http://dx.doi.org/10.5012/bkcs.2014.35.11.3389

Investigating the Regulatory Interaction of Linker Region of *Ciona intestinalis* Voltage-sensitive Phosphatase with Lipid Membrane

Sungjae Kim,[†] Md. Mahbubur Rahman,[‡] Kwangmo Noh,[‡] Jae-Joon Lee,[‡] and Young Jun Kim[†,‡,*]

[†]*Department of Applied Biochemistry and* [‡]*Nanotechnology Research Center, Konkuk University, Chung-ju 380-701, Korea*
[*]*E-mail: ykim@kku.ac.kr*
Received July 15, 2014, Accepted July 31, 2014

Key Words : Ci-VSP, Membrane Interaction, PTEN, Phosphoinositides, Lipid phosphatase

Ciona intestinalis voltage-sensitive phosphatase (Ci-VSP), which possesses the voltage-sensing domain (VSD) and phosphatase domain (PD), was discovered in sea squirt.[1] Members of the voltage-sensing phosphatase (VSP) family have an N-terminal membrane-embedded region known as the VSD and a C-terminal cytosolic catalytic domain called as the PD, which is a lipid phosphatase. VSD consists of four transmembrane segments that are activated by changes in cellular membrane potential.[2] A unique feature of VSPs in both invertebrate and vertebrate is that membrane depolarization induces phosphoinositide phosphatase activity[5] through the coupling of activated VSD.[3-5]

The catalytic domain of Ci-VSP contains the protein-tyrosine phosphatase (PTP) signature motif HCxxGxxR.[6,7] Few similarities exist between Ci-VSP and other transmembrane phosphatases, such as "transmembrane phosphatase with tensin" homology TPTE[8] and TPTE2[9] and phosphatase and tensin homolog (PTEN).[10,11] For instance, Ci-VSP and PTEN show 44% homology in catalytic domains. Furthermore, the residues that are important for catalysis are highly conserved.[12] There are few striking differences though; for example, the enzymatic activity of Ci-VSP increases under depolarizing membrane potentials, from −80 to 100 mV,[13,14] but PTEN does not have a VSD. Ci-VSP displays phosphatase activity toward phosphatidylinositol-3,4,5-trisphosphate ($PI(3,4,5)P_3$) and phosphatidylinositol-4,5-bisphosphate ($PI(4,5)P_2$), but PTEN is active against ($PI(3,4,5)P_3$) only. PTEN dephosphorylates $PI(3,4,5)P_3$ at the 3′-site of the inositol ring,[11] whereas Ci-VSP dephosphorylates $PI(3,4,5)P_3$ at the 5′-site.[15] The phosphatase activity was confirmed by *in vitro* measurements by site-specific radiolabeling of phosphate on phosphoinositides and $PI(4,5)P_2$.[16]

Ci-VSP has a linker region (18 amino acids, residues 240–257) located between VSD and PD, which plays a critical role in coupling of membrane potential to phosphatase activity.[17] At negative voltage, the VSD resides in a resting state, and the linker is distorted in an allosterically "tense" non-functional state. At positive voltage, the VSD is activated and the linker "relaxes" into the positive regulatory state that is necessary for activity of the isolated PD. The depletion of $PI(4,5)P_2$ by Ci-VSP activity leads to a destabilization of the activated state and uncoupling of the VSD from the PD, thus turning off the enzyme even though the membrane is still depolarized.[12] Based on this regulation model, linker residues K252 and R253 of Ci-VSP were shown to couple voltage changes with activity. These findings were supported by the crystal structures of Ci-VSP PD,[18] where the linker has a short α-helix (positions 248–252), a short β-sheet (positions 255–257), and other flexible regions. The residue K252 interacts with gating loop D400 through a salt bridge, whereas R253 coordinates with the backbone carbonyl of G365 at the active site of the apoenzyme. However, the residues R253 and K252 swap coordination with D400 and G365 upon binding inositol triphosphate (IP3).[18] Therefore, the linker region plays a pivotal role in the structural and functional integrity of PD[18] by membrane interaction[19] and voltage coupling.

The linker region is called as phospholipid or phosphoinositide binding motif (PBM). PTEN also harbors PBM in its N-terminal region, which binds phosphoinositides and facilitates docking[20] to membranes,[21,22] thereby regulating enzymatic activity.[23] This PBM is conserved in Ci-VSP and other Ci-VSP homologs. Recently, it was shown that voltage-dependent changes in enzymatic activities of VSP are lost in PBM due to mutations in the residues,[24] thereby suggesting a key role of this domain in coupling of VSD and PD.[18] Furthermore, this region is critical for catalytic activation of Ci-VSP homologs. PBMs from PTEN, TPTE, and Ci-VSP proteins carry positively charged residues in KRR that is highly conserved, and is important for binding, to negatively charged phosphoinositides membrane[19,25] (Figure S1) under physiological conditions. Thus, detailed understanding of how KRR residues affect membrane interaction and phosphatase activity of PD is required for a molecular description of the regulatory mechanism of Ci-VSP.

In this study, we constructed the ΔCi-VSP(248-576)-His wild-type (WT) and introduced A (Ala, neutral) or E (Glu, acidic) mutations in KRR residues (252–254) of PBM. Using these mutant derivatives of Ci-VSP, we measured *in vitro* phosphatase activity by using *para*-nitrophenyl phosphate (pNPP) and malachite green (MG) assay to confirm the effect of interdomain linker charge on the phosphatase activity and membrane interaction. We further tested the interaction between PBM and membrane, using a quartz crystal microbalance (QCM) method.

Initially, we measured the specific activity of ΔCi-VSP

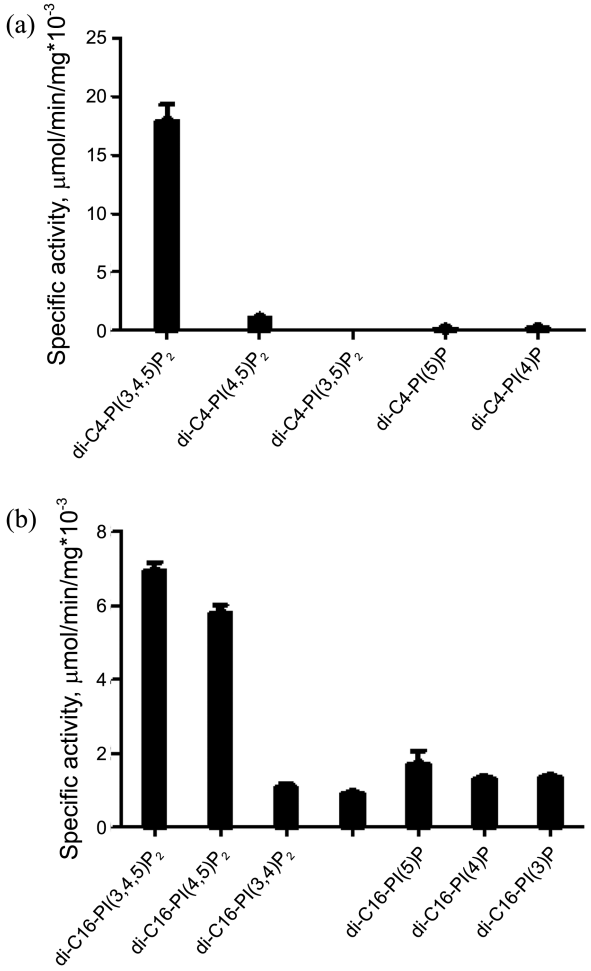

Figure 1. Specific activity of ΔCi-VSP(248-576)-His WT against (a) soluble di-C4 phosphoinositides and (b) membrane-supported di-C16 phosphoinositides determined by malachite green assay using 50 mM Tris (pH 8.0), 10 mM DTT, 0.5% NP-40, 200 μM substrate, and 1 μg protein incubated at 37 °C for 10 min.

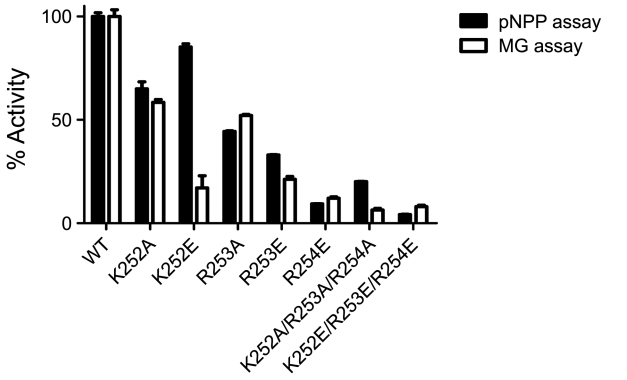

Figure 2. Percentage activity of ΔCi-VSP(248-576)-His WT and mutants in *p*NPP assay and MG assay on the basis of WT activity. *p*NPP assays were performed by incubating ΔCi-VSP mutants in 50 mM sodium acetate (pH 5.5), 50 mM *p*NPP, 2 mM DTT, and 1.5-3 μg protein at 37 °C for 30 min. MG assays were performed using 50 mM Tris (pH 8.0), 10 mM DTT, 0.5% NP-40, 200 μM di-C16-PI(3,4,5)P_3, 1 mM POPS, and 1~1.5~3 μg protein at 37 °C for 10 min.

(248-576) WT against di-C4-phosphoinositides and di-C16-phosphoinositides (Figure 1). Di-C4-phosphoinositides are amphipathic; therefore, they were immediately dissolved in the buffer containing detergent NP-40. However, hydrophobic di-C16-phosphoinositides require a lipid bilayer vesicles (see experimental conditions). Therefore, the difference between of water soluble and of membrane inserted substrates provides us how much membrane interaction of an enzyme is prominent. ΔCi-VSP showed maximal specific activity (~18 μmol/min/mg × 10^{-3}) against di-C4-PI(3,4,5)P_3 but negligible activity against the other di-C4-phosphoinositides. Additionally, it also showed a high specific activity (~7 μmol/min/mg × 10^{-3}) against di-C16-PI(3,4,5)P_3. Interestingly, Ci-VSP displayed high specific activity (~6 μmol/min/mg × 10^{-3}) against di-C16-PI(4,5)P_2 but had marginal specific activity against di-C4-PI(4,5)P_2. These results suggests that the membrane environment is required for the phosphoinositide phosphatase activity of Ci-VSP, and the residues in the linker region of K252, R253, and R254 are required for the phosphoinositide phosphatase activity of Ci-VSP. Therefore, we decided to investigate the interaction of the charged residues of Ci-VSP with the membrane by mutating them to A and E.

ΔCi-VSP(248-576)-His WT and mutated ΔCi-VSP(248-576)-His were expressed in *Escherichia coli* and purified using Ni-NTA affinity chromatography and size-exclusion chromatography. We obtained several milligrams of highly purified form of all mutants except R254A (Figure S2). Under optimized reaction conditions, we evaluated the effect of amino acid change on purified ΔCi-VSP-His WT and mutants by *p*NPP and MG assays (Figure 2). All ΔCi-VSP mutants showed reduced activities of *p*NPP dephosphorylation, perhaps representing loss in structural integrity. R254E and triple mutations showed the lowest activities, whereas K252A and K252E showed relatively higher activities. This finding implies that R254 is crucial for the structural integrity of Ci-VSP. Next, we tested the disruption of membrane interaction of the mutant derivatives of ΔCi-VSP by performing MG assay in the presence of di-C16-PI(3,4,5)P_3. All mutants showed reduced activity but K252E showed dramatic decrease in activity by MG assay unlike that of *p*NPP assay; this finding implies that the charge-inverted mutation of K252E interferes with membrane-binding of Ci-VSP.

In order to confirm that K252 is largely responsible for membrane interaction of Ci-VSP, we performed a QCM experiment. Initially, we immobilized liposomes containing phosphoinositides on the surface of Au-QCM, but it did not provide the desired results. Therefore, we applied 20 mM 11-mercaptoundecanoic acid (MUA) overnight, to obtain a reproducible electric sensorgram of QCM. The general procedure of membrane binding of ΔCi-VSP(248-576)-His WT and mutants using QCM is described in Figure 3(a), and the representative data of ΔCi-VSP(248-576)-His WT using QCM is shown in Figure 3(b). The experimental details of this procedure is described elsewhere (manuscript under

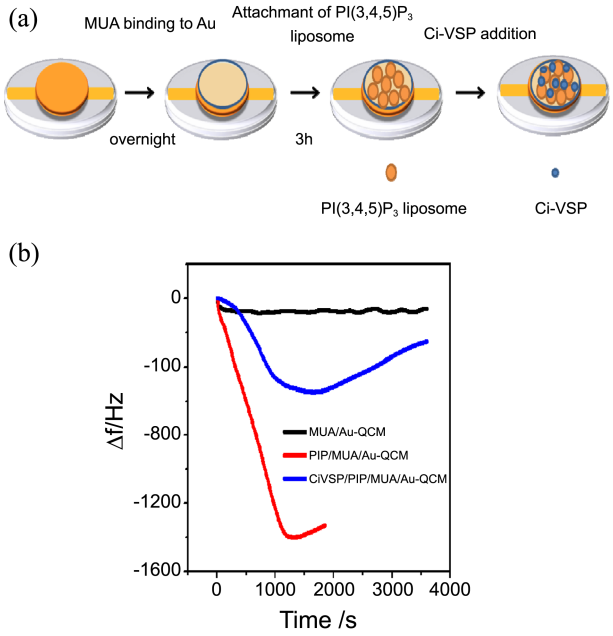

Figure 3. Measurement of membrane binding of ΔCi-VSP(248-576)-His WT and mutants by QCM (a) Procedure for preparation of the membrane binding assay (b) Representative electric sensorgram of the formation of Ci-VSP/PIP bilayer by using the QCM technique. Black line represents the binding of 11-mercaptoundecanoic acid (MUA) onto Au surface. Red line represents the attachment of $PI(3,4,5)P_3$ containing liposome onto MUA surface before Ci-VSP incubation. Blue line represents the binding of Ci-VSP onto liposome.

Table 1. Mass change slope obtained with 61ng of Ci-VSP(248-576)-His and mutants

Mutants	-Slope (-Hz/sec)
WT	0.1321 ± 0.0015
K252A	0.0475 ± 0.0008
K252E	0.0005 ± 0.0001
R253A	0.0641 ± 0.0009
R253E	0.1440 ± 0.0021
R254E	0.4891 ± 0.0119
K252A/R253A/R254A	0.1601 ± 0.0027
K252E/R253E/R254E	0.0000 ± 0.0001

preparation).

Using this novel membrane-binding assay, we optimized the amount of protein used to ~61 ng, to differentiate changes in signal due to mutant derivatives. We observed that addition of a larger amount of protein resulted in protein–protein interaction, leading to unfamiliar signals, while use of a smaller amount led to signals that were undetectable. We performed this membrane-binding assay for mutants (Figure S3) and then extrapolated the initial 5 s of the slope of electric sensorgrams, which is sensitive to intrinsic property of membrane interaction of Ci-VSP. The calculated slopes are summarized in Table 1. The mutants K252A, K252E, and K252E/R253E/R54E showed low values of the slope, whereas R253A, R253E, and K252A/R253A/R254E displayed similar profile;R254E interestingly showed a steep slope.

Taken together, these results imply that R253 and R254 mutants showed reduced activity and affect the structure and function of Ci-VSP, whereas K252 interacts with the membrane. Based on crystal structures of Ci-VSP PD,[18] K252 interacts with gating loop D400 and R253 coordinates with G365 of the active site in a resting state. Their interactions with D400 and G365 exchange positions in an IP_3-bound state, which is similar to the binding conformation of Ci-VSP and $PI(3,4,5)P_3$. Our result highlights a dynamic change of interaction of K252, R253, and R254, with a gating loop residue, an active site residue, and membrane phospholipids during coupling of voltage change and activity. In other words, the interaction of K252 with gating loop D400 could change into the interaction with membrane phospholipids.

We examined the effect of mutations in the linker region of Ci-VSP on the phosphoinositide phosphatase activity and membrane binding. Mutations of crucial amino acids (KRR) in the linker region of Ci-VSP tend to reduce the activity of the PD. On the one hand, R253 and R254 mutations resulted in structural deformations and did not affect membrane binding. On the other hand, K252 mutation decreased phosphoinositide phosphatase activity and membrane binding of Ci-VSP. Therefore, we suggest that K252 is an important residue for membrane binding and activation of Ci-VSP, while residues R253 and R254 influence the structural integrity. In conclusion, membrane binding of Ci-VSP is regulated by an electrostatic interaction of K252 with membrane lipid rather than R253 or R254.

Experimental Section

***p*NPP Phosphatase Assay.** Reaction mixtures (20 μL), containing 50 mM sodium acetate (pH 5.5), 50 mM *p*NPP, 2 mM DTT, and a suitable amount of WT or mutant proteins, were incubated at 37 °C for 30 minute. The reactions were quenched by addition of 80 μL of 0.25N NaOH. *p*NPP, released during the assay, was measured by reading the absorbance at 410 nm, using a Beckman Coulter™ DU 700 Series UV/Vis Spectrophotometer. The catalytically inactive mutant of ΔCi-VSP was used as a blank to correct for the background absorbance readings. The specific activity of proteins was calculated using molar absorptivity of 17,800 L/mol·cm.

Malachite Green Phosphatase Assay. di-C_{16}-Phosphoinositides and POPS were dried together in a SpeedVac and resuspended *via* vortexing for 15 min and sonication for 2 min in a sonicator in 19 μL of assay buffer [50 mM Tris (pH 8.0), 10 mM DTT, 0.5% NP-40] to final concentrations of 200 and 1000 μM, respectively. After sonication, vesicles were passed 17 times through a Liposofast microextruder containing a 100-nm polycarbonate filter.

The reactions were initiated by the addition of a suitable amount of WT or mutant proteins in a pre-warmed assay buffer at 37 °C for 5 min. For MG assay of soluble phospho-

inositides, 200 μM of di-C_4-phosphoinositides were added into reaction mixture, which was quenched after 5–30 min by the addition of 20 μL of 0.1 M *N*-ethylmaleimide. The sample was spun at 18,000 × *g* for 15 min to sediment the lipid aggregates. The supernatant (25 μL) was added to 50 μL of MG reagent and vortexed. Samples were allowed to sit for 30-40 min to develop color before measuring absorbance at 620 nm. The catalytically inactive mutant of ΔCi-VSP was used as the blank for measurements of absorbance. Inorganic phosphate release was quantified by comparison to a standard curve of KH_2PO_4 in distilled H_2O.[26]

Membrane Binding Assay using Quartz Crystal Microbalance. QCM experiments were performed using a CHI430A electrochemical workstation with an extra module for electrochemical quartz crystal microbalance testing (CH Instruments, USA). Prior to use, the Au/QCM electrode was cleaned with piranha solution (H_2SO_4:H_2O_2, 3:1, v/v) and rinsed with water (Safety note: the piranha solution should be used with extreme caution). The remaining active gold surface was incubated with 20 mM 11-mercaptoundecanoic acid (MUA) overnight so that the sulfhydryl groups could attach to the gold molecules. The MUA/Au-QCM electrode was washed three times with distilled water to remove any unbound MUA. Prepared liposomes containing di-C_{16}-phosphoinositides and POPS were attached on the MUA/QCM electrode by incubating them for 3 h. After three washes with buffer solution containing 50 mM Tris (pH 8.0), 10 mM DTT, the resulting PIP/MUA/QCM probe was blocked with 0.1% bovine serum albumin (BSA) solution to minimize non-specific adsorption. The frequency change (Δf) was monitored until a steady frequency was obtained by placing a suitable amount of WT or mutant protein on the PIP/MUA/QCM electrode. All experiments were performed three times.

Supporting Information. The materials and purification methods, figure S1, figure S2, and figure S3 are provided in the supplementary document.

References

1. Murata, Y.; Iwasaki, H.; Sasaki, M.; Inaba, K.; Okamura, Y. *Nature* **2005**, *435*, 1239-1243.
2. Mishina, Y.; Mutoh, H.; Knopfel, T. *Biophys. J.* **2012**, *103*, 669-676.
3. Sasaki, M.; Takagi, M.; Okamura, Y. *Science* **2006**, *312*, 589-592.
4. Lacroix, J. J.; Bezanilla, F. *Biophys. J.* **2012**, *103*, L23-25.
5. Hossain, M. I.; Iwasaki, H.; Okochi, Y.; Chahine, M.; Higashijima, S.; Nagayama, K.; Okamura, Y. *J Biol Chem* **2008**, *283*, 18248-18259.
6. Lee, J. O.; Yang, H.; Georgescu, M. M.; Di Cristofano, A.; Maehama, T.; Shi, Y.; Dixon, J. E.; Pandolfi, P.; Pavletich, N. P. *Cell* **1999**, *99*, 323-334.
7. Maehama, T.; Taylor, G. S.; Dixon, J. E. *Annu. Rev. Biochem.* **2001**, *70*, 247-279.
8. Tapparel, C.; Reymond, A.; Girardet, C.; Guillou, L.; Lyle, R.; Lamon, C.; Hutter, P.; Antonarakis, S. E. *Gene* **2003**, *323*, 189-199.
9. Walker, S. M.; Downes, C. P.; Leslie, N. R. *Biochem. J.* **2001**, *360*, 277-283.
10. Li, J.; Yen, C.; Liaw, D.; Podsypanina, K.; Bose, S.; Wang, S. I.; Puc, J.; Miliaresis, C.; Rodgers, L.; McCombie, R.; Bigner, S. H.; Giovanella, B. C.; Ittmann, M.; Tycko, B.; Hibshoosh, H.; Wigler, M. H.; Parsons, R. *Science* **1997**, *275*, 1943-1947.
11. Maehama, T.; Dixon, J. E. *J. Biol. Chem.* **1998**, *273*, 13375-13378.
12. Kohout, S. C.; Bell, S. C.; Liu, L.; Xu, Q.; Minor, D. L., Jr.; Isacoff, E. Y. *Nat. Chem. Biol.* **2010**, *6*, 369-375.
13. Murata, Y.; Okamura, Y. *J. Physiol.* **2007**, *583*, 875-889.
14. Oliver, D.; Lien, C. C.; Soom, M.; Baukrowitz, T.; Jonas, P.; Fakler, B. *Science* **2004**, *304*, 265-270.
15. Halaszovich, C. R.; Schreiber, D. N.; Oliver, D. *J. Biol. Chem.* **2009**, *284*, 2106-2113.
16. Sakata, S.; Hossain, M. I.; Okamura, Y. *J. Physiol.* **2011**, *589*, 2687-2705.
17. Villalba-Galea, C. A. *Cell Signal* **2012**, *24*, 1541-1547.
18. Liu, L.; Kohout, S. C.; Xu, Q.; Muller, S.; Kimberlin, C. R.; Isacoff, E. Y.; Minor, D. L., Jr. *Nat. Struct. Mol. Biol.* **2012**, *19*, 633-641.
19. Hobiger, K.; Utesch, T.; Mroginski, M. A.; Friedrich, T. *Biophys. J.* **2012**, *102*, 1313-1322.
20. Das, S.; Dixon, J. E.; Cho, W. *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 7491-7496.
21. Walker, S. M.; Leslie, N. R.; Perera, N. M.; Batty, I. H.; Downes, C. P. *Biochem. J.* **2004**, *379*, 301-307.
22. Shenoy, S.; Shekhar, P.; Heinrich, F.; Daou, M. C.; Gericke, A.; Ross, A. H.; Lösche, M. *PLoS One* **2012**, *7*, e32591.
23. Campbell, R. B.; Liu, F.; Ross, A. H. *J. Biol. Chem.* **2003**, *278*, 33617-33620.
24. Villalba-Galea, C. A.; Sandtner, W.; Dimitrov, D.; Mutoh, H.; Knopfel, T.; Bezanilla, F. *Biophys. J.* **2009**, *96*, L19-21.
25. Iijima, M.; Huang, Y. E.; Luo, H. R.; Vazquez, F.; Devreotes, P. N. *J. Biol. Chem.* **2004**, *279*, 16606-16613.
26. Taylor, G. S.; Dixon, J. E. *Methods Enzymol.* **2003**, *366*, 43-56.