Regulation of Cell Migration by the C2 Domain of the Tumor Suppressor PTEN

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PTEN is a tumor suppressor protein that dephosphorylates phosphatidylinositol 3,4,5 trisphosphate and antagonizes the phosphatidylinositol-3 kinase signaling pathway. We show here that PTEN can also inhibit cell migration through its C2 domain, independent of its lipid phosphatase activity. This activity depends on the protein phosphatase activity of PTEN and on dephosphorylation at a single residue, threonine^{383}. The ability of PTEN to control cell migration through its C2 domain is likely to be an important feature of its tumor suppressor activity.

The tumor suppressor gene *PTEN* is frequently mutated in human cancers, particularly gliomas, most of which have lost expression of PTEN protein (*1*). PTEN consists of a catalytic N-terminal phosphatase domain that acts on both protein and lipid substrates and a C-terminal C2 domain that interacts in a Ca^{2+}-independent manner with phospholipid substrates. The major physiological substrate of PTEN is phosphatidylinositol 3,4,5 trisphosphate [$PI(3,4,5)P_3$], and to a lesser extent $PI(3,4)P_2$, thereby antagonizing the phosphatidylinositol-3 (PI 3) kinase signaling pathway (*2*–*5*). Studies in *Caenorhabditis elegans*, *Drosophila*, and mammalian cells have revealed that PTEN and PI 3 kinase play major roles both during development and in the adult to control cell size, growth, and survival (*6*–*8*). The loss of PTEN in human tumors leads to an increase in $PI(3,4,5)P_3$ and the uncontrolled stimulation of growth and survival signals (*9*). PTEN has also been shown to play a key role in regulating cell migration (*10*, *11*). $PTEN^{-/-}$ mammalian fibroblasts, for example, exhibit an increased rate of migration that has been attributed to increased $PI(3,4,5)P_3$ levels, and the expression of wild-type PTEN inhibits this phenotype (*12*, *13*). However, a naturally occurring PTEN point mutant [$Gly^{129} \rightarrow Glu^{129}$ (G129E) (*14*)], which has lost its lipid phosphatase activity but not its protein phosphatase activity, inhibits the migration of some tumor cells, suggesting that other mechanisms may be involved (*13*, *15*).

To examine how PTEN regulates motility, we used an in vitro wound-healing assay (*16*) and three human glioma cell lines—U373, U138, and U87—that lack detectable PTEN protein as a result of genetic lesions (*1*). Cells were plated on coverslips and allowed to grow to confluency, and the monolayer was scratched with a glass microneedle to initiate cell migration. One hour later, cells at the leading edge of either side of the wound were microinjected with a control expression vector encoding green fluorescent protein (GFP) or with an expression vector encoding hemagglutinin (HA) epitope–tagged wild-type PTEN. U373 cells injected with control plasmid remained at the front of the migrating edge even after 16 hours, whereas cells expressing wild-type PTEN did not migrate and were overtaken by noninjected cells (Fig. 1). Wild-type PTEN also inhibited cell migration in U138 and U87 cells (*17*). To examine the requirement for PTEN's catalytic activity, cells were injected with expression vectors encoding either the PTEN(G129E) mutant that lacks lipid phosphatase activity but retains protein phosphatase activity (Fig. 1, C and G), or the PTEN(C124S) mutant that lacks both lipid and protein phosphatase activity (Fig. 1, D and H) (*18*). As reported, the lipid phosphatase activity of PTEN was not required for inhibition of migration, but the protein phosphatase activity of PTEN was required (*15*).

To investigate whether other regions of PTEN are required for inhibition of cell migration, several deletion mutants were tested in the same assay (Fig. 2A). A mutant in which the catalytic phosphatase domain was completely deleted (PTENΔN) was as efficient as wild-type PTEN in inhibiting the migration of U373 (Fig. 2), U138, and U87 (*17*). This result was confirmed by microinjecting U373 cells with recombinant PTENΔN protein made in *Escherichia coli* (*17*, *19*). Two additional deletions of the PTENΔN construct, one to remove the PDZ binding motif at the extreme C terminus [PTENΔN(ΔPDZBD)] and another to remove all sequences C terminal to the C2 domain (PTEN-C2), were also as efficient as wild-type PTEN in inhibiting the motility of U373 cells (Fig. 2, A and B). To check that not all C2 domains inhibit the migration of these cells, constructs expressing either the Ca^{2+}-dependent C2A domain or the Ca^{2+}-independent C2B domain of synaptotagmin were injected (*20*), and these had no inhibi-

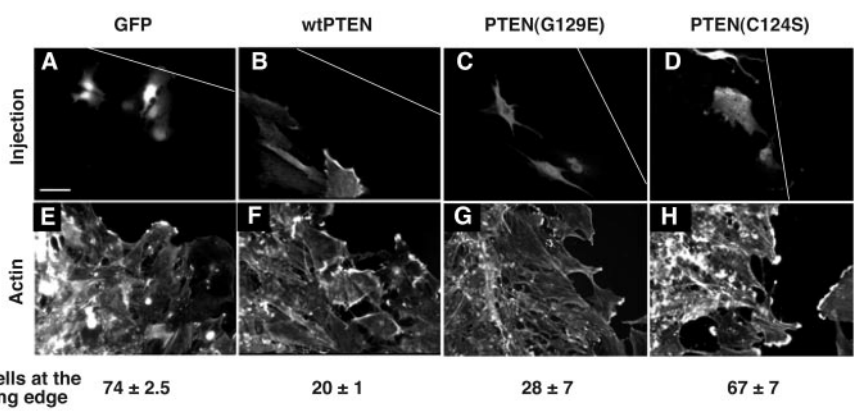

Fig. 1. The protein but not lipid phosphatase activity of PTEN is required to inhibit glioma cell migration. U373 cells at the leading edge of wounds were microinjected with expression vectors encoding GFP (**A** and **E**), HA-tagged wild-type (wt) PTEN (**B** and **F**), HA-tagged PTEN(G129E) (**C** and **G**), or HA-tagged PTEN(C124S) (**D** and **H**) and left to migrate for 16 hours. Expressing cells were detected after fixation by staining with rat antibody to HA [(B) to (D)], and all cells were visualized by staining for actin with rhodamine-conjugated phalloidin [(E) to (H)]. Scale bar, 50 μm. A white line indicates the edge of the wound (as visualized under phase). The number of cells expressing the various constructs that are still found at the edge of each wound 16 hours after injection of U373 cells was counted. Results are shown as a percentage of the total number of expressing cells. Data shown are the mean + SEM of three to five independent experiments in which an average of 120 cells (20 cells in each of six separate wound edges) were injected for each construct.

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tory effect on migration (Fig. 2, A and B). Thus, PTEN inhibited the migration of glioma cells specifically through its C2 domain.

Although the isolated C2 domain inhibited migration, catalytically inactive PTEN did not, suggesting that in the full-length protein, C2 domain activity is attenuated. PTEN contains five major phosphorylation sites and these are all located in the C-terminal 50–amino acid tail region (Ser^{370}, Ser^{380}, Thr^{382}, Thr^{383}, and Ser^{385}) and have been implicated in controlling PTEN activity (*21–24*). To examine whether phosphorylation at these sites affects the activity of the C2 domain, we first introduced four alanine substitutions at Ser^{380}, Thr^{382}, Thr^{383}, and Ser^{385} into full-length but catalytically inactive PTEN (Fig. 3A, PTEN(C124S)-A4). Removal of these four phosphorylation sites completely rescued the ability of catalytically inactive PTEN to inhibit migration of U373 cells (Fig. 3A). To identify which of the four sites is critical, individual residues were mutated to alanine (Fig. 3A). Mutation of the single residue Thr^{383} completely rescued inhibition of migration by PTEN(C124S), whereas mutation of any of the other three phosphorylation sites did not (Fig. 3, A and B). Thus, in the full-length protein, dephosphorylation of Thr^{383} is required to allow the C2 domain to inhibit migration, and this depends on the protein but not lipid phosphatase activity of PTEN. To confirm that dephosphorylation of Thr^{383} is critical for inhibition of glioma cell migration by PTEN, a phosphorylated Thr^{383} mimetic construct was generated (PTEN-T383D) by introducing an aspartic acid residue at this site in either a wild-type or a G129E mutant PTEN molecule [Fig. 3A (*17*)]. This substitution completely abrogated the ability of these two proteins to inhibit migration (Fig. 3A). It did not, however, affect the lipid phosphatase activity of wild-type PTEN, because PTEN-T383D was as efficient as wild-type PTEN in reducing PIP_3-mediated activation of the serine/threonine kinase AKT when transfected into HeLa cells (*17*).

The crystal structure of PTEN (lacking the C-terminal tail) reveals extensive intramolecular interactions between independently folding phosphatase and C2 domains (*25*). To examine whether phosphorylation at Thr^{383} might affect this interaction, we first examined whether the N terminus of PTEN can be immunoprecipitated with the

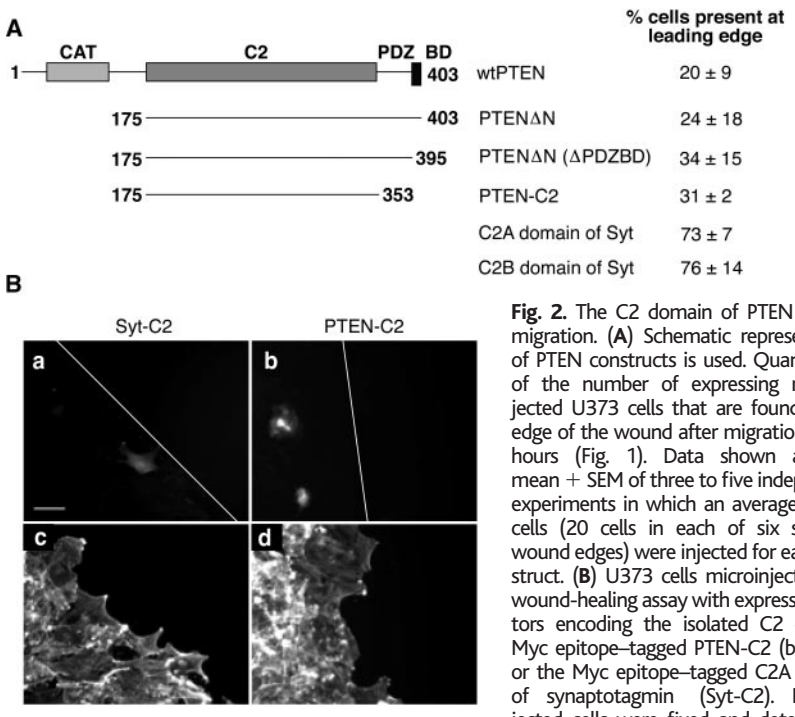

Fig. 2. The C2 domain of PTEN inhibits migration. (**A**) Schematic representation of PTEN constructs is used. Quantitation of the number of expressing microinjected U373 cells that are found at the edge of the wound after migration for 16 hours (Fig. 1). Data shown are the mean + SEM of three to five independent experiments in which an average of 120 cells (20 cells in each of six separate wound edges) were injected for each construct. (**B**) U373 cells microinjected in a wound-healing assay with expression vectors encoding the isolated C2 domain, Myc epitope–tagged PTEN-C2 (b and d), or the Myc epitope–tagged C2A domain of synaptotagmin (Syt-C2). Microinjected cells were fixed and detected by staining with 9E10 antibody to Myc (a and b). Cells were visualized by staining for actin with rhodamine-conjugated phalloidin (c and d). Scale bar, 50 μm. A white line is drawn to indicate the edge of the wound.

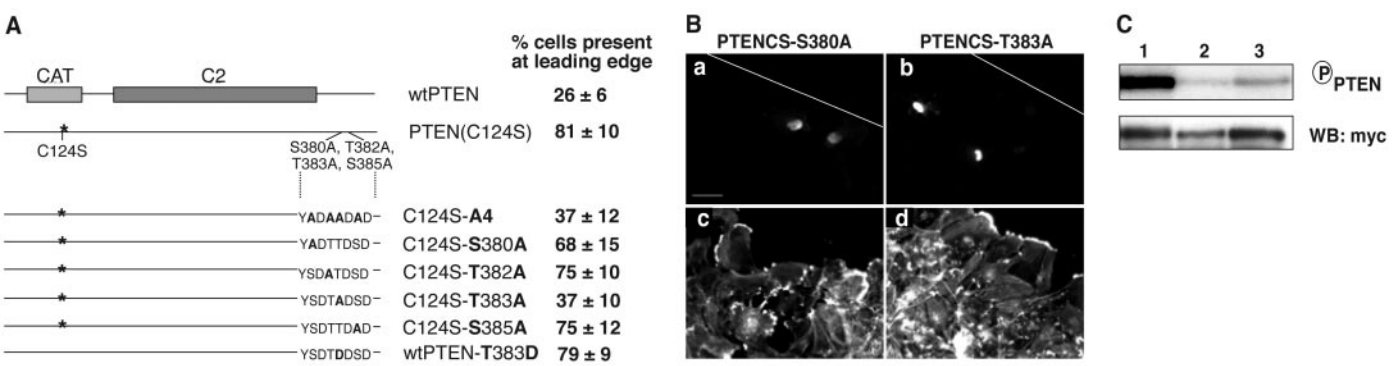

Fig. 3. The activity of the C2 domain is regulated by phosphorylation at Thr^{383}. (**A**) Schematic representation of the PTEN constructs used. Quantitation of the number of microinjected U373 cells that are found at the edge of the wound after migration for 16 hours (Fig. 1). Data shown are the mean + SEM of three to five independent experiments in which an average of 120 cells (20 cells in each of six separate wound edges) were injected for each construct. (**B**) U373 cells microinjected with expression vectors encoding Myc epitope–tagged PTEN(C124S)-S380A (a and c) or PTEN(C124S)-T383A (b and d). Microinjected cells were fixed and detected by staining with 9E10 antibody to Myc (a and b). Actin was visualized using rhodamine-conjugated phalloidin (c and d). Scale bar, 50 μm. A white line is drawn to indicate the leading edge of the wound. (**C**) COS-7 cells grown in [^{32}P]orthophosphate were transfected with Myc epitope–tagged versions of (i) catalytically inactive PTEN with residues Ser^{370}, Ser^{380}, Thr^{382}, and Ser^{385} mutated to aspartic acid [PTEN(C124S)-D4] (lane 1), (ii) wild-type PTEN with residues Ser^{370}, Ser^{380}, Thr^{382}, and Ser^{385} mutated to aspartic acid (PTEN-D4) (lane 2), and (iii) wild-type PTEN with residues Ser^{370}, Ser^{380}, Thr^{382}, Thr^{383}, and Ser^{385} all mutated (PTEN-S370D-A4) (lane 3). The 9E10 antibody to Myc was used to immunoprecipitate the PTEN constructs. Phosphorylation of the constructs was detected and quantitated by autoradiography using a phosphorimager. The 9E10 antibody to Myc was also used to control for the expression levels of the constructs immunoprecipitated. The circled P represents phosphorylated PTEN; WB, Western blot.

C terminus after expressing individual fragments in transfected COS-7 cells. Immunoprecipitation of the N terminus [residues 1 to 175; PTEN(C124S)ΔC] efficiently precipitated the C terminus [residues 175 to 403; PTENΔN] and vice versa (Fig. 4A). However, a C-terminal fragment lacking the 50–amino acid tail (residues 175 to 353; PTEN-C2) could not be immunoprecipitated with the N terminus (Fig. 4A), indicating that the C-terminal tail is essential for stable interaction. To determine whether phosphorylation of the tail affects this interaction, the N-terminal domain from a catalytically inactive version of PTEN [PTEN(C124S)ΔC] was coexpressed with a C-terminal domain containing either the four phosphorylation-site substitutions (PTENΔN-A4) at Ser^{380}, Thr^{382}, Thr^{383}, and Ser^{385} or the phosphomimetic substitution at Thr^{383} (PTENΔN-T383D). Both mutants interacted as well as the wild-type C terminus with the N-terminal domain, as determined by immunoprecipitation (Fig. 4B). Thus, phosphorylation and dephosphorylation of the C-terminal 50–amino acid tail of PTEN does not regulate the interaction of the carboxyl domain with the amino domain.

Direct examination of the phosphorylation state of Thr^{383} in PTEN is not straightforward, because PTEN is phosphorylated at five major sites within a 15-residue stretch and each with unknown stoichiometry. However, with respect to C2 domain activity, these sites can be functionally substituted with phosphomimetic Asp residues. We therefore constructed wild-type and catalytically inactive versions of PTEN, in which four of the five major phosphorylation sites at residues Ser^{370}, Ser^{380}, Thr^{382}, and Ser^{385} are replaced with Asp [PTEN-D4 and PTEN (C124S)-D4]. COS-7 cells grown in the presence of [^{32}P]orthophosphate were transfected with these constructs, and phosphorylated PTEN was immunoprecipitated. Catalytically inactive PTEN [PTEN (C124S)-D4] incorporated about 10 times as much phosphate as did catalytically active PTEN (PTEN-D4) (Fig. 3C, lanes 1 and 2). Furthermore, phosphate incorporation into the catalytically active PTEN-D4 mutant was the same as that incorporated into catalytically active PTEN in which all five major phosphorylation sites (i.e., including Thr^{383}) had been mutated (Fig. 3C, lanes 2 and 3). The catalytically inactive PTEN is, therefore, phosphorylated at Thr^{383}, whereas wild-type PTEN is not phosphorylated at this site.

We conclude that PTEN can inhibit the migration of human glioma cells through its C2 domain, independent of its effects on the PI 3 kinase pathway. The migration of U373 cells is unaffected by wortmannin (100 nM), whereas U87 migration is blocked by this inhibitor (*17*). It appears, therefore, that wild-type PTEN inhibits the migration of U373 cells solely through the activity of its C2 domain, whereas the inhibition of U87 migration occurs through both the C2 and the lipid phosphatase domains. The activity of the C2 domain is controlled by the phosphorylation state of Thr^{383}. When phosphorylated by an unknown kinase, the C2 domain is inactive, and when dephosphorylated the C2 domain is active and able to interfere with migration. Furthermore, the protein phosphatase activity of PTEN is required for dephosphorylation of Thr^{383}. Because the 12-residue peptide sequence surrounding Thr^{383} (YSDT**T**DSDPENE) is extremely acidic (five Asp or Glu and four phosphorylated Ser or Thr residues) and matches the optimal peptide substrates reported for PTEN, it is possible that dephosphorylation of Thr^{383} is mediated by an intramolecular autocatalytic activity (*18*). Alternatively, the protein phosphatase activity of PTEN could activate another protein phosphatase or inactivate a kinase, which would then lead to dephosphorylation of Thr^{383}. In tumors containing catalytically inactive PTEN, the antimigratory activity of the C2 domain may be lost along with the lipid phosphatase activity, and this could have important implications for tumor progression.

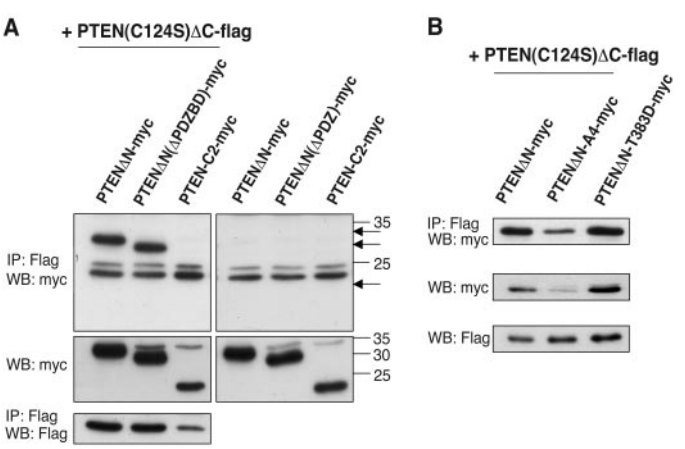

Fig. 4. The C-terminal tail interacts with the N-terminal catalytic domain of PTEN. (**A**) Transfected COS-7 cells expressing either Myc epitope–tagged PTENΔN, PTENΔN(ΔPDZBD), or PTEN-C2 with (lanes 1 to 3) or without (lanes 4 to 6) the flag epitope–tagged catalytically inactive N terminus of PTEN [PTEN (C124S) ΔC-flag]. PTEN(C124S)ΔC-flag was immunoprecipitated (IP) with antibody to flag and the precipitates were analyzed by Western blot with 9E10 antibody to Myc or M2 antibody to flag. One-tenth of the total lysate was also immunoblotted with 9E10 antibody to Myc. (**B**) Transfected COS-7 cells expressing flag epitope–tagged catalytically inactive N terminus of PTEN [PTEN(C124S)ΔC-flag] and either Myc epitope–tagged PTENΔN (lane 1), PTENΔN with residues Ser^{380}, Thr^{382}, Thr^{383}, and Ser^{385} all mutated to alanine (PTENΔN-A4-myc) (lane 2), or PTENΔN with residue Thr^{383} mutated to aspartic acid (PTENΔN-T383D-myc). The N terminus of PTEN was immunoprecipitated with an antibody to flag and the precipitates analyzed by Western blot (WB) with 9E10 antibody to Myc or M2 antibody to flag. One-tenth of the total lysate was also immunoblotted with 9E10 antibody to Myc.

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14. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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26. We thank N. K. Tonks for PTEN constructs, D. F. Cutler for synaptotagmin constructs, and A. Schmidt and A. B. Jaffe for helpful discussions. Supported by the Cancer Research UK and the Medical Research Council. M.R. is supported by a Medical Research Council Ph.D. studentship.

Supporting Online Material

www.sciencemag.org/cgi/content/full/303/5661/1179/DC1

Materials and Methods

1 October 2003; accepted 18 December 2003