Published in final edited form as:

Assay Drug Dev Technol. 2007 October ; 5(5): 645–653.

High yield expression of serine/threonine protein phosphatase type 5, and a fluorescent assay suitable for use in the detection of catalytic inhibitors

Li Ni^{*}, Mark S. Swingle^{*}, Austin C.B Bourgeois, and Richard E. Honkanen[¶]

Department of Biochemistry and Molecular Biology, College of Medicine, University of South Alabama, Mobile, AL 36688

Abstract

Protein phosphatase type 5 (PP5) belongs to the PPP-family of serine/threonine protein phosphatases and is expressed in most, if not all, human tissues. Although the physiological roles played by PP5 are not yet clear, PP5 is found in association with several proteins that influence intracellular signaling networks initiated by hormones (i.e. glucocorticoids) or cellular stress (i.e. hypoxia, oxidative stress). Recently, studies conducted with siRNA and antisense oligonucleotides indicate that PP5 plays an important role in the regulation of stress-induced signaling cascades that influence both cell growth and the onset of apoptosis. Therefore, the identification of small molecule inhibitors of PP5 is desired for use in studies to further define the biological/pathological roles of PP5. Such inhibitors may also prove useful for development into novel antitumor agents. Here we describe methods to express and purify large amounts of biologically active PP5c, an inhibitor-titration based assay to determine the amount of PP5 in solution, and a fluorescent phosphatase assay that can be used to screen chemical libraries and natural extracts for the presence of catalytic inhibitors.

Keywords

phosphatase; PP5; PPP5; DiFMUP; 6; 8-difluoro-4-methylumbelliferyl phosphate; umbelliferone; fluorescent; assay

Introduction

Reversible phosphorylation plays a key role in the regulation of many cellular signaling networks that control cell growth, differentiation, and programmed cell death (apoptosis). Phosphorylation occurs principally on serine, threonine and tyrosine residues, and for nearly all proteins the state of phosphorylation at any point in time reflects the combined actions of both protein kinases and protein phosphatases. Because reversible phosphorylation controls many signaling networks that regulate cell growth, a substantial effort has been devoted to the identification of inhibitors that act on “key” regulatory protein kinases. Indeed, several kinase inhibitors have already been developed for the medical management of human cancer (i.e. trastuzumab, imatinib, bevacizumab, gefitinib and cetuximab). In contrast, little progress has been made in the development of agents that regulate the actions of “key” protein phosphatases.

[¶]Please address correspondence to: Dr. Richard E. Honkanen, Department of Biochemistry and Molecular Biology, MSB 2362, University of South Alabama, Mobile AL 36688, phone 251 460-6859, email rhonkanen@jaguar1.usouthal.edu.

^{*}Both authors contributed equally to this manuscript

The lag in development of inhibitors that act on ser/thr protein phosphatases is due, in part, to difficulties associated with the expression of biologically active recombinant phosphatases and the identification of “key” phosphatases that control growth, differentiation or development. However, the advent of siRNA and antisense oligonucleotides capable of specifically suppressing individual phosphatases has greatly aided our understanding of the biological roles of ser/thr protein phosphatases. From such studies serine/threonine protein phosphatase type 5 (PP5) has emerged as an important regulator of hormone/stress-induced signaling networks that propagate cellular responses controlling growth and stress-induced apoptosis [1–7]. Nonetheless, the development of small molecule inhibitors of PP5 has been hampered because human PP5 expressed in bacteria has very low catalytic activity, and the [³²P]based phosphatase assays [8–10] commonly used to measure activity are not easily adaptable to high throughput screening assays. Here we describe the development of methods useful for the expression of large amounts of biologically active PP5 in *E. coli*, and a fluorescent phosphatase assay suitable for use in the detection of inhibitors.

Materials and methods

Materials

DiFMUP (6,8-difluoro-4-methylumbelliferyl phosphate), MUP (4-methylumbelliferyl phosphate), and tobacco etch virus (TEV) protease were obtained from Invitrogen. Histone (type-2AS) and protein kinase A (PKA) (3':5' – cyclic AMP dependent) were obtained from Sigma (St. Louis, MI). [g-³²P]-ATP was obtained from New England Nuclear. Okadaic acid, microcystin-LR and para-nitrophenyl phosphate (pNPP) were obtained from CalBichem.

Heterologous expression of PP5c

cDNA comprising the complete coding region of human PP5 (Accession **NM 006247**) was subcloned into pBlueScript. A prokaryotic expression vector for a N-terminally truncated mutant (residues Ser¹⁶⁹ to Met⁴⁹⁹) was then constructed by PCR amplification of this region of cDNA with primers complementary to 15 nucleotides in PP5. The 5' primer also incorporated a 5' EcoRI restriction site and a methionine start codon followed by a sequence encoding a hexahistidine affinity tag (MGGHHHHHHG). The 3' primer contained a PstI restriction site. Subsequent to PCR, the amplified fragment was subcloned into pKK223-3 (Pharmacia). Using a QuickChange Site-directed Mutagenesis Kit (Stratagene), a sequence encoding the peptide NLYFQGA (which forms part of a Tobacco Etch Virus protease recognition sequence) was inserted between the codon for Glu¹⁶⁸ and the codon for Ser¹⁶⁹ in the PP5 coding sequence. This insert was then subcloned into the pMal2cE vector (New England Biolabs). This new construct encodes an N-terminal Maltose Binding Protein (MBP) fusion with the catalytic domain of PP5 (residue Ser¹⁶⁹ to residue Met⁴⁹⁹; designated PP5c), plus a linker sequence (MGGHHHHHHGSSVVDSLDIENLYFQGA) between the fusion partners, which contains a hexahistidine affinity tag, a spacer sequence, and a tobacco etch virus (TEV) protease cleavage site (Figure 1). The TEV cleavage site is immediately N-terminal to the PP5 catalytic domain, and two vector-derived, non- native residues (codons encoding Gly-Ala were added by the introduction of an EheI/NarI restriction site into the expression plasmid to aid cloning) are left at the N-terminus of PP5c after protease digestion.

Expression and purification of PP5c

The MBP-PP5c fusion protein was expressed in transformed BL21(DE3) Tuner pLacI cells (Novagen). Cultures (27 L total) were grown in LB media containing 0.2% glucose, 1 mM MnCl₂, 200 µg/ml ampicillin and 34 µg/ml chloramphenicol. Cultures were grown with shaking (250 rpm) at 37°C to an OD₆₀₀ of ~2, then equilibrated to 25°C and induced for 25–27 h with IPTG at 300 µM. At the end of the induction period, EDTA was added (1 mM final

concentration) and the cells were collected by centrifugation at 5000 g for 20 minutes at 4°C. Cell pellets, totaling ~160 grams, were flash-frozen in liquid nitrogen and stored at -80°C.

The following purification procedures were carried out at ~4°C unless otherwise specified. The cells pellets were placed in a blender with 320 ml of chilled 2X lysis buffer (100 mM tris, pH 7.5 at 23°C, 4 mM EDTA and 0.2% β -mercaptoethanol), 160 ml of chilled deionized water and 320 μ l of a 1 M stock of PMSF (in DMF). The cells were partially thawed, puréed into a thick slush and allowed to thaw for another 30 minutes. Twenty ml of 33 mg/ml lysozyme and 320 μ l of 1 M PMSF were then added and mixed into the cell suspension with several 5-second bursts of the blender on its lowest setting. Lysozyme digestion was allowed to proceed for ~90 minutes with occasional gentle agitation. At this point, 10.8 ml of 30% Brij-35 was added to the suspension dropwise with stirring (final concentration of ~0.5%), followed by 320 ml of chilled 1X lysis buffer, 5 ml of 1 M $MgCl_2$ and DNase (final concentration of 10 μ g/ml), these last components being mixed in with short bursts of the blender. The crude lysate was then sonicated on ice with ten 15 W bursts of ~60 seconds duration and the DNase digestion was allowed to proceed for an additional 30 minutes before being stopped by the addition of 5 ml of 1 M EDTA. 100 μ l of 1 M PMSF was then added and the lysate was cleared by centrifugation at 20000 g for 1 h. The cleared lysate was diluted 2-fold with chilled fractionation buffer (50 mM tris, pH 7.5 at 23°C, 0.1 mM EDTA, 0.2 mM PMSF) and subjected to a 30–70% ammonium sulfate fractionation. Pellets from the ammonium sulfate fractionation were resuspended in 400 ml of chilled Ni-column loading buffer (20 mM imidazole, pH 8.0 at 23°C, 500 mM NaCl, 0.1 mM EDTA, 0.1 mM PMSF and 0.05% β -mercaptoethanol) and dialyzed against 4 L of the same buffer. The dialysate was cleared by centrifugation at 20000 g for 30 minutes and loaded onto a 100 ml (2.5cm i.d.) column of IDA-sepharose Fast Flow media (Pharmacia) charged with Ni^{2+} and pre-equilibrated with buffer A (20 mM imidazole, pH 8.0 at 23°C, 500 mM NaCl). The column was subsequently washed with 2.4 L of buffer A, and eluted (at 5 ml/min) with a step gradient: 1 L at 6.3% buffer B (500 mM imidazole, pH 8.0 at 23°C, 500 mM NaCl), then 500 ml at 16.7% buffer B, then 500 ml at 100% buffer B. 14 ml fractions were collected. The bulk of the pNPP phosphatase activity was found in the 50 mM imidazole peak (i.e. 6.3% buffer B/93.7% buffer A). SDS-PAGE of fractions indicated that the 50 mM peak had the greatest overall purity and that the first five fractions contained large amount of a high molecular weight contaminant. Thus, fractions 6–45 were pooled and dialyzed against buffer C (50 mM tris, pH 7.5 at 23°C, 0.1 mM EDTA, 0.1% β -mercaptoethanol). Dialysate (550 ml) was collected, equilibrated to room temperature, and TEV protease digestion was initiated with the addition of 5.5 ml of 100 mM DTT, 0.22 ml of 1 M EDTA, 10000 U of TEV protease (Invitrogen), and 0.55 ml of 100% NP-40. Proteolysis continued with gentle stirring at room temperature for 7 h and then for an additional 24 h at 4°C. The TEV protease digest was then cleared by centrifugation at 20000 g, for 20 minutes, then loaded at 10 ml/min onto 30 ml of Q-sepharose High Performance media (Pharmacia) pre-equilibrated with buffer C at 4°C. The Q-sepharose was washed with buffer C until the A280 fell to ~0.05, then washed with 100 ml of 5% buffer D (50 mM tris, pH 7.5 at 23°C, 0.1 mM EDTA, 0.1% β -mercaptoethanol, and 1 M NaCl)/95% buffer C at 2.5 ml/min (this was collected as fraction 0). Then a 1.25 L gradient from 5% to 35% buffer D was applied to the column. 14 ml fractions were collected, assayed for activity vs. pNPP and analyzed by SDS-PAGE. Fractions 0–3 were pooled and concentrated to ~20 mg/ml using Centriprep-YM10's and Centricon-YM30's (Millipore) according to the manufacturer's instructions. The concentrated protein was then subjected to diafiltration vs. storage buffer (40 mM tris, pH 7.5 at 23°C, 1 mM tris-carboxyethyl phosphine, and 1 mM Na_3N) using Centricon-YM30's (Millipore). The final concentration of protein was determined to be 20.2 mg/ml by Bradford assay (using BSA as a standard) and 300 μ M by microcystin-LR titration. The concentrated protein was then filtered to 0.1 μ m using Microcon spin-filters (Millipore), divided into 50 μ l aliquots, flash-frozen in liquid nitrogen, and stored at -80°C.

Determination of Phosphatase Activity with p-Nitrophenyl Phosphate

p-nitrophenyl phosphate phosphatase (pNPPase) activity was measured using an end-point assay. Aliquots of each sample tested were diluted to 50 ml with water (blanks consisted of 50 ml of water) and reactions were started by the addition of 50 ml of 2x assay buffer (100 mM Tris pH 8.3 at 37 °C, 100 mM MgCl₂, 100 mM pNPP, and 0.2% 2-mercaptoethanol) followed by incubation at 37 °C for 10 minutes. Reactions were stopped by the addition of 100 ml of 1M EDTA at pH 10, and absorbance was measured at 400 nm (stopped reactions with high absorbance can be diluted with water and remeasured). Active fractions (yellow) can also be noted by naked-eye observations. For inhibition studies, inhibitors were incubated with the enzymes for ~10 minutes prior to the addition of substrate.

Preparation of Phosphohistone Substrate and Determination of Phosphatase Activity with Phosphohistone

Phosphohistone, was prepared by the phosphorylation of bovine brain histone with 3':5'-cAMP-dependent protein kinase (PKA) from rabbit muscle in the presence of [³²P]ATP. Histone (type-2AS) was phosphorylated with PKA in a reaction containing 10 mg/ml histone, 2 mg/ml PKA, 6 mCi/ml [³²P]ATP (200 μM ATP), 10 μM cAMP, 40 mM PIPES pH 6.8 (at 37 °C), 7 mM MgCl₂, 0.1 mM EDTA, and 5 mM DTT as described previously [11]. Protein phosphatase activity against phosphoproteins was measured by the quantification of [³²P] liberated from phosphohistone, using an established protocol [8,10–12].

Measurement of PP5 activity using MUP (4-methylumbelliferyl phosphate) and DiFMUP (6,8-difluoro-4-methylumbelliferyl phosphate)

Stock solutions of purified PP5c (75 μM by microcystin-LR titration) were prepared in 10 mM Tris pH 7.5, 0.05% (v/v) β-mercaptoethanol, 50% glycerol, 0.1 mM MnCl₂ and stored at –20 °C. The dephosphorylation reactions were carried out in reaction buffer containing 100 mM MOPS pH 7.0, 0.1% (v/v) β-mercaptoethanol, 0.1 mg/ml BSA, and the indicated amount of DiFMUP or MUP. All reactions were at 30°C unless stated otherwise.

For Km and Vmax determinations, assays were carried out in black flat bottom 96 well plates using reaction volumes of 100 μL, and fluorescence was measured using a SpectraMax M5 microplate reader (Molecular Devices) after starting the reactions by the addition of enzyme (3.0 nM final concentration, estimated from microcystin-LR titration of PP5c stocks) plus buffer to previously dispensed solutions of buffer plus substrate to yield the indicated final concentrations (2.93 μM to 1500 μM) of DiFMUP. Progress curves for the hydrolysis reaction were obtained with 100 μM DiFMUP at several different enzyme concentrations ranging up to 75 nM. These, and all other experiments performed at a constant substrate concentration, were started by the addition of substrate to previously dispensed enzyme solutions prior to reading in the M5. Dose-response studies of okadaic acid and microcystin-LR vs 1.5 nM PP5c were carried out at 30°C with 100 μM DiFMUP in 96 well black flat bottom plates using reaction volumes of 100 μL and the inhibitors were incubated with the enzyme for ~10 minutes prior to the addition of substrate. For Z' value determinations, negative controls contained 100 mM MOPS pH 7.0, 0.1 mM EDTA, 0.1% (v/v) 2-mercaptoethanol, 0.1 mg/ml BSA, and either 100 μM DiFMUP (DiFMUP dissolves readily in DMF, which was used for 100 mM stock solutions were used, thus adding 1% DMF to the assays) or 25 μM MUP. Positive controls contained the components listed above plus 1.5 nM PP5c, and negative controls contained no PP5c. An assay volume of 100 μL was used for MUP and 200 μL was used for DiFMUP. The Z' value [13] for the DiFMUP-based assay was calculated from the rates (mRFU/min) of 95 negative controls and 96 positive controls and the Z' value for the MUP-based assay was determined from 48 negative controls and 48 positive controls. For all studies, reactions were typically monitored for 10–60 minutes at 28–60 sec intervals via fluorescence (em=450 nm, ex=333 nm for MUP and ex=358 nm for DiFMUP). Reaction rates (mRFU/min) were

determined by least-squares analyses of the linear portions of the reaction progress curves (typically the first 10–30 minutes of data, depending upon which concentrations of enzyme and substrate were used).

Determination of the Isosbestic Point of 4-Methylumbelliferone (4-MU)

Socks (2.5 μ M) of 4-MU were prepared in a four-component buffer system that has both a nearly constant ionic strength and a high buffering capacity over a broad pH range (25 mM glycine, 25 mM acetic acid, 25 mM MES, and 50 mM tris) at pH 4.0, 5.8, 6.8, 7.3, 7.8, 8.3, 8.8, 9.8, and 11. Aliquots (400 μ L) of 2.5 μ M 4-MU at each pH were placed in cuvettes and excitation spectra (excitation: 280 – 400 nm in 2 nm steps; emission: fixed at 450 nm) were obtained with a SpectraMax M5 (Molecular Devices) at room temperature. The spectrum at each pH was blanked with buffer of the appropriate pH. The raw data were then exported to Microcal Origin and fit with splines.

Results and discussion

Expression and purification of PP5c

In vivo, PP5 resides predominately in complexes with other proteins. Notably, PP5 has been shown to associate with several proteins that affect signal transduction networks [1–6,14–18]. When PP5 is displaced from these biological partners, phosphatase activity is greatly decreased. Similarly, PP5 purified from natural sources displays little activity when measured with conventional phosphatase assays, and the expression of PP5 in *E. coli* using methods developed for the expression of active PP1 results in a protein with little enzymatic activity. Recently the structure of PP5 has been solved [19,20]. These structural studies indicate that the N-terminal domain of PP5 is connected to the catalytic domain by a flexible 34 amino acid “linker” that allows a tripartite TPR (tetratricopeptide-repeat) motif within the N-terminal domain to adopt a conformation which occludes the active site via the formation of stabilizing interactions with the catalytic domain and an adjacent c-terminal J-helix. This N-terminal/C-terminal interaction is likely responsible for the low observed basal activity of “free” PP5, simply blocking substrate access to the catalytic site. The three TPRs within the N-terminal domain also mediate the association of PP5 with other proteins [16,20–22]. Therefore, when in a complex with other proteins a binding-induced conformational change appears to open the active site to substrates, “activating” PP5 [20,21].

Mutational studies indicate that PP5 can be partially activated by removal of 4–13 amino acids from the C-terminus [16], and a brief treatment of purified PP5 with proteases produces a ~35 kd fragment with phosphatase activity comparable to PP1 and PP2A. This suggested that the deletion of the N-terminal inhibitory-domain should be sufficient to produce a catalytic subunit (PP5c) suitable for use in screening efforts to identify catalytic-inhibitors of PP5. Because initial attempts to express PP5c in bacteria using methods similar to those reported for the structurally related phosphatases produced little active enzyme, other expression systems were tested. The system that proved most effective is the MBP-PP5c system. The fusion of the maltose binding protein to the N-terminus markedly improved the yield of soluble protein, with purified PP5c demonstrating activity against a phosphohistone substrate comparable to that of PP1c and PP2Ac. The inclusion of a TEV cleavage site and a His tag allowed for a simple two column purification scheme, with TEV digestion after the Ni-chelating column allowing the rapid purification of PP5c using ion exchange (Mono-Q) chromatography (Table 1; Figure 1).

Development of a non-radioactive PP5-based assay

Currently the most accurate and reliable methods for measuring the activity of serine/threonine protein phosphatases employs [32 P] labeled phosphoproteins as substrates [8,9,11,12,23].

Although such radiolabeled phosphoproteins closely mimic physiological substrates, they are expensive to produce and the procedures employed are not easily adaptable for use in a high throughput (HTP) screening effort. Chromogenic substrates have also been widely utilized to measure phosphatase activity, and one, para-nitrophenylphosphate (pNPP), is a good substrate for PP5. The dephosphorylation reaction yields para-nitrophenol, which in alkaline conditions is an intense yellow product that can be easily measured at 400–410 nM using a spectrophotometer. However, many natural product extracts are greenish-yellow in color. Accordingly absorbance by colored components contained in the extracts may require individual blanks for each extract. The commonly employed malachite green-based phosphatase assays are also complicated by colored components of natural product extracts and are affected by the presence of inorganic phosphates that may be contained in the samples or solutions.

Due to the limitations of the known chromogenic assays, we tested several fluorogenic phosphoesters to determine if they were suitable substrates for PP5. The hydrolysis of 4-methylumbelliferyl phosphate (MUP) to the fluorescent product 4-methylumbelliferone (MU), is widely used for the detection of alkaline phosphatase activity. Typically, the fluorescence of the MU anion ($\lambda_{\text{ex}}=360\text{nm}$, $\lambda_{\text{em}}=450\text{nm}$) is measured. However, the pKa of 4-methylumbelliferone is 7.8. Therefore, for use near physiological pH, endpoint basification is typically performed to enable maximal detection of the reaction products. However, as seen in Figure 2, protonated MU is also fluorescent when excited with light having a shorter wavelength ($\lambda_{\text{max ex}}=320\text{nm}$) producing a fluorescent signal about 2/3 that obtained at high pH with $\lambda_{\text{ex}}=360\text{nm}$ (λ_{max} of deprotonated MU). The intensity at the isosbestic point ($\sim 333\text{ nm}$) is about half the maximal intensity that can be achieved at high pH with $\lambda_{\text{ex}}=360\text{nm}$ and is constant throughout a wide pH range. Thus, instead of using the traditional endpoint assay, one may perform continuous assays with MUP, measuring fluorescence at $\lambda_{\text{ex}}=320\text{nm}$ at low pH or $\lambda_{\text{ex}}=333$ (isosbestic point) at any pH.

To improve upon MU as a fluorescent probe, Gee et al. [24] systematically fluorinated the nucleus of 4-methylumbelliferone and examined the pKa and photophysical properties (photostability and quantum yield) of the compounds produced. Their data indicate that the addition of fluorine on either side of the 7-hydroxyl group of 4-methylumbelliferone (i.e. the 6- and 8-positions) produced a compound with significantly enhanced photostability and quantum yield. The difluoro derivative also demonstrated a markedly lower pKa of 4.7. These properties suggest 6,8-difluoro-4-methylumbelliferyl phosphate (DiFMUP) should be a good small molecule substrate for PP5, having low detection limits and a product pKa that allows continuous monitoring of the highly fluorescent anion at neutral pH.

Here, in a continuous assay utilizing the same buffer system, both MUP and DiFMUP were found to be efficiently hydrolyzed into 4-methylumbelliferone or 6,8-difluoro-4-methylumbelliferone, respectively, by PP5 [MUP; $K_M 345.8 \pm 38.5 (\mu\text{M})$; DiFMUP $K_M 141 \pm 10.7 (\mu\text{M})$]. When compared to MUP at physiological pH, DiFMUP has a modestly lower K_M (Figure 2D). In order to determine appropriate concentrations of enzyme and substrate to use, reaction progress curves were obtained for a 1) a range of different DiFMUP concentrations at the same enzyme concentration (3.0 nM PP5c) and 2) a range of different enzyme concentrations at the same DiFMUP concentration (100 μM). Examination of Figure 2B reveals a good linear response throughout the entire 10-minute period for 3.0 nM PP5c up to about 100 μM DiFMUP, while Figure 2C indicates good linearity at 100 μM DiFMUP up to about 5 nM PP5c over a 30 minute period. For further characterization of the assay, a DiFMUP concentration of 100 μM and an enzyme concentration of 1.5 nM were used. 100 μM DiFMUP is close to the K_M and represents a reasonable compromise between activity and interference with the binding of competitive inhibitors. A PP5c concentration of 1.5 nM provides reasonable activity at 100 μM DiFMUP, good linearity (up to 30 minutes or more),

and is unlikely to interfere with the detection of small concentrations of high affinity ligands through inhibitor titration effects. To test the reproducibility and reliability of the assay in a multi-well format the reaction rates were determined for 95 (DiFMUP) or 48 (MUP) negative controls (no enzyme) and a similar number of positive controls (1.5 nM PP5c) by periodically measuring fluorescence in each well of a 96 well plate for a period of 10 minutes (MUP) or 30 minutes (DiFMUP) as described in methods. As seen in Figure 3A, using 1.5 nM PP5 and 100 μ M DiFMUP, the break down of DiFMUP to DiFMU in the absence of enzyme was minimal over the course of the experiment, and the variation between replicate control samples was small enough to predict the assay would be suitable for the detection of inhibitors in chemical libraries or natural product extracts ($Z'=0.873$).

Under similar reaction conditions with 1.5 nM PP5 and 25 μ M MUP and using the MU isosbestic point (333 nm) as the excitation wavelength, the breakdown of MUP to MU in the absence of enzyme was also minimal over the course of the experiment (Figure 3B). The variation between replicate control samples was again small enough to predict that the assay would be suitable for the detection of inhibitors in a HTP-screen ($Z'=0.812$). However, with MUP background fluorescence (presumably due to contaminating MU) was high (observed with two separately purchased batches; data not shown), which required using lower substrate concentrations (25 μ M) and increasing the PMT gain on the M5 plate reader in order to improve the signal to noise ratio. Thus, although the Z' value obtained with MUP suggests that it should be an adequate (and cheap) substrate for HTP-screening, overall DiFMUP represents a superior, albeit much more expensive, substrate.

Inhibitor sensitivity of PP5c

Native PP5 is potently inhibited by several natural toxins (e.g. okadaic acid, cantharidin and microcystin-LR)[23]. Mutational and structural studies indicate that both inhibitors bind in close proximity to the catalytic site, in a hydrophobic “pocket” that is highly conserved in PP1 [25,26] and PP2A [27]. To test the sensitivity of PP5c to inhibition by known inhibitors, dose-response studies were conducted using both [32 P]histone or DiFMUP as substrates (Figure 4). The inhibition of PP5c by microcystin-LR and okadaic acid (IC_{50} of ~ 2 and 1 nM, respectively) is similar to the inhibition of the native enzyme [23], and the inhibition profile obtained using DiFMUP was similar to that obtained using [32 P]phosphohistone for both inhibitors (compare Fig. 4A with 4B). These findings suggest that DiFMUP can indeed be employed as a substrate for the detection of catalytic inhibitors of PP5.

Conclusion

Here we describe and test methods useful for the production of biologically active PP5c, an inhibitor-titration based assay to determine the amount of PP5 in solution, and a fluorescent phosphatase assay that can be used to screen chemical libraries and natural extracts for the presence of inhibitors. Inhibitors that are identified from such screens will certainly be useful as research tools to study the biological roles of PP5 and may also be useful for the development of antitumor drugs that target PP5.

Acknowledgements

This work was supported in part by a grant from the National Institutes of Health (NCI grant CA-60750 to REH) and a summer research fellowship from the American Heart Association; South Eastern Affiliate (JCB). This investigation was conducted in a facility constructed with support from Research Facilities Improvement Program Grant (C06 RR11174) from the National Center for Research Resources.

Abbreviations

DiFMUP

6,8-difluoro-4-methylumbelliferyl phosphate

MUP

4-methylumbelliferyl phosphate

PP5

serine/threonine protein phosphatase type 5

TEV

tobacco etch virus

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Figure 1.

Purification of PP5c. A) Representative coomassie blue stained SDS-PAGE agarose gel showing the separation of PP5c obtained with anion exchange chromatography. Following affinity purification using a Ni^{2+} -chelating column, MBP-PP5c was treated with TEV. PP5c was then further purified via chromatography using a Mono Q column as described in methods. The aliquots of fractions containing phosphatase activity (19–32) were then separated using SDS-PAGE on 10% polyacrylamide gel, and protein was visualized by Coomassie blue staining. B) Representative coomassie blue staining of recombinant PP5c at different stages of the purification process following SDS-PAGE using 10% polyacrylamide gels. M; molecular weight protein standards, Lane 1, crude bacterial lysate after induction. Lane 2, active eluate obtained from the Ni^{2+} -chelating column. Lane 3, sample shown in lane 2 after TEV protease digestion. Lane 4, purified PP5c following anion exchange chromatography.

Figure 2.

Fluorescent substrates for PP5. A) pH-dependent fluorescence of 4-MU. Stocks (2.5 μM) of 4-MU were prepared in a four-component buffer system and diluted as described in Methods. Aliquots at each pH (4.0, 5.8, 6.8, 7.3, 7.8, 8.3, 8.8, 9.8, and 11) were placed in cuvettes and excitation spectra (excitation: 280 – 400 nm in 2 nm steps; emission: fixed at 450 nm) were obtained with a SpectraMax M5 (Molecular Devices) at room temperature. The spectrum at each pH was blanked with buffer of the appropriate pH. B) Substrate dependence. Reaction progress curves at fixed PP5c concentration (3.0 nM) and varying DiFMUP concentrations were obtained as described in methods. DiFMU fluorescence was measured at 30 sec intervals for 10 min. Labels on the right indicate the DiFMUP concentrations used for each set of curves (5 replicates at each DiFMUP concentration are shown). C) Enzyme dependence. Reaction progress curves at fixed DiFMUP concentration (100 μM) and varying PP5c concentrations were obtained as described in methods. DiFMU fluorescence was measured at 30 sec intervals for 30 min. Labels on the right indicate the PP5c concentrations used for each set of curves (8 replicates at each PP5c concentration are shown). D) Kinetic analysis of DiFMUP dephosphorylation by PP5. DiFMU fluorescence was measured for each reaction at 30 sec intervals for 10 min, as described in methods. Initial velocities of the reactions were then determined from the linear portions of the progress curves by least squares analysis and plotted against the substrate concentration. Data are shown for 5 replicates at each of the indicated DiFMUP concentrations (3 separate blanks were used at each substrate concentration). Error bars indicate standard deviation. K_m (141 $\pm$ 10.7 μM) and V_{max} (2169730 $\pm$ 49180 mRFU/min) were determined by fitting the data to the Michaelis-Menten equation.

Figure 3.

Z' estimation for DiFMUP and MUP assays. A) Rates of hydrolysis of DiFMUP (mRFU/min) were determined for 95 negative controls (no enzyme; diamonds) and 96 positive controls (1.5 nM PP5c; spheres) by measuring the fluorescence of each well every 60 seconds for a period of 30 minutes (total of 30 measurements per well). $Z' = 0.87$. B) Rates of hydrolysis of MUP (mRFU/min) were determined for 48 negative controls (no enzyme; squares) and 48 positive controls (1.5 nM PP5c; circles) by measuring the fluorescence of each well every 28 seconds for a period of 10 minutes (total of 21 measurements per well). $Z' = 0.81$.

Figure 4.

Comparison of inhibition curves of PP5c in the presence of microcystin-LR and okadaic acid using different substrates. PP5c activity was measured in the presence of the indicated concentrations of microcystin-LR (A) or okadaic acid (B) using 110 μ M DiFMUP (circles) or phosphohistone (squares). Each reaction contained $\sim$ 1.5 nM PP5c. The inhibitors were mixed with PP5c for 10 minutes prior to the initiation of the reaction with the addition of substrate. The data is expressed as a percentage of maximal activity of the controls $\pm$ S.D. (n=3).

Table 1

Purification of PP5c

Fraction	Micromoles of PP5c	Yield (percent)
Soluble Lysate	27	100
AS fractionation	16.7	61.9
IMAC/dialysis/TEV digestion	7.7	28.5
Q column	4.1	15.2
Diafiltration/concentration	2.99	11.1

The concentration of PP5c was determined by Microcystin-LR titration using pNPP assays (MBP-PP5c fusion protein and/or PP5c) in samples of: cleared lysate (Soluble Lysate), dialysate of the solubilized pellets from the ammonium sulfate fractionation (AS fractionation), and the final concentrated solution of high purity PP5c. Concentrations were then multiplied by total volumes to get the number of moles of PP5 recovered at each stage. The amount of PP5 in the cleared lysate was set as 100% yield.