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Expression and purification of the C-terminal fragments of TRPV5/6 channels

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ABSTRACT

The transient receptor potential vanniloid 5 and 6 (TRPV5 and TRPV6) Ca^{2+}-ion channels are crucial for the regulation of minute-to-minute whole body calcium homeostasis. They act as the gatekeepers of active Ca^{2+} reabsorption in kidney and intestine, respectively. In spite of the great progress in the TRP channels characterization, very little is known at the atomic level about their structure and interactions with other proteins. To the major extent it is caused by difficulties in obtaining suitable samples. Here, we report expression and purification of 36 intracellular C-terminal fragments of TRPV5 and TRPV6 channels, for which no structural information is reported thus far. We demonstrate that these proteins contain intrinsically disordered regions and identify fragments suitable for biophysical characterization. By combining bioinformatic predictions and experimental results, we propose several criteria that may aid in designing a scheme for large-scale production of difficult proteins.

Introduction

Calcium ions play a crucial role in various cellular processes, such as neuronal response, muscle contraction, enzyme activity, gene transcription, cell death, proliferation and differentiation at an organismal level. The concentration of Ca^{2+} throughout the body is tightly regulated by a concerted interplay of several organs, including bone, intestine and kidney [1]. Two highly homologous members of the transient receptor potential (TRP)[1] family, TRPV5 and TRPV6, are crucial for the regulation of minute-to-minute whole body calcium homeostasis. They act as the gatekeepers of active Ca^{2+} reabsorption in kidney and intestine, respectively [2,3].

In spite of the great progress in the TRP channels characterization [4], very little is known about their structure and interactions with other proteins at the atomic level. It is postulated that TRP channels contain six transmembrane domains and intracellular N- and C-termini. Functional TRPV5 and TRPV6 channels exist as tetramers and they are tightly regulated on different timescales [5].

There are three ways to control the activity of a TRPV5/6 channel: at the transcriptional level, at the level of trafficking the channel to the plasma membranes and at the level of gating the channel at the cell surface by intracellular Ca^{2+}, a process commonly denoted as negative feed-back regulation. The most fine tuning occurs through the latter channel gating mechanism: once the Ca^{2+}-concentration in the close vicinity of the channel pore of TRPV5/6 reaches a threshold concentration, it acts as a negative feedback regulator, inactivating the channel at the cell surface. Previously, it was shown that the C-terminal parts of TRPV5 and TRPV6 are crucial for this Ca^{2+}-dependent negative feedback regulation. Additionally, several auxiliary calcium-binding proteins, such as 80K-H protein, calbindin-D28K and calmodulin, were shown to bind *in vivo* to the C-terminus of TRPV5 and dramatically affect calcium reabsorption in kidney [6].

To understand the molecular machinery of the TRPV5/6 channel regulation, it is necessary to study interactions between the potential partners both *in vitro* and *in vivo*. In particular, data about structure and dynamics of the relevant complexes at the atomic level provide for crucial information complementary to the physiological data. Structural, dynamical and thermodynamic characterization of protein–protein complexes can be done in the first place by nuclear magnetic resonance (NMR) spectroscopy, X-ray crystallography and isothermal titration calorimetry (ITC). Unfortunately, these methods require quite large amounts of pure protein (milligrams), and it is becoming increasingly obvious that the success of a structural biological study completely relies upon the successful production of a suitable sample. Often, the effort put into sample preparation is much bigger than the effort required for the structure elucidation itself.

Here, we present a case study of expression and purification of intracellular C-terminal fragments of TRPV5 and TRPV6 channels, for which no structural information is reported thus far. We report

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[1] *Abbreviations used:* TRPV5 and TRPV6, transient receptor potential vanniloid 5 and 6; TRP, transient receptor potential; NMR, nuclear magnetic resonance; ITC, isothermal titration calorimetry; IPTG, isopropyl-β-D-thiogalactopyranozide; CD, circular dichroism.

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the expression results on 36 TRPV5/6 fragments, including those suitable for structural characterization. By combining bioinformatic predictions and experimental results, we propose several criteria that may aid in designing a scheme for large-scale production of difficult proteins.

Materials and methods

Materials

Oligonucleotide primers were synthesized by Eurofins MWG Operon (Germany). All enzymes and buffers for DNA manipulations were obtained from New England Biolabs (USA). pET28a vector was purchased from Novagen (USA), pGEX-6p-2 and pGEV2 vectors were a kind gift of Dr. Sjoerd Verkaart (Physiology Department, NCMLS, Radboud University Medical Centre, Nijmegen, The Netherlands) and Dr. Nina M. Link (Biozentrum, University of Basel, Switzerland), respectively. Synthetic genes were obtained from Mr. Gene GmbH (Germany). Expression *Escherichia coli* cells BL21(DE3), BL21(DE3)RIL and ArcticExpress™ (DE3) were from Stratagene (USA). IgG-Sepharose and MonoQ 5/50 column were purchased from GE Healthcare (USA). ^{15}N-ammonium chloride was obtained from Buchem BV (The Netherlands). Bradford reagent was from Bio-Rad (USA). The remainder of the chemicals were of the highest purity available from Sigma Aldrich (USA).

Bioinformatic predictions

Disordered regions of TRPV5 and TRPV6 C-terminal fragments were predicted by means of the Disorder Prediction MetaServer [7]. It provides the consensus result of the following disorder predictors: DISEMBL, DISOPRED2, DISpro, DRIPPRED, FoldIndex, FoldUnfold, GlobPlot2, IUPred, RONN and VSL2. Secondary structure prediction was performed using the same server based on the combination of the following predictors: ANCHOR, SignalP, TMHMM, SEG, PROFphd, PSIPred and coils.

Calmodulin binding regions were predicted based on the Calmodulin Target Database [8].

Solubility of different length constructs of TRPV5 and TRPV6 C-terminal fragments was predicted using a sequence-based protein solubility evaluator (PROSO) [9].

Cloning

The pGEX-6p-2 vector containing the C-terminal fragment of the rabbit TRPV5 channel with N-terminal GST-tag was used as a template. Full-size and short versions of the rabbit TRPV5 C-terminus were recloned in the pET28a vector at the NdeI/XhoI sites (Appendix A). The resulting constructs contained N-terminal His_{10}-tag, which could be cleaved by thrombin.

Synthetic genes of human TRPV5 (hTRPV5) and human TRPV6 (hTRPV6) C-terminal fragments were optimized for the expression in *E. coli*. The coding sequences corresponding to different fragments of the hTRPV5 and hTRPV6 were inserted into pGEV2 vector at the BamHI/XhoI sites. All constructs contained C-terminal His_6-tag and/or N-terminal GB1-tag which could be cleaved by thrombin (Appendix A). The nucleotide sequence of all the constructs was verified by DNA sequencing (Sequence Facility, Department of Human Genetics, Radboud University Medical Centre, Nijmegen, The Netherlands).

Protein expression and purification

The proteins were overexpressed in BL21(DE3), BL21(DE3)RIL and ArcticExpress™ (DE3) *E. coli* cells in LB medium containing 100 μg/ml ampicillin (for pGEV2 vector) or 50 μg/ml kanamycin (for pET28a vector). Cells were grown at various temperatures between 14 and 37 °C with shaking at 220 rpm. At a cell OD_{600} 0.6–0.8 the expression of a recombinant protein was induced by addition of isopropyl-β-D-thiogalactopyranozide (IPTG) to the final concentration 0.1–1.0 mM.

For the production of ^{15}N-labelled proteins the expression was performed in M9 minimal media containing 1 g/l ^{15}N-ammonium chloride as the sole source of nitrogen.

The fusion proteins were purified by affinity chromatography using IgG-Sepharose according to the manufacturer's instructions. The final purification step was performed via ion exchange chromatography at MonoQ 5/50 column. The GB1-tag was cut by thrombin according to the manufacturer's instructions, and the C-terminal fragments with or without His_6-tag were further purified by IgG-Sepharose.

Unless used immediately for experiments, the purified proteins were either stored frozen (−80 °C) in KCl 50 mM, $CaCl_2$ 10 mM, Tris 20 mM buffer pH 7.0 or dialyzed against 1/100 of the same buffer and then freeze-dried and stored at −20 °C.

Recombinant *Xenopus laevis* calmodulin (identical to mammalian) was expressed in *E. coli* AR58 cells carrying the pTNcoI2 plasmid (kind gift of Dr. C. Klee, NIH, Bethesda, MD, USA). Expression was induced by temperature shift from 30 °C to 42 °C and 3–4 h after induction cells were harvested and lysed. Calmodulin was purified by weak anion exchange (DEAE, GE Lifesciences) and affinity chromatography (Phenylsepharose, GE Lifesciences). Purified protein was dialyzed against 1/100 of KCl 50 mM, $CaCl_2$ 10 mM, Tris 20 mM buffer (pH 7.0), freeze-dried and stored at −20 °C.

Characterization of the purified proteins

The integrity of the recombinant proteins was checked by MALDI–TOF mass spectrometry. Coomassie blue-stained protein bands were cut from the SDS gel, washed and digested by trypsin according to the instructions for Trypsin profile IGD kit for in-gel digest (Sigma, USA). Positive-ion mass spectra were measured on a Biflex III mass spectrometer (Bruker Daltonics, Germany). The spectra of peptides obtained after tryptic digest were measured in reflection mode. α-Cyano-4-hydroxycinnamic acid in aqueous 50% acetonitrile-0.05% TFA (10 mg/ml) was used as matrix.

The purity and stability of the proteins was monitored by SDS–PAGE. All proteins were tested for stability at room temperature during 2 months.

Protein concentration was measured using standard Bradford assay with bovine serum albumin as standard.

Circular dichroism spectroscopy

Circular dichroism spectra were measured at Jasco 810 CD spectrometer (Jasco, USA). Hellma cuvette type 110-QS with the path length 1 mm was used (Elscolab B.V., The Netherlands). Prior to the measurements, all proteins were dialysed against 30 mM NaF 1 mM Tris (pH 7.0) to minimize absorption in the far UV range. Spectra were measured at 283 K, the temperature was controlled by a computer-operated Peltier device.

NMR spectroscopy

NMR samples were prepared in 50 mM KCl, 10 mM $CaCl_2$, 20 mM Tris pH 7.0 buffer. D_2O and NaN_3 were added to the NMR samples to the final concentrations of 5–7% (v/v) and 0.01% (v/v), respectively. The protein concentration in the samples was between 100 and 200 μM. Lyophilized calmodulin was dissolved in the same buffer as V6c7 and added to the latter at molar ratio 1:1.