The core domain as the force sensor of the yeast mechanosensitive TRP channel

Zhenwei Su,1 Andriy Anishkin,3 Ching Kung,1,2 and Yoshiro Saimi1

1Laboratory of Molecular Biology and 2Department of Genetics, University of Wisconsin-Madison, Madison, WI 53706
3Department of Biology, University of Maryland, College Park, MD 20742

Streth-activated conductances are commonly encountered in careful electric recordings. Those of known proteins (TRP, MscL, MscS, K, Kv, etc.) all share a core, which houses the ion pathway and the gate, but no recognizable force-sensing domain. Like animal TRPs, the yeast TRPY1 is polymodal, activated by stretch force, Ca2+, etc. To test whether its S5–S6 core senses the stretch force, we tried to uncouple it from the peripheral domains by strategic peptide insertions to block the covalent core–periphery interactions. Insertion of long unstructured peptides should distort, if not disrupt, protein structures that transmit force. Such insertions between S6 and the C-terminal tail largely removed Ca2+ activation, showing their effectiveness. However, such insertions as well as those between S5 and the N-terminal region, which includes S1–S4, did not significantly alter mechanosensitivity. Even insertions at both locations flanking the S5–S6 core did not much alter mechanosensitivity. Tryptophan scanning mutations in S5 were also constructed to perturb possible noncovalent core–periphery contacts. The testable tryptophan mutations also have little or no effects on mechanosensitivity. Boltzmann fits of the wild-type force–response curves agree with a structural homology model for a stretch-induced core expansion of ~2 nm² upon opening. We hypothesize that membrane tension pulls on S5–S6, expanding the core and opening the TRPY1 gate. The core being the major force sensor offers the simplest, though not the only, explanation of why so many channels of disparate designs are mechanically sensitive. Compared with the bacterial MscL, TRPY1 is much less sensitive to force, befitting a polymodal channel that relies on multiple stimuli.

INTRODUCTION

Mechanosensitive (MS) ion channels are major first-line force sensors in a variety of force-related physiological processes from osmotic regulation to hearing and touch sensations. Force-activated conductances have been observed in various preparations since its first demonstration in cultured muscle cells (Guharay and Sachs, 1984). MS channels of known sequences are widespread among diverse channel families, including the prokaryotic MscL and MscS (Sukharev et al., 1997; Booth et al., 2007; Kung et al., 2010) and the eukaryotic ENaC (Chalfie, 2009), K (Honoré, 2007), and TRP channels (Christensen and Corey, 2007). Several eukaryotic channels that are better known for sensing other stimuli are also found to be mechanosensitive under patch clamp, such as Kv (Gu et al., 2001; Schmidt and MacKinnon, 2008), Nav (Shcherbatko et al., 1999; Morris and Juranka, 2007; Beyder et al., 2010), Cav (Calabrese et al., 2002), HCN2 (Lin et al., 2007), BK (Zhao et al., 2010), NMDA receptor (Kloda et al., 2007), and even CFTR (Zhang et al., 2010). These disparate MS channels share no recognizable forcesensing motifs with conserved protein sequences comparable to the well-recognized voltage-sensing domain or various ligand-binding pockets.

How force activates MS channels is a crucial question in this field of study (Kung, 2005; Arnadóttir and Chalfie, 2010). Clearer molecular insights come from the study of prokaryotic MscL and MscS. They receive force through the membrane tension (Sukharev et al., 1997; Kung, 2005). They are homomultimers of subunits with two or three transmembrane helices forming the core domain that harbors the ion conduction pathway and the channel gate. Membrane tension pulls these transmembrane helices, triggering the core expansion and subsequent gate opening of MscL and MscS. There are no separate force-sensing domains besides the core.

On the other hand, it remains unclear how force activates eukaryotic MS channels in molecular terms (Arnadóttir and Chalfie, 2010). In principle, the source of force can either come from membrane bilayer, like MscL and MscS (Sukharev et al., 1997; Kung, 2005), or from extracellular matrix/cytoskeleton (Christensen and Corey, 2007; Arnadóttir and Chalfie, 2010), or can be indirectly conveyed by diffusible elements (Vriens et al., 2004). Cytoskeletons can negatively regulate the mechanosensitivity of MS channels by relaxing the local membrane tension surrounding MS channels (Sharif-Naeini et al., 2009; Sachs, 2010). There are reports of eukaryotic MS channels being reconstituted into pure lipids.
and still retaining mechanosensitivity, such as NMDA receptor (Kloda et al., 2007). Others, such as TRPY1 (Zhou et al., 2003), TRPV4 (Loukin et al., 2010) of the TRP channel family, TREK1 (Honoré, 2007) of the K_+ family, and CFTR (Zhang et al., 2010), have been shown to be mechanosensitive in excised patches in simple salt solutions and thus are most likely to receive force also from membrane lipids. This principle is also made clear with the gramicidin A model (Lundbaek et al., 2004, 2010). Common to all channels is the presence of the core domain that harbors the selectivity filter, ion pathway, and the gate. In want of a common force-sensing structure, the core domain of these eukaryotic channels may also serve as the membrane force sensor.

To explore this possibility, here we use the yeast MS TRP channel (Fig. S1), TRPY1, and quantitatively studied the molecular mechanism of its force activation. TRPY1 responds to hyperosmotic shocks in vivo (Denis and Cyert, 2002) and presents a 320-pS unitary conductance upon stretching of excised patches bathed in symmetrical 180-mM KCl (Zhou et al., 2005). Much like Kv, each TRP channel is likely a tetramer of subunits, each comprising six transmembrane helices (S1–S6), although crystal structures are not yet available. The four S5–S6s are presumed to converge to form the core domain, whereas S1–S4s likely form peripheral modules attached to the core. By analogy to the crystal structure of Kv in the open state, the core domain of these eukaryotic channels closely following KvAP structure resolved in the open conformation. The models (see Fig. 8) were based on the structural modeling

### Materials and Methods

#### Yeast strains and cultures

The wild-type parental strains were BY4742 (MATa his3Δ leu2Δ lys2Δ ura3Δ) and trpy1Δ mutant (chromosomal knockout strain YOR088W, trpy1Δ::kan). For electrophysiology, standard yeast media with yeast extract, peptone, and dextrose was used (Zhou et al., 2003; Su et al., 2009).

#### Mutagenesis

All site-directed mutagenesis were performed using the standard overlap extension PCR method (Ho et al., 1989). Different PCR fragments carrying the corresponding mutations were cloned into HindIII–XhoI sites of TRPY1 in a pYVC plasmid carrying a strong constitutively active promoter, the glyceraldehyde-3-phosphate dehydrogenase promoter, and a URA3 marker. All mutations were verified by sequencing. Sequenced plasmids were linearized by Eagl–MluI before transforming into trpy1Δ for gene integration into the chromosome. Transformed yeast mutant strains with correct markers were sequenced again from the genome to verify the trpy1 mutant integrants. All experiments were performed on these integrants. Wild-type control for ensemble current analysis was constructed the same way as the mutants. For wild-type single-channel analysis, a weak native promoter was used to replace the glyceraldehyde-3-phosphate dehydrogenase promoter to achieve low expression.

#### Solutions

For patch-clamp recordings, the pipette solution contained 150 mM KCl, 5 mM MgCl₂, 300 mM sorbitol, and 10 mM HEPES-KOH, pH 7.2. The bath solution contained 150 mM KCl, 5 mM MgCl₂, 300 mM sorbitol, 10 mM HEPES-KOH, pH 7.2, and different Ca²⁺ concentrations adjusted with CaCl₂ and EGTA. For [Ca²⁺] above 10⁻³ M, CaCl₂ was directly added to EGTA-free bath solution. For [Ca²⁺] below 10⁻³ M, appropriate amounts of CaCl₂ recommended by MaxChelator software were added to the bath solution containing 1–2 mM EGTA. For the 0 Ca²⁺ solution, 10 mM EGTA was added to the solution without additional Ca²⁺.

#### Electrophysiology

Yeast vacuoles were generated as previously described (Su et al., 2007; Zhou et al., 2007). All patch-clamp recordings were performed with Clampex 10.0 (Axon Instruments). Data were sampled at 5 kHz and filtered at 1–5 kHz. The data were further filtered at 1 kHz before analysis using a digital filter (Clampfit; Axon Instruments) as necessary. 100-µl glass pipettes (Drummond Scientific Company) were pulled with a micro-pipette puller (P-97, Sutter Instrument) to so-called bubble numbers strictly measured to be between 4.5 and 5.1 in ethanol (Sakmann and Neher, 1983). With negative pressure in the pipette, 2–3-GΩ seals were formed before being broken by transient voltage pulses to reach the whole-vacuole mode. Whole-vacuole mode patches were subsequently excised into the cytoplasmic-side-out mode. Data were acquired at −50 mV, with the cytoplasmic side being negative. Bath perfusion was used to change [Ca²⁺]. Mechanical stimulation was applied as pressure exerted with a 60-ml syringe, and the pressure was monitored with a pressure sensor (PX140; Omega). Experiments were performed at room temperature (21–23°C). Data were analyzed using Clampfit 10.0 (Axon Instruments). Leak currents measured by applying pressure at 0 [Ca²⁺] were negligible. For ensemble current analyses, saturation levels were the current maxima with much reduced noise level at high [Ca²⁺] and high pressure. In mutants with frequent flickers, saturations were judged by the lack of high pressure–induced additional current at high [Ca²⁺], assuming that these two energies are synergistically summed. Open probability (P_o) was calculated as the percentage of the saturation. In single-channel patches, P_o was also calculated from the flat-top events.

#### Statistical analysis

Data are presented as means ± SD. Curves were fitted with SigmaPlot 10.0 (Systat Software). A one-tail unpaired Student’s t test was performed with Excel (Microsoft) to examine statistical differences. P < 0.05 is considered significantly different.

#### Structural modeling

We used the ROSETTA software (Simons et al., 1997) to model the pore domain of TRPY1 (Zhou et al., 2007). The complete details on the modeling procedure, the features of the proposed models, and videos illustrating the suggested gating motion are presented in the web supplement to the original publication (Zhou et al., 2007). In brief, the models (see Fig. 8) were based on the remote homology to K_+ channels of the shaker family. The initial model was an open state with an architecture of the pore domain (S5–S6) closely following KvAP structure resolved in the open

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kB is the Boltzmann constant; and T is the absolute temperature.

Materials and methods for details. Although [Ca2+] laterally shifts the domain of [Ca2+]. Means ± SD (not SEM; indicating that [Ca2+] (10^−6) is the applied pressure; the channel at given [Ca2+] (Cui and Aldrich, 2000) and some of TRP channels allosteric activation of BK channels by both voltage and stimuli (Voets et al., 2005). We have previously shown that TRPY1 is also synergistically activated by stretch through its transmembrane helices and by Ca2+ binding in the cytoplasmic domain with a DDDD motif (Fig. 1 A; Su et al., 2009). This is most likely a result of the allosteric effect of membrane force and Ca2+, much like the allosteric activation of BK channels by both voltage and Ca2+ (Cui and Aldrich, 2000) and some of TRP channels by voltage, temperature, and chemicals (Latorre et al., 2007; Voets et al., 2007). Conceptually, TRPY1’s Po, is determined by the energy intrinsic to the channel’s structure at rest (ΔGo) as well as gating energies from different modalities; the ones from force (ΔGnaro) and Ca2+ (ΔGCa) are especially germane here. We directly measure TRPY1 activities in cytoplasmic-side-out patches excised from exposed vacuoles, clamped at −50 mV, and stretch the patch by measured pressures through the recording pipette. Without added pressure, the basal Po is governed by [Ca2+]. At extreme [Ca2+] (10^−6 or 10^−5 M) where the basal Po is very low or very high, force activation by pipette pressure remains, although limited in magnitude (Fig. 1 B; and see Fig. 3 A), whereas a wide range of wild-type force activation is best displayed at an intermediate [Ca2+] of 10^−5 M (Fig. 1 B; and see Fig. 3 A). [Ca2+] shifts the Boltzmann curve of Po versus pressure laterally but does not significantly alter its slope (Δα = ~0.015 mmHg−1; Fig. 1 B and Table I). Thus, the mechanosensitivity of TRPY1 is energy minimized, relaxed, and symmetry annealed in short molecular dynamics simulations.

Online supplemental material
Fig. S1 shows a sequence comparison and a phylogenetic tree, which indicate the deep roots and loose connections among TRP channel subfamilies. Online supplemental material is available at http://www.jgp.org/cgi/content/full/jgp.201110693/DC1.

RESULTS
Synergistic activation of TRPY1 by stretch force and cytoplasmic [Ca2+]
TRP channels are polymodal, responding to multiple stimuli (Voets et al., 2005). We have previously shown that TRPY1 is also synergistically activated by stretch through its transmembrane helices and by Ca2+ binding in the cytoplasmic domain with a DDDD motif (Fig. 1 A; Su et al., 2009). This is most likely a result of the allosteric effect of membrane force and Ca2+, much like the allosteric activation of BK channels by both voltage and Ca2+ (Cui and Aldrich, 2000) and some of TRP channels by voltage, temperature, and chemicals (Latorre et al., 2007; Voets et al., 2007). Conceptually, TRPY1’s Po, is determined by the energy intrinsic to the channel’s structure at rest (ΔGo) as well as gating energies from different modalities; the ones from force (ΔGnaro) and Ca2+ (ΔGCa) are especially germane here. We directly measure TRPY1 activities in cytoplasmic-side-out patches excised from exposed vacuoles, clamped at −50 mV, and stretch the patch by measured pressures through the recording pipette. Without added pressure, the basal Po is governed by [Ca2+]. At extreme [Ca2+] (10^−6 or 10^−5 M) where the basal Po is very low or very high, force activation by pipette pressure remains, although limited in magnitude (Fig. 1 B; and see Fig. 3 A), whereas a wide range of wild-type force activation is best displayed at an intermediate [Ca2+] of 10^−5 M (Fig. 1 B; and see Fig. 3 A). [Ca2+] shifts the Boltzmann curve of Po versus pressure laterally but does not significantly alter its slope (Δα = ~0.015 mmHg−1; Fig. 1 B and Table I). Thus, the mechanosensitivity of TRPY1 is

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**Figure 1.** Bimodal activation of TRPY1. (A) A diagram showing that TRPY1 senses membrane stretch force through the membrane-embedded domains and Ca²⁺ through the cytoplasmic domains. Energetically, the open probability is governed by ΔGo = ΔGnaro − ΔGCa; where ΔGo refers to the energy difference between the C and O state before the application of force or Ca²⁺. ΔGnaro includes the energies from structures, temperature, protonation, etc. (B) The Po, versus pressure plots at different [Ca²⁺]. For each of the three [Ca²⁺], Po from TRPY1 ensemble currents is plotted against the applied pressure and fitted with the following Boltzmann equation: Po = 1/[1 + a × exp(−Δα × P)], which is transformed from Po = 1/[1 + exp((ΔE − γ × ΔA)/kB × T)], where a and Δα are fitting parameters in the Po–pressure plot, with Δα (in millimeters of Hg⁻¹) being the slope parameter; P (in millimeters of Hg) is the applied pressure; ΔE is the free energy of the channel at given [Ca²⁺]; γ (in Newtons per meter) is the membrane tension; ΔA (in square meters) is the expansion coefficient; k₅ is the Boltzmann constant; and T is the absolute temperature. The transformation assumes that the radius of our patches, r (in meters), remains constant. We strictly controlled the sizes of our pipette tips in a narrow range with the bubble numbers 4.5–5.1 in ethanol (Sakmann and Neher, 1983) to approach the constancy of r. With this assumption, γ can be converted to P according to Laplace’s law. The saturation levels of channel activities were determined by applying high pressure at high [Ca²⁺] (10^−3 M). See Materials and methods for details. Although [Ca²⁺] laterally shifts P₀–pressure curves, all three fits have a similar Δα value (Table I), indicating that Δα defines the mechanosensitivity and is independent of [Ca²⁺]. Means ± SD (not SEM; n = 4 for 10^−3 M, n = 6 for 10^−5 M, and n = 4 for 10^−6 M Ca²⁺).

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**Table I**
<table>
<thead>
<tr>
<th>Constructs</th>
<th>[Ca²⁺] (M)</th>
<th>Slope parameter Δα (mean ± SD)</th>
<th>No. of patches</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>10^−3</td>
<td>0.015 ± 0.0043</td>
<td>4</td>
</tr>
<tr>
<td>WT</td>
<td>10^−5</td>
<td>0.016 ± 0.0050</td>
<td>6</td>
</tr>
<tr>
<td>WT</td>
<td>10^−6</td>
<td>0.018 ± 0.0031</td>
<td>4</td>
</tr>
<tr>
<td>358–486-12GGS</td>
<td>10^−5</td>
<td>0.012 ± 0.0033</td>
<td>4</td>
</tr>
</tbody>
</table>

The ensemble current plots of Po, versus pressure from wild type at different [Ca²⁺] (10^−6, 10^−5, and 10^−3 M) and 358–486-12GGS at intermediate [Ca²⁺] (10^−5 M) were fitted with the Boltzmann equation: Po = 1/[1 + a × exp(−Δα × P)] (Fig. 1 B). The slope parameters, Δα, of the wild type at different [Ca²⁺] are not significantly different (P = 0.38, 0.28, and 0.28 in a pairwise t test). Δα of 358–456-12GGS is also not significantly different from that of the wild type at 10^−5 M Ca²⁺ (P = 0.07).
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Figure 2. Single-channel analysis of the force activation indicating the C1 to burst transition step is mechanosensitive. (A, top) A single-channel recording at $10^{-6}$ M Ca$^{2+}$ in response to different pressures over 5.5 min. (middle) An expanded view of a short portion in the top trace showing different states: an interburst closed state (C1), a burst state comprising an open state (O), and an intraburst closed state (C2). The recording presented here has higher basal $P_o$ than average, which facilitated kinetics analysis. (bottom) A minimal kinetics model for TRPY1. (B) All-point histogram of A for dwell time analyses of different states without pressure (0 mmHg; top) and with 150 mmHg of pressure (bottom). The comparison shows that pressure mainly shortens C1 without much affecting O and C2. Also see Table II. (C) The normalized C1 dwell time to that of 0 mmHg of pressure is plotted against pressure at $10^{-6}$ M Ca$^{2+}$ and fitted with an exponential equation: normalized C1 dwell time $= b \times \exp(-\Delta \alpha \times P)$, similar to that described in Fig. 1 B. The slope parameter $\Delta \alpha$ (Table III) here corresponds to the slope parameter $\Delta \alpha$ from the ensemble current analysis in Figs. 1 B and 3 F. (D) A plot similar to C at $10^{-5}$ M Ca$^{2+}$. Means ± SD (not SEM; $n = 5$ for $10^{-6}$ M and $n = 3$ for $10^{-5}$ M Ca$^{2+}$).

Independent of [Ca$^{2+}$]. The mechanosensitivity of TRPY1, $\Delta \alpha$, should not be confused with the midpoint of the Boltzmann distribution or the threshold of channel activation, as these reflect the sum of $\Delta G_{m}$ and $\Delta G_{0}$ for TRPY1.

Kinetics analyses of single TRPY1 activities

Single-channel analyses show that TRPY1 opens in bursts. Its kinetics can be summarized as $C1 \leftrightarrow [C2 \leftrightarrow O]$), where the brackets delineate the burst state (Fig. 2 A). Within the burst, the order of O and C2 cannot be distinguished. Increasing pipette pressure mainly shortens the interburst closure, C1, with relatively small effects on the intraburst closure, C2, or the open dwell, O (Fig. 2 B and Table II). C1 shortens monotonously with pressure (Fig. 2, C and D). The plot of C1 dwell versus pressure yields a slope remarkably similar to the aforementioned $\Delta \alpha$ (Fig. 2, C and D; and Table III), indicating that the forward transition from C1 to the burst is the main mechanically sensitive step.

Insertions of long unstructured peptides after S6 effectively remove most of the Ca$^{2+}$ activation

Although there is yet no reported crystallographic structure of the TRP channel, TRP is expected to have a domain arrangement similar to Kv (Long et al., 2005b,
The cytoplasmic domain of TRPY1 harbors the Ca\textsuperscript{2+}-binding through a short S4–S5 linker peptide. Also, the C-terminal membrane domain connecting to the gate in the S5–S6 core between S6 and the C-terminal tails. Such insertions remove insertions is evident when placed in several positions be-

ular covalent structures in a protein. The potency of such insertions is near full range.

mutants so as to display the mechanosensitivity of the

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dees mechanically coupled to the gate (Long et al., 2005b). Sequence analyses suggest that Q359–K374 of TRPY1 forms an amphipathic helix that links S4 to S5, as in Kv

A 12-residue peptide, GGSGGSGGSGGS (henceforth called 12GGS), should produce an unstructured loop without a rigid or well-defined conformation. It is expected to greatly distort or disrupt all covalent

senses the membrane stretch before transmitting the force to the gate, we engineered strategic insertions or substitutions in an attempt to uncouple the S5–S6 core from the neighboring domains. Such mutations are near or in the predicted gating structure of the core and are therefore expected to result in channels that have abnormally high (gain of function) or low (loss of function) basal activities. Although mechanosensitivity remains in these mutants, the range can become limited at the intermediate [Ca\textsuperscript{2+}] of 10\textsuperscript{-5} M, the most suitable concentration in which to examine the wild-type channel. We therefore often adjusted the [Ca\textsuperscript{2+}] for most mutants so as to display the mechanosensitivity of the mutants in near full range.

A 12-residue peptide, GGSGGSGGSGGS (henceforth called 12GGS), should produce an unstructured loop without a rigid or well-defined conformation. It is expected to greatly distort, if not completely disrupt, any regular covalent structures in a protein. The potency of such insertions is evident when placed in several positions between S6 and the C-terminal tails. Such insertions remove nearly all the TRPY1’s sensitivity to Ca\textsuperscript{2+} (Su et al., 2009).

\begin{table}[h]
\centering
\caption{Dwell time analysis of the wild-type single channel showing the effect of membrane stretch force on different states}
\begin{tabular}{lccc}
\hline
[Ca\textsuperscript{2+}] & Normalized dwell times of different states & \multicolumn{2}{c}{No. of patches} \\
& under 150-mmHg pressure (mean ± SD) & C1 & C2 & O \\
\hline
M & & & & \\
10\textsuperscript{-5} & 0.21 ± 0.082 & 0.84 ± 0.24 & 1.3 ± 0.22 & 3 \\
10\textsuperscript{-6} & 0.13 ± 0.031 & 1.0 ± 0.59 & 1.9 ± 0.78 & 5 \\
\hline
\end{tabular}
\end{table}

The single-channel plots of normalized C1 dwell time versus pressure from the wild type at different [Ca\textsuperscript{2+}] (10\textsuperscript{-6} and 10\textsuperscript{-5} M) were fitted with an exponential equation: normalized C1 dwell time = b × exp(−Δα × P) (Fig. 2). The slope parameter Δα here is remarkably similar to the slope parameter Δα from the ensemble current analysis in Table I, indicating that the transition from C1 to B is the major MS step.

\begin{table}[h]
\centering
\caption{The slope parameters from the analysis of wild-type C1 dwell time}
\begin{tabular}{lccc}
\hline
[Ca\textsuperscript{2+}] & Slope parameter Δα (mean ± SD) & \multicolumn{2}{c}{No. of patches} \\
& mmHg\textsuperscript{-1} & & \\
\hline
M & & & \\
10\textsuperscript{-5} & 0.011 ± 0.0058 & 3 \\
10\textsuperscript{-6} & 0.017 ± 0.0086 & 5 \\
\hline
\end{tabular}
\end{table}

The CA2+ concentration in which to examine the wild-type channel. The single-channel plots of normalized C1 dwell time versus pressure from the wild type at different [Ca\textsuperscript{2+}] (10\textsuperscript{-6} and 10\textsuperscript{-5} M) were fitted with an exponential equation: normalized C1 dwell time = b × exp(−Δα × P) (Fig. 2). The slope parameter Δα here is remarkably similar to the slope parameter Δα from the ensemble current analysis in Table I, indicating that the transition from C1 to B is the major MS step.

Peptide insertions between S1–S4 and S5–S6

Given the dramatic effect of the 12GGS insertion at the C terminus of the S5–S6 core, we tested the effect of the same insertion at the N terminus of this core. In Kv, S1–S4 forms a peripheral domain connecting to the gate in the S5–S6 core through a short S4–S5 linker peptide. S1–S4 houses the voltage sensor, the movement of which is mechanically coupled to the gate (Long et al., 2005b). Sequence analyses suggest that Q359–K374 of TRPY1 forms an amphipathic helix that links S4 to S5, as in Kv (Long et al., 2005b, 2007). To test whether the S1–S4 peripheral domains likewise sense the membrane force before transmitting it to the gate through the linker, we inserted 12GGS at position 358 (358-12GGS) at the N terminus of the S4–S5 linker (Fig. 3 C). This insertion created a gain-of-function mutant, with a greatly increased basal Po. At intermediate [Ca\textsuperscript{2+}] (10\textsuperscript{-5} M), its basal Po is already ~40% (Fig. 3 E, squares), compared with 3% from wild type. At high [Ca\textsuperscript{2+}] (10\textsuperscript{-3} M), 358-12GGS opens near saturation, obscuring force activation (Fig. 3 C, right). Adjusting [Ca\textsuperscript{2+}] to lower levels revealed that 358-12GGS clearly remains sensitive to force (Fig. 3 C, left and middle traces).

To better uncouple the core domain, we inserted the aforementioned peptide at both sites flanking the core in an attempt to greatly distort or disrupt all covalent couplings between the core and the periphery. Like the 486-12GGS single insertant, this double-insertion mutant (358–486-12GGS) is almost insensitive to Ca\textsuperscript{2+} (Fig. 3 E, triangles). However, its basal activity is higher than that of wild type at lower [Ca\textsuperscript{2+}] (10\textsuperscript{-6} or 10\textsuperscript{-5} M), much like the 358-12GGS single insertant. Thus, the double-insertion mutant appears to show a compensatory effect from the two single insertions. Importantly, at intermediate [Ca\textsuperscript{2+}] (10\textsuperscript{-3} M), the 358–486-12GGS channel displays the full range of activation by force to near saturation (Fig. 3 D). The force activation of this double-insertion mutant was taken as a representative and analyzed in detail. Its Po relationship to applied pressure was indistinguishable from that of the wild type (Fig. 3 F). The Boltzmann fits yielded values of Δα for the wild type and 358–486-12GGS with no signifi-
cantly different (Table I).

The aforementioned results are not dependent on the specific nature of the GGSGGSGGSGGS peptide or the insertion site. We have tested 16 insertions of different
sequences, lengths, and locations (Fig. 4). Channels with insertions between S6 and the C-terminal cytoplasmic domain all showed symptoms similar to that of 486-12GGS with greatly reduced Ca\(^{2+}\)-induced basal activities. Compare basal activities at high \([\text{Ca}^{2+}]\) (10\(^{-3}\) M), where the basal activity is most suitable for the test of the force activation. Nonetheless, pressure-induced responses are robust. (C) 358-12GGS with the 12-residue peptide inserted in front of the S4–S5 linker increased basal activities. Compare basal activities with those in A. Responses to pressure pulses are robust, though partially masked by high basal activities. (D) 358–486-12GGS with the peptide inserted at both sides of the S5–S6 core; pressure responses remain robust. Although A–D show results from typical cases, those from other patches \((n = 6 \text{ for wild type and } n = 4 \text{ for 358–12GGS, 358–12GGS, and } 358–486-12GGS)\) are highly consistent. (E) Plots of \(P_o\) versus \([\text{Ca}^{2+}]\) showing a great reduction of the Ca\(^{2+}\) activation by the peptide insertion at position 486 (diamonds) and an elevation of the basal \(P_o\) by the insertion at position 358 (squares). 486-12GGS did not reach clear saturation even at high \([\text{Ca}^{2+}]\) and under high pressure. Its \(P_o\) is thus normalized to the highest level we observed and will result in an overestimation. Means ± SD \((n = 3 \text{ for all})\). (F) \(P_o\) versus pressure plots of wild type and 358–486-12GGS at 10\(^{-5}\) M Ca\(^{2+}\) fitted with the Boltzmann equation, the same as Fig. 1B. Means ± SD \((n = 6 \text{ for wild type and } n = 4 \text{ for } 358–486-12GGS)\). No clear difference in mechanosensitivity \(\Delta \alpha\) can be discerned. See Table I. The low or high spontaneous activity of 486-12GGS or 358-12GGS, respectively, limited the accurate estimate of \(P_o\) or the test range of mechanosensitivity and cannot be plotted here.
channels with decreased P_o at high [Ca^{2+}] (10^{-3} M; Fig. 5 A) compared with that of wild type at the same [Ca^{2+}] shown in Fig. 3 A are compared. (B) An over fivefold increase in nP_o observed in both the wild type and 374-12GGS upon 150-mmHg pressure. Because the saturation level could not be determined before lytic pressure even at high [Ca^{2+}] because of its shortened open durations and less stable seal under high pressures compared with wild type, the quantification was conducted using the fold increase of nP_o stimulated by 150-mmHg pressure from comparable basal activities between 374-12GGS and wild type at different [Ca^{2+}] (10^{-3} M for 374-12GGS and 10^{-5} M for wild type). No clear difference in mechanosensitivity can be discerned by this measure. P = 0.35. Means ± SD (n = 3 for both).

Peptide insertions between the S4–S5 linker and S5–S6 core

Similar to that of Kv, the amphipathic S4–S5 linker can associate with membrane, potentially sensing membrane force (Boukalova et al., 2010). An insertion at position 374, 374-12GGS, just after the S4–S5 linker where the S4–S5 linker contacts S6 in Kv, produced Figure 4. Various insertion mutants before or after the core domain do not affect force activation. (A and C) Diagrams showing insertions with different sequences, different lengths, and different locations. Insertion mutants with no observed functional channels are not depicted in the diagram, which include 369-6GGS, 372-6GGS, 373-6GGS, 375-6GGS, 376-6GGS, 377-6GGS, and 378-6GGS. (B and D) Similar diagrams and recording as in Fig. 3 of 486-11QTL and 358-24GGS, respectively.

Figure 5. Inserting unstructured peptides between the S4–S5 linker and S5–S6 core domain does not much affect force activation. (A) A diagram and recording from 374-GGS are presented as in Fig. 3. This insertion shortens the open duration and thus lowers basal activities, which is evident when the activity at 10^{-3} M Ca^{2+} here and that of the wild type at the same [Ca^{2+}] shown in Fig. 3 A are compared. (B) An over fivefold increase in nP_o observed in both the wild type and 374-12GGS upon 150-mmHg pressure. Because the saturation level could not be determined before lytic pressure even at high [Ca^{2+}] because of its shortened open durations and less stable seal under high pressures compared with wild type, the quantification was conducted using the fold increase of nP_o stimulated by 150-mmHg pressure from comparable basal activity between 374-12GGS and wild type at different [Ca^{2+}] (10^{-3} M for 374-12GGS and 10^{-5} M for wild type). No clear difference in mechanosensitivity can be discerned by this measure. P = 0.35. Means ± SD (n = 3 for both).
Perturbing possible noncovalent contacts between the S1–S4 and S5–S6 core by tryptophan and alanine substitutions does not significantly weaken TRPY1 force activation.

In the crystal structures of Kv channels (Long et al., 2005a,b, 2007) and especially that of the bacterial ligand-regulated cation channel (Clayton et al., 2008), there are noncovalent contacts between S1–S4 and S5, attaching S1–S4 to the core domain. If TRPY1 is constructed similarly, these contacts may transmit force. We next investigated such possible noncovalent coupling from S1–S4 to the S5 shell of the core domain. Tryptophan has a bulky side chain. A replacement of an amino acid with tryptophan likely perturbs not only its own contact discerned between 374-12GGS and the wild type. This finding makes it unlikely that this S4–S5 linker (the Q359–K374 amphipathic helix) acts as a force sensor. As position 374 is at the kink between the linker and S5, it might be a critical residue if there is an interaction between the linker and S6 just like the case of Kv. Thus, inserting 12GGS here would disrupt such a noncovalent interaction as well as any signal transduction from S1–S4 through the linker.

Overall, all functional TRPY1 insertion mutants (Figs. 3–5) retained their responses to membrane stretch in excised patches. In cases where quantifications allow, the insertion mutants retain much of the mechanosensitivity (Fig. 3 F, Fig. 5 B, and Table I).
Su et al. analyzed with alanine substitutions. Except for L385A, which still remained overly active, the other four alanine mutants, I388A, G389A, F390A, and G393A, showed overall similar force activations as the wild type (Fig. 6, B and C).

**DISCUSSION**

Few MS TRP channels have been quantitatively scrutinized at the single-channel level. We have improved the techniques in recording of the yeast vacuolar membrane, making a quantitative examination of the behavior of TRPY1 possible. An added advantage is the ease of yeast molecular genetics that makes gene replacement at the native chromosomal locus a routine. Such replacements make the wild-type mutant comparisons direct, avoiding complexities encountered in the plasmid expression or random chromosomal insertion.
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themselves (Fig. S1 B). In fact, TRPY1, the yeast TRP, was cloned by recognizing this sequence (Palmer et al., 2001). It expresses a 320-pS unitary conductance in the vacuolar membrane, to which we have ready patch-clamp access (Palmer et al., 2001; Zhou et al., 2003). Animal TRPs are polymodal, integrating multiple physical and chemical stimuli and summing the various gating energies to activate (Latorre et al., 2007; Voets et al., 2007). TRPY1 also responds to several stimuli (Bertl and Slayman, 1990; Zhou et al., 2003), of which mechanical force and cytoplasmic Ca$^{2+}$ are key (Fig. 1, A and B). These are not just parameters for biophysical evaluation but are biologically meaningful. Hyperosmotic shocks activate TRPY1 to release Ca$^{2+}$ from the vacuole into the cytoplasm, as indicated by the fact that trpy1 knockout shows no such release (Denis and Cyert, 2002). Osmotic activation of TRPY1 in vivo is readily explained by its activation by stretch force under patch clamp (Zhou et al., 2003). Ca$^{2+}$ activation apparently forms a positive feedback as in other Ca$^{2+}$-dependent Ca$^{2+}$ activations (Zhou et al., 2003). Previous (Su et al., 2009) and present experiments (Fig. 3, B and E; and Fig. 4, A and B) showed that Ca$^{2+}$ activation of TRPY1 requires coupling between the S5–S6 core domain and the C-terminal cytoplasmic domain containing Ca$^{2+}$-binding sites (Fig. 1 A; Su et al., 2009).

TRPY1 is clearly activated by membrane stretch (Figs. 1–7; Zhou et al., 2003, 2007; Su et al., 2007, 2009). Our kinetic analysis shows that the force-sensitive step is from the interburst closed state to the burst (Fig. 2 and Table III). Compared with the bacterial channels MscL and MscS (Martinac, 2004), however, the mechanosensitivity of TRPY1 is weak, as seen in the shallow slope in the P$_{o}$-pressure plot in Fig. 1 B. This weaker mechanosensitivity corresponds to the small $\Delta A$ during the core expansion in the homology model (Fig. 8 and see section $\Delta A$, the in-plane expansion of the core) and befits a polymodal channel that sums gating energies from several sources. Although TRPY1 is less impressive as a stretch-activated channel compared with those of bacteria, it is more representative of tetrameric cation channels, several of which have been shown to be stretch sensitive, including K$_{ga}$, Kv, Nav, etc. It is interesting to note that the interburst to burst transition step is also Ca$^{2+}$ sensitive (unpublished data).

The efficacy of (GGS)$_n$ insertions

The rationale in our dissection here is that mutations tend to be destructive instead of constructive. They may therefore interfere with mechanosensitivity if placed in the path of force transmission. Whereas point substitution likely has small effects, large insertions may be more effective. Although we cannot be certain, long peptides like GGSGGSGGS likely form unstructured loops that distort or disrupt $\alpha$, $\beta$, or other secondary structures. Insertion of unstructured peptides immediately before

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**Figure 8.** A homology model of the S5–S6 core domain of the TRPY1 channel to visualize the nature of the in-plane expansion upon opening. The computational model was constructed using the ROSETTA algorithm (Simons et al., 1997) based on limited homology between TRPY1 and K$^+$ channels (see supplemental information in Zhou et al., 2007). For clarity, only residues from 363 to 472 are shown as ribbons. Upon opening, both the external S5 helices (yellow) and the pore-lining S6 helices (blue) are modeled to bend, tilt, and expand driven by membrane tension.
or after the TM5–TM6 core does not disrupt the peptide-backbone continuity but should slacken the covalent linkage. The fact that these insertions do not alter mechanosensitivity indicates that the mechanical stimulus is transmitted not through the stressed backbone but through either membrane tension acting directly on the S5–S6 core or through secondary interdomain interactions. The probability that force transmission remains in such structures through noncovalent contacts at the two insertion junctions seems remote. Such peptides have been used successfully to disrupt the communication between the S5–S6 core and the cytoplasmic Ca\(^{2+}\)-binding domain of the BK channel (Niu et al., 2004). Ca\(^{2+}\) activation of TRPY1 depends on a similar arrangement (Fig. 1 A; Su et al., 2009). The almost complete removal of Ca\(^{2+}\) sensitivity after the 486-12GGS insertion (Fig. 3, B and E) demonstrates that 12GGS insertion is indeed effective in disrupting the covalent linkage structures. Similar removal of Ca\(^{2+}\) sensitivity by the insertions of different peptides at residue 486 or 490 (Fig. 4 A) reinforces this conclusion. However, peptide insertions at different points flanking S5–S6 do not significantly affect activation by stretch force (Figs. 3–5), although they do affect gating (basal P\(_{o}\) and kinetics) because of their proximity to the gate. The 358-24GGS result is especially impressive. The 24-residue peptide inserted is as long as an entire transmembrane helix. It is formally possible that these unstructured peptide loops are able to disrupt the gating force of C-terminal Ca\(^{2+}\) binding but do not disrupt the mechanical gating force from the S1–S4 domains. However, it is difficult to envision how the mechanics can be so different for the C- and N-terminal covalent peptides immediately adjacent to the core. An alternative possibility is that transmission of mechanical force is through certain noncovalent contacts between the S1–S4 periphery and the S5–S6 core tested here with tryptophan substitutions. The simplest interpretation, however, is that the core itself senses the mechanical force. In crystal structures, the core domain of the Kv channel is directly exposed to membrane lipids (Long et al., 2007). TRPY1 may be arranged similarly, allowing it to sense the membrane force.

The efficacy of the tryptophan substitutions
Although multiple helical interactions between S1–S4 and S5 might possibly tolerate single tryptophan perturbations to some extent, single tryptophan mutants have been shown to be effective enough to strongly perturb the noncovalent interactions between S1–S4 and S5 in Kv (Li-Smerin et al., 2000; Soler-Llavana et al., 2006). Multiple tryptophan substitutions in a channel without a crystal structure in the hope of disrupting a function are impractical. Therefore, to the extent that can be experimentally addressed, our results that all testable single tryptophan or alanine mutants on S5 retain much of the force activation are consistent with the hypothesis that the noncovalent periphery–core contacts, if any, are not likely to be required for the major force transmission in TRPY1.

The mechanosensitivity of insertion or substitution mutants was quantified in two different measurements. For 358–486-12GGS (Fig. 3 F) and some tryptophan mutants (Fig. 7 B), their saturation levels and P\(_{o}\) can be determined as with the wild type (Fig. 3 A) by their response to high pressure at high [Ca\(^{2+}\)] or by single-channel recordings. Thus, their \(\Delta A\) values are compared with that of wild type. For other mutants whose saturation level cannot be determined because of extremely low basal activities or drastic kinetics defects, we compared the nP\(_{o}\)-fold increases induced by 150 mmHg of pressure with that of wild type. These are the best quantifications allowed by this experimental system. Within the confidence imposed by variations of these experiments, we observed that all testable insertion and substitution mutants retain much of their mechanosensitivity and concluded that the core domain is the major force sensor of TRPY1.

\(\Delta A\), the in-plane expansion of the core
In the force activation, the product of the bilayer tension \(\gamma\) and the expansion coefficient \(\Delta A\) (Fig. 1) is the energy that works to open the channel; this is analogous to the voltage and gating charges. We deduced \(\Delta A\) from \(\Delta\alpha\) of the Boltzmann curve (Fig. 1 B and Table 1), where the transformation defines \(P \times \Delta A = \gamma \times \Delta\alpha / k_B \times T\) based on Laplace’s law (Fig. 1). We consistently observed the lytic pressure to be \(~300\) mmHg in the wild-type excised patches, and here we assume that it corresponds to the lytic tension \((~10\) mN/m\) for excised patches (Suchyna et al., 2009). Therefore, \(\Delta\alpha\) of 0.015 approximately corresponds to \(\Delta A\) of 1.8 nm\(^2\). In TREK1 that likely also senses membrane force directly, \(\Delta A\) is found to be \(~4\) nm\(^2\) (Maksaev et al., 2011). For MscL, \(\Delta A\) is reported as \(~20\) nm\(^2\) (Chiang et al., 2004), much larger than that of eukaryotic channels. In MscL, force tilts and expands the transmembrane helices of its core domain (Sukharev et al., 2001; Kung, 2005; Kung et al., 2010), resulting in an in-plane area change from a closed to open state seen in the crystal structure \(\Delta A_{structure}\). \(\Delta A_{structure}\) is predicted to be \(~23\) nm\(^2\) by structural models of MscL, which is consistent with its large \(\Delta A\) value. We used the ROSETTA software (Simons et al., 1997) to model the pore domain of TRPY1 (see Materials and methods; Zhou et al., 2007). Although explicit membrane tension was not included in building the models (and future refinements in explicit bilayer might modify the structures to some extent), the templates used for the closed and open conformations predict an expansion area of \(\Delta A_{structure} = ~2-4\) nm\(^2\) for the TRPY1 core, which is consistent with its experimental \(\Delta A\) of 1.8 nm\(^2\) that defines its mechanosensitivity. The general agreement of the
A common basis of mechanosensitivity?

Most biological membranes, when carefully examined, seem to have MS channels. Electrophysiologists often encounter force-activated channel currents of unknown origin. Many MS conductances, including stretch-activated channels, first described in 1984 (Guhray and Sachs, 1984), are of unclear molecular nature, although there are candidates (Coste et al., 2010). Although many eukaryotic MS channels underlying hearing, touch, or vision may or may not be of physiological use. This makes force sensing an anisotropic force environment of the lipid bilayer and to benefit polyvalent interaction with the cytoskeletons. We need to in-vestigate the mechanosensitive channel types thoroughly for a full understanding.

The authors thank Stephen H. Loukin and Xinliang Zhou for critical comments and advice throughout the work.

This work was supported by National Institutes of Health grants GM 54867 to Y. Saimi and RO3 GM125020 to C. Kung.

Kenton J. Swartz served as editor.

Submitted: 22 July 2011
Accepted: 7 November 2011

R E F E R E N C E S


Kenton J. Swartz served as editor.


