Three-dimensional Reconstruction Using Transmission Electron Microscopy Reveals a Swollen, Bell-shaped Structure of Transient Receptor Potential Melastatin Type 2 Cation Channel

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Transient receptor potential melastatin type 2 (TRPM2) is a redox-sensitive, calcium-permeable cation channel activated by various signals, such as adenosine diphosphate ribose (ADPR) acting on the ADPR pyrophosphatase (ADPRase) domain, and cyclic ADPR. Here, we purified the FLAG-tagged tetrameric TRPM2 channel, analyzed it using negatively stained electron microscopy, and reconstructed the three-dimensional structure at 2.8-nm resolution. This multimodal sensor molecule has a bell-like shape of 18 nm in width and 25 nm in height. The overall structure is similar to another multimodal sensor channel, TRP canonical type 3 (TRPC3). In both structures, the small extracellular domain is a dense half-dome, whereas the large cytoplasmic domain has a sparse, double-layered structure with multiple internal cavities. However, a unique square prism protuberance was observed under the cytoplasmic domain of TRPM2. The FLAG epitope, fused at the C terminus of the ADPRase domain, was assigned by the antibody to a position close to the protuberance. This indicates that the agonist-binding ADPRase domain and the ion gate in the transmembrane region are separately located in the molecule.

Transient receptor potential (TRP)2 channels, first described in the Drosophila phototransduction system (1), comprise a large family of cation channels (2–5). In mammals, nearly 30 TRP members have already been found, and are grouped into six subfamilies based on their amino acid sequences (TRPC, TRPV, TRPM, TRPA, TRPP, and TRPM5L). TRP channels have common structural features with voltage-gated cation channels: six transmembrane (TM) segments, a pore-forming region between the fifth and sixth TM segments, cytoplasmic N- and C-terminal regions, and tetrameric subunit stoichiometry (2–6). Outside of these commonalities, however, there are huge variations in amino acid sequence and sequence length, especially in the cytoplasmic regions. Corresponding to these variations, TRP channels can be activated by stimuli from various intracellular messengers, mechanical force, temperature change, tastes, hormones, and oxidative stress (2–5). Each TRP channel is basically regulated by multiple signals or by their combinations, so the TRP molecule is generally regarded as a multiple sensor.

TRP melastatin type 2 (TRPM2) is a voltage-independent, calcium-permeable non-selective cation channel ubiquitously expressed in various tissues (7, 8), and at higher levels in the brain, in insulin secreting cells (8, 9), and in immune cells including monocytic cells (10, 11), neutrophil granulocytes (12, 13), and microglia (14). Intracellular messenger molecules such as adenosine diphosphate ribose (ADPR), NAD2+, and cyclic ADPR (cADPR) have been proposed to act directly as activators/modulators for TRPM2 (8, 10, 15, 16). Reactive oxygen species such as H2O2 also act as potent activators/modulators of TRPM2 (8). H2O2-induced TRPM2 activation/modulation may occur directly via modification of TRPM2 at the membrane (13) and/or indirectly through production of the above intracellular messenger molecules (8, 17). It has been demonstrated that 2’-O-acetyl-ADP-ribose, produced by sir-tuin, binds to TRPM2 and modulates channel activity (18). Recently, Kolisek et al. (15) demonstrated that H2O2 and cADPR at lower concentrations can significantly potentiate the effects of ADPR and at higher concentrations can gate the channel directly, whereas Togashi et al. (16) described potentiation of TRPM2 activity by coapplication of cADPR and heat (>35 °C). Additionally, intracellular and extracellular Ca2+ appears to be an important modulator and cofactor of TRPM2 activation (8, 10, 19).
ADPR is the most intensively studied activator of the TRPM2 channel (10, 11, 20). ADPR binds to the motif named nucleoside diphosphate-linked moiety X-type motif 9 homology (NUDT9-H) domain, which has 39% identity on the amino acid sequence with mitochondrial NUDT9 ADPR pyrophosphatase (ADPRase) (10). The motif is located at the C-terminal end of the TRPM2 protein. Although the structure of the NUDT9-H domain is not clear yet, the crystal structure of the mitochondrial NUDT9 ADPRase was recently revealed (21). This can be the structural basis for interpreting the mechanism of the homologous NUDT9-H domain, however, the relationship between ADPRase and the TRPM2 channel still remains to be discussed. Because the catalytic activity of the NUDT9-H domain is lower than that of mitochondrial NUDT9 (10), the gating mechanism of TRPM2 has been investigated by site-directed mutagenesis, especially in relation to the catalytic activity of the NUDT9-H domain (17, 20). Because site-directed mutagenesis demonstrates that gating persists in double mutation (at 1408 and 1409 residues in the NUDT9-H domain), which is critical for ADPRase activity of mitochondrial NUDT9, catalytic activity does not seem necessary for ADPR-dependent gating (17). To understand the gating mechanisms of TRPM2, the entire three-dimensional structure including the NUDT9-H domain should be analyzed. Notably, the three-dimensional structure of the TRP canonical type 3 (TRPC3) channel, recently revealed using single particle analysis in combination with cryo-electron microscopy (cryo-EM) (22), represents the first structural depiction of a member of the TRP superfamily.

In this study, we have reconstructed the three-dimensional structure of TRPM2 from negatively stained EM images. The three-dimensional structure of TRPM2 was compared with that of the previously reconstructed TRPC3 to extract common structural features of the TRP superfamily, and detailed architecture specific to TRPM2.

**EXPERIMENTAL PROCEDURES**

**Recombinant Construction and Transfection of HEK293 Cells—**The full-length human TRPM2 coding sequence (GenBank accession number AB001535) was inserted into the pCMV-EXPERIMENTAL PROCEDURES

**Recombinant Construction and Transfection of HEK293 Cells—**The full-length human TRPM2 coding sequence (GenBank accession number AB001535) was inserted into the pCMV-Tag4A vector (Stratagene) to introduce a FLAG tag sequence at the C terminus. Human embryonic kidney HEK293 cells were grown in Dulbecco’s modified Eagle’s medium, supplemented with 10% fetal bovine serum and 100 µg/ml kanamycin, and incubated at 37 °C in a 5% CO2 humidified atmosphere. The HEK293 cells were transfected with 0.18 µg/cm2 of the TRPM2 expression vector for 6 h using the calcium phosphate precipitation method (23) or SuperFect Transfection Reagent (Qiagen). After 48 h, the cells were washed twice with a phosphate-buffered saline solution (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na2HPO4, 1.5 mM KH2PO4, pH 7.4), harvested with a cell scraper, and collected by centrifugation. Cells were stored at −80 °C for further protein purification.

**Measurement of Intracellular Ca2+ Change and Current Recording—**HEK293 cells on coverslips were transfected similarly, and were loaded with fura-2 by incubation in Dulbecco’s modified Eagle’s medium containing 1 µM fura-2/AM (Dojindo Laboratories, Kumamoto, Japan), 10% fetal bovine serum, 30 units/ml penicillin, and 30 µg/ml streptomycin at 37 °C for 40 min. The coverslips were then placed in a perfusion chamber mounted on the stage of the microscope. Fluorescence images of the cells were recorded and analyzed with a video image analysis system (AQUACOSMOS, Hamamatsu Photonics, Hamamatsu, Japan). Fura-2 fluorescence at an emission wavelength of 510 nm (bandwidth: 20 nm) was observed at room temperature by exciting fura-2 alternately at 340 and 380 nm (bandwidth: 11 nm). The 340:380 nm ratio images were obtained on a pixel-by-pixel basis. These images were converted to Ca2+ concentrations by in vivo calibration (8). H2O2 was diluted to the final concentration (100 µM) in a buffer containing 107 mM NaCl, 6 mM KCl, 1.2 mM MgSO4, 2 mM CaCl2, 11.5 mM glucose, and 20 mM HEPES (pH 7.4), and applied to the cells by perfusion.

Whole cell currents were recorded at room temperature using the conventional whole cell mode of the patch clamp technique (24) with EPC9 amplifier (HEKA, Pfaiz, Germany). The pipette solution was 40 mM CsCl, 105 mM CsOH, 2 mM MgCl2, 1.3 mM CaCl2, 5 mM EGTA, 2 mM Na2ATP, 105 mM L-aspartate, and 5 mM HEPES (adjusted to pH 7.2 with CsOH). The 2 mM Ca2+-NaCl solution contained 125 mM NaCl, 1.2 mM MgCl2, 2 mM CaCl2, 10 mM glucose, 11.5 mM HEPES, and 51 mM mannitol (adjusted to pH 7.4 with NaOH). All data are expressed as mean ± S.E. The statistical analyses were performed using the Student’s t test.

**Protein Purification—**The transfected cells were homogenized with a Potter Teflon homogenizer in Tris-buffered saline (20 mM Tris-HCl, pH 7.4, at 4 °C, 140 mM NaCl) containing 50 mM n-dodecyl β-D-maltoside (DDM) (Sigma), protease inhibitors (500 µM 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride, 400 nM aprotinin, 25 µM bestatin, 7.5 µM E-64 protease inhibitor, 10 µM leupeptin hemisulfate, and 5 µM pepstatin A) (Wako Pure Chemicals, Osaka, Japan), and 0.02% sodium azide. All procedures were performed on ice or at 4 °C. Cell debris was precipitated from the homogenate by centrifugation for 20 min at 14,000 × g. The supernatant containing FLAG-tagged TRPM2 was applied to an anti-FLAG M2 affinity gel (Wako, Osaka, Japan), and 0.02% sodium azide. The bound proteins were eluted with the same buffer containing 125 µg/ml FLAG peptide (Sigma). The eluate was concentrated 20 times with a Microcon centrifuge filter unit YM-100 (Millipore, Billerica, MA). Further purification was performed using Superose 6 (3.2/30) gel filtration chromatography in a Smartsystem (GE Healthcare) with Tris-buffered saline containing 1 mM DDM, 750 mM MgCl2, 15% glycerol, and 0.02% sodium azide. The elution of protein was profiled at 280 nm, and subfractionated into 20-µl fractions. Protein concentrations were determined with the BCA method (25).

**SDS-PAGE and Chemical Cross-linking—**SDS-PAGE was carried out using the standard method of Laemmli (26). Samples were mixed with a SDS-sample buffer (final concentrations...
of 62.8 mM Tris-HCl, pH 6.8, 10% glycerol, 1% SDS, 1% 2-mercaptoethanol, and 0.0005% bromphenol blue), and heated at 60 °C for 15 min. 5–20% polyacrylamide gradient gel was used for electrophoresis. Proteins were visualized by silver staining (Wako Pure Chemicals) or Western blotting. For Western blots, proteins were transferred to the polyvinylidene difluoride membrane and detected with alkaline phosphatase-labeled anti-FLAG antibodies (Sigma) or with two different kinds of rabbit polyclonal antibodies that recognize the N-terminal region between residues 650 and 700 and the C-terminal region between residues 1200 and 1250 of human TRPM2 (Bethyl Laboratories, Montgomery, TX).

For chemical cross-linking, the buffer components were substituted in advance with phosphate-buffered saline solution containing 1 mM DDM, 750 mM MgCl2, 15% glycerol, and 0.02% sodium azide. Glutaraldehyde was added to a final concentration of 12.5 mM, and incubated at 25 °C for 30 min. The cross-linking reaction was terminated by the addition of the SDS-sample buffer. Samples were incubated at 60 °C for 15 min, and analyzed in 2–15% polyacrylamide gel.

**Native Gel Electrophoresis**—Native gel electrophoresis was performed by the method of Davis (27). Purified TRPM2 protein was mixed with an equal volume of sample buffer (62.5 mM Tris-HCl, pH 6.8, 25% glycerol, and 0.01% bromphenol blue), and electrophoresed at 4 °C at 3.4 V/cm in 2–15% polyacrylamide gel with a running buffer containing 25 mM Tris-HCl (pH 8.4), 192 mM glycine, and 0.5 mM DDM. Proteins were visualized by silver staining.

**Transmission Electron Microscopy**—Purified proteins were adsorbed to thin carbon films rendered hydrophilic by glow discharge in low-pressure air and supported by copper mesh grids. Samples were washed with 10 drops of double-distilled water, negatively stained with 2% uranyl acetate solution for 30 s twice, blotted, and dried in air. Micrographs of negatively stained particles were recorded using Kodak SO163 electron microscopy film (Eastman Kodak, Rochester, NY) using a JEOL 100CX transmission electron microscope (JEOL, Tokyo, Japan) at ×41,000 magnification with 100 kV acceleration voltage. Micrographs were digitized with a Scitex Leafscan 45 scanner (Leaf system Inc., Westborough, MA) with a pixel size of 2.44 Å at the specimen level.

**Automated Particle Selection and Image Analysis**—We developed a single particle image analysis method using neural network (28–30) and simulated annealing (31, 32) named SPINNS (33). For three-dimensional reconstruction, image analysis was performed using SPINNS and the IMAGIC V algorithm (34). TRPM2 projections were first picked up using the auto-accumulation method with simulated annealing (SA) (31). 344 particles in 240 × 240 pixel subframes were selected and used to train a three-layer pyramid-type neural network (28, 29). Using the trained neural network, 3,832 particles were selected. After background subtraction, these 3,832 particles were aligned rotationally and translationally (35, 36) by the reference free method (29). The aligned images were sorted into 150 classes by the modified growing neural gas network method (30). Their class averages were adopted as new references, and this cycle from alignment to averaging was repeated 25 times.

The Euler angles of the class averages were automatically determined by the echo-correlated three-dimensional reconstruction method with SA (32) assuming a C4 symmetry, and used to calculate a primary three-dimensional structure by the SIRT method (37). The reprojections from the volume were employed as references for multi-reference alignment, and raw images in the library were aligned and further clustered, providing improved cluster averages. From the averages, a new three-dimensional map was generated by the reconstruction method using SA without a three-dimensional reference.

The three-dimensional map was further refined by the projection matching method (38) followed by the echo-correlated reconstruction. This cycle was repeated 17 times. Particle images correlating poorly with the three-dimensional projections were automatically rejected using the cross-correlation function. The final reconstruction included 3,604 particles, 94.1% of all the selected images. The resolution of the final three-dimensional map was assessed using the Fourier shell correlation (FSC) function (39) at the intersecting point between signal and noise curves of 3σ, or at the threshold of 0.5.

**Formation of the Channel-Antibody Complex**—Anti-FLAG antibodies were added to the purified TRPM2 protein in antibody binding buffer (Tris-buffered saline containing 1 mM DDM, 350 mM MgCl2, 15% glycerol, and 0.02% sodium azide), and incubated at 4 °C for 1 h. Unbound antibodies were then separated from the TRPM2-antibody complex using Superose 6 gel filtration chromatography (40). Samples were negatively stained and imaged as described above.

Fab fragments of anti-FLAG antibodies were generated by papain digestion using a commercial kit (Pierce). Fab fragments were conjugated with colloidal gold (5 nm in diameter) (BB International), and non-reacted Fab fragments were removed using 10–30% glycerol gradient centrifugation. The Fab-gold conjugates were mixed with the purified TRPM2 in antibody binding buffer, and incubated at 4 °C for 1 h. TRPM2–Fab-gold complexes were separated from unbound Fab-gold conjugates by Superose 6 gel filtration chromatography, then negatively stained and observed by EM.

**RESULTS**

**Expression and Purification of TRPM2 Protein**—As endogenously produced TRPM2 is scarce and not sufficient for purification, we constructed recombinant FLAG-tagged TRPM2. Within the splice variants of TRPM2 reported in the human body, one is the full-length wild type, whereas the others contain different deletions in their sequences (7, 13, 41, 42). The full-length sequence was fused with FLAG tag at the C terminus and expressed in HEK293 cells. The function of the FLAG-tagged TRPM2 channel was measured by the intensity of fura-2 fluorescence and by the whole cell current, and compared with measurements from wild type TRPM2. The H2O2-induced intracellular Ca2+ rise was similar to that of the wild type (Fig. 1A), and the activation by ADPR was also demonstrated to be similar by whole cell patch clamp experiment (Fig. 1, B–D): FLAG-tagged TRPM2 displays a linear voltage-current relationship that is characteristic of the TRPM2 channel (Fig. 1C). These experiments confirmed that the FLAG-tagged TRPM2 protein forms a fully functional channel.
Structure of the TRPM2 Channel

For protein purification, FLAG-tagged TRPM2 proteins were transiently expressed in the HEK293 cells, and their membranes were solubilized with nonionic detergent DDM. The solubilized proteins were applied to an anti-FLAG immunoprecipitation column and competitively eluted by FLAG peptides. The solubilized TRPM2 proteins before and after this chromatography were further separated by SDS-PAGE under the reduced conditions, and visualized by silver staining (Fig. 2A) and Western blotting using three different antibodies (anti-FLAG, anti-N terminus, and anti-C terminus of human TRPM2) (Fig. 2E, F, and G). In both peaks I and II, the majority of the protein species was TRPM2. In other peaks (1.76 and 2.09 M), the band of TRPM2 was not observed by silver staining, or by Western blotting. Furthermore, degraded fragments of TRPM2 were not detected by Western blotting using three different kinds of antibodies. The difference between peaks I and II is speculated to result from the different stoichiometries of TRPM2, and the intensities of TRPM2 protein in the gel corresponded well to the height of the peaks in gel filtration chromatography.

Biochemical Analyses of Purified TRPM2—Subunit stoichiometry of purified TRPM2 was examined by chemical cross-linking. The proteins in peaks I and II were treated with glutaraldehyde to form intramolecular cross-links, then analyzed by SDS-PAGE and visualized by silver staining (Fig. 3A) or by Western blotting using the anti-FLAG antibody (Fig. 3B). Control samples were adopted without glutaraldehyde treatment. Even after glutaraldehyde treatment, the antibody readily detected TRPM2 protein. After cross-linking, the band of TRPM2 in peak I was shifted from monomer size (~160 kDa) to much larger than the 204-kDa marker. This demonstrates that the cross-linked proteins were multimers. In contrast, the band of TRPM2 in peak II did not significantly change even after cross-linking. The mobility was slightly slower than that obtained from monomers, but clearly faster than that of dimers. The reduced speed in electrophoresis after cross-linking could be caused by tight intramolecular cross-links within each subunit that is dissociated as a monomer. The cross-links may decrease the flexibility of TRPM2 proteins to decrease the mobility of the polypeptides in the gel matrix. In the control samples without glutaraldehyde treatment, smear bands were observed at higher molecular weights, especially in Western blotting. These smear bands are speculated to be aggregations of TRPM2, which is frequently observed when TRPM2 proteins are treated with SDS. The difference in the intensities between silver staining and Western blotting could be attributed to the transfer efficiency from the polyacrylamide gradient gel to polyvinylidene difluoride membrane because the large aggregations are transferred more easily to the polyvinylidene difluoride membrane than the smaller sized monomers.

Subunit stoichiometry was further analyzed by native PAGE without cross-linking (Fig. 3C). Proteins in peak I exhibited a predominant band at a higher level than the 669-kDa marker.
monomers in peak II. This agrees with the fact that some kinds of functional TRP molecules are also tetramers (43), like voltage-gated cation channels. In the following study, we analyze the tetrameric TRPM2 in peak I.

**Electron Microscopy and Three-dimensional Reconstruction of TRPM2—Purified TRPM2 protein was blotted onto a glow-discharged carbon grid, negatively stained with 2% uranyl acetate, and imaged using an electron microscope at ×41,000 magnification. Various shaped particles of uniform size were observed (Fig. 4A). Most particles were isosceles triangles or trapezoids with round corners; the minority were squares with round corners. The variation in shapes is interpreted to reflect different orientations of the same molecule on the grid. The square-shaped particles seem to imply top views of the tetrameric form; the isosceles triangles would be side views. The overall shape of the TRPM2 molecule appears to be swollen and bell-like. In the vicinity of some corners of the square and isosceles particles, small blobs (6–8 nm in diameter) were frequently observed (Fig. 4B). Various shaped large aggregates, presumably denatured TRPM2 proteins, were also found in several images.

Because TRPM2 has large N and C termini in the cytoplasm, we tentatively assume that the larger domain is cytoplasmic. To confirm this hypothesis, the FLAG-fused C terminus was labeled with anti-FLAG antibody, negatively stained, and visualized using EM (Fig. 4C). The particle has antibody decorations in the large domain (Fig. 4C, panels 1, 2, 4, and 5). Because the FLAG-fused C terminus is cytoplasmic, the large domain is indicated to locate inside the cell. In the labeled images, TRPM2 bearing multiple antibodies is frequently observed (Fig. 4C, panels 4 and 5). This agrees well with the tetrameric subunit stoichiometry of TRPM2, which allows a maximum of four antibody bindings. Viewed from the top, antibody binding sites locate near corners of the large square (Fig. 4C, panel 3). To make the antibody-labeled image clearer, we prepared Fab fragments from anti-FLAG antibodies and conjugated them with colloidal gold (5
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FIGURE 3. Biochemical analysis of the purified TRPM2 molecule. A, chemical cross-linking of the purified TRPM2. Proteins in peaks I and II from the gel filtration chromatography were treated with or without glutaraldehyde (GA). Samples were separated by SDS-PAGE under the reduced condition and visualized by silver staining. B, Western blot analysis of the cross-linked TRPM2. Cross-linked TRPM2 proteins (same sample as shown in A) were detected by alkaline phosphatase-labeled anti-FLAG antibodies. C, native gel electrophoresis of the purified TRPM2. Proteins in peaks I and II were electrophoresed using the method of Davis (27). TRPM2 proteins in peak I were detected just above the 669 kDa of the standard, which is slightly larger than the estimated size of the tetramer (689 kDa), whereas those in peak II were at the size of the monomer (172 kDa). Cross-linking reaction was not introduced in native gel analysis. Bars indicate the monomer and tetramer of TRPM2. The total amount of proteins applied to each lane were: 0.14, 0.06, 0.04, 0.02, 0.32, and 0.07 μg for A, peaks I and II; B, peaks I and II; and C, peaks I and II, respectively.

FIGURE 4. Electron microscopy of negatively stained TRPM2 molecules. A, after adsorption to hydrophilic carbon film, negatively stained particles were imaged using a JEOL 100CX electron microscope at ×41,000 magnification with 100 kV acceleration voltage. Particles observed in the image represent variously shaped but uniformly sized projections of the molecule, as demonstrated by the arrows. For statistical analysis, 3,832 particles were picked up by a combination of the auto-accumulation method (31) and the three-layered neural network auto-picking system (28, 29). The following image analysis was performed in three steps. First, the 3,832 selected images were aligned rotationally and translationally (35, 36) using the reference free method (29). The aligned images were then classified into 150 clusters using the modified growing neural gas network method (30). Images in each cluster were averaged, and the averages were used as new references. This cycle, from alignment to averaging, was repeated until convergence. Second, Euler angles of the class averages were determined using the echo-correlated three-dimensional reconstruction method with SA (32) assuming C4 symmetry, because of the tetrameric subunit stoichiometry of TRPM2. A primary three-dimensional density map was calculated from the determined angles by the SIRT method (37). The reprojections from the volume were employed as new references for multireference alignment. Each image in the library was aligned and classified, providing improved cluster averages, then a new three-dimensional map was generated by the echo-correlated method without a three-dimensional reference. Third, the density map was further refined by the projection matching (38) followed by echo-correlated reconstruction. This cycle was repeated until convergence. The final reconstruction included 3,604 particles, 94.1% of all the selected images (Fig. 5A, third row). Representative raw images are presented (Fig. 5A, first row), with their corresponding class averages (Fig. 5A, second row), with surface representations (Fig. 5A, third row), and with their projections (Fig. 5A, fourth row). Reproductions from the final volume (Fig. 5A, fourth row) are consistent with the raw images and the class averages, reflecting consistency between the three-dimensional reconstruction and the experimental averages.

A plot of the Euler angles of the 128 adopted class averages (Fig. 5B) shows that TRPM2 is almost randomly oriented on the grid surface. According to the FSC function (39), the resolution...
is 2.8 nm at the intersecting point of signal and noise curves of 3σ, or 3.7 nm if FSC > 0.5 was used as the resolution criterion (Fig. 5C). Considering the absence of channel activators such as H2O2, ADPR, or temperatures greater than 35 °C, this structure is speculated to represent TRPM2 in the closed state.

**Structural Features of the TRPM2 Molecule**—The surface representation demonstrates that TRPM2 is a long, bell-shaped molecule (Fig. 6). From the top view, TRPM2 is a square with round corners, 17 nm in side length and 18 nm diagonally (Fig. 6, panel 1). The molecular height, estimated from the side views, is 25 nm (Fig. 6, panels 3 and 4). For surface representation, the three-dimensional map is contoured at an isosurface containing a volume corresponding to 896 kDa. This is 130% of the tetrameric TRPM2 mass (689 kDa) calculated from the amino acid composition, assuming a protein density of 1.37 g/cm³. The additional volume seems attributed to the glycosylation and the attached lipids and detergents.

The TRPM2 molecule can be separated into three parts (Fig. 6, panels 3 and 4). Between a small hemispheric (half-dome) extracellular domain and a large intracellular domain, a putative TM region is shown by two white lines ~3 nm apart. The TM region has a continuous surface (Fig. 7C, panels 2–5), as in other ion channels (44–46). At the bottom of the large intracellular domain, there is a protrusion consisting of a square prism with round edges measuring 6 nm in width and 5 nm in height (Fig. 6, panels 3 and 4). The prism is rotated almost 30° from the large intracellular domain about a quadrilaterally symmetrical axis (Fig. 6, panel 5). In the final volume, the TM region occupies up to 23%, whereas the extracellular and cytoplasmic regions are 6 and 71%, respectively (Fig. 7B, left). This ratio is somewhat different from the prediction from the amino acid sequence (TM, 8%; extracellular, 6%; cytoplasmic region, 86%) (Fig. 7A), which might be due to lipids and detergents bound to the hydrophobic TM helices.

Sections parallel to the membrane plane are presented to demonstrate the internal structure of the bell-shaped molecule. The images are presented at 1.22-nm intervals throughout the molecule and at 0.97-nm intervals around the TM region (Fig. 7, B, left, and C). The top of the molecule, including both the TM and extracellular domains, has a dense inner structure surrounded by a continuous outer shell (Fig. 7C, panels 1–6). The dense inner structure at the TM region is interpreted as an ion permeation apparatus; probably in the closed state (Fig. 7C, panel 5). In contrast, the large cytoplasmic domain has a sparse structure consisting of delicate pillars and beams, which are spatially disposed at regular intervals (Fig. 7C, panels 8, 9, 15, and 16). At its center are intricate columnar densities (Fig. 7C,
Structure of the TRPM2 Channel

A

B

C

FIGURE 7. Horizontal sections through TRPM2 parallel to the membrane plane. A, a schematic diagram of TRPM2 from the amino acid sequence. The TRPM2 monomer has a long cytosolic N-terminal region, six TM segments (yellow columns, S1–S6), and a cytosolic C-terminal region that includes the TRP domain (magenta box), coiled-coil region (green column), NUDT9-H domain (red box), and terminally fused FLAG tag (blue box). A pore forming region is predicted between SS and S6. According to the topology, the size of the extracellular, TM, and cytoplasmic domains of TRPM2 was calculated to be 6, 8, and 86%, respectively. B, side view of the surface representation of TRPM2 molecule. Positions of cross-sections at 1.22-nm intervals throughout the channel, and at 0.97-nm intervals around the TM region, are indicated by numbers 1–21. Insert, a side view of the surface representation of TRPC3 is shown at half-scale. Two blue lines indicate the putative position of the lipid bilayer. C, sections normal to the symmetric axis of the molecule reveal the nested-box structure of TRPM2. In the cytoplasmic domain, an intricate stem architecture was observed (indicated by white arrows in slice 9, 11, and 16), from under which protrudes a prism with round edges (indicated by black arrows in slices 18 and 20). Scale bars represent 10 nm.

panels 7–16). This structure creates considerable amounts of water-filled space inside (Fig. 7C, panels 7–17). The four vertical edges of the large domain inclined to the quadrilaterally symmetrical axis include large columnar densities inside (Fig. 7C, panels 9–13), which become fainter in the lower sections (Fig. 7C, panels 14–16). TRPM2 has a double layered structure consisting of an outer shell and an inner skeletal core. Both layers are constituted of multiple columns specific to TRPM2, although the double layered structure itself is common to voltage-sensitive sodium channel and inositol 1,4,5-trisphosphate receptor (44, 45). Sections of the prism-like density at the bottom of the molecule (Fig. 7C, panels 18–21) demonstrate somewhat higher densities inside. The protrusion is surrounded by very faint densities (Fig. 7C, panels 18–21), but these disappeared after isosurface contouring. The positions of these faint densities correspond to the blobs in Fig. 4B, the tops of which seem positioned in various directions from the square bottom corners of the cytoplasmic domain.

DISCUSSION

In this study we purified TRPM2 tetramer, and reconstructed a three-dimensional structure at 2.8 nm resolution from EM images. This is the first three-dimensional reconstruction of a member of the TRPM family. TRPM2 has a swollen, bell-like shape with a prism protruding from the cytoplasmic end. The small extracellular domain and the transmembrane region have a dense structure. Inside the molecule, a long inner layer continues from the extracellular domain to the large, sparse cytoplasmic domain.

Within the tetrameric TRPM2 molecule, subunits carry the NUDT9-H domains, which can bind ADPR for gating and show ADPRase activity. In this study, we assigned the NUDT9-H domain by the proximal antibody binding. The epitopes are located near the corners of the large, square cytoplasmic domain, which suggests three candidates for the NUDT9-H domain: (i) the prism protruding from the cytoplasmic end, (ii) the faint, doughnut-like density surrounding it, or (iii) the four pillars at the side corners of the cytoplasmic domain. In the above hypothesis, the NUDT9-H domains are contacting each other in density (i); whereas they are separated in densities (ii) and (iii) above (Fig. 7C). In electrophysiology, the dose-response curve for ADPR-induced gating of TRPM2 demonstrates cooperative ADPR concentration dependence (10, 11). Multiple binding of the antibodies also suggests cooperative ADPR binding to NUDT9-H domains through interactions with each domain, whereas homologous mitochondrial NUDT9 ADPRase can work as a monomer only in terms of catalytic reaction (47). This leads to the conclusion that the quadrilaterally symmetrical protrusion at the cytoplasmic end (density (ii)) is the most promising candidate for the NUDT9-H domain, and densities (ii) and (iii) are unlikely this domain. The hypothesis of contacts among NUDT9-H domains is also supported by the fact that the neighboring coiled-coil region is only 30 residues distant from the NUDT9-H domain (Fig. 7A). These coiled-coil regions of TRPM2 molecule are reported to be essential for subunit-subunit contact (48, 49), implying that four NUDT9-H domains are spatially arranged in the close proximity.

Recently, the crystal structure of the mitochondrial NUDT9 that is homologous to the NUDT9-H domain has been determined (Protein Data Bank 1Q33) (21). Taking into account the dimensions of this structure, the monomeric NUDT9-H domain is estimated to be 3 × 4 × 7 nm if observed at the 2.8-nm resolution assessed in this study. In the isosurface of TRPM2 contoured at 130% of the molecular mass, the dimensions of the prism-shaped protrusion (6 × 6 × 5 nm) seem insufficient to harbor the tetrameric NUDT9-H domain (estimated as 7 × 7 × 7 nm). The unassigned region of the tetrameric domain, especially the top, may harbor in the bottom part of the large cytoplasmic domain.

We have recently reconstructed TRPC3 from cryo-EM images (Fig. 7B, right) (22). TRPC3 has an expanded cytoplasmic domain (density (iii)) is the most promising candidate for the NUDT9-H domain, whereas densities (ii) and (iii) are unlikely this domain. The hypothesis of contacts among NUDT9-H domains is also supported by the fact that the neighboring coiled-coil region is only 30 residues distant from the NUDT9-H domain (Fig. 7A). These coiled-coil regions of TRPM2 molecule are reported to be essential for subunit-subunit contact (48, 49), implying that four NUDT9-H domains are spatially arranged in the close proximity.

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mic structure with large molecular dimensions (20 × 20 × 24 nm), much larger than the size estimation from its amino acid sequence (388 kDa as a tetramer). A globular inner chamber surrounded by delicate wireframe structures includes a large amount of water-filled space. These expanded frames may simultaneously sense various signals, including diacylglycerol, receptor-activated phospholipase Cγ (50, 51), and/or calcium store depletion (52, 53), which are different from those for TRPM2. The overall structure of TRPM2 resembles that of TRPC3 in that TRPM2 has similar dimensions (17 × 17 × 25 nm), but TRPM2 has almost twice the molecular mass (689 kDa as a tetramer) of TRPC3. For both types of channels, four prominent thick pillars are located at side corners of the cytoplasmic domain.

Besides the structural similarities to TRPC3, a detailed structural analysis revealed features unique to TRPM2. First, TRPM2 has a more intricate architecture: the swollen cytoplasmic domain consists of delicate stems, which might be developed to accommodate its various kinds of ligands. The N-terminal region characteristic of the TRPM family could be attributed to these intricate structures, because this region is long enough (752 amino acids in TRPM2) to form this structure (2–5). In contrast, TRPC3 has a relatively shorter N-terminal region (381 amino acids) than TRPM2. Second, TRPM2 has the football-shaped protuberances on the cytoplasmic end that protrusion, TRPC3 has a short receptor consisting of four small regions (NUDT9-H and inositol 1,4,5-trisphosphate receptor). In both channels (TRPM2 and TRPC3), control functions, such as ligand binding and gating, further improve-activity for ADPR (19), which is likely to be mediated by calmodulin bound to the N-terminal residues 406–416 of TRPM2 (54). Furthermore, these signals synergically regulate the gating of the TRPM2 channel. For example, ADPR-dependent gating of TRPM2 is accelerated by cADPR at low concentrations (15). The intricate, expanded cytoplasmic domain of TRPM2 is interpreted to be a sensor with multidocking ports that simultaneously receive various stimuli, as is the case with TRPC3 (22). The expanded structure also enables TRPM2 to integrate gating signals, further modulating ADPR-dependent gating, through its conformational changes, because the NUDT9-H domain seems structurally integrated to the intricate stem-pil-lar architecture of the cytoplasmic domain. Because the expanded cytoplasmic structure is shared among TRP channels, this architecture may be common to TRP channels as a multimodal sensor.

In this study, we have presented the closed state of TRPM2 from negatively stained EM images. To understand the channel functions, such as ligand binding and gating, further improve-

ment in resolution is required. Structural analysis at improved resolution should be performed in the future using cryo-EM single particle analysis.

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REFERENCES

1. Montell, C., and Rubin, G. M. (1989) Neuron 2, 1313–1323
2. Harteneck, C., Plant, T. D., and Schultz, G. (2000) Trends Neurosci. 23, 159–166
3. Minke, B., and Cook, B. (2002) Physiol. Rev. 82, 429–472
4. Clapham, D. E. (2003) Nature 426, 517–524
5. Montell, C. (2005) Sci. STKE 2005, re3
6. Mio, K., Ogura, T., Hara, Y., Mori, Y., and Sato, C. (2005) Biochem. Biophys. Res. Commun. 333, 768–777
7. Naganine, K., Kudoh, J., Minoshima, S., Kawashita, K., Asakawa, S., Ito, F., and Shimizu, N. (1998) Genomics 54, 124–131
8. Hara, Y., Wakamori, M., Ishii, M., Maeno, E., Nishida, M., Yoshihara, T., Yamada, H., Shimizu, S., Mori, E., Kudoh, J., Shimizu, N., Kurose, H., Okada, Y., Imoto, K., and Mori, Y. (2002) Mol. Cell. 9, 163–173
9. Inamura, K., Sano, Y., Mochizuki, S., Yokoi, H., Miyake, A., Nozawa, K., Kitada, C., Matsushima, H., and Furuchi, K. (2003) J. Membr. Biol. 191, 201–207
10. Perraud, A. L., Fleig, A., Dunn, C. A., Bagley, L. A., Launay, P., Schmitz, C., Stokes, A. J., Zhu, Q., Bessman, M. J., Penner, R., Kinet, J. P., and Scharenberg, A. M. (2001) Nature 411, 595–599
11. Sano, Y., Inamura, K., Miyake, A., Mochizuki, S., Yokoi, H., Matsushima, H., and Furuchi, K. (2001) Science 293, 1327–1330
12. Heiner, I., Eisfeld, J., Halaszovich, C. R., Wehage, E., Jüngling, E., Zitt, C., and Lückhoff, A. (2003) Biochem. J. 371, 1045–1053
13. Wehage, E., Eisfeld, J., Heiner, I., Jüngling, E., Zitt, C., and Lückhoff, A. (2002) J. Biol. Chem. 277, 23150–23156
14. Kraft, R., Grimm, C., Grosse, K., Hoffmann, A., Sauerbruch, S., Kettenmann, H., Schultz, G., and Harteneck, C. (2004) Ann. J. Physiol. 286, C129–C137
15. Kolisek, M., Beck, A., Fleig, A., and Penner, R. (2005) Mol. Cell. 18, 61–69
16. Togashi, K., Hara, Y., Tominaga, T., Higashi, T., Konishi, Y., Mori, Y., and Tominaga, M. (2006) EMBO J. 25, 1804–1815
17. Perraud, A. L., Takahashi, C. L., Shen, B., Kang, S., Smith, K. M., Schmitz, C., Knowles, H. M., Ferraris, D., Li, W., Zhang, J., Stoddard, B. L., and Scharenberg, A. M. (2005) J. Biol. Chem. 280, 6138–6148
18. Grubisha, O., Rafty, L. A., Takahashi, C. L., Xu, X., Tong, L., Perraud, A. L., Scharenberg, A. M., and Denu, J. M. (2006) J. Biol. Chem. 281, 14057–14065
19. McHugh, D., Fleming, R., Xu, S. Z., Perraud, A. L., and Beech, D. J. (2003) J. Biol. Chem. 278, 11002–11006
20. Kühn, F. J., and Lückhoff, A. (2004) J. Biol. Chem. 279, 46431–46437
21. Shen, B. W., Perraud, A. L., Scharenberg, A., and Stoddard, B. L. (2003) J. Biol. Chem. 282, 385–393
22. Mio, K., Ogura, T., Kiyonaka, S., Hiroaki, Y., Tominaga, T., Furuichi, K., Ito, Y., Mori, Y., and Sato, C. (2007) J. Biol. Chem. 282, 385–393
23. Graham, F. L., and van der Eb, A. J. (1973) J. Mol. Biol. 81, 404–427
24. Ogura, T., and Sato, C. (2001) J. Struct. Biol. 136, 227–238
25. Ogura, T., and Sato, C. (2004) J. Struct. Biol. 145, 63–75
26. Ogura, T., Iwakawa, K., and Sato, C. (2003) J. Struct. Biol. 143, 185–200
27. Ogura, T., and Sato, C. (2004) J. Struct. Biol. 146, 344–358
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32. Ogura, T., and Sato, C. (2006) J. Struct. Biol. 156, 371–386
33. Yazawa, M., Ferrante, C., Feng, J., Mio, K., Ogura, T., Zhang, M., Lin, P. H., Pan, Z., Komazaki, S., Kato, K., Nishi, M., Zhao, X., Weisleder, N., Sato, C., Ma, J., and Takeshima, H. (2007) Nature 448, 78–82
34. van Heel, M., Harauz, G., Orlova, E. V., Schmidt, R., and Schatz, M. (1996) J. Struct. Biol. 116, 17–24
35. Frank, J. (2006) Three-Dimensional Electron Microscopy of Macromolecular Assemblies: Visualization of Biological Molecules in Their Native State, Oxford University Press, New York
36. van Heel, M., Gowen, B., Matadeen, R., Orlova, E. V., Finn, R., Pape, T., Cohen, D., Stark, H., Schmidt, R., Schatz, M., and Patwardhan, A. (2000) Q. Rev. Biophys. 33, 307–369
37. Penczek, P., Radermacher, M., and Frank, J. (1992) Ultramicroscopy 40, 33–53
38. Penczek, P. A., Grassucci, R. A., and Frank, J. (1994) Ultramicroscopy 53, 251–270
39. Harauz, G., and Van Heel, M. (1986) Optik 73, 146–156
40. Sato, C., Sato, M., Iwasaki, A., Doi, T., and Engel, A. (1998) J. Struct. Biol. 121, 314–325
41. Zhang, W., Chu, X., Tong, Q., Cheung, J. Y., Conrad, K., Masker, K., and Miller, B. A. (2003) J. Biol. Chem. 278, 16222–16229
42. Uemura, T., Kudoh, J., Noda, S., Kanba, S., and Shimizu, N. (2005) Biochem. Biophys. Res. Commun. 328, 1232–1243
43. Hoenderop, J. G., Voets, T., Hoefs, S., Weidema, F., Prelen, J., Nilius, B., and Bindels, R. J. (2003) EMBO J. 22, 776–785
44. Sato, C., Ueno, Y., Asai, K., Takahashi, K., Sato, M., Engel, A., and Fujioyoshi, Y. (2001) Nature 409, 1047–1051
45. Sato, C., Hamada, K., Ogura, T., Miyazawa, A., Iwasaki, K., Hiroaki, Y., Tani, K., Terauchi, A., Fujioyoshi, Y., and Mikoshiba, K. (2004) J. Mol. Biol. 336, 155–164
46. Miyazawa, A., Fujioyoshi, Y., Stowell, M., and Unwin, N. (1999) J. Mol. Biol. 288, 765–786
47. Perraud, A. L., Shen, B., Dunn, C. A., Rippe, K., Smith, M. K., Bessman, M. J., Stoddard, B. L., and Scharenberg, A. M. (2003) J. Biol. Chem. 278, 1794–1801
48. Tsuruda, P. R., Julius, D., and Minor, D. L., Jr. (2006) Neuron 51, 201–212
49. Mei, Z. Z., Xia, R., Beech, D. J., and Jiang, L. H. (2006) J. Biol. Chem. 281, 38748–38756
50. Nishida, M., Sugimoto, K., Hara, Y., Mori, E., Morii, T., Kuroskii, T., and Mori, Y. (2003) EMBO J. 22, 4677–4688
51. van Rossum, D. B., Patterson, R. L., Sharma, S., Barrow, R. K., Kornberg, M., Gill, D. L., and Snyder, S. H. (2005) Nature 434, 99–104
52. Zhu, X., Jiang, M., Peyton, M., Boulay, G., Hurst, R., Stefani, E., and Birnbaumer, L. (1996) Cell 85, 661–671
53. Vazquez, G., Lievremont, J. P., Bird, G. S. I., and Putney, J. W., Jr. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 11777–11782
54. Tong, Q., Zhang, W., Conrad, K., Mostoller, K., Cheung, J. Y., Peterson, B. Z., and Miller, B. A. (2006) J. Biol. Chem. 281, 9076–9085