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Research article

TRPM7 N-terminal region forms complexes with calcium binding proteins CaM and S100A1

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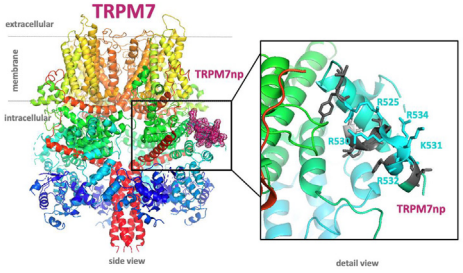
GRAPHICAL ABSTRACT



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ABSTRACT

Transient receptor potential melastatin 7 (TRPM7) represents melastatin TRP channel with two significant functions, cation permeability and kinase activity. TRPM7 is widely expressed among tissues and is therefore involved in a variety of cellular functions representing mainly Mg^{2+} homeostasis, cellular Ca^{2+} flickering, and the regulation of DNA transcription by a cleaved kinase domain translocated to the nucleus. TRPM7 participates in several important biological processes in the nervous and cardiovascular systems. Together with the necessary function of the TRPM7 in these tissues and its recently analyzed overall structure, this channel requires further studies leading to the development of potential therapeutic targets. Here we present the first study investigating the N-termini of TRPM7 with binding regions for important intracellular modulators calmodulin (CaM) and calcium-binding protein S1 (S100A1) using in vitro and in silico approaches. Molecular simulations of the discovered complexes reveal their potential binding interfaces with common interaction patterns and the important role of basic residues present in the N-terminal binding region of TRPM.

1. Introduction

The transient receptor potential melastatin subfamily member 7 (TRPM7) is ubiquitously expressed chanzyme mediating divalent cations Ca^{2+}, Mg^{2+}, Zn^{2+} transport. TRPM7 is involved in a number of cellular functions and is critically associated with cell proliferation, growth, apoptosis, Mg^{2+} homeostasis, Ca^{2+} signalling, including receptor tyrosine kinase mediated pathways [1, 2]. TRPM7 kinase requires Mn^{2+} or

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Mg^{2+} for its activity and mainly uses Mg-ATP for phosphorylation [3]. Abundant auto-phosphorylation of TRPM7 increases kinase activity and substrates recognition [4, 5]. Tissue specific deletion of TRPM7 in thymocytes or macrophages, as well as inactivation of kinase activity, emphasized the importance of this channel in immune system function [6]. TRPM7 α-kinase can also be cleaved and translocated to the nucleus to modulate gene expression [7, 8]. Despite the many similarities shared by TRPM6 and TRPM7, they still modulate cell functions differently and their responses cannot be compensated by each other [9].

The cryo-EM structure of TRPM7 (lacking the α-kinase domain) revealed a similar overall architecture as the other TRPM members [10]. However, the conformation of the N-terminal cytosolic melastatin homology regions (MHR) differ between TRPM7 and another structurally characterized melastatin TRP member, TRPM4 channel. In both TRPM7 and TRPM4, the C-terminal stretcher helix penetrates through the MHR regions to the TRP box domain which could lead to signal transfer from the N-terminal MHR domains to the S6 pore gating helix. Such structural information suggests more complex modulation mechanisms in TRPM channels. It has been discovered by electrophysiology methods that TRPM7 activity is inhibited by increasing cytosolic Mg^{2+}/Mg-ATP and PIP2 hydrolysis [11, 12]. Negative modulation of TRPM7 by Ca^{2+} is at least partially mediated by Ca^{2+}/CaM-dependent kinase II [13, 14]. Possible direct or indirect and more complex regulation of TRPM7 by CaM/Ca^{2+} still needs to be explored. The closest member to TRPM7, the TRPM6 member has already revealed overlapping binding epitopes for CaM and S100A1 at the N-termini [15].

Calcium as a universal second messenger involved in fundamental physiological processes including fertilization, proliferation, neurotransmission, muscle contraction, bone formation, apoptosis, etc. [16, 17] can also act through protein mediators. Calcium-binding proteins (CBPs) represent family that is part of important multifunctional Ca^{2+} dependent messengers expressed in most of eukaryotic cells [18]. Representatives of the CBPs family are the well-described monomeric calmodulin (CaM) [19] and the dimeric protein S100 calcium-binding A1 (S100A1) [20, 21]. In many regulatory pathways of ion channels, Ca^{2+}-induced modulation is controlled by the interaction of these intracellular CBPs to the channel binding regions exposed to the intracellular environment [22, 23]. These types of interactions are often created by multicomplex machineries and can lead to many variations of activation, inhibition or non-trivial interplay activities in the channel regulation.

We identified novel CaM and S100A1 binding regions present at the N-terminus of TRPM7. The binding regions of TRPM7 involved in the interactions with the ligands was investigated using fluorescent spectroscopy method accompanied by an atomistic explanation of the binding interfaces addressed by molecular modelling. To support the evidence for significant amino acids of the TRPM7 binding regions participating on TRPM7/CaM and TRPM7/S100A1 complex formations, we characterized these amino acids using multiple sequence analysis of TRPM binding regions. This analysis was supported by molecular models of the identified complexes built on the TRPM7 structure [10]. The common mechanisms of interaction of CaM and S100A1 with the TRP binding region have been validated and may help to design potential therapeutic targets.

2. Materials and methods

2.1. Design of TRPM7 N-termini binding regions

The human TRPM7 cDNA sequence was analyzed to detect a potential CaM/S100A1 binding motif using the Calmodulin Target Database [24]. To demonstrate the presence of CaM and S100A1 binding region at the N-terminal of TRPM7, we selected the TRPM7np UniProtKB/SwissProt: Q96QT4, position T523-L535. TRPM7np was synthesized as a peptide and interactions to CaM and S100A1 were investigated by *in vitro* fluorescence anisotropy and *in silico* molecular modelling approaches.

2.2. TRPM7np peptide synthesis

Wild-type TRPM7np sequence was synthesized as a peptide by GenicBio Limited (Shanghai, China) and supplied in lyophilized powder. The peptide was C-terminally labelled with fluorescein-5-isothiocyanate (FITC, PubChem CID: 18730). The purity of the peptide was >90%, checked by HPLC (GenicBio Limited, Shanghai, China). Peptide probes were dissolved in 50 mM Tris-HCl buffer (pH 7.5) containing 500 mM NaCl and 1 mM $CaCl_2$.

2.3. CaM and S100A1 expression and purification

CaM and S100A1 cDNAs were subcloned into the expression vector pET28b and were expressed and purified according to our standard purification protocol [25].

2.4. Steady-state fluorescence anisotropy measurements

Steady-state fluorescence anisotropy measurements were performed using PC1 photon counting spectrometer (ISS Inc., Champaign, Illinois, USA) at RT. The samples were titrated in a 2-mm cuvette with increasing aliquots of 100 μM protein. Fluorescence was excited at 495 nm; the emission in parallel ($I_{\parallel}$) and perpendicular ($I_{\perp}$) orientations to the direction of the polarised excitation was gained at 520 nm by switching the emission polariser. The steady-state fluorescence anisotropy value (r) was calculated from the equation $r = (I_{\parallel} - I_{\perp})/(I_{\parallel}+2I_{\perp})$. Further analysis was based on the mean anisotropy value calculated from five independent measurements for each protein addition. The fractions of bound (FB) CaM/S100A1 were expressed as [26]:

$$FB = (r_{obs} - r_{min}) / [(r_{max} - r_{obs}) Q + (r_{obs} - r_{min})], \quad (1)$$

where r_{max} is the anisotropy of a saturated binding complex, r_{min} stands for the anisotropy of a peptide probe without a ligand and r_{obs} is the anisotropy at a particular protein concentration. Q represents the quantum yield ratio of the bound to the free peptide, calculated from fluorescence lifetimes (τ) according to equation

$$Q = Q_{bound}/Q_{free} = \tau_{bound}/\tau_{free}. \quad (2)$$

To determine the equilibrium dissociation constant (KD), FB was plotted as a function of protein concentration and fitted by a single-binding-site model [27]:

$$FB = \frac{KD + [P1] + [P2] - \sqrt{(KD + [P1] + [P2])^2 - 4[P1][P2]}}{2[P1]}, \quad (3)$$

where [P1] is the peptide concentration and [P2] is the protein concentration. Nonlinear data fitting was performed using SigmaPlot 11.0 (Systat Software Inc., San Jose, USA).

2.5. TRPM7np molecular model built from TRPM7 structure

The TRPM7np molecular model was built from the original Cryo-EM structure (3.28 Å) of mouse TRPM7 (PDB: 5ZX5) [28]. The initial structure of TRPM7np (UniProtKB/SwissProt: Q96QT4, positions: T523-L535) was completely defined by positions T523-L535 extracted in PDB coordinates from TRPM7 structure (PDB: 5ZX5) [10]. The additional criteria for TRPM7np structure were applied according to geometry requirements of the potential CaM- and S100A1-binding regions for TRPM7np based on known experimental structures of CaM and S100A1 with receptor derived peptide complexes (PDB: 3SUI, 2K2F). The specific attention was paid to the positions of the basic and hydrophobic residues in TRPM7np which should be exposed to the solvent. Consequently the TRPM7np model was optimised keeping coordinates of backbone atoms constrained by MOE optimization algorithm [29]. The final structure was assessed by STING Millennium [30] and ProSA-web [31].

2.6. TRPM binding regions analysis

To predict the basic amino acid residues of TRPM7np involved in the interactions with CaM/S100A1, we performed multiple sequence alignment of already *in vitro* characterized TRPM binding regions for CaM and S100A1 using CLUSTAL 1.2.4. software [32].

2.7. Models of TRPM7np complexes with CaM and S100A1

Docking of CaM and S100A1 into the TRPM7np structural model was performed using the ClusPro platform [33, 34, 35]. The CaM and S100A1 templates were selected from the structures of the TRPV1 complexes with CaM/Ca^{2+} (PDB: 3SUI) and RyR1 with S100A1/Ca^{2+} (PDB: 2K2F). Molecular models of the TRPM7np/CaM and TRPM7np/S100A1 complexes were built with respect to predicted binding interfaces [29]. Schematic representations of all four complexes were generated using Discovery Studio Visualizer [36].

3. Results

3.1. Characterization of the N-terminal binding region of TRPM7

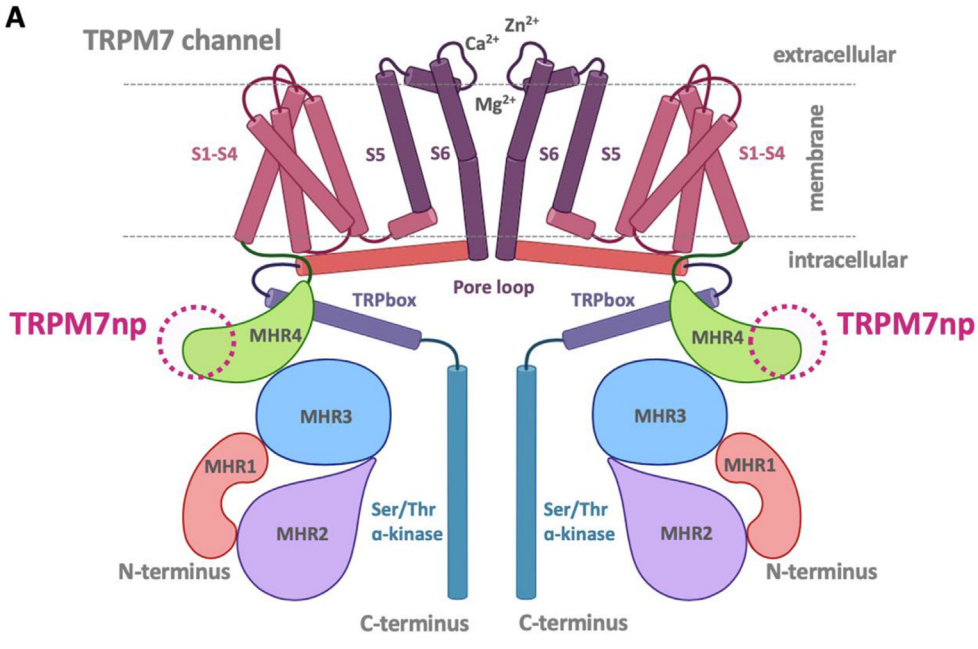
The human TRPM7 sequence was analyzed in silico for potential CaM-binding region using the Calmodulin Target Database [24]. The TRPM7 binding region was identified in the proximal N-terminus of the channel between position T523 and L535 (Figure 1A–B). TRPM7np (see Material and Methods) contains aromatic and hydrophobic amino acids (Y524–Y528–F533) arranged in 1-5-10 CaM-binding motif, and five interspaced basic amino acids (R525, R530, K531, R532 and R534). Due to the known shared binding epitopes of CaM and S100A1 at e.g., ryanodine (RyR) or TRP receptors [22, 25, 37, 37, 38], we also decided to investigate binding site of S100A1 at TRPM7np. The position of TRPM7np in the TRPM7 structure (PDB: 5ZXZ) proves the accessibility of the binding region to intracellular environment and the basic amino acid residues of TRPM7np are exposed out of the TRPM7 structure backbone which suggest suitable condition for complex formation with the ligands (Figure 2A–C).

3.2. TRPM7np forms a complex with CaM

Evaluation of CaM binding to TRPM7np was performed by measuring steady state fluorescence anisotropy with FITC-labelled TRPM7np (Figure 3A). Fluorescence anisotropy of the TRPM7np peptide was measured during CaM titration. TRPM7np about c = 1μM concentration was incubated (1 min) with a gradually increasing volume of CaM about stock solution c = 118 μM led to a reduction in its rotational diffusion, which led to an apparent increase in fluorescence anisotropy. The ratio of the quantum yield (Q) of CaM bound to the free peptide was calculated from the corresponding fluorescence lifetimes. The Q value was used to determine the CaM-bounded fraction (FB) of TRPM7np at each CaM titration point. Finally, FB was plotted against a specific concentration of CaM and the equilibrium dissociation constant (KD) for the TRPM7np/CaM complex was determined. The CaM-binding affinity of TRPM7np is within the micromolar range and the KD was calculated to be 6.1 (SD 0.4) μM (Figure 3B).

3.3. TRPM7np forms a complex with S100A1

S100A1 recognizes transient receptor potential (TRP) channels binding motifs in a manner similar to CaM. The S100A1 and CaM binding regions often overlap, and S100A1/CaM can compete for the same binding site on the target protein [22, 39]. Based on our previous experience of shared CaM binding regions with S100A1 [25, 37, 37, 38, 39] we also investigated the possible binding of S100A1 to the identified CaM-binding epitope to TRPM7np (Figure 3A). The interaction of S100A1 with TRPM7np was confirmed by steady state fluorescence



B

TRPM binding domain TRPM7np	sequence $_{523}$TYRCTYTRKRFRL$_{535}$

Figure 1. Shared CaM and S100A1 binding region on TRPM7. (A) Common membrane topology of the TRPM7 (homodimer in scheme) with the location of the TRPM7np binding epitope (purple dashed circle) shown in both monomeric units; the biological formation of TRPM7 channel appears commonly in homo-tetramer formation. TRP channels consist of six transmembrane helices (S1–S6) with a pore loop and a pore helix between S5 and S6. The long cytoplasmic N-terminus contains melastatin homology regions (MHR 1–4), the C-terminus contains TRP box, Ser/Thr and alpha-kinase regions. The cytosolic regions often contain specific binding regions for modulatory molecules. (B) Amino acid sequence of the TRPM7np (UniProtKB/SwissProt: Q96QT4, position T523-L535) binding epitope with red highlighted basic residues investigated as novel CaM/S100A1 binding sites.

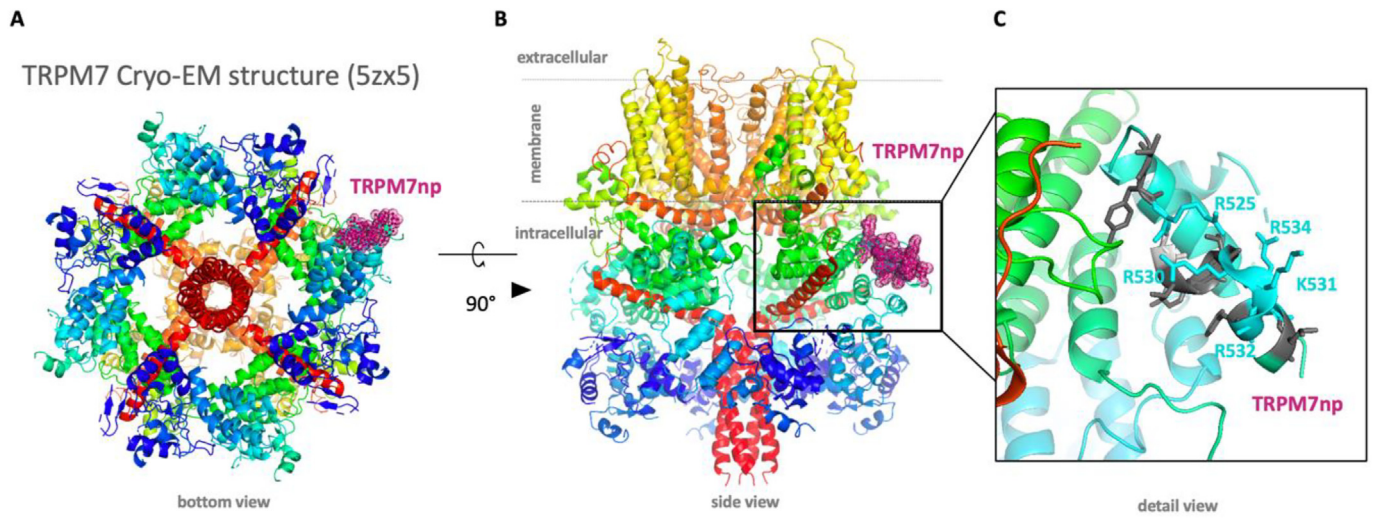
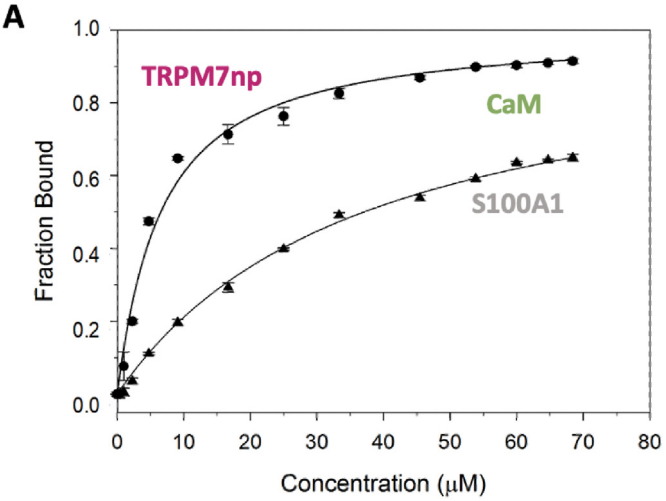


Figure 2. TRPM7np localization in the TRPM7 channel (PDB: 5ZX5). (A) Bottom view of the whole TRPM7 structure with the purple TRPM7np localization on the right. (B) Side view of the whole TRPM7 structure. The frame localizes TRPM7np binding site. (C) The corresponding region of TRPM7 known to bind CBDs in TRPM6. Detail of the TRPM7np potential binding site for CaM and S100A1 displaying the accessible amino acid side chains of TRPM7np potentially significant for the interactions. Grey sticks indicate the hydrophobic amino acids and blue sticks indicate the basic amino acids R525, R530, K531, R532, R534 predicted to be responsible for the interactions with ligands.

anisotropy similar to that described above for CaM binding experiments. TRPM7np about c = 1μM concentration was incubated (1 min) with a gradually increasing volume of S100A1 about stock solution c = 112 μM led to a reduction in its rotational diffusion, which led to an apparent increase in fluorescence anisotropy. FB TRPM7np as a function of S100A1 concentration according to equation (Equation 2) provided the expected binding isotherm. The KD value evaluated by steady state fluorescence anisotropy experiments was 38.0 (SD 2.0) μM for the TRPM7np/S100A1 complex (Figure 3B). The binding of TRPM7np to S100A1 indicated a slightly weaker affinity than for CaM. Differences in binding affinities between CaM and S100A1 could suggest the mechanism of ligands with different binding modes of interaction.



B

TRPM7np	K_D (SD) μM
CaM / Ca^{2+}	6.1 (0.4)
S100A1 / Ca^{2+}	38 (2)

Figure 3. TRPM7np interacts with CaM and S100A1. The bound fractions (F_B) of (A) TRPM7np as a function of CaM and S100A1 concentration were obtained from steady-state fluorescence anisotropy experiments. Binding isotherms (solid lines) were generated from the best fit using Eq. (2) (Materials and Methods). Five independent measurements were performed for each gradually increasing CaM/S100A1 concentrations (stock solutions c (CaM) = 118 μM, c (S100A) = 112 μM) and the calculated SD is represented by error bars. (B) The equilibrium dissociation constants (KD) of CaM/S100A1 interactions with TRPM7np together with corresponding standard deviations (SD).

3.4. Analysis of TRPM7np binding specificity by multiple sequence alignment

TRP interactions with CaM or S100A1 are maintained by hydrophobic and basic amino acid residues. Alanine scanning mutagenesis of basic residues in the TRP binding region prevented the complex formation in many studied complexes [25, 37, 40, 41]. Therefore, it has been concluded that non-covalent interactions play the most significant role in these complex formations. TRP binding regions carry typical clusters of positively charged residues [25, 37]. The multiple sequence alignment analysis of TRPM binding regions revealed consensus sequences of basic amino acid residues (RxxxxR/K, where x is any amino acid) in analysed regions (Figure 4) suggested the importance of R525, R530, K531, R532 and R534 in TRPM7np. This analysis of multiple TRPM sequences was

TRPM	peptide sequence	AA
TRPM1np	-------VKLRRQLEKHI--------	14
TRPM4np1	VLQTWLQDLLRRGLVRAAQ-------	19
TRPM5np	----WLRDVLRKGLVK----------	12
TRPM4np2	-----FGECYRSSEVRAARLLLRRCP	21
TRPM6np	----LIGRAYRSNYTRKHFR------	16
TRPM7np	--------TYRCTYTRKRFRL-----	13
	* :	

Figure 4. Multiple alignment of selected CaM binding regions in TRPM channels. CLUSTAL 1.2.4 multiple sequence alignment of CaM/S100A1 binding regions of TRPMs N-termini [15, 25, 37, 38, 40, 41]. The sequence alignment shows a first perfect match (indicated by an asterisk) of arginine residues and second almost accurate match (indicated by dots) of basic residues across the TRPM sequences. Red colour stands for hydrophobic, pink for basic, blue for acidic amino acids; all others amino acids stand for green. The number on the right side of the sequence marks its amino acid length.

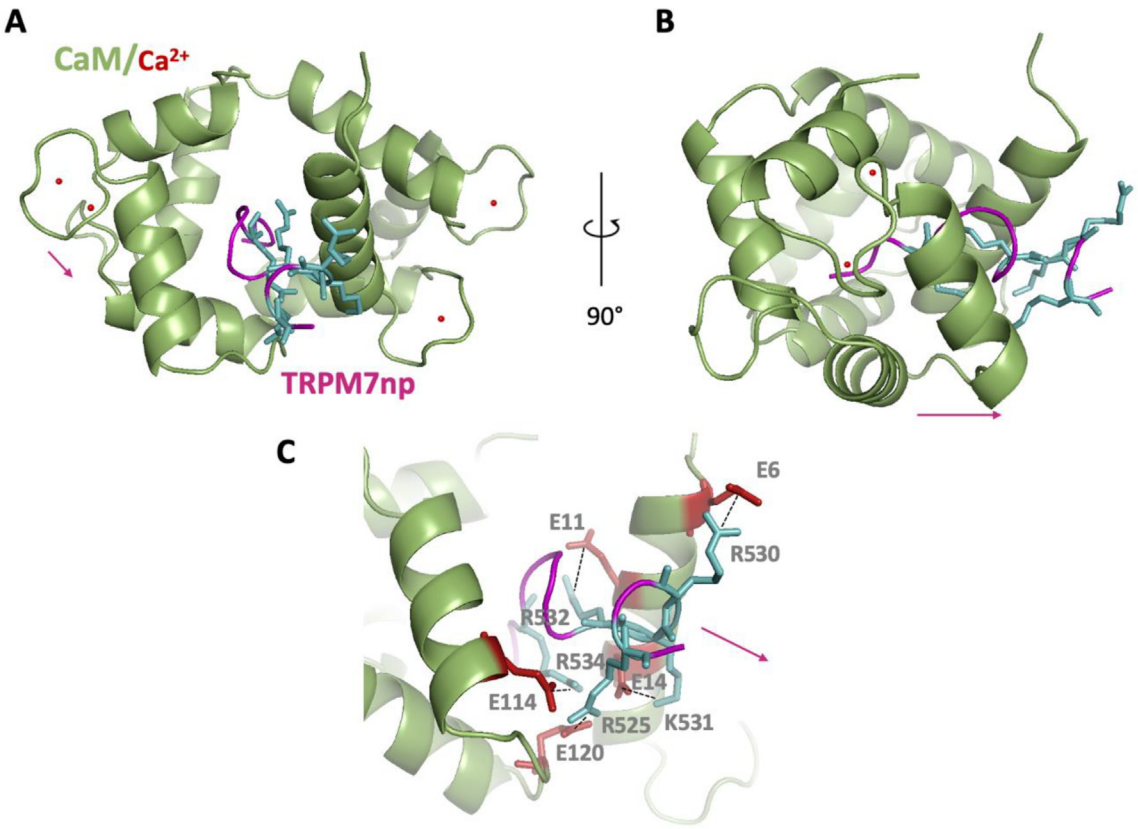


Figure 5. In silico analysis of the TRPM7np/CaM complex interface. (A) Top view of the TRPM7np/CaM-Ca^{2+} complex representation 1:1. TRPM7np (backbone in violet) and CaM (backbone in green, red balls represent calcium ions) composing the binding interface, the blue side chains represent basic amino acids of TRPM7np interacting with CaM. (B) Side view of TRPM7np/CaM complex ribbon representation with the same binding interface. (C) Detailed representation of TRPM7np positively charged residues R525, R530, K531, R532 and R534 (blue sticks) with negatively charged residues of CaM E6, E11, E14, E114 and E120 (red sticks) predicted to be involved in the non-covalent bonding. The colour convention was used same as in the A representation. The violet arrow symbolizes the direction of the helix (pseudo-helix) for a clear visualization of the orientation of the TRPM7np in the complex.

performed from *in vitro* experimentally characterized TRPM binding regions for CaM and S100A1 [25, 37, 38, 40, 41].

The highest sequence similarity was identified for TRPM7np and TRPM6np. The binding regions share an identical 1-5-10 hydrophobic binding motif at positions Y524–Y528–F533 (TRPM7np) and Y525–Y529–F534 (TRPM6np). TRPM7np and TRPM6np contain four basic residues R525-R530-K531-R534 in TRPM7 and R526-R531-K532-R535 in TRPM6 in the same position with respect to the hydrophobic motif. Alignment revealed a strong agreement for TRPM7np R525 and R530 with all TRPM binding epitopes. In addition, TRPM7np K531 and R534 are consistent with the basic residues of TRPM6np, as expected, due to their closest TRP member. Only TRPM7np R532 did not correspond to consensus with other TRPM-binding epitopes. Therefore, we assume that the TRPM7np cluster R525, R530, K531 and R534 has the highest tendency to engage in the interactions with CaM/S100A1.

3.5. *TRPM7np/CaM and TRPM7np/S100A1 complexes in silico analysis*

The general strategy was to use ClusPro2.0 as the ligand docking procedure for CaM and S100A1 [34]. The final TRPM7np/CaM-Ca^{2+} complex (Figure 5A, B) was chosen from results of ClusPro docking using the highest compliance with the previously published structure of the TRPV1p/CaM complex (PDB: 3SUI) [25, 37, 38, 42]. The TRPM7np/S100A1- Ca^{2+} complex (Figure 6A, B) was built by same approach using crystal structure of the RyR1P12/S100A1 complex (PDB: 2K2F) [43]. Finally, we optimised the identified binding modes by conjugate gradient energy minimisation implemented by MOE software constraining all backbone atoms of CaM/S100A1 with full relaxation of the peptide and side chains of CaM/S100A1 [29].

Further, the ligand docking analysis of TRPM7np/CaM and TRPM7np/S100A1 complexes predicted that R525, R530, K531, R532 and R534 of TRPM7np are involved in the interactions with negatively charged residues of CaM and S100A1. Specifically, the negatively charged residues of CaM involved in TRPM7np interactions were identified as: E6, E11, E14, E114 and E120 which correspond to CaM negative residues involved in the interactions in TRPV6/CaM complex [44]. The S100A1 residues D46, D52, N86 involved in TRPM7np interactions correspond to the negative cluster of S100A1 involved at TRPM6np/S100A1 complex formation [15]. The ligand docking supported the proposal of high participation of TRPM7np R525, R530, K531, R532 and R534 basic residues in the complex formations with CaM and S100A1 as predicted by multiple sequence alignment of TRPM binding regions.

4. Discussion

TRP channels are commonly known to be modulated by many intracellular and extracellular modulators [45, 46, 47, 48, 49]. CaM belongs to a significant game player in overall cellular metabolism and signalling with a dominant function in regulations in calcium homeostasis [17]. While countless CaM binding regions in TRP channels have been characterized [42, 50, 51], the regulatory or signalling functions of CaM in such ion transports remains still under necessary investigation. We are currently beginning to understand how protein machines, such as transmembrane receptors, are regulated, and it is necessary to discover every piece of the puzzle to understand the complexity involved in these processes. In recent years, number of publications mentioning more binding sites and more complex processes of interactions in

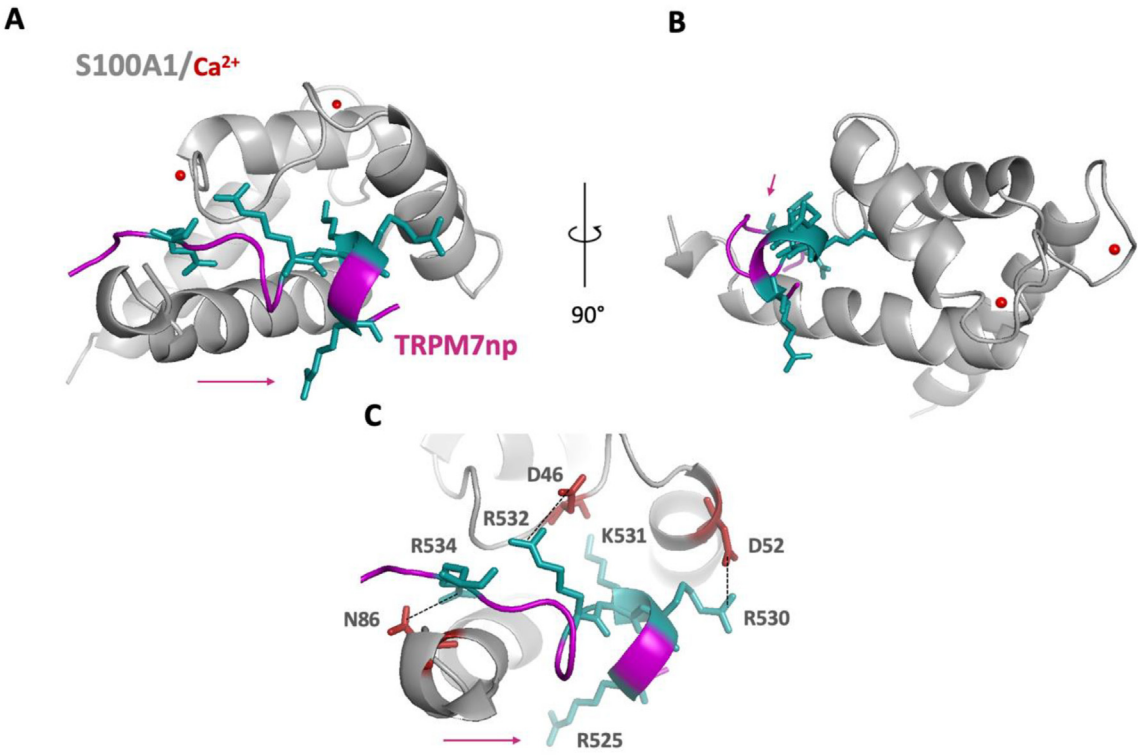


Figure 6. In silico analysis of the TRPM7np/S100A1 complex interface. (A) Top view of the TRPM7np/S100A1-Ca^{2+} complex 1:1. TRPM7np (backbone in violet) and S100A1 (backbone in grey, red balls represent calcium ions) composing the binding interface, the blue side chains represent basic amino acids of the TRPM7np interacting with S100A1. (B) Side view of TRPM7np/CaM complex ribbon representation with the same binding interface. (C) Detailed representation of TRPM7np positively charged residues R525, R530, K531, R532 and R534 (blue sticks) with negatively charged residues of S100A1 D46, D52 and N86 (red sticks) predicted to be involved in the non-covalent bonding. The colour convention was used as in the A representation. The violet arrow symbolizes the direction of the helix (pseudo-helix) for a clear visualization of the orientation of the TRPM7np in the complex.

ligands/receptors complexes have begun to rise [50]. The whole mechanism of modulation of membrane receptors has been expected to be much more complex than protein functions and interactions explained in the past.

In this work, we contribute by identification and characterization new CaM and S100A1 binding sites at the N-terminal intracellular tail of the TRPM7 channel. The provided insight into the understanding of the mechanisms of TRPM7 interactions with intracellular potential modulatory CBPs [51, 52, 53] was investigated based on our theoretical and experimental experience with the identification and characterization of similar interactions of TRPM members with CaM and S100A1 [25, 37, 38, 41]. Based on current knowledge, we hypothesize that shared CaM/S100A1 binding epitopes are a common phenomenon across all members of TRPs [22, 25, 37]. We identified a new TRPM7np T523-L535 binding region revealing the ability to bind CaM and S100A1. Our biophysical and computational characterization of TRPM7np complexes with modulatory ligands CaM and S100A1 may help to elucidate the mechanism of TRPM7 channel function [54]. The structural resolution from CryoEM analysis revealed an accessible TRPM7np binding region located throughout the TRPM7 channel. The dissociation constants of both identified complexes TRPM7np/CaM and TRPM7np/S100A1 range in typical micromolar levels for these TRP interactions. Both complexes are formed upon Ca^{2+} presence [25, 37]. Multiple sequence alignment of TRPM binding regions indicated the importance of basic residues in TRPM7np. The interface formation of the TRPM7np/CaM and TRPM7np/S100A1 complexes based on non-covalent interactions was also supported by *in silico* ligand docking analysis. Insight into the interface of the complexes provided by molecular docking with atomistic resolution can also help us understand the potential initiation of channel dysfunctions associated with severe disorders [1, 2, 14]. Therefore, we expect the information provided in the current study and in our previous TRPM projects [25, 37, 37, 38, 41] may help to clarify a deeper understanding of TRPM channel function in general and may further assist in the design of new therapeutics for ion channel modulation.

Declarations

Author contribution statement

Kristyna Bousova: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Monika Zouharova: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Petr Herman: Performed the experiments; Analyzed and interpreted the data.

Veronika Vetyskova: Performed the experiments.

Katerina Jiraskova: Analyzed and interpreted the data.

Jiri Vondrasek: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

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Data availability statement

Data included in article/supplementary material/referenced in article.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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