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FAST TRACKS

PTEN Enters the Nucleus by Diffusion

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Abstract Despite much evidence for phosphatidylinositol phosphate (PIP)-triggered signaling pathways in the nucleus, there is little understanding of how the levels and activities of these proteins are regulated. As a first step to elucidating this problem, we determined whether phosphatase and tensin homolog deleted on chromosome 10 (PTEN) enters the nucleus by passive diffusion or active transport. We expressed various PTEN fusion proteins in tsBN2, HeLa, LNCaP, and U87MG cells and determined that the largest PTEN fusion proteins showed little or no nuclear localization. Because diffusion through nuclear pores is limited to proteins of 60,000 Da or less, this suggests that nuclear translocation of PTEN occurs via diffusion. We examined PTEN mutants, seeking to identify a nuclear localization signal (NLS) for PTEN. Mutation of K13 and R14 decreased nuclear localization, but these amino acids do not appear to be part of an NLS. We used fluorescence recovery after photobleaching (FRAP) to demonstrate that GFP-PTEN can passively pass through nuclear pores. Diffusion in the cytoplasm is retarded for the PTEN mutants that show reduced nuclear localization. We conclude that PTEN enters the nucleus by diffusion. In addition, sequestration of PTEN in the cytoplasm likely limits PTEN nuclear translocation. J. Cell. Biochem. 96: 221–234, 2005.

Key words: phosphatase; phosphatidylinositol; nucleus; signal transduction; FRAP

Phosphatase and tensin homolog deleted on chromosome 10 (PTEN) was originally cloned as a tumor suppressor for gliomas [Li and Sun, 1997; Li et al., 1997; Steck et al., 1997]. We now know that PTEN is deleted or inactivated in many tumor types, including endometrial, melanoma, prostate, and breast, identifying PTEN as an important tumor suppressor [Simpson and Parsons, 2001]. The PTEN protein is a phosphatidylinositol phosphate (PIP) phosphatase specific for the 3-position of the inositol ring [Maehama and Dixon, 1998]. Although PTEN can dephosphorylate PI(3)P, PI(3,4)P_2, or PI(3,4,5)P_3, it is likely that PI(3,4,5)$_3$ is the most important substrate in vivo. The balance between PTEN and phosphoinositide 3-kinase (PI3K) determines PI(3,4,5)P_3 levels at the plasma membrane [Iijima and Devreotes, 2002], which in turn, regulates numerous cell processes. PTEN lowers PI(3,4,5)P_3 levels, thereby, decreasing Akt kinase activity and inducing apoptosis [Furnari et al., 1997; Myers et al., 1998]. Loss of PTEN increases cell motility and invasiveness [Liliental et al., 2000; Li et al., 2002; Raftopoulou et al., 2004]. Loss of PTEN also activates the Tor pathway, increasing protein biosynthesis and cell size [Backman et al., 2002; Bjornsti and Houghton, 2004].

In addition to its well-established role at the plasma membrane, PTEN may function in the nucleus. Although PTEN is cytoplasmic in

Abbreviations used: PTEN, phosphatase and tensin homolog deleted on chromosome 10; NLS, nuclear localization signal; PI(3,4,5)P_3, phosphatidylinositol (3,4,5)-trisphosphate; PH, plekstrin homology; PIP, phosphatidylinositol phosphate; PHD, plant homeodomain; PK, pyruvate kinase; FRAP, fluorescence recovery after photobleaching; NES, nuclear export signal.

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some cell types [Li and Sun, 1997; Gu et al., 1998], we and others have found that PTEN is both cytoplasmic and nuclear [Sano et al., 1999; Lachyankar et al., 2000; Perren et al., 2000; Zhang and Steinberg, 2000; Marshall et al., 2001; Tsao et al., 2003; Shoman et al., 2005]. In MCF-7 cells, nuclear PTEN is more prominent in the G_0–G_1 segment of the cell cycle [Ginn-Pease and Eng, 2003], and introduction of PTEN into isolated nuclei enhances apoptosis-induced DNA fragmentation [Ahn et al., 2004]. Nuclear PIPs, including $PI(4,5)P_2$ and $PI(3,4,5)P_3$, have been detected by immunohistochemical techniques and chemical analyses of nuclear extracts [Mazzotti et al., 1995; Caramelli et al., 1996; Boronenkov et al., 1998; Lu et al., 1998] and may regulate plekstrin homology (PH) domain proteins in the nucleus. For example, Akt is activated at the plasma membrane and then translocates to the nucleus [Meier et al., 1997]. A PIP-binding protein abundant in brain is targeted to the nucleus [Tanaka et al., 1999], and Bruton's tyrosine kinase shuttles between cytoplasm and nucleus [Mohamed et al., 2000]. In addition, nuclear PIPs may regulate proteins that do not bear PH domains. The candidate tumor suppressor, ING2, is a nuclear protein that binds PIPs through a plant homeodomain (PHD) finger [Gozani et al., 2003], and $PI(4,5)P_2$ activates the BAF chromatin remodeling complex [Zhao et al., 1998]. Hence, it is clear that PIP-regulated proteins are present in the nucleus, but the biological consequences are still being elucidated.

Some tumor cells have cytoplasmic, but not nuclear, PTEN, and loss of nuclear PTEN correlated with increased tumorigenicity [Gimm et al., 2000; Perren et al., 2000; Tachibana et al., 2002; Whiteman et al., 2002; Zhou et al., 2002]. The effects of nuclear PTEN on tumor progression may be partly due to interactions with the p53 tumor suppressor. PTEN and p53 form a nuclear complex that prevents p53 degradation and increases p53 transcriptional activity [Freeman et al., 2003; Su et al., 2003]. Despite the potential importance of nuclear PTEN in tumor development, the mechanism of PTEN nuclear localization is not known.

To test whether PTEN enters the nucleus by passive diffusion through the nuclear pores or nuclear localization signal (NLS)-mediated transport, we carried out four series of experiments. First, we used the tsBN2 cell line, which shows temperature-dependent Ran-mediated nuclear transport. At the nonpermissive temperature, the distribution of GFP-PTEN was normal, even though GFP-PTEN linked to an NLS showed decreased nuclear localization. Second, we prepared various PTEN fusion proteins and determined whether the molecular weight of these fusion proteins affects nuclear translocation. This is a critical experiment because diffusion through nuclear pores is limited to proteins of approximately 60,000 Da or less [Boulikas, 1993]. We found that the largest PTEN fusion proteins showed decreased nuclear localization. Third, we mutated positively charged amino acids, seeking to identify an NLS or other sequences that regulate nuclear localization for PTEN. Although mutation of K13 and R14 decreased nuclear localization of PTEN, these amino acids do not appear to be part of a classical NLS. Fourth, we used fluorescence recovery after photobleaching (FRAP) to demonstrate that GFP-PTEN translocates to the nucleus. In addition, diffusion in the cytoplasm is retarded for the PTEN mutants that show reduced nuclear localization. Based on these four sets of experiments, we conclude that PTEN enters the nucleus by diffusion. In addition, sequestration of PTEN in the cytoplasm likely influences the nuclear/cytoplasmic distribution of PTEN.

MATERIALS AND METHODS

Cell Culture

U87MG cells (human glioblastoma), HeLa S3 cells (human cervical carcinoma) and tsBN2 cells (hamster kidney) were maintained in DMEM supplemented with 10% fetal bovine serum (FBS). LNCaP cells (human prostate carcinoma) were cultured in RPMI 1640 medium supplemented with 10% FBS. The tsBN2 cells have a temperature-sensitive defect in Ran-dependent nuclear transport [Tachibana et al., 1994]. At 33.5°C (permissive), nuclear transport is normal, but at 39.5°C (nonpermissive), nuclear transport is greatly reduced. These cells were routinely maintained at 33.5°C. For experiments at the nonpermissive temperature, they were placed at 39.5°C for 8 h.

Preparation of Plasmids

The coding sequence for human PTEN was cloned into the BamHI and EcoRI sites of the pcDNA3 plasmid (Invitrogen, Carlsbad, CA).

An enhanced GFP sequence [Anderson et al., 1996] was inserted at the 5′ end, using the HindIII and BamHI restriction enzyme sites. Mutant PTEN plasmids were prepared using the Quick-Change site-directed mutagenesis kit from Stratagene, following the manufacturer's instructions (Stratagene, La Jolla, CA). In addition, an NLS-PTEN construct was prepared with a strong NLS attached to the N-terminus of GFP-PTEN. This sequence included a tandem repeat of SPKKKRKV, which is a strong NLS based on the SV40 large T antigen, followed by AAA as a linker to the N-terminus of GFP-PTEN. The NLS-PTEN construct was then mutated to create the NLS-C124S construct. PTEN proteins with the C124S mutation are phosphatase-inactive. All plasmids were confirmed by automated DNA sequencing.

The pcDNA3 GFP-GFP and pcDNA3 GFP-GFP-PTEN plasmids were prepared by insertion of the GFP sequence into the HindIII sites of the pcDNA3 GFP and pcDNA3 GFP-PTEN plasmids.

The chicken pyruvate kinase (PK)-PTEN expression vector was prepared by ligating the PTEN sequence into the KpnI and NotI sites of pcDNA3-myc-PK plasmid [Siomi and Dreyfuss, 1995]. We also introduced stop codons into PK-PTEN with the Quick-Change kit (Stratagene), creating constructs for expression of PK fused to the first 20 or 173 PTEN amino acids.

Microscopic Analysis of PTEN Subcellular Distribution

To examine the distribution of endogenous PTEN, HeLa cells were plated on glass coverslips and on the following day, the cells were fixed at 4°C for 30 min with 4% formaldehyde (Ted Pella, Inc.) in cytoskeletal buffer (300 mM sucrose, 100 mM NaCl, 3 mM $MgCl_2$, 1 mM EGTA, 10 mM PIPES, pH 6.8). The cells were permeabilized at 4°C for 5 min with 0.5% Triton X-100 in cytoskeletal buffer and blocked at 4°C for 30 min with 0.1% (w/v) bovine serum albumin, 0.05% (v/v) Tween-20, 0.2% (w/v) glycine, 150 mM NaCl, 3 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris, pH 7.7. The cells were incubated at 4°C for 1 h with 0.5 μg/ml of 6H2.1 anti-PTEN mouse monoclonal antibody (Cascade Immunology Corp., Springfield, OR) in blocking buffer. After washing, the cells were incubated with 1:2,000 goat anti-mouse Alexa-488 secondary antibody (Molecular Probes, Eugene, OR).

For transient transfections, cells were plated on glass coverslips in 24-well plastic plates, and on the following day, the cells were subjected to transfection. For HeLa, U87MG and tsBN2 cells, 94 μl DMEM, 6 μl Fugene 6 (Roche, Indianapolis, IN), and 2 μg of plasmid were mixed, and 10 μl was added to each well. LNCaP cells were treated with 0.4 μg of plasmid per coverslip and Lipofectamine Plus (Invitrogen) according to the manufacturer's instructions. After 24 h, the cells were washed with PBS and fixed with methanol for 20 min at −20°C and then mounted in Vectashield (Vector Laboratories). The cells were examined either with a Leica SP1 confocal microscope and a Plan Apo 100× objective (1.4 n.a.) or a Zeiss Axioskop microscope, Apochromat 100× objective (1.4 n.a.), AxioCam digital camera and OpenLab Software (Improvision, Lexington, MA). Average intensities of nuclear and cytoplasmic fluorescence were calculated using OpenLab software. For each cell, the intensities were measured for three z-axis sections (thickness about 0.5 μm) at the maximum radius of the nucleus. We used average intensities rather than total fluorescence for these calculations, because integrated intensities would be greater for sections with larger areas. By this method, the nuclear/cytoplasmic ratio was quite reproducible. Montages of micrographs were prepared using Adobe Photoshop.

The integrity of GFP-PTEN fusion proteins was tested by Western blotting with anti-GFP mouse monoclonal antibody (1:1,000, Covance).

HeLa cells were transfected with the PK-PTEN plasmids and after 24 h, the cells were washed, fixed with methanol, and blocked with 5% horse serum in Tris-buffered saline. Cells were incubated for 1 h with anti-myc-FITC-antibody (1:500, Invitrogen). The cells were washed three times and mounted with Vectashield (Vector Laboratories, Burlingame, CA).

PTEN Phosphatase Assay

U87MG cells in 60-mm cell culture dishes were subjected to transfection with 1.0 μg pcDNA3 GFP-PTEN (or pcDNA3 GFP as a control) plasmid, 0.4 μg HA-Akt plasmid (HA, hemagglutinin epitope), 0.4 μg PI3K plasmid with a CAAX sequence to induce prenylation and 0.2 μg pcDNA3 GFP plasmid per dish. After 48 h, the cells were lysed with 0.5 ml of buffer per dish (1% NP-40, 10% (v/v) glycerol, 100 mM NaCl, 1 mM EDTA, 2 μl/ml PIC protease

inhibitor mix (Sigma, St. Louis, MO), 2 mg/ml β-glycerophosphate, 2 mg/ml sodium fluoride, 50 mM Tris, pH 7.5). Cell debris was eliminated by centrifugation (17,800 × g) for 10 min at 4°C. To prepare immunoprecipitates, we incubated 1 μg of anti-HA antibody (monoclonal HA.11 from Covance, Inc., Princeton, NJ) with 450 μl of cell extract for 1 h at 4°C. The antibody complexes were collected with Protein G Sepharose beads (Sigma) and analyzed by Western blotting with rabbit anti-phospho-Akt antibody (Cell Signaling, Beverly, MA) and anti-HA antibody.

In vitro activity of GST-PTEN proteins was assessed as described [Maehama et al., 2000; Campbell et al., 2003]. In brief, release of free phosphate from 45 μM PI(3,4,5)P_3 was detected using BIOMOL GREEN (BIOMOL, Plymouth Meeting, MA).

FRAP Assays

Twenty-four hours after transfection, cells were transferred to and maintained in a 37°C FCS2 live cell chamber (Bioptechs, Butler, PA) mounted on a Leica SP1 confocal microscope with an objective heater. Using low laser intensity (5% of maximum), two images were taken. After the bleach, 20–40 images were recorded at intervals of 1.7 or 20 s, depending on the rate of recovery. To destroy GFP-PTEN fluorescence, the 488 nm laser line parked in a selected area was increased to maximum intensity for 3–10 s. The beam diameter was about 1.5 μm. The fluorescence intensities of the bleached area and the whole cell were measured with Leica Confocal Software (Version 2) at all time points. For analysis, these data were transferred into a Microsoft Excel spreadsheet. The relative fluorescence intensity (I_{rel}) in the previously bleached area was calculated: $I_{rel} = T_0 I_t / T_t I_0$ with T_0 being the total cellular intensity before bleach, T_t the total cellular intensity at time t, I_0 the intensity in the bleached area before bleach, and I_t the intensity in the previously bleached area at time t [Phair and Misteli, 2000].

For statistical analysis of the data, the measured recovery curves were subjected to fits using Kaleidagraph 3.5 (Synergy Development, Reading, PA). Various equations were tested, but the best fits for the cytoplasmic recoveries were obtained for $y = a/(1 + \exp(-a \times (b \times t + c)))$. Half times of recovery were obtained from individual fitted recovery curves when the relative recovery reached half of the plateau value minus the initial value after the bleach. Full recovery was achieved when fluorescence reached 99% of the plateau value. Statistical significance was tested by the Student's *t*-test.

RESULTS

Influence of Molecular Weight on Nuclear Localization

For our initial experiments, we utilized tsBN2 cells, which have normal Ran-dependent nuclear transport at the permissive temperature (33.5°C) but not at the nonpermissive temperature (39.5°C). The distribution of GFP-PTEN was the same at both temperatures (Fig. 1A,B,E). As a positive control for active

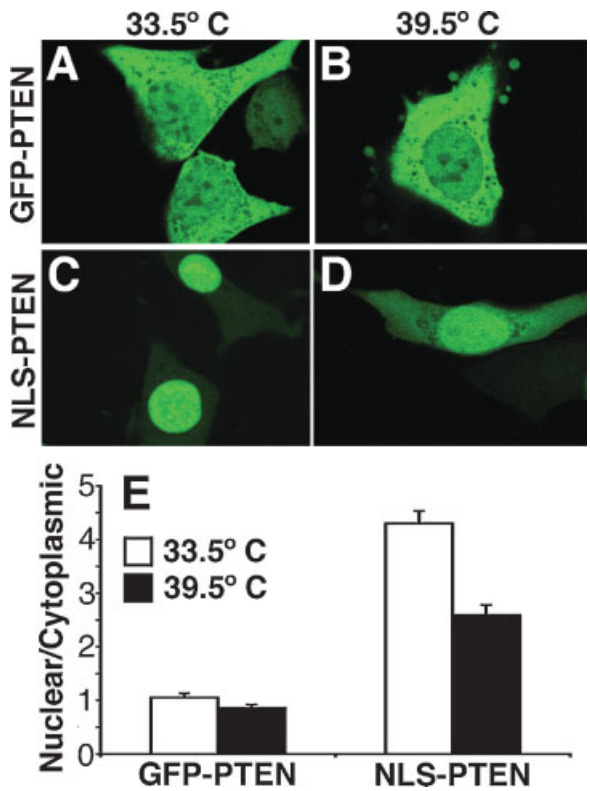

Fig. 1. Nuclear localization of phosphatase and tensin homolog deleted on chromosome 10 (PTEN) fusion proteins in tsBN2 cells. GFP-PTEN (**A**–**B**) or nuclear localization signal (NLS)-PTEN (**C**–**D**) were expressed in tsBN2 cells and incubated at 33.5°C (permissive for Ran-dependent nuclear transport, A and C) or 39.5°C (nonpermissive for Ran-dependent nuclear transport, B and D). Micrographs were recorded using a conventional fluorescence microscope. The ratio for average nuclear intensity to average cytoplasmic intensity were calculated (**E**, average ± SEM). The nuclear localization for NLS-PTEN, but not GFP-PTEN, is decreased at the nonpermissive temperature. These data suggest that NLS-PTEN, but not GFP-PTEN, is actively transported into the nucleus.

nuclear transport, we analyzed NLS-PTEN, which bears a strong NLS. Nuclear localization of NLS-PTEN was less for tsBN2 cells at 39.5°C than for cells at 33.5°C (Fig. 1C–E). Finally, as a negative control, we analyzed GFP. There was no apparent difference for GFP at the permissive and nonpermissive temperatures (data not shown).

We wanted to use HeLa cells for additional experiments because they are efficiently transfected and their flat morphology aids subcellular localization. As a first step, we tested whether HeLa cells express PTEN. By RT-PCR, we detected a PTEN mRNA, and sequencing revealed a wild type PTEN sequence (data not shown). This result is consistent with a report that PTEN is infrequently mutated in cervical carcinomas [Holway et al., 2000]. We analyzed the PTEN distribution by immunofluorescence microscopy, and found that PTEN was present in both the nucleus and cytoplasm (Fig. 2).

Having determined the distribution of endogenous PTEN, we tested a series of PTEN fusion proteins for nuclear localization. GFP (27,000 Da) and GFP-PTEN (74,000 Da) showed both cytoplasmic and nuclear localizations (Fig. 3A,B). In contrast, GFP-GFP (54,000 Da) and GFP-GFP-PTEN (101,000 Da) showed no apparent nuclear localization (Fig. 3C,D). We also used constructs with chicken PK. PK forms a tetramer (240,000 Da) that does not enter the nucleus [Siomi and Dreyfuss, 1995]. However, PK fused to an NLS efficiently localizes in the nucleus. PK, PK-20, PK-173, and PK-PTEN showed minimal nuclear localization (Fig. 3E–H). PK-20 and PK-173 include the first 20 and 173 amino acids of PTEN, respectively. The 20-amino acid N-terminal sequence was chosen because as will be described, mutations of residues 13–14 decrease nuclear localization of GFP-PTEN. The 173 amino acid N-terminal sequence was chosen because in the three-dimensional structure of PTEN [Lee et al., 1999], residues 13–14 are near the positively charged TI-loop (residues 160–169). Based on the data in Figures 1–3, the distribution of

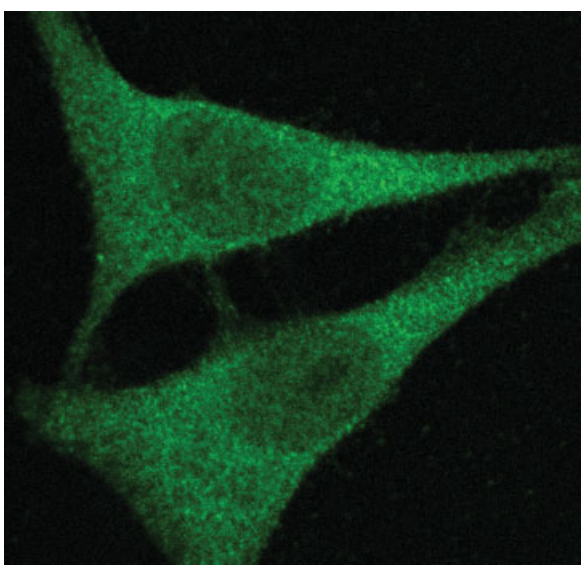

Fig. 2. Distribution of endogenous PTEN in HeLa cells determined by immunofluorescence microscopy. PTEN was present in both the cytoplasm and nucleus.

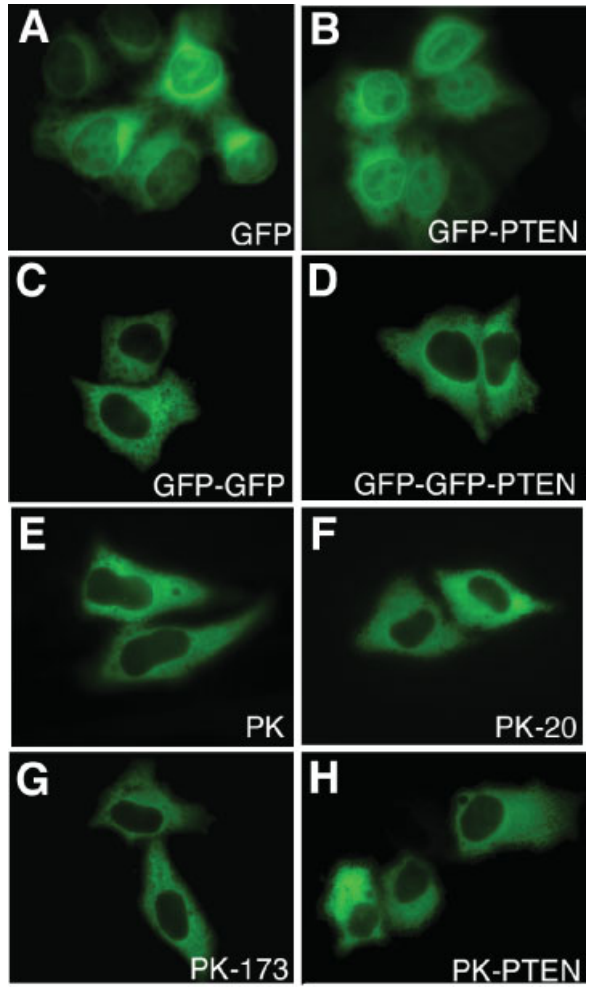

Fig. 3. Nuclear localization of PTEN fusion proteins in HeLa cells. Twenty-four hours after transfection, cells were fixed, and micrographs were recorded with a conventional fluorescence microscope. The lack of nuclear localization for GFP-GFP-PTEN, PK-20, PK-173, and PK-PTEN suggests that PTEN is not actively transported into the nucleus. **A**: GFP. **B**: GFP-PTEN. **C**: GFP-GFP. **D**: GFP-GFP-PTEN. **E**: PK. **F**: PK-20. **G**: PK-173. **H**: PK-PTEN.

GFP-PTEN resembles that of endogenous PTEN, and there does not appear to be a PTEN NLS.

A related question is whether PTEN has a nuclear export signal (NES). The PTEN primary sequence does not have an obvious NES. To further address this question, we treated GFP-PTEN-expressing HeLa cells with or without 10 ng/ml leptomycin B, which is an inhibitor of CRM1-dependent nuclear export. After a 4-h treatment, we determined the subcellular localization of GFP-PTEN and found no significant effect of leptomycin B, providing additional evidence that PTEN does not have a functional NES.

PTEN Mutants and Nuclear Localization

As we were carrying out the studies described in the first section of the Results, we also sought PTEN mutants that showed decreased nuclear localization. Our approach was to mutate PTEN sequences with multiple positively charged amino acids, which are frequently associated with NLSs. We expressed wild type and mutant PTENs as GFP-PTEN fusion proteins in HeLa cells and assessed subcellular localization by confocal microscopy. GFP-PTEN showed both cytoplasmic and nuclear localization (Fig. 4A). In the nucleus, GFP-PTEN was excluded from the nucleoli. PTEN mutants were examined by the same procedure (Fig. 4B–F), and for these as well as additional mutants not shown in Figure 4B–F, the ratios of nuclear to cytoplasmic fluorescence intensity were determined (Fig. 4H). Mutation of amino acids 13 and 14 significantly reduced nuclear localization compared with wild type PTEN ($P < 0.001$). Even the highly conservative K13R mutation

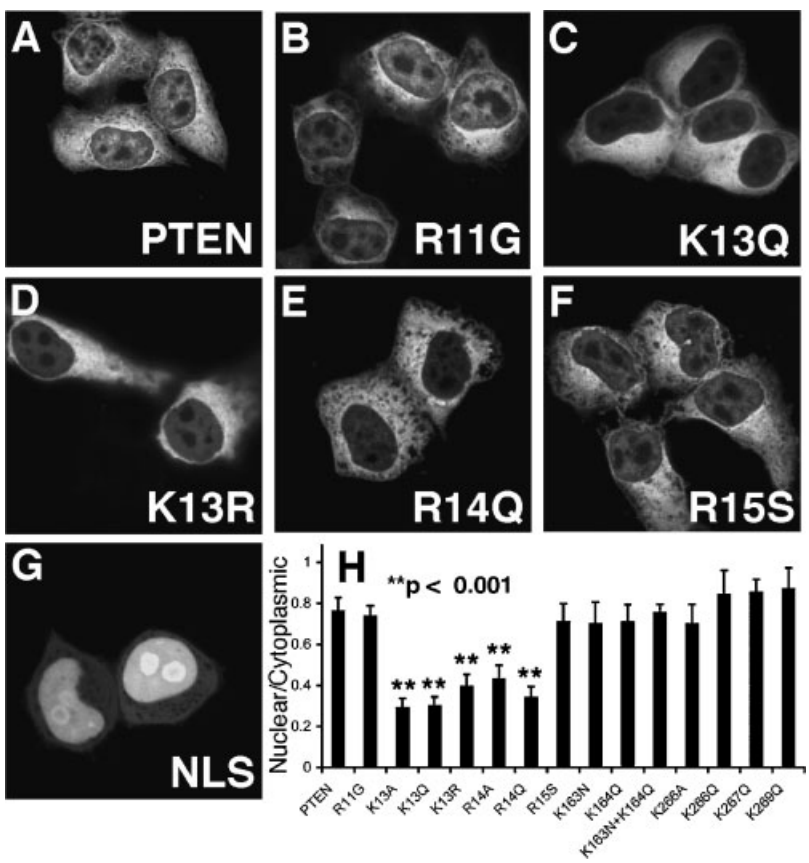

Fig. 4. Mutational analysis of nuclear localization of GFP-PTEN expressed in HeLa cells. **A**: Confocal microscopy image of HeLa cells transiently expressing GFP-PTEN. **B**: R11G mutant. **C**: K13Q mutant. **D**: K13R mutant. **E**: R14Q mutant. **F**: R15S mutant. **G**: NLS-PTEN. **H**: Quantification of nuclear/cytoplasmic intensity ratios for mutants shown in panels (A)–(G) as well as additional mutants (see Materials and Methods for quantification procedure). These results are representative of at least four experiments for each mutant PTEN. The averages are based on measurements from 20–21 cells in each category. The mutants that are significantly different from wild type PTEN are marked with **($P < 0.001$). All of the mutations that reduce GFP-PTEN nuclear localization are found in the N-terminus (amino acids 13–14). The ratio for NLS-PTEN is 4.13 ± 0.42 and is not shown in the bar graph.

decreased nuclear PTEN. Mutation of additional positively charged residues at the N-terminus (R11 and R15) as well as other parts of the PTEN protein (K163, K164, K266, K267, and K269) did not significantly affect nuclear localization. The NLS-PTEN fusion protein showed robust nuclear localization (Fig. 4G). The ratio of nuclear to cytoplasmic fluorescence intensity was 4.13 ± 0.42 (not shown in Fig. 4H).

The integrity of the GFP-PTEN fusion proteins was critical for the interpretation of the results shown in Figure 4. Extracts from cells expressing GFP-PTEN and the mutants were analyzed by Western blotting with anti-GFP antibody. We detected fusion proteins with apparent molecular weight of 80 kDa (data not shown). The PTEN mutations did not affect the expression levels of GFP fusion proteins. The lack of lower molecular weight bands and, in particular, free GFP demonstrated that proteolysis was not a significant problem. In addition, in each of these cultures, the cells that were not transfected served as an internal negative control. Quantification showed that these dim cells had negligible fluorescence compared with the GFP-PTEN-expressing bright cells. Hence, the fluorescence measured in these studies was due to GFP-PTEN and not autofluorescence.

To further test the role of amino acids 13–14 in nuclear localization of PTEN, we expressed wild type GFP-PTEN as well as N-terminal mutants in U87MG and LNCaP cell lines. In contrast to HeLa cells, these lines lack functional PTEN [Davies et al., 1999; Ishii et al., 1999]. As with HeLa cells, mutations of amino acids 13–14 reduced nuclear localization by two to three fold (Fig. 5A,B). Hence, the role of amino acids 13–14 on PTEN nuclear localization is not peculiar to any one cell line or tumor type.

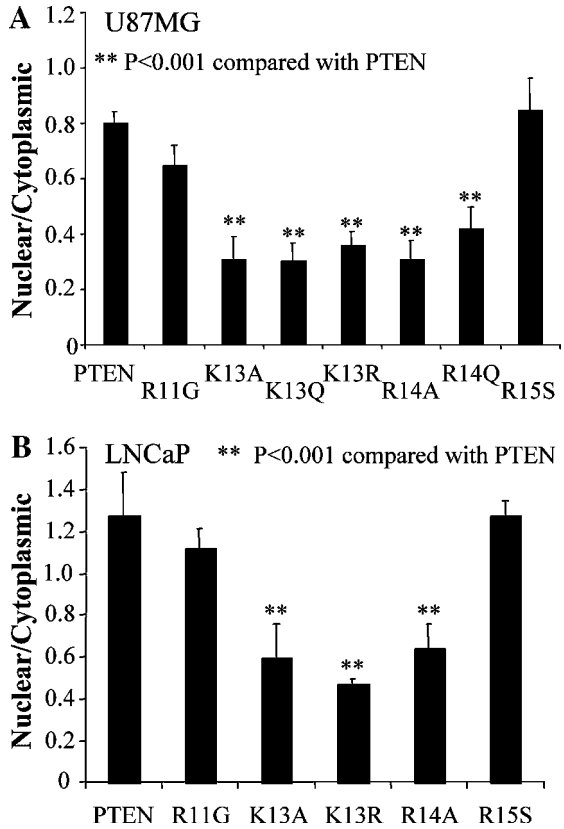

Fig. 5. Mutational analysis of GFP-PTEN nuclear localization in U87MG and LNCaP cells. We examined only the mutants that showed reduced nuclear localization in HeLa cells (Fig. 4). The ratios of nuclear to cytoplasmic intensities were calculated by the same procedure, and the mutants that are statistically significantly different from wild type PTEN are marked with **($P < 0.001$). As noted for HeLa cells, mutations of amino acids 13–14 reduce nuclear localizations in U87MG and LNCaP cells.

Phosphatase Activity of PTEN Mutants

Because mutation of R15 greatly reduces PIP phosphatase activity [Furnari et al., 1998], we were concerned whether mutation of amino acids 13–14 would also diminish phosphatase activity. We assessed PTEN activity by co-transfecting the PTEN expression plasmids with PI3K and HA-Akt. We used U87MG cells for this assay because they lack functional endogenous PTEN and are efficiently transfected. We measured phosphorylation of immunoprecipitated HA-Akt by Western blotting with anti-phospho-Akt antibody (Fig. 6A–D). In this assay, active PTEN decreases Akt phosphorylation. The K13A and K13Q mutants showed little or no phosphatase activity, but the K13R mutant showed normal activity. Mutants R14A and R14Q were inactive. As expected [Furnari et al., 1998], the R15S mutant was inactive.

We also tested additional mutations of positively charged amino acids. K163N, K164Q, K163N + K164Q, K266A, K266Q, and K269Q showed normal activity. However, K267Q showed decreased activity. NLS-PTEN only modestly decreased phosphorylation of Akt, consistent with current views that Akt phosphorylation occurs at the plasma membrane. NLS-C124S PTEN, which is a widely used phosphatase-dead mutant, had no apparent effect on Akt phosphorylation.

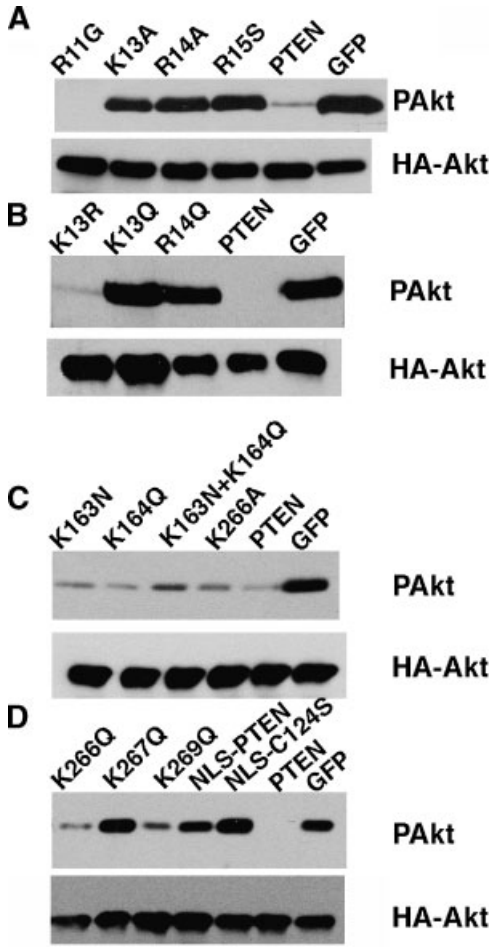

Fig. 6. Measurements of PTEN activity by phospho-Akt levels in U87MG cells. The GFP-PTEN mutants (or GFP as a negative control), HA-tagged Akt and a constitutively activated phosphoinositide 3-kinase (PI3K) were transiently expressed in U87MG cells. The HA-Akt protein was immunoprecipitated with anti-HA antibody, and phosphorylation levels were determined by Western blotting with anti-phospho-Akt. Relative levels of expression were determined by Western blotting with anti-HA antibody. These results are representative of three independent experiments. In this assay, active PTEN decreases phosphorylation of Akt. **A**: PTEN and R11G are active, but K13A, R14A and R15S show little or no activity. **B**: The K13R PTEN mutant is active, but K13Q and R14Q show little activity. **C**: Mutants in the TI loop of PTEN (K163N, K164Q and K163N plus K164Q) as well as K266A in the C2 domain are active. **D**: The K267Q mutant in the C2 domain is inactive, but the neighboring mutants (K266Q and K269Q) are active. PTEN coupled to a strong NLS showed decreased activity, and no activity was evident for the NLS-C124S PTEN mutant. These results demonstrate that amino acids 13–15 and 267 are important for phosphatase activity. However, the highly conservative substitution K13R did not eliminate activity.

Using an in vitro assay, we tested GST-PTEN and N-terminal mutant proteins for phosphatase activity. In good agreement with the phospho-Akt assay, the R11G and K13R mutant proteins were active, but the K13A, R14A, and R15S mutant PTENs were inactive (Fig. 7). Collectively, the results in this section demonstrate the importance of the N-terminus for both subcellular localization and phosphatase activity.

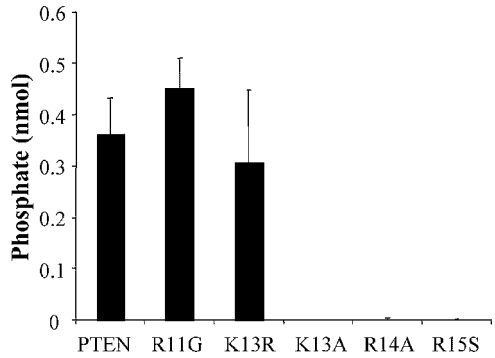

Fig. 7. In vitro assays for PTEN phosphatase activity. GST-PTEN proteins were assayed using a malachite green assay and PI(3,4,5)P_3 as substrate. The phosphatase reactions were terminated after a 20-min incubation at 37°C. PTEN and the R11G and K13R mutants are active, but the K13A, R14A and R15S mutants are inactive.

FRAP Measurements of PTEN Mobility

To measure mobility, we expressed GFP-PTEN in HeLa cells. First, we sought to measure the rate of nuclear import. To this end, we bleached a spot of approximately 1.5 μm in the nucleus for three (not shown) or 10 s (Fig. 8A,B). For both bleach times, no spot was evident immediately after the bleach, but instead fluorescence was greatly reduced for the whole nucleus (Fig. 8B). This result suggests that nuclear PTEN moves so fast that almost all of the GFP-PTEN molecules traverse the laser beam within 3 s. Having determined that diffusion of GFP-PTEN in the nucleus is rapid, we measured translocation of GFP-PTEN into the nucleus. Because it is easier to measure translocation for nuclei depleted of nearly all fluorescence, we used 10 s bleaches and then allowed nuclear fluorescence to recover (Fig. 8C–E). In panel E, we show the slow nuclear recovery for a representative cell. We also attempted to measure nuclear recovery for the K13R and R14Q PTEN mutants. As shown earlier, the initial nuclear fluorescence for these mutants was much lower than for PTEN. Accordingly, absolute recovery for these mutants was so slight that we could not reliably quantify the rate of nuclear translocation for these mutants (data not shown). We also

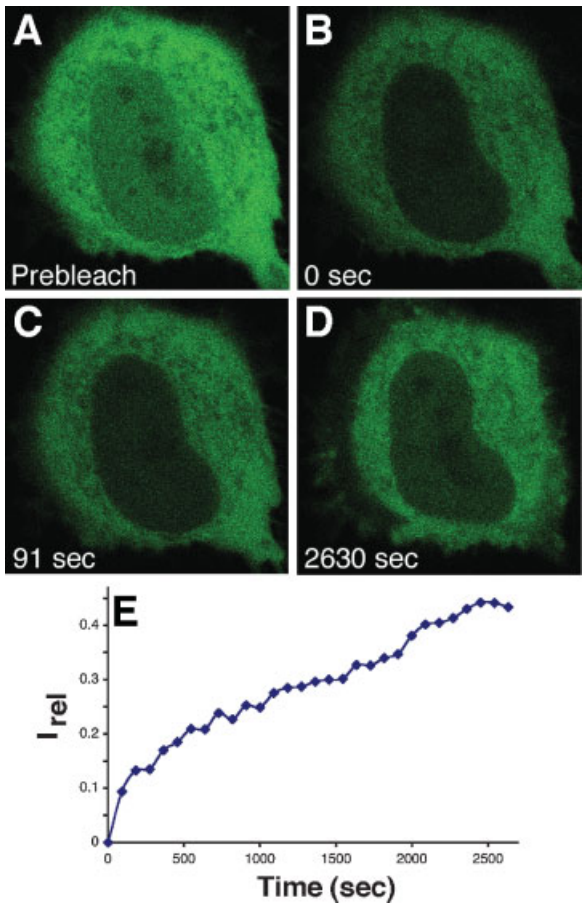

Fig. 8. Recovery of GFP-PTEN fluorescence after photobleaching of the nucleus in live HeLa cells. Micrographs were recorded prior to the bleach (**A**), immediately after the bleach (**B**) and at subsequent times ((**C**), 91 s; (**D**), 2630 s). The time-course of recovery is presented in (**E**).

photobleached cells expressing GFP. Consistent with an earlier study [Wei et al., 2003], recovery of nuclear fluorescence for GFP was much more rapid than for GFP-PTEN (data not shown). Hence, GFP-PTEN can slowly translocate from the cytoplasm to the nucleus, and mutation of K13 and R14 reduced nuclear translocation.

We next measured GFP-PTEN mobility in the cytoplasm. We bleached a spot of 1.5 μm in the cytoplasm for 3 s. Unlike the nuclear bleaches, the bleached spot was evident in the first image collected after the bleach. Recovery was faster for GFP-PTEN than GFP-PTEN (K13R) and GFP-PTEN (R14Q) (Fig. 9, Table I). We also photobleached cells expressing GFP, but the cytoplasmic recovery was too rapid to reliably quantify using our microscopy settings (data not shown). These results suggest that GFP-PTEN in the cytoplasm is constrained, and mutation of amino acids 13–14 can further restrict movement of GFP-PTEN.

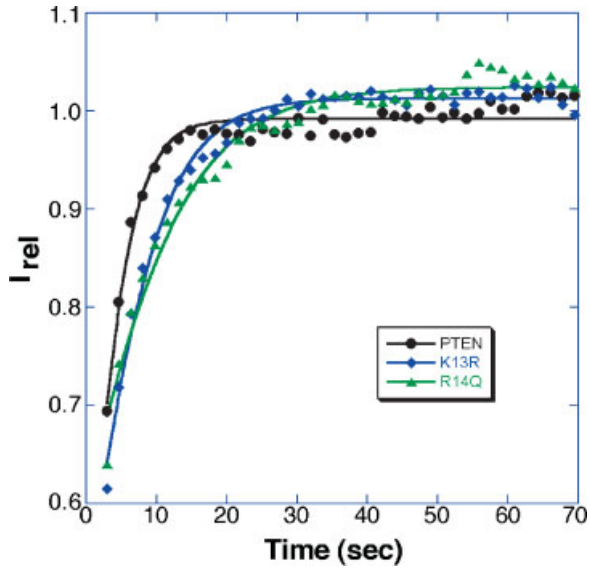

Fig. 9. Recovery of GFP-PTEN fluorescence in a photobleached spot in the cytoplasm of live HeLa cells. The averages of multiple recovery curves for GFP-PTEN (n = 9), GFP-PTEN (K13R mutant)(n = 8) and GFP-PTEN (R14Q)(n = 7) are shown. For statistical analyses, see Table I.

DISCUSSION

Many PIP-associated signaling proteins have been detected in the nucleus, including PTEN [Sano et al., 1999; Lachyankar et al., 2000; Perren et al., 2000; Zhang and Steinberg, 2000; Marshall et al., 2001; Ginn-Pease and Eng,

TABLE I. Cytoplasmic Fluorescence Recovery of GFP-PTEN and Mutants K13R, and R14Q in HeLa Cells*

Protein	Half time for recovery (s)	Time for full (99%) recovery (s)
GFP-PTEN (n = 9)	6.3 ± 0.8	16.6 ± 3.2
GFP-PTEN (K13R) (n = 8)	7.2 ± 0.7[a]	22.2 ± 2.8[b]
7GFP-PTEN (R14Q) (n = 7)	9.5 ± 1[a]	26.9 ± 3.7[b]

*Recovery curves were analyzed for time for 50% relative recoveries and for full recovery were calculated and then averaged (±SEM).
[a]By the Student's *t*-test, $P < 0.04$.
[b]By the Student's *t*-test, $P < 0.06$.

2003; Tsao et al., 2003], PI3K [Zini et al., 1996; Boronenkov et al., 1998; Lu et al., 1998], PIP-specific phospholipase Cβ [Manzoli et al., 1997; Sun et al., 1997; Neri et al., 1998], Akt [Andjelkovic et al., 1997; Meier et al., 1997], Bruton's tyrosine kinase [Mohamed et al., 2000] and ING2 [Gozani et al., 2003]. Furthermore, introduction of PTEN into nuclei enhances apoptosis-induced DNA fragmentation [Ahn et al., 2004], and $PI(4,5)P_2$ activates the BAF chromatin remodeling complex [Zhao et al., 1998]. Despite substantial evidence for PIP signaling in the nucleus, there is little understanding of how the levels and activities of these nuclear proteins are regulated. As a first step to elucidating this problem, we analyzed the mechanism by which PTEN enters the nucleus and found that PTEN diffuses through nuclear pores into the nucleus. In addition, it appears that tethering of PTEN in the cytoplasm limits nuclear translocation.

Using several experimental approaches, we tested the mechanism of nuclear translocation for GFP-PTEN. Based on photobleaching of GFP-PTEN in the nucleus, GFP-PTEN can pass through nuclear pores (Fig. 8). This translocation could be mediated by active transport or passive diffusion. We used tsBN2 cells in which the major active transport system (Ran-dependent) into the nucleus is inactivated at nonpermissive temperatures. [Tachibana et al., 1994]. We found that the distribution of GFP-PTEN, but not NLS-PTEN, in tsBN2 cells was the same for permissive and nonpermissive temperatures (Fig. 1). Also, we assessed the subcellular localization of a series of PTEN fusion proteins, noting that in HeLa cells, GFP-PTEN was present in both the nucleus and cytoplasm. This distribution was similar to that of endogenous PTEN (Fig. 2). In contrast, GFP-GFP-PTEN and PK-PTEN did not enter the nucleus (Fig. 3). Large proteins can enter the nucleus by active transport but not by passive diffusion [Siomi and Dreyfuss, 1995]. Collectively, these experiments lead us to conclude that GFP-PTEN enters the nucleus by passive diffusion.

But is nuclear diffusion consistent with the relatively large size of GFP-PTEN? Many authors have concluded that there is an upper limit of 60,000 Da for nuclear diffusion [Peters, 1986], and GFP-PTEN has a predicted molecular weight of 74,000 Da. For three reasons, we believe that GFP-PTEN's size does not preclude diffusion into the nucleus. First, the cut-off for nuclear diffusion is not a simple function of molecular weight. For example, hemoglobin (68,000 Da), but not bovine serum albumin (67,000 Da), can enter the nucleus in HeLa cells [Stacey and Allfrey, 1984]. In this study, GFP-GFP (54,000 Da) showed little or no nuclear localization (Fig. 3). Second, the nuclear uptake of GFP-PTEN is slow (Fig. 8), consistent with a molecular weight near the cut-off. Third, PTEN has an elongated, prolate shape [Lee et al., 1999], which might aid transit through the nuclear pores. Finally, based on GFP-PTEN's slow diffusion into the nucleus, it is likely that the relatively small PTEN protein readily diffuses between the cytoplasm and nucleus. It also should be noted that one could not predict passive diffusion of PTEN into the nucleus based only on PTEN's molecular weight. A number of proteins have molecular weights less than the cut-off for diffusion through nuclear pores and are nonetheless transported into the nucleus by NLS-mediated active transport [Kurz et al., 1997; Jäkel et al., 1999; Baake et al., 2001].

We also observed that mutations of K13 and R14 reduce nuclear localization. At first glance, this finding suggests that K13 and R14 are part of an NLS and seems to contradict our conclusion that GFP-PTEN diffuses into the nucleus. Classical NLSs consist of short sequences (5–20 amino acids) containing multiple lysines and arginines [Boulikas, 1993]. The sequence 11-R-N-K-R-R-15 could be an NLS. However, mutation of R11 and R15 did not reduce nuclear localization (Figs. 4 and 5) so these two amino acids are not part of an NLS. It is unlikely that K13 and R14 without additional positively charged amino acids could act as an NLS. In addition, fusion proteins of PK and the first 20 or 173 amino acids of PTEN did not enter the nucleus (Fig. 3E–H). Given these results, we conclude that K13 and R14 are not part of a classical NLS and must consider other models to explain how these mutations affect nuclear transport.

Our favorite model is cytoplasmic sequestration in which interactions of PTEN with cytoplasmic molecules prevent nuclear translocation. Nuclear translocation of both MAPK [Fukuda et al., 1997; Brunet et al., 1999; Zuniga et al., 1999] and β-catenin [Wheelock and Johnson, 2003] is limited by cytoplasmic sequestration. Cytoplasmic sequestration is consistent

with the decreased cytoplasmic motility of the two PTEN mutants (Fig. 9). It is intriguing that some cancer cells have cytoplasmic PTEN but not nuclear PTEN [Gimm et al., 2000; Perren et al., 2000; Tachibana et al., 2002; Whiteman et al., 2002; Zhou et al., 2002]. Our working hypothesis is that these tumor cells have enhanced cytoplasmic tethering.

Because the N-terminus of PTEN likely binds $PI(4,5)P_2$ [Campbell et al., 2003; McConnachie et al., 2003; Iijima et al., 2004; Walker et al., 2004], we considered $PI(4,5)P_2$ as a candidate for the cytoplasmic tethering molecule. However, mutations of K13 and R14 would likely decrease, not increase, PTEN binding to $PI(4,5)P_2$ [McLaughlin et al., 2002] and, therefore, $PI(4,5)P_2$ cannot be the tethering molecule. Another possible model is that mutation of K13 and R14 might alter the structure of the phosphatase domain and, in turn, alter the structure and lipid binding of the C2 domain. There is an extensive interface between the two domains [Lee et al., 1999] that might allow cross-domain regulation [Wishart and Dixon, 2002]. An additional model is that these amino acids interact with nuclear pore proteins during diffusion. Even for passive nuclear transport, interactions with pore proteins can influence the rate of translocation [Shibayama et al., 2002]. Finally, we considered whether mutations of K13 and R14 reduce nuclear translocation by altering phosphatase activity. For example, the protein phosphatase activity of calcineurin regulates nuclear translocation [Shibasaki et al., 1996]. This is not the case for PTEN. The K13R mutant protein is active but shows reduced nuclear localization (Figs. 4 and 5). The R15S and K267Q mutant proteins lack phosphatase activity but show normal nuclear localization.

The apparent tethering of PTEN to cytoplasmic structures raises an intriguing question relating to cytoplasmic functions of PTEN. Does this tethering enhance PTEN activity by placing PTEN in the vicinity of substrate or does the tethering prevent PTEN from acting at the plasma membrane? In this regard, it is interesting that the subcellular localization of $PI(3,4,5)P_3$ is disputed. Studies with GFP coupled to $PI(3,4,5)P_3$-specific PH domains suggest that $PI(3,4,5)P_3$ is primarily located at the plasma membrane [Balla et al., 2000; Czech, 2000]. However, $PI(3,4,5)P_3$ was detected in endomembranes by immunostaining with anti-$PI(3,4,5)P_3$ antibodies [Chen et al., 2002]. Utilizing fluorescence resonance energy transfer, $PI(3,4,5)P_3$ also was detected in endomembranes [Sato et al., 2003]. Although considerable more work is required, it is likely that cytoplasmic sequestration affects both nuclear and cytoplasmic functions of PTEN.

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