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Recombinant expression and refolding of the extracellular loops of Claudin-1

Ying Wang

Department of Cell Biology, College of Life Science and Technology, Jinan University, Guangzhou 510632, China

1197622570@qq.com

Abstract

Claudin-1 forms the backbone of tight junction chains and plays a crucial role in regulating permeability of paracellular channels and maintaining cell polarity. In addition, Claudin-1 also plays a role in the subsequent steps of HCV invasion into host cells by interacting with CD81.However, the three-dimensional structure of the two extracellular loops (EL) of Claudin-1 is still unclear. In this study, we constructed CLD1 fusion protein by fusing EL1 and EL2 of Claudin-1 with a linker. The CLD1 fusion protein was expressed as an insoluble form in Escherichia coli. Upon Ni NTA affinity purification and oxidative refolding, it was successfully prepared. The RP-HPLC results show that the fusion protein forms a certain conformation. The CD results show that the secondary structure of CLD1 fusion protein in solution is α/β Structure, mainly composed of β-sheet. This provides a useful basis for further understanding the structure and molecular interactions of Claudin-1.

Keywords

Claudin-1, extracellular loop, recombinant protein, oxidative refolding, tight junction.

1. Introduction

Tight junctions (TJs) are the most apical component of the junction complex in epithelial and endothelial cells composed of integral membrane proteins and cytoplasmic proteins [1-3]. The former consists of Occludin, Claudins, and junctional adhesion molecules (JAMs), and the latter consists of ZO-1, ZO-2, ZO-3 and so on [4-6]. TJs have two classical functions, the barrier function and the fence function [5]. The former regulates the passage of ions, water and various molecules through paracellular space. The latter maintains cell polarity by forming a fence to prevent intermixing of molecules in the apical membrane with those in the lateral membrane. Recently, two novel aspects of tight junctions have been reported [7]. One is their involvement in signal transduction. The other is that the fact that tight junctions are considered to be a crucial component of innate immunity. Claudin-1 is the first discovered member of the Claudin family, encoded by the *CLDN1* gene and composed of 211 amino acids, with a molecular weight of approximately 22 kDa. Claudin-1 is integral to the structure and function of tight junctions with four membrane-spanning regions, which includes two extracellular loops (EL), N- and C-terminal cytoplasmic domains [8]. The extracellular loops are highly conserved. It was previously reported that certain residues in the extracellular loops of Claudin-1 participate in cell adhesion and affect the permeability and charge selectivity of paracellular pathways, however, the structural basis for how it forms tight junction with itself or other claudin subtypes is still unclear.

Hepatitis C virus (HCV) is an enveloped, positive-strand RNA virus and is a major cause of acute chronic liver disease, cirrhosis, hepatocellular carcinoma and liver failure [9]. As an essential HCV entry factor, Claudin-1 contributes to the post-binding steps of HCV entry by interacting

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with CD81, which facilitates virus internalization [10,11]. Although the lifestyle of HCV has been understood and many HCV co-receptors have been identified, how they work together in time ang space remains a problem.

In this study, we performed recombinant expression, oxidative refolding, and preliminary structural characterization of the fusion protein of two extracellular loops of Claudin-1 without transmembrane regions. Circular dichroism spectroscopy reveals that the fusion protein after oxidative folding adopts β-sheet structure. This may help us understand the structure of Claudin-1 and its interactions with other molecules.

2. Methods

2.1. Design of fusion protein

The cDNA sequences coding for EL1 domain (amino acid Glu-29 to Arg-81) and EL2 domain (amino acid Thr-137 to Glu-163) of human CLD1 (UniProKB accession number Q95832) were connected by the cDNA sequence coding for a linker peptide, with His_6 tag fusion at its C termini. It was subcloned into NcoI/XhoI sites of pET-15b vector for the preparation of CLD1-His_6. In short, the EL1 and EL2 of CLD1-His_6 were fused by the linker and denoted as CLD1 fusion protein.

2.2. Protein expression and affinity purification

The recombinant plasmid harboring the target genes was transformed into *Escherichia coli* BL21 (DE3) host cells for large scale protein production. Specifically, the fusion protein was grown in Luria Bertani medium, at 37 ℃ for 6 h after induction with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) when OD_{600} reached 0.4-0.6. Cells were harvested by centrifugation, resuspended in lysis buffer containing 20 mM Tris, 500 mM NaCl, pH 8.0.and homogenized using a cell disruptor (JNBIO). Inclusion bodies were collected by centrifugation and washed by suspension in washing buffer containing 20 mM Tris, 500 mM NaCl, 0.1% Triton X-100, pH 8.0. By centrifugation again, the wet paste was stored at -80 °C. Inclusion bodies were solubilized (as a 10% (w/v) solution) in dissolution buffer containing 8 M urea, 40 mM glycine, 100 mM Tris, pH 8.0 as well as 20 mM dithiothreitol (DTT). After 10 times dilution, the supernatant of reduced inclusion bodies was purified using a Histrap FF Chelating column (GE Heathcare) according to the procedures of Ni NTA affinity chromatography. According to the protein elution peaks on the graph and SDS-PAGE analysis, collected fractions were merged, concentrated and exchanged into the buffer which did not contain imidazole.

2.3. Oxidative refolding of protein and RP-HPLC purification

Refolding was achieved by diluting the supernatant of reduced inclusion bodies to a final protein concentration of 0.1 mg/ml in 2 M urea, 100 mM Tris, 10 mM glycine, 0.4 mM DTT, and 1 mM 2-Hydroxylethyl disulfide, pH 8.0. After slow stirring for 2 h at room temperature, the reaction was stopped by acidification to pH 3.0 with HCl. The refolding fusion protein was monitored by 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and reversed-phase HPLC (RP-HPLC). The refolding fusion protein was purified by a RP-HPLC step, employing a Globalsil C18-BIO column (25 mm diameter x 10 cm long, 300 Å pore size, 10 μm bead size) and a linear gradient from 20% to 60% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid (TFA) over 40 min at a flow rate of 1 ml/min. Final fractions were freeze-dried overnight on Christ Alpha 1-2 LDplus according to SDS-PAGE analysis.

2.4. Circular dichroism spectroscopy

CD spectra was measured with a CD spectrometer (Chirascan, Applied Photophysics). Lyophilized protein from the previous step was prepared at a concentration of 0.06mg/ml was dissolved in 10 mM HCl. Protein concentrations were determined by the UV absorbance. A

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quartz cell with 1-mm path length was used. The instrument parameters were set as follows: 25 °C, 1nm band width, 190-260 nm scanning range, 100 nm min^{-1} scanning rate, and 1nm wavelength step. Two accumulations were recorded for each spectrum, and a blank containing the same buffer was subtracted as a reference. CD results were expressed in millidegrees (mdeg). Analysis of the experimental data was performed using the software Chirascan's Pro-data viewer. The spectrum was further analyzed using BeStSel software to determine secondary structure content[12].

3. Results

3.1. Expression and purification of CLD1 fusion protein

After induction with IPTG, the expression of CLD1 fusion protein was detected by SDS-PAGE analysis (Fig 1 B). A prominent protein band was observed ~15 kDa confirming that CLD1 fusion protein was successfully expressed in BL21 which was mainly expressed as insoluble inclusion body protein (Lane 3). As described in 2.2, the solubilized fusion protein was performed on Ni NTA affinity chromatography. Fig 1A showed s single elution peak, which is confirmed the peak of CLD1 fusion protein by SDS-PAGE (Fig 1B).

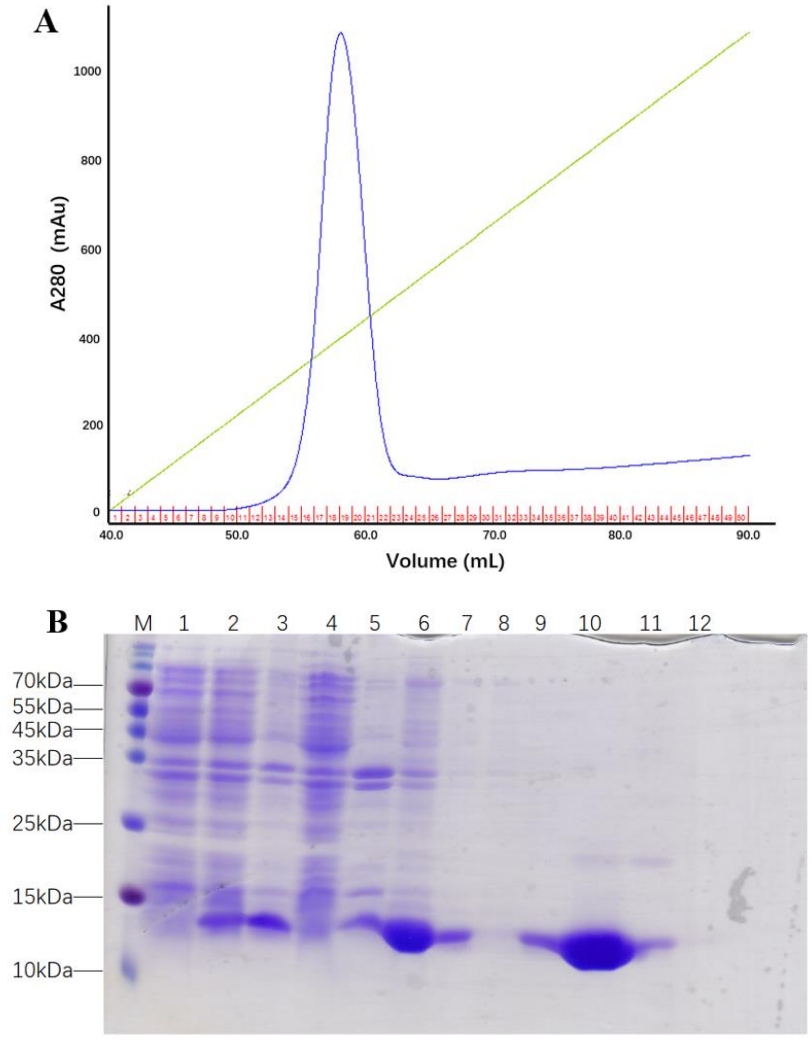

Fig 1. Analysis of CLD1 fusion protein by Affinity chromatography and SDS-PAGE.

(A) Affinity chromatography chromatogram of CLD1 fusion protein. (B) SDS-PAGE analysis results of expression and affinity purification of CLD1 fusion protein. Lane M, protein marker; Lane 1, Uninduced bacterial lysate; Lane 2, IPTG-induced bacterial lysate; Lane 3, Precipitate after crushing; Lane 4, Supernatant after crushing; Lane 5, Precipitate after denatured

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dissolution; Lane 6, Supernatant after denatured dissolution; Lane 7, Supernatant after denatured dissolution, dilution, and filtration; Lane 8, Unbound fractions after affinity purification; Lane 9, Collected fraction #15; Lane10, Collected fraction #19; Lane 11, Collected fraction #24; Lane 12 , Abandoned fractions after affinity purification.

3.2. Oxidative refolding and RP-HPLC purification of CLD1 fusion protein

The purified CLD1 fusion protein was subject to oxidative refolding in the presence of DTT and 2-Hydroxyethyl disulfide. The refolded protein still exists in the supernatant according to the SDS-PAGE analysis (Fig 2). As shown in the chromatogram obtained from C18 RP-HPLC, there was a difference in the retention time between the peak of denatured protein and that of refolded protein when the sample size is 0.2 mg. (Fig 3). Specifically, the refolded protein peak appeared 5 min earlier than the solubilized denatured protein peak. When the loading of refolded fusion protein was increased by three times, that is, 0.6 mg, there were two peaks in the chromatogram (Fig 4). By SDS-PAGE analysis, peak a is the target protein peak. Thus, the results confirmed that refolded CLD1 fusion protein was successfully prepared.

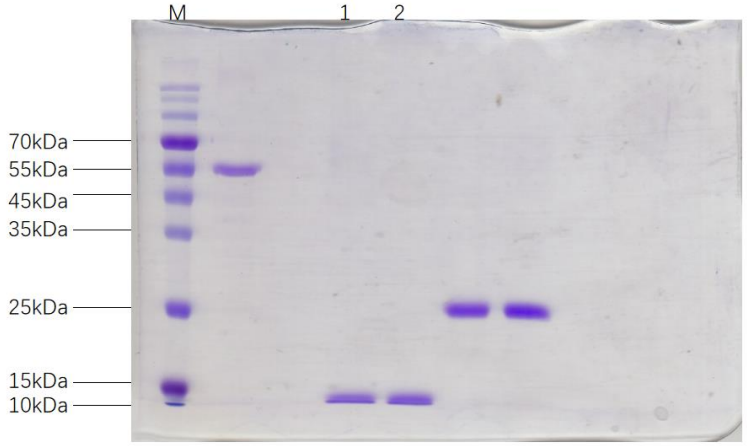

Fig 2. SDS-PAGE analysis results after oxidative refolding of CLD1 fusion protein.
Lane M, protein marker; Lane 1, Supernatant of denatured protein; Lane 2, Supernatant of refolded protein.

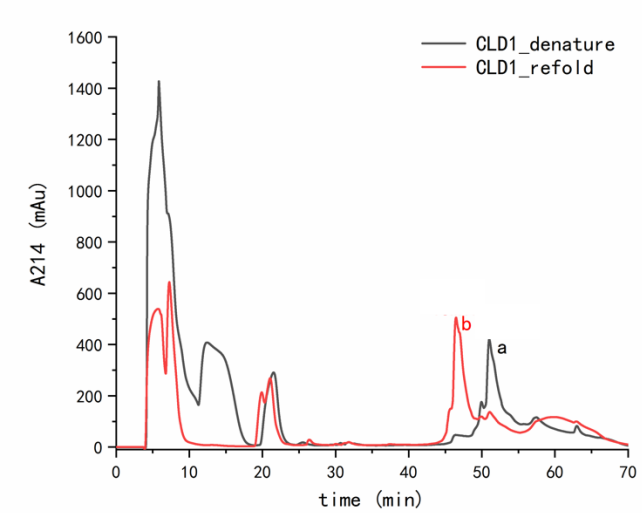

Fig 3. Comparison of RP-HPLC purification chromatograms of CLD1 fusion protein.
Peak a, Elution component at 46 min; Peak b, Elution component at 51 min.

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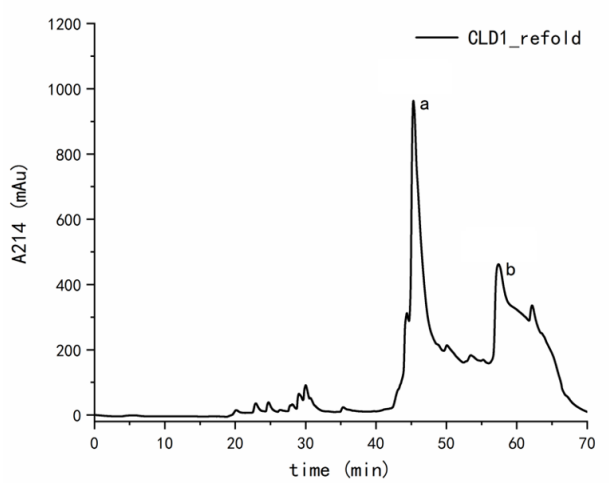

Fig 4. RP-HPLC purification chromatogram of Claudin-1 fusion protein.
Peak a, Elution component at 45 min; Peak b, Elution component at 57 min.

3.3. Conformational analysis of fusion protein using CD Spectroscopy

The secondary structural characteristics of refolded CLD1 fusion protein was studied by far-UV CD analysis at 25 ℃ (Fig 5). In the far-UV regions, the CD spectrum of CLD1 fusion protein featured negative bands at around 216 nm, a characteristic of a structure rich in β-sheet. The CD spectra of CLD1 fusion protein was analyzed by BeStSel to determine its secondary structure proportion. The normalized root mean square deviation (NRMSD) values was widely used to evaluate the quality of CD data and it should be lower than 0.05. The NRMSD value of CLD1 fusion protein in our measurement is lower than 0.05, indicating good fits. The result showed that CLD1 fusion protein contained 8.6% α-helix, 55% β-sheet, among which contained 22.2% antiparallel β-sheet and 32.8% parallel β-sheet.

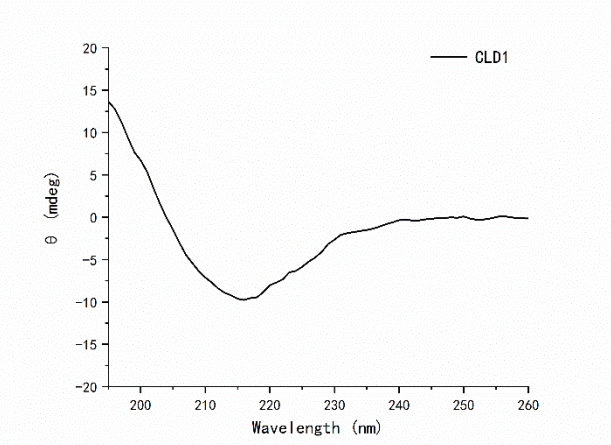

Fig 5. Circular dichroism analysis of CLD1 fusion protein after oxidative refolding.

4. Conclusion

In this study, the construction method of CLD1 fusion protein had not been reported yet. We chose prokaryotic expression system to express the new construct. CLD1 fusion protein was successfully prepared in soluble form through denatured affinity purification and oxidative refolding. CD analysis found that the secondary structure of the fusion protein in solution was mainly β-sheet. The finding provides a basis for further research into the structural biology of Claudin-1.

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Acknowledgments

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