Molecular Bases of Multimodal Regulation of a Fungal Transient Receptor Potential (TRP) Channel*

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Background: Multimodality of TRP channels underlies their diverse physiological functions.

Results: We identified a fungal multimodal TRP channel whose cytosolic domain (CTD) mediates various channel regulation.

Conclusion: CTD has an oligomerization module critical for osmoreception, yet its flexible structure allows dynamic regulations with other functional modalities.

Significance: This work proposes structural and biophysical principles for multimodality of a TRP channel family member.

Multimodal activation by various stimuli is a fundamental characteristic of TRP channels. We identified a fungal TRP channel, TRPGz, exhibiting activation by hyperosmolarity, temperature increase, cytosolic Ca2+ elevation, membrane potential, and H2O2 application, and thus it is expected to represent a prototypic multimodal TRP channel. TRPGz possesses a cytosolic C-terminal domain (CTD), primarily composed of intrinsically disordered regions with some regulatory modules, a putative coiled-coil region and a basic residue cluster. The CTD oligomerization mediated by the coiled-coil region is required for osmoreception, yet its flexible structure allows dynamic regulations with other functional modalities.

Transient receptor potential (TRP) channels are tetrameric non-selective Ca2+-permeable cation channels existing in a wide variety of fungi and animals (1). The TRP channels exhibit relatively diverse primary sequences and are divided into seven subfamilies: TRPC, TRPV, TRPM, TRPML, TRPN, TRPP, and TRPA (2). With regard to their physiological functions, TRP channels serve as sensors for various environmental stimuli, such as temperature, chemical substances, and osmolality (3). The physiological relevance of TRP channels has long been implicated in store-operated calcium entry (4), although this aspect still remains controversial. TRP channels also act as voltage-gated cation channels (5). Notably, many important physiological functions are achieved by a single type of TRP channel that can respond to both physical and chemical stimuli. For example, TRPV1 is activated by both high temperature (∼=42 °C) and capsaicin (6); TRPV4 is activated by both warm temperature (∼=27–42 °C) (7, 8) and high osmolality (9, 10); and TRPA1 is activated by nocuous cold temperature (∼=17 °C) (11), pungent chemical substances, such as mustard oils (12, 13), and O2 (14). The “multimodality” of the channel regulation is one of the most remarkable features of the TRP channel functions.

The TRP channel core domain consists of six transmembrane (TM) regions, with a central channel pore composed of a re-entrant loop between TM5 and TM6 from each protomer in the tetramer, which is conserved among not only the TRP channels but also the voltage-gated channel superfamilies (15). On the other hand, the molecular architectures of the N and C termini of the cytosolic domains are highly diverse among the TRP family members. The domains usually consist

*This work was supported by the Funding Program for Next Generation World-Leading Researchers (NEXT Program) from the Japan Society for the Promotion of Science (JSPS), the Council for Science and Technology Policy (CSTP) (to A.Y.), and the Targeted Proteins Research Program (TPRP) from the Ministry of Education, Culture, Science and Technology (MEXT), Japan (to A.Y. and M.K.) and Grants-in-aid for Scientific Research from MEXT (to S.H. and N.U.).

The atomic coordinates and structure factors (code 3VVI) have been deposited in the Protein Data Bank (http://wwpdb.org/).

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3 The abbreviations used are: TRP, transient receptor potential; CC, coiled-coil; CTD, C-terminal cytosolic domain; FSEC, fluorescence-detection size-exclusion chromatography; HMOC, heteronuclear multiquantum correlation; HSQC, heteronuclear single-quantum correlation; PI, phosphatidylinositol; PIP, phosphatidylinositol phosphate; TM, transmembrane; TEV, tobacco etch virus; Tricine, N-(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)glycine; TCEP, tris(2-carboxyethyl)phosphine.
Multimodal Regulation of a Fungal TRP Channel

of multiple functional domains or regions for channel regulation (16), such as ankyrin repeats as bait for protein or ligand interactions (17), a coiled-coil region that probably functions in channel assembly (18), an EF-hand motif (19) or a calmodulin binding motif (20) for Ca\(^{2+}\)-mediated regulation, and a phosphatidylinositol phosphate (PIP) binding domain for regulation (21). The structural information for TRP channels is limited to a few individual functional modules (22–28), and low resolution electron microscopy structural studies of entire TRP channels revealed large cytosolic domains with chamber-like structures (29–32, 77). However, the molecular mechanisms by which the functional modules in the cytosolic domains regulate the channel activity have remained unclear. Furthermore, in order to attain the multimodality, each module should be functionally compatible in a single channel; for example, in some cases, several modules should operate simultaneously. The molecular bases of the module integration in the cytosolic domains, enabling the mutual compatibility between the modules, have not been investigated extensively.

Among the TRP channel family members, those possessing cytosolic domains with relatively small and thus simple structures were identified in fungi (33–35). The TRP channel from the yeast Saccharomyces cerevisiae, Yvc1 or TRPY1, has been most extensively characterized (33, 34, 36, 37). TRPY1 is a Ca\(^{2+}\) release channel in yeast vacuoles that functions upon hyperosmotic shock (33, 34). TRPY1 is reportedly activated by cytosolic Ca\(^{2+}\) elevation (34, 38, 39) and membrane stretching (37), and its function is affected by the vacuolar PIPs level (40). Therefore, the fungal TRP channels are also likely to possess similar channel regulation multimodality as the other mammalian TRP channels, despite their smaller cytosolic domains.

In this study, we addressed the multimodality of TRP channels by analyzing the fungal TRP channels. We identified a TRP channel homolog from Gibberella zeae, which shares sequence similarity with TRPY1 but exhibits substantially higher responses to multiple stimuli, as a good model for investigation. The integrated approaches with biochemical, physiological, crystallographic, and NMR spectroscopic analyses revealed the structural and functional correlations between the modular characteristics of the cytosolic domain and the multimodal regulation of the channel activities.

**EXPERIMENTAL PROCEDURES**

**Materials**—Ultrapure water from MilliQ synthesis (Millipore) with resistance of >18.3 megaohms/cm and <3 ppb total organic carbon was used for all research, including preparation of culture media, if not mentioned otherwise. For general purposes, the highest purity chemical reagents available from either Nacalai Tesque, Wako Pure Chemical, Kanto-Chemical, or Sigma-Aldrich Japan were used. The peptide TRPGz-CC, corresponding to residues 600–620, was obtained from either Hokkaido System Science or RIKEN Research Resource Center, and its selenomethionine derivative was purchased from Dr. Yoshitaka Takano (Kyoto University). The gene encoding TRPY1 (NM_001183506.1) was provided by Prof. Ching Kung (University of Wisconsin).

**Vector Preparation for Functional Assays of TRPGz and TRPY1**—For yeast expression constructs, the p416GALL (ATCC87340) and pDR195 (42) vectors were used. The multiple cloning sites of both vectors were modified to Xhol and SpeI sites, placed at the 5′- and 3′-ends of multiple cloning sites, respectively. For the construction of yeast expression vectors for the C-terminal GFP fusion proteins (p416GALL-TRPGz-GFP and TRPY1-GFP; pDR195-TRPGz-GFP and TRPGzΔCC-GFP), the coding regions of TRPGz (Met\(^1\)–Asn\(^{692}\)), TRPGzΔCC (Met\(^1\)–Asn\(^{692}\) with the deletion of Val\(^{690}\)–Thr\(^{692}\)), TRPGzΔ600− (Met\(^1\)–Glu\(^{599}\)), and TRPGzΔ621− (Met\(^1\)–Thr\(^{620}\)), followed by a TEV protease digestion site and the yEGFP (43), provided by Prof. Alistair Brown (University of Aberdeen), were inserted between Xhol and SpeI sites of the modified pDR195 and p416GALL vector. For the construction of yeast expression vectors for the C-terminal His tag fusion proteins (pDR195-TRPGz, TRPY1, TRPGzΔCC, TRPGzΔ600−, TRPGzΔ621−, TRPGzM607D/L610D, TRPGzL610R, and TRPGzE606P), the corresponding genes, followed by a TEV protease digestion site and a hexahistidine tag, were inserted into modified pDR195 vector between the Xhol and SpeI sites. The M607D, L610D, and E606P mutations were introduced by an inverse PCR-mediated method. For the construction of Escherichia coli expression vectors for the His-tagged C-terminal domains (pET-19b-TRPGz-CTD, TRPGz-CTDΔCC, TRPGz-CTD-M607D/L610D, TRPGz-CTD-L610R, and TRPGz-CTD-E606P), the genes encoding the wild type TRPGz C-terminal cytosolic domain (CTD, Ala\(^{539}\)–Asn\(^{692}\)) or with the corresponding mutations, followed by a TEV protease digestion site and the hexahistidine tag, were inserted in the pET-19b (Novagen) vector between the Ncol and Xhol sites. For the construction of E. coli expression vectors for the N-terminal glutathione S-transferase (GST) fusion proteins (pET42-TRPGz-CTD, pET42-TRPGz-CTDΔC, pET42-TRPGz-CTDΔN, and pET42-TRPGz-CTDΔC), the coding sequences of TRPGz-CTD, TRPGz-CTDΔN (Ala\(^{539}\)–Thr\(^{620}\)), and TRPGz-CTDΔC (Leu\(^{614}\)–Asn\(^{692}\)), with the TEV protease digestion site at their 5′ termini, were inserted in the pET-42b (Novagen) vector between the Spel and Xhol sites.

**Transformation and Culture of Yeast Cells**—Transformation of S. cerevisiae cells was performed according to the method optimized by Akada et al. (44). Briefly, S. cerevisiae cells were cultured on a YPD agar plate overnight, the day before transformation. Immediately before the transformation, the yeast cells were collected from the YPD agar plate by suspension in sterilized water and pelleting by a tabletop centrifuge for 30 s. The cells were washed once with transformation buffer composed of 667 μl of 60% PEG 4000, 100 μl of 1× DTT, 50 μl of 4 M lithium acetate, and 183 μl of MilliQ water, and they were resuspended in transformation buffer. Usually, the cells from an overnight culture on one 90-mm YPD plate were suspended into 1 ml of transformation buffer, and the volume of the yeast cell pellet was usually about 200–400 μl. A 50–50% portion of the competent yeast cells was added to the of DNA mixture (50 μg of heat denatured carrier DNA and 50–500 ng of DNA for
transformation) and incubated for 2–3 h at 42 °C. After this period of incubation, the yeast cells were diluted 2-fold in the sterile water. The diluted cells were spread on agar plates containing synthetic complete medium lacking specific nutrients, such as l-leucine for the pEVP11/AEQ vector (see below) or uracil for the pDR195 and p416GALL vectors, for transformant selection.

The transformants were inoculated in the appropriate culture media and cultured at 30 °C. Unless otherwise stated, the YKO11863 strain transformed with p416GALL-based expression vectors was cultured in the Sc-dropout medium (−Ura, −Leu) supplemented with 1% (w/v) galactose and raffinose. The SH1007 strain transformed with pDR195-based expression vectors was cultured in the same medium supplemented with 2% (w/v) glucose.

Confocal Microscopic Observations of Localization in S. cerevisiae Cells—The yeast TRPY1 knock-out strain YKO11863 was used for microscopic observations. The yeast cells were transformed with plasmids encoding either p416GALL-TRPGrz-GFP or p416GALL-TRPY1-GFP. Yeast vacuoles were stained with CellTracker Blue CMAC dye (Invitrogen) according to the manufacturer’s recommendations, and the fluorescent signals from both the GFP and CMAC dye were monitored with a Zeiss thermomodule of the thermal cycler for PCR, and after the thermodenaturation was achieved by ZAP pulse (20 ms, 1.2 V). The pipette solution contained 100 mM KCl, 1 mM MgCl2, 200 mM sorbitol, 10 mM Tris-MES, pH 7.5, to facilitate the access of the patch pipette to the vacuole membrane. The whole-cell configuration was achieved by ZAP pulse (20 ms, 1.2 V). The pipette solution contained 100 mM KCl, 1 mM MgCl2, 200 mM sorbitol, and 10 mM Tris-MES, pH 7.5. The bath solution was the same unless otherwise stated. To determine the Ca2+ dependences of TRPGrz, patch clamp recording in the cytoplasmic side-out excised patch configuration was performed. Measurements were performed with the same pipette solutions as described above, and the bath solution was the same except for the Ca2+ concentration. To prepare a low concentration of free Ca2+, a Ca2+-EGTA buffered solution was employed. For [Ca2+] = 10 µM, 1 mM EGTA and 1.058 mM CaCl2 were used, for [Ca2+] = 1 µM, 1 mM EGTA and 0.966 mM CaCl2 were used, and for [Ca2+] = 0.1 µM, 1 mM EGTA and 0.701 mM CaCl2 were used. The currents were amplified by a patch clamp amplifier (CEZ2400, Nihon-Koden) and recorded by a digital data recorder (EX-RP10, Sony). All recording was performed using the standard patch clamp technique at 25 °C.

Fluorescence-Detection Size-Exclusion Chromatography (FSEC) (48)—Yeast cells (SH1007) transformed with pDR195-TRPGrz-GFP and pDR195-TRPGrzΔCC-GFP were cultured overnight in conventional YPD medium and then harvested by centrifugation at 5,000 × g for 2 min at room temperature. The cells were resuspended in 500 µl of lysis buffer (50 mM Tris, pH 7.5, 200 mM NaCl, 20 mM KCl, supplemented with cOmplete protease inhibitor mixture (Roche Applied Science)) in a 2.0-ml polypropylene test tube. After approximately the same volume of precooled glass beads was added, the mixture was vortexed for 15 min at 4 °C and then centrifuged at 15,000 × g for 5 min at 4 °C. After the supernatant was recovered, a 500-µl portion of lysis buffer was added, and the previous procedure was repeated. The supernatants were combined and ultracentrifuged at 50,000 rpm for 30 min, using a TLA55 rotor at 4 °C, to obtain the yeast total membrane fraction.
Multimodal Regulation of a Fungal TRP Channel

The total membrane fractions were solubilized with 40 mM digitonin by an incubation for 3 h at 4 °C and then subjected immediately to the FSEC analyses. FSEC was performed with a SEC-5 column (500-Å pore size, 4.6 x 300 mm, Agilent), using a Shimadzu isocratic HPLC system with FSEC buffer, composed of 50 mM Tris, pH 7.5, 200 mM NaCl, 20 mM KCl, and 1 mM digitonin. The fluorescence of yEGFP was monitored, with then 1.0-ml aliquots were treated with 0.1 mM disuccinimidyl glutarate for 0.5 h at ambient temperature. After this incubation, the reaction mixtures were centrifuged at 13,000 g for 5 min, and a 10-μl aliquot was subjected to SDS-PAGE (SuperSepAce 15–20% protein at a concentration of 0.75 g/ml in 5 ml of lysis buffer. The cell pellets were then mixed with SDS-PAGE sample loading buffer and separated on 3–8% NuPAGE Tris-acetate SDS-polyacrylamide gels (Invitrogen). The gels were subjected to electroblotting onto nitrocellulose membranes with an iBlot system (Invitrogen), and the protein bands of interest were detected using an HRP-conjugated anti-penta-His antibody (Qiagen) and Immobilon Western Chemiluminescent HRP substrate (Millipore).

Protein Preparation—GST fusion proteins were expressed in E. coli cells expressing TRPGz-CTD were disrupted in phosphate-buffered saline composed of 50 mM sodium phosphate, 250 mM NaCl, 250 mM KCl, and 2 mM TCEP (pH 8.0) supplemented with cOmplete protease inhibitor mixture. The lysate was applied to a Ni²⁺-NTA Superflow (Qiagen) column packed in house (~10 ml in bed volume), which was washed with the same buffer without protease inhibitor mixture. The proteins of interest were eluted by elution buffer, composed of 20 mM sodium phosphate, 250 mM NaCl, 250 mM KCl, 1 mM TCEP, and 250 mM imidazole (pH 8.0). The eluted protein was treated with TEV protease, prepared in house according to van den Berg et al. (50), at 4 °C for 8–12 h, and then dialyzed against ~20 volumes of the dialysis buffer (20 mM Tris, 1 mM TCEP, pH 8.0) for 3–4 h to reduce the salt concentration of the samples for the following anion exchange chromatography. The partially desalted proteins were applied to a Source Q (GE Healthcare) anion exchange column and purified by elution with a 0–40% (B) linear gradient of the following buffer combinations (A, 20 mM Tris, 1 mM TCEP, pH 8.0; B, 20 mM Tris, 500 mM NaCl, 500 mM KCl, 1 mM TCEP, pH 8.0). The identities of the purified proteins were confirmed by MALDI-TOF or electrospray ionization-Q-TOF-MS.

Lipid Binding Assays—For the lipid overlay assays, membrane lipid strips and PIP Strips (Echelon) were used, according to the supplier’s recommended protocols. The strips were blocked by Blocking One (Nacalai Tesque) for 1 h at room temperature and then probed with the GST fusion of the test protein at a concentration of 0.75 µg/ml in 5 ml of lysis buffer. The strips were incubated overnight (~12 h) at 4 °C with gentle shaking. Detection of lipid binding was performed using an HRP-conjugated anti-GST antibody (MBL) at a 1:5,000 dilution and Immobilon Western Chemiluminescent HRP substrate (Millipore).

For the liposome co-sedimentation experiments, PolyPiPosome (Echelon) was used. A mixture of 5 µg of the GST-fused test proteins, 15 µl of PolyPiPosome, and 1 ml of Tris-buffered saline (50 mM Tris (pH 7.5), 200 mM NaCl, and 20 mM KCl) containing 0.05% IGepal CA-630 was incubated for 10 min with gentle agitation at 25 °C. The mixture was then centrifuged for 10 min at 21,500 × g and 25 °C. The supernatant was recovered for analysis, and the precipitate was mixed with 250 µl of Tris-buffered saline and centrifuged for 10 min at 21,500 × g and 25 °C. This washing procedure was performed three times, and the precipitate obtained after the washing step was recovered for analysis. For Western blotting analyses, 20 µl of 2X loading buffer for SDS-PAGE was added to the precipitated fractions. The mixtures were heated at 95 °C for 5 min, and a 10-µl portion was subjected to SDS-PAGE (SuperSepAce 15–20% Tris-Tricine gels, Wako). For the supernatant fractions, an 8-µl portion of the supernatant fractions was mixed with 2 µl of 5X
Ultrascan 3 package.

Analytical Ultracentrifugation—The sedimentation equilibrium experiment was performed at 4 °C using an Optima XL-I system (Beckman Coulter), equipped with an An-60 Ti rotor. TRPGz-CTD and its mutants were dialyzed against 50 mM Tris (pH 7.5), 200 mM NaCl, 20 mM KCl, and 1 mM TCEP. The absorbance of the test protein solutions at 280 nm was adjusted to 0.5, 1.0, and 2.0, corresponding to ∼0.5, 1.0, and 2.0 mg/ml protein, respectively. A 100-μl portion of the test protein solution was placed in the six-channel Epon charcoal-filled centerpiece, with 110 μl of optical blank. Centrifugation was performed at speeds of 8,000, 12,000, and 16,000 rpm. Data were collected using interference optics and processed using the XL-A/ XL-I data analysis software version 6.04 (Beckman Instruments) using the self-association model. The obtained association constants were converted to molar units with a conversion factor of 3.26 fringes/mg/ml/1.2 cm, with the molecular weight calculated from the primary sequence of the test protein. Partial specific volumes and solvent density were calculated by the Ultrascan 3 package.

NMR Spectroscopy—The [1-13C]Met-, U-15N-labeled TRPGz-CTD samples were concentrated to 0.2 mM protein in 5 mM HEPES buffer (pH 7.5), containing 50 mM NaCl, 50 mM KCl, 2 mM TCEP, 0.01% sodium 2,2-dimethyl-2-silapentane-5-sulfonate, and 1% D2O. The NMR measurements were performed using an Avance900 spectrometer equipped with a TCI cryogenic probe (Bruker Biospin) at 25°C. In the two-dimensional 1H-15N heteronuclear single quantum correlation (HSQC) experiments, the data size and spectral width were 256 (τ1) × 2048 (τ2) and 3300 Hz (ω1, 15N) × 14,400 Hz (ω2, 1H), respectively. The carrier frequencies of 1H and 15N were 4.7 and 117 ppm, respectively. The number of scans/free induction decay was 16. In the two-dimensional 1H-13C heteronuclear multiquantum correlation (HMQCC) experiments, the data size and spectral width were 128 (τ1) × 2048 (τ2) and 2,700 Hz (ω1, 13C) × 14,400 Hz (ω2, 1H), respectively. The carrier frequencies of 1H and 13C were 4.7 and 14 ppm, respectively. The number of scans/free induction decay was 16. All NMR spectra were processed with TopSpin version 3.1 software (Bruker Biospin).

RESULTS

TRPGz Is a TRP Channel Homolog in the Fungal Vacuolar Membrane That Responds to Various Extracellular and Cytoplasmic Stimuli—G. zeae is a plant pathogenic ascomycete responsible for Fusarium head blight on wheat and barley as well as being an important model organism for biological studies (57). The G. zeae-derived TRP channel homolog, hypothetical protein FG04178.1, which we named TRPGz, consists of 692 amino acid residues and shares sequence similarity with TRPY1 (with 40% amino acid identity among 533 core residues; Fig. 1). A BLAST search using the homolog sequence against human proteins produced TRPC6, -7, and -3 as the highest scoring hits, with 23% sequence identity (among 280 core residues) with TRPC6. The important residues for TRPY1 channel gating that are conserved in other TRP channels, such as Phe380 in the putative TM5 and Tyr458 in the putative TM6 (58), are also conserved in this homolog (Fig. 1). These results implied that TRPGz shares a similar architecture and functions with other TRP channels, especially TRPY1.

We cloned the TRPGz gene and analyzed its molecular functions by expressing the protein in TRPY1 knock-out strains of S. cerevisiae. Fluorescent microscopy revealed that TRPGz localized at the vacuolar membrane (Fig. 2A). Furthermore, TRPGz responded to extracellularly applied hyperosmotic shock, resulting in an increase in the cytosolic calcium concentration to an even larger extent than that observed with TRPY1 (Fig. 2B). The response was observed upon the addition of 1.2 M sorbitol and became saturated at about 1.4 M (Fig. 2C). These results strongly indicated that, like TRPY1 (33), TRPGz is a vacuolar membrane protein responsible for Ca2+ release from the vacuole to the cytosol upon osmotic shock.

Whole-yeast vacuole patch clamp recording (41) revealed that TRPGz evoked a voltage-dependent cation current. The current amplitude was also increased by the supplementation of Ca2+ to the cytoplasmic side (Fig. 2D). The channel activation by cytosolic Ca2+ was observed in a concentration-dependent manner, with an effective concentration of at least 1 μM Ca2+ (Fig. 2E). We also found that TRPGz responded to an extracellularly applied oxidizing reagent, hydrogen peroxide, which evoked cytosolic Ca2+ elevation (Fig. 2F) in a concentration-dependent manner, with a similar range between 0.2 and 5.0 mM (Fig. 2G), as reported by Popa et al. (59). The level of responses was not saturated within the range of the tested H2O2 concentration. Interestingly, TRPGz-expressing yeast cells showed a marked cytosolic Ca2+ increase upon rapid temperature elevation from 25 to 40 °C (Fig. 2H). It should be noted that the responses were not significant in the cases of stepwise temperature elevation (Fig. 2I), implying the possibility that the observed response is not necessarily derived from the direct temperature sensing but also a consequence of the effect caused by rapid temperature increase.

These results indicated that the TRPGz channel is essential for responses to various multiple cytoplasmic and extracellular stimuli, such as cytosolic Ca2+, membrane potential, extracellular oxidizer application, and temperature change as well as...
osmotic upshock. Therefore, TRPGz probably plays key roles in Ca\textsuperscript{2+}/H\textsubscript{\textsuperscript{1001}} signaling, inducing reactions against various cell stresses, as proposed for TRPY1 in \textit{S. cerevisiae} (33). Importantly, the above mentioned stimuli are the ones commonly known to activate other eukaryotic TRP channels. Therefore, we expected TRPGz to serve as a prototypic TRP channel and addressed its molecular bases for multimodal regulation.

The Putative Coiled-coil Region in TRPGz-CTD Is Essential for the Hyperosmotic and Temperature Responses but Not for Channel Formation and Responses to Other Stimuli

We searched for the essential regions for the above described various regulations of the TRPGz channel activities. We focused on the CTD located downstream of the channel pore-forming TM helix S6, the region from Ala\textsuperscript{539} to the C terminus, and assessed the channel functions of serial deletion mutants (Fig. 1). We first confirmed that most of the tested deletion mutants of TRPGz displayed the proper vacuolar localization, although the deletion of the entire CTD resulted in defective trafficking (data not shown). The mutants exhibiting the proper localization were subjected to further functional analyses.

Notably, deletion mutants lacking the region between residues 600 and 621 (CC and 600–617) did not show any response to hyperosmotic shock, in contrast to the mutant retaining the

\[ \text{FIGURE 1. Multiple sequence alignment of TRPGz and other TRP channels.} \]

The amino acid sequences of TRPGz (XP_384354), TRPY1 (NP_014730), human TRPC3 (NP_001124170), and human TRPV1 (NP_542436) were obtained from NCBI database and aligned using the ClustalW2 algorithm (74) with the BLOSUM 62 matrix using Geneious software version 5.5 (75), where initial multiple sequence alignment was built with the default parameter setting given by the software, and then the multiple sequence alignment was divided into three parts (N-terminal, core TM, and C-terminal parts) and realigned separately in the same manner as described above. The combined multiple sequence alignment was further adjusted manually. Conserved residues with 100% similarity based on PAM250 matrix were colored yellow with a red background. The TRPGz-CTD is highlighted in green, where the symbols indicating its structural characteristics are shown above or below the TRPGz sequence as follows. The presumed coiled-coil region (residues 600 – 621; the CC region) is depicted by the pink cylinder, the residues subjected to mutation in this study are marked with closed triangles, and the three methionine residues, Met\textsuperscript{575}, Met\textsuperscript{607}, and Met\textsuperscript{613}, which were analyzed by NMR (Fig. 7, C–E), are boxed in green. The residues referred to in this work are pinpointed with blue stars. Positions of the estimated transmembrane and pore helix domain are shown as reported elsewhere (34, 76).
region from residue 600 to 621 (Δ621−) (Fig. 3A). Furthermore, the responses to rapid temperature increase were also diminished by the deletion of the region from residue 600 to 621 (Fig. 3B), in a manner similar to the hyperosmotic shock responses (Fig. 3A). The amino acid sequence analysis revealed that the region from residue 600 to 621 is predicted to be a coiled-coil domain (Fig. 1). These results suggested that the CC region in the CTD is required for osmotic and temperature shock reception by TRPGz.

Because coiled-coil modules are present in numerous TRP channels and are considered to be important for homo- or heterotetrameric channel assembly in many cases (18), we examined whether the CC region is responsible for the oligomerization of the entire channel region of TRPGz. FSEC analyses of the C-terminal GFP fusion protein of wild type TRPGz and its CC deletion mutant (TRPGz-ΔCC) revealed that both proteins exclusively eluted as presumable tetramers (Fig. 3C) without any apparent additional elution peaks for lower molecular weight species.
Multimodal Regulation of a Fungal TRP Channel

FIGURE 3. Functional roles of the CC region in TRPGz-CTD. A, normalized maximum responses of the TRPGz deletion mutants, Δ600−, Δ621−, and ΔCC (Δ600−621), by hyperosmotic shock generated by 2 M sorbitol. B, normalized maximum responses of TRPGz WT, Δ621−, Δ600−, and ΔCC (Δ600−621) to rapid temperature elevation from 25 to 40 °C. C, fluorescence detection size exclusion chromatography of the digoxin-solubilized C-terminal GFP fusion protein of full-length TRPGz (WT) and its CC region deletion mutant (ΔCC). The predicted elution volumes for tetramer (T), dimer (D), and monomer (M) are indicated with closed triangles. D, in vivo cross-linking of full-length TRPGz (WT) and its CC region deletion mutant (ΔCC). The lanes indicated with minus and plus signs represent the absence and presence of cross-linking reactions, respectively. E, CaCl2 activation of the voltage-dependent current of the CC deletion mutant (ΔCC) of TRPGz. Shown are representative traces of membrane currents recorded before (tan) and after (black) the addition of 1 mM CaCl2. F, normalized maximum responses of TRPGz WT, Δ621−, Δ600−, and ΔCC to 1 mM H2O2 treatment. The bars in B, C, and G show the means of the peak heights of the responses by each sample, with the error bars representing the S.E. values (n = 4–7).

weightization of the entire channel molecule was also verified by in vivo cross-linking experiments using protein samples without GFP, in which both the wild type and CC deletion mutant generated similar cross-linking profiles, with protein bands corresponding to the tetramer (Fig. 3D). Therefore, the tetramization of TRPGz itself is most likely to occur independently of the CC region.

Interestingly, whole-vacuole patch clamp measurements revealed both Ca2+ and voltage-dependent activations, even for the mutant lacking the CC region, to similar extents as those for the wild type protein (Fig. 3E). Furthermore, the activation by H2O2 application was not significantly affected by the deletion of the CC region (Fig. 3F). These results verified that TRPGz lacking the CC region retains the intact channel architecture and suggested that the CC region is not required for the cytosolic Ca2+, membrane potential change, and H2O2 responses. The results also implied that the Ca2+, membrane potential, and hyperoxic activations of the TRPGz channel occur by a modality different from those for the hyperosmotic and temperature change activations, which require the CC region.

The C-terminal Region of the TRPGz-CTD, Downstream from the CC Region, Is Responsible for Channel Regulation by PIPs—We next addressed the roles of the other regions in the CTD for the TRPGz functions. It is notable that the C-terminal deletion mutant of TRPGz, Δ621−, gave significantly higher responses upon all stimuli in the assays described above, such as osmotic upshock, temperature increase, and H2O2 treatment (Fig. 3, A, B, and F). We found a cluster of basic residues in the C terminus (Fig. 1), which is expected to interact with acidic phospholipids, such as PIPs. To clarify the interaction between the TRPGz-CTD and phospholipids, we performed protein-lipid overlay assays. The results revealed that the TRPGz-CTD interacted strongly with all PIPs and phosphatidic acid, whereas it showed weaker interactions with other acidic lipids, such as cardiolipin, lysophosphatidic acid, and sphingosine 1-phosphate (Fig. 4A). Among the PIPs, no binding specificity for the phosphorylation sites in the inositol moiety was observed. Similar reaction patterns were observed for the C-terminal half of the CTD, whereas the N-terminal half lacked reactivity to any phospholipids. The PIP binding by the CTD was also confirmed by liposome co-precipitation assays (Fig. 4B). These results indicated that the C-terminal basic cluster in the TRPGz-CTD specifically binds to general PIPs.

Furthermore, the electrophysiology also revealed that the application of water-soluble phosphatidylinositol 3-phosphate and phosphatidylinositol 3,5-diphosphate analogues to the cytoplasmic side significantly reduced the whole-vacuole currents of TRPGz (Fig. 4C), whereas the currents from TRPGz-D621 were not affected by the application of PIPs (Fig. 4D). These results clearly indicated that the interaction between the C-terminal basic cluster of TRPGz-CTD and the PIPs on the vacuolar membrane is responsible for the channel regulation, by inhibiting the channel activity. It should be noted that hyperosmotic shock dynamically increases the PIP levels in S. cerevisiae, mediated by proteins including vacuole-associated...
The TRPGz-CTD Is Primarily Composed of Intrinsically Disordered Regions with a Central Tetrameric Parallel Helix Bundle—In order to elucidate the structural basis of the channel regulation by the CTD, we analyzed its structural characteristics. Unfortunately, our attempts to crystallize the CTD failed, for not only the entire region but also several patterns of deletions in the N- or C-terminal regions. The $^1$H-$^{13}$N HSQC NMR spectrum of the CTD revealed that most of the CTD regions showed the characteristics of random coil structures (Fig. 5) and are considered to be intrinsically disordered regions. Nevertheless, we successfully crystallized the presumed coiled-coil region, TRPGz-CC, consisting of Val$^{600}$–Thr$^{620}$, and solved the x-ray crystal structure at 1.25 Å resolution (Table 1).

TRPGz-CC formed a left-handed parallel coiled-coil-like tetrameric assembly with an approximate length of 40 Å (Fig. 6A). At the intermolecular interface inside the molecular assembly, Val$^{600}$, Leu$^{603}$, Met$^{607}$, Leu$^{610}$, Leu$^{614}$, and Leu$^{617}$, the residues at the canonical “a” and “d” positions in the presumed coiled-coil region (Figs. 1 and 6B) form extensive hydrophobic interactions devoid of water molecules. At the surface, numerous salt bridges and hydrogen-bond networks were observed, such as between Glu$^{606}$ and Glu$^{609}$ from one protomer and Arg$^{604}$, Glu$^{608}$, and Lys$^{611}$ from another protomer, further reinforcing the interprotomer interaction (Fig. 6C).

The oligomerization topology observed in TRPGz-CC, however, deviates notably from the canonical four-stranded coiled-coil topologies. Structural analyses using the TWISTER program (62) revealed that the coiled-coil pitches of TRPGz-CC were 138.6 ± 16.7 and 138.3 ± 17.7 Å for the two different coiled-coil assemblies in the asymmetric unit of the crystal. These values are significantly smaller than those observed for other four-stranded left-handed parallel coiled-coil structures, such as 205.4 Å for the tetrameric mutant of the GCN4 leucine zipper (63) and 211.3 Å for a de novo designed synthetic four-stranded coiled coil (64). The results indicated that TRPGz-CC forms an assembly with a larger twisting angle, as compared with the typical four-stranded coiled-coils. This “acute twist” leads to the positional shift of the intermolecular interaction site between the uppermost protomer (protomer 1; Fig. 6A) and the lowermost protomer (protomer 4; Fig. 6A), resulting in the loss of the 4-fold rotational symmetry observed in canonical coiled-coil assemblies. Consequently, the intermolecular site between protomers 1 and 4 maintains fewer interactions (Fig. 6D), as compared with those between the other protomers, such as 1 and 2 (Fig. 6C), 2 and 3, and 3 and 4. The observation was further supported by a structural analysis with the SOCKET program (65), which indicated the absence of the typical “knobs-into-holes” packing for a coiled-coil assembly (66, 67) between protomers 1 and 4, although several are observed...
Multimodal Regulation of a Fungal TRP Channel

TABLE 1
X-ray data collection and refinement statistics

|                | Native (beamline BL41XU, 1.000-Å wavelength) | Se-Met (beamline BL26B2) |
|----------------|-----------------------------------------------|--------------------------|
|                | Rmerge (°)                                     | Rmerge (°)               |
|                | 35.290, 120.237                               | 35.215, 120.212          |
|                | 35-1.25                                       | 35-2.0                   |
|                | 6.8 (58.2)                                    | 19.4 (44.4)              |
|                | 45.3 (3.8)                                    | 33.1 (10.9)              |
|                | 10.1 (6.7)                                    | 10.6 (8.5)               |
| Completeness (%) | 99.8 (99.5)                                   | 99.9 (100)               |
| Complementary completeness (%) | 99.70                                        | 99.9 (100)               |
| Space group    | p4_1                                          | p4_3                     |
| Reflections    | 38548                                         | 19.17                    |
| Rmerge (%)     | 14.6/20.0                                     | 14.6/20.0                |
| Rmerge (%)     | 14.6/20.0                                     | 14.6/20.0                |
| Average Φ factor | 19.17                                         | 19.17                    |
| All atoms      | 1792                                          | 1792                     |
| Root mean square deviation, bonds (Å) | 0.017                                        | 0.017                    |
| Root mean square deviation, angles (degrees) | 1.990                                        | 1.990                    |

* Numbers in parentheses are the data in the highest resolution shells.
* Rmerge = \( \sum |I| - \langle |I| \rangle / \sum |I| \), Rmerge is the R-value for a subset of 5% of the reflection data, which were not included in the crystallographic refinement.

between the other protomers. Therefore, the structure is not actually a tetrameric coiled-coil but a four-helix bundle assembled in an offset spiral, with weaker interprotomer interactions.

TRPGz-CTD Exists in Association and Dissociation Equilibrium in the Physiological State—We next asked whether the tetrameric assembly observed in the TRPGz-CC crystal structure actually corresponds to the native state of the protein and is not simply achieved by the crystal packing of the small fragment. To address this, we first analyzed the oligomerization state of the entire CTD, including not only the CC region but also the upstream/downstream flanking regions (Fig. 1), by sedimentation equilibrium analytical ultracentrifugation experiments. The results indicated that the entire CTD is in equilibrium between monomer, dimer, and tetramer, with an apparent molecular mass of 56,000 Da, corresponding to a 3.1-mer (Table 2). On the other hand, the CTD lacking the CC region (ΔCC) was observed as a monomer (Table 2). In order to clarify the relationship between CTD oligomerization and assembly at the CC region, we introduced point mutations in the CC region to disrupt the intermolecular interactions. Based on the crystal structure, residues such as Met607 and Leu610, lining the hydrophobic intermolecular interface (Fig. 6B), and Glu606, on the hydrophilic interface (Fig. 6C), were replaced with either charged amino acids or proline as a helix breaker. As expected, the oligomerization states of the CTD were significantly affected by the mutations to various extents; almost all of the mutants, including the double mutant M607D/L610D, exhibited diminished multimer formation, and L610R displayed weakened oligomerization, whereas E606P had less influence on oligomerization (Table 2). These results clearly indicated that the oligomerization observed in the CC crystallographic structure is responsible for the oligomerization in the entire CTD and thus the native state of the full-length protein. In addition, the moderate interprotomer affinity of the CTD is consistent with the weaker packing characteristics observed in the crystal structure, as compared with the cases of canonical four-stranded coiled coils, which exclusively exist as tetramers judged by analytical centrifugation experiments with lower protein concentrations than those used in this study (63, 64).
The dynamic oligomerization state, between association and dissociation, is further supported by NMR analyses of the entire CTD. In the 1H-15N HSQC spectrum, the number of observed amide peaks was smaller than that expected from the primary sequence (Fig. 5). In the HSQC spectrum of M607D/L610D, extra amide peaks were detected in addition to those observed for the wild-type CTD (Fig. 7A), which were undetectable for a deletion mutant lacking the CC (Fig. 7B). Taken together, the amide signals in the CC region undergo exchange broadening, in agreement with the dynamic oligomerization. The same trend was also observed for the methyl signals of methionine residues. Significant line broadening was observed for the peaks of Met607 and Met613 in the CC region (Fig. 1) in the 1H-13C HMBC spectrum of the wild type (Fig. 7C), whereas the line broadening was no longer manifested in the M607D/L610D mutant (Fig. 7D) or the CC deletion mutant (Fig. 7E). In summary, the CTD exists in equilibrium between monomer, dimer, and tetramer association and dissociation, mediated by the central CC region with weak assembly characteristics.

The CC Region-mediated TRPGz-CTD Oligomerization Positively Correlates with the Levels of Hyperosmotic Responses—To further clarify the relationship between the CC region-mediated CTD assembly and the channel activities, we examined the hyperosmotic responses of the above-described point mutants with disrupted CTD assembly at the CC region. Almost all of the mutants exhibited either weak or minimal responses. Notably, the amplitudes of the responses of the mutants clearly exhibited a positive correlation with the association constants of the CTDs, as determined by the sedimentation equilibrium experiments (Fig. 8A and Table 2). These results strongly indicated that the hyperosmotic activation (and probably also the temperature increase activation) occurs on the TRPGz molecules with the CTD regions oligomerized through the CC regions (Fig. 8B).

DISCUSSION

Multimodal regulation is one of the most characteristic functions of TRP channels. In this study, we determined that a fungal TRP channel, TRPGz, is activated by various extracellular and cytosolic stimuli, such as hyperosmolarity, H2O2, application, temperature increase, cytosolic Ca2+ elevation, and membrane potential change. We identified the multiple regulatory modules of TRPGz within its C-terminal cytosolic domain that mediate at least part of the multimodal channel functions, such as the middle presumed coiled-coil region necessary for activation by hyperosmolarity and temperature increase and the C-terminal phospholipid binding module for PIP-dependent channel inhibition. From another point of view, the results clarified that the TRPGz activities are regulated by multiple independent modalities, including at least those that require the CTD assembly at the CC region (hyperosmotic and temperature activations) and those independent from the CTD assembly (Ca2+, membrane potential, and H2O2 activations) (Fig. 8B).

Coiled-coil modules are present in numerous TRP channels and are considered to be important for homo- or heterotetrameric channel assembly in many cases (18). However, we found that the assembly at the CC region, the presumable coiled-coil region of TRPGz, is not necessary for tetrameric channel formation and some channel modalities, such as Ca2+ activation (Fig. 3E). Nevertheless, the CC region is indispensable for the other modalities, such as hyperosmotic responses (Fig. 3A). The results clearly implied that the CTD oligomerization through the CC region positively correlates with the channel opening for hyperosmotic and temperature increase activation (Figs. 3A and 3B and 8A). There are several reported examples indicating that the assemblies of the cytosolic domains mediated by structural modules, including coiled-coils, are not necessary for tetrameric channel formation but are required for some channel functions. For example, cyclic AMP binding to the C-terminal domain of a hyperpolarization-activated, cyclic nucleotide-modulated channel, which reportedly shares structural similarity with the TRPV1 channel (68), was suggested to shift the association equilibrium of the domain to the tetramer, with the resultant conformational change responsible for channel opening (69).

In the case of TRPGz, what is the relevance of the CTD assembly to the channel activities arising from hyperosmotic shock or temperature increase? It is noteworthy that both are physical stimuli, and we hypothesize that one of the possible candidates underlying the activities is mechanosensitivity, arising from membrane stretching. Zhou et al. reported the mechanosensitive channel activities of TRPY1 and other fungal TRPY1 orthologs and suggested that they can explain the response to hyperosmotic shock, which causes dehydration of the cytosol and vacuole, resulting in transient osmotic pressure on the vacuolar membrane (35, 37). With regard to the response to rapid temperature increase observed with TRPGz, the responses could also be caused by vacuolar membrane tension resulting from thermal expansion of the solutions in the cytosol and vacuoles upon rapid temperature elevation, in addition to the possibilities of direct temperature sensing and/or changes in the physical properties of the vacuolar membrane. Further analyses are necessary to clarify the mechanism. Because the tetrameric channel pore exists between the TM helices S5 and S6, the CTD assembly just beneath the pore-lining S6 might serve as a fulcrum for the pore opening by membrane tension or as an anchor to maintain the appropriate topology and positioning for the pore opening even under conditions with membrane tension. We attempted to measure the force-activated vacuolar currents, but no significant conductance was observed by applying pressure up to ~0.5 kilopascal (data not shown). Because a pressure threshold for TRPGz activation is likely to exist (Fig. 2C), further analyses are needed, although the application of higher pressure is not fea-

### Table 2

Summary of the sedimentation equilibrium analytical ultracentrifugation results

|        | Calculated mass | Apparent mass | $K_{d1}$ | $K_{d2}$ |
|--------|-----------------|---------------|----------|----------|
| WT     | Da              | Da            | $4.75 	imes 10^{-5}$ | $3.69 	imes 10^{-2}$ |
| ΔCC (Δ600–620) | 15,384         | 15,000        | ND       | ND       |
| M607D/L610D | 17,800         | 20,000        | $2.87 	imes 10^{-2}$ | $3.54 	imes 10^{-2}$ |
| L610R  | 17,857          | 22,000        | ND       | ND       |
| E606P  | 17,782          | 54,000        | $3.18 	imes 10^{-7}$ | $1.12 	imes 10^{-4}$ |

$K_{d1}$ and $K_{d2}$ are the dissociation constants for dimer-monomer and tetramer-dimer, respectively.
sible under the current experimental conditions, due to the fragility of the yeast vacuolar membrane (37).

What is the relevance of the CTD structure to the TRPGz channel activities that are independent of the CC region, such as cytosolic Ca²⁺ elevation, membrane potential shift, and H₂O₂ activation or PIP-dependent inhibition? The offset spiral assembly at the CC region observed in the x-ray crystallographic structure disrupts the interstrand interactions between the uppermost and lowermost protomers and explains the moderate interstrand affinity observed by analytical ultracen-
trifugation and confirmed by NMR analyses. The weaker assembly, as compared with the canonical coiled-coil, enables the association and dissociation of the CTD during various functions and thus is considered to be compatible with the multimodal regulation mechanisms. Some require the assembly for activation, as in the cases of hyperosmotic shock and temperature increase, whereas others, supported by other regulatory modules, might require sufficient structural flexibility with less restraint, allowing access to their stimulants/ligands and the resultant conformational change for regulation. The structural characteristics of the entire CTD, which primarily consists of intrinsically disordered regions, are also probably favorable for regulation by multiple stimulants/ligands. In TRPY1, the calcium binding sites for channel activation are reportedly located in the C-terminal domain (70), and a similar acidic residue cluster also exists in TRPGz upstream of the CC region (Fig. 1). Several TRP channels are considered to act as sensors for the cellular redox state and are equipped with oxidizing/reducing sites (e.g. Cys residues) in cytosolic domains (e.g. as described by Takahashi et al. (14)). The PIP-binding module was also found in the TRPGz-CTD downstream of the coiled-coil, as a regulatory module that probably functions in suppressing channel gating and maintaining Ca$^{2+}$ homeostasis under conditions of cell stress, which in many cases induces elevated PIP levels. These dynamic interaction properties of the CTD with various ligands suggest that multiple conformational substates of CTD exist (Fig. 8B). With regard to the oligomerization of the CTD, the $\Delta G$ for TRPGz-CTD oligomerization is $-7.9$ kcal/mol (estimated from the equilibrium constant in Table 2). On the other hand, the nonspecific PIP-binding motif, such as the MARCKS effector domain, probably exhibits strong electrostatic interaction with PIPs, with a $\Delta G$ of $-8$ kcal/mol (estimated from the value reported by Arbuzova et al. (71)). Notably, the activation energy of TRPV1 was reported as $7$ kcal/mol (estimated from the value reported by Arbuzova et al. (71)). Notably, the activation energy of TRPV1 was reported as $7$ kcal/mol (estimated from the value reported by Arbuzova et al. (71)).

Acknowledgments—We thank Yoshitaka Takano for the first-strand cDNA preparation from G. zeae; Ching Kung for the TRPY1 gene; Patrick Masson for the aequorin expression vector; Naoko Takahashi and Koji Takio for the N-terminal sequencing analysis; Go Ueno and Nobutaka Shimizu for support with x-ray data collection at the SPring-8 beamlines BL26B2 and BL41XU; Toshiro Oda and Susumu Uchiyama for assistance with and advice on the analytical ultracentrifugation experiments; Atsuo Miyazawa for help with the microscopy observations; Toshiki Ishi for advice on the lipid-binding experiments; Naoko Ono, Takashi Yamada, Fumie Iwabuki, Noriko Matsuura, and Junko Nakamura for technical assistance; and Yuichiro Maeda, Yuji Ashikawa, and the participants in the NIPS Research Meeting “TRP Channel Conference 2012” for discussions.

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J. Biol. Chem. 2013, 288:15303-15317.  
doi: 10.1074/jbc.M112.434795 originally published online April 3, 2013

Access the most updated version of this article at doi: 10.1074/jbc.M112.434795

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