The Phosphoinositide Phosphatase Sac1 Is Required for Midline Axon Guidance

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Sac1 phosphoinositide (PI) phosphatases are important regulators of PtdIns(4)P turnover at the ER, Golgi, and plasma membrane (PM) and are involved in diverse cellular processes including cytoskeletal organization and vesicular trafficking. Here, we present evidence that Sac1 regulates axon guidance in the embryonic CNS of *Drosophila*. Sac1 is expressed on three longitudinal axon tracts that are defined by the cell adhesion molecule Fasciclin II (Fas II). Mutations in the *sac1* gene cause ectopic midline crossing of Fas II-positive axon tracts. This phenotype is rescued by neuronal expression of wild-type Sac1 but not by a catalytically-inactive mutant. Finally, *sac1* displays dosage-sensitive genetic interactions with mutations in the genes that encode the midline repellent Slit and its axonal receptor Robo. Taken together, our results suggest that Sac1-mediated regulation of PIs is critical for Slit/Robo-dependent axon repulsion at the CNS midline.

INTRODUCTION

Phosphoinositides (PIs) are phosphorylated derivatives of the membrane lipid phosphatidylinositol (PtdIns) that play essential roles in a number of cellular processes, including actin cytoskeleton organization and intracellular membrane trafficking (Di Paolo and De Camilli, 2006). These regulatory roles depend on their ability to recruit and/or activate various effector proteins that possess specific PI-binding domains (Lemmon, 2008). Accumulating data suggest that each PI displays a unique sub-cellular distribution (Di Paolo and De Camilli, 2006). For example, PtdIns(4)P is localized primarily to Golgi membranes, while PtdIns(3)P is concentrated in endosomal membranes. The compartment-specific distribution of PI pools is established and maintained through the precise temporal and spatial regulation of multiple PI kinases and phosphatases.

One family of PI phosphatases is characterized by the presence of a conserved Sac phosphatase domain, which is about 400 amino acids in length and contains a signature Cys-X₅-Arg-Thr/Ser (CX₅RT/S) catalytic motif (Hughes et al., 2000). The

Sac-domain phosphatases in yeast and mammals can be classified into two subfamilies (Blagoveshchenskaya and Mayinger, 2009). The first subfamily includes yeast Sac1 and Fig4 and related mammalian proteins in which an N-terminal Sac domain is followed by a C-terminal region without any recognizable phosphatase domains. The second subfamily consists of yeast and mammalian synaptojanins, in which the N-terminal Sac phosphatase module is immediately followed by a C-terminal PI 5-phosphatase domain.

The Sac1 PI phosphatase has been shown to be an important regulator of PtdIns(4)P turnover. Biochemical analysis in yeast has demonstrated that PtdIns(4)P is the major substrate for Sac1 *in vivo* (Foti et al., 2001; Tahirovic et al., 2005). A similar substrate specificity has been reported for *Drosophila* and mammalian Sac1 proteins (Nemoto et al., 2000; Yavari et al., 2010). Yeast and mammalian Sac1 proteins are integral membrane proteins that localize to the endoplasmic reticulum (ER) and Golgi complex (Nemoto et al., 2000; Rohde et al., 2003; Tahirovic et al., 2005; Whitters et al., 1993). In yeast, loss of the *sac1* gene causes pleiotropic defects, such as cold sensitivity for growth (Novick et al., 1989), inositol auxotrophy (Whitters et al., 1993), multiple drug sensitivity (Hughes et al., 1999), cell wall defects (Schorr et al., 2001), and abnormal vacuole morphology (Foti et al., 2001). In *Drosophila*, *sac1*-null mutations cause an abnormal activation of Jun kinase, which is paralleled with dorsal closure defects and embryonic lethality (Wei et al., 2003), and Hedgehog signaling (Yavari et al., 2010). In mice, deletion of the *sac1* gene produces preimplantation lethality (Liu et al., 2008). In mammalian cells, RNAi-mediated depletion of Sac1 causes defects in Golgi morphology and mitotic spindle organization (Liu et al., 2008).

In this study, we show that Sac1 is highly expressed in the developing nervous system. Loss of *sac1* function causes ectopic midline crossing of Fasciclin II (Fas II)-positive CNS axons, which normally do not cross the midline. We also find that the phosphatase activity of Sac1 is required in neurons for midline axon repulsion. Finally, we show that *sac1* genetically interacts with *slit* and *robo*, which encode the midline repellent Slit and its receptor Robo, respectively. Thus, this study establishes a

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novel role for Sac1 in midline axon repulsion.

MATERIALS AND METHODS

Molecular biology

Full-length *sac1* cDNA was obtained from the *Drosophila* Genomic Resource Center (clone ID: GH08349; USA). The entire 1,779-bp cDNA fragment was amplified by PCR and subcloned into pBluescript II KS- (pBS; Stratagene, USA). To generate *pGEX-sac1-N* (encoding amino acids 18-100) and *pUAS-HA-sac1-WT*, *sac1* cDNA fragments of interest were PCR-amplified and inserted into *pGEX6P1* (Amersham Pharmacia, USA) and *pUAST-HA*, a derivative of the *pUAST* vector (Brand and Perrimon, 1993). The *sac1-C388S,T395A* mutation was introduced into *pBS-sac1* with the QuickChange Site-Directed Mutagenesis Kit (Stratagene, USA). The following mutagenic primers were used: for *sac1-C388S*, 5'-GTGTCTTCCGAACG AATTCTATCGACTGTCTCGATAG-3' and 5'-CTATCGAGAC AGTCGATAGAATTCTCGGAAGACAC-3'; and for *sac1-T395A*, 5'-CGACTGTCTCGATAGGCGAACGTCGTGCAGA G-3' and 5'-CTCTGCACGACGTTCCGCCCTATCGAGACAGT CG-3'. The *sac1-C388S,T395A* insert was introduced into *pUAST-HA* to generate *pUAS-HA-sac1-CS-TA*.

Genetics

The wild-type strain used in this study was *w¹¹¹⁸*. Two P-element insertions in the *sac1* locus (G8721 and G4263) were obtained from GenExel (Korea) and imprecisely mobilized to generate independent excision mutants, *sac1¹* and *sac1²*, respectively. The EMS-induced *sac1²¹⁰⁷* allele was kindly provided by Nicholas Harden (Simon Fraser University, Canada). UAS transgenic lines were generated in the *w¹¹¹⁸* background using standard procedures and expressed under the control of either the ubiquitous *da-GAL4* driver (Wodarz et al., 1995) or the panneuronal *elav-GAL4* driver (Lin and Goodman, 1994).

Cell culture and double-stranded RNA interference

Drosophila S2 cells were grown at 25°C in Schneider's medium supplemented with 10% heat-inactivated (56°C for 30 min) fetal bovine serum (FBS) and transfected in six-well plates using Cellfectin (Invitrogen, USA) according to the manufacturer's instructions.

For double-stranded RNA interference (dsRNAi) in S2 cells, DNA fragments containing coding sequences of *sac1* and *gfp* were PCR-amplified using the following primers, which contain upstream T7 promoter sequences: *sac1* sense primer, 5'-CGAATGGAGGAGATGAGTTGCTG-3', *sac1* antisense primer, 5'-CAAGTGTCTGGCGTAGCAGTCGC-3'; *gfp* sense primer, 5'-ACGTAAACGGCCACAAGTTC-3', *gfp* antisense primer, 5'-GTCCTCCTTGAAGTCGATGC-3'. The PCR products were used as DNA templates to generate dsRNAs by *in vitro* transcription. S2 cells were treated with dsRNA at a final concentration of 37 nM, as described previously (Lee et al., 2007).

Antibodies and immunohistochemistry

An N-terminal region of *Drosophila* Sac1 (amino acids 18-100) was expressed as a GST fusion protein in *Escherichia coli*, purified by glutathione-Sepharose 4B column chromatography (Amersham Pharmacia, USA), and used for the immunization of rats.

Whole-mount staining of embryos was performed as previously described (Lee et al., 2000; Spencer et al., 1998). Monoclonal antibodies against Fasciclin II (1D4) and Futsch (22C10) were purchased from the Developmental Studies Hybridoma Bank (USA) and used at a dilution of 1:5. FITC- and cyanine 3

(Cy3)-conjugated secondary antibodies (Jackson ImmunoResearch, USA) were used at 1:200.

Phosphatase activity assay

Sac1-catalyzed dephosphorylation of PtdIns(4)P was assayed in 25 μ l of reaction buffer containing 200 mM sodium acetate, 100 mM Bis-Tris, 100 mM Tris-base (pH 6.0), 20 μ g/ml porcine gelatin (Sigma, USA), 500 μ M diC₁₈-phosphatidylserine (Sigma, USA), 200 μ M diC₈-PtdIns(4)P (Echelon, USA), and 1 μ g of purified recombinant GST-Sac1-WT or GST-Sac1-CS-TA. Lipid suspension in the reaction buffer was prepared as described previously (Maehama et al., 2000). Reactions were incubated at 37°C for 1 h, stopped by adding 20 μ l of 100 mM *N*-ethylmaleimide (NEM), and centrifuged at 14,000 $\times$ *g* at 4°C for 30 min. Twenty-five microliters of the resultant supernatant was transferred to a 96-well plate and incubated with 50 μ l of malachite green solution for 20 min at room temperature. Phosphate release was measured by a microplate reader (MP-100, Bio-Rad) at 620 nm.

RESULTS AND DISCUSSION

Sac1 protein is expressed in the nervous system

As the first step toward studying the neuronal function of Sac1, we wished to examine whether it is expressed in the developing nervous system of the embryo. We therefore generated a polyclonal antibody against an N-terminal region of Sac1 (amino acids 18-100). On western blots of *Drosophila* S2 cells, this antibody detected a single band of ~65 kDa, corresponding to the predicted size of Sac1 (Fig. 1A). Levels of the 65 kDa protein were significantly decreased in cells treated with *sac1* dsRNA but not in cells treated with *gfp* dsRNA (Fig. 1A), suggesting the specificity of the antibody.

Immunohistochemical analysis of whole-mount wild-type embryos using anti-Sac1 revealed that the Sac1 protein is abundantly expressed during early stages of embryogenesis (e.g., stage 3) (Fig. 1B), suggesting a strong maternal contribution. We observed the most prominent expression in posteriorly located pole cells (Fig. 1B, asterisk). However, at stages 13 and 16, Sac1 expression was highly restricted to the developing central nervous system (CNS) and peripheral nervous system (PNS) (Figs. 1C-1E, arrowheads and brackets, respectively). In stage 13 embryos, strong Sac1 immunoreactivity was also found in the dorsal epidermis (Fig. 1C, arrow), consistent with its reported role in dorsal closure (Wei et al., 2003). The immunoreactivity was substantially decreased in *sac1*-null (*sac1¹*/*sac1²*, see below) mutant embryos at stage 16 (Fig. 1F), confirming the specificity of anti-Sac1 signals.

The expression of Sac1 in the nervous system was further analyzed by performing double labeling with anti-Sac1 and mAb 22C10 (anti-Futsch), a marker that labels a subset of interneurons and motor neurons in the CNS as well as all sensory neurons in the PNS (Fujita et al., 1982; Hummel et al., 2000). In the PNS of wild-type embryos at stage 16, anti-Sac1 signals perfectly overlapped with 22C10 (Figs. 1G-1I), revealing the strong expression of Sac1 in all sensory neurons. Like 22C10 (Hummel et al., 2000), Sac1 was detected in the axon, cell body, and dendrite of sensory neurons. In the CNS at stage 16, anti-Sac1 signals partially overlapped with 22C10, clearly labeling a subset of longitudinal axon fascicles (Figs. 1J-1L). The identity of these axon fascicles was investigated by double labeling with anti-Sac1 and mAb 1D4 (anti-Fasciclin [Fas] II), which labels three major longitudinal axon pathways (i.e., the medial, intermediate, and lateral Fas II pathways) in the CNS (Lin et al., 1994) as well as all motor axons in the PNS (Vactor

Fig. 1. Sac1 is highly expressed in the embryonic nervous system. (A) Cellular extracts of S2 cells treated with *sac1* or *gfp* dsRNA were subjected to Western blot analysis using anti-Sac1. (B-F) Whole-mount wild-type (B-E) and *sac1*¹/*sac1*² (F) embryos stained with anti-Sac1. (B) Lateral view of a stage 3 embryo shows ubiquitous expression of Sac1, with the highest levels in the posterior pole cells (asterisk). (C) Lateral view of a stage 13 embryo shows Sac1 expression in the developing sensory neurons (bracket) and epidermal cells around the amnioserosa (arrow). (D, E) Lateral (D) and ventral (E) views of stage 16 embryos show Sac1 enrichment in the CNS (arrowheads) and PNS (brackets). (F) Ventral view of a stage 16 *sac1*¹/*sac1*² embryo. (G-R) Higher magnification images of stage 16 embryos doubly stained with anti-Sac1 and either 22C10 (G-L) or 1D4 (M-R). (G-I) Lateral views of a stage 16 embryo. Sac1 (green) is expressed in all sensory neurons in the PNS. The morphology of sensory neurons is outlined by 22C10 staining (red). (J-L) Ventral views of a stage 16 embryo. In the CNS, Sac1 (green) partially overlaps with 22C10 (red). (M-R) Ventral views of embryos at stages 16 (M-O) and 17 (P-R). In the CNS, Sac1 (green) is detected on the longitudinal axon tracts, which are marked by 1D4 staining (red). Scale bars: in (E) 50 μ m for (B-F); in (E), 10 μ m for (G-R). Anterior is left.

et al., 1993). The Sac1-positive fascicles were also labeled with 1D4 in embryos at stage 16 or 17 (Figs. 1M-1R), suggesting that Sac1-positive CNS axons contribute to the formation of the Fas II pathways.

Sac1 is required for midline axon guidance

Given the enrichment of Sac1 in a subset of embryonic neurons, we wondered if it might be required for some aspects of neuronal morphogenesis. To investigate this point genetically, deletion mutants of *sac1* were generated via imprecise excision of

two P-element insertions (G4263 and G8721) located within or near the *sac1* gene (Fig. 2A). The *sac1*¹ allele, derived from G8721, has a 2,222-bp deletion (-17 to 2,205 from the translation initiation site), while the *sac1*² allele, derived from G4263, has a smaller deletion (-294 to 1,562). Animals homozygous for each of these deletions died at early larval stages, suggesting that *sac1* is an essential gene.

To characterize the effects of *sac1* loss on axon extension or guidance, we examined *sac1*-null mutant embryos. In the PNS of *sac1*¹/*sac1*² embryos stained with 22C10, sensory axon

Fig. 2. *sac1* is required for accurate midline guidance of CNS axons. (A) Genomic organization of the *sac1* gene at 61F4 on chromosome III. The insertion sites of two P-element lines G4263 and G8721 within *sac1* are indicated by inverted triangles. The extents of deleted regions in *sac1*¹ and *sac1*² are indicated. Shown below is the intron-exon organization of *sac1* and its neighboring genes. Untranslated regions are indicated by white boxes and translated regions by black boxes. (B-F) Several CNS segments of wild-type (B and D) and *sac1*¹/*sac1*² mutant (C, E, and F) embryos stained with mAb 1D4. Anterior is up. (B, C) Early stage 13 embryos. (B) In wild-type embryos, the pCC axon (arrow) extends anteriorly on its own side of the midline. (C) In *sac1*¹/*sac1*² mutant embryos, the pCC axon (arrows) ectopically crosses the midline. (D-F) Stage 16-17 embryos. (D) In wild-type embryos, 1D4 clearly visualizes three longitudinal axon pathways on each side of the midline. (E, F) In *sac1*¹/*sac1*² mutant embryos, 1D4-positive CNS axons ectopically cross the midline. (E) In segments in the mild category, the innermost tract displays midline crossing defects without impairing the entire morphology of the CNS axon scaffold. (F) In segments in the severe category, all three Fas II-positive tracts aberrantly cross the midline, altering the morphology of the CNS axon scaffold. Scale bar, 10 μm. (G) Quantification of the percentage of segments with ectopic midline crossing in each of the indicated genotypes (*sac1*¹/+, n = 600 segments; *sac1*²/+, n = 184; *sac1*¹/*sac1*², n = 1856; *sac1*¹/*sac1*²¹⁰⁷, n = 312).

morphology appeared normal (data not shown). We also found no defects in the extension and guidance of motor axons, as determined by 1D4 staining (data not shown). However, we did observe a defect in the midline guidance of CNS axons in *sac1*-null mutant embryos stained with 1D4. In stage 13 wild-type embryos, the 1D4-positive pCC axons pioneer the medial Fas II pathway (Fig. 2B, arrow), which normally runs parallel to the midline (Lin et al., 1994). In *sac1*¹/*sac1*² embryos, these axons often crossed the midline (Fig. 2C, arrow). The midline crossing phenotype of Fas II-positive CNS axons was more evident at later stages of development. In wild-type embryos at stage 16, axons in the Fas II-positive pathways never crossed the midline (Fig. 2D). Heterozygous *sac1*¹/+ and *sac1*²/+ embryos displayed none or very mild midline phenotypes (Fig. 2G). However, in comparable *sac1*¹/*sac1*² embryos, a varying range of Fas II-positive CNS axons aberrantly crossed the midline. In segments in the mild class, only the medial Fas II pathway ectopically crossed the midline, and the entire morphology of

the CNS axon scaffold was fairly normal (Fig. 2E). In segments in the severe class, axons in the medial, intermediate, and lateral Fas II pathways often crossed the midline, collapsing the CNS axon scaffold (Fig. 2F). In *sac1*¹/*sac1*² embryos, approximately 6% and 12% of segments displayed the midline crossing phenotypes in the mild and severe classes, respectively (Fig. 2G). A similar CNS defect was also found when *sac1*¹ and *sac1*² were placed in *trans* over either another lethal *sac1* allele *sac1*²¹⁰⁷ (Wei et al., 2003) or *Df(3L)Fpa2*, a deficiency disrupting *sac1* (data not shown).

The phosphatase activity of Sac1 is essential for accurate midline axon guidance

To confirm whether the loss of Sac1 is responsible for the CNS phenotype associated with *sac1* mutants, we performed genetic rescue experiments in the *sac1*¹/*sac1*² background. Neuronal expression of *UAS-HA-sac1-WT* in all postmitotic neurons using *elav-GAL4* substantially rescued the midline crossing

Fig. 3. The PI phosphatase activity of Sac1 is required for accurate midline axon guidance. (A) The sequences flanking motif 6 within the Sac domain are compared using CLUSTALW software. The sequence data for *Drosophila*, rat, and human Sac1 are available from GenBank (accession numbers AAF47460, AAG29810, and AAH16559, respectively). Sequence identities are indicated by white letters with a black background. Sequence similarities are indicated by black letters with a gray background. The highly conserved catalytic core sequences (CX₅RT) are indicated by the horizontal line. The double substitution (C388S-T395A) used in this study is also indicated. (B) Dephosphorylation of PtdIns(4)P by GST-Sac1-WT or GST-Sac1-CS-TA was determined using phosphatidylserine lipid vesicles (see "Materials and Methods"). (C) Quantification of the midline crossing phenotype in each of the indicated genotypes (*sac1¹/sac1²*, n = 1856 segments; *sac1¹;elav-GAL4/sac1²*, *UAS-HA-sac1-WT*, n = 496; *sac1¹;elav-GAL4/sac1²*, *UAS-HA-sac1-CS-TA*, n = 832). The percentages of segments with ectopic midline crossing are shown. (D) Quantification of adult viability in each of the indicated genotypes (*sac1¹;da-GAL4/sac1²*, *UAS-HA-sac1-WT*, n = 168 flies; *sac1¹;da-GAL4/sac1²*, *UAS-HA-sac1-CS-TA*, n = 170). The numbers of eclosed *sac1¹/sac1²* flies to the adult are expressed as percentages of the expected viability.

phenotype (Fig. 3C), suggesting that Sac1 is required, at least in part, in neurons for midline axon repulsion of Fas II-positive CNS axons.

Fig. 4. *sac1* genetically interacts with *slit* and *robo*. The percentages of segments with ectopic midline crossing are given for each of the indicated genotypes (*slit²/+*, n = 220 segments; *robo²/+*, n = 446; *slit²/+*; *sac1¹/+*, n = 384; *robo²/+*; *sac1¹/+*, n = 392).

Drosophila, rat, and human Sac1 proteins commonly contain a Sac phosphatase domain that consists of seven conserved motifs (Hughes et al., 2000). In particular, the sequence CXDCLDRT within the sixth motif defines the catalytic core of Sac1 phosphatases (Fig. 3A, horizontal line) (Foti et al., 2001). Replacing the first conserved cysteine with serine has been shown to impair the phosphatase activity of human Sac1 (Rohde et al., 2003). In addition, other conserved residues in the core sequence have been shown to be essential for the activity of the Sac domain in yeast Sac1 (Kearns et al., 1997). Consistent with these studies, a variant of *Drosophila* Sac1 carrying a double-point mutation (C388S,T395A) within the catalytic core sequence impaired its phosphatase activity toward PtdIns(4)P (Fig. 3B). To determine whether the phosphatase activity of Sac1 is essential for midline axon guidance, we generated a UAS transgene expressing the C388S,T395A mutation of Sac1 (*UAS-HA-sac1-CS-TA*). Neuronal expression of *UAS-HA-sac1-CS-TA* using *elav-GAL4* failed to rescue the midline crossing phenotype in *sac1¹/sac1²* embryos (Fig. 3C). When driven by *elav-GAL4*, the distribution and expression levels of the *UAS-HA-sac1-CS-TA* transgene were not significantly different from those of *UAS-HA-sac1-WT* (data not shown), suggesting that the phosphatase activity of Sac1 is essential for midline avoidance of CNS axons.

The importance of phosphatase activity for the *in vivo* function of Sac1 was further investigated. The ubiquitous expression of *UAS-HA-sac1-WT* under the control of the *da-GAL4* driver was also able to substantially rescue the lethality associated *sac1* mutations, while expression of *UAS-HA-sac1-CS-TA* using the same GAL4 driver failed to do so (Fig. 3D). Thus, the phosphatase activity of Sac1 is essential for animal survival during development.

***sac1* genetically interacts with the Slit/Robo repellent pathway at the midline**

The midline repellent Slit and its Robo receptor play a critical role in axon repulsion from the CNS midline (Kidd et al., 1998; 1999). Therefore, we investigated whether there is a functional link between Sac1 and the Slit/Robo repellent pathway. For this,

we examined dosage-sensitive genetic interactions between *sac1* and *slit* and *robo*. In heterozygous *slit*^{1/+} and *robo*^{2/+} embryos, we observed weak midline crossing phenotypes: 9% mild and 1% severe, respectively (Fig. 4). When we removed one copy of *sac1* in a *slit*^{1/+} or *robo*^{2/+} background, a dramatic enhancement of the *slit*^{1/+} or *robo*^{2/+} phenotype was observed: 2% mild and 48% severe in *slit*^{1/+}; *sac1*^{1/+} embryos and 13% mild and 11% severe in *robo*^{2/+}; *sac1*^{1/+} embryos (Fig. 4). These dosage-sensitive genetic interactions support a role for *sac1* in Slit/Robo-mediated axon repulsion from the midline.

In summary, we have demonstrated for the first time that the PI phosphatase Sac1 plays an essential role in midline axon guidance in the developing CNS. The protein is expressed in the developing nervous system. In embryonic sensory neurons, the distribution of Sac1 is almost identical to that of Futsch, a fly homolog of the vertebrate microtubule-associated protein MAP1B. However, Sac1 expression does not strongly overlap with Futsch in the embryonic CNS but largely labels Fas II-positive longitudinal axon tracts, which do not cross the midline. We also show that Sac1 functions in neurons to regulate midline avoidance of Fas II-positive axons. This regulatory role of Sac1 depends on its PI phosphatase activity, as evidenced by the observation that a phosphatase-defective variant of Sac1 fails to rescue the midline crossing phenotype observed in *sac1* mutants. Genetic interactions between *sac1* and *slit* or *robo* support a potential role for Sac1 in Slit/Robo-dependent axon repulsion at the midline. Because PtdIns(4)P, the primary *in vivo* substrate for Sac1, has been shown to be an important regulator of anterograde membrane trafficking from the Golgi complex (D'Angelo et al., 2008), it is highly possible that Sac1 regulates the anterograde trafficking of the Robo receptor by modulating PtdIns(4)P levels at the Golgi complex. Alternatively, Sac1-mediated regulation of PtdIns(4)P metabolism at the plasma membrane could affect Robo signaling directly or indirectly. Future analysis of Sac1 may reveal new insights into the functional link between PtdIns(4)P metabolism and Robo signaling.

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