Suppression of a phosphatidylinositol 3-kinase signal by a specific spliced variant of Drosophila PTEN

Tomohiko Maehama[a,*], Natsuki Kosaka[a,b], Fumiaki Okahara[a,c], Ken-ichi Takeuchi[d], Masato Umeda[d], Jack E. Dixon[e,f], Yasunori Kanaho[a]

[a]*Department of Pharmacology, Tokyo Metropolitan Institute of Medical Science, 3-18-22 Honkomagome, Tokyo 113-8613, Japan*
[b]*Department of Molecular Biology and Biochemistry, Graduate School of Humanities and Sciences, Ochanomizu University, Ohtsuka, Tokyo 112-8610, Japan*
[c]*Department of Biological Sciences, Graduate School of Science, Tokyo Metropolitan University, Hachiohji, Tokyo 192-0397, Japan*
[d]*Division of Molecular Biology and Information, Institute for Chemical Research, Kyoto University, Gokasho Uji, Kyoto 611-0011, Japan*
[e]*Departments of Pharmacology and Cellular and Molecular Medicine, School of Medicine, University of California at San Diego, La Jolla, CA 92093, USA*
[f]*Department of Chemistry and Biochemistry, University of California at San Diego, La Jolla, CA 92093, USA*

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Abstract Drosophila PTEN (dPTEN) plays indispensable roles in the development of *Drosophila melanogaster* by controlling cell size and number. Although three potential spliced forms of dPTEN have been isolated, functional distinction among these forms remains elusive. In this study, we demonstrate that all spliced forms of dPTEN dephosphorylate phosphatidylinositol 3,4,5-trisphosphate ($PI(3,4,5)P_3$); however, $PI(3,4,5)P_3$-dependent activation of Drosophila Akt is suppressed specifically by one of three spliced forms, dPTEN3. Further, dPTEN3 dramatically changes its expression during the Drosophila development, while the other forms are expressed throughout the development. Our results suggest that dPTEN3 is the predominant spliced form that participates in $PI(3,4,5)P_3$-mediated signaling pathways.

Keywords: PTEN; Drosophila; Phosphoinositide; Phosphatase; Phosphoinositide 3-kinase

1. Introduction

PTEN has been originally identified as a tumor suppressor gene, which is mutated in a number of human tumors [1,2]. The following studies have unveiled that human PTEN dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate ($PI(3,4,5)P_3$), which is produced by phosphatidylinositol 3-kinase (PI3K) upon growth factor stimulation [3]. Therefore, PTEN antagonizes PI3K actions, such as promoting cell proliferation and migration and preventing cells from apoptosis [4–6]. Bioinformatic approaches have shown that *PTEN* is evolutionary conserved in a wide variety of species, from yeast to mammals. Genetic studies in model organisms have revealed that these PTEN orthologs also fundamentally antagonize PI3K actions [7–13].

Drosophila PTEN (dPTEN) plays indispensable roles in the development of the fruit fly, *Drosophila melanogaster* [8–10]. Homozygous mutation in the coding region of *dPTEN* results in embryonic lethality and ubiquitous overexpression of dPTEN in the fly also exhibits lethality. In mosaic Drosophila flies, *dPTEN*$^-$ cells proliferate faster than their heterozygous siblings, resulting in increased cell size and organ size [8–10]. On the other hand, organ-specific overexpression of dPTEN results in opposite phenotype, as shown by reduced organ size [8–11]. The phenotypes from the loss of dPTEN function are suppressed by a mutation in the $PI(3,4,5)P_3$ target, Drosophila *Akt* (*dAkt*) [11,14]; in addition, the overexpression of dPTEN suppresses the phenotypes induced by the overexpression of Drosophila *PI3K* (*dp110*) or its upstream activator, *Inr* [8–10]. These observations indicate that dPTEN counteracts signaling downstream of the dp110 to control cell growth and proliferation as human PTEN does; however, no biochemical studies on dPTEN have been yet provided, therefore underlying biochemistry of dPTEN actions still remains poorly understood. Further, at least three alternatively spliced forms of dPTEN have been identified, varying their extended carboxyl termini [15]; however, distinction of these potential spliced variants in their functions and biochemical properties remains unknown.

In this study, we employ biochemical approaches to understand the molecular functions of three dPTEN-spliced variants, and we demonstrate that dPTEN3, among these variants, functions as a negative regulator for a dp110-mediated signal by dephosphorylating $PI(3,4,5)P_3$, while the other forms do not. In addition, we also demonstrate that the expression of dPTEN3 is firmly regulated during the development of the Drosophila.

* Corresponding author. Fax: +81-3-3823-5284.
E-mail address: tmaehama@rinshoken.or.jp (T. Maehama).

Abbreviations: dPTEN, Drosophila PTEN; $PI(3,4,5)P_3$, phosphatidylinositol 3,4,5-trisphosphate; $PI(3,4)P_2$, phosphatidylinositol 3,4-bisphosphate; PI(3)P, phosphatidylinositol 3-phosphate; PI3K, phosphatidylinositol 3-kinase; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; GFP, green fluorescent protein; GST, glutathione *S*-transferase

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2. Materials and methods

2.1. Plasmids

Complementary DNAs encoding dPTEN spliced variants (dPTEN1–3) were amplified by RT-PCR using RNA isolated from Schneider-2 cells and cloned into pGEX-6P1 (Amersham Pharmacia) to create dPTEN1/pGEX, dPTEN2/pGEX, and dPTEN3/pGEX. Site-directed mutagenesis, to substitute glycine-135 (GGA) to glutamic acid (GAA), was carried out with a PCR-based strategy using dPTEN2/pGEX as the template. In order to generate Drosophila expression vector, pDEX, the promoter region of Drosophila actin 5C was amplified by PCR from Schneider-2 cell genomic DNA and subcloned into *Sac*II site of pTRE (Clontech). The cDNA encoding full-length dAkt was amplified by RT-PCR using RNA isolated from Schneider-2 cells. The sequence encoding the FLAG epitope tag was included in the forward primer. The FLAG-dAkt cDNA insert was subcloned into pDEX to produce FLAG-dAkt/pDEX. To create N-terminally green fluorescent protein (GFP)-tagged dPTEN expression vectors, pDEX-GFP, dPTEN1/pDEX-GFP, dPTEN2/pDEX-GFP, and dPTEN3/pDEX-GFP, dPTEN cDNA inserts excised from dPTEN/pGEX vectors were cloned into pDEX with GFP cDNA insert from pEGFP-C1 (Clontech). Nucleic acid sequences were confirmed by conventional sequencing methods.

2.2. Preparation of recombinant dPTEN proteins

Expression and preparation of recombinant glutathione *S*-transferase (GST)-fused dPTEN proteins were performed essentially as described previously [3,16]. GST–dPTEN proteins were recovered in elution buffer (50 mM Tris–HCl (pH 8), 1 mM Na-EDTA, 2 mM dithiothreitol, 300 mM NaCl and 10 mM glutathione), concentrated using Centricon-30 (Amicon), and stored at −80 °C until use. To determine the protein concentration of GST–dPTEN proteins, protein samples were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). The samples were stained with Coomassie brilliant blue R250 or immunoblotted with anti-GST antibody (Cell Signalling Technology) after being transferred to FluoroTrans membrane filter (Pall). Signals were visualized using Luminol Reagent (Santa Cruz Biotechnology) according to the manufacturer's recommended protocol. Bovine serum albumin and GST were used as standards for Coomassie brilliant blue R250-staining and immunoblotting, respectively.

2.3. Phosphatase assay

Standard assay was performed in 10 μl of the reaction mixture consisting of 100 mM Tris–HCl (pH 8), 10 mM dithiothreitol, 0.5 mM dioleoyl phosphatidylserine (Sigma), 50 μM dipalmitoyl-PI(3,4,5)P_3, 0.2% NP-40, and 30–60 ng of GST–dPTEN protein. In some experiments (Table 1 and Fig. 2), dipalmitoyl phosphatidylinositol 3-phosphate (PI(3)P) or dipalmitoyl phosphatidylinositol 3,4-bisphosphate (PI(3,4)P_3) was used instead of PI(3,4,5)P_3. All phosphoinositides were purchased from Cayman Chemicals. After incubation at 28 °C, the amount of inorganic phosphate released from phosphoinositides was determined as described previously [16].

2.4. Cell culture and transfection

Drosophila Schneider-2 cells (ATCC) were maintained as described previously [17]. For the transfection, cells (1×10^7) were washed with K-PBS (8.1 mM Na_2HPO_4, 1.46 mM KH_2PO_4, 30.8 mM NaCl, 120.7 mM KCl, and 10 mM $MgCl_2$), suspended in 0.6 ml of K-PBS, and transferred into 0.4-cm cuvette with 20 μg of plasmid DNA. After the electroporation (500 μF, 320 V) was performed, cell suspension was added to 4 ml of complete medium, and incubated for 2 days to allow protein expression.

2.5. dAkt in vitro kinase assay

Cells (2×10^6) transfected with 5 μg of FLAG-dAkt/pDEX and 15 μg of pDEX-GFP, dPTEN1/pDEX-GFP, dPTEN2/pDEX-GFP, or dPTEN3/pDEX-GFP were serum-starved for 2 h. After stimulating with 10% fetal bovine serum for 15 min at 24 °C, cells were lysed in 1 ml of ice-cold lysis buffer (50 mM Tris–HCl (pH 7.5), 120 mM NaCl, 1% NP-40, 1 mM Na-EDTA, 1 mM Na-EGTA, 25 mM NaF, 25 mM β-glycerophosphate, 15 mM Na-pyrophosphate, 1 μM microcystin LR, 1 mM Na-orthovanadate, 0.02% β-mercaptoethanol, 2 μg/ml of aprotinin, 2 μg/ml of pepstatin A, 1 mM phenylmethylsulfonyl fluoride, and 1 mM benzamidine–HCl). After removing debris by centrifugation, anti-FLAG M2 Agarose beads were added to cleared lysate, and the mixture was incubated for 2 h at 4 °C with gentle agitation. Beads were washed with 1 ml of lysis buffer containing 0.5 M NaCl and 1 ml of kinase reaction buffer (50 mM Tris–HCl (pH 7.5) and 1 mM dithiothreitol), then 30 μl of kinase reaction buffer, 10 μl of myelin basic protein (2.5 mg/ml), and 10 μl of ATP solution (50 mM $MgCl_2$, 0.25 mM ATP, and 10 μCi of [γ-^{32}P]ATP) were added to start kinase reaction. After incubation at 30 °C for 1 h, the reaction was terminated by the addition of Laemmli sample buffer, then samples were subjected to SDS–PAGE. Radioactivity incorporated into myelin basic protein was determined by BAS1500 Bio Imaging Analyzer (Fuji Photo Film). To analyze the expression of FLAG-dAkt and GFP–dPTEN proteins, the transfected cells were lysed in Laemmli sample buffer and immunoblotting using anti-FLAG M2 antibody (Sigma) and anti-GFP antibody (Zymed) was performed as described in Section 2.2.

2.6. Confocal microscopy

Cells transfected with 20 μg of pDEX-GFP, dPTEN1/pDEX-GFP, dPTEN2/pDEX-GFP, or dPTEN3/pDEX-GFP were seeded onto glass-bottomed dishes and incubated for one day to allow cells to attach. Fluorescence analysis was performed by confocal microscopy (Zeiss Axiovert S100 equipped with Yokogawa confocal scanner unit CSU21). Fluorescent intensity along major axis of the cell on captured image was measured using NIH Image 1.62 and averaged from 10 different images.

2.7. Analysis for the expression of dPTEN transcripts

Complementary DNA was synthesized using MoMLV reverse transcriptase from total RNA isolated from each developmental stage of *D. melanogaster* Canton S strain. For the amplification of dPTEN transcripts, PCR (35 cycles of 94 °C, 15 s; 48 °C, 20 s; 68 °C, 70 s + 10 s/cycle) was performed using 0.5 μg of cDNA and AccuTaq polymerase (Sigma). Primers used were TTTCGAAAGATCAGACTATGACAGTT and TTTACAAATTCGAATAGATTAGCCAA. For the amplification of Act5C transcripts, PCR (25 cycles of 94 °C, 30 s; 55 °C, 20 s; and 72 °C, 45 s) was performed using 0.1 μg of cDNA and recombinant Taq polymerase (Takara). Primers used were CGTCTTCCCATCGATTGTG and GATGCCAGGGTACATG- GTG. PCR products were resolved on agarose gel and signals were visualized by ethidium bromide staining.

Table 1
Phosphatase activity of dPTEN spliced variants towards D3-phosphoinositides

Substrate	Specific activity (nmol/min/mg)		
	dPTEN1	dPTEN2	dPTEN3
PI(3)P	4.4 ± 3.3	4.5 ± 0.5	7.1 ± 4.8
PI(3,4)P_2	72.7 ± 3.8	92.5 ± 16.3	72.0 ± 4.2
PI(3,4,5)P_3	136 ± 15	669 ± 30	583 ± 22

Specific activity was calculated from the amount of released phosphate from PI(3)P, PI(3,4)P_2, or PI(3,4,5)P_3 after 60-min, 10-min, or 3-min incubation, respectively. All values represent means and standard deviations from four independent determinations.

3. Results

3.1. Phosphoinositide phosphatase activity of dPTEN

The phosphatase domain of dPTEN is highly similar to that of human PTEN with identity of 58% and the catalytic core motif including two invariant lysine residues (HCKAGKGRT) that are critical for the PI(3,4,5)P_3 phosphatase activity of human PTEN [18] is completely conserved in dPTEN. These features strongly suggest that dPTEN functions as a PI(3,4,5)P_3 phosphatase; however, because of difficulty in the preparation of recombinant dPTEN proteins, direct evidence that dPTEN catalyzes dephosphorylation of PI(3,4,5)P_3 has

not been provided yet. Therefore, we first prepared recombinant proteins of three potential dPTEN spliced variants as GST-fusion proteins (Figs. 1A and B) and examined their phosphatase activities towards PI(3,4,5)P_3. As shown in Fig. 1C, all spliced variants dephosphorylated PI(3,4,5)P_3 and approximately five times higher activity of dPTEN2 and dPTEN3 than dPTEN1 was observed (Fig. 1C and Table 1). We also tested the other D3-phosphoinositides for substrates of dPTEN, since human PTEN utilizes PI(3,4)P_2 and PI(3)P as its substrates. PI(3,4)P_2 was dephosphorylated by all spliced variants with almost same magnitude, while PI(3)P was an extremely poor substrate for dPTEN (Table 1).

Mutational analyses of human PTEN have revealed that the phosphatase activity is essential for its biological function [6]. Most of tumor-associated mutations result in a loss of the phosphatase activity. In *D. melanogaster*, several *dPTEN* mutant alleles have been isolated ([8–10,19], and see FlyBase FBgn0026379), and *dPTEN*^{c494} encodes a missense mutant, of which glycine-135 in the catalytic core motif was replaced by glutamic acid (G135E) [9]. As reflecting absolute requirement of the glycine residue for maintaining catalytic pocket architecture, G135E mutation resulted in complete loss of the phosphatase activity (Fig. 2), suggesting that the phosphatase activity is essential for dPTEN function in the Drosophila development.

3.2. Effect of dPTEN spliced variants on dAkt activity

It has been reported that dAkt is a major downstream target of dp110/PI(3,4,5)P_3 in Drosophila [11,14]. Moreover, knock down of dPTEN by RNA interference results in significant enhancement of dAkt activity in Drosophila Schneider-2 cells [17]. Although these observations clearly indicate that dPTEN participates in PI(3,4,5)P_3-dependent dAkt activation as a negative regulator, it remains unknown which spliced form of

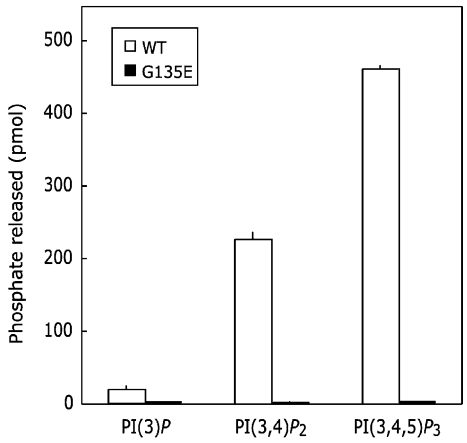

Fig. 2. Effect of G135E mutation on the phosphatase activity. Phosphatase assay for indicated D3-phosphoinositides were performed with 50 ng of GST–dPTEN2 (wild type, open column and G135 mutant, closed column). Reactions were terminated after incubation for 30 min, then released inorganic phosphate was determined. Bars represent differences between duplicate determinations in a typical study.

dPTEN is responsible for this signaling pathway. In order to analyze the contribution of dPTEN spliced variants in this signal, we overexpressed each variant with dAkt in Schneider-2 cells and assessed the effects of these variants on serum-induced dAkt activation (Fig. 3). In GFP-expressing control cells, dAkt activity was increased (2.5-fold) upon serum stimulation (Fig. 3B). dPTEN3 abolished the dAkt activation completely and dPTEN1 partially suppressed the dAkt activation (Fig. 3B). Surprisingly, dPTEN2 that exhibited highest phosphatase activity in the in vitro assay (Table 1) showed no effect on the dAkt activation (Fig. 3B), even though much

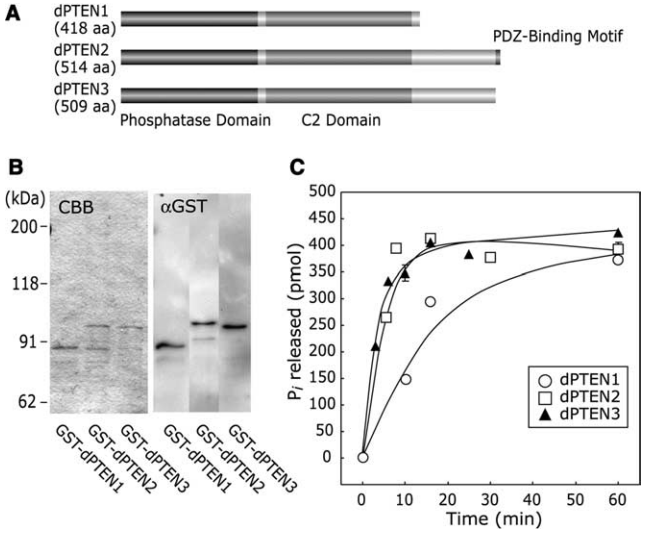

Fig. 1. Recombinant dPTEN dephosphorylates PI(3,4,5)P_3. (A) Schematic representation of dPTEN spliced variants. (B) Recombinant GST–dPTEN proteins (1 μg each) were subjected to SDS–PAGE, stained by Coomassie brilliant blue R250 (Left) and immunoblotted with anti-GST antibody (Right). (C) Time course of dPTEN-catalyzed dephosphorylation of PI(3,4,5)P_3. Reactions with each GST–dPTEN protein (50 ng) were terminated at indicated times, then released inorganic phosphate was determined. Bars represent differences between duplicate determinations in a typical study.

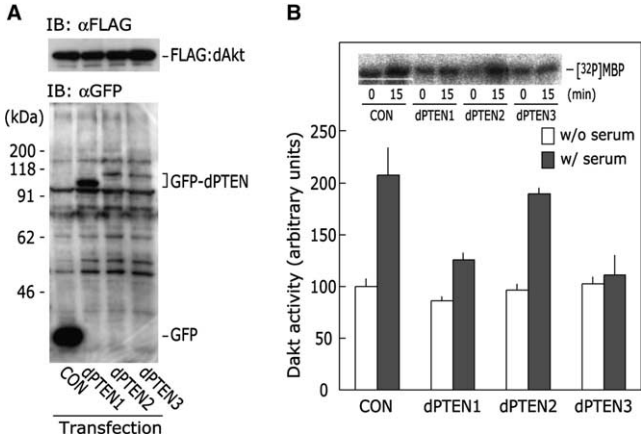

Fig. 3. Suppression of serum-induced dAkt activation by dPTEN. (A) Ectopic expression of FLAG-dAkt and GFP–dPTEN proteins were confirmed by immunoblotting with anti-FLAG antibody (top) and anti-GFP antibody (bottom), respectively. (B) Serum-starved Schneider-2 cells expressing FLAG-dAkt with either of GFP, GFP-dPTEN1, GFP-dPTEN2, or GFP-dPTEN3 were incubated with (closed column) or without (open column) 10% fetal bovine serum, then FLAG-dAkt was immunoprecipitated and subjected to in vitro kinase assay. Phosphorylation of myelin basic protein (MBP) was visualized by an autoradiography (inset) and quantitated as described in Section 2. Bars represent differences between duplicate determinations. Similar results were obtained in a repeated experiment.

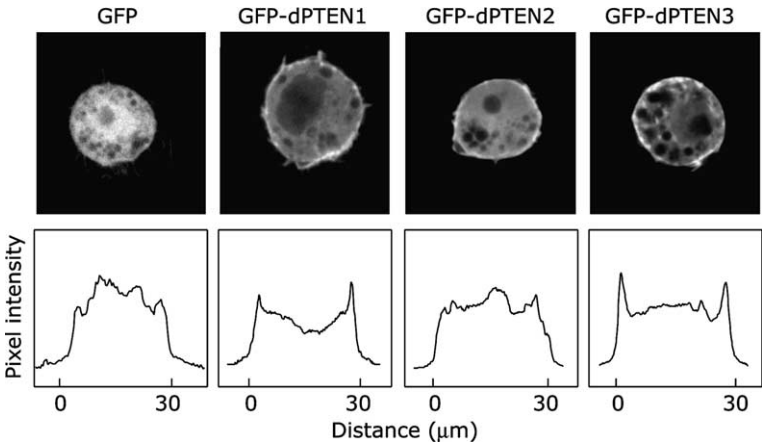

Fig. 4. Subcellular localization of dPTEN-spliced variants. Localization of GFP, GFP-dPTEN1, GFP-dPTEN2, and GFP-dPTEN3 proteins expressed in Schneider-2 cells was analyzed by confocal microscopy. Represented are typical images (top) and averaged pixel intensities along the major axis of cells (bottom).

higher protein expression level than that of dPTEN3 was observed (Fig. 3A).

PI(3,4,5)P_3 that activates dAkt is localized to the plasma membranes, indicating that dPTEN must be recruited to the plasma membranes to suppress the dAkt activation. This idea led us to analyze intracellular localization of dPTEN variants in Schneider-2 cells (Fig. 4). Both dPTEN1 and dPTEN3 were predominantly localized to the plasma membranes, while dPTEN2 was mainly localized in the cytoplasm and partially to the plasma membrane. The dissociation of dPTEN2 from the plasma membranes may partially account for the loss of suppressive activity against the dAkt activation. In addition, as dPTEN1 and dPTEN3 showed almost identical cellular localization, it is more likely that partial suppressive effect of dPTEN1 on the dAkt activation could be due to its low PI(3,4,5)P_3 phosphatase activity (Table 1).

3.3. Expression of dPTEN during the Drosophila development

Genetic studies have shown that both mutation and overexpression of dPTEN fundamentally result in embryonic lethality [8–11], suggesting the requirement of dPTEN and its strictly controlled expression in the Drosophila development. Studies described above indicate that dPTEN3 is the most responsible spliced form for the PI(3,4,5)P_3-mediated signal at least in Schneider-2 cells. We next analyzed the expression of dPTEN by RT-PCR with discriminating its three spliced variant forms during the development. Either dPTEN1 or dPTEN2, that showed small or no effect on dAkt activation (Fig. 3), was expressed evenly in each developmental stage (Fig. 5). On the other hand, the expression of dPTEN3 was dramatically altered during the development. In early embryonic stage, no expression of dPTEN3 was observed, and the expression was increased at late embryonic stage, decreased at pupal stage, then disappeared in adult flies. Intriguingly, *dPTEN* mutant flies start to die at stages of late embryo and early larva that are consistent with the time when dPTEN3 transcript appeared [9,10]. These observations, taken together, would suggest that dPTEN3, which exhibited robust suppressive activity towards the dAkt activation in Schneider-2 cells, plays a crucial role in the transition from embryos to larvae.

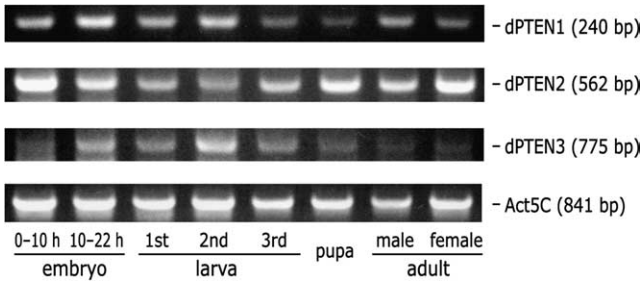

Fig. 5. Developmental profile of three dPTEN transcripts in *D. melanogaster*. RT-PCR analyses were performed on RNAs isolated from Drosophila animals of different developmental stages as indicated. PCR products corresponding to each dPTEN variants were distinguished based on their sizes as indicated.

4. Discussion

In this study, we first demonstrated that recombinant dPTEN proteins dephosphorylated PI(3,4,5)P_3. Its human counterpart, PTEN, specifically hydrolyzes phosphate group at D3 position of PI(3,4,5)P_3. The specificity is determined by highly strict coordination of the phosphate groups on the inositol ring in the catalytic pocket [18]. Lysine-125 and lysine-128 play critical roles in the coordination, and replacement of these lysine residues with the neutral amino acid, methionine, affects the substrate specificity of human PTEN [18]. Methionine substitution for lysine-136 (K136M) of dPTEN that is corresponding to K128M mutation of human PTEN provided same effect as human K128M mutant does, in which PI(3,4,5)P_3 phosphatase activity was specifically eliminated (data not shown). Moreover, dPTEN released approximately 1 mol of phosphate from 1 mol of substrate (Fig. 1C). These results indicate that dPTEN essentially employs same catalytic mechanism as human PTEN does. In addition, it should be noted that dPTEN1 showed lower PI(3,4,5)P_3 phosphatase activity when compared to dPTEN2 and dPTEN3, while PI(3,4)P_2 phosphatase activity of dPTEN1 was almost identical to theirs (Table 1). The major structural difference between dPTEN1 and dPTEN2/3 is in their extended carboxyl-terminal regions; dPTEN1 lacks most of this region (see Fig. 1A),

suggesting the requirement of the carboxyl-terminal region for the recognition of PI(3,4,5)P_3 as the substrate. Another striking structural difference found in dPTEN-spliced variants is the existence of a potential PDZ-binding motif at the carboxyl-terminus of dPTEN2 (see Fig. 1A). Human PTEN also has a PDZ-binding motif and several PDZ proteins are known to bind to human PTEN through this motif [20–23]. These PDZ proteins may recruit human PTEN to the plasma membrane and facilitate its function as the PI(3,4,5)P_3 phosphatase. In the case of dPTEN2, its PDZ-binding motif appears to have the opposite function, inhibiting the recruitment of dPTEN2 to the plasma membranes and its biological activity. Further study will be required to understand the biological significance of the PDZ-binding motif of dPTEN2 and to identify its binding partner(s).

Our results also have shown that dPTEN3 may be the most responsible form in the regulation of PI(3,4,5)P_3-mediated signaling pathway in Schneider-2 cells. It has also been reported that overexpression of dPTEN1 or dPTEN2 has severe impact on Drosophila flies, resulting in reduced organ size and lethality [9,10], although these variants did not significantly affect the dAkt activation in cultured Schneider-2 cells. These observations raise the possibility that dPTEN1 and dPTEN2 employ unidentified activation machinery that lacks in Schneider-2 cell to suppress dAkt-mediated signals in Drosophila animal. It is also conceivable that dPTEN1 and dPTEN2 are involved in the phosphoinositide-mediated but dAkt-independent signaling process that also controls cell size and organ size, such as Drosophila S6 kinase-mediated signals [24,25].

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References

[1] Steck, P.A. et al. (1997) Nat. Genet. 15, 356–362.
[2] Li, J. et al. (1997) Science 275, 1943–1947.
[3] Maehama, T. and Dixon, J.E. (1998) J. Biol. Chem. 273, 13375–13378.
[4] Di Cristofano, A. and Pandolfi, P.P. (2000) Cell 100, 387–390.
[5] Cantley, L.C. and Neel, B.G. (1999) Proc. Natl. Acad. Sci. USA 96, 4240–4245.
[6] Maehama, T., Taylor, G.S. and Dixon, J.E. (2001) Annu. Rev. Biochem. 70, 247–279.
[7] Ogg, S. and Ruvkun, G. (1998) Mol. Cell 2, 887–893.
[8] Goberdhan, D.C., Paricio, N., Goodman, E.C., Mlodzik, M. and Wilson, C. (1999) Genes Dev. 13, 3244–3258.
[9] Huang, H., Potter, C.J., Tao, W., Li, D.M., Brogiolo, W., Hafen, E., Sun, H. and Xu, T. (1999) Development 126, 5365–5372.
[10] Gao, X., Neufeld, T.P. and Pan, D. (2000) Dev. Biol. 221, 404–418.
[11] Scanga, S.E. et al. (2000) Oncogene 19, 3971–3977.
[12] Funamoto, S., Meili, R., Lee, S., Parry, L. and Firtel, R.A. (2002) Cell 109, 611–623.
[13] Iijima, M. and Devreotes, P. (2002) Cell 109, 599–610.
[14] Stocker, H., jelkovic, M., Oldham, S., Laffargue, M., Wymann, M.P., Hemmings, B.A. and Hafen, E. (2002) Science 295, 2088–2091.
[15] Smith, A., Alrubaie, S., Coehlo, C., Leevers, S.J. and Ashworth, A. (1999) Biochim. Biophys. Acta 1447, 313–317.
[16] Maehama, T., Taylor, G.S., Slama, J.T. and Dixon, J.E. (2000) Anal. Biochem. 279, 248–250.
[17] Clemens, J.C., Worby, C.A., Simonson-Leff, N., Muda, M., Maehama, T., Hemmings, B.A. and Dixon, J.E. (2000) Proc. Natl. Acad. Sci. USA 97, 6499–6503.
[18] Lee, J.O. et al. (1999) Cell 99, 323–334.
[19] Oldham, S., Stocker, H., Laffargue, M., Wittwer, F., Wymann, M. and Hafen, E. (2002) Development 129, 4103–4109.
[20] Adey, N.B., Huang, L., Ormonde, P.A., Baumgard, M.L., Pero, R., Byreddy, D.V., Tavtigian, S.V. and Bartel, P.L. (2000) Cancer Res. 60, 35–37.
[21] Wu, Y., Dowbenko, D., Spencer, S., Laura, R., Lee, J., Gu, Q. and Lasky, L.A. (2000) J. Biol. Chem. 275, 21477–21485.
[22] Leslie, N.R., Bennett, D., Gray, A., Pass, I., Hoang-Xuan, K. and Downes, C.P. (2001) Biochem. J. 357, 427–435.
[23] Tolkacheva, T., Boddapati, M., Sanfiz, A., Tsuchida, K., Kimmelman, A.C. and Chan, A.M. (2001) Cancer Res. 61, 4985–4989.
[24] Lizcano, J.M., Alrubaie, A., Kieloch, A., Deak, M., Leevers, S.J. and Alessi, D.R. (2003) Biochem. J. 374, 297–306.
[25] Miron, M., Lasko, P. and Sorenberg, N. (2003) Mol. Cell. Biol. 23, 9117–9126.