

Award Number: W81XWH-05-1-0029

TITLE: Identification and Validation of PTEN Complex, Associated Proteins

PRINCIPAL INVESTIGATOR: Rosalia Rabinovsky, Ph.D.

CONTRACTING ORGANIZATION: Dana Farber Cancer Institute
Boston, MA 02115

REPORT DATE: November 2005

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. **PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.**

| | | | | | |
|--|-------------------------|---|-----------------------------------|---|---|
| 1. REPORT DATE (DD-MM-YYYY) 01-11-2005 | | 2. REPORT TYPE Annual Summary | | 3. DATES COVERED (From - To) 1 Nov 2004 – 31 Oct 2005 | |
| 4. TITLE AND SUBTITLE Identification and Validation of PTEN Complex, Associated Proteins | | | | 5a. CONTRACT NUMBER | |
| | | | | 5b. GRANT NUMBER W81XWH-05-1-0029 | |
| | | | | 5c. PROGRAM ELEMENT NUMBER | |
| 6. AUTHOR(S) Rosalia Rabinovsky, Ph.D. E-mail: rosalia_rabinovsky@dfci.harvard.edu | | | | 5d. PROJECT NUMBER | |
| | | | | 5e. TASK NUMBER | |
| | | | | 5f. WORK UNIT NUMBER | |
| 7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Dana Farber Cancer Institute Boston, MA 02115 | | | | 8. PERFORMING ORGANIZATION REPORT NUMBER | |
| 9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012 | | | | 10. SPONSOR/MONITOR'S ACRONYM(S) | |
| | | | | 11. SPONSOR/MONITOR'S REPORT NUMBER(S) | |
| 12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited | | | | | |
| 13. SUPPLEMENTARY NOTES | | | | | |
| 14. ABSTRACT <p>The PTEN/MMAC1/TEP1 is a tumor suppressor gene, targeted for biallelic somatic inactivation in a variety of cancers including advanced prostate adenocarcinomas among many others malignancies. PTEN is a phosphatase and has an important role in regulation of the PI3K/AKT signaling pathway, which plays a key role in regulating cellular functions including proliferation, apoptosis, glucose homeostasis, cell size, nutrient response and DNA damage. Furthermore, PTEN functions in the cell to restrict both growth and survival in absence of growth signals.</p> <p>Studies performed in our laboratory indicated that in addition to its 47Kda form, PTEN could be detected as a part of a >600Kda complex. Further, we have also shown that PTEN acts as an antagonist of the PI3K/AKT signaling, only when it is unphosphorylated and recruited into the large protein complex.</p> <p>To purify the complex and study the dynamics of the phosphorylated versus the unphosphorylated forms of PTEN in the cell we have generated a novel antiserum that selectively recognizes the unphosphorylated form of PTEN. This new antibody (T382/383) will allow us to study and purify the lower abundance of unphosphorylated form of PTEN present in the PTEN associated complex (PAC). In addition, using our previously generated high affinity anti PTEN antibody (C54) that recognizes both the phosphorylated and unphosphorylated forms of PTEN we generated an immunoaffinity column (C54-DSS-Protein A) that maintains all the qualities of the C54 antibody, i.e. the high ability to bind and precipitate the endogenous PTEN. The immunoaffinity columns will be used additional step in our biochemical purification of the PAC.</p> | | | | | |
| 15. SUBJECT TERMS Tumor suppressor gene, PTEN, PTEN-associated complex, Phosphoinositide-3 kinase, Signal Transduction | | | | | |
| 16. SECURITY CLASSIFICATION OF: | | | 17. LIMITATION OF ABSTRACT | 18. NUMBER OF PAGES | 19a. NAME OF RESPONSIBLE PERSON USAMRMC |
| a. REPORT U | b. ABSTRACT U | c. THIS PAGE U | | | 19b. TELEPHONE NUMBER (include area code) |
| | | | UU | 18 | |

Table of Contents

| | |
|--|--------------|
| Cover..... | 1 |
| SF 298..... | 2 |
| Table of Contents..... | 3 |
| Introduction..... | 4 |
| Body..... | 5-14 |
| Key Research Accomplishments..... | 14 |
| Reportable Outcomes..... | 14 |
| Conclusions..... | 14-15 |
| References..... | 15-18 |

Introduction

Prostate cancer is the second leading cause of cancer related deaths in American men resulting in nearly 31,000 deaths per year [1]. Despite widespread adoption of early detection strategies based upon prostate specific antigen test screening, a significant number of men relapse after local therapy and progress to metastatic prostate cancer. Hormone-withdrawal therapy typically induces a response, however, nearly all patients treated in this fashion will eventually develop hormone-refractory disease.

The *PTEN/MMAC1/TEP1* tumor suppressor gene [2-4] maps to chromosome 10q23, a region frequently altered by loss-of-heterozygosity in a variety of tumors including advanced, metastatic prostate adenocarcinomas [2, 5-10]. Up to 70% of primary prostate tumors lose one PTEN allele [11-13]. Alterations in the remaining allele of PTEN leading to biallelic inactivation, including nonsense, frame-shift, deletion or insertion mutations, (reviewed in [14]) are observed in prostate cancer, as well as in endometrial cancer, glioblastoma and melanoma (reviewed in [15]). Previously, the Sellers lab and others have shown that loss of PTEN is associated with more aggressive forms of prostate cancer in man. This data is complimented by a number of genetic experiments in mice [10, 16-18]. Thus, PTEN is an important tumor suppressor gene whose loss of function contributes to tumorigenesis and the development of aggressive forms of prostate cancer.

The PTEN protein is a phosphatase that interacts with and dephosphorylates the D3 position of phosphatidylinositol-3,4,5-trisphosphate (PIP₃) [19]. It is also capable of interacting and dephosphorylating other phosphoinositides, like PI-3-P, PI-3,4-P₂, Ins-1,3,4-P₃, but with a lower affinity (reviewed in [14]). PTEN likely functions as a tumor suppressor primarily through its lipid phosphatase activity and its ability to negatively regulate signaling through the PI3K/AKT pathway (reviewed in [20]), which is involved in the regulation of cell proliferation and survival.

PTEN has several well-characterized domains and motifs. There is a N-terminal phosphatase domain with the canonical HCXXXXXR active site motif. This active site is larger in comparison to other known protein phosphatases to fit the size of the phosphoinositol lipid [21]. It is followed by a lipid-binding-C2 domain (C2D), a C-terminal 50-amino acid "Tail", two PEST homology regions and a PDZ binding domain (PDZbd), at the extreme C-terminus (reviewed in [14]). Recent studies reveal that the N-terminus phosphatase domain is essential for membrane binding, whereas the C2D, although known to be involved in cell signaling and membrane trafficking [21], plays an indirect role. Moreover, it was shown that through the N-terminus, PTEN can bind to PI-3,4-P₂, which may also serve as a putative partner for membrane binding [22, 23]. Our group was the first to demonstrate that PTEN is modified by phosphorylation at the C` terminus by Casein Kinase-2 (CK2), a serine/threonine kinase [24].

Gel-filtration experiments over Sephacryl S-300 column using rat liver extracts revealed two major peaks of endogenous PTEN protein. The first peak eluted with a molecular weight greater than 600 kDa while the second eluted with a molecular weight of 44-100 kDa. In this experiment while unphosphorylated PTEN migrated as both a monomeric and higher molecular weight form (>600 kDa), the phosphorylated form of PTEN was found only in the monomeric peak (44-100 kDa) [25]. Lastly, additional data from our lab showed that the non-phosphorylated form of PTEN strongly cooperated with MAGI-2 to block Akt activation, in addition to PTEN interaction with other PDZ domain containing proteins [25]. These data suggested that PTEN phosphorylation might regulate its ability to form associations with a larger protein complex and that PTEN activity is regulated through this association [25].

In the past year additional evidence has accumulated in support of the notion that PTEN forms a multi protein complex. A number of new putative PTEN interactors have been suggested: among which are LKB1, a nuclear serine/threonine kinase and a known tumor suppressor protein [26]; and MSP58/MCRS1, an additional PDZ domain containing protein and an oncogene [27]. By using a yeast two-hybrid system it was also suggested that PTEN might interact indirectly with β -catenin though direct binding to MAGI-1b [28].

In aggregate, these data provide additional evidence that PTEN forms a complex that contributes to its function as a tumor suppressor.

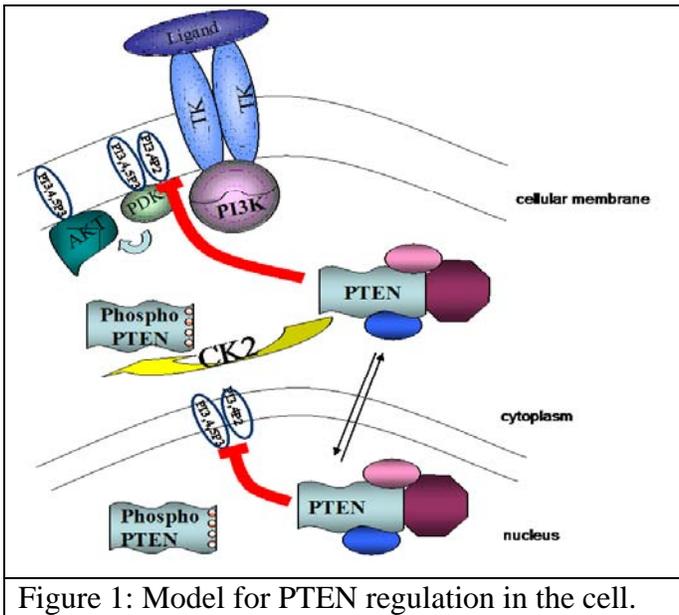
Objectives

The specific aims of my on going research are:

1. Identification of the PTEN associated complex (PAC) by biochemical purification.
2. Identification of the PTEN associated proteins by Tandem Affinity Purification.
3. To determine whether the interactions of PTEN with components of the PAC are necessary for PTEN function as tumor suppressor gene.

Body

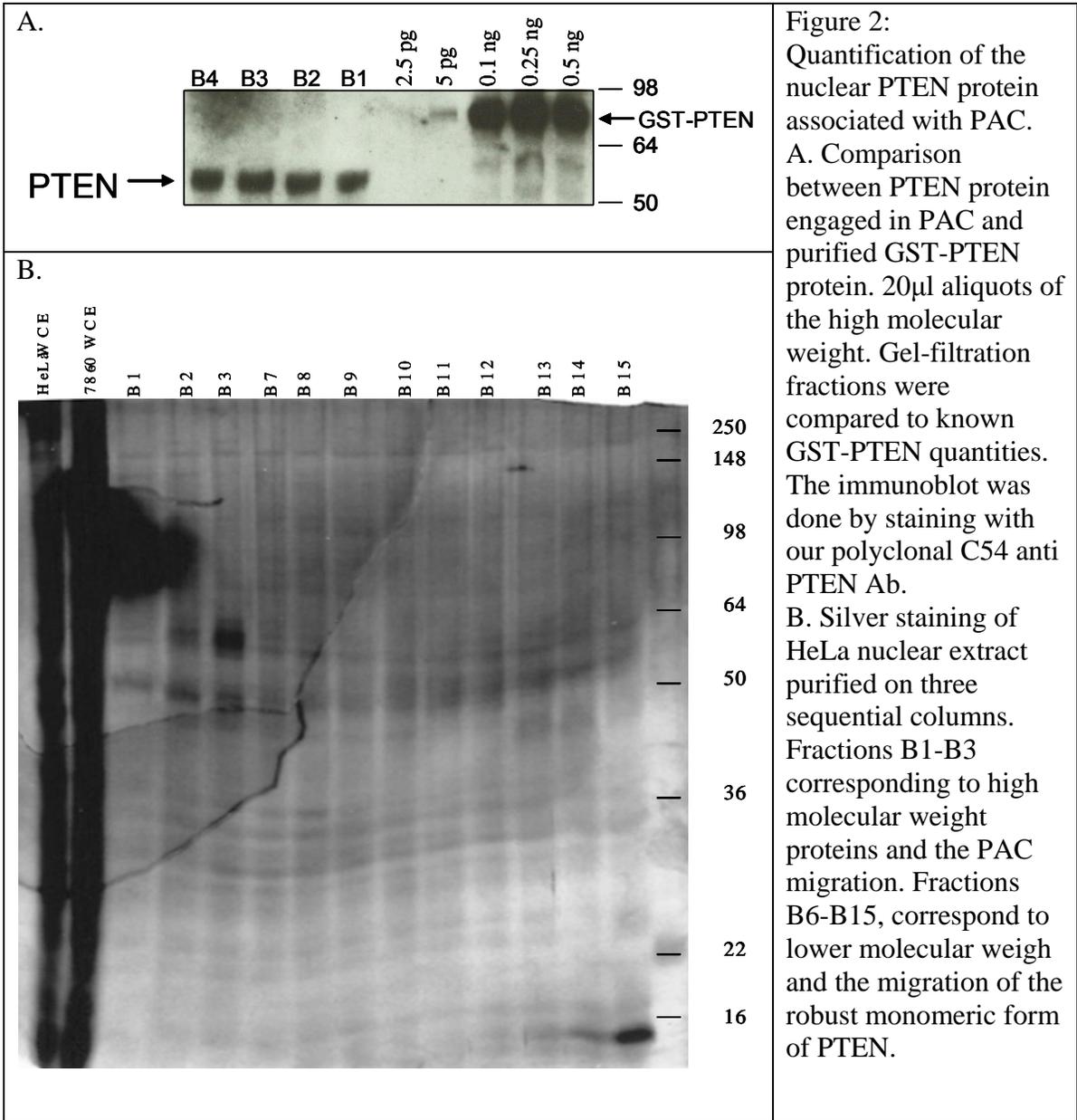
Specific Aim 1: Identification of the PTEN associated complex (PAC) isolated from distinct cellular compartments by biochemical purification.



We have chosen chromatographic purification strategy to purify the PAC. As described in our original report our purification is based on using three sequential columns: P11, DEAE, Superose 6, which differ on their biochemical properties. We detected the monomer form of PTEN in B7-B15 gel filtrations fractions corresponding to low molecular weight proteins (~ 44 kDa -100 kDa). In addition another pick of PTEN was detected at B1-B3, corresponding to high molecular weigh proteins (~ 670 kDa).

Our pilot purification of HeLa cytoplasmic and nuclear subcellular fraction suggested the presence of a uniform complex in both cellular fractions. The membrane fraction PAC appeared to have similar biochemical behavior to the nuclear and cytoplasmic fractions. We believe that the same PAC migrates between the different cellular compartments, as presented in Figure 1. Under our purification conditions, the most robust signal for the PAC was observed in the nucleus. Thus we choose to evaluate the PAC by analyzing, the sequentially purified HeLa nuclear extract. We have examined aliquots of the high molecular weight gel filtration fraction (fractions B1 to B4). As observed previously these were positive for endogenous PTEN by immunoblotting (Figure 2A). To evaluate the levels of PTEN present in these fractions we compared them against known quantities of purified GST-PTEN protein (Figure 2A). In addition, larger quantities of the same samples were separated on a 4%-16% gradient PAGE,

followed by silver staining (Figure 2B) to determine if this quantity of proteins would allow us to identify potential bands for analysis by tandem mass spectrometry (ms/ms). The fractions, corresponding to the high molecular weight (B1-B3), the PAC, were compared to the fractions corresponding to the low molecular weight proteins (B7-B15), the monomer PTEN. Although we could detect the PAC by immunoblotting (Figure 2A), we were unable to detect any specific bands corresponding to either PTEN or putative PAC protein on the silver stained gel (Figure 2B). Our immunoblot quantification indicated that the amount of PTEN was less than 100pg. These results suggest that a very large scale (80-100 liters) purification of HeLa cells will be required, to yield sufficient quantities of PAC for mass spectrometry based identification.



Scaling up to purify PTEN associated proteins:

Accordingly, a large-scale purification is now underway starting with 80L of HeLa cell extract obtained through the National Cell Culture Center as frozen pellets. The cells were lysed and fractionated into membrane, cytoplasmic and nuclear fractions, resulting in almost 150mg of cytoplasmic, 250 mg of nuclear and 20mg of membrane proteins. The subcellular fractionation was performed by using a hypotonic non-denaturing buffer to lyse the cells. The cytoplasm and the membrane fraction were separated from the nuclei by low-speed centrifugation and soluble nuclear proteins were extracted from isolated nuclei in non-ionic detergent buffer and insoluble material was removed by high-speed centrifugation. The membranes were separated from the cytoplasmic fraction by ultracentrifugation at 100,000G, followed by sucrose gradient. The purity of the fractionation was determined by staining a sample of each of the fractions for α Tubulin expressed only in the cytoplasm (Figure 3). The cytoplasmic and membrane fractions were loaded (separately) on P11-phosphocellulose column and eluted first with 0.1M KCl, to wash off all the proteins that did not bind to the column, followed by 0.3M KCl elution, a concentration shown in our pilot experiment with 20L of HeLa cells to elute the PAC (See Figure 3 in the original report). Currently the two fractions are being concentrated to be loaded on the DEAE column, for further purification. The proteins that bind to the DEAE column will be eluted with 0.35M KCl, and loaded on gel-filtration Sepharose 6 column, for further purification. Fraction positive for PTEN and corresponding to high molecular weight will be concentrated by IP on an anti PTEN immuno-column.

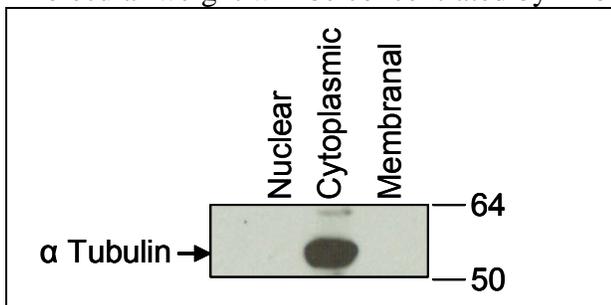


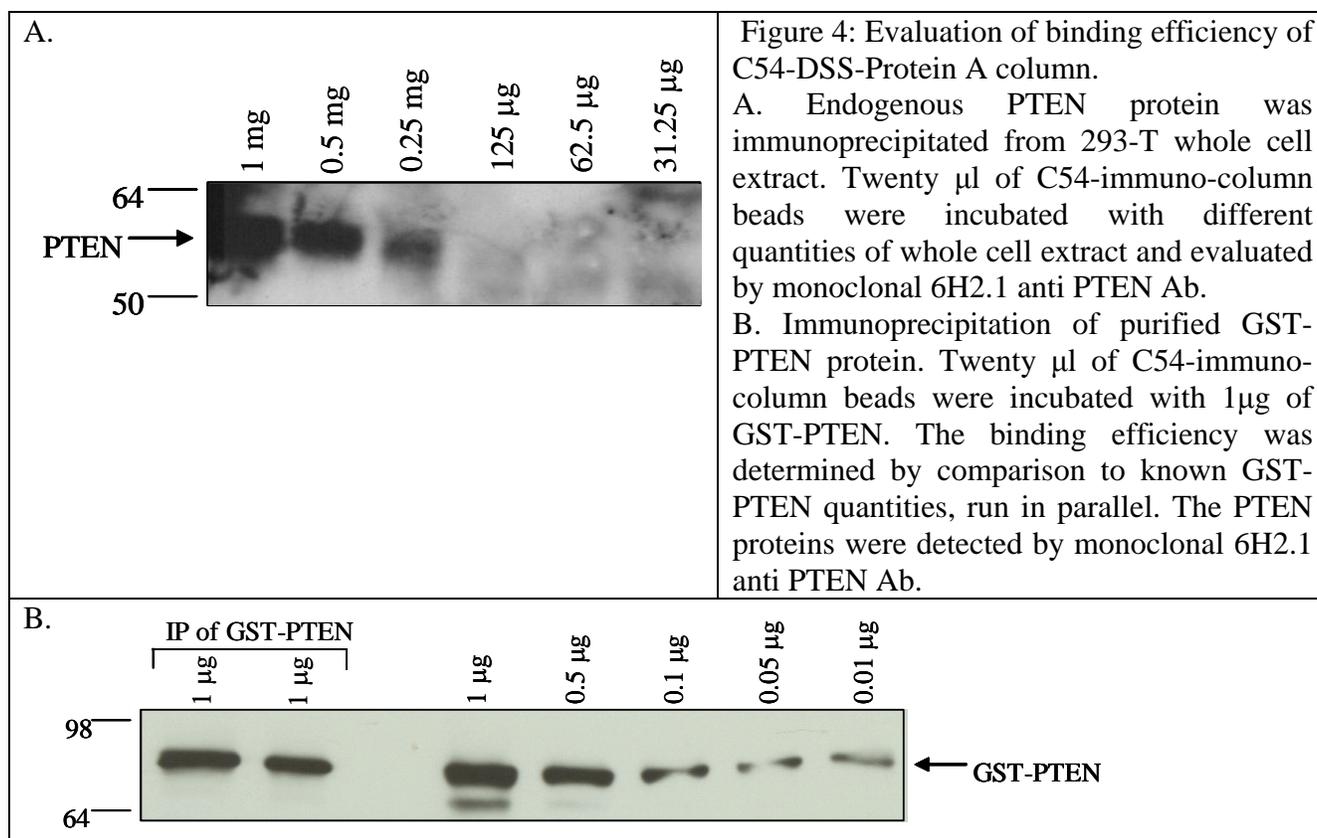
Figure 3: HeLa whole cell extract was fractionated into membranal cytoplasmic and nuclear fractions. The purity of the fractions was determined by α -Tubulin.

Proteins associating with the PAC and detected by silver stain will be identified by ion-trap mass spectrometry and amino acid sequence determined by tandem mass spectrometry (ms/ms).

Optimization of immunoaffinity purification:

The last step in our purification process will be an immunoaffinity purification based on anti PTEN immuno-column. To this end, we have generated a column, based on our own antiserum C54, covalently coupled to agarose beads. Glycine will be used to elute the PAC from the immunoaffinity column, a strategy that was successfully applied to this antibody previously (See figure 4 in the original report). The C54 antibody (Ab) was first purified on a Protein A column, the high titer Ab was subsequently collected and used for generating the immuno-column. For this purpose we used the IgG Plus Orientation kit (Pierce Biotechnology). Briefly, C54 purified Ab was incubated over night with protein A-agarose beads, then activated Disuccinimydyl suberate (DSS) cross-linker was used to covalently couple between the Fc of the Ab and the protein A-agarose beads.

To evaluate the efficiency of our immuno-column 20 μ l of the beads coupled to C54, were incubated with different quantities of either 293-T whole cell extract (Figure 4A) or with 1 μ g of purified GST-PTEN protein, produced in E. coli (Figure 4B), varying known quantities of GST-PTEN were run in parallel, to determine the binding capacity of 20 μ l of coupled Ab. Twenty μ l of C54-DSS- Protein A can bind approximately 0.5 μ g of purified GST-PTEN protein (figure 4B) while maintaining the C54 Ab efficiency to immunoprecipitate the endogenous PTEN (figure 5A).



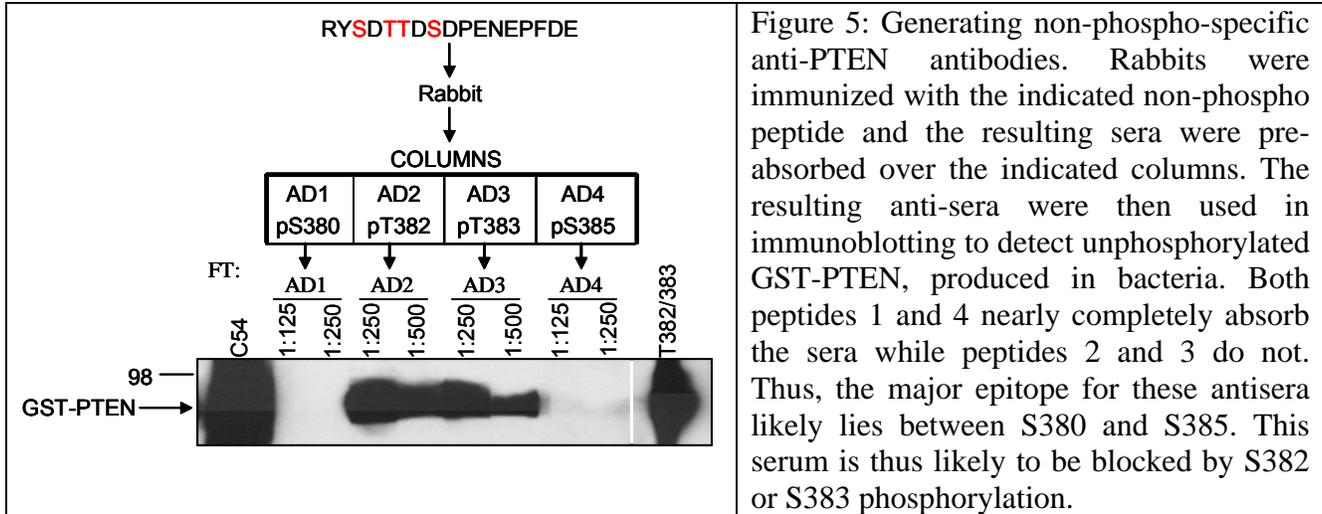
Anticipated results: We believe that this column will enrich for PAC by eliminating all unrelated proteins that run the same size on a gel-filtration column as the PAC.

The eluted proteins from the immunoaffinity column will be separated by gel electrophoresis and detected by silver stain. Bands that co-elute with PTEN in the PAC will be excised and sent for mass spectrometry at the Taplin Biological Mass Spectrometry Facility at Harvard Medical School, Directed by Steven Gygi (<http://deer.med.harvard.edu/facility/>). In the course of this work, we will also be mindful of proteins that are already reported to interact with PTEN.

Generating non-phospho-specific anti PTEN antibodies:

To study the less abundant, unphosphorylated form of PTEN present in the PAC, we require Abs that selectively recognize the unphosphorylated form of PTEN. To address this, we generated anti unphosphorylated PTEN specific Abs. We were successful in generating high-titer non-phospho specific antibodies to the C` terminus "tail".

The new antibodies were generated by immunizing rabbits with a KLH coupled PTEN peptide (RYSDDTSDPENPFDE) containing the S380, T382, T383 and S385 residues in their unphosphorylated form. Sera from these rabbits were then split into aliquots and independently pre-absorbed against 4 mono-phosphorylated columns each phosphorylated on a different Ser or Thr (Figure 5). Here, the hope was that Abs capable of recognizing the phosphorylated epitope will bind to the columns, thereby allowing Abs against the non-phosphorylated residue to pass through the column. This should result in presenting us with 4 types of antisera, named AD1, AD2, AD3, AD4, that will recognize only non-phosphorylated S380, T382, T383 and S385 residues, respectively. The resulting pre-absorbed antisera were used to detect recombinant, unphosphorylated GST-PTEN protein (Figure 5).



As shown in Figure 5, antisera pre-absorbed against the AD2-pT382 and the AD3-pT383 columns robustly detected GST-PTEN in immunoblots, while antisera pre-absorbed against the AD1-pS380 and the AD4-pS385 columns failed to immunoblot GST-PTEN. Thus, it appears that our immunization strategy produced an antiserum with a dominant epitope (₃₈₁DTTD₃₈₄). For simplicity, here on, we will refer to the unabsorbed antiserum as anti-T382/T383.

To evaluate the efficiency of binding of the new Ab we compared its binding ability to the C54 Ab (Figure 6). C54 Ab, has high affinity both to phosphorylated and non-phosphorylated forms of PTEN, i.e. it detects the total PTEN in the cell. To do so, we compare the immunoblotting efficiency of different dilution of the two Abs against constant concentration of GST-PTEN. GST-PTEN was loaded and run lengthwise on electrophoresis gel. The transfected blot was cut into equal strips and incubated with different dilutions of the C54 or T382/383 Abs, ranging from 1:500 to 1:10,000 (Figure 6A). In our second approach we ran known quantities of purified GST-PTEN protein and immunoblotted with a constant (1:1000) dilution of the two Abs (Figure 6B). These experiments showed us that the new T382/382 Ab is nearly as robust and sensitive as our C54 total Ab. Even at the dilution of 1:10,000 both C54 and T382/383 were able to detect GST-PTEN protein (Figure 7A). From our second approach we learned that although C54 is 10 times more sensitive than T382/383, the new Ab was still successful in detecting at least 0.5ng of protein under standard ECL conditions (Figure 7B).

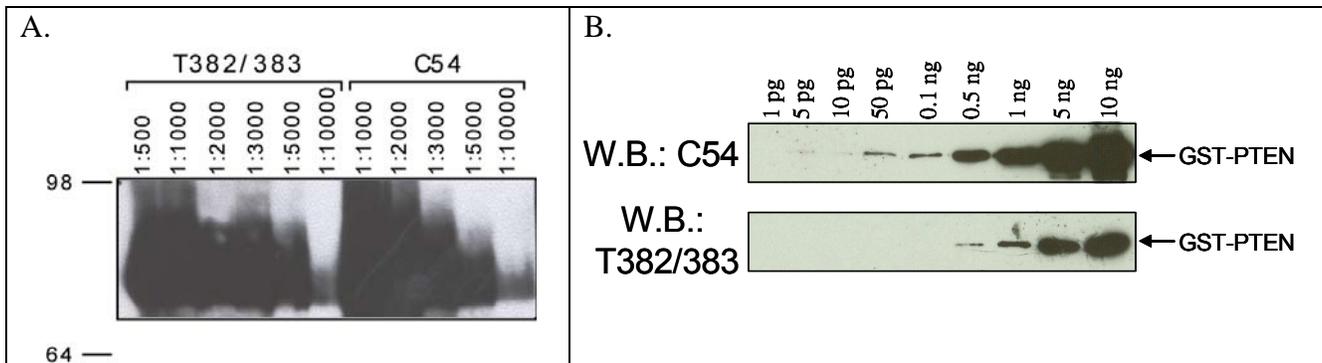
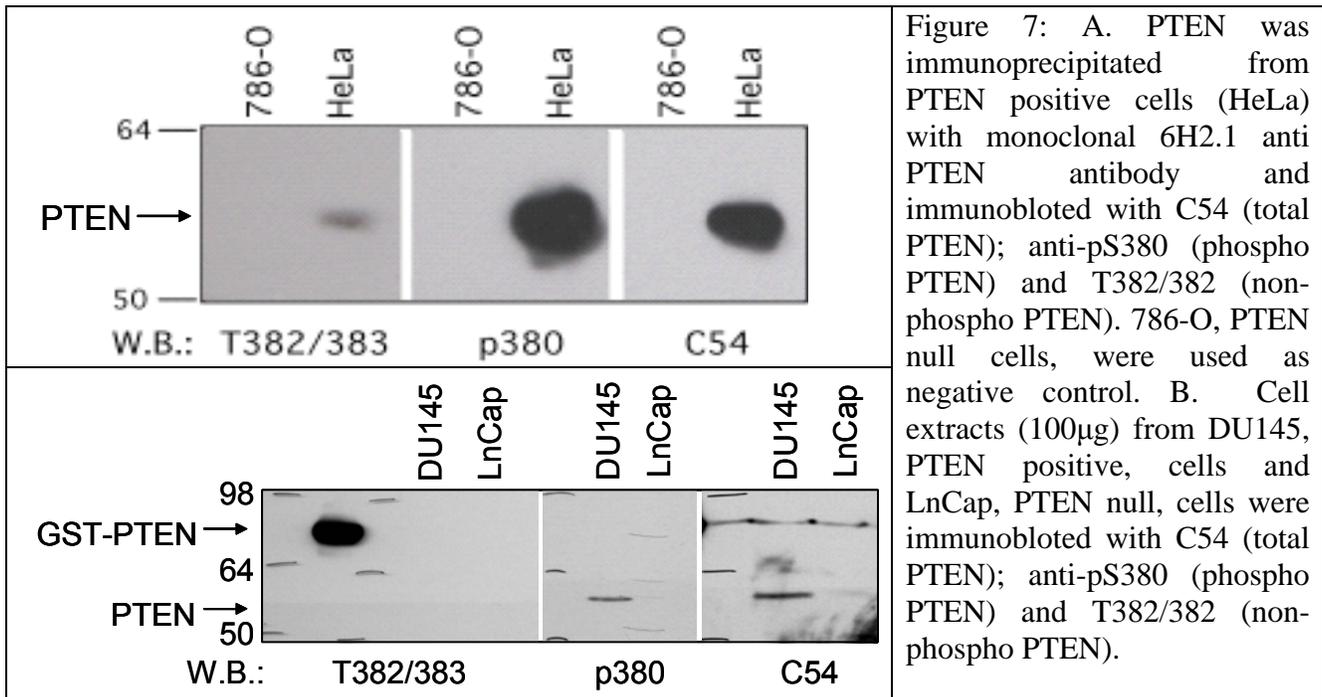


Figure 6: A. comparison between C54 and T382/383 on GST-PTEN detection; B. Quantification of PTEN detected by C54 in comparison to BSA; C. Quantification of PTEN detected by T382/383 in comparison to BSA.

Next, we evaluated the T382/383 Ab for its ability to recognize the endogenous PTEN. One mg of HeLa PTEN positive whole cell extract was immunoprecipitated with monoclonal 6H2.1 anti total PTEN Ab and immunoblotted for total PTEN, with C54 Ab; for phospho PTEN, with anti p380; and for non-phospho PTEN, with T382/383 Ab (Figure 7A). As expected, the quantity of the non-phosphorylated form of PTEN in the cell is less abundant in comparison to the phosphorylated form and to the total PTEN (Figure 7A). Results presented in Figure 7B emphasize again that the phosphorylated state is the abundant form of PTEN in the cell. Whole cell extracts (100µg) from PTEN positive and negative prostate cell lines were analyzed for the different forms of PTEN in the cell. The endogenous PTEN was detected in PTEN positive cell line (DU145) both by C54 and by the phospho-specific p380 Ab, identifying this protein as primarily phosphorylated (Figure 7B). T382/383 failed to detect the non-phosphorylated form of the protein, due to the low PTEN quantities in the samples.



The 4 pre-absorbed sera of T382/383 were also evaluated for their ability to immunoprecipitate the non-phosphorylated form of PTEN. Since endogenous non-phosphorylated form of PTEN present at low levels in the cells, for these experiments we used purified GST-PTEN, produced in *E. coli* and phosphorylated versus non phosphorylated proteins transcribed and translated *in vitro*. All the pre-absorbed sera were able to immunoprecipitate GST-PTEN at some level. Apparently, the AD3 corresponding to the ³⁸¹DTTD₃₈₄ epitope worked best in immunopercipitation. Surprisingly AD4 that was a weak immunoblot Ab nonetheless seemed to immunoprecipitate the GST-PTEN protein. AD1 antisera, on the other hand did not recognize the protein (Figure 8). Although, this experiment sugested that C54 Ab had a better binding ability in comparison to the new Abs, it should be noted that C54 is a Protein A purified antibody, where AD1, AD2, AD3, AD4 are pre-absorbed sera that are at least 4 times more diluted than T382/383. When more of the new pre-absorbed sera were used, the immunopercipitation was more effective (Figure 8). Next, PTEN wild type construct, was transcribed and translated in Rabbit reticulocyte lysate transcription/translation system to generate a phosphorylated protein. In parallel, the same

construct was transcribed and translated using a wheat germ lysate transcription/translation system to generate an unphosphorylated protein, in the presence of radioactively labeled methionine. The aliquots of proteins were incubated with the 4 pre-absorbed sera or C54, as positive control, and evaluated by exposure to film. The pre-absorbed sera did not bind to the protein translated in Rabbit reticulocytes lysate transcription/translation system. C54 on the other hand, successfully immunoprecipitated the same protein. Although the transcription of the PTEN protein was less efficient using the wheat germ lysate transcription/translation, system the new antisera immunoprecipitated the protein as well as the C54 Ab, especially AD2.

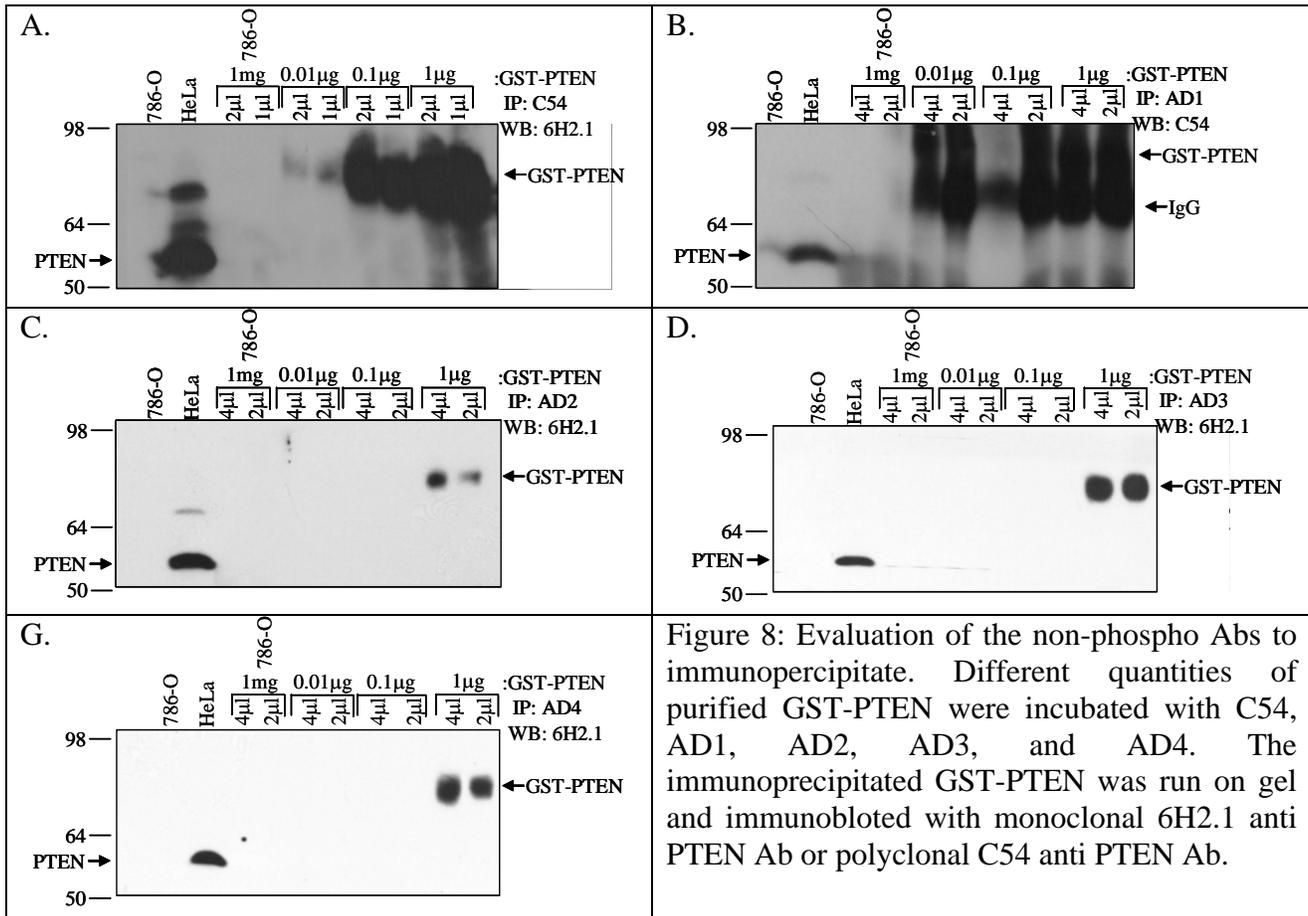
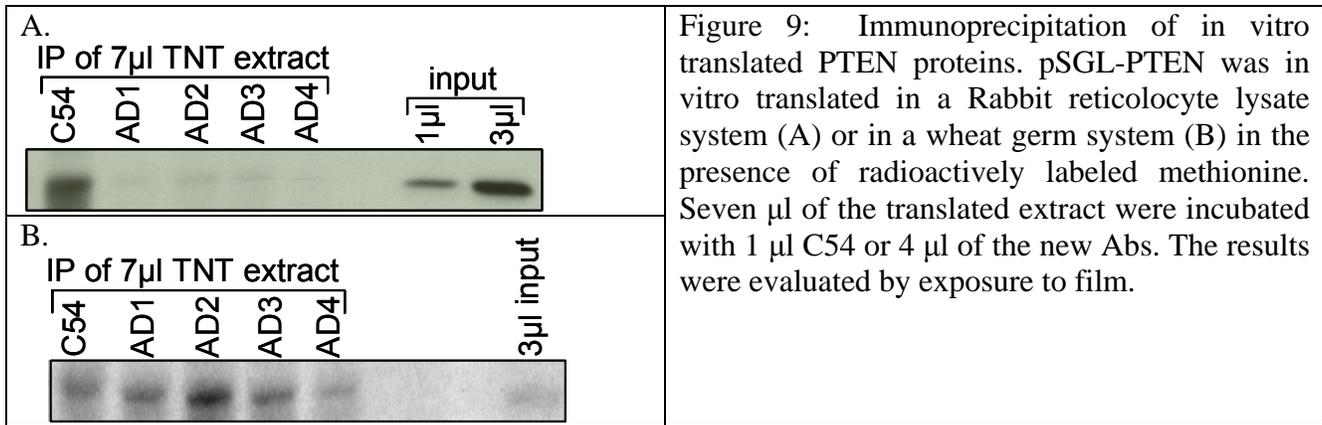


Figure 8: Evaluation of the non-phospho Abs to immunopercipitate. Different quantities of purified GST-PTEN were incubated with C54, AD1, AD2, AD3, and AD4. The immunoprecipitated GST-PTEN was run on gel and immunoblotted with monoclonal 6H2.1 anti PTEN Ab or polyclonal C54 anti PTEN Ab.



This reagent is of particular interest, as alanine mutations of the T382 or T383 residues have been associated with increased efficacy in cell-cycle arrest assays, accelerated degradation, increased membrane affinity, and increased activity in regulating migration [22, 24, 29-33]. Furthermore, this reagent would allow the detection of the unphosphorylated form of PTEN and used to purify and study the regulation of the PAC. These reagents will have precisely the same utility as phosphospecific antibodies when the latter are used to study the appearance of the lower abundance phosphorylated proteins during signaling. The difference here is that the unphosphorylated form is the less abundant regulated isoform that we wish to study.

Generating an immunoaffinity column based on the non-phospho Ab T382/383

It appears that we have succeeded in generating an antiserum that selectively recognizes the unphosphorylated form of PTEN. Moreover, this antiserum is generated against a peptide and the epitope has been mapped through the pre-absorption strategies to fall within residues 381-384.

This allows us to consider the following strategy: immunoaffinity purification of non-phosphorylated PTEN on an anti-T382/T383 antibody column followed by peptide elution. We will use similar conditions and protocol to generate the T382/383 column, as was successfully used for the C54-DSS-Protein A column. First, we will cross-link anti-T382/T383 to protein-A beads using the IgG Plus Orientation kit and cross-link with DSS (Pierce Biotechnology). The new column will be tested for its coupling efficiency and then small aliquots of the column will be used to determine the PTEN binding capacity using purified GST-PTEN or whole cell extract, as was done for the C54-DSS-Protein a column.

Caveats: It is clear that non-phospho antibodies have limited discriminatory power. We have discussed non-phosphorylated PTEN as if it is a single entity. Of course, this may not be true and individual phosphorylation sites may be regulating specific cell phenotypes. Gel filtration of the rat-liver protein extract showed that the most abundant monomeric form of PTEN is heavily phosphorylated while PTEN present in the PAC is nearly completely unphosphorylated [25].

Another caveat is that the PAC or unphosphorylated PTEN may be enriched in the membrane fraction, and if this is found in our up scale purification we will focus on isolation of the PAC from this cellular compartment.

Specific Aim 2: Identification of the PTEN associated proteins by Tandem Affinity Purification (TAP) purification.

In addition to the chromatographic sequential purification the Tandem Affinity Purification (TAP) system will be used to purify putative PAC proteins. The TAP system has proved itself to be a useful tool in large-scale identification of protein complexes in yeast and mammalian systems [34-36]. This is a two-step purification system based on a retroviral pZome vectors (generated from pBabe), that enables near endogenous levels of fusion proteins to be expressed in cell cultures. In pZome there is a leader sequence that contains the Protein A gene, a sequence for the TVE protease digestion and the gene for calmodulin binding protein (CBP). The TAP method produces a fusion protein in which a calmodulin binding domain and a protein-A fusion are generated. Thus proteins tagged with these fusions can be isolated on IgG beads, cleaved off with a TEV protease, re-isolated on Calmodulin-coated beads, and eluted by calcium ions.

We have generated the appropriate plasmid that expresses TAP-PTEN. Our goal is to determine whether this PTEN fusion protein can participate in the PAC. TAP fusion proteins are expressed in a retroviral system. To prepare the virus, 293-T cells were transfected with pZome-TAP-PTEN wild type construct and the packaging construct. The mature virus supernatant was collected and 786-O and PC3, both PTEN null cell lines, were infected. The cell lines were treated with Puromycin and selected for stable clones. To detect the ectopic expression of TAP-PTEN fusion protein the cells resistant to Puromycin were harvested, lysed and separated by gel electrophoresis, to be detected by immunoblot.

We were unable to detect the expression of the TAP-PTEN fusion protein in the cells, although the cells were resistant to the antibiotics. Ectopic expression of wild-type PTEN in PTEN-null tumor cell lines results either in G1 growth arrest, anoikis, or apoptosis, depending on the cell type [37-40]. Introducing the TAP-PTEN fusion protein into 786-O and PC3, both PTEN null cell lines resulted in cell growth arrest and delay, (specially in the case of PC3 cells), and loss of the PTEN expression.

We have chosen the TAP system as a tool that will allow us to introduce and evaluate the function of PTEN mutants and in particular the non-phosphorylated form PTEN^{A4}, in which S380, T382, T383 and S385 residues were substituted with alanine, that, based on our biochemical purification strategy is expected to be integrated into the PAC. If in fact, TAP-PTEN^{A4} can participate in the PAC and as predicted we will proceed as planned with TAP-purification.

Alternative approach: We will try to express the TAP-PTEN fusion protein in a cell line expressing endogenous PTEN. The exogenous TAP-PTEN is readily distinguished by size from the endogenous. Hopefully expression of TAP-PTEN in these cells will not engage in cell cycle arrest or apoptosis. If this approach will fail we will re-clone the ProteinA-CBP-PTEN fusion protein under a Tetracycline conditional expression using the "Gateway" cloning system (Invitrogen).

Once expressed, TAP-PTEN extracts will be pooled from multiple plates of cells transfected with the relevant plasmid or control vector only. Extracts will be separated by gel-filtration on a Superose 6 column and eluted fractions will be immunoblotted with an anti-Pan-PTEN antibody.

Specific Aim 3: To determine whether the interactions of PTEN with components of the PAC are necessary for PTEN function as tumor suppressor gene.

The central question of this aim is whether the identified associated proteins influence PTEN tumor suppressor activity. The work anticipated under this aim, will follow the completion of aim 1 and 2.

Key Research Accomplishments:

1. To date, we have optimized the conditions for scale up of our biochemical purification of the PAC.
2. We have generated a functional high affinity immuno-column. The C54-DSS-Protein A is a stable column that maintains the ability to bind and precipitate the endogenous PTEN. We anticipate that this column will be a useful tool as an additional step in our biochemical purification strategy.
3. We have been successful in generating an antiserum that selectively recognizes the unphosphorylated form of PTEN.

Reportable outcomes

1. Rabinovsky R, Levi H, Dripkin R, Duke-Cohen J, Sellers WR. Functional analysis of the PTEN tumor suppressor gene.* DF/HCC prostate/Renal Cancer SPORE Retreat, 2004 October. * The Poster was awarded the certificate of “Poster session Honorable Mention” of the retreat.
2. anti unphosphorylated PTEN antibodies: T382/383; AD2; AD3; AD4.

Conclusions

We have generated innovative and unique tools that will contribute to our success to purify the PAC.

As an additional and significant step to our biochemical purification strategy we generated an immunoaffinity column, based on highly potent C54 Ab covalently coupled to Protein A agarose beads. C54 Ab is a pan antibody with high affinity and specificity for both phosphorylated and unphosphorylated endogenous forms of PTEN. The C54-DSS-Protein A is a stable column that maintains all the qualities of the C54 Ab, i.e. the high ability to bind and precipitate the endogenous PTEN.

To study and purify the lower abundance of unphosphorylated form of PTEN present in the PAC, we generated the T3832/383, AD1, AD2, AD3, AD4 Abs that selectively recognize the unphosphorylated form of PTEN. Not only these reagents would allow the detection of the unphosphorylated form of PTEN in the PAC, but we also can use them to study the regulation of the transition between phosphorylated and non-phosphorylated forms PTEN. These reagents will have precisely the same utility as phosphospecific antibodies when the latter are used to study the

appearance of the lower abundance phosphorylated proteins during signaling. The difference here is that the unphosphorylated form is the less abundant regulated isoform that we wish to study. This reagent is of particular interest as alanine mutations of the T382 or T383 has been associated with increased efficacy in cell-cycle arrest assays, accelerated degradation, increased membrane affinity, and increased activity in regulating migration [22, 24, 29-33].

References

1. Jemal, A., et al., *Cancer statistics, 2004*. CA Cancer J Clin, 2004. **54**(1): p. 8-29.
2. Li, J., et al., *PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer*. Science, 1997. **275**(5308): p. 1943-7.
3. Li, D.M. and H. Sun, *TEP1, encoded by a candidate tumor suppressor locus, is a novel protein tyrosine phosphatase regulated by transforming growth factor beta*. Cancer Res, 1997. **57**(11): p. 2124-9.
4. Steck, P.A., et al., *Identification of a candidate tumour suppressor gene, MMAC1, at chromosome 10q23.3 that is mutated in multiple advanced cancers*. Nat Genet, 1997. **15**(4): p. 356-62.
5. Sellers, W.R. and C.A. Sawyers, *Somatic Genetics of Prostate Cancer: oncogenes and Tumor Suppressors*, in *Prostate Cancer Principles and Practice*, P.W. Kantoff, Editor. 2002, Lippincott Williams & Wilkins: Philadelphia.
6. Liu, W., et al., *PTEN/MMAC1 mutations and EGFR amplification in glioblastomas*. Cancer Res, 1997. **57**(23): p. 5254-7.
7. Liu, T.C., et al., *Mutation analysis of PTEN/MMAC1 in acute myeloid leukemia*. Am J Hematol, 2000. **63**(4): p. 170-5.
8. Dicuonzo, G., et al., *Colorectal carcinomas and PTEN/MMAC1 gene mutations*. Clin Cancer Res, 2001. **7**(12): p. 4049-53.
9. Birck, A., et al., *Mutation and allelic loss of the PTEN/MMAC1 gene in primary and metastatic melanoma biopsies*. J Invest Dermatol, 2000. **114**(2): p. 277-80.
10. Bayascas, J.R., et al., *Hypomorphic Mutation of PDK1 Suppresses Tumorigenesis in PTEN(+/-) Mice*. Curr Biol, 2005. **15**(20): p. 1839-46.
11. Suzuki, H., et al., *Interfocal heterogeneity of PTEN/MMAC1 gene alterations in multiple metastatic prostate cancer tissues*. Cancer Res, 1998. **58**(2): p. 204-9.
12. Feilotter, H.E., et al., *Analysis of PTEN and the 10q23 region in primary prostate carcinomas*. Oncogene, 1998. **16**(13): p. 1743-8.
13. Hermans, K.G., et al., *Loss of a small region around the PTEN locus is a major chromosome 10 alteration in prostate cancer xenografts and cell lines*. Genes Chromosomes Cancer, 2004. **39**(3): p. 171-84.
14. Maehama, T., G.S. Taylor, and J.E. Dixon, *PTEN and myotubularin: novel phosphoinositide phosphatases*. Annu Rev Biochem, 2001. **70**: p. 247-79.
15. Sansal, I. and W.R. Sellers, *The biology and clinical relevance of the PTEN tumor suppressor pathway Sansal I and Sellers WR. Journal of Clinical Oncology 2003 (in press)*. J Clin Oncol, 2004. (**in press**)(July).
16. Suzuki, A., et al., *High cancer susceptibility and embryonic lethality associated with mutation of the PTEN tumor suppressor gene in mice*. Curr Biol, 1998. **8**(21): p. 1169-78.

17. Di Cristofano, A., et al., *Pten is essential for embryonic development and tumour suppression*. Nat Genet, 1998. **19**(4): p. 348-55.
18. Podsypanina, K., et al., *Mutation of Pten/Mmac1 in mice causes neoplasia in multiple organ systems*. Proc Natl Acad Sci U S A, 1999. **96**(4): p. 1563-8.
19. Maehama, T. and J.E. Dixon, *The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate*. J Biol Chem, 1998. **273**(22): p. 13375-8.
20. Vazquez, F. and W.R. Sellers, *The PTEN tumor suppressor protein: an antagonist of phosphoinositide 3-kinase signaling*. Biochim Biophys Acta, 2000. **1470**(1): p. M21-35.
21. Lee, J.O., et al., *Crystal structure of the PTEN tumor suppressor: implications for its phosphoinositide phosphatase activity and membrane association*. Cell, 1999. **99**(3): p. 323-34.
22. Das, S., J.E. Dixon, and W. Cho, *Membrane-binding and activation mechanism of PTEN*. Proc Natl Acad Sci U S A, 2003. **100**(13): p. 7491-6.
23. Walker, S.M., et al., *The tumour suppressor function of PTEN requires an N-terminal lipid binding motif*. Biochem J, 2004. **Pt**.
24. Vazquez, F., et al., *Phosphorylation of the PTEN tail regulates protein stability and function*. Mol Cell Biol, 2000. **20**(14): p. 5010-8.
25. Vazquez, F., et al., *Phosphorylation of the PTEN tail acts as an inhibitory switch by preventing its recruitment into a protein complex*. J Biol Chem, 2001. **276**(52): p. 48627-30.
26. Mehenni, H., et al., *LKB1 interacts with and phosphorylates PTEN: a functional link between two proteins involved in cancer predisposing syndromes*. Hum Mol Genet, 2005. **14**(15): p. 2209-19.
27. Okumura, K., et al., *Cellular transformation by the MSP58 oncogene is inhibited by its physical interaction with the PTEN tumor suppressor*. Proc Natl Acad Sci U S A, 2005. **102**(8): p. 2703-6.
28. Kotelevets, L., et al., *Implication of the MAGI-1b/PTEN signalosome in stabilization of adherens junctions and suppression of invasiveness*. Faseb J, 2005. **19**(1): p. 115-7.
29. Torres, J. and R. Pulido, *The tumor suppressor PTEN is phosphorylated by the protein kinase CK2 at its C terminus. Implications for PTEN stability to proteasome-mediated degradation*. J Biol Chem, 2001. **276**(2): p. 993-8.
30. Torres, J., et al., *Phosphorylation-regulated cleavage of the tumor suppressor PTEN by caspase-3: implications for the control of protein stability and PTEN-protein interactions*. J Biol Chem, 2003. **278**(33): p. 30652-60.
31. Tolkacheva, T., et al., *Regulation of PTEN binding to MAGI-2 by two putative phosphorylation sites at threonine 382 and 383*. Cancer Res, 2001. **61**(13): p. 4985-9.
32. Raftopoulou, M., et al., *Regulation of cell migration by the C2 domain of the tumor suppressor PTEN*. Science, 2004. **303**(5661): p. 1179-81.
33. Georgescu, M.M., et al., *The tumor-suppressor activity of PTEN is regulated by its carboxyl-terminal region*. Proc Natl Acad Sci U S A, 1999. **96**(18): p. 10182-7.
34. Rigaut, G., et al., *A generic protein purification method for protein complex characterization and proteome exploration*. Nat Biotechnol, 1999. **17**(10): p. 1030-2.
35. Bouveret, E., et al., *A Sm-like protein complex that participates in mRNA degradation*. Embo J, 2000. **19**(7): p. 1661-71.

36. Pijnappel, W.W., et al., *The S. cerevisiae SET3 complex includes two histone deacetylases, Hos2 and Hst1, and is a meiotic-specific repressor of the sporulation gene program*. Genes Dev, 2001. **15**(22): p. 2991-3004.
37. Wu, X., et al., *The PTEN/MMAC1 tumor suppressor phosphatase functions as a negative regulator of the phosphoinositide 3-kinase/Akt pathway*. Proc Natl Acad Sci U S A, 1998. **95**(26): p. 15587-91.
38. Myers, M.P., et al., *The lipid phosphatase activity of PTEN is critical for its tumor suppressor function*. Proc Natl Acad Sci U S A, 1998. **95**(23): p. 13513-8.
39. Lu, Y., et al., *The PTEN/MMAC1/TEP tumor suppressor gene decreases cell growth and induces apoptosis and anoikis in breast cancer cells*. Oncogene, 1999. **18**(50): p. 7034-45.
40. Davies, M.A., et al., *Regulation of Akt/PKB activity, cellular growth, and apoptosis in prostate carcinoma cells by MMAC/PTEN*. Cancer Res, 1999. **59**(11): p. 2551-6.
41. Puig, O., et al., *The tandem affinity purification (TAP) method: a general procedure of protein complex purification*. Methods, 2001. **24**(3): p. 218-29.
42. Gavin, A.C., et al., *Functional organization of the yeast proteome by systematic analysis of protein complexes*. Nature, 2002. **415**(6868): p. 141-7.

ABBREVIATIONS

Ab; antibody

Abs; antibodies

C2D; C2 domain

CBP; calmodulin binding protein

CK2; Casein Kinase-2

FT; flow through

IP; immunoprecipitation

MS; mass spectrometry

NES; nuclear export sequence

NLS; nuclear localization sequence

PAC; PTEN associated complex

PDZbd; PDZ binding domain

PI3K; Phosphatidylinositol-3-kinase

PI3,4,5P3/PIP3; phosphoinositide 1-3,4,5-triphosphate

PI3,4P2; phosphoinositide 1-3,4,-bisphosphate

PTEN; Phosphatase with homology to Tensin located on chromosome 10

TAP; Tandem affinity purification system

WT; wild type