domain (amino acids Pro-122 to Thr-217) of human TSPAN12 (UniProKB accession number O95859) is encoded by cDNA sequences that were connected with His6-tags at its N termini. It was subcloned into NcoI/XhoI sites of the pET-15b vector to produce TSPAN12-LEL.

2.2. Protein expression

To produce protein on a large scale, *Escherichia coli* BL21 (DE3) host cells were transformed with the recombinant plasmid containing the target genes. After induction with 0.5 mM isopropyl β-D-1-thiogalactopyranoside, the cells were grown in LB medium for 4 hours at 37 ℃ until OD600 reached 0.4-0.6. Cells were harvested by centrifugation, resuspended in lysis buffer containing 5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 8.0, and then homogenized by a cell disruptor. The insoluble inclusion bodies were obtained by high-speed centrifugation, which were then resuspended in inclusion dissolution buffer containing 20 mM Dithiothreitol, 8 M Urea, 0.1 M Tris-HCl, pH 8.0, and agitated for 40 min-1 h at room temperature for full dissolution. The supernatant of inclusion bodies was collected by centrifugation, diluted tenfold with inclusion dissolution buffer, and then purified using Ni-NTA affinity chromatography.

2.3. Oxidative refolding and SEC purification in 0.5 mM SDS

The protein was diluted to a final protein concentration of 0.1 mg/ml in order to begin oxidative refolding. The refolding system consisted of 0.5 mM SDS, 2 M Urea, 0.1 M Tris-HCl, 0.4 mM DTT, and 1 mM 2-hydroxylethyl disulfide, pH 8.0. Then the solution was agitated for 2 h at room temperature to accelerate protein refolding. After the Oxidative refolding, the protein was purified by SEC using HiLoad 16/600 SuperdexTM 75 (GE Healthcare). SEC buffer consisted of 0.5 mM SDS, 0.3M Urea, 20 mM NaCl, 0.1 M Tris-HCl, and pH 8.0.

2.4. Circular dichroism analysis

Protein samples from SEC purification were solvent exchanged into 0.5 mM SDS, 20 mM NaF, 5 mM Tris-HCl, pH 8.0 for CD analysis. The CD spectroscopy was measured by a Chirascan CD spectrometer. The experiment was performed by using a 0.1 cm light-diameter quartz cuvette. The protein was scanned at 25 ℃ in the wavelength range of 190-260 nm, with a scan rate of 100 nm/min and both steps and bandwidth setting to 1 nm. The secondary structure proportions of protein were calculated by BeStSel software.

3. Results

3.1. Oxidative refolding of TSPAN12-LEL in 0.5 mM SDS

TSPAN12-LEL was successfully expressed in BL21 and was mainly produced as insoluble inclusion bodies. The inclusion bodies were dissolved in inclusion dissolution buffer and

ISSN: 1813-4890

purified by affinity chromatography. The protein was then added to the refold system, which contained 0.5 mM SDS, 2 M urea, 0.4 mM DTT, 1 mM 2-hydroxylethyl disulfide, 0.1 M Tris-HCl, pH 8.0, to initiate oxidative refolding. The SDS-PAGE result of oxidative refolding is shown in Fig. 1. The result showed that the protein existed in the supernatant after oxidative refolding (Lane 1) and can be efficiently concentrated (Lanes 2-5).

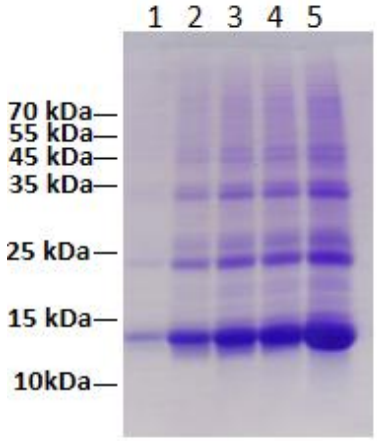

Figure 1. SDS-PAGE analysis of TSPAN12-LEL refolding in 0.5 mM SDS.
Lane 1, refolding protein; Lane 2, concentrate 7 times of refolding protein; Lane 3, concentrate 14 times of refolding protein; Lane 4, concentrate 18 times of refolding protein; Lane 5, concentrate 35 times of refolding protein.

3.2. SEC purification in 0.5 mM SDS and SDS-PAGE analysis

Following oxidative refolding, the TSPAN12-LEL was concentrated for SEC purification. SEC chromatogram is displayed in Fig. 2. The SEC chromatogram showed that there were three peaks with different elution volumes, namely 47 ml, 72 ml, and 111 ml (Fig. 2). Analytical SDS-PAGE was used to determine the peak components. The SDS-PAGE result showed that the peak components at 47 ml and 72 ml were TSPAN12-LEL protein and the peak components at 111 ml were not protein (Fig. 3). The lines with BME were able to disrupt the disulfide bond of the protein molecule, whereas the lines without BME were unable to do so. This was the difference between the lanes with and without BME. The peak components at 47 ml were mainly composed of TSPAN12-LEL polymers with high molecular weight and the polymers were mainly connected by intermolecular disulfide bonds (Lane 2, 9). In comparison, the peak components at 72 ml were TSPAN12-LEL oligomers (Lanes 3-5, 10-12).

3.3. Secondary structure of TSPAN12-LEL

Peak components of TSPAN12-LEL at 72 ml were taken for circular dichroism spectroscopy analysis. By using far-UV CD at 25℃, the secondary structural of TSPAN12-LEL was determined (Fig. 4). The CD spectra showed positive bands at around 192 nm and negative bands at around 208 and 222 nm in the far-UV ranges of 190-260 nm. This is a typical characteristic of α-helix. BeStSel software was used to analyze the CD spectra data and calculate the secondary structure composition (Table 1). The normalized root mean square deviation (NRMSD) score of TSPAN12-LEL was lower than 0.05, indicating the result was reliable. The result showed that 43% of TSPAN12-LEL was a helix, 10% was β-sheet, 9.8% was turns, and 37.2% was unordered.

ISSN: 1813-4890

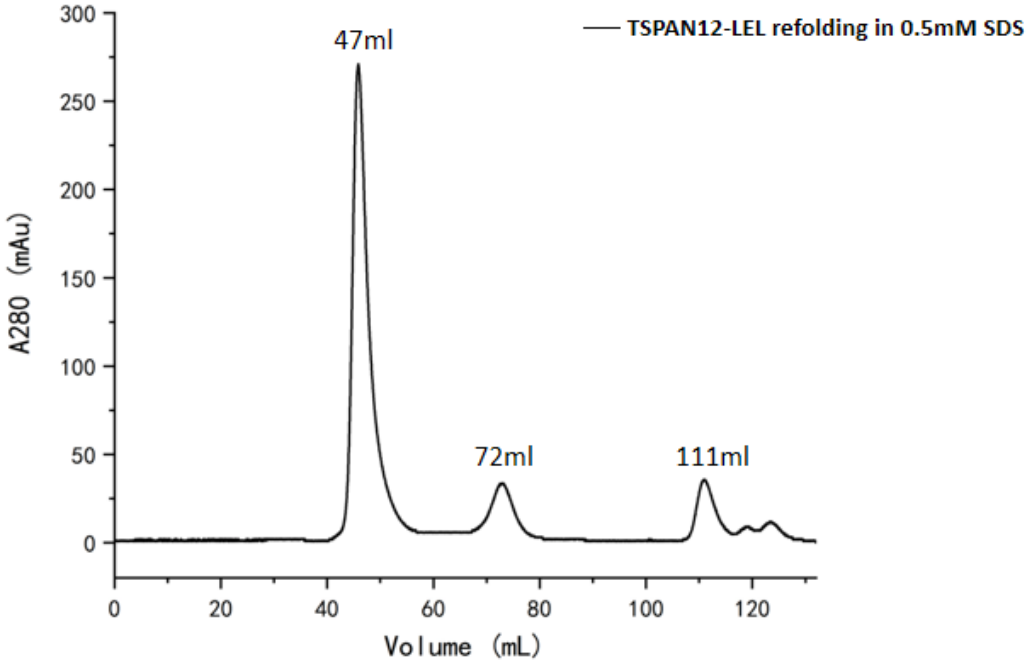

Figure 2. SEC chromatogram of TSPAN12-LEL refolding in 0.5 mM SDS

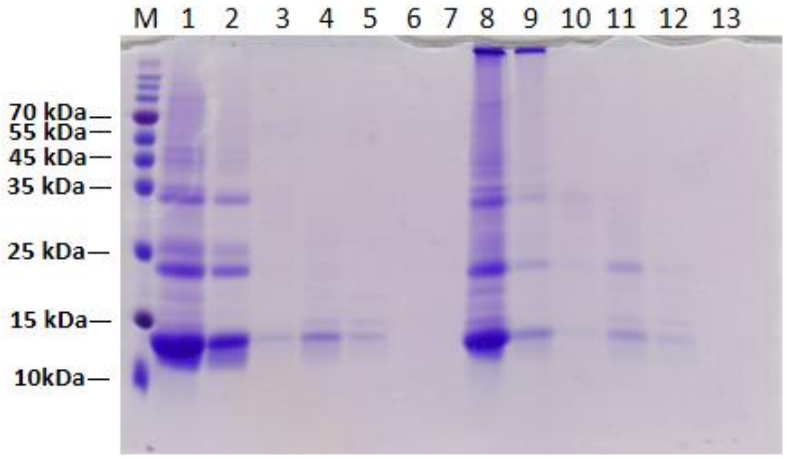

Figure 3. SDS-PAGE analysis of peak components of SEC purification in 0.5 mM SDS. Lane M, marker; Lane 1, SEC loading sample of TSPAN12-LEL; Lane 2, peak component of 46 ml of TSPAN12-LEL SEC chromatography; Lane 3-5, peak component of 73 ml of TSPAN12-LEL SEC chromatography; Lane 6, peak component of 111 ml of TSPAN12-LEL SEC chromatography; Lane 7, empty; Lane 8-13, the same as lanes 1-6. (Lanes 1-6 with BME, Lanes 8-13 without BME.)

ISSN: 1813-4890

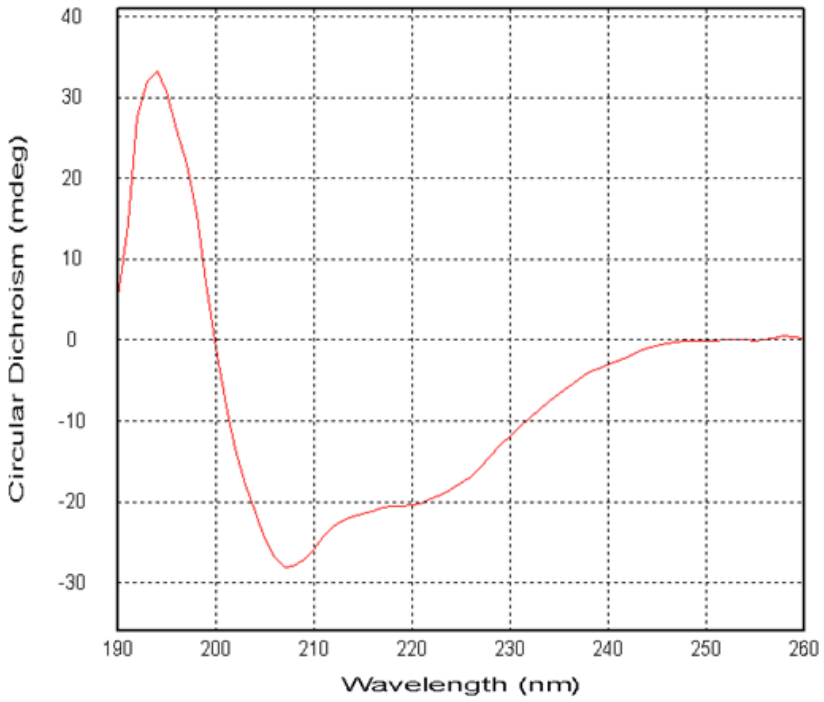

Figure 4. CD mapping of TSPAN12-LEL refolding in 0.5 mM SDS

Table 1. The secondary structure composition of TSPAN12-LEL refolding in 0.5 mM SDS

Recombinant proteins	Helix (%)	Antiparallel (%)	Parallel (%)	Turns (%)	Unordered (%)	RMSD	NRMSD
TSPAN12-LEL	43.0	5.6	4.4	9.8	37.2	0.4133	0.03925

4. Conclusion

In this study, TSPAN12-LEL was produced as inclusion bodies and was prepared through oxidative refolding and SEC purification in 0.5 mM SDS. The composition of the secondary structure was determined by a CD spectrometer. The circular dichroism (CD) spectroscopy results revealed that the secondary structure of TSPAN12-LEL refolding in 0.5 mM SDS was mainly composed of α-helix. This study provides a useful basis for further research on the structural biology of TSPAN12.

Acknowledgments

The author is grateful to other members of the laboratory for their assistance. The author declares no conflict of interest.

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