Voltage-sensing phosphatases (VSPs) consist of a voltage-sensor domain and a cytoplasmic region with remarkable sequence similarity to phosphatase and tensin homolog deleted on chromosome 10 (PTEN), a tumor suppressor phosphatase. VSPs dephosphorylate the 5′ position of the inositol ring of both phosphatidylinositol 3,4,5-trisphosphate [PI(3,4,5)P3] and phosphatidylinositol 4,5-bisphosphate [PI(4,5)P2] upon voltage depolarization. However, it is unclear whether VSPs also have 3′ phosphatase activity. To gain insights into this question, we performed in vitro assays of phosphatase activities of Ciona intestinalis VSP (Ci-VSP) and transmembrane phosphatase with tensin homology (TPTE) and PTEN homologous inositol lipid phosphatase (TPIP; one human ortholog of VSP) with radiolabeled PI(3,4,5)P3.

**Results**

**3′ Phosphatase Activity of VSP in Vitro.** Previous studies showed that Ci-VSP dephosphorylates the 5′ phosphate of the inositol ring of PI(3,4,5)P3 and PI(4,5)P2 (17, 18). However, it remains unknown whether Ci-VSP dephosphorylates the 3′ phosphate from PI(3,4,5)P3 because phosphatase activity of Ci-VSPs toward PI(4,5)P2 could have masked potential 3′ phosphatase activity toward PI(3,4,5)P3 in previous studies. To address this issue, the cytoplasmic region of Ci-VSP or TPTE (the human VSP ortholog) was reacted with PI(3,4,5)P3 that had a radiolabeled phosphate on the 3′, 4′, or 5′ (D3, D4, or D5) position of the inositol ring. The number of phosphoinositides with distinct numbers of phosphates was quantified by TLC. When the 4′ phosphate on the inositol ring of PI(3,4,5)P3 was labeled with 32P, radioactive signal appeared at the position corresponding to phosphatidylinositol monophosphate, indicating that both the D3 position phosphate and the D5 position phosphate were removed from PI(3,4,5)P3 (Fig. 1A). PI(3,4,5)P3 with radioactive phosphate

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**Author contributions:** T.K., S.H., K.I.H., T.S., and Y.O. designed research; T.K., S.T., S.S., S.Y., and Y.O. performed research; T.K., S.T., S.S., and S.Y. analyzed data; and T.S. and Y.O. wrote the paper.

The authors declare no conflict of interest.

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that Ci-VSP has enzymatic activity toward PI(3,4)P2, with a peptide of the Ci-VSP cytoplasmic region. The results showed that Ci-VSP exhibited phosphatase activity toward PI(3,4,5)P3 and PI(4,5)P2. The GST-fused poly- 
peptide of PI(3,4,5)P3 was not moved by VSP. Most of the radiolabeled PI(3,4,5)P3 was not dephosphorylated in this analysis, as shown in the quantification of radioactive spots (Fig. S1), providing evidence against the possibility that VSP dephosphorylates the D3 position phosphate of PI(3,4,5)P3. 

Table 1. Kinetic parameters of the malachite green assay of the GST-Ci-VSP polypeptide (residues 248-576) with PI(3,4)P2 and PI(3,4,5)P3 as substrate

<table>
<thead>
<tr>
<th>Substrate</th>
<th>(K_m), (\mu M)</th>
<th>(V_{max}), nmol min(^{-1}) (\mu g^{-1})</th>
<th>(K_{cat}), min(^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>PI(3,4)P2</td>
<td>30</td>
<td>0.369 ± 0.042</td>
<td>23.4 ± 2.66</td>
</tr>
<tr>
<td>PI(3,4,5)P3</td>
<td>20</td>
<td>0.121 ± 0.015</td>
<td>7.7 ± 0.95</td>
</tr>
</tbody>
</table>

Measurement was done in six tubes per each concentration of phosphoinositide.

Fig. 1. (A and B) Purified recombinant TPIP was assayed for phosphatase activity toward PI(3,4,5)P3 radiolabeled by \(^{32}\)P specifically at D3, D4, or D5 phosphate \([\text{PI}(3,4,5)\text{P}_3]\), \([\text{PI}(3,4)\text{P}_2]\), or \([\text{PI}(3,4,5)\text{P}_3\text{P}_1]\). A representative radio-TLC image (A) and quantitative estimation of PI(3,4,5)P3 phosphatase activity (B Left) are shown. Note that \(^{32}\)P-labeled PI(3,4,5)P3 was detectable only when PI(3,4,5)P3 was used as substrate. (B Right) Ci-VSP displayed similar activity to that of TPIP. (C) Malachite green assay with the GST-fused Ci-VSP cytoplasmic region using C16-PI(3,4)P2. (Left) Plot of initial rate against PI(3,4)P2 concentration. \(V_{max}\) and \(K_m\) were estimated as 0.121 nmol min\(^{-1}\) \(\mu g^{-1}\) and 20 \(\mu M\), respectively. (Right) Plot of initial rate against PI(3,4,5)P3 concentration. \(V_{max}\) and \(K_m\) were estimated as 0.369 nmol min\(^{-1}\) \(\mu g^{-1}\) and 30 \(\mu M\), respectively.

on the D5 position did not give rise to any band shift after incubation with Ci-VSP or TPIP (Fig. 1B), indicating that the D3 position phosphate of the inositol ring of PI(3,4,5)P3 is not removed by VSP. Most of the radiolabeled PI(3,4,5)P3 was not dephosphorylated in this analysis, as shown in the quantification of radioactive spots (Fig. S1), providing evidence against the possibility that VSP dephosphorylates the D3 position phosphate of PI(3,4,5)P3. PI(3,4,5)P3 was rapidly dephosphorylated into phosphatidylinositol 4-phosphate [PI(4)P]. These findings indicate that Ci-VSP and TPIP dephosphorylate the 3′ phosphate of PI(3,4,5)P3 but not that of PI(3,4,5)P3.

To confirm the phosphatase activity toward PI(3,4)P2, a malachite green assay was also performed with the GST-fused polypeptide of the Ci-VSP cytoplasmic region. The results showed that Ci-VSP has enzymatic activity toward PI(3,4)P2, with \(K_m=20 \mu M\) and \(V_{max}=0.121 \text{nmol min}^{-1} \text{g}^{-1}\) (Fig. 1C and Table 1). This \(K_m\) value was in a similar range to our previously obtained value for PI(3,4,5)P3 (36 \(\mu M\)) (9), suggesting that VSP has similar affinity toward PI(3,4)P2 and PI(3,4,5)P3. The \(V_{max}\) value indicates that the turnover rate for PI(3,4)P2 is about one-third of that for PI(3,4,5)P3.

### 3 Phosphatase Activity of VSP in Cells

To test whether voltage-dependent dephosphorylation of PI(3,4)P2 by VSP occurs in live cells, the PI(3,4)P2 level was monitored in Xenopus oocytes by using the GFP-fused pleckstrin homology (PH) domain of tandem PH domain-containing protein (TAPP1) (PH\_TAPP1-GFP) (19) that selectively binds to PI(3,4)P2 (20). In oocytes expressing PH\_TAPP1-GFP with Ci-VSP (Fig. 2A), fluorescence intensity increases with depolarization to a level more positive than −40 mV (Fig. 2B), consistent with 5′ phosphatase activities toward PI(3,4,5)P3 (21). Depolarization up to 60 mV led to reduction of GFP fluorescence (Fig. 2B), suggesting that the PI(3,4)P2 level decreases. Pooled data from multiple cells at distinct voltages (Fig. 2C) showed a bell-shaped pattern of voltage sensitivity: an increase of fluorescence at depolarizations ranging from −40 mV to 0 mV, saturation of the signal intensity at 30 mV (Fig. 2C), and then a decrease from the basal level at 60 mV (Fig. 2B). This decrease of the PH\_TAPP1-GFP signal at 60 mV, compared with the increase at 0 mV, is also illustrated in the bar graph in Fig. S2E. The fluorescence change at 60 mV often showed two phases: an early transient increase and a later decrease.

We noticed that oocytes expressing only PH\_TAPP1-GFP also showed some change of fluorescence upon depolarization (Fig. S2). This activity seemed to depend on some endogenous phosphatase because pervanadate suppressed the fluorescence change (Fig. S2 C and D). Xenopus ortholog VSP mRNA has been
reported to be present in gonads (12). The change of PH\textsubscript{TAPP1-GFP} in the absence of VSP differs from that based on heterologously expressed VSP in that the fluorescence signal does not increase at less than 10 mV and that it shows no reduction of fluorescence at high depolarization.

We also used oocytes from the Japanese newt \textit{Cynops pyrrhogaster} as a system for heterologous expression (22). Newt oocytes overexpressing only PH\textsubscript{TAPP1-GFP} exhibited no voltage-dependent change of fluorescence (Fig. S3). Newt oocytes expressing Ci-VSP and PH\textsubscript{TAPP1-GFP} recapitulated phenotypes of \textit{Xenopus} oocytes with Ci-VSP and PH\textsubscript{TAPP1-GFP}; they showed an increase of fluorescence signal with mild depolarization, saturation, and sometimes reduction of signal at higher depolarization (Fig. 2 D and E). This finding verifies that changes of PH\textsubscript{TAPP1-GFP} upon activation of VSP phosphatases observed in \textit{Xenopus} oocytes were not significantly affected by endogenous enzyme activities. Variations in voltage-dependent profile of change of PH\textsubscript{TAPP1-GFP} signal were more remarkable in newt oocytes than in \textit{Xenopus} oocytes, probably because of larger variations of expressed Ci-VSP proteins in cell membranes. In later studies, we used \textit{Xenopus} oocytes.

To test whether the above pattern of PI(3,4)\textsubscript{P2} change could be attributable to a possible bell-shaped voltage dependence of phosphatase activity toward PI(3,4,5)\textsubscript{P3}, we measured the change of PH\textsubscript{Btk-GFP} upon activation of VSP phosphatases in different membrane potentials (Fig. 2 F).

**Discussion**

An increase in PI(3,4,5)\textsubscript{P3} was observed upon activating VSP phosphatases, but the increase was not attributable to a bell-shaped voltage dependence of PI(3,4,5)\textsubscript{P3}.

A newt oocyte expressing Ci-VSP and PH\textsubscript{TAPP1-GFP} showed a negative change of PH\textsubscript{Btk-GFP} fluorescence intensity at high depolarization.

**Conclusion**

The increase in PI(3,4,5)\textsubscript{P3} was not attributable to a bell-shaped voltage dependence of PI(3,4,5)\textsubscript{P3}.

**Experiment**

To test whether the above pattern of PI(3,4)\textsubscript{P2} change could be attributable to a possible bell-shaped voltage dependence of phosphatase activity toward PI(3,4,5)\textsubscript{P3}, we measured the change of PH\textsubscript{Btk-GFP} upon activation of VSP phosphatases in different membrane potentials (Fig. 2 F).

**Discussion**

An increase in PI(3,4,5)\textsubscript{P3} was observed upon activating VSP phosphatases, but the increase was not attributable to a bell-shaped voltage dependence of PI(3,4,5)\textsubscript{P3}.

**Conclusion**

The increase in PI(3,4,5)\textsubscript{P3} was not attributable to a bell-shaped voltage dependence of PI(3,4,5)\textsubscript{P3}.
particular, suppressing phosphatase activity toward PI(3,4,5)P3 (10). In vitro malachite green assays show that the double mutant G365A/E411T exhibits reduced phosphatase activity toward PI(4,5)P2 but retains activity toward PI(3,4,5)P3 (Fig. S8B). Confocal microscopic fluorimetry of the GFP-fused PH domain of phospholipase C6 (PHPLC-GFP) fluorescence shows less decrease of signal with G365A/E411T than with wild-type Ci-VSP (Fig. 3B and C). When G365A/E411T Ci-VSP was coexpressed with PHTAPP1-GFP in Xenopus oocytes, an increase of fluorescence signal was observed at 0 mV, indicating that G365A/E411T retains 5′ phosphatase activity toward PI(3,4,5)P3. In addition, a late phase of reduction of PI(3,4,5)P3 level followed upon depolarization to 0 mV or 60 mV.

Discussion

Our previous results of in vitro measurements of Ci-VSP suggested that VSP has phosphatase activity toward PI(3,4,5)P3 (9, 10). In the present study, we showed that VSP has 3′ phosphate phosphatase activity toward PI(3,4,5)P3 and 4′ phosphate phosphatase activity toward PI(4,5)P2, both by in vitro measurements and by live-cell imaging. It is unlikely that the voltage-dependent decrease of PI(3,4,5)P3 in live cells could be indirectly induced by alterations of other phosphoinositide species. Two versions of VSPs, G365A/E411T Ci-VSP and a chimeric protein harboring the cytoplasmic region of chick VSP (Ci-Gg-VSP), show reduced phosphatase activities toward PI(3,4,5)P3. These exhibited a voltage-dependent decrease of PI(3,4,5)P3 as did the wild-type Ci-VSP. Detailed quantitative analyses are hampered by the presence of endogenous voltage-sensitive activities that increased the level of PI(3,4,5)P3, which could be because of endogenous VSP activities given that Xenopus oocytes show reduced phosphatase activities toward PI(3,4,5)P3 still increased in the presence of endogenous voltage-sensitive activities (12). However, these endogenous activities were resistant to antisense DNAs against Xenopus VSP transcripts. Newt oocytes that do not exhibit endogenous voltage-sensitive changes in the level of PI(3,4,5)P3 still showed similar bell-shaped voltage dependence of the level of PI(3,4,5)P3 as in Xenopus oocytes.

How can the 3′ phosphatase activity of the VSP be interpreted in light of recent resolved structural information (10)? The substrate-binding pocket of VSP is slightly smaller than that of PTEN, at least in part because of the presence of glutamic acid at residue 411 instead of threonine (which is the residue in the corresponding site of PTEN) (10). Modeling of the docking of inositol 1,3,4,5-tetrakisphosphate [Ins(1,3,4,5)P4] suggests that the residue histidine 332 interacts with the 4′ phosphate to stabilize binding of phosphoinositides that contain 4′ phosphate such as PI(4,5)P2, PI(3,4)P2, and PI(3,4,5)P3 (10). PI(4,5)P2 and PI(3,4,5)P3 present their 5′ phosphate of the inositol ring on the side of cytochrome 363 in the active center for dephosphorylation. This orientation is more favorable for docking of PI(3,4,5)P3 than the flipped orientation with the axis of the 1′ to 4′ position is in part because of steric hindrance and/or electrostatic repulsion by E411. Unlike PI(3,4,5)P3, PI(4,5)P2 may be able to dock to the substrate-binding pocket in the flipped orientation where the 3′ phosphate could position next to cytochrome 363, with the 4′ phosphate positioning next to histidine 332.

Interestingly, the decrease of PI(3,4,5)P3 was observed only at high voltages. PI(4,5)P2 binding to the phosphoinositide-binding motif between the VSD and the cytoplasmic region has been suggested by previous studies to play role in coupling the VSD to the cytoplasmic region (24). Two versions of VSPs with reduced PI(4,5)P2 phosphatase activities showed saturation or decrease of PI(3,4,5)P3 level upon large depolarization, and the level of PI(3,4,5)P3 monitored by PHPLC-GFP did not show blunted phosphatase activity at high depolarization, ruling out the possibility that a potential regulatory role of PI(4,5)P2 in VSP...
enzymatic activities underlies the bell-shaped voltage dependence of PI(3,4)P2 change. Then how is dephosphorylation of PI(3,4)P2 seen more remarkably at higher depolarization? One possibility is that PI(3,4,5)P3 and PI(3,4)P2 compete for the substrate-binding site of VSP and the relative availability of PI(3,4)P2 versus PI(3,4,5)P3 as substrate for dephosphorylation by VSP is biased by membrane potential. Given that the headgroup of PI(3,4,5)P3 has two more negative charges than that of PI(3,4)P2, the effect of electric field across the cell membrane on the phosphoinositide headgroup will be more potent for PI(3,4,5)P3 than for PI(3,4)P2. Alternatively, substrate preference could be based on the distinct conformation of the VSP’s enzyme region coupled to the distinct activated state of the VSD. More studies will be necessary to reveal mechanisms by which substrate preference depends on membrane potential.

PI(3,4)P2 plays a key role in cell morphology and cell adhesion by regulating signaling in the formation of podosomes (25) or lamellipodia through binding to Tks5/FISH (26), lamellipodin (27, 28), and TAPPI (19). VSP/TPTPE is expressed in testis of ascidian, chick, mouse, and human. VSP is also expressed in blood cells, the nervous system, and epithelium of the developing intestine of ascidian (29) and in the epithelium of chick kidney (16). Patterns of membrane-potential changes may lead to alteration of cell morphology in these VSP-expressing cells, and the bidirectionality of the regulation of PI(3,4)P2 levels may be important for fine-tuning cell morphology that depends on membrane potential. Ci-VSP is also expressed in sperm where membrane-potential change takes place upon exposure to an egg-derived chemoattractant called sperm activating and attracting factor (SAAF) (30). Future studies examining membrane-potential changes in VSP-expressing cells such as sperm, blood cells, or developing cells, are necessary to gain insights into physiological roles of activities of VSP.

Materials and Methods
cDNA Cloning and Plasmids. For human VSP (TPTP) cDNA, human testis RNA purchased from Clontech was reverse-transcribed, and cDNA was amplified by PCR. Ci-Hs-VSP (ascidian–human VSP) chimera was generated by using the cDNA encoding residues 1–257 of Ci-VSP and 215–522 of TPP as a template and subcloned into the XhoI NotI site of pSD64 vector (kindly gifted by Terry Snutch, University of British Columbia, Vancouver, Canada) for Xenopus oocyte expression. The Ci-Gg-VSP (ascidian–chick VSP) chimera was generated by using the cDNA encoding residues 1–257 of Ci-VSP and 197–511 of Gg-VSP and subcloned into pSD64 vector. Point mutants of G36SA/E41IT were Ci-VSP were generated by PCR (QuickChange kit; Stratagene). The PHδtyr/GFP construct was made by modifying the PHδtyr/YFP construct kindly provided by Dario Alessi (Medical Research Council, Dundee, United Kingdom). A constitutively active, membrane-bound form of P3-kinase was constructed by introduction of a mutation of K227E into p110CAAX (p110CAAX-K227E; kindly gifted by Thomas Franke, New York University, New York, NY) subcloned into pSD64.

In Vitro Phosphatase Assay. For malachite green assay, dipalmitoyl-phosphatidylcholine (Wako and Echelon BioSciences) and phosphatidylserine (Sigma) were used. The reactions were initiated by the addition of 2 μg of GST-Ci-VSP (residues 248–576) and incubated at 23 °C. BIOMOL Green reagent was added to the supernatants, and OD620 was measured. Time-dependent activity was measured with different substrate doses up to 200 μM. Data were fit with IgorPro. Initial rate, Vo, of Ci-VSP-catalyzed dephosphorylation of PI(3,4,5)P3 and PI(3,4)P2 was determined from reactions with various concentrations of substrate. Data were fit by the equation: Vo = Vmax[substrate]/(Km + [substrate]).

TLC Assay. For TLC assay, assay buffer (Tris HCl and DTT) was added to the mixture of phosphatidylserine, nonradioactive PI(3,4,5)P3 and radioactive PI(3,4,5)P3. [3-32P]PI(3,4,5)P3 was obtained by incubating dipalmitoyl-

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Fig. 4. Decrease of PI(3,4)P2 at 60 mV depolarization in Xenopus oocyte expressing ascidian–chick VSP chimera (Ci-Gg-VSP). (A) Sensing currents of Ci-Gg-VSP (Lower), a chimera of Ci-VSP VSD and chick VSP phosphatase region compared with those of Ci-VSP (Upper). (B) PI(4,5)P2 phosphatase activities of Ci-Gg-VSP by confocal microscopy measurements of PHPLC–GFP fluorescence upon depolarization to 0 mV and 60 mV. Note that Ci-Gg-VSP has milder PI(4,5)P2 phosphatase activity than Ci-VSP (Fig. 3B). (C) Magnitude of sensing charges. Sensing charges were obtained by integrating OFF sensing currents that are evoked by repolarization to −60 mV after 200-ms depolarization to 140 mV. (D) Extent of voltage-induced change of PHPLC–GFP fluorescence at 0 mV, standardized by sensing charge (reflecting the surface expression level) of VSP. Sensing currents and PHPLC–GFP fluorescence were measured from the same cells. The maximum intensity during depolarizing pulse normalized by the fluorescence intensity just before depolarization was divided by the magnitude of sensing currents in individual cells. (E) Magnitude of voltage-dependent change of fluorescence of PHPLC–GFP standardized by the magnitude of sensing currents in individual cells. (F) Time course of fluorescence from PHδtyr/GFP upon depolarization to four distinct voltage levels. Ci-Gg-VSP shows decrease of PI(3,4)P2 signal at 60 mV. (G) Scheme of VSP phosphatase activities. The transition proposed in this study is in red.
Pi(4,5)P$_2$ with $[\gamma$-$^{32}$P]ATP in the presence of PI3-kinase, FLAG-tagged iSH2-p110$. [D4-$^{32}$P]Pi(3,4,5)P$_3$ and [D5-$^{32}$P]Pi(3,4,5)P$_3$ were prepared from [D4-$^{32}$P]Pi(4,5)P$_2$ and [D5-$^{32}$P]Pi(4,5)P$_2$, respectively (see SI Materials and Methods for more detailed descriptions). Reaction was performed with purified GST-Ci-VSP at 23 °C.

Electrophysiology and Live-Cell Imaging of Phosphoinositides. Xenopus laevis and Japanese newt, C. pyrhogaster, were anesthetized by immersion in water containing 0.1–0.2% Tricaine. Experiments were performed according to the guidelines of the Animal Research Committees of the Graduate School of Medicine of Osaka University. The oocytes were incubated at 18 °C in ND96 solution (31).

Sensing current was recorded under the two-electrode voltage clamp with a “bath-clamp” amplifier (OC-725C; Warner Instruments) (7). Linear and symmetrical current were subtracted by a P–8 procedure. Stimulation and data acquisition were performed with Digidata 1440A AD/DA converter with pCLAMP software (Molecular Devices). The bath solution contained 96 mM N-methyl-D-glucamine-methanesulfonate, 3 mM MgCl$_2$, and 5 mM Hepes (pH 7.4) or ND96. The level of PI(4,5)P$_2$ or PI(3,4)P$_2$ was monitored by imaging of PHPLC glucamine-methanesulfonate, 3 mM MgCl$_2$, and 5 mM Hepes (pH 7.4) or ND96. The level of PI(4,5)P$_2$ or PI(3,4)P$_2$ was monitored by imaging of PHPLC.

Note Added in Proof. A decline of PH$_{TAPP1}$-GFP fluorescence upon activities of CI-VSP has been reported also in a recent paper (Liu at al. (2012) A glutamate switch controls voltage-sensitive phosphatase function. Nature Struct and Mol Biol, 10.1038/nsmb.2289).

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**Supporting Information**

**Kurokawa et al. 10.1073/pnas.1203799109**

**SI Materials and Methods**

**cDNA Cloning and Plasmids.** For cloning of human voltage-sensitive phosphatase (VSP) [TPTE and PTEN homologous inositol lipid phosphatase (TPIP)] cDNA, total human testis RNA purchased from Clontech was reverse-transcribed, and cDNA was amplified with the primer set 5'-GAGCTTACACATCTCATCA-CAGGC-3' and 5'-GCATCATCAGGAAGACTTTAGG-3'. The PCR product was cloned into pCR4-TOPO (Invitrogen). The Cl-Hs-VSP chimera was then generated by PCR using the cDNA encoding residues 1–257 of *Ciona intestinalis* VSP (Ci-VSP) and 215–522 of TPIP as a template and subcloned into the XhoI/NcoI site of pSD64 vector. For Xenopus oocyte expression.

To isolate the chick (*Gallus gallus domesticus*) VSP cDNA, Ci-VSP amino acid sequence was used to survey databases. We found an annotated mRNA sequence (GenBank accession no. XM_417079) whose deduced amino acid sequence showed a similarity to Ci-VSP through overall sequences (47.7%). To provide this information, we performed RT-PCR with cDNA derived from 1-d-old chick brains as a template and then obtained a series of cDNA fragments containing the full-length coding region. The deduced amino acid sequence of the cDNA matched that deduced from the annotated sequence in the database except for a few amino acid substitutions (glycine 34 with aspartic acid and arginine 287 with histidine). Chick VSP was termed Gg-VSP. The nucleotide sequences of primers used were as follows: Gg-VSP sense, 5'-GGGCCGTGCTGAGAAGACAA-3'; Gg-VSP antisense, 5'-TTACAGGTGAGGGTTATCCAGCTCGTT-3'. The resulting PCR products were cloned into pGEM-T Easy Vector (Promega). The CI-Gg-VSP chimera was generated by PCR using the cDNA encoding residues 1–257 of Ci-VSP and 197–511 of Gg-VSP as a template and subcloned into the XhoI/NcoI site of pSD64 vector for Xenopus oocyte expression. Point mutants of G365A/E411T into Ci-VSP were generated by PCR (QuickChange kit; Stratagene).

**GFP-tandem pleckstrin homology (PH) domain-containing protein (TAPP1) fusion construct (PHTag-GFP) for live-cell imaging in *Xenopus* oocyte was made by modifying the PH-TAPP1 YFP construct kindly provided by Dario Alessi (Medical Research Council, Dundee, UK). The cDNA fragment encoding the PH domain of TAPP1 was amplified by PCR from the TAPP1-YFP plasmid as the template and subcloned into to EGFP-C1 (Clontech), resulting in PH-TAPP1-GFP construct, which was then subcloned into pSD64 vector.

**Malachite Green Assay.** Dipalmitoyl-phosphatidinositols (Wako and Echelon Biosciences) and phosphatidylycerine (Sigma) were dried up and dispersed in the phosphatase assay buffer [100 mM Tris-HCl (pH 8.0) and 10 mM DTT] to final concentrations of 0.2 and 1 mM, respectively. The reactions were initiated by the addition of 2 μg of GST-Ci-VSP (residues 248–576) diluted in assay buffer and incubated at 23 °C. Reactions were quenched after 60 min by the addition of 100 mM N-ethylmaleimide and centrifugation. BIOMOL Green reagent was added to the supernatants, and OD_{600} was measured with H2O as a reference. Time-dependent phosphatase activity was measured with substrate doses of 0 μM, 5 μM, 10 μM, 20 μM, 30 μM, 40 μM, 50 μM, 100 μM, and 200 μM. Data were fit by the Hill plot equation with IgorPro. Initial rate, V_0, of Ci-VSP-catalyzed dephosphorylation of phosphatidylinositol 3,4,5-trisphosphate [PI(3,4,5)P_3] and phosphatidylinositol 3,4-bisphosphate [PI(3,4)P_2] was determined from reactions with various concentrations of substrate. Data were fit by the equation: V_0 = V_{max}[substrate]/(K_{cat} + [substrate]). Turnover rate, K_{cat}, was calculated from V_{max}, the maximum rate.

**TLC Assay.** [D3-32P]PI(3,4,5)P_3 was obtained by incubating dipalmitoyl-phosphatidylinositol 4,5-bisphosphate [PI(4,5)P_2] with [γ-32P]ATP in the presence of PI3-kinase, FLAG-tagged iSH2-p110, [D3-32P]PI(3,4,5)P_3, and [D5-32P]PI(3,4,5)P_3 were prepared from [D3-32P]PI(4,5)P_2 and [D5-32P]PI(3,4,5)P_2, respectively. [D4-32P]PI(4,5)P_2 was obtained by incubating dipalmitoyl-phosphatidylinositol 5-phosphate [PI(5)P] with [γ-32P]ATP in the presence of PI(5)P 4-kinase, GST-tagged human PIPKII (GenBank accession no. NM000528). [D5-32P]PI(4,5)P_2 was isolated by TLC and then phosphorylated on the D3 position with non-radioactive ATP by PI3-kinase, FLAG-tagged iSH2-p110. [D3-32P]PI(4,5)P_2 was obtained by incubating dipalmitoyl-phosphatidylinositol 4-phosphate [PI(4)P] with [γ-32P]ATP in the presence of PI(4)P 5-kinase, FLAG-tagged mouse PIPKIα (GenBank accession no. NM000846). [D5-32P]PI(4,5)P_2 was isolated by TLC and then phosphorylated on the D3 position with non-radioactive ATP by PI3-kinase, FLAG-tagged iSH2-p110. In TLC purification of some radiolabeled substrate, silica gel 60 plates were pre-treated with 1.2% (wt/vol) potassium oxalate in methanol/water (2:3), and phosphoinositides were developed under the condition of chloroform/methanol/acetic acid/acetic acid/water (7:5:2:2/2). Radiolabeled PI(3,4,5)P_3 was isolated from the plate, extracted by the Bligh–Dyer method, and evaporated, then used as substrate for phosphatase activities of VSP. For enzyme reaction, the mixture of phosphatidylserine (50 μg), nonradioactive PI(3,4,5)P_3 (5 μg), and radioactive PI(3,4,5)P_3 was evaporated, and 50 μL of assay buffer (50 mM Tris-HCl (pH 8) and 2 mM DTT) was added, and mixture was obtained by sonication. This was reacted with 100 μL of purified GST-Ci-VSP (5 μg) or buffer (as negative control) at 23 °C for 2 h. Lipid was purified and extracted by TLC by the Bligh–Dyer method. Proportions of PI(3,4,5)P_3, PI(4,5)P_2, and phosphatidylinositol phosphate were determined by Fujifilm Image Analyzer FLA-5000. The amount of PI(3,4,5)P_3 input was calculated from buffer control.

**Electrophysiology and Live-Cell Imaging of PI(3,4)P_2, PI(4,5)P_2, and PI(3,4,5)P_3.** For most of experiments, *Xenopus laevis* was used. In some experiments (Fig. 2 *D* and *E*), the Japanese newt *Cynops pyrrhogaster* was also used. The animals were anesthetized by immersing in water containing 0.15% (wt/vol) Tricaine. Isolated oocytes were obtained by treatment with type I collagenase (1 mg/mL; Sigma-Aldrich) and injected with ~50 nL of cRNA solution. Newt oocytes were isolated manually after mild digestion with collagenase (1). Experiments were performed according to the guidelines of the Animal Research Committees of Graduate School of Medicine of Osaka University. cRNAs were synthesized from linearized plasmid DNA with a mMESSAGE mMACHINE transcription kit (Ambion). The injected oocytes were incubated for 2 d at 18 °C in ND96 solution (2).

Sensing current was recorded under the two-electrode voltage clamp using a “bath-clamp” amplifier (OC-725C; Warner Instruments) (3). Linear and symmetrical current were subtracted by a P-8 procedure. The Q–V curve was fitted by a Boltzmann relation, Q = 1/[1 + exp{(V − V_{1/2})/k}] where k is the Boltzmann constant and e is the elementary electric charge. Stimulation and data acquisition were performed with Digidata 1440A AD/DA converter with pCLAMP software (Molecular Devices). The bath solution contained 96 mM N-methyl-d-glucamine-methanesulfono-
nate, 3 mM MgCl$_2$, and 5 mM Hepes (pH 7.4). Intracellular glass microelectrodes were filled with 3 M KCl (pH 7.2), and their resistance was ranged from 0.1 to 0.6 MΩ.

PI(4,5)P$_2$ or PI(3,4)P$_2$ level was monitored by imaging of the GFP-fused PH domain of phospholipase Cδ (PH$_{PLCδ}$-GFP) or PH$_{TAPP1}$-GFP in Xenopus oocyte and newt oocyte by XY-T or XT mode of a confocal microscopy system (FV300; Olympus) with an upright microscope. PI(3,4,5)P$_3$ level was monitored by imaging the GFP-fused PH domain from Btk (PH$_{Btk}$-GFP; kindly provided by M. Fukuda, Tohoku University, Sendai, Japan). To increase the level of resting PI(3,4,5)P$_3$ level in cells for imaging of PH$_{Btk}$-GFP, a constitutively active, membrane-bound PI3-kinase, p110CAAX-K227E, was expressed. After insertion of two microelectrodes, cell was kept to −60 mV without depolarizing episode or confocal imaging for at least more than 1 min, which was needed for phosphoinositide levels to reach steady state upon change of membrane potential. Image was taken by a 20× objective lens using FluoView software (Olympus). In all experiments of confocal imaging, cells were voltage-clamped with OC-725C (Warner Instruments) without bath perfusion. Membrane potential and current were monitored with Digidata 1440A AD/DA converter under the control of pCLAMP software (Molecular Devices). The bath solution contained 96 mM N-methyl-D-glucamine-methanesulfonate, 3 mM MgCl$_2$, and 5 mM Hepes (pH 7.4).


**Fig. S1.** Quantification of relative radioactivity as photostimulated luminescence value resolved by TLC assay of dephosphorylated products from three forms of $^{32}$P-labeled PI(3,4,5)P$_3$. Photostimulated luminescence values were read by an imaging analyzer.

**Fig. S2.** Endogenous voltage-sensitive phosphatase activity in Xenopus oocyte. (A) Representative time course of fluorescence intensity of PH$_{TAPP1}$-GFP from an oocyte that was only microinjected with cRNA encoding PH$_{TAPP1}$-GFP. (B) Relative increase of fluorescence measured at the last 10 s of depolarizing pulse plotted against the membrane potential. (C) Sensitivity of fluorescence change to pervanadate (red). The same oocyte was recorded before and after application of 300 μM pervanadate. (D) Comparison of fluorescence change between the absence (control) and presence of pervanadate. (E) Ratio of fluorescence deviation at 60 mV ($F_{60\text{ mV}}$) versus that at 0 mV ($F_{0\text{ mV}}$).
Fig. S3. Absence of endogenous voltage-sensitive phosphatase activity in newt oocyte as shown by PH$_{TAPP1}$-GFP. Membrane potential was held to $-60 \text{ mV}$ under the two-electrode voltage clamp, and step was applied to 0, 30, or 60 mV.

Fig. S4. Design of chimeric VSP constructs used in this study. S4 region and phosphoinositide-binding motif (PBM) are underlined.

Fig. S5. PI(4,5)P$_2$ phosphatase activity of the chimeric protein (Ci-Hs-VSP) consisting of the voltage-sensor domain (VSD) of Ci-VSP and the enzyme region of the human ortholog of VSP (TPIP) or Hs-VSP. (A) Depolarization induced reduction of PI(4,5)P$_2$ with Ci-Hs-VSP as indicated by PH$_{PLC\delta}$-GFP. (B) Summary of sensing charges. (C) Extent of fluorescence change normalized by sensing charge (equivalent to the density of VSP molecules on the cell surface).
Fig. 56. Conserved phosphoinositide phosphatase activities in human VSP. (A) Representative data of intensity of PH\textsubscript{TAPP1}-GFP fluorescence in a voltage-clamped Xenopus oocyte expressing a chimeric protein, Ci-Hs-VSP, harboring the cytoplasmic region of human ortholog of VSP (TPIP). Records from a single Xenopus oocyte at four membrane potentials are shown. (B) Pooled data of voltage-dependent change in PH\textsubscript{TAPP1}-GFP fluorescence intensity from Xenopus oocytes expressing Ci-Hs-VSP ($n$ = 3–5). The mean intensity during the last 10 s of depolarizing pulse was calculated and normalized by the intensity just before depolarizing pulse in individual Xenopus oocyte. (C) Comparison of the magnitudes of the maximum “sensing” charge between Ci-VSP and Ci-Hs-VSP. The values of sensing charges were obtained by taking saturated level of the charge in the charge–voltage plot, which was constructed by integrating OFF sensing currents upon repolarization to −60 mV from 140 mV, giving almost saturated magnitude of charge movement. (D) The increase of PH\textsubscript{TAPP1}-GFP fluorescence measured at 0 mV normalized by the sensing charge. Both sensing currents and PH\textsubscript{TAPP1}-GFP fluorescence were measured from the same Xenopus oocytes.
**Fig. S7.** Comparison of voltage dependence of charge movements of the voltage sensor between Ci-VSP (n = 10) and Ci-Hs-VSP (n = 12) in Xenopus oocyte.

**Fig. S8.** (A) Malachite green assay with four species of phosphoinositides of chick VSP (Gg-VSP). (B) Malachite green assay with G365A/E411T Ci-VSP mutant.

**Fig. S9.** Comparison of voltage dependence of charge movements of the voltage sensor between Ci-VSP (n = 10) and Ci-Gg-VSP (n = 10) in Xenopus oocyte.