Voltage-Sensing Phosphatase: Its Molecular Relationship With PTEN

Voltage-sensing phosphoinositide phosphatase (VSP) contains voltage sensor and cytoplasmic phosphatase domains. A unique feature of this protein is that depolarization-induced motions of the voltage sensor activate PtdIns(3,4,5)P₃ and PtdIns(4,5)P₂ phosphatase activities. VSP exhibits remarkable structural similarities with PTEN, the phosphatase and tensin homolog deleted on chromosome 10. These similarities include the cytoplasmic phosphatase region, the phosphoinositide binding region, and the putative membrane interacting C2 domain.

Phosphoinositides and PTEN

Phosphoinositides constitute up to 4% of the total membrane phospholipids and are located at the inner leaflet of biological membranes. The headgroup of phosphoinositides is composed of an inositol ring with phosphate(s). Phosphoinositides are key second messengers for intracellular signaling, with distinct phosphoinositides having different signaling roles depending on the position and the number of phosphates on the inositol ring.

PTEN (phosphatase, tensin homolog, deleted on chromosome TEN) was first identified as a tumor suppressor gene by mapping homozygous deletions on human chromosome 10q23.3 (23, 24, 45). PTEN exhibits overall homology to protein tyrosine phosphatases and weak homology to tensin, a cell adhesion molecule, and auxilin, a protein involved in synaptic vesicle transport. Because the phosphatase domain of PTEN shows a signature Cys-x₅-Arg (CX(₅)R) motif that is conserved in protein tyrosine phosphatases (PTPs), PTEN was first thought to be a protein phosphatase (24). However, PTEN exhibited only low catalytic activity toward phosphoproteins and peptides. It showed the highest activity toward negatively charged phosphorylated polymers such as Glu-Tyrₙ, but this activity was low relative to other protein phosphatases (23, 36). PTEN was shown by the Dixon laboratory to dephosphorylate PtdIns(3,4,5)P₃, making it the first phosphatase identified to utilize a phosphoinositide as its substrate (29, 30). A large number of studies with gene knockdown or conditional gene knockout mice have shown that PTEN plays a critical role in diverse biological events, including development, cell growth, cell size, and morphology, through negative regulation of PtdIns(3,4,5)P₃.

PTEN consists of two major domains (the phosphatase domain and the C2 domain) and three short regulatory regions [the NH₂-terminal phosphoinositide binding motif (PBM), the COOH-terminal tail region, and the COOH-terminal PDZ binding motif] (see FIGURE 1). The phosphatase domain of PTEN has a similar structure to that of non-metal-regulated phosphatases, including PTPs and dual-specificity phosphatases (22). A study of the crystal structure of PTEN (22) showed that the phosphatase domain has a deeper substrate binding pocket than PTPs and dual-specificity phosphatases, accounting for a much higher preference for phosphoinositides than protein substrates.

The structure of the C2 domain of PTEN is similar to the C2 domain in other proteins, including phospholipase C and phospholipase A (22). Although the C2 domain of PTEN does not bind calcium, as predicted from the absence of conserved amino acids in the calcium binding loops that face membranes (22), it has membrane binding affinity (22) through nonspecific electrostatic interactions (32). Furthermore, the C2 domain of PTEN interacts with the phosphatase domain through multiple hydrogen bonds (22). The regulatory functions of the C2 domain in PTEN seem to be multi-fold. First, the C2 domain helps target the protein to the membrane through its electrostatic affinity. The GST fusion protein of the human PTEN C2 domain can bind to multi-lamellar lipid vesicles in vitro (7). PTEN constructs with mutations in several basic amino acids in the C2 domain showed reduced affinity to phospholipid vesicles and low tumor suppressing activities. However, the addition of a myristoylation signal to those C2 domain mutants does not rescue the tumor suppressing activities of PTEN (13). These suggest that membrane targeting is not the only functional role of the C2 domain in PTEN (13). Truncation or mutation of the C2 domain makes the protein unstable and accelerates protein degradation (7, 12,
PTEN constructs with point mutations in the loop regions of beta-sheets of the C2 domain showed reduced tumor suppressing activity but retained catalytic activity toward water-soluble PtdIns(3,4,5)P3 (13). Furthermore, many of the residues involved in interdomain contacts are mutated in human cancers (22), suggesting that the C2 domain regulates the phosphatase domain through maintaining the protein stability.

PTEN has three short regions for enzyme regulation. The NH2-terminal region contains a conserved phosphoinositide-binding motif (called PBM) with multiple basic residues. In particular, the KRR sequence at residues 13–15 is highly conserved among PTEN orthologs from slime mold to human. PBM seems to preferentially bind PtdIns(4,5)P2. A 21-amino acid polypeptide containing the PBM of PTEN physically binds to PtdIns(4,5)P2 (43). K13E (9, 14, 27) and R15A (11, 45) are reported as human cancer mutations. The K13E PTEN mutant does not bind to PtdIns(4,5)P2 in vitro as examined by FRET between tryptophan fluorescence and a pyrene label on phosphatidylethanolamine (PE) (43). K13E of human PTEN and the Dictyostelium protein lacking the whole PBM region showed remarkably lower phosphatase activities toward PtdIns(3,4,5)P3 (5, 53) and reduced tumor suppressing activities (17, 53). The K13A mutation of human PTEN (5) also showed reduced phosphatase activity. In Dictyostelium, PTEN plays a key role in directed migration during chemotaxis (16). PTEN downregulates PtdIns(3,4,5)P3 at the leading edge of migrating cells, and ablation of the PTEN gene in Dictyostelium abolishes chemotaxis (16). Introduction of the PBM-deleted version of PTEN into PTEN-knockout Dictyostelium cells cannot rescue the cell migration in response to chemo-attractant (17). With mutations in PBM or on depletion of PtdIns(4,5)P2, PTEN does not efficiently localize to cell membranes, suggesting that PtdIns(4,5)P2 binding to PBM is required for efficient membrane anchoring (41). Other mechanisms by which PtdIns(4,5)P2 binding to PBM regulates PTEN activities have also been proposed. Devreotes’s group suggests that PBM intrinsically binds to the catalytic site to shield it from substrate in the membrane (41). In this model, binding of PBM to phosphoinositide releases the catalytic site to allow enzyme activity. It has also been suggested that binding of PtdIns(4,5)P2 to PBM allosterically regulates protein conformation of the phosphatase domain of PTEN (43).

The region downstream of the C2 domain contains the COOH-terminal tail (C-tail) and the PDZ binding motif (see FIGURE 1). The C-tail consists of a region of ~60 amino acids and contains sites that are phosphorylated by casein kinase, glycogen synthase 3b (2), and RhoA-associated kinase (25). Phosphorylation of the C-tail silences phosphatase activities (38, 41). The phosphorylated C-tail region directly binds to the rest of the protein, thereby masking enzyme activity (41). This interaction does not occur when four phosphorylation sites in the C-tail are replaced by alanine (41). Binding of the C-tail to the rest of the protein also depends on protein conformation of the phosphatase domain and the C2 domain, because the C-tail region does not bind PTEN containing a mutation in the cysteine of the CX(5)R motif or a mutation in the calcium binding region 3 (CBR3) loop, thought to be involved in membrane interaction. The PDZ binding motif most likely stabilizes the proteins and facilitates protein anchoring to cell membranes through its binding to membrane-anchored PDZ proteins.

**FIGURE 1.** Scheme of VSP, PTEN, tensin, myotubularin, PTPMT1, and Inpp4

Phylogenetic trees show a close relationship between human PTEN and human VSP, with human PTEN diverging from the human VSP before human VSP diverged from VSP orthologs in other species. VSP protein contains a transmembrane domain and a cytoplasmic phosphatase region. The ascidian VSP, Ci-VSP, has been most intensively characterized. This gene was identified from the genome of a marine invertebrate, Ciona intestinalis (39), but it is conserved from sea squirts to human. Human orthologs (52) and murine orthologs (54) of VSP had been characterized as PTEN-related phosphoinositide phosphatases without considering the function of the transmembrane domain. The cytoplasmic region of VSP has a remarkable sequence similarity to PTEN but lacks the region corresponding to the COOH-terminal region and the PDZ-binding motif.
FIGURE 2. Cartoon of VSP vs. voltage-gated ion channels and voltage-gated proton channel
Cartoon of VSP (left) vs. voltage-gated ion channels (right) and voltage-gated proton channel (middle). Green indicates VSD. In VSP, the phosphatase region with similarity to PTEN is located downstream of VSD. In voltage-gated ion channels such as voltage-gated potassium channels (right), four repeats assemble to form an ion permeation pathway (blue). Cytoplasmic regions play a role in subunit assembly and regulation of channel gating. In the voltage-gated proton channel (Hv channel), proteins assemble as a dimer, and individual subunits provide a permeation pathway.

Voltage sensing

The structure of VSD of VSP shares signature features with voltage-gated ion channels. It consists of four transmembrane segments from S1 to S4. S2 and S3 have conserved acidic residues, and S4 has periodically aligned basic residues in a pattern similar to S4 of voltage-gated ion channels. Motions of the voltage sensor of VSP have been characterized by voltage clamp both by measuring nonlinear capacitative currents similar to gating currents of voltage-gated ion channels (33, 51) and detection of local environments around single amino acid residues using the method of site-directed fluorometry (20, 21, 51). VSP is the first example in which a voltage sensor functions in a different context than regulation of ion permeation through the pore domain. VSD of VSP lacking the cytoplasmic region still shows “gating” currents (15, 33) that are also called “sensing currents” (51). This provides compelling evidence that VSD is a self-contained sensor module consistent with the protein structures of voltage-gated potassium channels (26). A part of the voltage sensor of VSP can replace a corresponding region of the VSD of a voltage-gated potassium channel without losing channel activities (3). A protein that only consists of VSD also exists, and it functions as a voltage-gated proton channel (42, 44) (see FIGURE 2).

The voltage sensor of VSP shares many features with that of voltage-gated ion channels. Amino acid replacement in VSD of VSP leads to a shift of voltage dependence of the voltage sensor motions (8, 15, 21, 33). VSP’s “gating” currents or sensing currents show peaks with clear delay following the initiation of voltage step. These indicate that multiple states exist in the transitions of activation of the voltage sensor. In voltage-gated ion channels from many species, the voltage dependence of the voltage sensor motions is shifted to a negative direction when the membrane potential is held to a depolarized level. This phenomenon correlates with the development of slow inactivation of voltage-gated ion channels. Villalba-Galea et al. found a similar shift of voltage-dependence by altering holding potentials in Ci-VSP: the voltage sensor can move within a more negative range of voltages when the membrane is held at a potential in which the sensing charges have moved to the active position of the voltage sensor (51). In the voltage clamp fluorometry of tetramethylrhodamine (TMR) dye attached to G214C of Ci-VSP, it was shown that a slower component of the change of fluorescence traces the transition between the active state and the relaxed state. These findings indicate that with persistent depolarization the voltage sensor of Ci-VSP enters into the third state, the so called “relaxed state,” distinct from the resting and activated state, characteristics shared by various types of voltage-gated ion channels.

Enzymatic activities

Remarkable sequence similarity of Ci-VSP to PTEN suggested that the major enzymatic reaction of Ci-VSP is to dephosphorylate PtdIns(3,4,5)P3. However, electrophysiological measurements in Xenopus oocytes heterologously expressing Ci-VSP proteins showed remarkable depletion of PtdIns(4,5)P2 on depolarization of the cell membrane (34), which cannot be explained by the phosphatase activity toward PtdIns(3,4,5)P3. Imaging of PtdIns(4,5)P2 using green fluorescent protein (GFP)-tagged pleckstrin homology (PH) domain from phospholipase C δ subunit (PLC-δ) showed that PtdIns(4,5)P2 concentration is reduced during depolarization (34). A malachite green assay showed that the cytoplasmic region of VSP not only dephosphorylates PtdIns(3,4,5)P3 but also PtdIns(4,5)P2 in vitro (18) (see FIGURE 3).
Thin-layer chromatography with isotope labeling showed that the 5′ phosphate is removed from PtdIns(4,5)P₂ (18). Imaging studies with total internal reflection fluorescence (TIRF) in mammalian cells using a PtdIns(4)P-sensitive probe, oxysterol binding protein (OSBP)-pleckstrin homology (PH) domain-GFP, and PtdIns(3,4)P₂-sensitive probe, tandem PH-domain-containing protein 1 (TAPP1)-PH domain-YFP, showed that PtdIns(4)P and PtdIns(3,4)P₂ are produced by the activities of Ci-VSP, consistent with the results of in vitro assays (18). Dephosphorylation of PtdIns(4,5)P₂ and PtdIns(3,4,5)P₃ was also shown with a teleost ortholog of VSP (Dr-VSP) (15). It still remains to be established whether PtdIns(3,4,5)P₃ is dephosphorylated by VSP to PtdIns(4,5)P₂ as well as to PtdIns(3,4)P₂.

Thus Ci-VSP has a different substrate specificity from PTEN that does not dephosphorylate PtdIns(4,5)P₂ and is highly selective for the phosphate on the 3′ position. Such a distinct substrate specificity of Ci-VSP partly depends on a glycine residue in the active site (the fourth residue of HCKGGKGR) (18), because mutation of G365 to A abolishes phosphatase activity toward PtdIns(4,5)P₂. However, it is possible that other residues also contribute to the substrate specificity of Ci-VSP (18).

In vitro malachite green assay of the whole cytoplasmic region of Ci-VSP with PtdIns(3,4,5)P₃ as substrate showed that \( V_{\text{max}} \) and \( K_{\text{m}} \) values are 0.292 nmol·min⁻¹·μg⁻¹ and 36 μM, respectively (33), which are within the range of those reported for PTEN. The turnover rate is estimated at ~3.2 s. No similar analysis has been available for PtdIns(4,5)P₂ so far.

Voltage sensor-enzyme coupling

The relationship between enzyme activity and membrane voltage was examined by electrophysiological methods (33) and imaging studies (34). PtdIns(4,5)P₂-activated potassium channels serve as indirect readouts of PtdIns(4,5)P₂ phosphatase activities. Inward rectifier K⁺ channels and KCNQ2/3 channels were coexpressed with Ci-VSP in heterologous expression systems (34). By analyzing current magnitudes or kinetics of those K⁺ channels on distinct pulse protocols of voltage commands, it was established that depolarization drives the enzymatic activity of Ci-VSP (see FIGURE 4).

By electrophysiological studies of potassium channels with distinct PtdIns(4,5)P₂-sensitivities, it was suggested that enzyme activity is tuned over a wide range of membrane potential. Depolarization-induced enzyme activity is detected at ~40 mV and enhanced at a more positive level when GIRK channels (Kir3) were used as the phosphoinositide-sensor (see FIGURE 4). The current decay of KCNQ2/3 channel continues to become sharper over 50 mV where the charges carried by the voltage sensor still do not saturate. These changes of K⁺ channel activities over a wide range of membrane potentials are consistent with the idea that enzymatic activities are correlated with the motions of the voltage sensor. More detailed study will be necessary to address how enzymatic activities are quantitatively related to the motions of the voltage sensor, especially because KCNQ2/3 channels have intrinsic voltage sensor. It also remains unknown whether the phosphatase activity is completely shut down at hyperpolarized voltage.

Interaction between two protein modules is often known to be bidirectional. Motions of the voltage sensor on suppression of enzyme activity with mutation of the phosphatase domain or treatment with phosphatase inhibitor were studied by measuring “gating” or sensing currents of teleost VSP in a mammalian cell expression system (15). They were accelerated when enzyme activities were suppressed by phosphatase inhibitors such as pervanadate or hydrogen peroxide, or when a cysteine residue, a site critical for enzyme activity, was mutated to serine (15). Altered patterns of voltage sensor motions were also seen when Ci-VSP with a mutation, D331 in the phosphatase domain corresponding to D92 in PTEN, was expressed in Xenopus oocyte (20). These retrograde effects from the phosphatase region to the voltage sensor suggest that the operation of the voltage sensor is influenced by the state of the phosphatase domain and...
that coupling between the two modules is very tight.

Coupling is diminished when the linker region between VSD and the phosphatase region is deleted, suggesting critical roles of the linker region in coupling (33). The linker contains a conserved phosphoinositide-binding motif (PBM), which is similar to the motif at the NH2-terminus of PTEN (5, 17, 53). Two recent studies have addressed how a mutation in this region of Ci-VSP alters motions of the voltage sensor (20, 50). Mutations in the PBM of Ci-VSP accelerate the speed of “gating” or sensing current during repolarization, shift the voltage dependence of the charge movement to positive potentials, and cause a “deeper” relaxation of the voltage sensing domain (50). Effects of PBM mutation on the motions of the voltage sensor of Ci-VSP were also studied by measuring fluorescence of TMR attached to the external end of S4 (20). Motions of the voltage sensor are decelerated when D331 of the phosphatase domain is mutated (20). Such a retrograde effect of the phosphatase domain on the voltage sensor is abolished when PtdIns(4,5)P2 levels are depleted either by the activation of phospholipase C (PLC) or rapamycin-induced activation of an exogenous 5′ phosphatase, Inp54p. Effect of PtdIns(4,5)P2 depletion on the retrograde coupling was not seen when PBM was mutated, consistent with the idea that PtdIns(4,5)P2 acts on the PBM of the linker region (20). These suggest a model that a PtdIns(4,5)P2-PBM interaction is required to link the voltage sensor and the phosphatase domains, whereas the release of PtdIns(4,5)P2 from PBM uncouples the two modules. This idea needs to be tested by further studies, including a demonstration of direct binding of PtdIns(4,5)P2 to PBM.

Biological roles of VSP

VSP is expressed in testis in both ascidians and mammals (see Table 1). Localization of native VSP proteins has been studied in the ascidian Ciona intestinalis, where it is expressed on sperm tail membranes (33). Sperm plasma membrane has diverse types of ion transporting membrane proteins such as voltage-gated calcium channels (6), CatSper channels, and voltage-gated proton channels. It will not be surprising if VSP plays a role, together with sperm membrane proteins, in sperm physiology such as motility or maturation. Perhaps functions of multiple types of PtdIns(4,5)P2-dependent membrane proteins on sperm membranes can be acutely shut down by downregulating PtdIns(4,5)P2 via depolarization-dependent phosphatase activities of VSP. A recent report showed that the chicken VSP gene is transiently expressed in mesonephros, suggesting its role in cell differentiation or kidney function (37). Possible neural expression of mammalian ortholog of VSP, called TPIP (52), and weak expression of Ci-VSP in the nervous system of adult ascidian (33) raise a possibility that VSP may play role in phosphoinositide-regulated events in neural cells such as vesicle turnover and regulation of membrane excitability. Future studies to define detailed protein expression patterns and gene knockout or knockdown in model organisms will shed light on physiological roles of VSPs.

VSP Serves as a Molecular Tool

PtdIns(4,5)P2 regulates many biological processes such as dynamics of the cytoskeleton, vesicle turnover, and activities of membrane proteins (46). Because the levels of phosphoinositides can be acutely altered by VSP’s enzyme activity through a
simple jump of membrane potential, VSP serves as a tool to study the detailed kinetics and functional roles of phosphoinositides. Recently, kinetics of PtdIns(4,5)P$_2$ metabolism and regulation of ion channels have been studied using VSP as a molecular tool to acutely deplete PtdIns(4,5)P$_2$ (10, 19). Residence time of PtdIns(4,5)P$_2$ on KCNQ2/3 channels have been studied using VSP as a molecular tool to study the detailed kinetics and functional roles of phosphoinositides. However, roles of phosphoinositides in the regulation of neuronal voltage-gated calcium channels have remained unclear. A recent study with VSP as a tool to acutely deplete PtdIns(4,5)P$_2$ has demonstrated regulatory roles of PtdIns(4,5)P$_2$ in voltage-gated calcium channel (47). Mutational modification of the properties of VSP, such as voltage threshold, activation kinetics, and substrate turnover, will help to expand the range of such applications.

VSP also provides a tool to develop a protein-based voltage probe. Measuring the electrical activity of a specific group of neurons simultaneously is important for understanding the principles of neuronal networks. Several voltage-sensitive probes have been designed utilizing VSDs from voltage-gated ion channels (4, 31) without any successful application for imaging electrical activities. VSP has several advantages for developing a voltage probe. VSD of voltage-gated ion channels does not exhibit gating currents if the pore domain is deleted, thus making it difficult to optimize the voltage-sensitive properties by electrophysiology. In contrast, voltage sensitivity of VSD of VSP is accurately measured. The voltage sensor of VSP shows robust “gating” or sensing currents even when the protein lacks the cytoplasmic region (15). Furthermore, overexpression of a VSP-based probe is less likely to perturb native cellular functions because of its monomeric organization (21) and low native expression in excitable tissues such as neurons and muscle. Voltage reporter proteins based on conventional voltage-gated ion channels may perturb cellular functions by interfering with native channel proteins. However, it should be noted that cellular properties are not completely unchanged when a VSP-based probe is overexpressed. On heterologous expression of such a probe, capacitative currents need to be supplied when the voltage sensor of VSP is moved. This mimics the effect of the addition of cell capacitance, thus affecting intrinsic electrical properties of native cells (1). It should also be considered that overexpression of membrane proteins may cause ER stress leading to alteration of the cell state.

Several voltage-sensitive probes have been designed by fusing the VSD of Ci-VSP with fluorescent proteins (8, 28, 35, 40, 49). A mutation was introduced into the S4 segment to optimize the range of voltage sensitivity. In one such example, the protein named “mermaid,” two coral-derived fluorescent proteins as a bright and pH-resistant FRET pair were fused to VSD of Ci-VSP. This protein exhibits a large change in emission ratio (49) and enabled non-averaged imaging of membrane potential changes in isolated cardiac muscle cells and cortical neurons (49). Recently, mermaid was stably expressed in cardiac muscle cells of zebrafish, and propagation of an electrical signal within the heart was visualized in live fish (48). Trials of modifying properties (magnitude, voltage range, and speed of activation) by site-directed mutagenesis or by utilizing other fluorescent proteins (40) are in progress in different laboratories.

### Summary and Future Directions

VSP consists of the ion-channel-like voltage sensor and a PTEN-like phosphatase region. The voltage sensor of VSP is a self-contained module that activates, on depolarization, a PtdIns(3,4,5)P$_3$/PtdIns(4,5)P$_2$ phosphatase region. Interaction between the two protein modules in VSP is bidirectional; there is a retrograde effect of the phosphatase module on the voltage sensor. Given that VSP shares structural similarities with other phosphatases, understanding how the voltage sensor regulates phosphatase activities of VSP will lead to deeper insights into operating mechanisms of phosphoinositide metabolism.
PTEN and other phosphatases. One important clue is that the linker between VSD and the cytoplasmic phosphatase region has a conserved PBM that potentially binds to PtdIns(4,5)P₂ to regulate their coupling (29, 50). In addition, whether the role of the C2 domain of VSP is similar to that of PTEN is also an open question. To further understand these findings, characterization of VSP would include studies of both the voltage sensor and the phosphoinositide phosphatase.

Note: Ci-VSP has recently been shown to be expressed in blood cells of adults and juveniles (Ogasawara Y, et al. Gene Expr Patterns. In press).

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