A glutamate switch controls voltage-sensitive phosphatase function

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The Ciona intestinalis voltage-sensing phosphatase (Ci-VSP) couples a voltage-sensing domain (VSD) to a lipid phosphatase that is similar to the tumor suppressor PTEN. How the VSD controls enzyme function has been unclear. Here, we present high-resolution crystal structures of the Ci-VSP enzymatic domain that reveal conformational changes in a crucial loop, termed the ‘gating loop’, that controls access to the active site by a mechanism in which residue Glu411 directly competes with substrate. Structure-based mutations that restrict gating loop conformation impair catalytic function and demonstrate that Glu411 also contributes to substrate selectivity. Structure-guided mutations further define an interaction between the gating loop and linker that connects the phosphatase to the VSD for voltage control of enzyme activity. Together, the data suggest that functional coupling between the gating loop and the linker forms the heart of the regulatory mechanism that controls voltage-dependent enzyme activation.

Ci-VSP is the founding member of a recently discovered membrane protein family in which a transmembrane VSD controls the activity of a C-terminal cytoplasmic enzyme domain that has sequence similarity to the tumor suppressor phosphatase PTEN (protein lipid phosphatase and tensin homolog deleted on chromosome 10) rather than an ion channel pore1–3. This VSD-PTEN protein class is found in frogs, fish, mice and humans4 and provides a direct means to couple membrane potential changes with phosphatase action1,5,6. Despite extensive biophysical characterization, the structural underpinnings of how voltage controls Ci-VSP function have remained unclear. Two distinct conceptual models have been proposed to explain how the VSD response to voltage changes drives the enzymatic activity of the cytoplasmic domain: simple movement of active site to the membrane and a voltage-dependent conformational change in the active site2.

To define elements that influence Ci-VSP function, we determined six Ci-VSP cytoplasmic domain high-resolution X-ray crystal structures of different conformations and variants and one substrate analog complex. These reveal an enzymatic domain loop, the gating loop, that differs substantially from its PTEN counterpart, the ‘TI loop’, and show three distinct conformations that open or close active site access by controlling the position of gating loop residue Glu411. Functional investigation of a battery of structure-based mutants evaluated in the isolated enzymatic domain in vitro and full-length protein in live cells show that Glu411 contributes to activity. Further, our data suggest that the linker region that connects the VSD to the enzymatic domain and is known to be important for function6,7 couples voltage sensing to enzyme activity by controlling gating loop action. Together, the results provide a new model in which gating loop conformational changes have an essential role in how voltage regulates Ci-VSP activity.

RESULTS

Ci-VSP cytoplasmic domain high-resolution structure

We crystallized several Ci-VSP cytoplasmic domain constructs in different conditions and crystal habits (Table 1 and Supplementary Fig. 1). The highest-resolution diffraction data came from a construct that encompassed the VSD-phosphatase linker and the entire N-terminal phosphatase domain (residues 241–576, denoted ‘241’) bearing the catalytic site mutant, C633S, (Form 1, Table 1). Molecular replacement with a PTEN-based model showed that 241 Form I crystals contained two essentially identical copies in the asymmetric unit (all-atom r.m.s. deviation equals 0.48 Å, Supplementary Table 1). Because of the superior electron density, we use copy B for description and comparisons. As anticipated from the PTEN sequence similarity (Fig. 1a), the Ci-VSP cytoplasmic domain (Fig. 1b) comprises an N-terminal phosphatase domain (residues 263–431) that has the canonical phosphatase fold found in protein tyrosine phosphatase 1B (PTP1B)8–11 (Supplementary Fig. 2a) and a C-terminal C2 domain (residues 432–576)12 (the PTEN Cα r.m.s. deviation equals 1.60 Å, Fig. 1c and Supplementary Table 1).

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Multiple Ci-VSP structures reveal gating loop movement

The core regions of the structures of two other 241 C363S crystal forms, Forms II and III (Table 1 and Supplementary Fig. 1b,c), and a C363S construct having a truncated linker domain, denoted ‘256’ (residues 256–576, C363S) (Table 1 and Supplementary Fig. 1d), were similar to 241 Form I (Fig. 2a,b and Supplementary Table 1). However, the linker and gating loop had notable differences. The extent of α-helical structure in the linker varied in the three 241 structures, whereas the β-hairpin remained. In 256, the visible part of the linker common to 241 and 256 (residues 258–263) had no regular secondary structure (Fig. 2b). The gating loop conformations in 241 Form I and 241 Form III were similar, whereas this region adopted a different conformation in the 241 Form II and 256 structures (Fig. 2c,d).

Further comparison revealed that even though the gating loop N-terminal end showed a range of conformations (Fig. 2a,b), some of which participate in minor crystal lattice interactions (Supplementary Table 2), a four-residue C-terminal segment (residues 409–412) adopted just two conformations (Fig. 2c,d) that placed Glu411 in either the hydrophobic α456 pocket (241 Forms I and III) (Fig. 2c) or the active site (241 Form II, both chains, and 256) (Fig. 2c,d). This second conformation, similar to that seen

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Table 1 Data collection and refinement statistics

|                  | 241–576 (C363S) (Form I) | 241–576 (C363S) (Form II) | 241–576 (C363S) (Form III) | 241–576 (ΔA401–405, C363S) | 256–576 (C363S) | 256–576 (E411F) (C363S + IP₃) |
|------------------|--------------------------|---------------------------|----------------------------|-----------------------------|----------------|--------------------------------|
| Resolution (Å)   | 100–1.10                 | 100–1.30                  | 100–1.50                   | 100–1.72                    | 100–1.60       | 100–1.95                        | 100–1.85 |
| Redundancy       | (1.12–1.10)              | (1.33–1.30)               | (1.54–1.50)                | (1.78–1.72)                 | (1.65–1.60)    | (2.02–1.95)                      | (1.92–1.85) |
| Space group      | P₂₁                     | P₂₁                       | P₁                        | P₂                          | P₂             | P₂                            | P₂           |
| Cell dimensions  | a, b, c (Å)              | 50.97, 85.71, 50.75, 80.37, 50.86, 83.86, 50.86, 83.86, 50.56, 76.80, 50.85, 38.60, 50.98, 76.59, 50.66, 79.85, 84.33, 83.64, 83.60, 84.0, 89.83, 84.95, 87.93 |
| α, β, γ (°)      | 90, 90.96, 90, 90.93, 12.90, 90, 86.55, 89.55, 89.37, 90, 89.27, 90, 90.93, 90, 90.93, 90, 90.93, 90, 90.93, 90, 90.93, 90, 90.93, 90, 90.93, 90, 90.93, 90 |
| Rₚ (%), I/σ (%)  | 5.4 (47.5), 8.4 (58.4), 5.5 (29.0), 11.6 (74.0), 7.0 (67.2), 11.2 (38.3), 11.1 (66.0) |
| Total reflections| 2,445,254                | 4,385,060                 | 3,194,799                  | 1,081,730                   | 606,240        | 532,266                         | 1,952,687 |
| Unique reflections| 291,443                  | 153,889                   | 176,659                    | 68,903                      | 43,750         | 24,720                          | 59,840      |
| Completeness (%) | 99.6 (96.9), 94.1 (90.1), 80.5 (83.1), 100.0 (100.0), 97.7 (82.2), 99.9 (99.8), 99.9 (99.6) |
| Redundancy       | 4.3 (2.9), 12.5 (7.0), 2.6 (1.4), 6.9 (3.7), 5.8 (4.1), 5.6 (3.5), 4.7 (3.5) |

Values in parentheses are for the highest-resolution shell. Five percent of randomly selected reflections were used for R_free monitoring during refinement.
by others,\(^\text{13}\), docks Val410 rather than Glu411 in the α456 pocket (Fig. 2c,d). Having Glu411 in the α456 pocket leaves the active site wide open (‘open’) (Fig. 2e), whereas having Glu411 in the active site (‘closed’) leaves only a small pocket occupied by a sulfate ion in the 256 structure (Fig. 2f) and should preclude substrate binding. Refinement of 241 Form I revealed a minor conformation in which Glu411 has a similar position to that in 256. The reduced active site pocket size in the closed form is notable given prior evidence that Glu411 has a similar position to that in 256. The reduced active site pocket size in the closed form is notable given prior evidence that Glu411 has a similar position to that in 256. The reduced active site pocket size in the closed form is notable given prior evidence that Glu411 has a similar position to that in 256.

Active site architecture

The Ci-VSP P-loop signature motif (residues His362–Arg369) adopts the canonical structure of protein tyrosine phosphatases (Supplementary Fig. 2a,b) and PTEN (Fig. 1c,d), and is exceptionally well ordered, having B-factors among the lowest in the structure (Supplementary Fig. 2c–e). The active site pocket is lined by a loop that contributes general acid-base residues for catalysis, (residues 330–333, the ‘D331 loop’) in a conformation identical to PTEN (Fig. 1d). The D331 loop is joined by Tyr522, which closes off a shallow pocket occupied by solvent in PTEN (Fig. 1d and Supplementary Fig. 2f) and whose hydroxyl is oriented in a manner seemingly ideal for substrate interactions. The 241 Form I electron density also revealed a phosphate ion coordinated by Arg369, the P-loop element commonly used to bind substrate phosphates\(^\text{11}\) (Fig. 1d). This phosphate position coincides with a sulfate ion in 256 and substrate phosphate in PTP1B phosphatase–substrate complexes\(^\text{8,16}\) (Supplementary Fig. 2b). Thus, it probably marks the position of the phosphate that Ci-VSP would cleave from its substrates.

Residues Ser363–Gly366, which are central to the active site, adopt two distinct conformations (Fig. 3a). The 256 active site matches the structure described in ref. 13 (Fig. 3b), whereas the 241 Form I and Form III active sites match those of the G365A mutant (Fig. 3c) and are similar to PTEN, which has an alanine at the 365-equivalent position. The 241 Form II and the 248 (ref. 13) active sites adopt a conformation hybrid between the 241 Form I and 256 structures. These differences are uncorrelated with gating loop status (Supplementary Table 3). Nevertheless, this active site mobility may influence catalysis. Together, the multiple structures point to active site and gating loop conformational changes that we set out to investigate in turn.

Roles of Gly365 and Tyr522 in enzymatic activity

Ci-VSP cleaves the 5-phosphate of phosphatidylinositol-3,4,5-trisphosphate (PI(3,4,5)P\(_3\)) and phosphatidylinositol 4,5-bisphosphate (PI(4,5)P\(_2\))\(^{17–19}\) and has weaker activity against the 3-phosphate\(^{17}\). By contrast, PTEN acts solely on the PI(3,4,5)P\(_3\) 3-phosphate\(^{17,20,21}\). These specificity differences have been suggested to originate from an alanine-to-glycine difference at Ci-VSP active site residue 365 (refs. 4, 17); however, the 241 Form I structure shows that Ci-VSP and PTEN active sites are identical (r.m.s. deviation equals 0.29 Å and 0.77 Å, Cα and all atoms, respectively) (Fig. 1d and Supplementary Table 1). To investigate whether the alanine-to-glycine difference might affect substrate specificity, we conducted a series of functional assays on full-length Ci-VSP G365A in live *Xenopus laevis* oocyte membranes in the context of a second mutation, G214C, that serves as a fluorescent label attachment site at the S4 transmembrane segment external end to assess membrane expression and VSD conformational changes\(^5\).

We coexpressed Ci-VSP with Kir2.1 R228Q (Kir2.1Q), a potassium channel activated by PI(4,5)P\(_2\) (refs. 1, 5, 7, 18), and used Kir2.1Q activity as a reporter of Ci-VSP–induced changes in PI(4,5)P\(_2\) levels.

Figure 1 Structure of the Ci-VSP catalytic domain and comparison with PTEN. (a) Ci-VSP intracellular domain (241–576) and PTEN sequence comparison. Secondary structure elements from 241 Form I are indicated. Linker, phosphatase and C2 domains are in orange, violet blue and purple, respectively. The gating loop is indicated by beige. Dashed lines indicate residues not modeled or sequence alignment gaps. (b) 241 Form I Ci-VSP intracellular domain ribbon diagram. N-terminal linker, orange; phosphatase domain, violet blue; gating loop, beige; C2 domain, purple. C363S and Glu411 are shown as sticks. (c) Superposition of Ci-VSP 241 Form I (colored as in panel b with the 522 loop shown in dark blue) and PTEN (light blue). (d) Structural comparison of Ci-VSP 241 and PTEN active sites. The superscripts indicate origin: C, Ci-VSP; P, PTEN. The orientation is similar to that in panel e. (e) View of the α456 pocket. The view is 90° to the orientation in panels c and d.
**Figure 2** Comparison of Ci-VSP intracellular domain structures reveals gating loop movements. (a) Superposed cartoon diagrams of Ci-VSP, residues 241–576. Form I, violet blue; Form II, chain A, lime green; Form II, chain B, orange; Form III, blue. (b) Superposed cartoon diagrams of Ci-VSP 241 Form I (violet blue) and 256 (magenta). Active site C363S, Glu411, 241 and 256 linker and gating loops are indicated. (c) Details of the gating loop and α456 pocket from Ci-VSP, residues 241–576, Form I (violet blue); Form II, chain A (lime green); Form II, chain B (orange); Form III (blue). Val410, Glu411 and Thr412 are shown as sticks and are labeled. (d) Details of the gating loop and α456 pocket from 241 Form I (violet blue) and 256 (magenta). Val410, Glu411 and Thr412 are shown as sticks and are labeled. (e) Surface view of the Ci-VSP 241 Form I active site. Ci-VSP elements are colored as follows: N-terminal linker, orange; phosphatase (PPase) domain, violet blue; gating loop, beige; C2 domain, purple; 522 loop, dark blue. The active site phosphate is shown in sticks. The dashed oval indicates the open active site pocket. (f) Surface view of the Ci-VSP 256 active site. Ci-VSP elements are colored as follows: phosphatase domain, magenta; gating loop, beige; C2 domain, light violet; 522 loop, dark blue. The active site sulfate is shown in sticks. The dashed circle indicates the closed active site pocket. The colors of the C2 domains differ in e and f to distinguish the 241 Form I (e) structure from the 256 (f) structure.

Comparison with the wild type and the catalytically inactive mutant C363S showed that Ci-VSP G365A produces a depolarization-induced reduction of Kir2.1Q current (Fig. 4a), indicating that the glycine-to-alanine change does not make Ci-VSP an exclusive 3-phosphatase like PTEN.

To test specificity further, we used a set of green fluorescent protein (GFP)-tagged pleckstrin homology (PH) domains that bind to different phosphoinositides: a PI(4,5)P2 probe, the phospholipase C (PLC) PH domain (GFP-PLC-PH)18,19 (Fig. 4b); a PI(3,4,5)P3 probe, the tandem PH domain–containing protein 1 (TAPP-1) PH domain (GFP-TAPP-PH)19 (Fig. 4d), and a phosphatidylinositol-3,4-disphosphate (PI(3,4)P2) probe, the general receptor for phosphoinositides-1 (GRP1) PH domain (GFP-GRP-PH) (Fig. 4c); and a phosphatidylinositol-3,4,5-disphosphate (PI(3,4,5)P3) probe, the tandem PH domain–containing protein 1 (TAPP-1) PH domain (GFP-TAPP-PH)19 (Fig. 4d). For the PI(4,5)P2 and PI(3,4,5)P3 probes (Fig. 4b,c), following depolarization, both WT and G365A, but not C363S, caused similar probe mobilization, albeit with different magnitudes, suggesting that WT and G365A have similar substrate selectivity. For the PI(3,4)P2 probe (Fig. 4d), WT caused a fluorescence increase that was followed by a decrease. These changes correspond to the probe moving to the membrane because of PI(3,4)P2 generation from PI(3,4,5)P3, and then away from the membrane, indicating PI(3,4)P2 destruction. In contrast, only a small fluorescence decrease was observed with G365A, indicating preservation of some 3-phosphatase activity against PI(3,4)P2. Consistent with prior work17, all assays showed that G365A had reduced activity relative to WT, as indicated by smaller ΔF amplitudes. This reduction is consistent with the idea that active site flexibility is important for function and is restricted by the introduction of the side chain methyl group.

To test whether Tyr522 influences Ci-VSP function, we examined the Y522A mutant. Measurements of Kir2.1Q current (Fig. 4a) and depolarization-induced membrane fluorescence changes for the PI(4,5)P2 probe (Fig. 4b) indicated that Y522A had little effect on PI(4,5)P2 5-phosphate dephosphorylation. However, Y522A produced a marked augmentation in the depolarization-induced fluorescence of the PI(3,4)P2 probe (Fig. 4d inset and Supplementary Fig. 4a,b), indicating increased PI(3,4)P2 accumulation. Additionally, Y522A eliminated the transient nature of the rise (Fig. 4d inset and Supplementary Fig. 4a,b). Such behavior suggests that Y522A slows dephosphorylation at the PI(3,4)P2 3-phosphate.

**Figure 3** Comparison of Ci-VSP active site P-loop conformations. (a) The 241 (violet blue) and 256 (magenta) conformations; (b) The 241 (violet blue), 256 (magenta) and 236 (light blue)13 conformations. (c) The 241, 256 and 248 G365A (lime green) conformations13. Crucial residues are labeled. The superscripts indicates origin. Residues with common positions have no superscript.
We used a malachite green assay 1,17,17 to measure activity of purified WT and Y522A 241 cytoplasmic domain constructs against PI(3,4,5)P3 and PI(3,4)P2. Circular dichroism measurements showed that Y522A does not cause a loss of structure (Supplementary Fig. 4c). Y522A modestly reduced steady-state activity against PI(3,4)P2 relative to WT, but without a significant effect on Vmax (Fig. 4e, Supplementary Fig. 4d and Supplementary Table 4), indicating no major change to the isolated enzyme fragment substrate specificity. However, Y522A accelerated VSD activation and deactivation rearrangements in voltage-clamp fluorometry (VCF) measurements of tetramethyl-rhodamine-6-maleimide (TMRM)-labeled full-length protein (Fig. 4f). This is reminiscent of how other catalytic site mutations affect VSD motion 6,7,18, and together with the effects on GFP-probe behavior, it supports the idea that Tyr522 has a role in Ci-VSP function.

Gating loop conformation and Ci-VSP function

To address the hypothesis that gating loop mobility has a role in controlling enzyme activity, we made a set of Glu411 hydrophobic substitutions (E411L, E411I and E411F) that we expected to favor residue 411 occupancy of the hydrophobic α456 pocket and stabilize the open conformation. We also made a deletion (Δ401–405) that removed gating loop residues having the greatest structural variation to create a Ci-VSP gating loop of equal length to the PTEN T1 loop (Fig. 5a).

Malachite green assays showed that all three hydrophobic mutants reduced PI(3,4,5)P3 dephosphorylation (Fig. 5b), with E411F causing the greatest reduction. Δ401-405 resulted in nearly complete loss of activity (Fig. 5b). All three Glu411 hydrophobic mutants and Δ401-405 eliminated the activity of full-length Ci-VSP measured in oocytes by using the Kir2.1Q reporter assay (Fig. 5c). In contrast, polar substitutions (E411D, E411Q and E411T) had a milder impact on the isolated enzymatic domain and full-length protein (Fig. 5c).

We determined the structures of the two gating loop mutants that caused the most marked functional losses: E411F and Δ401-405 (Table 1 and Fig. 5d–g). E411F crystallized in a 256 background that bore a native active site residue Cys363, whereas Δ401-405 crystallized in the 241 C363S background. The overall structures of 256 E411F and 241 Δ401-405 are very similar to the 256 and 241 Form I structures, respectively (Supplementary Table 1). The similarity of 256 E411F to the other structures demonstrates that, as expected from phosphatase structural studies 8, the C363S mutation has a negligible impact on conformation.

In the 256 E411F structure, the gating loop segment is in the open conformation that has E411F in the hydrophobic α456 pocket (Fig. 5e and Supplementary Table 2). By contrast, the Δ401–405 structure shows that the gating loop N-terminal deletion yields a closed conformation (Fig. 5f and Supplementary Table 2). Both structures have a catalytic site conformation identical to that of 241 Form I, providing additional evidence that the conformation of this region and the gating loop are uncorrelated (Supplementary Table 3). Notably, neither gating loop conformation matches the equivalent PTEN region in which Thr167, the 411 counterpart, is midway between open and closed conformations (Fig. 5g). This, together with the functional data showing that E411F (open) and Δ401-405 (closed) markedly impair activity of the isolated domain and full-length protein (Fig. 5b, c), suggests that the structures represent trapped forms of two crucial 410–412 segment conformations. The structures further suggest that gating loop movement, centered on the exchange of residues 410 and 411 in the α456 hydrophobic pocket, is part of the Ci-VSP catalytic cycle.

To probe the gating loop further, we examined E411T and E411F in the full-length protein expressed in X. laevis oocytes using the GFP-PH domain assay and VCF. E411F did not generate detectable PI(3,4,5)P3 (Fig. 5h). By contrast, E411T caused an unexpected increase in GFP-TAPP-PH membrane fluorescence that lacked the subsequent late-declining phase seen with the WT (Fig. 5h). This was accompanied by a slowing of the fluorescence increase (Supplementary Fig. 4a, b), suggesting that E411T moderately slows PI(3,4,5)P3 5-phosphate dephosphorylation and strongly decreases PI(3,4)P2 3-phosphate dephosphorylation. Kinetic studies on the isolated cytosolic domain confirmed the decrease in 3-phosphate dephosphorylation, showing an ~50% reduction in Vmax for the E411T cytosolic domain relative to WT (Supplementary Fig. 4d and Supplementary Table 4). VCF measurements showed that both E411T and E411F...
altered VSD movement (Fig. 5i). E411T shifted the activation curve to the left (–13.2 mV) and slowed deactivation, consistent with resting-state stabilization. Conversely, E411F shifted the activation curve to the right (+11.3 mV) and slightly accelerated deactivation, consistent with active-state stabilization. Together, these data on the sensitivity to changes at Glu411 support the idea that gating loop motion is crucial to Ci-VSP function and suggest that Glu411 may have a role in substrate recognition.

A gating loop conformation similar to that of PTEN

The structure of a complex of a soluble substrate mimic, inositol 1,4,5-trisphosphate (IP3) and the 241 C363S construct (Table 1) revealed an IP3 molecule in the active site (Fig. 6a,b) and a gating loop in which Glu411 is between the open and closed positions (Fig. 6c). Here, Val410 remains in the α456 pocket (Fig. 6d), and the active site conformation is identical to 241 Form I (Supplementary Table 3). The active site IP3 clashes sterically with the Glu411 closed conformation (Fig. 6b–d) and provides direct evidence that the gating loop must move to accommodate substrate binding. The remainder of the gating loop is very similar to the closed 241 Form II chain B. Notably, the Val410 and Glu411 conformations are exceptionally similar to the equivalent PTEN positions, including the alignment of Ci-VSP residues Thr412 and Glu411 with the PTEN Thr167 across the Cα-Cγ positions (Fig. 6d).

The IP3 has its 1-phosphate at the position of the active site phosphate and sulfate from the 241 and 256 structures, respectively (Fig. 6a). In native Ci-VSP inositol phosphate substrates, the
Fig. 7 Ci-VSP linker–gating loop coupling. (a) Cartoon diagram of 241 Form I linker–gating loop region. Phosphatase domain, linker and gating loop are colored violet blue, orange and beige, respectively. Side chains are shown as sticks. Select residues are labeled. (b) Comparison of linker and gating loops from the 241 Form I (violet blue) and IP₃ complex structures (teal). Crucial residues and IP₃ are shown as sticks. The superscripts indicate origin. (c) Malachite green assay with PS-PI(3,4,5)P₃ vesicles for Ci-VSP 241 and indicated mutants. Error bars are ± s.e.m, n = 3. (d) Full-length Ci-VSP activity measured by Kir2.1Q coexpressed with WT, C363S, D400K or D400R in the G214C background. Error bars are ± s.e.m, n = 10–38. (e) Representative TMRM fluorescence trace during a step from a −80 mV holding potential to +200 mV for labeled G214C and indicated mutants. Phosphatase (blue oval) and C2 domain (light purple square) are depicted. Phosphatase domain green and white elements indicate the active site and α456 pocket, respectively. Orange hexagon, IP₃.

1-phosphate links the inositol ring to a lipid and is thus unlikely to be cleaved. Nevertheless, a counterclockwise rotation of the IP₃ can be made to place the 5-phosphate in the active site and the 4-phosphate in a position where it could interact with Glu411.

The structure also revealed a second IP₃ bound to cationic residues Arg281, Arg286, Arg313 and Lys364, proximal to the active site (Supplementary Fig. 5). As this region is likely to appose the lipid bilayer, this second IP₃ site may represent a position for nonspecific interactions with the membrane or may have a more specific role in affecting substrate movement into or out of the active site.

Gating loop–linker coupling
To probe how voltage changes drive Ci-VSP function, we investigated the linker–gating loop relationship. Inspection of the N-terminal linker in 241 Form I, where this element is most completely defined (Fig. 7a), suggested residues that might mediate gating loop–linker coupling: the conserved gating loop residues 398–400 (Supplementary Fig. 3) and linker residues Lys252 and Arg253, known to couple voltage changes to activity₆. In 241 Form I, gating loop Asp400 interacts through a salt bridge with Lys252, and linker residue Arg253 coordinates the Gly365 backbone carbonyl of the active site. Notably, Lys252 and Arg253 exchange positions between the 241 Form I and IP₃-bound structures (Fig. 7b), Lys252 goes from a solvent-exposed interaction with Asp400 to a position that forms a hydrogen bond with the Gly365 carbonyl. Conversely, Arg253 goes from coordinating the Gly365 carbonyl to a solvent-exposed interaction with Asp400. This exchange is also present in the closed structure 241 Form II chain B (Supplementary Movie 1). Gating loop Arg398 has hydrogen bond interactions with the active site loop (Fig. 7a) in all of the structures. Based on the structures and evidence that K252Q and R253Q drastically reduce voltage-dependent activity and uncouple the VSD from the active site⁷, we changed Asp400 to asparagine, alanine, lysine and arginine. All were detrimental to enzymatic domain function (Fig. 7c) and full-length protein function (Fig. 7d). None affected the structure, as assessed by circular dichroism (Supplementary Fig. 6).

We next asked if Asp400 has a role in coupling the VSD and active site. Earlier analysis of VSD–active site coupling showed that linker residues Lys252 and Arg253 are critical for voltage-dependent activity and for coupling catalytic site properties to the VSD⁶. Asp400 mutants D400K and D400R that abolished enzymatic domain activity almost eliminated the marked influence of catalytic site mutation D311A on VSD deactivation rate, which is consistent with disruption of VSD–active site coupling (Fig. 7e). Together, the data suggest that voltage-dependent rearrangements of the VSD couple to the enzyme active site through interactions between linker residues Lys252 and Arg253 and their gating loop partner Asp400.

DISCUSSION
Understanding how transmembrane voltage changes influence the activity of diverse membrane proteins remains a challenge. In voltage-gated phosphatases, two distinct models could explain how membrane depolarization regulates membrane lipid dephosphorylation. In one model, analogous to the PTEN diffusion-based process where the N-terminal domain interacts with the membrane, depolarization would cause the Ci-VSP linker to interact with membrane phospholipids and place a constitutively active catalytic site within reach of substrate⁶. In a second model, depolarization would drive a
conformational change that gates the catalytic activity\(^7\). Our studies strongly support the second model.

The crystal structures show that gating loop residues 409–412 form a switch that adopts three distinct conformations: open, closed and bound. The gating loop movement between the open and closed conformations is extreme (Supplementary Movie 1). Glu411 moves from a hydrophobic cavity formed by the \(\alpha_4\), \(\alpha_5\) and \(\alpha_6\) helices in the open state into the active site in the closed state, where it is in direct steric competition with substrate. Repositioning Glu411 to the bound conformation, which overlaps well with the analogous PTEN \(T1\) loop, relieves the direct steric competition between Glu411 and substrate in the closed form (Fig. 6). Thus, gating loop movement is a central aspect of Ci-VSP function. These conformational changes may account entirely for voltage-dependent regulation of activity but may also be accompanied by active site movement relative to the membrane.

Two mutants markedly demonstrate the importance of Ci-VSP gating loop movement for function. Ci-VSP \(\Delta 401-405\), with the gating loop truncated to a PTEN-equivalent length, is inactive owing to Glu411 active site occupation. Conversely, E411T favors the open gating loop conformation and a wide open active site similar to PTEN (Fig. 5d,e). This arrangement should offer unfettered substrate access to the active site, yet it disrupts Ci-VSP function (Fig. 5b,c). This seeming paradox suggests that the gating loop does more than simply control active site access. Indeed, E411T influences both substrate selectivity and VSD movement (Fig. 5f,i). Thus, the data point to a mechanism in which the gating loop facilitates active site entry or exit of substrate, product or both, and underscores a central role for Glu411 in this process.

Burial of a glutamate in a hydrophobic cavity, as seen with Glu411 in the \(\alpha 456\) pocket, is unusual but not unprecedented\(^{23,24}\). There are well-characterized examples where hydrophobic pockets interact with charged or polar ligands\(^{25–27}\). Nevertheless, such interactions have an energetic penalty that is likely to be an important component of gating loop function. Indeed, hydrophobic mutations of Glu411, which should partition into the \(\alpha 456\) hydrophobic cavity, markedly reduce activity (Fig. 5b,c). This result fits with the concept of a trade-off between stability and function for enzymatic residues involved in substrate binding and catalysis\(^28\) and suggests that gating loop mobility depends on the hydrophilic character of residue 411.

Our findings also highlight the interdomain linker connecting the VSD and enzyme\(^1,6,7\). Structure-based mutational analysis indicates that the two linker residues Lys252 and Arg253 and gating loop residue Asp400 are crucial to linker–gating loop coupling. This is substantially different from the PTEN\(^{29–31}\)–membrane recruitment mechanism\(^6\) and instead supports a mechanism driven by voltage-dependent conformational change in the active site\(^7\). Notably, the structural plasticity of the gating loop N-terminal end contrasts with the switch region conformational properties. Interactions of this portion of the gating loop with the lipid bilayer, absent from the structures, may be important for mediating VSD–switch region coupling.

The data presented here uncover a previously unknown element required for Ci-VSP function, the gating loop. The gating loop switch region, residues 409–412, adopts three distinct conformations that we propose are part of the catalytic cycle (Fig. 7f), in which substrate is given active site access (open), occluded from the active site (closed) or selected (bound) by Glu411. The ability of the gating loop to access these conformations is controlled by the VSD and involves linker residues Lys252 and Arg253 and gating loop residue Asp400. Together, these results suggest that a mobile gate whose conformation is modulated by the linker that connects the cytoplasmic domain to the voltage-sensing S4 segment of the VSD mediates the voltage dependence of Ci-VSP enzyme activity.

**METHODS**

Methods and any associated references are available in the online version of the paper.

**Accession codes.** Coordinates and structure factors have been deposited in the Protein Data Bank with the following accession codes: 241 Form I, 3V0D; 241 Form II, 3V0E; 241 Form III, 3V0G; 241 AA01–405, 3V0J; 241 iP3, complex, 3V0H; 256, 3V0E; and 256–E411F, 3V0I.

**Note:** Supplementary information is available in the online version of the paper.

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**AUTHOR CONTRIBUTIONS**

L.L., S.C.K., S.M., E.Y.I. and D.L.M. conceived the study. L.L., S.C.K., Q.X., S.M. and C.K. conducted the experiments and analyzed data. E.Y.I. and D.L.M. analyzed data and provided guidance and support throughout. L.L., S.C.K., E.Y.I. and D.L.M. wrote the paper.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

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ONLINE METHODS

Con structs and protein expression. Structural and in vitro activity studies used two backgrounds, Ci-VSP241–256 and Ci-VSP256–257, denoted ‘241’ and ‘256’, respectively, cloned into pET-28b(+) using Ndel and HindIII to yield a fusion bearing an N-terminal His tag and tobacco etch virus (TEV) protease site. Proteins were expressed in Escherichia coli Rosetta (DE3) at 65°C in 2–3 h, harvested at 200–300 mg/ml in lysis buffer (50 mM NaCl, 1 mM DTT, 10% glycerol, 30 mM imidazole, 1 M PMSE, 25 µg ml−1 DNase, 5 mM MgCl2, 20 µg ml−1 lysozyme and 0.05% Triton X-100, 10 mM HEPES, pH 7.4). After centrifugation (40,000g), supernatant was incubated with 5 µl of Talon Co3+ resin (Clontech) for 1 h on a rotary shaker at 4 °C. Resin was packed into a column and washed with 100 ml buffer A (50 mM NaCl, 30 mM imidazole, 10 mM HEPES, pH 7.4), followed by 100 ml buffer B (buffer A plus 450 mM NaCl) and 50 ml buffer A. Protein was eluted with 30–ml stepwise increments of 50 mM and 300 mM imidazole in buffer A. A 1-mL portion of 500 mM EDTA (pH 8.0) was added to the Ci-VSP fraction (50 mM imidazole) and dialyzed overnight (Amicon, MWCO 10,000) against 41 of buffer C (50 mM NaCl, 3 mM DTT, 50 mM Tris, pH 8.0). For crystallization, TEV protease was added into samples in buffer CDuring the dialysis, at a molar ratio of TEV:Ci-VSP = 1:5 at 4 °C overnight with slow rocking. Samples were centrifuged (30,000g) to remove precipitates, loaded onto Hi-load Q Sepharose (241 constructs) (GE Healthcare), and eluted using a 0–70% gradient of buffer C and buffer D (buffer C + 950 mM NaCl). Ci-VSP fractions were pooled, concentrated to less than 1 ml by Centriprep or Centricon (Amicon, 10,000 MWCO) and run on a Superdex 200 column in buffer C. Ci-VSP fractions were pooled and concentrated to 10 mg ml−1 for crystallization. Concentrations were determined by absorbance32. For circular dichroism, protein was exchanged into circular dichroism buffer (10 mM Na phosphate, pH 7.3, 50 mM NaCl, 3 mM DTT) by passage over a Superdex 200 column (GE Healthcare). All proteins gave single bands on SDS-PAGE and single, monodisperse gel-filtration peaks.

Circular dichroism. Circular dichroism spectra were measured on an Aviv model 215 spectrometer (Aviv Biomedical) from 300 nm to 200 nm at 4 °C using a cuvette with 0.1-cm path length. Five scans of the same sample were averaged. Molar ellipticity was calculated as [θ] = 100µm/Cdl, where m is the circular dichroism signal in millidegrees, C is protein concentration in millimolar, n is the number of residues and l is path length in centimeters.

In vitro phosphatase assay. Chloroform: methanol:water (1:2:0.8) solutions of 1,2-dioleoyl-sn-glycerol-3-phospho-1-serine and either Pi(3,4,5)P3 or Pi(3,4)P2 lipids (Avanti Polar Lipids) were prepared as described previously7. Final concentrations were 150 or 250 µM for Pi(3,4,5)P3 or Pi(3,4)P2, and 750 µM or 1.25 mM for PS. Steady-state reactions were initiated by vesicle addition (40 µl at 75 µM Pi(3,4,5)P3 or purified Ci-VSP (10 µl at 2.44 µM), incubated at 25 °C and stopped by addition of 50 µl of 100 mM N-ethylmaleimide. Phosphate was measured with the Malachite Green Phosphate Assay Kit (Cayman). Stopped reactions were centrifuged. A 50-µl volume was transferred to a 96-well plate. A 5-µl volume of malachite green (MG) acidic solution was added per well, gently mixed by tapping and incubated at room temperature for 10 min. A 15-µl volume of MG blue solution was added to each well and gently mixed by tapping and incubated at room temperature for 20 min to develop color. Absorption was determined at 620 nm with an Infinite 200 PRO microplate reader (Tecan). Phosphate concentrations were determined by comparison to a KH2PO4 standard curve. Reaction with lipids alone was used as a control. Kinetic reactions were measured at substrate concentrations of 10, 20, 30, 40, 50, 100, 150 and 200 µM over an 80-min time period using the Biomol Green reagent (Biomol International, ENZO). Initial velocities were fit for each concentration and plotted versus substrate concentration. Velocity versus substrate plots were fit with V = (Vmax × [S])/(Km + [S]).

Crystallization and data collection. Hanging-drop crystallization screens (Hampton Research and Qiagen) were done using a Mosquito (TPP Labtech) and optimized with Limbo plates (Hampton Research). The 256 crystals were obtained from 2.1–2.3 M ammonium sulfate, 0.1 M HEPES, pH 7.0–7.5. 256 E411F crystals were obtained in a similar condition with 2.5 M ammonium sulfate. The 241 Form I and A401–405 crystals were grown with 17.5–22.5% PEG 2000, 0.1 M ammonium dihydrophosphate, 0.1 M Tris-HCl, pH 8.0. The 241 Form II crystals were obtained by soaking 241 Form I crystals in 25% PEG 2000, 0.1 M Tris-HCl, pH 8.0, using six serial dilutions over three days. The 241 Form III crystals were obtained by soaking form I crystals in 30% PEG 2000, 0.1 M ammonium dihydrophosphate, 0.1 M Tris-HCl, pH 8.0, overnight. The 241–IP3 complex crystals were prepared by soaking 241 Form II in 25% PEG 2000, 0.1 M Tris-HCl, pH 8.0, 2.5 mM IP3, for three days.

Diffraction data were collected at 100 K from crystals flash frozen in liquid nitrogen, beamline 8.3.1 (ALS, Berkeley, CA), by oscillation at ω = 1.116 Å, with an ADSC Quantum 315r detector. For cryoprotection, crystals were passed through their corresponding well solution, with the addition of 18% glycerol for ammonium sulfate conditions or with PEG 2000 concentrations increased to 30% for PEG 2000 conditions. Data were processed using HKL2000 (ref. 33).

Structural determination and refinement. Structures were determined by molecular replacement using MolRep41 and PTEN (PDB 1DSR10) with non-conserved residues replaced by alanine as a search model for the solution of 241 Form I and 256. The 241 Form I and 256 structures were used for the other crystal forms, depending on the beginning residues. COOT33 was used for graphical modeling. Structures were refined with Refmac5, and 5% of the data, randomly selected, was used for Rfree. Typically, after the molecules were located in the asymmetric unit cell, rigid-body refinement was conducted at 3.5 Å (this refinement was redone later, as needed), followed by restrained refinement. TLS refinement was applied. For resolution better than 1.5 Å, B-factors were refined anisotropically; for the 241 Form I dataset, unrestrained refinement was carried out at the end.

Molecular biology for full-length assays. Ci-VSP in the pSD64TF vector was provided by Y. Okamura. GFP-PLC-PH, GFP-TAPP-PH and GFP-GRP-PH were provided by T. Meyer, T. Balla and J. Falke, respectively. Each was subcloned into pGEMHE. Kir2.1 was provided by E. Revezy. Mutations were made by QuikChange (Stratage) and confirmed by sequencing. RNA was transcribed using T7 and SP6 mMessage mMachine (Ambion) kits.

Voltage clamp fluorometry. VCF was carried out as described previously7. Briefly, X. laevis oocytes were injected with 50 nl mRNA at 0.02–2.0 mg ml−1 depending on the experiment. Cells were then incubated in ND-96 (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl2, 1 mM MgCl2, 50 µg ml−1 gentamicin, 2.5 mM Na pyruvate and 5 mM HEPES, pH 7.6) at 18 °C for 24–48 h. Light was filtered through the following filter sets (excitation and emission, respectively): HQ535/50 and HQ610/75 for TMRM; and HQ450/50 and HQ510/50 for GFP (Chroma Technology).

On the day of the experiment, cells were treated as described previously7. The recording solution was a modified ND-96, ND-96 (without the gentamicin or pyruvate). Cells were constantly perfused with ND-96 for all experiments.

Fluorescence measures of activity. RNA ratios of 2:1 (µg ml−1) were used for Ci-VSP and each PH domain (total RNA ~1.2 µg ml−1). Cells were recorded in perfused ND-96’. GFP-PLC-PH, GFP-GRP-PH and GFP-TAPP-PH were used to detect PI(3,4,5)P3, PI(3,4)P2 and PI(3,4)P2, respectively. For GRP and TAPP PH domains, 8 µM insulin was added to the ND-96’ to promote PI3K activity and boost PI(3,4)P2 levels. Catalytically inactive Ci-VSP C363S showed TAPP–PH domain background activity, presumed to come from the native X. laevis VSP, Xi-VSP38. Therefore, we subtracted an average trace obtained from 19 oocytes expressing C363S showed TAPP–PH domain background activity. The cell was clamped with standard conditions, except that the cell was preincubated with 0.2 µM TAPP–PH domain (total RNA ~1.2 µg ml−1). Cells were monitored by detecting Kir2.1 R228Q (Kir2.1Q) activation by PI(4,5)P2 (ref. 5). The cell was hyperpolarized to ~100 mV, where Ci-VSP is off, until a steady-state current was established (5–7 min) followed by depolarization to +60 mV to activate Ci-VSP.

Electrophysiological measures of activity. Ci-VSP catalytic activity was measured by detecting Kir2.1 R228Q (Kir2.1Q) activation by PI(4,5)P2 (ref. 5). The cell was hyperpolarized to ~100 mV, where Ci-VSP is off, until a steady-state current was established (5–7 min) followed by depolarization to +60 mV to activate Ci-VSP.
until a steady state was re-established (2–7 min). Resulting current changes between the Ci-VSP off and on states were measured and expressed as percent activity. Currents were leak subtracted by assuming a voltage-independent linear leak. Current was measured at −100 mV after the test holding potential, and leak was measured at +50 mV where the Kir2.1Q channels should be blocked. Leak-subtracted (LS) current was calculated as: \( I_{LS} = I_{-100mV} + 2I_{+50mV} \). Percent activity was calculated as:

\[
\frac{\Delta I}{I_{max}} = \frac{I_{+60mV \text{first}} - I_{+60mV \text{last}}}{I_{+60mV \text{first}}}
\]

A 50-nl volume of mRNA was injected into *X. laevis* oocytes at a 40:1 to a 100:1 (\( \mu g \mu l^{-1} \)) ratio of Ci-VSP to Kir2.1Q (total RNA ~0.9–2 \( \mu g \mu l^{-1} \)). Ci-VSP expression levels were confirmed using TMRM labeling. Cells were incubated in ND-96 at 18 °C for 24–36 h. Recording solutions contained 90 mM K methanesulfonic acid, 3 mM MgCl\(_2\), 2 mM CaCl\(_2\), 10 mM HEPES, pH 7.4. Other conditions were the same as for VCF.

**Data analysis.** Kinetic and steady-state traces were analyzed using Clampfit (Molecular Devices), IGOR Pro (WaveMetrics) and Excel (Microsoft). Steady-state voltage-dependent traces were fit with Boltzmann equations. Data were normalized to the amplitude of the Boltzmann fits. Error bars indicate the s.e.m. Statistical significance was determined using the Student’s t-test.

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