Enzyme Domain Affects the Movement of the Voltage Sensor in Ascidian and Zebrafish Voltage-sensing Phosphatases**

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The ascidian voltage-sensing phosphatase (Ci-VSP) consists of the voltage sensor domain (VSD) and a cytoplasmic phosphatase region that has significant homology to the phosphatase and tensin homolog deleted on chromosome TEN (PTEN). The phosphatase activity of Ci-VSP is modified by the conformational change of the VSD. In many proteins, two protein modules are bidirectionally coupled, but it is unknown whether the phosphatase domain affects the movement of the VSD in VSP. We addressed this issue by whole-cell patch recording of gating currents from a teleost VSP (Dr-VSP) cloned from Danio rerio expressed in tsA201 cells. Replacement of a critical cysteine residue, in the phosphatase active center of Dr-VSP, by serine sharpened both ON- and OFF-gating currents. Similar changes were produced by treatment with phosphatase inhibitors, pervanadate and orthovanadate, that constitutively bind to cysteine in the active catalytic center of phosphatases. The distinct kinetics of gating currents dependent on enzyme activity were not because of altered phosphatidylinositol 4,5-bisphosphate levels, because the kinetics of gating current did not change by depletion of phosphatidylinositol 4,5-bisphosphate, as reported by coexpressed KCNQ2/3 channels. These results indicate that the movement of the VSD is influenced by the enzymatic state of the cytoplasmic domain, providing an important clue for understanding mechanisms of coupling between the VSD and its effector.

Voltage-gated ion channels play an important role in electrical activities and cell signaling of muscles and nerves. The first four transmembrane regions (S1–S4) are conserved among all the voltage-gated channels and operate as the voltage sensor, thus called the voltage sensor domain (VSD)4 (1). The VSD regulates the operation of the downstream pore domain, consisting of the two transmembrane segments (S5–S6) and a loop that provides an ion permeation pathway. The VSD has several positively charged residues interspersed with two hydrophobic residues in the fourth transmembrane segment, called S4, that plays critical roles in voltage sensing (1–4). Recently resolution of the crystal structure of voltage-gated potassium channels (5, 6) and biophysical measurements of the movement of specific sites of the VSD (3) have led to proposed models for the operation of the VSD (7). However, the VSD has long been studied as a structure unique to voltage-gated ion channels.

We have recently identified a protein, Ci-VSP, that contains the VSD but not the pore domain (8). Ci-VSP has the following two modules in its structure: the transmembrane spanning region that corresponds to the VSD of voltage-gated ion channels and the cytoplasmic region with homology to the phosphatase and tensin homolog deleted on chromosome TEN (PTEN), a PtdIns(3,4,5)P3 phosphatase (9). The VSD of Ci-VSP exhibits gating currents that indicate the conformational change in response to membrane voltage, and the phosphoinositide phosphatase activity is voltage-dependently regulated (8). This is the first example where an effector other than an ion pathway is regulated by the VSD. Furthermore, another protein that contains the VSD but lacks the pore domain was identified as the long sought molecular correlate of voltage-gated proton channels (10, 11). These findings indicate that the VSD is a protein module that operates as a self-contained functional unit.

Recently, detailed relationships between voltage-sensor movement and phosphatase activity of Ci-VSP were examined using several types of phosphoinositide sensors (12). This revealed that phosphatase activity changes over a range of

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4 The abbreviations used are: VSD, voltage sensor domain; VSP, voltage-sensing phosphatase; RT, reverse transcription; GST, glutathione S-transferase; NMDG, N-methyl-D-glucamine; MΩ, meqohm; PtdIns(3,4,5)P3, phosphatidylinositol 3,4,5-trisphosphate; PtdIns(4,5)P2, phosphatidylinositol 4,5-bisphosphate; Ci-VSP, ascidian voltage-sensing phosphatase; Dr-VSP, D. rerio VSP; pC, picocoulomb; pF, picofarad.
membrane voltages as the voltage-sensor movement is increased. A shift of the voltage dependence of the VSD leads to a shift of the voltage dependence of the phosphatase activity to the same direction (12), verifying that VSD confers voltage sensitivity to phosphatase activities of the cytoplasmic region. Deletion of 8 amino acids in the linker region between the VSD and the phosphatase domain eliminates coupling between the two modules (8). The above findings indicate that VSD of VSP is tightly coupled with the downstream effector, as for voltage-gated ion channels. A recent study showed that after replacement of a part of the VSD of a voltage-gated potassium channel by that of Ci-VSP or a voltage-gated proton channel, the voltage-gating is still retained, indicating that the mechanisms of voltage sensing are conserved between conventional voltage-gated ion channels and voltage sensor domain proteins (13). Furthermore, a recent study with single molecule photobleaching has shown that Ci-VSP operates as a monomer (14), indicating similar stoichiometry than voltage-gated ion channels. Thus VSP serves as a simple model to understand how VSD is coupled with its effector.

In this study, a VSP ortholog gene was identified from zebrafish, Danio rerio (named Dr-VSP). Dr-VSP shares most properties with Ci-VSP, including gating currents and voltage-sensitive phosphoinositide phosphatase activity. Dr-VSP showed much more robust gating currents in tsA201 cells than Ci-VSP, providing us with a unique opportunity to explore how the phosphatase module interacts with the VSD. We found that inhibition of phosphatase activity, either by the introduction of a point mutation or by pharmacological inhibition with vanadate, accelerates the movement of the VSD, providing evidence that the enzyme activity of the phosphatase domain influences the VSD movement.

**EXPERIMENTAL PROCEDURES**

*cdDNAs and Mutagenesis*—The zebrafish ortholog of Ci-VSP was cloned by RT-PCR. Rat KCNQ2/3 clones in the Xenopus expression vector were provided by Dr. Nakajo (National Institute for Physiological Sciences, Okazaki, Japan). The same inserts were subcloned into pIRE82-EGFP (BD Biosciences) for transfection into HEK293 cells.

To identify the zebrafish VSP cDNA, the mouse VSP protein sequence was used to survey zebrafish translated by expressed sequence tag (EST) data base. Two EST clones were found, each of which putatively covers the middle and C-terminal region of VSP. Based on this information, we designed two primers, TTCTCAGATTTCGTCAAAAACCTGA and TTGAATTCAAGGCTAGTGAAGAAG, and performed RT-PCR with cDNA from 24- to 60-h embryos as template. A 1.186-kb PCR product was obtained. Because this lacked the 5’ end of zebrafish VSP, 5’- and 3’-rapid amplification of cDNA ends were performed with the primers CTCACATACGTCA-CAGAAGGA and GTCCAGATCCACCTACCTTT. These two clones were ligated to reconstitute the entire coding sequence of VSP and cloned into pSD64TF expression vector (provided by Dr. Terry Snutch, University of British Columbia) between EcoRV and SpeI sites. Dr-VSP was subcloned into the pIRE82-EGFP expression plasmid between NheI and SacII restriction sites. For electrophysiology of Ci-VSP, the coding region of the wild type and the enzyme-defective mutant, C363S (8), were cloned into pIRE82-EGFP at PstI and SacII sites. Point mutations were introduced using the QuikChange site-directed mutagenesis kit (Stratagene). Oligo primers in both directions were synthesized with 10–15-bp correct sequences in both sides of the modified sequence(s). The mutations were confirmed by sequencing the entire insert with the Beckman Coulter sequencer. Primers used for the mutation, C302S, were G6TTAATTGCCCATCCACACGAAAGAGG-GAAAGG (forward) and C6CTTCCCTTGGTGTG-GATGGCAATAAC (reverse). For mutation of T156R, the primers C6CCAGGTGTTGAGGTTCGTCT (forward) and AGAACCAGAAACCTCAACCCTCTGGG (reverse primer) were used. The double mutation I165R/T156R was further constructed from Dr-VSP-T156R using the following primers: CTGAGGATCTTAAGGCTGTA-CGATTC (forward) and GATGGTGACTAGGACTT-TCCCTG (reverse). For mutation of R153Q, the primers were G6CCAGTCTGATTCCCAGGTGTGACATT-CCTG (forward) and C6AGAGTGCACCACCTGGGGA-ATCACAAGCTGG (reverse).

*In Vitro Phosphatase Assay*—For expression of the phosphatase domain of Dr-VSP, GST was fused to the N terminus of the 332-amino acid cytoplasmic region. The cDNA was amplified by PCR and subcloned into pGEX4T3 (GE Healthcare) at EcoRI and XhoI sites. The GST fusion protein was synthesized in Escherichia coli JM109 and purified by glutathione-Sepharose 4B (GE Healthcare). The malachite green assay (16) was performed by using 1 nmol of 1-α-phosphatidyl-d-β-myoinositol 3,4,5-triphosphate and 10 nmol of phosphatidylinositol (Wako) per microcentrifuge tube and dispersed in the phosphatase assay buffer following lyophilization. 1 μg of purified protein was added to each reaction tube containing substrate solution and incubated at 31 °C. At various time points, the reaction was stopped by adding N-ethylmaleimide. The supernatant was collected by centrifugation. BIOMOL GREEN™ (Biomol) was added to the supernatant and incubated at room temperature for ~20 min. The amounts of phosphate were determined by measurements of absorption at 620 nm. H2O2 was used for reference reading. The values of released phosphate from Dr-VSP(WT), Dr-VSP(C302S), and GST alone were calculated using a standard curve according to the manufacturer's instructions (Biomol).

*Generation of Antibody and Western Blot Analysis of Recombinant Dr-VSP Proteins*—A polyclonal antibody against Dr-VSP was raised in rabbit against the protein of the whole C-terminal cytoplasmic region of Dr-VSP. A cDNA fragment corresponding to the cytoplasmic region (amino acid residues Glu-180 to Pro-511) was subcloned into a vector, pGEX-4T-3, that has a thrombin cleavage site after the GST sequence. After the GST fusion protein was produced and purified from E. coli using a glutathione-Sepharose 4B column (GE Healthcare), GST was removed from the cytoplasmic region of Dr-VSP by treatment with thrombin. Purified protein was injected into rabbit six times.

Wild type and C302S mutant Dr-VSP DNA was transfected into tsA201 cell with pIRE82-EGFP plasmid (BD Biosciences) as a transfection marker. The lysates were centrifuged at 15,000
Interaction between Enzyme Domain and Voltage Sensor in VSP

rpm for 30 min at 4 °C to collect a membrane fraction. 20 μg of each sample was separated on 12% SDS-PAGE under reducing conditions. The membrane was reacted with the Dr-VSP antiserum (1/500) and then horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody (1/2000) (GE Healthcare). The labeled band was detected using ECL Plus (GE Healthcare).

Mammalian Cell Expression and Whole-cell Patch Recording—tsA201 cells, a derivative of HEK293 cells that stably express SV40, were used for most of studies. HEK293 cells were used for coexpression of Dr-VSP with KCNQ2/3 channels, because HEK293 cells exhibited more robust K+ currents than tsA201 cells. Cells were cultured in Dulbecco’s modified Eagle’s medium (Sigma) with 10% fetal bovine serum in an incubator with 5% CO2 at 37 °C. The cDNAs were transfected using Polyfect (Qiagen) reagent following the manufacturer’s protocol. Whole-cell patch clamp recording was done using Axopatch 200B (Molecular Devices) with tsA201 cells. The external bath solution was 75 mM N-methyl-D-glucamine (NMDG), 1 mM CaCl2, 1 mM MgCl2, 180 mM HEPES, 0–10 mM glucose (pH 7.0). The internal solution was 65 mM NMDG, 3 mM MgCl2, 1 mM EGTA, 100 mM HEPES (pH 7.0). The pH and osmolality were adjusted with methanesulfonic acid and glucose, respectively.

For stimulation and data acquisition, Pclamp Digitdata 1322A 16-bit data acquisition system was used. Drumond 100-μl calibrated pipettes were fabricated with 8–15-Ω resistance and used as patch pipettes. Macroscopic current records were filtered at 5-kHz low pass Bessel filter/80 dB. Symmetrical and linear currents were subtracted by P/5 procedure to isolate the gating current. Total carried charge was calculated by Clampfit 9.0 software. Kinetics of gating currents were examined by measuring half-decay times, rather than fitting with exponentials. In some cases, gating currents were fitted by single exponentials, giving basically similar results. More than a single exponential was required for fitting the curves in many cases, consistent with the view that more than single transition occurs in the activation of the voltage sensor of Ci-VSP as examined by profiles of voltage-induced movements of fluorescence-labeled amino acid residues of S4 in Ci-VSP (14).

The Q-V curve was fitted by the Boltzmann equation $Q = 1/(\exp(z(e(V - V_{50})/kT))$, where the parameter $e$ is elementary electric charge; $k$ is Boltzmann constant; $z$ is the effective valance; and $T$ is the absolute temperature.

Because NMDG and methanesulfonate in the pipette solution have low mobility, pipette resistance was not less than 8 MΩ even when the inner tip diameter was as large as 1–1.5 μm. In this study, we did not perform compensation of series resistance in most of measurements. Therefore, changes in gating current kinetics of less than ~1 ms, or gating currents larger than 1 nA, were not interpretable. Data presented in Fig. 7 with extremely fast and robust gating currents of the S4 double mutant were not analyzed in detail.

Oocyte Preparation and cRNA Expression—Oocytes were isolated by surgery from Xenopus laevis. Animals were anesthetized by immersion in 1.5% ethyl 3-aminobenzoate methanesulfonate salt (Sigma) for about 10 min and placed on ice. The excised ovary was rinsed with ND96 (containing 96 mM NaCl, 2 mM KCl, 1.8 mM CaCl2, 1 mM MgCl2, 5 mM HEPES (pH 7.5) with NaOH) and was agitated for an hour in a solution containing 10 mM collagenase (Nitta Gelatin). The isolated oocytes were incubated at 18 °C. In vitro transcription was performed to synthesize cRNA by capped transcription kit (mMESSAGEmMACHINE®, Ambion). The oocyte expression vector, pSD64TF, containing the Dr-VSP insert, was linearized with XbaI, and SP6 RNA polymerase was used for transcription. Total cRNA of 0.1–0.5 ng was microinjected into oocytes a day after harvesting. The cRNA-injected oocytes were incubated in ND96 containing gentamycin and pyruvate (18). The incubation temperature was maintained at ±2 °C. Recording was carried out 2–3 days after injection.

Two-electrode Voltage Clamp with Oocyte Expression—KCNQ2/3 current was measured by bath clamp amplifier OC-725C (Warner Instrument Corp.). The bath solution for gating current was 96 mM NaCl, 2 mM KCl, 1.8 mM CaCl2, 1 mM MgCl2, 5 mM HEPES (pH 7.5) with NaOH. The bath solution was 2 mM KCl, 92 mM NaCl, 3 mM MgCl2, 4 mM NaOH, and 5 mM HEPES (pH 7.4). The pipette solution contained 3 mM KCl. The microelectrode resistance was 0.1 to 0.6 MΩ. Output current was filtered at 1 kHz by a four-pole Bessel filter. A holding potential during intervals (10 s) was set to −70 mV for hyperpolarization and 70 mV for depolarization. KCNQ2/3 currents were evoked by a depolarizing step to 60 mV, 50 ms. The sampling frequency was 13–27.7 kHz. Data were acquired by ITC-16 AD/DA converter with pulse software (HEKA) on a Macintosh computer. The recordings were performed at 23–27 °C. Data were analyzed with Igor pro (WaveMetrics, Inc.) and presented as mean ± S.D.

Drugs—Sodium pervanadate (Na3VO4) was applied to inhibit the phosphatase activity of the phosphatase domain. Sodium orthovanadate (Na3VO4) (Sigma) was dissolved in water to make 20 mM stock solution. 20 mM hydrogen peroxide (H2O2) (Wako) was added in a final volume of 200 ml, and the solution was incubated at room temperature for 30 min. Then catalase (Sigma, 100 unit) was added. The solution was then kept on ice until used for recording. Catalase was dissolved in water to make a stock solution of 10 mg/ml. For intracellular perfusion of orthovanadate, orthovanadate was diluted to 200 μM in the internal solution for patch pipette.

RESULTS

Identification and Electrophysiological Characterization of Dr-VSP—Our bioinformatics search showed that ortholog genes of Ci-VSP exist in genomes of zebrafish, Xenopus, chick, mouse, rat, and human. To clone a teleost Ci-VSP ortholog cDNA, RT-PCR was performed using RNA isolated from 24- to 60-h stage embryos of zebrafish, D. rerio. The amino acid sequence of mouse VSP was used as a query against the zebrafish data base of expressed sequenced tag. By subcloning RT-PCR products, a single species of cDNA that matches the sequence of the EST clone was obtained. The deduced amino acid sequence of the full-length cDNA of Dr-VSP showed significant similarity to Ci-VSP (31% identity in the transmembrane regions and 53% in the cytoplasmic region) and human PTEN (26% identity in the cytoplasmic region).

Like Ci-VSP, Dr-VSP shows a pattern of positively charged residues interspersed with hydrophobic residues in the 4th
transmembrane segment (S4), which is a signature motif conserved among all voltage-gated ion channels. However, the position of positive charges in Dr-VSP is slightly distinct from Ci-VSP. Dr-VSP has isoleucine, at a site corresponding to arginine (Arg-229) of Ci-VSP (Fig. 2A).

The cytoplasmic phosphatase domain of VSP has a sequence, HCKGGKGRGTG, similar to that of PTEN in the phosphatase active site. This includes cysteine that is known to exist at the active site of all spectrums of phosphoinositide phosphatase (9) and protein-tyrosine phosphatases to provide thiol-phosphate bonding during catalysis. The downstream region of the cytoplasmic domain has high sequence homology to the C2 domain of human PTEN. Like Ci-VSP, Dr-VSP lacks the PDZ domain which is contained in the C terminus of PTEN (supplemental Fig. S1).

To examine whether the VSD of Dr-VSP senses membrane potential like Ci-VSP (8), we measured gating currents as an indication of the movement of the voltage sensor (1). A tsA201 cell line was used as the heterologous expression system. On transient transfection of Dr-VSP cDNA, cells produced highly robust ON- and OFF-gating currents (Ig-ON and Ig-OFF) (Fig. 1A). The total and standardized curve of the charge versus voltage (Q-V) showed that charge movement was almost equal between Ig-ON and Ig-OFF over a wide range of membrane potential (Fig. 1B and inset), consistent with the interpretation that both ON-charges and OFF-charges are derived from the same structural elements. Decay kinetics of Ig-ON and Ig-OFF were examined at distinct voltage levels by measuring half-decay times (Fig. 1, C and D). Both Ig-ON and Ig-OFF decayed at a fixed depolarizing membrane potential became slower as depolarizing pulse became more positive (Fig. 1C).

Ci-VSP was also expressed in tsA201 cells to compare with gating currents of Dr-VSP. Gating currents from Dr-VSP were more robust than from Ci-VSP (Fig. 1A, bottom); maximum carried charge was 2.89 ± 0.27 pC/pF (n = 6) for Dr-VSP versus 1.08 ± 0.1 pC/pF (n = 6) in Ci-VSP. The voltage-charge relationship was more positive for Dr-VSP (voltage for half-activation \( V_{1/2} \), 94.27 ± 6.83 mV, \( n = 5 \)) than Ci-VSP (\( V_{1/2} \), 62.9 mV ± 4.5 mV, \( n = 6 \)). The Q-V curve of Ci-VSP was less steep than Dr-VSP (Fig. 1B); the effective valence, Z values of Ig-OFF for Ci-VSP and Dr-VSP were 1.01 ± 0.15, \( n = 6 \), and 1.61 ± 0.15, \( n = 5 \), respectively (see Table 1). Such properties of voltage sensing of Ci-VSP are similar to those previously characterized in Xenopus oocytes (8).

In voltage-gated ion channels, positive charges in S4 play critical roles in voltage sensing. To test if positive charges on S4 are critical for the voltage-sensing nature of Dr-VSP, arginine,
Arg-153 (Fig. 2A), was replaced by the uncharged residue, glutamine, and gating currents were measured. Although gating currents of R153Q were less robust (1.58 ± 0.64 pC/pF, \(n = 12\)) than for the wild type Dr-VSP, the kinetics of ON- and OFF-currents were significantly faster (Fig. 2B). Ig-OFF exhibited two phases of decay. The Q-V curve was shifted leftward by about 80 mV (\(V_{1/2}\) was 16.18 mV ± 8.94 mV, \(n = 12\)) compared with the wild type (Fig. 2C). This is consistent with the finding that a similar mutant of Ci-VSP with replacement by glutamine at a site, R217Q, corresponding to Arg-153 of Dr-VSP, showed a leftward shift of voltage dependence as shown by voltage clamp fluorometry (14). In addition, voltage dependence was significantly less steep than the wild type (valence, \(Z\), for OFF-gating currents was 0.77 ± 0.30 versus 1.61 ± 0.15 for R153Q and wild type, respectively), consistent with the idea that positive charge on Arg-153 contributes to charge movement upon voltage-triggered conformational change of the voltage sensor.

When the S4 amino acid sequences of Ci-VSP and Dr-VSP were aligned, residue 165 was different, arginine for Ci-VSP and isoleucine for Dr-VSP (Fig. 2A). To test if the distinct voltage dependence of charge movements between Ci-VSP and Dr-VSP is derived from the amino acid sequence in the VSD, Ile-165 was replaced by arginine, and gating currents were compared. In Dr-VSP I165R, Ig-OFF was faster, and the Q-V curve was shifted leftward as compared with the wild

### TABLE 1

|                  | \(V_{1/2}\) (ON) | \(V_{1/2}\) (OFF) | \(Z\) (ON) | \(Z\) (OFF) | Maximum charge (OFF) | Current density |
|------------------|-----------------|-----------------|-----------|-----------|---------------------|---------------|
|                  | \(mV\)          | \(mV\)          | \(pC\)    | \(pC/pF\) | \(pC\)              | \(\mu A/\mu F\) |
| Dr-VSP(WT), \(n = 5\) | 96.70 ± 7.07   | 94.00 ± 6.13   | 1.45 ± 0.04 | 1.61 ± 0.15 | 4.57 ± 0.29         | 2.89 ± 0.27    |
| Dr-VSP(R153Q), \(n = 12\) | 13.06 ± 7.60   | 16.18 ± 8.94   | 0.60 ± 0.10 | 0.77 ± 0.30 | 1.14 ± 0.62         | 1.58 ± 0.64    |
| Dr-VSP(C302S), \(n = 6\) | 107.16 ± 7.82  | 96.48 ± 5.98   | 1.54 ± 0.25 | 1.77 ± 0.17 | 2.25 ± 0.51         | 1.13 ± 0.52    |
| Dr-VSP(DM), \(n = 9\) | 21.25 ± 2.58   | 25.26 ± 2.51   | 1.42 ± 0.25 | 1.52 ± 0.21 | 1.93 ± 0.55         | 1.23 ± 0.78    |
| Dr-VSP(TM), \(n = 3\) | 31.62 ± 5.35   | 36.28 ± 4.10   | 1.49 ± 0.11 | 1.42 ± 0.10 | 1.28 ± 0.30         | 0.99 ± 0.09    |
| Ci-VSP(WT), \(n = 6\) | 55.26 ± 5.19   | 44.83 ± 4.16   | 0.90 ± 0.07 | 1.01 ± 0.14 | 5.24 ± 1.17         | 1.08 ± 0.10    |
| Ci-VSP(C363S), \(n = 3\) | 93.47 ± 9.64   | 35.46 ± 0.14   | 0.65 ± 0.08 | 1.01 ± 0.15 | 1.75 ± 0.65         | 0.12 ± 0.07    |

**FIGURE 2.** A, amino acid sequence of S4 segment among Shaker potassium channel, Ci-VSP, and Dr-VSP. At position 156 and 165 (asterisk), arginine is missing in Dr-VSP as compared with Shaker potassium channel. B, gating currents of R153Q mutant of Dr-VSP. C, Q-V relationship of Ig-OFF of R153Q in comparison with that of wild type (dotted curve).
This Q-V curve has a similar profile to that of Ci-VSP (Fig. 3C). We also introduced arginine 156, and this mutation accelerated kinetics and exhibited a leftward shift of the Q-V curve (data not shown), although the shift was less remarkable than that of I165R. These findings indicate that positively charged residues of S4 play principal roles in determining voltage dependence of voltage sensor of VSP.

PTEN is known to dephosphorylate phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P3) (9), and Ci-VSP exhibits similar activity (8). A fusion protein of C-terminal cytoplasmic domain with glutathione S-transferase (GST) of Dr-VSP was synthesized in *E. coli*, and phosphatase activity was measured by the malachite green assay as described previously (8). Released phosphate increased in a time-dependent manner and became saturated around at 60 min from the starting point (Fig. 4B). The concentration of released phosphate was estimated as 500 pmol with 1 nmol of substrate PtdIns(3,4,5)P3. This value was much higher than the low constant level when PtdIns(3,4,5)P3 was incubated with GST alone. Cysteine is a critical residue for phosphoinositide phosphatase activity (9), and mutating the corresponding site to serine in Ci-VSP abolished its phosphatase activity (8). No free phosphate (P) was detected when the cytoplasmic region of Dr-VSP(C302S) was incubated with PtdIns(3,4,5)P3 (Fig. 4B).

To test if the phosphatase activity of Dr-VSP is voltage-sensitive, we took a similar approach to that utilized for the analysis of Ci-VSP (8). Phosphoinositide-sensitive potassium channels were coexpressed with Dr-VSP in *Xenopus* oocytes, and their voltage dependence was examined following the method previously established (8). The KCNQ2/3 potassium channel that mediates M current and is best characterized for its sensitivity to PtdIns(4,5)P2 concentration (19) was coexpressed with Dr-VSP, and outward current was measured at different membrane potential levels. In cells that were microinjected with only KCNQ2/3 cRNAs, the current activated during a test pulse to 60 mV following an interval for 10 s at +70 mV (inset in Fig. 4C) and slightly increased its amplitude with time (Fig. 4C, upper panel). This is consistent with our previous finding (8) and probably occurs because of the actions of the intrinsic voltage sensor of KCNQ2/3 channels. In oocytes that coexpressed Dr-VSP and the KCNQ2/3 potassium channel, the current amplitude of KCNQ2/3 channel decreased drastically with time at 70 mV and increased with time at +70 mV (Fig. 4, C, lower panel, and D). An enzyme-null mutant (Dr-VSP:C302S) did not induce such change of KCNQ2/3 channel currents, and exhibited similar patterns as those in oocytes that only express KCNQ2/3 channels (Fig. 4D). Therefore, Dr-VSP is a voltage-sensing phosphatase in which the cytoplasmic phosphatase is regulated by the operation of the VSD as in Ci-VSP. This also indicates that a functional VSP is not restricted to invertebrates but is more ubiquitously present in chordates.

**Alteration of Gating Currents by Inhibition of the Phosphatase**—In our previous report (8), the version of Ci-VSP lacking the whole C-terminal cytoplasmic region showed gating currents, indicating that the VSD is a self-contained functional unit. In that experiment, it was noted that the kinetics of gating currents of the C-terminal truncated version were significantly faster than those of the full-length protein. This preliminary finding on Ci-VSP motivated us to measure gating currents also from the version of Dr-VSP lacking the whole cytoplasmic region. Kinetics of the currents were significantly faster than the full-length Dr-VSP (supplemental Fig. S2). Because interaction between two modules is often bidirectional, we suspected that faster kinetics of gating currents of the C-terminal truncated version could be due to the absence of phosphatase activity. To test this idea, gating currents were measured from the enzyme-defective mutant, Dr-VSP-C302S, in which Cys-302 in the active center of the phosphatase domain was changed to serine. Both Ig-ON and Ig-OFF showed...
faster kinetics in Dr-VSP-C302S than those of the wild type (Fig. 5A). Half-decay times \( t_{\text{decay-1/2}} \) of Ig-ON and Ig-OFF were significantly faster than those of the wild type (Fig. 5, D and E). In contrast with Ig-OFF from the wild type Dr-VSP, decay kinetics of Ig-OFF of Dr-VSP-C302S were almost constant irrespective of the depolarizing level (Fig. 5F, square). Total charge movement of C302S was 1.13 ± 0.52 pC/pF, \( n = 6 \), which is smaller than that of wild type (2.89 ± 0.27 pC/pF, \( n = 5 \)). Q-V curve was also similar, and half-activation \( V_{1/2} \) for wild type and C302S was not significantly different (94.0 ± 6.1 mV, \( n = 5 \), and 96.5 ± 6.0 mV, \( n = 6 \), for the wild type and C302S, respectively) (Fig. 5C).

To verify that the C302S mutant protein is expressed as the full-length protein, Western blot analysis was performed (Fig. 5B). A polyclonal antibody against the cytoplasmic region of Dr-VSP detected a positive band for the wild type and C302S mutant of expected molecular size (about 57 kDa).

To test if such changes in gating currents in the enzyme-defective mutant are a conserved feature among molecular species of VSP, gating currents were also measured from the version of Ci-VSP with serine on cysteine 363 that corresponds to cysteine 302 in Dr-VSP and is known to be critical for phosphoinositide phosphatase activity (8). Ci-VSP-C363S showed faster kinetics of Ig-OFF than wild type Ci-VSP (supplemental Fig. S3), indicating that acceleration of the voltage sensor movement in the enzyme-defective mutant is not restricted to Dr-VSP.

To test if pharmacological inhibition of phosphatase activity also induces acceleration of gating currents, we used pervanadate (Na3VO8), which is known to inhibit the activity of protein-tyrosine phosphatases (20) by binding to a cysteine residue in the active center of phosphatases. Pervanadate was applied to the external bath solution. Both Ig-ON- and Ig-OFF appeared sharper than before pervanadate treatment (Fig. 6A). The total carried charge remained unchanged before and after application of pervanadate (Fig. 6B). The kinetics of Ig-ON as measured by time of half-activation \( t_{\text{decay-1/2}} \) against membrane potential is smaller with pervanadate than before its application (Fig. 6E). In the presence of pervanadate, \( t_{\text{decay-1/2}} \) of Ig-OFF is smaller than before its application (Fig. 6D).
over a wide range of depolarizing membrane potential and does not change with depolarization, phenocopying results with C302S mutant. Application of pervanadate did not alter kinetics of gating currents of the C302S Dr-VSP (Fig. 6C). Acceleration of gating currents by pervanadate is partially because of the actions of residual H$_2$O$_2$ that was contained in pervanadate solution, because slight acceleration of kinetics of gating currents was also seen when the solution containing H$_2$O$_2$ and catalase but not orthovanadate was used in the patch pipette or applied to the bath chamber (data not shown).

Acceleration of Gating Currents by the Inhibition of Phosphatase Activity Occurs Additively to the Effect of Increased Positive Charges on S4—Modification of gating currents is known to occur with mutation of S4 in voltage-gated ion channels (21, 22). The above results with I165R mutant (Fig. 3) suggest that positive charges on S4 play a critical role in the movement of the voltage sensor. We tested if the effect of acceleration of gating currents by the inhibition of phosphatase activity is additive with acceleration of gating currents by addition of positive charges into S4. Arginines were introduced into Ile-165 and Thr-156, so that seven basic amino acid residues were periodically aligned interspersed with pairs of hydrophobic residues. This mutant, I165R/T156R, referred to as “double mutant” (DM), exhibited remarkably fast gating currents (Fig. 7A). It also showed a remarkable shift of the Q-V curve to a negative direction (Fig. 7C).

Gating currents were compared before and after pervanadate application (Fig. 7B). Because the movement of gating charge of the double mutant is partially activated at $-60$ mV, the holding potential was set to $-110$ mV. In the presence of pervanadate, the Q-V curve is significantly shifted in a negative direction; $V_{1/2}$ values before and after treatment with pervanadate were $-20.9 \pm 4.3$ mV ($n = 3$) and $-39.2 \pm 10.2$ mV ($n = 3$), respectively (Fig. 7C). In addition, the kinetics of Ig-ON became slightly faster (Fig. 7, B and D). When orthovanadate was included in the patch pipette, a similar shift of the Q-V curve was seen (data not shown).

Gating currents were also measured from I165R/T156R with a cysteine-to-serine mutation at residue 302 (called “triple mutant”) and compared with the double mutant. The Q-V curve of the triple mutant is significantly shifted to the negative direction as compared with that of the double mutant ($V_{1/2}$ val-
These results indicate that acceleration of gating currents by the inhibition of phosphatase activity occurs additively to the acceleration by the addition of positive charges in S4, indicating that mechanisms of acceleration of gating currents upon inhibition of phosphatase activity are distinct from those with the addition of positive charges to the moving segments of the VSD.

**Alteration of Gating Currents by VSP Enzyme Activity Is Not Because of Decreased PtdIns(4,5)P$_2$**—Our recent study (12) indicated that depletion of PtdIns(4,5)P$_2$ by phosphatase activity during depolarization might change electrostatic environment around the voltage-sensing region, which then indirectly modifies movement of the VSD. To test if the alteration of kinetics of gating currents upon inhibition of enzyme activity is because of altered phosphoinositide concentration, we examined kinetics of gating currents at the decreased level of PtdIns(4,5)P$_2$. One efficient method to induce depletion of PtdIns(4,5)P$_2$ would be to utilize depolarization-induced phosphatase activity of VSP itself.

We tested if Dr-VSP can deplete PtdIns(4,5)P$_2$ by monitoring the activity of heterologously expressed KCNQ2/3 channels. HEK293 cells transfected only with KCNQ2/3 channel genes exhibited slowly rising and persistent outward currents with threshold of activation around $-40$ mV. These properties are characteristic of KCNQ2/3 channels (26). Expression of KCNQ2/3 channel was also verified by its sensitivity to a specific inhibitor, XE991 (data not shown). Current magnitude continued to increase as the voltage step increased over 80 mV (Fig. 8A, upper panel). When expressed with Dr-VSP, KCNQ2/3 current showed current decay when the step pulse exceeded 40 mV (Fig. 8A, lower panel). The extents of current decay were compared among cells by measuring fractions of the outward current remaining at the end of a 5-s, 80-mV depolarizing step relative to the peak amplitude during the whole series of depolarizing episodes (from $-60$ mV to 100 mV by 20 mV increment). The values were $0.50 \pm 0.16$ ($n = 7$) and $0.92 \pm 0.16$ ($n = 6$), for cells coexpressing Dr-VSP and KCNQ2/3 and those expressing only KCNQ2/3, respectively (Fig. 8C). In most cases, the I-V curve also exhibited a bell-shaped pattern; current amplitude decreased as the voltage step reaches over 20 or 30 mV (Fig. 8B). These patterns are similar to those previously reported for Ci-VSP in *Xenopus* oocyte (12). In addition, upon application of pervanadate, current decay was much milder and peak amplitude continued to increase up to 100 mV (supplemental Fig. S5). These results indicate that current decay of KCNQ2/3 current occurs as a result of voltage-induced enzyme activity of Dr-VSP and that phosphatase activity of Dr-VSP activated by a step pulse to 80 mV is sufficient to induce PtdIns(4,5)P$_2$ depletion during a 5-s time scale.
To address if gating currents of Dr-VSP show distinct kinetics between conditions with PtdIns(4,5)P$_2$-depleted state and normal state, gating currents were compared either with a prepulse of 10 s, 80 mV (depleted condition) or without a prepulse (undepleted condition). Fig. 8D shows representative results; shapes and magnitudes of gating currents were independent of the prepulse. Similar results were obtained from two other cells. These findings suggest that accelerated gating currents are not because of any secondary effect of distinct electrostatic environments by altered phosphoinositide levels. We infer that conformational change of the phosphatase module influences the movement of the VSD through its tight coupling.

**DISCUSSION**

In this study, the vertebrate ortholog of VSP cloned from zebrafish, Dr-VSP, was expressed in mammalian cells. Dr-VSP exhibited extremely robust gating currents, enabling us to study detailed kinetics under distinct enzyme states. Dr-VSP showed faster kinetics of gating currents under inhibition of phosphatase activity either by cysteine-to-serine mutation or by vanadate treatment. We propose that conformational change of the phosphatase module influences the movement of the VSD through tight coupling.

How does the kinetics of voltage sensor movement depend on distinct enzyme states? Gating of voltage-gated ion channels is known to be affected by local electrostatic environments based on the concentrations of ions, phospholipids, and sugars surrounding the voltage sensor (27–30). Phosphoinositides have negative charges on phosphate groups. Dephosphorylation of phosphoinositides by the VSP phosphatase activity might change electrostatic environment around the voltage sensor, which then indirectly modifies movement of the VSD. However, this is unlikely for the following reasons. First, kinetics of gating currents was compared under distinct levels of PtdIns(4,5)P$_2$, which were induced by voltage-dependent activation of VSP phosphatase activities with various magnitudes or durations of depolarization (Fig. 8). In this experiment, no clear change in kinetics and magnitude of gating currents was detected. Second, bath perfusion of pervanadate to cells expressing wild type Dr-VSP induced rapid change of kinetics of gating currents even when they were kept voltage-clamped to −60 mV where enzyme activity is not active (8).

The cytoplasmic regions of VSP and PTEN share a conserved structure called movable XXD loop/WPD loop with protein-tyrosine phosphatase. The crystal structure of protein-tyrosine phosphatase showed that aspartic acid residue of movable loop/WPD loop is located near the C$\times_5$R loop in the opposite site of cysteine that forms a thiol-phosphate bond during catalysis (31). In the case of the protein-tyrosine phosphatase 1B, the relative position of the P-loop facing the active enzyme center against the movable loop/WPD loop changes when the substrate-binding residue, cysteine, is mutated to serine, whereas global structure is unchanged (32, 33). In the PTPase ortholog YopH, a protein-tyrosine phosphatase, the frequency of switching between the “closed” state and “open” state of the movable loop/WPD loop decreases when the phosphatase active site cysteine was mutated to serine (34). Both Ci-VSP and Dr-VSP have a con-
served aspartic acid residue in the corresponding region of the WPD loop/movable loop (Asp-270 in Dr-VSP and Asp-331 in Ci-VSP, respectively). One possible scenario is that upon change of cysteine to serine, the region corresponding to the WPD loop/movable loop of protein-tyrosine phosphatases may reduce its dynamics or flexibility, leading to a situation in which the VSD can more easily move. Identification of other sites in the cytoplasmic domain that are sensitive to the VSD movement may give us a hint to understand how two protein modules are functionally coupled in VSPs.

Retrograde regulation of voltage sensor movement by the cytoplasmic region gives mechanistic insights into coupling between VSD and its effector. The recently resolved crystal structures of voltage-gated potassium channels (5) demonstrated that VSD is a self-contained structure, isolated from the central pore domain, locating at the periphery of the channel proteins. Quantitative comparison between voltage sensor movement and phosphatase activities indicates that the voltage range of tuning of enzyme activity matches well with the range of voltage-induced charge movement (12). This suggests that the VSD of VSP is not a simple accessory that confers weak voltage-dependent modification to constitutively active enzyme, but rather operates as a switch to activate phosphatase. Retrograde regulation of voltage sensor movement by the enzyme domain strongly suggests that the VSD is tightly linked to the phosphatase, reinforcing the above idea.

In the voltage-gated potassium channel (21), a model has been proposed in which the S4-S5 linker that couples the voltage sensor and pore domain mechanically pulls down S5, causing channel opening (7). Ig-OFF of the Kv1.5 channel decays more sharply in the presence of permeating ions in the pore than in the absence of permeant ions (17), suggesting that a conformational change in the pore domain could back-propagate to influence the state of voltage sensor movement in voltage-gated ion channels. Future investigation of how mechanisms of bidirectional interaction between the VSD and its effector are distinct or common between VSPs and voltage-gated ion channels may shed light on the general principle of voltage sensor operation.

**FIGURE 8. Kinetics of gating currents is unchanged upon depletion of PtdIns(4,5)P2.** A, representative traces of potassium currents when the KCNQ2/3 channel was expressed without (upper traces) or with Dr-VSP (lower traces). Red and blue indicate traces evoked at 100 and 80 mV, respectively. Dr-VSP induces clear current decay. Holding potential was −80 mV. Currents were elicited at depolarizing steps from −60 to 100 mV in 20-mV increments. B, I-V curve of KCNQ2/3 currents when KCNQ2/3 was expressed alone (circle, n = 6) or with Dr-VSP (square, n = 7). C, comparison of current decay with and without Dr-VSP. The amplitude of KCNQ2/3 outward current evoked at the end of depolarizing pulse (5 s, 80 mV) was divided by the maximum outward current obtained throughout a series of voltage steps up to 100 mV in individual cells. D, superimposition of gating currents measured with and without a prepulse. The prepulse was applied to 80 mV for 10 s. A–C show data from HEK293 cells.
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