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Expression, Purification, and Secondary Structure Characterization of Recombinant KCTD1

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Abstract—Potassium channel tetramerization domain containing 1 (KCTD1) contains a BTB domain, which can facilitate protein–protein interactions that may be involved in the regulation of signaling pathways. Here we describe an expression and purification system that can provide a significant amount of recombinant KCTD1 from *Escherichia coli*. The cDNA encoding human KCTD1 was amplified and cloned into the expression vector pET-30a(+). The recombinant protein was expressed in *E. coli* BL21(DE3) cells and subsequently purified using affinity chromatography. To confirm that KCTD1 was correctly expressed and folded, the molecular weight and conformation were analyzed using mass spectroscopy, Western blot, and circular dichroism. Optimizing KCTD1 expression and investigating its secondary structure will provide valuable information for future structural and functional studies of KCTD1 and KCTD family proteins.

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Key words: KCTD1, circular dichroism spectroscopy, secondary structure

KCTD1 encoded by the *KCTD1* gene is a member of the potassium channel tetramerization domain containing (KCTD) protein family [1]. The KCTD family contains 21 human proteins that bear a conserved domain at their N-terminus [2] that contain a bric-a-brac, tramtrak, and broad complex (BTB) motif, which is similar to the tetramerization domain (T) of some voltage-gated potassium channels [3, 4]. KCTD proteins are responsible for the ordered assembly of homologous proteins and interactions with other macromolecules [1, 5, 6]. BTB/pox virus and zinc finger (POZ) domain proteins have been demonstrated to be involved in a variety of biological processes including protein degradation, ion channel assembly, cell proliferation, apoptosis, and human cancer [7-11].

Unlike other BTB family proteins, KCTD1 does not contain any other motif, this indicating that KCTD1 may have special cellular functions. Ding et al. initially reported that KCTD1 functioned as a transcriptional repressor of the AP-2 family, particularly of AP-2a [1]. Recent studies of KCTD in protein degradation produced a surprisingly unifying view. In spite of their marked sequence difference, a large number of KCTD proteins carry out a similar task: recruiting target proteins to cullin-based E3 ubiquitin ligases [12-14]. This realization naturally leads to the question of whether KCTD proteins might have shared functions. We have demonstrated that KCTD1 interacts with the prion protein that is a conformational conversion of a normal cell surface glycoprotein (PrPC) into a pathogenic isoform (PrPSc) [15]. Then, what function does KCTD1 play in recruiting the prion protein to form a macromolecular complex? To understand the different functions of the KCTD1 protein, a comprehensive collection of KCTD1 protein structures must be

Abbreviations: BLAST, Basic Local Alignment Search Tool; BTB domain, bric-a-brac, tramtrak, and broad complex domain; CD, circular dichroism; IPTG, isopropyl thio-β-D-galactopyranoside; KCTD, potassium channel tetramerization domain containing; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; ORF, open reading frame; POZ domain, pox virus and zinc finger domain; SDS-PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate.

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obtained. As a step toward these structural studies of the KCTD1 protein, we need to express the KCTD1 protein. The overall aim of this study was to explore the expression and purification conditions to obtain pure and active KCTD1 protein for structural and functional studies. The successful purification of KCTD1 and our circular dichroism (CD) results are important steps toward further structural and functional analyses.

MATERIALS AND METHODS

Materials. The bacterial expression vector pET-30a(+) was obtained from Novagen (USA). Oligonucleotide primers for KCTD1 were purchased from Invitrogen Life Technologies (USA). Restriction endonucleases, isopropyl thio-β-D-galactopyranoside (IPTG), and T4 DNA ligase were from MBI. Proteins standard used as SDS-PAGE markers were from Sigma Chemical (USA). All chemicals used were of analytical grade.

Cloning and expression vector construction. Recombinant DNA techniques were performed using standard protocols. The human KCTD1 gene open reading frame (ORF) was amplified using forward (5′-GGGAATCCATATGTCAAGACCTCTGATCAC-3′) and reverse (5′-CCGCTCGAGGTCCAGAGGCTCTTGCTTTAC-3′) primers. The amplified DNA encoding KCTD1 was ~774 bp in length. The DNA encoding KCTD1 was inserted into vector pET-30a (+) via the NdeI and XhoI sites. The plasmid pET-30a-KCTD1 was amplified by transformation into *E. coli* (DH5a), and the cloned plasmid was identified by restriction analysis and sequencing. Sequencing data were treated by the Basic Local Alignment Search Tool (BLAST) from the National Center for Biotechnology Information network (http://www.ncbi.nlm.nih.gov). Sequencing of the cloned vectors indicated the open reading frame of KCTD1, in addition to a cluster of six histidine residues for protein purification by Ni^{2+}-metal chelate affinity chromatography. The expression vector pET-30a-KCTD1 was extracted from the transformants using the a QIAprep spin Miniprep kit (Qiagen, Germany), and transformed *E. coli* BL21(DE3)-competent cells. A total of 500 μl of an overnight culture of DE3 was diluted into 500 ml LB medium containing kanamycin (50 μg/ml). The culture was grown at 37°C until the optical density at 600 nm reached 0.6.

Expression and purification of recombinant KCTD1. The culture was induced with 1 mM IPTG and was incubated for an additional 6 h at 16 or 37°C. Then the culture was harvested by centrifugation for 10 min at 4°C and 6000*g*. The pelleted cells were resuspended in binding buffer (20 mM sodium phosphate, 0.5 M NaCl, 50 mM imidazole, pH 7.4) and disrupted at 4°C for 30 min by sonication. After sonication, the suspension was centrifuged for 20 min at 4°C and 12,000*g* to separate the cell pellet and resuspended. KCTD1 expression was analyzed by SDS-PAGE. At the same time, the expressed KCTD1 was purified using immobilized metal affinity chromatography on Ni-NTA His-Bind Resin (Novagen, USA). The supernatant was incubated with 2 ml Ni-NTA His-Bind Resin and equilibrated at room temperature for 30 min. The resin was poured into a column and washed with 10 column volumes of binding buffer at a flow rate of 0.5 ml/min. The protein was then eluted with five column volumes of elution buffer (20 mM sodium phosphate, 0.5 M NaCl, 500 mM imidazole, pH 7.4), and the eluted fractions were collected. The eluted protein was desalted by centrifugal filtration on a 10-kDa molecular weight cut-off filter (Millipore, USA). Protein concentration was determined by the Bio-Rad Protein Assay Kit (Bio-Rad, USA). Protein was quantified by Coomassie staining of SDS-PAGE gels.

Analytical size-exclusion chromatography (SEC). SEC was performed on an AKTA Explorer chromatographic system (GE Healthcare, UK) using Superdex 75 prep-grade HR 30/10 columns (GE Healthcare) at flow rate of 1 ml/min. The column was equilibrated in the elution buffer (20 mM sodium phosphate, 0.5 M NaCl, pH 7.4), and 200 μl of the sample at a concentration of 0.4 mg/ml was loaded. Absorption was monitored at 280 nm. Purity of the eluted protein was also determined by SDS-PAGE. Membranes were immunoblotted with KCTD1 polyclonal antibodies. Blots were revealed using the ECL reagent (Pierce, USA) using a Fusion FX7 image acquisition system (France).

Characterization of KCTD1 using mass spectrometry. Mass spectroscopic analysis was carried out using an Ultraflex II TOF/TOF spectrometer (Bruker, Germany). The proteins were deposited on a metal target as co-crystals with 3,5-dimethoxy-4-hydroxycinnamic acid, and the mass spectrum was analyzed in the positive ion mode.

Secondary structural analysis by far-UV circular dichroism (CD). Far-UV CD measurements of KCTD1 were performed on a chiroptical spectrometer (Jasco, Japan) at 25°C. Thermal stability of the KCTD1 secondary structure was monitored from 25 to 85°C. The protein sample used for CD measurements was 0.5 mg/ml in 30 mM KCl. Measurements were carried out with a 1-mm pathlength cuvette over the wavelength range from 195 to 260 nm at 1-nm intervals. Multiple scans were averaged for the collected spectrum. The spectrum was processed by subtracting the buffer spectrum. The ellipticity of the far-UV CD spectrum was analyzed using the DICROPROT software.

RESULTS AND DISCUSSION

The KCTD1 protein, which contains a BTB domain, can facilitate many possible binding interactions to itself,

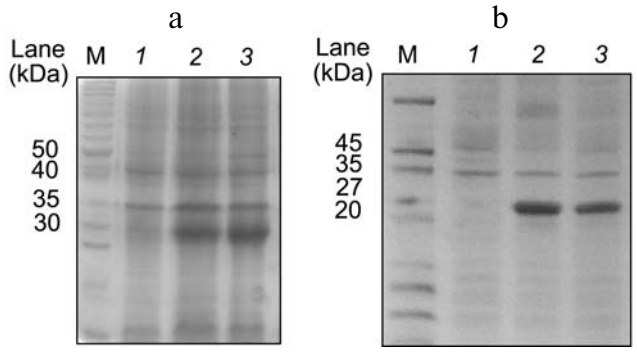

Fig. 1. SDS-PAGE analysis of KCTD1 expression in *E. coli*. a) KCTD1 expression at different temperatures. Lanes: M, molecular weight marker; *1*) cell lysate before IPTG induction; *2*) cell lysate after IPTG induction for 4 h at 16°C; *3*) cell lysate after IPTG induction for 4 h at 37°C. b) Supernatant after sonication. Lanes: M, molecular weight marker; *1*) before IPTG induction; *2*) after IPTG induction for 4 h at 16°C; *3*) after IPTG induction for 4 h at 37°C.

to other proteins, or to other domains of the full-length channel. To study KCTD1 structure and function, an expression plasmid pET30-KCTD1 containing the full-length human KCTD1 was constructed. The pET30a vector, which contains T7 promoter and a lac operator, is a high-yield production system. This system allowed us to overexpress KCTD1 in *E. coli* cells under the induction of IPTG. To optimize the condition for soluble KCTD1 expression in *E. coli*, we tested KCTD1 expression at different temperatures. Total KCTD1 expression was evaluated from whole cell lysate, and the soluble KCTD1 expression was evaluated in the supernatant of the cell lysate after sonication (in buffer containing lysozyme, DNase, 20 mM sodium phosphate, 0.5 M NaCl, and 50 mM imidazole, pH 7.4) and centrifugation. SDS-PAGE analysis showed that the expressed KCTD1 protein was visible as a 29.4-kDa protein band regardless of the temperatures (Fig. 1). While the total KCTD1 expression at 16 and 37°C were comparable (Fig. 1a), the soluble KCTD1 protein was more abundant at 16 than 37°C (Fig. 1b). So KCTD1 was expressed at 16°C for large-scale KCTD1 preparation. After sonication and centrifugation, the supernatant was incubated with Ni-NTA His-Bind resin and equilibrated for 30 min at room temperature, and the resin containing the bound protein was loaded onto a column. After washing with 10 column volumes of binding buffer, the pure protein was eluted using a high concentration of imidazole. Further purification of KCTD1 was achieved by gel filtration using Superdex G75 medium (Fig. 2a), resulting in successful purification of KCTD1 with only a single peak. SDS-PAGE and Western blot confirmed that the collected fraction is KCTD1 (Figs. 2b and 2c). The collected fraction was concentrated and stored for further investigation.

To confirm that KCTD1 was correctly expressed at the molecular weight of the native protein, recombinant KCTD1 was analyzed by MALDI-TOF mass spectrometry (Fig. 3), and the observed molecular weight of the recombinant KCTD1 was found to be 30 kDa, which closely matches the predicted molecular weight of 29.4 kDa, and the purity of the recombinant KCTD1 was greater than 99%. The purity of the recombinant KCTD1 protein will facilitate further research.

KCTD1 expression and purity evaluation were performed for the study of its structure and function. Here, we investigated the secondary structure of KCTD1 by far-UV CD spectroscopy, because the far-UV region (195-250 nm) is sensitive to the secondary structure of the protein. The far-UV CD spectrum of KCTD1 at pH 4.0 and room temperature (Fig. 4a) displayed two strong minima at 222 and 208 nm, suggesting the presence of a significant amount of α-helix, and the overall shape of the spectrum indicates the presence of a certain amount of β-structure as well. This suggested that the purified KCTD1 has an ordered conformation with a significant amount of

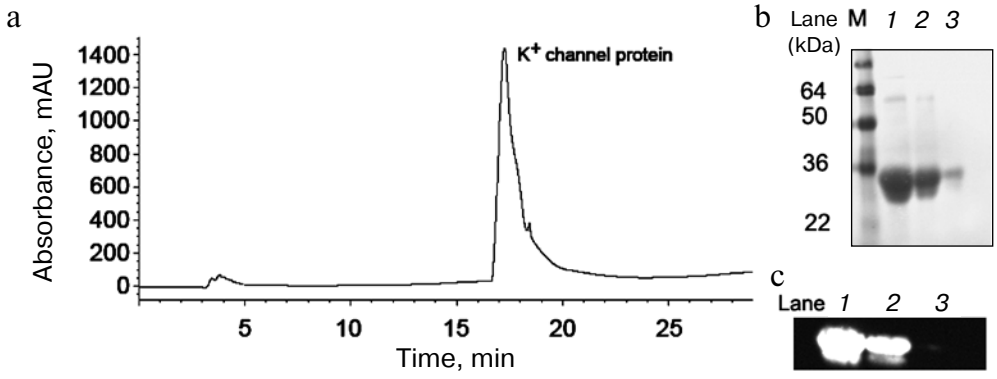

Fig. 2. a) Superdex G75 chromatography of KCTD1. The eluted sample (0.4 mg/ml, 200 μl) was monitored by absorbance at 280 nm. b) SDS-PAGE analysis of KCTD1 purified by chromatography. c) Western blot analysis of the eluted KCTD1. The protein concentration in panels (b) and (c): 0.6 μg (lane *1*); 0.3 μg (lane *2*); 0.15 μg (lane *3*).

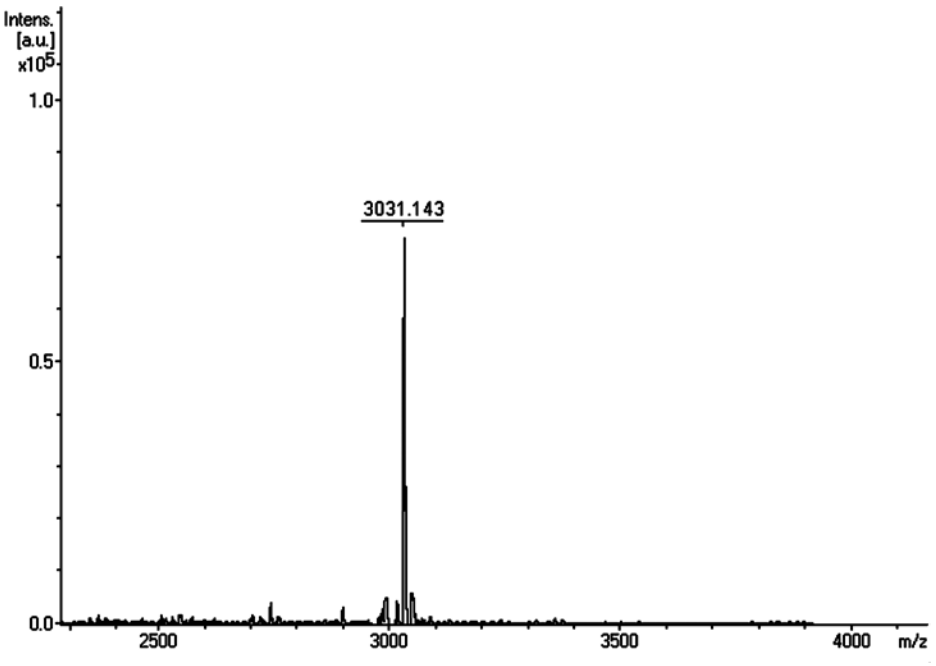

Fig. 3. MALDI-TOF mass spectrum of purified KCTD1. The KCTD1 was excised from the gel (Fig. 2b, lane *1*).

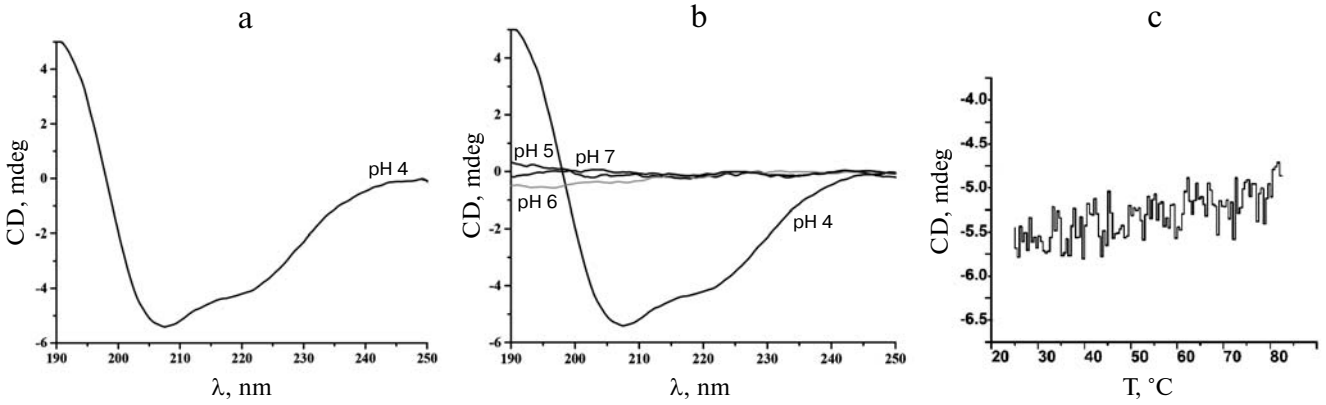

Fig. 4. Secondary structural characterization of KCTD1 in 30 mM KCl. a) Far-UV spectrum of KCTD1 at pH 4.0 and 25°C. b) Change in the secondary structure at different pH values and 25°C. c) Thermal stability at pH 4.0: CD signal was followed at 208 nm at different temperatures.

regular secondary structure, implying native conformation of the protein within the cells.

Analysis of the molar ellipticity values by the method of Woody [16] provided an estimation of the secondary structural components at pH 4.0 (α-helix: 22.5). We also found that the secondary structure of KCTD1 was dependent on the pH (Fig. 4b). The results showed KCTD1 has an ordered conformation at pH 4.0 and 25°C; however, it is unexpected that the same concentration of KCTD1 is highly unstructured at pH 5.0, 6.0, and 7.0 in 30 mM KCl solution. As we know, the lysosome allows protein to work near pH 4.5 [17]. Considering recent studies indicating that KCTD plays an important role in protein degradation, these CD results suggest that KCTD1 may also have the same function in protein degradation as other KCTD proteins, though no finding so far has linked KCTD1 protein with the lysosome/ubiquitin system, and reported studies indicate that the KCTD1 is localized mostly in the nucleus to function as a transcriptional repressor of the AP-2 family [1]. Our experiment of pEGFP-N1/KCTD1 transient expression in transfected HeLa cells, which showed localization of EGFP-KCTD1 in both the cytoplasm and the nucleus (data not shown), at least partially supports the hypothesis.

Circular dichroism studies at different temperatures can be used to provide information on the stability of proteins; therefore, we investigated the stability of the KCTD1 secondary structure at different temperatures. The CD signal of KCTD1 at 208 nm was monitored at temperatures from 25 to 85°C at pH 4.0 in 30 mM KCl solution, the α-helical structure of KCTD1 did not change noticeably with increasing temperature (Fig. 4c). And the thermal stability of KCTD1 is similar to that of KCTD5 [12]. The ability of KCTD1 to adopt stable conformations at different temperatures may be essential to its function as a protein–protein interaction/signaling domain to modulate the function of the intact KCTD1 macromolecular complex.

Taken together, we established an *in vitro* system to overexpress and purify the KCTD1 protein in *E. coli*, and we demonstrated that the purified KCTD1 protein had the expected molecular weight and ordered conformation with a significant amount of regular secondary structure. These studies provide a useful basis for future biophysical studies of KCTD1, including the structural basis of its function, especially in future studies involving the KCTD1 protein in complex with the prion protein.

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