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Determinants of the tumor suppressor INPP4B protein and lipid phosphatase activities

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ABSTRACT

The tumor suppressor INPP4B is an important regulator of phosphatidyl-inositol signaling in the cell. Reduced INPP4B expression is associated with poor outcomes for breast, prostate, and ovarian cancer patients. INPP4B contains a CX_5R catalytic motif characteristic of dual-specificity phosphatases, such as PTEN. Lipid phosphatase activity of INPP4B has previously been described. In this report we show that INPP4B can dephosphorylate para-nitrophenyl phosphate (pNPP) and 6,8-difluoro-4-methylumbelliferyl (DiFMUP), synthetic phosphotyrosine analogs, suggesting that INPP4B has protein tyrosine phosphatase (PTP) activity. Using mutagenesis, we examined the functional role of specific amino acids within the INPP4B C_{842}KSAKDR catalytic site. The K843M mutant displayed increased pNPP hydrolysis, the K846M mutant lost lipid phosphatase activity with no effect on PTP activity, and the D847E substitution ablated PTP activity and significantly reduced lipid phosphatase activity. Further, we show that INPP4B but not PTEN is able to reduce tyrosine phosphorylation of Akt1 and both the lipid and PTP activity of INPP4B likely contribute to the reduction of Akt1 phosphorylation. Taken together our data identified key residues in the INPP4B catalytic domain associated with lipid and protein phosphatase activities and found a robust downstream target regulated by INPP4B but not PTEN.

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1. Introduction

Deregulation of phosphatidyl inositol signaling plays an important role in various disorders. These pathways are stimulated by phosphatidyl 3, 4, and 5 kinases (PI3K, PI4K, PI5K) and inhibited by lipid phosphatases such as PTEN, INPP4B, and SHIP [1]. These phosphatases dephosphorylate the phosphatidyl inositol ring on the 3rd, 4th, and 5th position respectively. Loss of INPP4B is a poor prognostic factor for breast, ovarian, and prostate cancers [2–4]. Similar to PTEN, INPP4B contains a dual specificity phosphatase (DuSP) domain with a characteristic DuSP CX_5R motif, C_{842}KSAKDR$_{848}$. Residue C_{842} is required for INPP4B enzymatic activity [3,5]. There are three known INPP4B substrates: phosphatidylinositol 3,4-bisphosphate (PI(3,4)P2), phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2), and inositol 1,3,4-trisphosphate (Ins(1,3,4)P3) [5]. Substrates of INPP4B lipid phosphatase activity are important second messengers in pathways regulating cellular proliferation and metastasis and have been implicated in prostate cancer progression [6,7]. PI(4,5)P2 is a substrate for both INPP4B and phospholipase C, an enzyme implicated in cellular motility and tumor dissemination in cancer [8,9]. PI(3,4)P2 is present at low levels on the cell membrane and accumulates at the sites of invadopodia [10], cellular appendages responsible for focal pericellular proteolysis of the extracellular matrix necessary for cellular invasion and metastasis [11]. PI(3,4)P2 binds to the pleckstrin homology domains of Akt and PDK1, recruiting them to the plasma membrane, leading to Akt phosphorylation on T308 and S473, and the activation of Akt. In addition to T308 and S473, tyrosine (Y) phosphorylation of Akt is emerging as an important regulatory mechanism for Akt signaling. Recruitment to the cell membrane and activation of Akt are regulated by its phosphorylation on several Y residues [12–14]. Elevated Y176 phosphorylation promotes Akt recruitment to the plasma membrane and increases

Abbreviations: PI3K, by phosphatidyl 3-kinase; PI4K, phosphatidyl 4-kinase; PI5K, phosphatidyl 5-kinase; INPP4B, inositol polyphosphate 4-phosphatase type II; PTP, protein tyrosine phosphatase; pNPP, para-nitrophenyl phosphate; DifMUP, 6,8-difluoro-4-methylumbelliferyl phosphate; PTEN, phosphatase and tensin homolog; DuSP, dual specificity phosphatase; PI(34)P2, phosphatidylinositol 3,4-bisphosphate; PI(45)P2, phosphatidylinositol 4,5-bisphosphate; Ins(13,4)P3, inositol 1,3,4-trisphosphate.

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phosphorylation of the Akt residues T308 and S473 [12]. In breast cancer patients, increased levels of Y176 phosphorylation correlate with poor overall survival [13].

In this report, we show that INPP4B can dephosphorylate para-nitrophenyl phosphate (pNPP) and 6,8-difluoro-4-methylumbelliferyl (DifMUP), analogs of phosphotyrosine, suggesting that INPP4B has protein tyrosine phosphatase (PTP) activity. Mutation of four key residues in the active site identified distinct residues that contribute to INPP4B lipid and protein phosphatase activities. Finally, we found that INPP4B reduced Akt1 phospho-Y levels in Hek293T cells. Co-expression of Akt1 with wild-type (WT) and mutant INPP4B proteins revealed that both lipid and PTP activities contribute to downregulation of Akt1 phospho-Y levels. PTEN overexpression did not reduce phosphorylation of Akt1 on Y residues, suggesting distinct downstream signaling for INPP4B and PTEN tumor suppressors.

2. Materials and methods

2.1. Constructs

p3xFLAG-CMV-10-INPP4B-C842S, p3xFLAG-CMV-10-INPP4B-K843M, p3xFLAG-CMV-10-INPP4B-K846M, and p3xFLAG-CMV-10-INPP4B-D847E were generated by site directed mutagenesis of p3xFLAG-CMV-10-INPP4B [4]. Site-directed mutagenesis was conducted using the Stratagene QuikChange II XL Site-Directed Mutagenesis Kit from Agilent Technologies (La Jolla, CA) and the primers used are listed in Table 1. FLAG-HA-Akt1 construct was a kind gift of William Sellers (Addgene plasmid 9021) [15]. FLAG-PTEN was generated by PCR amplification GACAAGCTT GCGGCCGCAACAGCCA, GATGAATTCGCGGC CGCTCAGACTTT) of *PTEN* from DU145 cells and cloned into p3xFLAG-CMV-10 (Sigma–Aldrich), (St. Louis, MO). Constitutively active *Src* was obtained by PCR amplification (ACCCAAGCTG GCTAGCACCATGGG TAGCAACAAGA, AAGTTTAAACGCTAGCCTAGTACTGGGGCTCGGT) of *Src* [16] from PC-3 cells and insertion into pCR3.1. All cloning was performed using the Infusion HD cloning kit from Clontech (Mountain View, CA). All expression constructs were fully sequenced.

2.2. Cell culture and reagents

Hek293T cell line was purchased from ATCC (Manassas, VA) and maintained in Dulbecco's Modified Eagle Medium (DMEM), 5% FBS, and 1% penicillin–streptomycin. Media and antibiotics were purchased from Life Technologies (Carlsbad, CA). 3xFLAG peptide was purchased from Sigma–Aldrich. PI(3,4)P2 DiC8, PI(4,5)P2 DiC8, Ins(1,3,4)P3 and Malachite Green were purchased from Echelon Biosciences (Salt Lake City, UT). pNPP was obtained from New England Bio Labs (Ipswich, MA).

2.3. Protein expression and immunoprecipitation

Hek293T cells were transfected with 20 μg of p3xFLAG-CMV10-WT and indicated mutant expression constructs using Lipofectamine 2000 reagent from Life Technologies. Three days later cell lysates were prepared as previously described [4]. Cleared lysates were immunoprecipitated using EZview Red ANTI-FLAG M2 Affinity Gel from Sigma–Aldrich (St. Louis, MO). Affinity agarose gel beads were washed with ice cold TBS and 100 mM Tris–HCl pH 8.0. Proteins were eluted with 100 μl of 3xFLAG elution buffer [100 mM Tris–HCl pH 8.0, 1 mM EDTA, 30 mM NaCl, 0.01% Triton-X 100, 10 mM DTT, 150 ng/μL 3xFLAG peptide]. Eluted protein was used for lipid phosphatase assay, protein phosphatase assay, and western blotting.

Table 1
INPP4B active site mutations and associated primer sequences used in this study.

INPP4B mutation	Primers
C842S	5′-tggtattcgtttcacctgtagtaaaagtgccaaagacag-3′ 5′-ctgtctttggcacttttactacaggtgaaacgaatacca-3′
K843M	5’-ctgaatggtattcgtttcacctgttgtatgagtgccaaagacagg-3′ 5′-cctgtctttggcactcatacaacaggtgaaacgaataccattcag-3′
K846M	5′-acctgttgtaaaagtgccatggacaggacatcgatgtcag-3′ 5′-ctgacatcgatgtcctgtccatggcacttttacaacaggt-3′
D847E	5′-acctgttgtaaaagtgccaaagagaggacatcgatg-3′ 5′-catcgatgtcctctctttggcacttttacaacaggt-3′

2.4. Western blot analysis

Approximately 2% of eluted proteins were resolved on 7.5% SDS–PAGE and transferred using an iBlot Western Blotting transfer device (Invitrogen, Carlsbad, CA). Immunoblotting was performed using the following antibodies: anti-FLAG M2 (1:5000) (Sigma–Aldrich), PTEN (1:1000), phospho-Tyrosine (1:1000) (Cell Signaling Technology, Beverly, MA). Images were visualized with SuperSignal West Pico chemiluminescent substrate from Thermo Scientific (Pittsburg, PA) and Kodak Gel Logic 2200 imaging system and Carestream software (Carestream, Rochester, NY).

2.5. Lipid phosphatase assay

Eighteen μL of eluates were mixed with 2 μL of 1 mM PI(3,4)P2, PI(4,5)P2, or Ins(1,3,4)P3 and incubated for five minutes at 37 °C. One hundred μL of Malachite Green was added to each reaction or lysis buffer for calorimetric detection of free phosphate groups. Reactions were then measured at 660 nm using a FLUOstar Omega plate reader (BMG Labtech, Durham, NC).

2.6. Phosphotyrosine phosphatase assay

Thirty μL of eluates were diluted with 100 mM Tris–HCl pH 8.0 and pNPP added to a final concentration of 100 mM. Reactions were incubated at 25 °C and absorbance at 405 nm measured at two minute intervals using a FLUOstar Omega plate reader. Alternatively, the same amount of eluate was incubated with 200 μM 6,8-difluoro-4-methylumbelliferyl phosphate (DiFMUP). Excitation/emission of the substrate, DiFMU, was measured every 2 min at 358/452 nm on a FLUOstar Omega plate reader.

For determination of the Michaelis constant, km, 70 μL of WT INPP4B eluate or elution buffer as a background control were incubated at 25 °C with 10 mM, 25 mM, 50 mM, 75 mM, and 100 mM of pNPP in a total reaction volume of 100 μL. Absorbance at 405 nm was recorded with a FLUOstar Omega plate reader. km was calculated from five independent experiments using Michaelis–Menten steady state kinetics.

3. Results

3.1. INPP4B has PTP activity

Since the INPP4B catalytic site is characteristic of DuSP, we tested whether INPP4B has PTP activity. 3xFLAG-WT INPP4B and a phosphatase dead mutant, 3xFLAG-C842A, were expressed in Hek293T cells, purified by immunoprecipitation, and incubated with pNPP. WT-INPP4B could dephosphorylate pNPP, but not the phosphatase dead mutant, C842A, ruling out coimmunoprecipitated phosphatase activity (Fig. 1A). INPP4B protein phosphatase activity was confirmed using 6,8-difluoro-4-methylumbelliferyl (DiFMUP) (Fig. 1B). To evaluate substrate affinity we incubated immunoprecipitated INPP4B with pNPP at 10 mM, 25 mM,

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