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Molecular and topological membrane folding determinants of transient receptor potential vanilloid 2 channel

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ABSTRACT

Transient Receptor Potential (TRP) channels are related to adaptation to the environment and somatosensation. The transient receptor potential vanilloid (TRPV) subfamily includes six closely evolutionary related ion channels sharing the same domain organization and tetrameric arrangement in the membrane.

In this study we have characterized biochemically TRPV2 channel membrane protein folding and transmembrane (TM) architecture. Deleting the first N-terminal 74 residues preceding the ankyrin repeat domain (ARD) show a key role for this region in targeting the protein to the membrane. We have demonstrated the co-translational insertion of the membrane-embedded region of the TRPV2 and its disposition in biological membranes, identifying that TM1-TM4 and TM5-TM6 regions can assemble as independent folding domains. The ARD is not required for TM domain insertion in the membrane. The folding features observed for TRPV2 may be conserved and shared among other TRP channels outside the TRPV subfamily.

1. Introduction

Transient Receptor Potential (TRP) channels are polymodal cation channels with physiological key roles represented in six subfamilies in mammals [1]. The vanilloid subfamily in mammals consists of 6 members (TRPV1-6) with a very defined topology of two cytosolic domains flanking a membrane-embedded region. The TRPV1-4 subgroup has been defined as non-selective cation channels, whereas TRPV5-6 are calcium selective. The closest homologs are TRPV5 and TRPV6, sharing a 75% sequence identity [2]. The most studied member of the TRPV subfamily is TRPV1, the capsaicin receptor, which structure has been recently solved at high resolution. This structure showed a tetrameric arrangement of 6 transmembrane (TM) helices per monomer in TRPV1 with a topology and tetrameric arrangement similar to sodium channels [3], which is likely to be shared among the different TRPV members. The TRPV1-4 subgroup has a low sequence identity (TRPV1 and TRPV2 are the closest homologs with about 50% sequence identity), although evolutionary pressure patterns are similar [4].

Translocation of TRPV2 towards the plasma membrane has been observed in the presence of physical stimuli, growth factors or chemotactic peptides [5]. Recently, the possibility has arisen that TRPV2 functions as an intracellular or plasma membrane ion channel in a cell type/tissue dependent manner [6]. Thus it is key to define which TRPV2 domains are in charge for the sub-cellular localization and the proper folding of the channel.

Most membrane-embedded proteins are inserted and assembled in the ER membrane at sites termed translocons [7]. During this process, the translocon mediates the integration of TM sequences into the non-polar core of the membrane and delivers hydrophilic cytoplasmic and luminal domains to the appropriate

Abbreviations: ARD, ankyrin repeat domain; EGFP, enhanced green fluorescent protein; TM, transmembrane; TMD, transmembrane domain; TMHMM, TM hidden Markov model; TRP, transient receptor potential; TRPV, transient receptor potential vanilloid subfamily.

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compartments. Simultaneously, the nascent protein may undergo covalent modifications (e.g. *N*-glycosylation) and fold properly to eventually adopt its native state. *N*-glycosylation is cotranslationally performed in the lumen of the ER by the enzyme oligosaccharyltransferase, which is adjacent to the translocon [8]. TRPV2 has canonical *N*-glycosylation sites, $N_{571}N_{572}ST$, thus integration of the protein into ER-derived membranes can be monitored by *N*-glycosylation.

In this study we have applied a combination of biochemical approaches focusing on key molecular aspects of the TRPV2 channel related to membrane location, transmembrane architecture and folding. Some of the conclusions derived from this study can be relevant for the other members of the TRPV subfamily and other ion channels.

2. Materials and methods

2.1. Plasmids

cDNA sequences for rat TRPV2 were cloned into a pCDNA3 vector. The TRPV2 ORF was encoded in frame with an EGFP tag and an 8XHis-tag at the C-terminus. The N-terminus truncations for TRPV2 were obtained by classical PCR cassette reaction and ligated into pCDNA3 within NdeI and NotI sites.

2.2. Cell culture and transfection

HEK293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 100 units/mL penicillin and 100 μg/mL streptomycin. Transfection was performed using polyethyleneimine (PEI, Polysciences, 23966). HEK293 cells overexpressing the transfected constructs were harvested 48 h after transfection and cells were lysed and membrane proteins solubilized for 15 min at 4 °C in detergent-containing buffer (50 mM Tris–HCL pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% Triton, 5% glycerol, 1 mM benzamidine and EDTA-free protease inhibition cocktail, ROCHE 11873580001). The total fractions were centrifuged at 14000 g for 10 min. The supernatant was labeled as soluble (S) and the non-solubilized pellet was dissolved using a volume of buffer equal to the supernatant's volume and labeled as non-solubilized (P). EGFP-containing cell extracts could be visualized in an SDS-PAGE using a blue light box to monitor EGFP emission. Quantification of bands has been carried out using ImageJ gel analyzer tool [9].

2.3. MTT viability assay

24 h after transfecting HEK293T cells, cells were plated at 40,000 cells/well density in a 96 well plate. 48 h transfected cells were incubated with 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) reagent (Invitrogen, M6494) for 3.5 h at 37 °C and the absorbance at 590 nm was measured with a FluoStar Optima microplate reader (BMG Labtech).

2.4. Biotinylation assay

Transfected HEK293 cells were washed with phosphate buffered saline (PBS) and incubated with sulfo-NHS biotin (0.5 mg/ml) for 30 min at 4 °C in an orbital shaker. After incubation cells were washed with PBS, quenched with a solution of 50 mM glycine and 50 mM ammonium chloride in PBS and washed with PBS. Cells were lysed in IP buffer pH = 7.4 (PBS, 1% Triton, 5% glycerol, 2.5 mM calcium chloride, 1 mM magnesium chloride, 5 mM EDTA, 5 mM EGTA, 10 mM sodium pyrophosphate, 50 mM sodium fluoride, 1 mM NaVO3, 1 mM benzamidine and EDTA-free protease inhibition cocktail, ROCHE). Cell extracts were incubated for 15 min at 4 °C and centrifuged at 14,000× *g* for 10 min. 500 μg of total protein from the cell extracts were incubated for 3 h at 4 °C with 50 μL neutravidin beads. Immunoprecipitated complexes were denatured with SDS-PAGE sample buffer (90 °C for 5 min), separated by SDS-PAGE and analyzed by immunoblotting. Scanned films were quantified using ImageJ gel analyzer tool [9]. For exocytosis inhibition, transfected HEK293 cells were treated with 50 μM Exo1 (SantaCruz, sc-200752) 2 h prior to biotinylation treatment. To allow the comparison of protein levels between the samples, all samples were loaded into the same gel.

2.5. Immunoblotting

Lysates and inmunoprecipitates were loaded into SDS-PAGE and transferred to a nitrocellulose membrane. Membranes were blocked in TBS-T with 5% (w/v) non-fat dry milk powder and incubated with primary anti His-Tag antibody from Developmental Studies Hybridoma Bank (DHSB mouse P5A11) at 1:1000 dilution and secondary antibody (Santa Cruz, anti mouse sc-2031) at 1:3000 dilution. Detection was carried out with Crescendo reagent (Millipore, WBLUR0100).

2.6. Microscopy and image analysis

Transfected cells on glass bottom microwell dishes (MATTEK) were in vivo visualized using a Leica TCS SP5 confocal microscope with a lens PL APO 40x/1.25–0.75. Cells were incubated 5 min with CellMask Deep Red Plasma Membrane (Life Technologies, C-10046) and HOESCH dyes (Life Technologies, H1399) before visualization. At least five fields were captured for each sample, yielding a total of $\geq$40 cells visualized.

2.7. Transmembrane topology experiments

TRPV2 truncated constructs were obtained by using forward primers that include the SP6 promotor sequence at the 5′ end and reverse primers at defined positions with an N-glycosylation C-terminal tag followed by tandem stop codons (Table S1) [10]. *In vitro* transcription and translation were performed as previously reported [11]. After membrane pelleting, samples were analyzed by SDS-PAGE, and gels were visualized on a Fuji FLA3000 phosphorimager using the ImageGauge software. The membrane insertion efficiency of a given truncated form was calculated as the quotient between the intensity of the singly glycosylated band divided by the summed intensities of the non-glycosylated and singly glycosylated bands.

2.8. TRPV2 modeling and docking into the TRPV2 EM map

The rat TRPV2 homology model was built using the Modeller [12] built-in in UCSF Chimera [13] using the rat TRPV1 coordinates (PDB code 3j5p). The membrane-embedded domain of TRPV2 was restrained to the topology experiments. TRPV2 homology model docking into the electron microscopy (EM) map of TRPV2 (code EMDB-5688, [14]) was carried out in UCSF Chimera. The TRPV2 EM map was flipped horizontally to show the same handedness of the TRPV1 structure.

3. Results and discussion

3.1. N-terminus of TRPV2 is involved in cell membrane localization

To characterize structural and physiological aspects related to the N-terminus of TRPV2 we studied two N-terminal truncations of