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Expression of the voltage-sensing phosphatase gene in the chick embryonic tissues and in the adult cerebellum

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Abbreviations: VSD, voltage sensor domain; VSP, voltage-sensing phosphatase; Gg-VSP, chick voltage-sensing phosphatase; PTEN, phosphatase and tensin homolog deleted on chromosome TEN; HH, Hamburger and Hamilton; RT-PCR, reverse transcription-polymerase chain reaction; GST, glutathione S-transferase; Ci-VSP, ascidian voltage-sensing phosphatase; FRET, fluorescence resonance energy transfer; PtdIns(3,4)P2, phosphatidylinositol 3,4-bisphosphate.

Voltage-sensing phosphatase (VSP) consists of a transmembrane voltage sensor domain (VSD) and the cytoplasmic domain with phosphoinositide-phosphatase activities. It operates as the voltage sensor and directly translates membrane potential into phosphoinositide turnover by coupling VSD to the cytoplasmic domain. VSPs are evolutionarily conserved from marine invertebrate up to humans. Recently, we demonstrated that ectopic expression of the chick ortholog of VSP, Gg-VSP, in a fibroblast cell line caused characteristic cell process outgrowths. Co-expression of chick PTEN suppressed such morphological change, suggesting that VSP regulates cell shape by increasing PI(3,4)P_2. However, the *in vivo* function of Gg-VSP remains unclear. Here, we showed that in chick embryos Gg-VSP is expressed in the stomach, mesonephros, pharyngeal arch, limb bud, somites, floor plate of neural tube, and notochord. In addition, both Gg-VSP transcripts and the protein were found in the cerebellar Purkinje neurons. These findings provide an insight into the physiological functions of VSP.

V*SP* genes are widely conserved among deuterostome genomes.[1,2] Previous studies have indicated the possible role of VSP in fertilization and development in ascidians.[3,4] There is evidence suggesting that VSP may play a role in the fertilization of frog eggs.[5] Moreover, VSP is expressed in tissues other than reproductive organs.[6-8] This indicates that VSP plays a role in various aspects of development. Although intensive studies have been done on the molecular mechanisms of VSP as a simple voltage sensor protein, little is known about the physiological functions of VSP.

Recently we reported that ectopic expression of the chick VSP ortholog, Gg-VSP, in DF-1 fibroblast cell line caused characteristic cell process outgrowths. Co-expression of chick PTEN, which can reduce the amount of PtdIns(3,4)P_2, suppressed the morphological changes induced by Gg-VSP. In addition, Gg-VSP overexpression changed the distribution of PtdIns(3,4)P_2. These findings suggested that Gg-VSP could play a role in the regulation of cell shape by increasing PtdIns(3,4)P_2.[9]

In this study, to gain insight into the physiological role of Gg-VSP, we investigated the expression pattern of Gg-VSP in chick embryos. Domestic chicks of the Cobb strain (*Gallus gallus domesticus*) were used. Fertilized eggs (3M, Nagoya, Japan) were incubated at 37°C until embryos reached the appropriate stage. Embryos were staged according to Hamburger and Hamilton.[10] In a previous study, gene expression of a chick ortholog of VSP, which the authors termed cTPTE, was reported in the epithelial cells of the developing kidney.[8] In contrast, we observed Gg-VSP gene expression with a method that uses tyramide-based amplification techniques to amplify weak signals for a more comprehensive study.[11] We could not detect clear positive signals in 2-day chick embryos (HH stage 13). However, in the 4-day chick embryos (HH stage 24) we detected Gg-VSP transcripts in the

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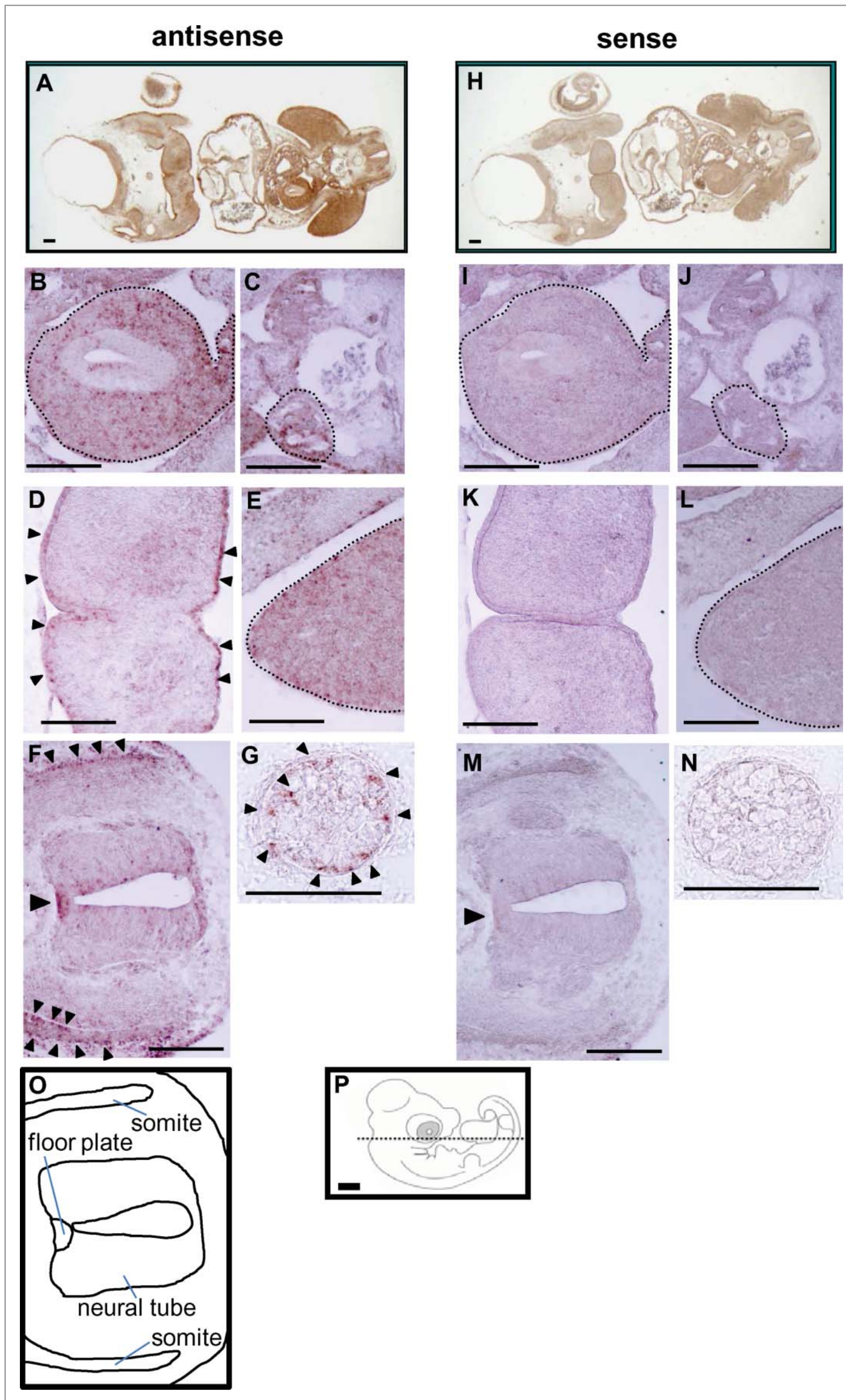

Figure 1. Expression patterns of Gg-VSP in the chick embryo. *In situ* hybridization of chick embryos was performed as previously described,[22] with some modification. To synthesize the probe, we cloned a partial Gg-VSP cDNA fragment corresponding to +1168-1470 base pairs of the annotated mRNA sequences (Genbank accession number; XM_417079). (**A**-**N**) Sections of 4-day embryos stained with antisense probe (**A**-**G**) and sense probe (**H**-**N**). The entire view of the sections (**A**, **H**), stomach (**B**, **I**), mesonephroses (**C**, **J**), pharyngeal arches (**D**, **K**), limb buds (**E**, **L**), somite and neural tube (**F**, **M**), and notochord (**G**, **N**) are shown. (**O**) Diagram of the position of somites, neural tube, and floor plate for results shown in F and M. (**P**) Diagram of the lateral view of a 4-day chick embryo for results shown in (**A**-**N**). The sections were prepared along the dotted line. Scale bars indicate 1 mm. The positions of the stomach (**B**, **I**), mesonephrose (**C**, **J**), and limb bud (**E**, **L**) are shown with dotted line. Triangles indicate positive signals (**D**, **F** and **G**). The anterior side is always shown in left.

stomach (**Fig. 1B, I**), mesonephros (**Fig. 1C, J**), surface layer of the pharyngeal arch (**Fig. 1D, K**), limb bud (**Fig. 1E, L**), and the somites (**Fig. 1F, M, O**). We also detected Gg-VSP expression in the floor plate; however, clear signals were not detected in other regions of the neural tubes (**Fig. 1F, M, O**). The cells in the outside layer of the notochord expressed Gg-VSP (**Fig. 1G, N**). Gene expression in the mesonephros was consistent with a previous study.[8] Gg-VSP transcripts were not uniformly distributed in these tissues. *In situ* hybridization and reverse transcription-polymerase chain reaction (RT-PCR) of the brain tissue of 1-day embryos revealed that Gg-VSP was expressed in the brain.[9] RT-PCR showed that gene expression was most abundant in the cerebellum.[9] Therefore, we tested if *in situ* hybridization could detect gene expression in the cerebellum. Positive signals were detected in Purkinje neurons (**Fig. 2A, B**). Immunostaining of sections of the chick cerebellum was also performed using an antibody raised against Gg-VSP protein. Anti-Gg-VSP rabbit antibody was raised against the affinity-purified GST-tagged cytoplasmic region of Gg-VSP, corresponding to amino acids residues 187-511. The antibody was affinity-purified using this antigen polypeptide. Western blot analysis revealed that anti-Gg-VSP had specifically reacted with the Gg-VSP protein in the lysate of DF-1 fibroblast cells (**Fig. 2E**). Positive signals for Gg-VSP were detected in Purkinje neurons (**Fig. 2C, D**). Notably, positive signals were not detected in all Purkinje neurons as in the results of *in situ* hybridization (**Fig. 2A, B**). Within Gg-VSP-positive Purkinje neurons, signal was observed in both cell surface and cytoplasm, suggesting the localization of proteins in both plasma and intracellular membranes.

Expression of VSP in Chick Embryos and its Physiological Implications

There is substantial evidence suggesting that electric fields are present in developing animal tissues and play an important role as a mediator of morphogenetic information in many processes of embryonic development.[12,13] For example, currents and endogenous voltage gradients were shown to be present during chick development, and

shunting the current caused morphological abnormalities in the tail and limb bud.[14,15] However, it remains unknown how the membrane potential derived from the electric field is transduced into cytoplasmic molecular signaling. In this study, we detected Gg-VSP gene expression in the stomach, mesonephros, surface layer of the pharyngeal arch, limb bud, somites, floor plate in the neural tube, and notochord in the 4-day chick embryo (HH stage 24). Interestingly, some of these tissues are the sites where membrane potential has been reported in developing chick embryos: mesonephric nephrons of chick embryos were reported to exhibit measurable transepithelial potential differences,[16] and ion current flowing out of the limb buds and reversing it causes major limb abnormalities.[15] These expression patterns suggest that Gg-VSP can function as a voltage-sensitive membrane phosphatase during embryonic development in these tissues.

A previous study demonstrated that Gg-VSP expression was restricted to the developing kidney in embryos from HH stage 18 to stage 35.[8] In contrast, when we applied a method that uses tyramide-based amplification techniques to amplify weak signals, we were able to detect Gg-VSP transcripts in the stomach, surface layer of the pharyngeal arch, limb bud, somites, floor plate, and notochord in embryos at HH stage 24. In future, this amplification method will be useful in addressing the issue of whether Gg-VSP expression can be detected in these tissues in later stage embryos.

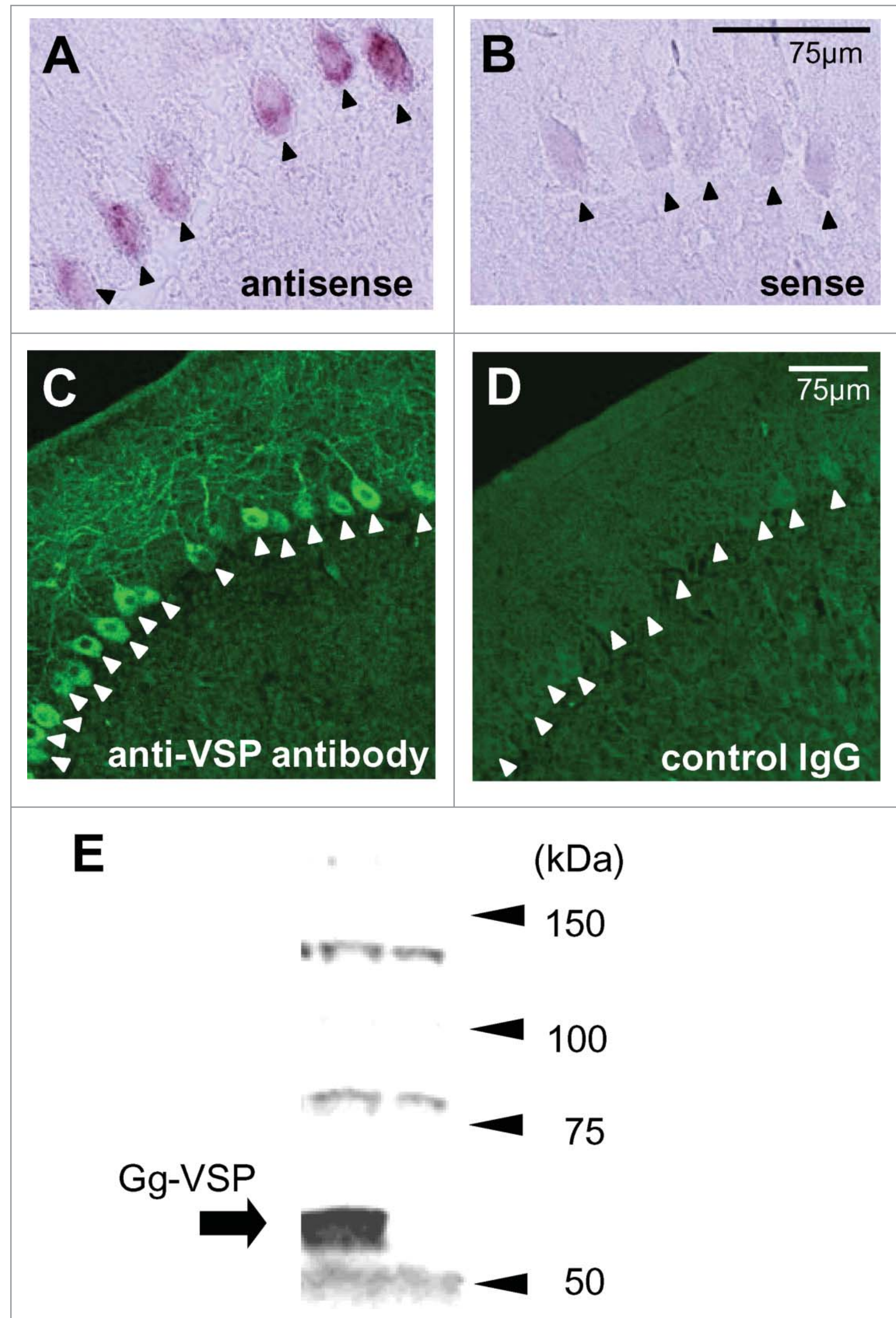

Figure 2. Expression patterns of Gg-VSP in 1-day-old chick cerebellum. (**A**, **B**) *In situ* hybridization for Gg-VSP. Sections of the cerebellum were probed with antisense or sense (control) RNA probe. (**C**, **D**) A section of the cerebellum stained with anti-Gg-VSP antibody or control IgG. Triangles point to positive signals in (**A**-**D**). (**E**) Western blot analysis of DF-1 cells expressing Gg-VSP by using anti-Gg-VSP antibody (1:1000). Left Lane, DF-1 cells transfected with pIRES-Gg-VSP; right lane, DF-1 cells without transfection. Each lane contained 50 μg of protein. The arrow indicates the position of Gg-VSP. Scale bars indicate 75 μm.

Expression of VSP in Neurons and Physiological Implications

We demonstrated that Gg-VSP was expressed in Purkinje neurons in the cerebellum. Gg-VSP may be involved in the differentiation of Purkinje neurons. Ci-VSP has been reported to be expressed in the ascidian nervous system,[3] and the mouse VSP ortholog has been reported to be expressed in embryonic brain.[17] However, our present study provides the first evidence at the cellular level among all animal species that VSP is expressed in excitable cells. In VSP, the single VSD is linked to the cytoplasmic phosphatase of which the activity is tightly coupled to voltage sensing over a wide range of voltages.[18] This molecular nature of VSP differs from that of voltage-gated ion channels where a sharp increase of ion conductance is achieved in response to a small change of membrane

voltage through cooperativity among 4 voltage sensors within one molecule. The molecular properties of VSP are rather suited for a gradual change in membrane voltage. In fact, Ci-VSP is expressed in the developing stomach and blood cells where rapid changes of membrane potential such as action potentials in excitable cells is not expected.[19] It is unlikely that the activity of VSP follows individual action potentials, given that the voltage sensor motion of Gg-VSP, as seen by sensing currents,[9] occurs in a more positive range than the range of common action potentials. In addition, a depolarization at 20 mV for few milliseconds is not sufficient to activate the enzyme activity of Ci-VSP, which has a higher sensitivity to depolarization than that of Gg-VSP (data not shown). It is more likely that VSP activity is tuned by the persistent change of subthreshold voltage or senses frequency of spikes, rather than individual action potentials. Several studies that used voltage probes, whose design was based on the voltage sensor of Ci-VSP, showed that a florescence resonance energy transfer (FRET) signal from a cytoplasmic pair of 2 fluorescent proteins increases in response to action potential frequency.[20,21] Similar "decoding" of action potential frequency may naturally occur in chick Purkinje neurons. Notably, the use of both *in situ* hybridization and immunohistochemistry demonstrated that not all Purkinje neurons express Gg-VSP. It will be interesting to see whether gene expression of Gg-VSP is induced by preceding neural activities or the ontogenic history of neurons.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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