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Stabilization and Productive Positioning Roles of the C2 Domain of PTEN Tumor Suppressor

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ABSTRACT

PTEN is a tumor suppressor frequently inactivated in brain, prostate, and uterine cancer. It acts as a phosphoinositide phosphatase and consists of an amino-terminal phosphatase domain tightly linked to a COOH-terminal C2 domain involved in lipid membrane-binding. We investigated the functions of the C2 domain and their relevance for tumor growth. To discriminate between PTEN C2 domain ability to recruit or to position the active site to the membrane, we artificially membrane-targeted PTEN by a myristoylation signal. This modification increased wild-type PTEN growth inhibition but did not rescue a C2 mutant defective in lipid-binding, suggesting a model in which PTEN C2 domain positions the active site productively with respect to the membrane-bound phosphoinositide substrate. When tumor-derived mutations in the loops that connect the C2 β-strands were analyzed, we found that these generally destabilized the protein but had variable effects on the phosphatase activity and tumor growth. The magnitude of these effects was dependent on the presence of the COOH-terminal PEST sequences and on the cell type where the mutant proteins were expressed, suggesting the existence of fluctuating structural defects of the mutant protein. One of the C2 loop mutants induced a total loss of PTEN tumor-suppressor function, most likely by affecting both the membrane binding and the protein stability. These data support a double role for PTEN C2 domain in protein stability and in productive orientation of the catalytic site.

INTRODUCTION

The *PTEN* tumor suppressor gene located on human chromosome 10q23.3 is frequently deleted in glioblastoma, endometrial cancer, prostate, and small-cell lung cancer (1–4). Germ-line mutations of *PTEN* are the cause of Cowden's disease, an autosomal-dominant hamartoma syndrome with increased risk for the development of tumors in a variety of tissues (5).

The *PTEN* gene encodes a 403-amino acid phosphatase that acts on both polypeptide and PIP_3[3] substrates (6, 7). The generation of PIP_3 by the PI-3 kinase is the key event in activating multiple downstream pathways (reviewed in Ref. 8). These include the activation of PKB/Akt promoting survival signals, of p70S6-kinase involved in G_1 cell cycle transition, of Tec-family nonreceptor tyrosine kinases inducing the activation of PLC-γ and calcium release from intracellular stores, and of the small G protein Rac mediating cytoskeletal rearrangements. The inactivation of both *PTEN* alleles in glioblastoma causes constitutive activation of the PKB/Akt downstream effector of the PI-3 kinase (9). The degree of PKB/Akt activation correlates to the transformed phenotype in glioblastoma cells (10) and use of a constitutively active PKB/Akt form in PTEN-suppressed breast cancer cells rescues these cells from apoptosis (11), suggesting that PTEN exerts its tumor-suppressor function by negatively regulating the PI-3 kinase-PKB signaling pathway.

The biological effects of PTEN on tumor cells are different depending on the cell type. In glioblastoma cells, PTEN induces cell-cycle arrest in G_1 (12, 13), whereas in LNCaP prostate cancer cells, it induces apoptosis (14); both of these effects depend on the phosphatase activity of the protein.

In addition to the amino-terminal phosphatase domain, the PTEN crystal structure revealed the presence of a C2 COOH-terminal domain (15). C2 domains bind phospholipid membranes (16), and we have shown that the PTEN C2 domain that is formed by two antiparallel β-sheets associates with lipids by a Ca^{2+}-independent novel mechanism involving stretches of basic residues situated in two regions (CBR3 and Cα2) connecting the β-strands (15). The mutagenesis of clusters of these residues impaired the tumor-suppressor function of PTEN supporting the lipid-binding role of the C2 domain (15). Because the disruption of the lipid-binding function would require multiple hits, there are no reported combined mutations of these residues in tumors. However, a subset of the tumor-derived mutations are situated in the loops interconnecting the β strands of the C2 domain, and in this study we present a detailed analysis of the functional and biological effects of these mutations. We also propose a role for the PTEN C2 domain in the productive positioning of the phosphatase active site with respect to the phosphoinositide substrate.

MATERIALS AND METHODS

Plasmid Construction. PTEN wild-type and the mutants PTEN-T319Δ, PTEN-L345Q, PTEN-351, PTEN-H93A, M-Cα2, and M-CBR3 in the pCX retroviral vector were described previously (10, 15). In M-Cα2, the amino acid sequence 327-KANKDKANR-335 is replaced with 327-AAGADAANA-335 and in M-CBR3 the sequence 263-KMLKKDK-269 is changed to 263-AAGAADA-269. The myristoylation signal of v-*Src*, MGSSKSKPKDPSQR (17), was added by PCR at the NH_2 terminus of wild-type PTEN and of M-CBR3, resulting in the Myr-PTEN and Myr-CBR3 constructs, respectively. The mutants PTEN-Y68H, PTEN-L186V, PTEN-P204S, PTEN-S227F, PTEN-G251C, PTEN-K289E, and PTEN-D331G were obtained by PCR with mutated primers and cloned with an *N*-terminal *Myc* tag in the pCX vector. The L345Q mutation was introduced by PCR in the mutant M-CBR3 cDNA, resulting in the double mutant CBR3-L345Q. In the mutants PTEN-T319Δ, *PTEN*-G251C, and PTEN-D331G, the COOH-terminal PEST sequences were deleted by inserting a stop codon after the amino acid 351. This truncation resulted in the deletion constructs T319Δ-351, G251C-351, and D331G-351. For transient transfections, the point mutants PTEN-P204S and PTEN-G251C were expressed in pFLAG-CMV-2 vector (Kodak).

Cell Growth Assays. U-87 MG glioblastoma cell line (American Type Culture Collection), COS-7, and Bosc23 cells were grown in DMEM growth medium with 10% FCS. The LNCaP prostate cancer cell line (gift of Makoto Sumitomo) was maintained in RPMI 1640 supplemented with 10% FCS.

The protocols for transfection, retroviral infection with amphotropic defective retroviruses, stable expression of PTEN proteins, cell proliferation, and soft agar colony assays were described in detail elsewhere (10).

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[3] The abbreviations used are: PIP_3, phosphatidylinositol 3,4,5-trisphosphate; PI-3 kinase, phosphatidylinositol-3-OH kinase; PLC, phospholipase C; PKB/Akt, protein kinase B; PEST sequences, sequences rich in proline, glutamic acid, serine, and threonine; PDZ domains, domains present in PSD-95/Dlg/Zo-1 proteins.

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Protein Analysis. Cell lysis, immunoprecipitation, immunoblotting, and pulse-chase assays were performed as described previously (10, 18). Antibodies were obtained as follows: anti-PTEN A2B1 monoclonal antibody from Chemicon; M2 anti-FLAG from Kodak; anti-*Myc* from Invitrogen; anti-*Myc* 9E10 from Calbiochem; and anti-phosphoS473 Akt/PKB and anti-Akt/PKB from New England Biolabs.

Phosphatase Assay. The phosphatase reactions with water-soluble diC_8-PIP_3 (Echelon) were performed with immunoprecipitated proteins from cells as described (10).

Immunofluorescence. U87-MG cells (2×10^4) were plated on poly-D-lysine-coated glass coverslips (Becton Dickinson) in growth medium and left to adhere overnight. The cells were fixed in 3% formaldehyde for 30 min, permeabilized with 0.2% Triton X-100 in PBS for 5 min, blocked with 50 mM glycine in PBS for 10 min, incubated with the anti-PTEN antibody for 1 h and with the FITC-conjugated anti-mouse secondary antibody (Jackson ImmunoResearch Laboratories) for 1 h. All of the incubations were performed at room temperature. The cells were washed twice in PBS between the incubations and four times for 5 min in PBS containing 2 mg/ml gelatin after the incubations with the antibodies. The cells were mounted in Vectashield (Vector Laboratories) and pictures were taken with a Nikon immunofluorescence microscope coupled to a charge-coupled device color camera.

RESULTS

Recruitment *versus* Productive Positioning to the Membrane by PTEN C2 Domain. We have shown previously that the COOH-terminal region of PTEN folds into a lipid-binding C2 domain (15). In the crystal structure, there are three loops exposed on the membrane-facing surface of the C2 domain: (*a*) CBR3 between the fifth and sixth β-strands; (*b*) Cα2 between the seventh and eighth β-strands; and (*c*) Cβ1/2 between the first and second β-strands (Fig. 1). We have shown previously that the mutations of the basic and hydrophobic residues at the tip of the CBR3 and Cα2 loops affect the lipid membrane-binding and the tumor-suppressor function of PTEN (15).

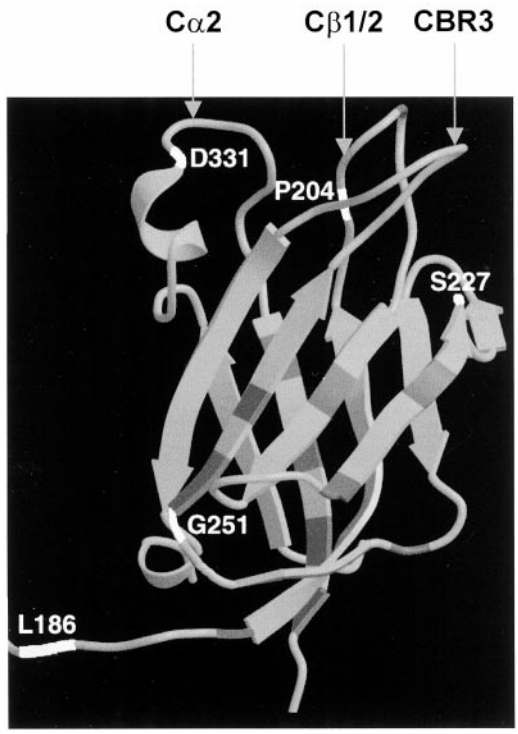

Fig. 1. Three-dimensional structure of PTEN C2 domain showing the localization of tumor-derived missense mutations. The mutations in the β-strands or in the loops connecting the β-strands of the C2 domain are shown in the *darker shade* or in *white* (for those that were engineered in the PTEN cDNA). The loops situated on the same face as the phosphatase active site are indicated with *arrows*.

To investigate the function of the lipid-binding C2 loops that could serve to recruit the phosphatase domain to the membranes and also to position and orient it with respect to the substrate, we expressed in U87-MG cells a myristylated form of the membrane binding-deficient mutant M-CBR3, Myr-CBR3. We compared the tumor suppressor ability of Myr-CBR3 with those of M-CBR3 and of the myristylated wild-type PTEN, Myr-PTEN (Fig. 2). The expression of the myristylated forms of both wild-type PTEN and M-CBR3 changed the cell morphology and the cellular localization of the protein (Fig. 2*A*). The cells appeared flattened and, in contrast to the nonmyristylated proteins that were localized mostly in the perinuclear cytoplasm, the myristylated proteins were distributed all over the cell, including the fine processes extending from the cell body. The perinuclear granular staining of the myristylated forms may be derived from Golgi and/or endosomal localization. The myristoylation signal added to wild-type PTEN induced a drastic proliferation arrest, increasing PTEN tumor-suppressor function probably by more efficient membrane targeting (Fig. 2*B*). In contrast, its addition in Myr-CBR3 altered moderately the growth effect of M-CBR3 (Fig. 2*B* and *C*). Although Myr-CBR3 suppressed the tumor growth more efficiently than did M-CBR3 (Fig. 2*C*), the full tumor-suppressor ability of wild-type PTEN was not rescued by membrane targeting, suggesting that the CBR3 loop might function to orient productively the active site toward the substrate.

Tumor-derived Mutations in C2 Domain Loops Can Affect Protein Stability and Occasionally the Membrane-Binding Function. We have shown that tumor-derived mutations of residues buried in the C2 β-strands destabilize the protein and inactivate its tumor-suppressor function (10). The loops connecting the β-strands are also a target for missense mutations in tumors, and we engineered some of these mutations in PTEN to analyze the role of the C2 domain (shown in Fig. 1). The mutants PTEN-L186V, PTEN-P204S, PTEN-S227F, PTEN-G251C, PTEN-K289E, and PTEN-D331G, which each contained a change in a different loop connecting C2 β-strands, were expressed stably in U87-MG glioblastoma cells (Fig. 3*A*). They had decreased expression levels compared with wild-type PTEN except for PTEN-K289E, indicating that mutations situated in the loops of the C2 domain affect the protein stability. To ascertain that mutations in the C2 domain loops affect the protein stability by increasing the degradation rate similarly to mutations in the C2 domain β-strands (10), pulse-chase analysis of protein turnover was performed for PTEN-P204S and PTEN-G251C loop mutants (Fig. 3*B*). The rate of degradation, represented by the slope of the curve, was considerably higher for the loop mutants than for wild-type PTEN, showing that protein degradation is the cause for the reduced protein stability of the C2 domain mutants.

Because we observed that mutations within the C2 domain decrease the protein stability, we investigated whether a similar effect is induced by mutations predicted to disrupt the core of the phosphatase domain. The Y68 residue belongs to a β strand that is part of the phosphatase domain hydrophobic core and is frequently mutated in cancer to hystidine (19) or cysteine (20). Most importantly, from all of the investigated mutations, Y68 is also replaced with hystidine in the *PTEN* pseudogene (21, 22). The Y68H mutation reduced PTEN stability similarly to C2 domain core mutations (Fig. 3*C*) and decreased the tumor-suppressor activity of PTEN (not shown), indicating that misfolding of both domains has a destabilizing effect on the entire protein.

In contrast to the effects on the protein stability, the ability to dephosphorylate a water-soluble PIP_3 analogue was preserved for most of the C2 loop mutants (Fig. 3*D*). Only two of the loop mutants, PTEN-P204S and PTEN-G251C, which had also the lowest expression level, completely lost phosphatase activity, indicating profound structural changes of the whole protein.

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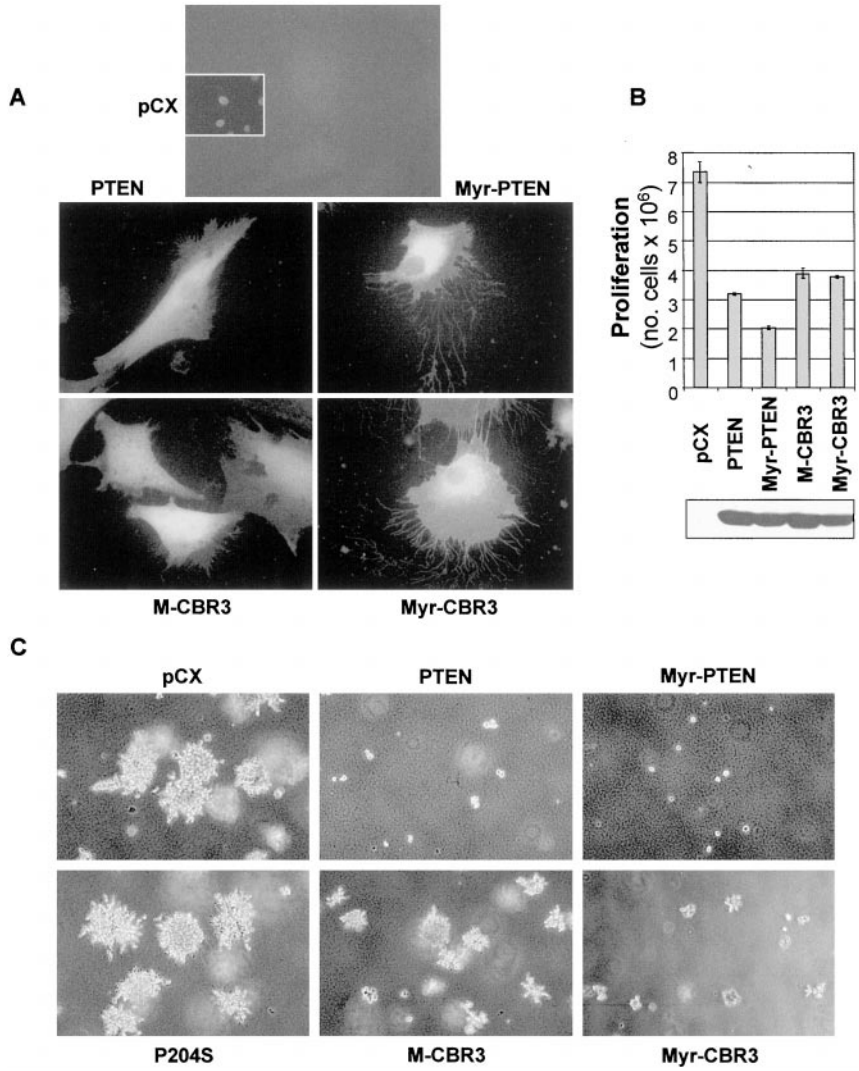

Fig. 2. Functional analysis of PTEN membrane-association. *A*, wild-type PTEN and the lipid-binding deficient M-CBR3 mutant were targeted to the membrane by the addition of a myristoylation signal (*Myr*). Immunofluorescence of control pCX vector-transfected U87-MG cells (*inset* shows DAPI staining of nuclei) or of cells expressing PTEN and the indicated variants showing membrane localization of myristylated proteins. The cells were labeled with anti-PTEN antibody. *B*, the proliferation of U87-MG cells stably expressing PTEN and membrane-targeted mutants represents the number of retrovirus-infected U87-MG cells growing on plate after completion of the drug selection. For the protein expression, proteins (50 μg) from lysates of stably transfected U87-MG (U87) cells were resolved by SDS-PAGE and analyzed by immunoblotting with anti-PTEN antibody. *C*, soft agar colony assay showing the anchorage-independent growth ability of PTEN and membrane-targeted mutants. U87-MG cells stably expressing PTEN proteins were seeded in soft agar under drug selection and allowed to form colonies. Photographs of the colonies were taken after 18 days of growth at $\times$10 magnification.

The loop mutants showing phosphatase activity had a slightly higher proliferation advantage in U87-MG cells relative to PTEN, but their tumor growth in soft agar was rather similar to PTEN (Fig. 4). The two mutants lacking phosphatase activity had impaired tumor-suppressor abilities. PTEN-G251C showed an intermediate tumor growth phenotype, behaving similarly to mutants in β-strands (PTEN-L345Q) or to the lipid-binding deficient mutants (M-Cα2 and M-CBR3; Fig. 4). The PTEN-P204S mutant presented a complete inability to suppress tumor growth (Figs. 2*C* and 4), providing the first example of a total loss of function for a C2-domain point mutant.

To explain the effects of these mutations, we positioned the amino acids in the three-dimensional structure (Fig. 1). We observed that L186 is entirely solvent when exposed, and has no apparent structure-stabilizing role consistent with its reduced destabilizing effect (Fig. 3*A*). S227 is situated on the edge of the β sheet, and because its side chain is involved in stabilizing one of the β-strands, its mutation to phenylalanine would be expected to cause local structural defects. The K289 residue belongs to a loosely structured loop that was not included in the crystallization construct. Although the K289E mutation reversed the charge of the amino acid, the mutant protein had a high expression level, suggesting no alteration of the structure. The D331G mutation significantly reduced the expression level of the protein consistent with its structuring role by stabilizing the turn at the end of the Cα2 helix (Fig. 3*A*). In the case of the G251C mutation, its localization near the interface between the phosphatase and the C2 domain (15) most likely explains the destabilization of the domains and the phosphatase activity loss (Fig. 3).

P204 is positioned in the β1/2 loop that packs with both CBR3 and Cα2 loops. Because its side chain reaches into the hydrophobic core, its replacement with a polar one in the P204S mutant would disrupt the packing of the CBR3 and Cα2 loops and possibly more of the structure. On the basis of these putative effects on the structure, we attempted to explain the complete loss of tumor suppression for PTEN-P204S as a consequence of two concurrent defects: one of protein folding coupled to one of membrane binding. If this hypothesis were correct, these defects which, taken apart, affect only partially the tumor-suppressor function, when combined, would sum up and entirely abrogate the tumor suppression. To demonstrate this, we introduced the L345Q destabilizing mutation into the membrane binding-deficient mutant M-CBR3 and analyzed the tumor growth of the double mutant CBR3-L345Q. The presence of both defects totally inactivated the tumor-suppression function of PTEN similarly to PTEN-P204S (Fig. 4).

Partial Recovery of Tumor Suppression for C2 Domain P204S Mutant But Not for Phosphatase Domain Mutant in LNCaP Cells. The C2 domain mutant P204S differed from the other C2 domain point mutants by presenting a total loss of tumor suppression in the U87-MG glioblastoma cells similarly to mutants in the phosphatase domain (Fig. 4). Surprisingly, when we expressed this mutant in

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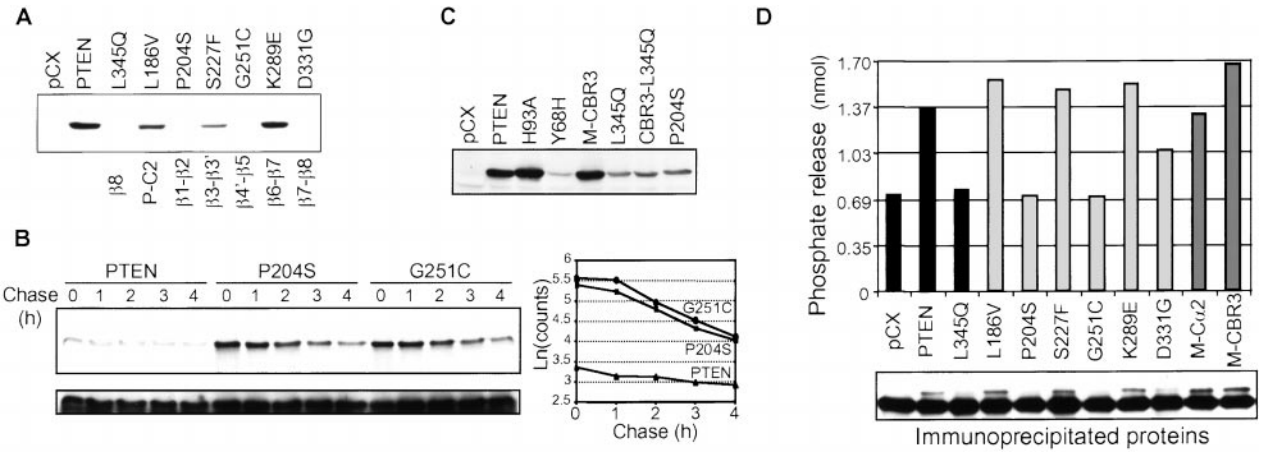

Fig. 3. *A*, expression level of the C2 loop mutants. Proteins (50 μg) from lysates of stably transfected U87-MG (U87) cells expressing PTEN or the indicated mutants (names of mutants are abbreviated by the *mutation*) were resolved by SDS-PAGE and analyzed by immunoblotting with anti-PTEN antibody. The loop between two β-strands corresponding to each mutation is indicated *under the panel*. *B*, pulse-chase assay showing rapid degradation of C2-domain loop mutants. COS-7 cells transiently expressing FLAG-tagged PTEN and mutants were pulse-labeled for 30 min and chased for the time periods indicated. Proteins were immunoprecipitated from the lysates with M2 antibody, and the filter was exposed first (*upper panel*) and then immunoblotted with the M2 antibody (*lower panel*) to show protein levels. The phosphorimager densitometric analysis is shown in the graph. *C*, decreased PTEN stability by mutations affecting the hydrophobic core of the phosphatase or C2 domains. The immunoblotting was performed with anti-*Myc* antibody. *D*, phosphatase activity of the PTEN C2 domain mutants. Phosphatase assay using water-soluble PIP_3 and proteins immunoprecipitated with the anti-*Myc* 9E10 antibody from lysates of U87-MG cells expressing *Myc*-tagged PTEN and mutants. The *lower panel* shows the amount of the immunoprecipitated proteins that, at the end of the phosphatase reaction, were resolved on SDS-PAGE and analyzed by immunoblotting with anti-*Myc* antibody. The amount of free phosphate released in the reaction was measured in a colorimetric assay and compared with a standard curve. This experiment was repeated at least three times with similar results.

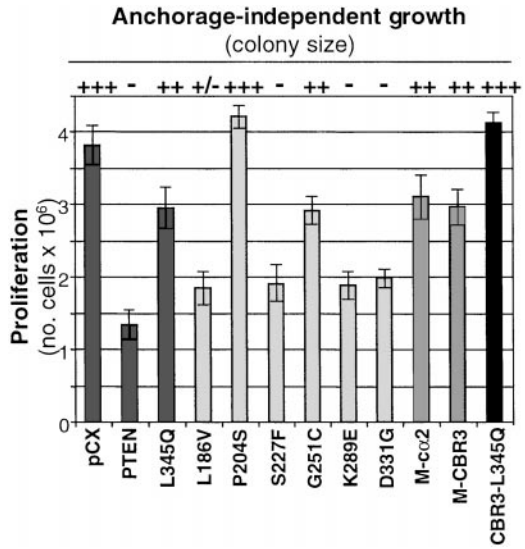

Fig. 4. Variable effects of the C2 domain loop mutants on the tumor growth of U87-MG cells. The ability to form colonies in soft agar of retrovirus-infected U87-MG cells stably expressing PTEN or the indicated mutants was evaluated by the size of the colonies and scaled from no growth (−) to maximum growth as in the vector-transfected cells (+++). The proliferation represents the number of retrovirus-infected U87-MG cells growing on the plate after completion of the drug selection.

LNCaP prostate cancer cells, it presented partial tumor suppressor ability, inducing cell death and a decrease in proliferation (Fig. 5). These results suggested that the P204S mutation induced more or less severe structural defects depending on the cellular context. The PTEN-H93A mutant in the phosphatase domain maintained its loss of tumor suppression in LNCaP cells almost to the level of the vector control consistent with the proposed role of H93A as a catalytic site residue.

Deletion of the COOH-terminal PEST Sequences Increases Growth Suppression in C2 Domain Mutants. The last 50-residue stretch of PTEN contains two PEST sequences, and we have shown that their deletion in the mutant PTEN-351 does not stabilize the protein (10) as would have been expected from the role of PEST sequences in targeting proteins for degradation (23). Because of its high susceptibility to proteolysis, indicating a loose structure, this region was not included in the construct used for crystallization, and its position relative to the rest of the protein structure is not known. Because the C2 mutants have lower stability than the wild-type protein, we investigated the possibility of their stabilization by deleting the PEST sequences. This deletion in the PTEN-T319Δ β7-strand mutant as well as in the PTEN-G251C and PTEN-D331G loop mutants had no effect on their stability (Fig. 6*A*).

The deletion of the PEST sequences in wild-type PTEN reduced slightly its tumor-suppressor activity. In contrast, the PEST-deletion mutants T319Δ-351, G251C-351, and D331G-351 presented increased tumor suppression compared with their nondeleted counterparts in both proliferation and soft agar assays (Fig. 6*A*), suggesting a structural role for the PEST region.

We have shown that the level of PKB/Akt activation correlates to the tumor-suppressor phenotype of PTEN mutants in U87-MG cells (10). For the PEST-deletion mutants, we also found correlation between PKB/Akt activation and tumor-growth (Fig. 6*B*). Depending on the integrity of the C2 domain, the deletion of the PEST sequences from wild-type PTEN or from C2-domain mutants had opposite effects on the activation of PKB/Akt. For the lipid-binding-deficient mutants M-Cα2 and M-CBR3, which had phosphatase activity toward water-soluble PIP_3 but lost the ability to suppress tumor growth, the activation of PKB/Akt correlated to the growth phenotype (Fig. 6*B*), indicating decreased *in vivo* lipid phosphatase activity.

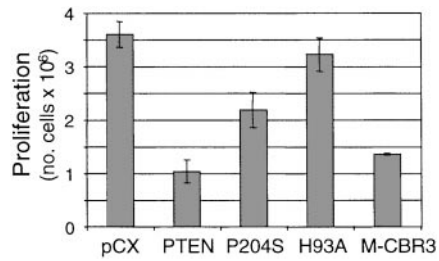

Fig. 5. Tumor suppression by C2 domain mutants in the prostate cancer LNCaP cell line. LNCaP cells were infected with retroviruses carrying wild-type PTEN and mutants, and the proliferation of cells was assayed as in Fig. 4.

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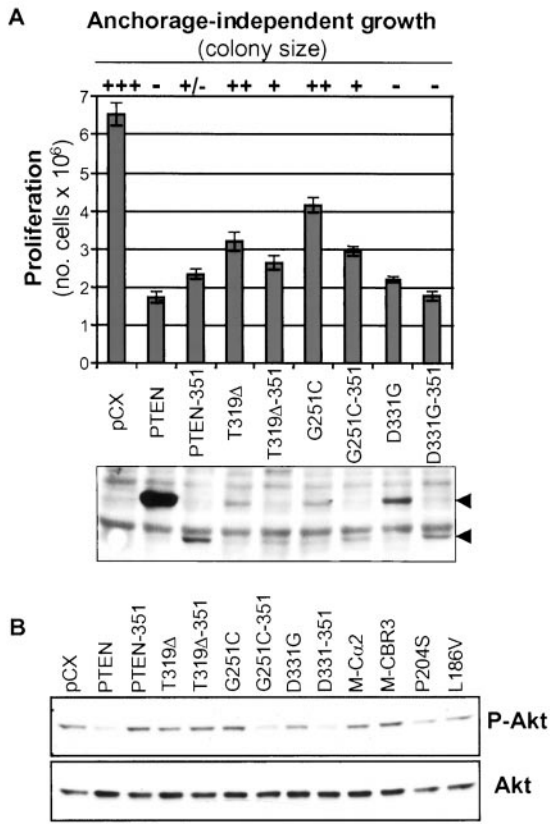

Fig. 6. *A*, tumor growth and expression levels for C2 domain point mutants with or without the deletion of the COOH-terminal PEST sequences. The lysates were processed identically as in Fig. 3, and immunoblotting was done with anti-*Myc* antibody recognizing *Myc*-tagged PTEN and mutants. *Arrowheads* indicate the position of full-length and truncated variants of PTEN. These experiments were repeated at least three times with similar results. *B*, Akt/PKB phosphorylation in U87-MG cells stably expressing PTEN and the indicated mutants. Proteins (50 μg) from total lysates were analyzed by immunoblotting with anti-PKB/Akt antibodies recognizing total levels of PKB/Akt or only the phosphorylated form (P-Akt).

DISCUSSION

In this study we analyzed structural elements of the PTEN C2 domain required for the tumor-suppressor function of the protein. This function relies on the ability of PTEN to dephosphorylate PIP_3 (12, 24). The mechanism by which PTEN accesses its phospholipid substrate depends on its C2 domain. Two of the three C2-domain loops exposed on the same face of the protein as the active site contain basic residues that are important for *in vitro* membrane binding (15). Unlike the majority of C2 domains, which, through acidic residues, bind Ca^{2+} that bridges them to the membrane (25, 26), the existence of basic residues predicts a direct contact of PTEN with the membrane. To discriminate whether this contact serves for simple membrane recruitment or for the precise positioning and orientation of the phosphatase domain, we tagged with a membrane-targeting motif a lipid-binding defective PTEN variant that was also deficient in its tumor-suppressor activity. Because the artificial membrane-targeting did not fully rescue the tumor-suppressor function of this mutant, we inferred that the role of the C2 domain is to productively orient the phosphatase site onto the lipid substrates. The relation between the C2 and the catalytic domain of PTEN connected through a rigid interface (15) is very similar to that observed between the corresponding domains of PLCδ (25). For membrane binding, in addition to the C2 domain, PLCδ also has a PH domain that is flexibly connected to the rest of the enzyme. The mechanism that was proposed for PLCδ membrane attachment involved membrane tethering by the PH domain and then protein fixation to the membrane by the C2 domain (25). In the case of PTEN, a similar mechanism could operate, and we showed here that the C2 domain does more than simple membrane recruitment and is likely involved in the productive positioning of the active site in a manner analogous to that proposed for the C2 domain of PLCδ. A role for membrane tethering could be postulated for the PDZ-binding motif, but additional evidence is needed to ascertain the cooperation between the C2 domain and the PDZ motif in membrane association.

The tumor-suppressor function of PTEN is impaired in tumors by mutations occurring in the phosphatase or in the C2 domains. To assess the importance of the membrane-binding function of PTEN C2 domain for tumor evolution we analyzed the biological effect of C2 tumor-derived missense mutations. These mutations fall in two categories: (*a*) mutations disrupting the hydrophobic β-sheet core; and (*b*) mutations at the level of the loops connecting the β-strands of the C2 domain. We have previously shown that the disruption of the C2 core by mutations in the β-strands impairs the tumor-suppressor function by decreasing the phosphatase activity of the protein (10). From a structural perspective, the mutations in the hydrophobic core of the C2 domain represent significant changes that could lead to the destabilization of the folded state of the C2 domain with consequences on the entire protein. On the other hand, most of the mutations in the loops are not expected to cause large-scale destabilization of the folded state of PTEN. Rather, it is likely that these mutations will create local structural defects involving small shifts in local elements or the local loss of structure. We introduced tumor-derived mutations in almost every loop of the C2 domain and found that they variably affect the tumor-suppressor function. Almost all of the changes also destabilized the protein to different extents, indicating an alteration of the folding. However, four of six examined loop mutations (L186V, S227F, K289E, and D331G) did not significantly change the PTEN growth-suppression in the U87-MG glioblastoma cell line. Only two of the loop mutations, G251C and P204S, impaired PTEN tumor-suppressor function.

The loss of the tumor-suppression for PTEN-G251C was partial and comparable with that induced by mutations affecting the C2 hydrophobic core. These mutants with intermediate tumor-growth phenotype lacked *in vitro* phosphatase activity toward PIP_3 but also toward PI 3,4-bisphosphate and PI 3,5-bisphosphate (not shown). Because they appeared to suppress slightly the activation of PKB/Akt, it is likely that these mutants may have a residual phosphatase activity within the cells that cannot be detected *in vitro*. Unlike the other C2 mutants, PTEN-P204S mutant had a total loss of tumor suppression. On the basis of structural predictions, we assumed that both phosphatase activity impairment and membrane-binding loss are the cause. By concomitantly introducing distinct mutations ablating each of these functions, we observed that they confer an additive effect on tumor suppression. This double inactivation may also be the cause of the total loss of tumor suppression observed for C2 truncation mutations (10), which are the most frequent genetic events affecting the C2 domain.

The evidence that a mutation in the C2 domain does not determine a fixed structural defect came from the P204S mutant that altered differently the cell growth in different cell types. The observed variations in phenotype could be caused by interactions between PTEN and cell-type specific factors resulting in structural changes affecting its stability and phosphatase activity. Very recently, two groups cloned two PDZ domain-containing proteins that, upon binding to PTEN, stabilized the protein and modulated its ability to inactivate PKB/Akt (27, 28). It will be interesting to investigate whether the interaction between PTEN and PDZ domain-containing proteins is the one responsible for the variations in phenotype that we observed.

The differences in tumor suppression with the same mutant protein

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(PTEN-P204S) in different cell lines suggested a possible explanation for the lack of a significant tumor suppression loss for four of six C2-loop mutants. With the exception of PTEN-P204S, which was detected in glioblastoma (29), the other mutations were detected in endometrial carcinoma, D331G and S227F (30), breast carcinoma, L186V (20), lung cancer, G251C (31, 32), or Cowden's disease, K289E (33). Because all these mutants that developed in heterogeneous tissues were tested in a single cell line, it is reasonable to conceive that their real tumor-suppressor ability might be slightly different from the one we detected.

Another indication about the possibility to modulate the tumor-suppressor function for PTEN C2 domain mutants came from the analysis of the deletion of the last 53 amino acids of PTEN. This region contains two PEST sequences and a terminal three-residue PDZ-binding motif. The deletion of the PDZ-binding motif alone had no effect on the tumor growth in U87-MG cells, whereas the deletion of the whole region decreased slightly the tumor-suppressor function (10). Surprisingly, the same deletion in three C2-domain mutants increased the tumor-suppressor function without increasing the expression level of the proteins. The mechanism appears to be related to an increase in the *in vivo* phosphatase activity of these mutants, as shown by the decrease in the PKB/Akt activation. While this paper was under review, a report showed that constitutive phosphorylation of three residues situated in the second PEST sequence decreases PTEN tumor-suppressor function (34). Removal of these residues in the C2-domain mutants most likely accounts for the increased tumor suppression. It seems that the alleviation of the structural defects in PTEN C2-domain mutants by modifying the PEST tail might be a possible conformational intervention similar to the one undertaken for the p53 tumor suppressor (35).

Our study showed that the PTEN C2 domain functions to tightly regulate the catalytic activity of the protein. This regulation depends upon structural constraints that are altered by tumor-derived mutations and modified in different cellular contexts.

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Stabilization and Productive Positioning Roles of the C2 Domain of PTEN Tumor Suppressor

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