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Abstract

Mutations in the tumor suppressor gene PTEN (MMAC1/TEP1) are associated with a large number of human cancers and several autosomal-dominant disorders. Mice mutant for PTEN die at early embryonic stages and the mutant embryonic fibroblasts display decreased sensitivity to cell death. Overexpression of PTEN in different mammalian tissue culture cells affects various processes including cell proliferation, cell death and cell migration. We have characterized the Drosophila PTEN gene and present evidence that both inactivation and overexpression of PTEN affect cell size, while overexpression of PTEN also inhibits cell cycle progression at early mitosis and promotes cell death during eye development in a context-dependent manner. Furthermore, we have shown that PTEN acts in the insulin signaling pathway and all signals from the insulin receptor can be antagonized by either Drosophila or human PTEN, suggesting a potential means for alleviating symptoms associated with altered insulin signaling.
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SUMMARY

Mutations in the tumor suppressor gene PTEN (MMAC1/TEP1) are associated with a large number of human cancers and several autosomal-dominant disorders. Mice mutant for PTEN die at early embryonic stages and the mutant embryonic fibroblasts display decreased sensitivity to cell death. Overexpression of PTEN in different mammalian tissue culture cells affects various processes including cell proliferation, cell death and cell migration. We have characterized the Drosophila PTEN gene and present evidence that both inactivation and overexpression of PTEN affect cell size, while overexpression of PTEN also inhibits cell cycle progression at early mitosis and promotes cell death during eye development in a context-dependent manner. Furthermore, we have shown that PTEN acts in the insulin signaling pathway and all signals from the insulin receptor can be antagonized by either Drosophila or human PTEN, suggesting a potential means for alleviating symptoms associated with altered insulin signaling.

Key words: Drosophila development, PTEN tumor suppressor, Cell size, Proliferation, Apoptosis, Insulin-signaling

INTRODUCTION

The tumor suppressor gene PTEN is one of the most frequently mutated genes involved in the development of human cancer (Cantley and Neel, 1999; Li et al., 1997a; Steck et al., 1997). PTEN mutations are found in a wide variety of tumors such as glioblastomas, endometrial carcinomas, advanced prostate cancers and melanoma cells (Cairns et al., 1997; Guldberg et al., 1997; Li et al., 1997a; Liu et al., 1997; Rasheed et al., 1997; Risinger et al., 1997; Wang et al., 1997). Germ-line mutations in PTEN are linked to three rare autosomal dominant syndromes: Cowden Disease, Bannayan-Zonana syndrome and Lhermitte-Duclose disease (Liaw et al., 1997; Marsh et al., 1997). A common feature of these syndromes is a predisposition for the development of hamartomas, benign tumors that have differentiated but disorganized cells.

The PTEN protein contains the phosphatase signature motif HCXXGXXRS/T that is found in all protein tyrosine phosphatases (Denu et al., 1996; Li and Sun, 1997; Li et al., 1997a; Steck et al., 1997). This phosphatase domain is the most common site of germline or sporadic PTEN mutations (Myers and Tonks, 1997). In vitro studies have demonstrated that PTEN can dephosphorylate phosphotyrosine, phosphoserine and phosphothreonine residues on artificial substrates (Li and Sun, 1997; Myers et al., 1997). Recently, PTEN has been shown to have strong in vitro and in vivo activity for the 3′ position of phosphatidylinositol 3,4,5 trisphosphate (PIP3) (Maehama and Dixon, 1998). PIP3 is produced by the catalytic activity of phosphatidylinositol 3 kinase (PI3K) and can act as a membrane-embedded second messenger for the activation of a variety of signaling molecules. A number of Pten mutant mice have been described (Di Cristofano et al., 1998; Podsypanina et al., 1999; Suzuki et al., 1998). Although the spectrum of disorders affecting the Pten mutant mice varies between the different strains, all homozygous Pten mutants exhibit early embryonic lethality (E7.5-E9.5) and the heterozygotes display a predisposition to tumor development (Di Cristofano et al., 1998; Podsypanina et al., 1999; Suzuki et al., 1998). Consistent with the role for PTEN as a PIP3 phosphatase, Pten mutant cells exhibit increased PIP3 signaling (Stambolic et al., 1998; Sun et al., 1999).

The C. elegans gene daf-18 encodes a distant PTEN homolog (Gil et al., 1999; Mihaylova et al., 1999; Ogg and Ruvkun, 1998; Rouault et al., 1999). In addition to a conserved phosphatase domain, daf-18 encodes a large non-homologous C-terminal region (Ogg and Ruvkun, 1998). Daf-18 mutants can suppress the mutant phenotypes of daf-2, the C. elegans insulin-like receptor, and age-1, the C. elegans PI3K (Gil et al., 1999; Mihaylova et al., 1999; Ogg and Ruvkun, 1998; Rouault et al., 1999).
A variety of cellular functions have been reported for PTEN using different mammalian systems. Overexpression of PTEN in PTEN− glioblastoma cells inhibits cell cycle progression (Cheney et al., 1998; Furnari et al., 1997). Furthermore, homozygous Pten mutant mice exhibit regions of increased cell proliferation (Suzuki et al., 1998). These results suggest a role for PTEN in regulating cell division. On the other hand, overexpression of PTEN in PTEN− breast and prostate cancer cells causes apoptosis (Li et al., 1998; Myers et al., 1998), and immortalized Pten mutant fibroblasts exhibit an elevated resistance to apoptosis (Stambolic et al., 1998). Thus, PTEN could have a distinct role in regulating apoptosis. Furthermore, PTEN has been reported to inhibit cell migration by directly dephosphorylating focal adhesion kinase (FAK) and She (Gu et al., 1999; Tamura et al., 1998). In C. elegans,daf-18 has been shown to be involved in dauer formation (Gil et al., 1999; Mihaylova et al., 1999; Ogg and Ruvkun, 1998; Rouault et al., 1999).

To explore the various roles of PTEN during development, we have isolated and characterized the Drosophila PTEN homolog. We show that fly PTEN functions in the insulin signaling pathway, and both human and fly PTEN elicit similar effects during Drosophila eye development. Using this Drosophila system, we demonstrate that PTEN affects cell proliferation and apoptosis in a developmental context-dependent manner. Furthermore, we show that PTEN plays an essential role in the regulation of cell size.

MATERIALS AND METHODS

Molecular biology

The low stringency condition used in cDNA library screening was as described (Tao et al., 1999). Three dPTEN cDNA were isolated and sequenced. A 1.8 kb dPTEN cDNA, a wild-type human PTEN cDNA and the human PTENC124S mutant cDNA were cloned into pUAST for P-transformation (Brand and Perrimon, 1993). The dPTEN genomic region was isolated from F1 clones Ds01978, Ds02806 and Ds05299 (Berkeley Drosophila Genome Project). A 6.2 kb SpeI genomic fragment was cloned into pW8 for rescue experiments.

Fly genetics

dPTENg94 is an EMS-induced mutation isolated from a screen for lines that failed to complement the Df(2L)J1 b tti l deficiency. Clones of dPTEN mutant cells were generated by X-ray irradiation of w; P[w+; ry+]30C P[ry+; hs-neo; FRT]40A (Fig. 1A). To isolate mutations in dPTEN, we carried out a genetic screen and isolated ten lethal complementation groups using the Df(2)J1 deficiency, which removes dPTEN as well as other genes (Fig. 1A). The lethality of one group consisting of a single allele (dPTENg94) can be fully rescued by P-element transformants carrying dPTEN genomic DNA (Fig. 1A). Two lines of evidence indicate that this mutation is a null or strong dPTEN allele. First, sequence analysis reveals a G135E change in the coding region (Fig. 1B). This is an invariant residue in the active-site motif of the catalytic domain and is required for phosphatase activity (Denu et al., 1999). Indeed, an identical change in human PTEN has been previously found in human melanomas (Guldberg et al., 1999). Moreover, both dPTENg94 homozygotes and dPTENg94/Df(2)J1 trans-heterozygotes die at a similar late embryonic/early first instar larval stage. Thus, dPTEN encodes a vital function that is needed for Drosophila early development.

Loss of dPTEN affects cell size but not patterning during eye development

To gain insight into the functions of dPTEN during development, we examined clones of cells that lack dPTEN in the Drosophila eye. The compound eye provides a useful model for studying cell proliferation and differentiation and yet it is dispensable for viability. dPTENg94 mutant ommatidia in the mosaic eyes appear larger in size compared to their neighboring wild-type ommatidia (Fig. 2A). Sections of these mosaic eyes revealed that the mutant ommatidia have normal photoreceptor cell composition and orientation (Fig. 2B,D). However, the sizes of the individual mutant cells are much larger than their neighboring wild-type cells (the average size of a mutant ommatidium is approximately 2.5 times that of dissimilar, and at least 15,000 cells were analyzed for each sample. We found no difference between human and fly PTEN transgenes tested.

Histology and immunofluorescence

Eye disc fixation and staining, scanning electron microscopy (SEM) and adult eye sections were performed as described (Xu and Harrison, 1994). Acridine orange staining was performed according to Spreij (1971). For labeling of cells in S phase, eye discs were dissected in PBS solution, incubated in M3 solution (Sigma) containing 500 μg/ml BrdU (Sigma) for 60 minutes at room temperature, and fixed in 4% paraformaldehyde in PBST (PBS with 0.3% Triton X-100) for 30 minutes at room temperature. Signals were detected with anti-BrdU antibody (1:50, Becton Dickinson). Samples were collected using confocal microscopy (BioRad, MRC-1024).

RESULTS AND DISCUSSION

Isolation and characterization of the Drosophila PTEN gene

We have identified a Drosophila PTEN (dPTEN) homolog by screening an imaginal disc cDNA library using a human PTEN probe at low stringency (Materials and Methods; Fig. 1). The fly and human PTEN gene products are similar in length and share sequence similarities in both the N-terminal phosphatase domain and the C-terminal region (overall 44% amino acids identical; Fig. 1B). We determined the genomic organization for dPTEN and mapped the gene to the cytological region 31C-D (Fig. 1A). To isolate mutations in dPTEN, we used a genetic screen and isolated ten lethal complementation groups using the Df(2)J1 deficiency, which removes dPTEN as well as other genes (Fig. 1A). The lethality of one group consisting of a single allele (dPTENg94) can be fully rescued by P-element transformants carrying dPTEN genomic DNA (Fig. 1A). Two lines of evidence indicate that this mutation is a null or strong dPTEN allele. First, sequence analysis reveals a G135E change in the coding region (Fig. 1B). This is an invariant residue in the active-site motif of the catalytic domain and is required for phosphatase activity (Denu et al., 1999). Indeed, an identical change in human PTEN has been previously found in human melanomas (Guldberg et al., 1999). Moreover, both dPTENg94 homozygotes and dPTENg94/Df(2)J1 trans-heterozygotes die at a similar late embryonic/early first instar larval stage. Thus, dPTEN encodes a vital function that is needed for Drosophila early development.

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PTEN function during Drosophila eye development

A wild-type one; Fig. 2B-D). Careful examination of the chimeric ommatidia that contain both mutant and wild-type cells revealed that the PTEN mutant phenotype is cell autonomous, as every mutant cell has an enlarged cell body while its immediate wild-type neighbors have normal cell body sizes (Fig. 2C). Unlike the Drosophila tumor suppressor genes such as lats that we have previously analyzed (Tao et al., 1999; Theodosiou et al., 1998; Xu et al., 1995), we did not observe an obvious overproliferation defect in PTEN mutant clones in the eye. This could be because dPTEN may not normally

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**Fig. 1. Drosophila PTEN (dPTEN) genomic organization and predicted amino acid sequence.** (A) The genomic region and dPTEN transcript map are shown with the dPTEN coding region labeled in black. The deficiency utilized to isolate the dPTEN mutant and the genomic region used to generate a dPTEN transgene are indicated above the genomic map. Bg, BgIII; B, BamHI; S, SpeI. (B) Alignments of the PTEN homologs among human, Drosophila and C. elegans. Only amino acids from Drosophila and C. elegans that are identical to human PTEN (hPTEN) are highlighted. The G→E amino acid substitution found in the dPTENc594 mutant is noted above the alignment. The GenBank accession number for Drosophila PTEN cDNA is AF144232.
function in the eye in regulating cell proliferation, or alternatively, there may be redundancy for \( PTEN \)-like molecules in the eye.

**Overexpression of \( PTEN \) inhibits cell proliferation during eye development**

To explore other potential roles of \( dPTEN \) during eye development, we used the \( UAS/GAL4 \) system to overexpress both human and fly \( PTEN \) in \textit{Drosophila} (Brand and Perrimon, 1993). Interestingly, the phenotypes caused by overexpression of human \( PTEN \) and \( dPTEN \) are indistinguishable, suggesting that the functions of the two homologs are conserved. For simplicity, we will use the name \( PTEN \) in text while specifying between human \( PTEN \) and \( dPTEN \) in figures when it is necessary. To verify whether the effects that we observed with expression of \( PTEN \) are related to its enzymatic activity, we also tested a construct, \( UAS-PTENC124S \), in which a critical residue in the phosphatase domain has been mutated. This change also leads to elimination of catalytic activity in vitro (Li and Sun, 1997; Li et al., 1997b). While ectopic expression of wild-type \( PTEN \) causes specific phenotypes (see below), expression of \( PTENC124S \) under the same conditions has no effect. Together with the \( PTEN^{c494} \) mutant, these experiments demonstrate that phosphatase activity is necessary for \( PTEN \) to exert its functions during development.

Since ubiquitous expression of \( PTEN \) directed by heat-shock induction of GAL4 causes lethality during embryonic and larval stages, we expressed \( PTEN \) specifically in the developing eye using the \textit{eyeless} GAL4 line (\textit{EYE-GAL4}). The \textit{eyeless} enhancer directs gene expression in the young developing eye disc where cells are actively proliferating.
Overexpression of PTEN function during Drosophila eye development

**Fig. 3.** Overexpression of PTEN in proliferating cells of the eye disc resulted in dramatic reduction of eye sizes in a dosage-dependent manner (Fig. 3C). In fact, multiple copies of UAS-PTEN can completely eliminate the eye (Fig. 3D). This small adult eye phenotype could be caused either by the inhibition of cell proliferation by PTEN, which would result in eye discs of smaller than normal size, or by the failure of ommatidium differentiation in eye discs of normal size. The sizes of eye discs dissected from third instar larvae carrying EYE-GAL4/UAS-PTEN were dramatically reduced (Fig. 4C,H), suggesting a defect in cell proliferation. Consistent with other studies, Acridine orange staining shows few apoptotic cells in a wild-type eye disc (C) and massive cell death in the posterior region of the dPTEN-expressing disc (D). The GMR-GAL4/UAS-dPTEN adult eye phenotype can be largely rescued by coexpression of p35. The slight disorganization of the eye in (F) is also seen in flies with GMR-p35 alone (Hay et al., 1994). Arrowheads indicate the MF, and anterior is to the left. Similar results were obtained with human PTEN (data not shown). (A,C) Wild type. (B,D) GMR-GAL4/++; UAS-dPTEN/UAS-dPTEN. (E) GMR-GAL4/++; UAS-dPTEN/+ . (F) GMR-GAL4/++; UAS-dPTEN/UAS-p35.

**Fig. 4.** Overexpression of PTEN affects cell-cycle progression in proliferating cells. While the BrdU-staining patterns are indistinguishable between wild-type and GMR/dPTEN discs (A,D), the number of staining cells are reduced in the anterior region of the EYE/dPTEN third instar eye disc (C). BrdU incorporation is abolished in the GMR/p21 disc posterior to the MF (B). Arrows indicate the MF, and anterior is to the left. Confocal images of third instar eye discs stained with anti-Elav antibody showing that neural differentiation is relatively normal in PTEN-overexpressed small third instar eye disc (H). (F,G) Propidium iodide staining shows that in a wild-type eye disc few post-replication cells with bright nuclear staining can be seen along regions anterior and posterior to the MF (G is an enlarged image of the boxed region in F). (I,J) The anterior region of a EYE/dPTEN-expressing disc accumulates many bright staining cells with a DNA content of 4C (arrow in J, which is an enlarged region of the boxed image in I). Histograms of FACS analysis display DNA content and cell numbers (K,L). Similar results were obtained from three repeated experiments. (A, E-G,K) wild type. (C,H-L) EYE-GAL4/++; UAS-dPTEN/UAS-dPTEN. (B) GMR-GAL4/+; UAS-dPTEN/UAS-dPTEN. (D) GMR-GAL4/++; UAS-dPTEN/UAS-dPTEN.

**Fig. 5.** Overexpression of PTEN causes cell death in the differentiating cells of the eye disc. Anti-Elav staining shows that the pattern of photoreceptor differentiation in dPTEN-expressing third instar eye disc (B) is comparable to that of a wild-type disc (A). Acridine orange staining shows few apoptotic cells in a wild-type eye disc (C) and massive cell death in the posterior region of the dPTEN-expressing disc (D). The GMR-GAL4/UAS-dPTEN adult eye phenotype can be largely rescued by coexpression of p35. The slight disorganization of the eye in (F) is also seen in flies with GMR-p35 alone (Hay et al., 1994). Arrowheads indicate the MF, and anterior is to the left. Similar results were obtained with human PTEN (data not shown). (A,C) Wild type. (B,D) GMR-GAL4/++; UAS-dPTEN/UAS-dPTEN. (E) GMR-GAL4/++; UAS-dPTEN/+ . (F) GMR-GAL4/++; UAS-dPTEN/UAS-p35.
Overexpression of PTEN in differentiating cells triggers apoptosis

Overexpression of PTEN under the direction of GMR results in adult eyes that are not only smaller but also rough (Fig. 3B). Sections of these adult eyes revealed a complex phenotype with varied degrees of severity. While the apical lenses of the ommatidia are smaller (Fig. 3B), the sizes of photoreceptor cells in the cross sections are larger than normal (Fig. 3F,G). Furthermore, these GMR/PTEN adult eyes have a flattened appearance with a narrowed retina (Fig. 3A,B; data not shown), suggesting the mutant phenotype could result from changes in both cell shape and size. In addition, different from the phenotype caused by inactivation of PTEN, the rhodobemers of the PTEN-overexpressed phoretceptors are reduced in size (Fig. 3G). Moreover, these sections showed that some ommatidia had missing pigment or photoreceptor cells (Fig. 3F; data not shown). We therefore stained third instar eye discs with the anti-Elav antibody to monitor retinal neuron differentiation (Robinow and White, 1991). Elav staining revealed a neuronal differentiation pattern comparable to wild type (Fig. 5B), indicating that photoreceptor differentiation is not affected. To pinpoint the cause of the phenotype, we further assessed whether cell death might have contributed to the GMR-GAL4/UAS-PTEN eye phenotype by staining these eye discs with acridine orange. Unlike wild-type third instar eye discs which have few dying cells (Wolff and Ready, 1991), the GMR-GAL4/UAS-PTEN eye discs have a substantially increased amount of cell death in the posterior region of the eye disc, where GMR-GAL4 is expressed (Fig. 5D). To further verify the contribution of cell death to the eye phenotype, GMR-GAL4 was used to coexpress PTEN with the p35 baculovirus gene, which can block apoptotic cell death (Hay et al., 1994; White et al., 1996). The eye phenotype induced by GMR/p35 was largely rescued by coexpression of p35 (Fig. 5F).

These experiments suggested the possibility that cell death might also contribute to the EYE/PTEN small eye phenotype. However, we found that EYE/PTEN eye discs do not have increased acridine orange staining and that the EYE/PTEN small eye phenotype was not suppressed by coexpression of p35 (data not shown). Thus, cell death appears not to be a major factor in PTEN-mediated inhibition of cell proliferation. Instead, these results suggest that cell death is a consequence of cell cycle arrest at the G2 or G2/M phase.
of cells at different developmental stages in response to the PTEN product.

**PTEN negatively regulates insulin signaling in Drosophila**

We have further examined the relationship between PTEN and the Drosophila PI3 kinase (D-PI3K) gene, Dp110 (Leevers et al., 1996). At 25°C, flies do not survive to adult when wild-type D-PI3K is expressed with EYE-GAL4. Interestingly, this lethality can be rescued by coexpression of PTEN. Furthermore, the small eye phenotype of PTEN overexpression is suppressed by overexpression of wild-type D-PI3K and enhanced by overexpression of dominant negative D-PI3K (Fig. 6A,B). These results clearly indicate that PTEN and PI3K function antagonistically in Drosophila.

The recent characterization of chico, a Drosophila IRS1-4 homolog, showed that chico, D-PI3K and insulin receptor (Inr) act as positive elements in a Drosophila insulin signaling pathway to regulate cell proliferation and cell size (Bohni et al., 1999). Consistent with the role of PTEN as a negative regulator in this insulin pathway, removal of one copy of the chico gene genetically enhances the EYE/PTEN eye phenotype (Fig. 6D). Overexpression of Inr (EYE/Inr) causes lethality at 25°C. At room temperature, few animals survive with overproliferated eyes (Fig. 6E). Strikingly, cooverexpression of PTEN completely rescues lethality and the overproliferation phenotype (Fig. 6F). This suggests that all signals from the insulin receptor can be antagonized by PTEN function. Together with the previous findings that mammalian and C. elegans PTEN molecules interact with components of the insulin pathway (Furnari et al., 1998; Maehama and Dixon, 1998; Myers et al., 1998; Ogg and Ruvkun, 1998; Stambolic et al., 1998; Sun et al., 1999), our genetic data argues that PTEN functions as a major conserved negative regulator in the insulin signaling pathway.

The role of PTEN in tumor suppression is not fully understood. Results from experiments involving different mammalian cell systems favor different theories. In this study, we have taken a genetic approach to analyze PTEN's function in Drosophila. Sequence analysis has shown that Drosophila PTEN is a close homolog of human PTEN, sharing high sequence similarity in both the N-terminal phosphatase domain and in the C-terminal region. Furthermore, expressing human and fly PTEN in Drosophila elicits similar effects during development. These results indicate that Drosophila is a useful model for studying PTEN functions. Phenotypes caused by overexpression of PTEN depend on the developmental status of the cells: overexpression of PTEN arrests cell-cycle progression in proliferating cells while promoting apoptosis in differentiating cells during eye development. These results suggest that PTEN may suppress tumorigenesis by preventing damaged cells from dividing and/or promoting a response to apoptotic signals in a cell context-dependent manner. Although it has been reported that PTEN affects cell migration in mammalian cell cultures (Tamura et al., 1998), we did not observe effects of PTEN on cell migration in Drosophila (H. H. and T. X., unpublished data). In addition to previously reported effects, we have shown for the first time that PTEN function is essential in the regulation of cell size. The finding that PTEN acts in the Drosophila insulin pathway, together with previous reports that Inr, chico and PI3K affect cell size (Bohni et al., 1999; Leevers et al., 1996), supports a pivotal role for the insulin signaling pathway in cell-size regulation. The conservation of this pathway raises the possibility that it may be involved in the regulation of cell size in different organisms. Finally, the observation that all signals from the insulin receptor can be antagonized by either Drosophila or human PTEN indicates that modulation of PTEN activity could be an effective means for alleviating symptoms associated with altered insulin signaling.

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