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A Dynamic View of Protein Phosphatase 1 Interaction with its Inhibitors

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Abstract

Protein phosphatase 1 (PP1) is well known for their role in signal transduction and protein function. Together with its inhibitors 1 and 2, it regulates wide variety of cellular activities. We have amplified catalytic subunit of PP1 (PP1c) and its inhibitors 1 (I-1) and 2 (I-2) from dog cardiac mRNA by rt-PCR and cloned into bacterial expression vector pCRT7. Cloned genes were expressed in *E. coli* BL21 (DE3) pLysS by IPTG induction. Functional positive clones were identified by western blotting of bacterial lysate and polymerase chain reaction. Double transformed bacterial cells were also generated by transforming PP1c clone with either I-1 or I-2. Activity of PP1 was analyzed in whole bacterial lysate by measuring dephosphorylation of phos b. Activities of inhibitors were analyzed by their capability to inhibit PP1 from dephosphorylation of phos b. Our findings indicate differential regulation of PP1 by I-1 and I-2. Both expressed recombinant inhibitors 1 and 2 have high potency for inhibition of PP1 activity. Interestingly, I-2 co-expression caused increase in PP1c expression but no change in expression was observed when co-expressed with I-1.

Keywords: Protein phosphatase; Inhibitor; Gene expression; Cloning

Introduction

A well-controlled regulation of protein phosphatase and protein kinase is required to maintain the phosphorylation status of proteins. These proteins are responsible for modulating metabolic pathways as per their phosphorylation state. There are at least 4 types of ser-thr protein phosphatases, protein phosphatase1 (PP1), protein phosphatase 2A (PP2A), protein phosphatase 2B (PP2B (Calcineurin) and protein phosphatase 2C (PP2C), which along with multiple types of protein kinases regulate phosphorylation status of metabolically active proteins. Protein phosphatase-1 (PP1) is a major ser-thr phosphatase which comprises of a catalytic subunit and a variable regulatory subunit. Catalytic subunit is a functional unit which is directed to subcellular compartments on the basis of binding to regulatory subunit [1]. PP1 can modulate various functions like, neuronal signaling, muscle contraction, protein synthesis, glycogen metabolism, cell cycle etc. [2]. PP1 has 3 specific heat stable inhibitors-inhibitor-1, inhibitor-2, inhibitor-3 and DARPP-2. Inhibitor-1 and DARPP-2 are phosphorylation dependent and requires phosphorylation by PKA at Thr-35 and Thr-34, respectively. Inhibitor-2 does not need phosphorylation to inhibit PP1 activity. Instead, calcium channel mediated phosphorylation of Ser-43 of Inhibitor-2 relieves PP1 from its inhibition [3].

PP1 mediated phosphorylation state of many proteins are known to altered in pathological conditions including Heart failure where increased PP1 expression [4,5] caused cardiac dilation and premature death [6]. Limited gene manipulation studies with I-1 have shown promising results in normalizing cardiac function [7].

In view of these facts, a clear understanding of PP1 interactions with its inhibitors is required. We do not have enough information available about interaction of PP1 and its inhibitors and detailed knowledge will provide the information to manipulate metabolic pathways for treatment of wide spectrum of diseases wherever PP1 is involved.

In the current study, we have cloned the genes of PP1, inhibitor-1 and inhibitor-2 and expressed them in BL21 (DE3) cells by Isopropyl-D thiogalactoside (IPTG) induction. We analyzed the activity of PP1 and compared the inhibition of PP1 by inhibitor-1 and inhibitor-2. Since both inhibitors are potent inhibitors of PP1, they can be used as therapeutic agents in diseases where activity of PP1 is enhanced.

Materials and Methods

Materials

Cloning vector pcrT7-topo and bacterial expression system consist of *E. coli* BL21 (DE3) pLysS cells, mini plasmid isolation kit, IPTG and ampicillin were purchased from Invitrogen. Agar and LB media was from Bio 101 Systems (QBiogene, Carlsbad, CA).

Cloning of PP1, inhibitor-1 and Inhibitor-2

Using custom primers (Table 1), cDNA from dog heart, was amplified by PCR and cloned in to pCR-T7-topo vector according to manufacturer's instruction (Gene Bank Accession Nos. AY062037, AY063765 and EU170432). The recombinant plasmid containing gene for PP1 and inhibitors were isolated and purified. Purified recombinant plasmids were used to transform *E. coli* BL21(DE3) pLysS cells for expression studies.

Induction of gene expression

The recombinant proteins were expressed in BL21 (DE3) cells by IPTG induction according to the instructions provided by supplier (Invitrogen). Briefly, fresh LB medium was inoculated by overnight culture of cloned PP1 or inhibitors transformed in BL21 (DE3) cells. After growing the cultures for 2 hours, IPTG was added up to 1mM of final concentration. Cultures were further allowed to grow up to desired time (~4 hrs.). Bacterial cells were harvested by centrifugation and resuspended in 50 mM Tris-HCl (pH 7.4), 0.5 mM sodium EDTA (pH 7.0), 0.3 M sucrose, and protease inhibitors (0.8 mM benzamidine, 0.8 mg/l each aprotinin and leupeptin, and 0.4 g/l antipain).

PP1 activity Assay

Bacterial lysate was prepared by homogenization in the same

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Gene	Sequence	Size (bp)
Inhibitor-1	5'-CCATggAgCAAgACAACAg-3'	602
	5'-CCCAAAAgTgAAggAATAAgAA-3'	
Inhibitor-2	5'-CCAATggCggCCTCgACggCCTC-3'	697
	5'-TgAAgAACAAgAAgCAACgTACTA-3'	
PP1c	5'-gCCATgTCCgACAgCgAgAAg-3'	1122
	5'-TCCATgTTCCCCgTgACAggTg-3'	

Table 1: Primers used for polymerase chain reaction and sizes of amplified products.

buffer it was resuspended (Tris-sucrose buffer). With the use of [^{32}P] phosphorylase *b* as the substrate, PP activity was determined in the bacterial lysate. The assay was performed in a 60 µl aliquot that consisted of 50 mM Tris-HCl (pH 7.4), 5 mM caffeine, 0.5 mM EGTA, 0.5 mM EDTA, 50 mM β-mercaptoethanol 100 ng of aprotinin (protease inhibitor), 1 µg of the cell lysate and 550 pmol ^{32}P-phosphorylase *a*. The assay was initiated by adding the phos a mix and carried out for 10 min at 34°C. Incubation was rapidly stopped by addition of 20 µl of 60% TCA and 20 µl of BSA (50 mg/ml). Tubes were held in ice for 10 min and then centrifuged at 12,000 g for 5 min. After centrifugation, ^{32}P radioactivity was counted in 80 µl of clear supernatant in 5 ml of liquid scintillation fluid. PP1 activity was calculated by comparing with samples with no IPTG induction. Activity in each sample was expressed as pmol ^{32}P released/min/mg of protein.

Inhibitor activity assay

Activity of inhibitor-1 and inhibitor-2 was measured by mixing bacterial lysate of inhibitor expressions with lysate from PP1 expression before adding phos a mix to measure phosphatase activity. Briefly, 10 µl (1 µg) of PP1 and 30 µl (15 µg or 30 µg) of inhibitor-1 or inhibitor-2 was mixed in the assay tube and kept in ice for 5 min. Reaction was initiated by adding 20 µl phos a mix and was incubated at 34°C for 10 min. Reaction was terminated by adding 20 µl of 60% TCA and 20 µl of BSA (50 mg/ml). Inhibitor activity was calculated by comparing released ^{32}P with the samples without inhibitor.

Western blotting

To determine the protein expression of PP1C, Inhibitor-1 and inhibitor-2, western blotting was performed on SDS extracts of the bacterial extracts. Bacterial culture was centrifuged at 6000 rpm for 15 min. Pellet was resuspended in Tris-Sucrose homogenization buffer and homogenized for 2 × 15 sec using homogenizer. Extract was prepared by adding 1/10th volume of 20% SDS in the homogenized sample followed by incubating in boiling water for 10 min. After boiling samples were allowed to cool at room temperature and centrifuged at 12,000 × g for 15 min. Supernatant was collected in the fresh tubes and used for western blotting. Protein amount was measured according to Lowery's method [8]. Equal amount of protein was subjected to electrophoresis on 4-20% SDS-polyacrylamide gel (Bio-Rad), and the separated proteins were electrophoretically transferred to a nitrocellulose membrane [9,10]. Accuracy of the electro transfer was confirmed by staining the membrane with 0.1% ponceau S. For the immunoreaction, the nitrocellulose blot was incubated with diluted primary antibody (monoclonal or polyclonal) based on the supplier's instructions. Antibody-binding protein was visualized by autoradiography after treating the blot with horseradish peroxidase-conjugated secondary antibody (anti-mouse for PP1 and Inhibitor-2 and anti-rabbit for inhibitor-1) and enhanced chemiluminescence color-developing reagents according to the supplier (NEN).

Co-transformation of *E. coli*

Clones of PP1c and inhibitors (either inhibitor-1 or inhibitor-2) were mixed in equal concentration of 5 ng/µl. One µl of this mixture was added into chemically competent BL21 pLysS cells and transformed by heat shock at 42°C. Transformed cells were selected on amp-LB agar plate. 10 colonies were grown in 10 ml culture and induced by IPTG for gene expression. Dual-positive clones were selected by western blot analysis for PP1 and inhibitor-2. Phosphatase activity and western blots were carried out on these samples according to the methods described in previous sections.

Results

Expression of recombinant proteins

To analyze the expression of recombinant PP1, culture was incubated for different time points (1hr-4 hrs) after induction with 0.5 mM IPTG. Both western blot and activity shows increasing trend with time (Figure 1). Similarly, cultures for inhibitor-1 and inhibitor-2 were incubated for 1-4 hrs. after adding 1 mM IPTG. Increase in time of expression after induction with 1 mM IPTG showed increasing trend in expression and PP1 inhibiting activity for both I-1 and I-2 (Figures 2 and 3).

Effect of heat on inhibitor-1 and inhibitor-2

Naturally both of these inhibitors are heat and acid stable. We tested expressed inhibitor-1 and inhibitor-2 for heat resistance. After treatment both inhibitors not only maintained their activity but also showed 10-20% enhancement in their activity (Figure 4).

Effect of co-expression

I-1 and I-2 were separately co-expressed with PP1c inside single bacterium to study their effect on each other. I-1 co-expression had no effect on amount of gene expression of PP1 or vice versa but I-2 co-expression with PP1c resulted in higher expression of both genes when compared to their individual expression (Figures 5 and 6).

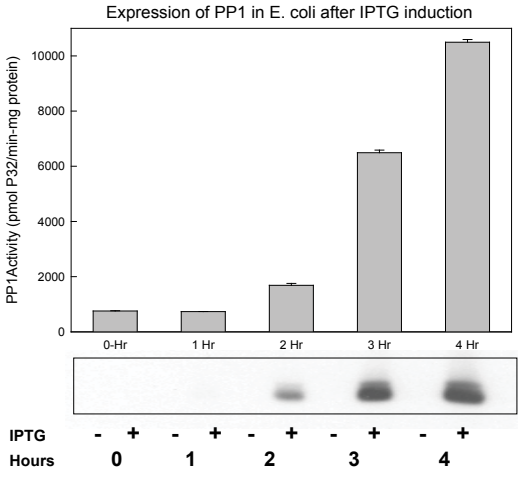

Figure 1: Gene expression at different time intervals after induction with 0.5 mM IPTG. A: PP1 activity in bacterial homogenate and B: western blot of PP1c in uninduced and induced samples.

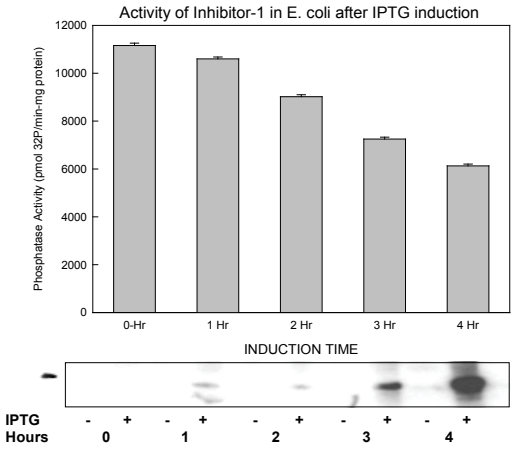

Figure 2: Gene expression at different time intervals after induction with 1.0 mM IPTG. A: PP1 activity in bacterial homogenate and B: western blot of I-1 in un-induced and induced samples.

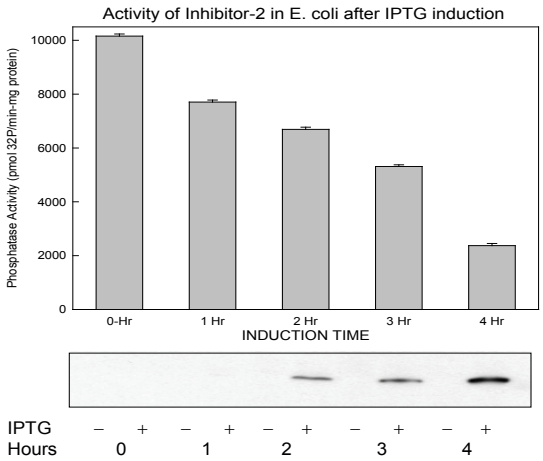

Figure 3: Gene expression at different time intervals after induction with 1.0 mM IPTG. A: PP1 activity in bacterial homogenate and B: western blot of I-2 in un-induced and induced samples.

Discussion

We have cloned and analyzed the expression of canine PP1 and inhibitors in bacterial system. The purpose of study was to characterize these genes for future gene manipulation studies. Our bacterial expression system utilizes T7 promoter which results in robust expression of mammalian genes in bacteria. We used BL21(DE3) pLysS cells for expression of canine PP1 and inhibitors genes. These cells maintain T7 RNA polymerase under suppressed conditions to minimize basal level expression. An inducer IPTG is required to relieve T7 RNA polymerase from suppression and start gene expression [11]. We analyzed the amount and activity of expressed products up to 4 hours after addition of IPTG. Samples were collected for analysis after each hour. Our results showed that gene expression is associated with time of incubation after addition of IPTG. PP1 and both inhibitors continuously increased up to 4 hrs. It indicates that the products are stable and active at least up to 4 hrs. and they can be used for further analysis.

Analysis of gene function showed maximum PP1 activity in extract of 0.5 mM IPTG induced cells in comparison to extracts prepared after incubation with other concentrations of IPTG (data not shown), thus this concentration of IPTG was used for further experiments. The decrease in activity at higher concentrations of IPTG may be due to aggregation of enzyme at very high concentrations [12,13]. Maximum activity of both inhibitors were observed at 1mM IPTG concentration (data not shown), thus selected for further experiments.

In the earlier studies several workers have reported about dependence of PP1 on Mn^{2+} [14]. There are other reports also that Mn^{2+} is required for activation of PP1 [15]. The role of Mn^{2+} in activation of PP1 is not clear. However, the PP1 described in this manuscript is Mn^{2+} independent.

PP1 inhibitors 1 & 2 are acid and heat stable and mostly exist in random coil structure [16-19]. This unique structure makes them resistant to heat but sensitive to proteases. Inhibitors acquire flexible conformation and they bind to PP1 at multiple sites with a long range distribution on the surface of the enzyme [20]. We tested the PP1 inhibition activity of both inhibitors after heat treatment and observed slight increase in PP1 inhibition after treatment. This increase in inhibition may be due to heat-induced dissociation of some interacting proteins from inhibitors. This separation might make them free and available to interact with PP1.

Co-expression of Inhibitors with PP1c gives an opportunity to study their interaction. Selective increased expression of both genes during co-expression of I-2 and PP1c indicates their unique interaction which needs to be explored. It looks like that inhibition of PP1 activity by inhibitor-2 somehow inducing more expression of PP1. This extra PP1 requires extra inhibitor-2, which induces expression of more inhibitor-2. This feedback mechanism may be the cause of increased

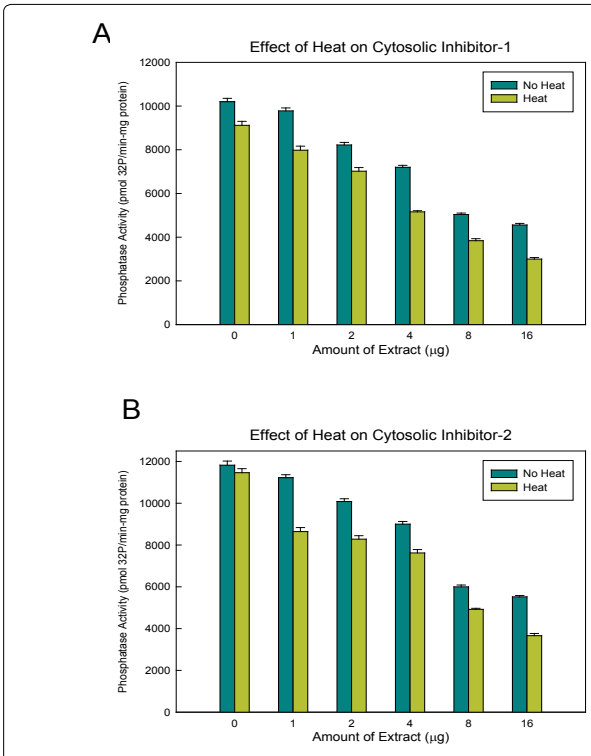

Figure 4: Effect of Heat on inhibitors. Inhibition of phosphatase activity by different amount of I-1 (A) and I-2 (B) induced bacterial extract.

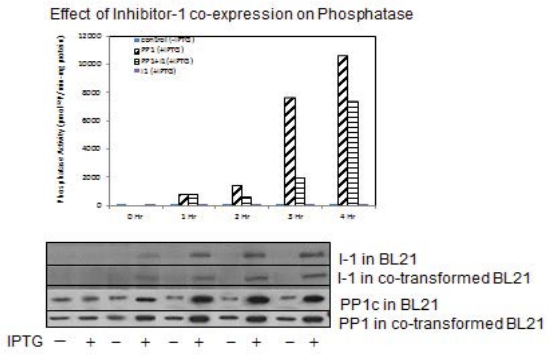

Figure 5: Co-expression of PP1 and inhibitor-2. Top: Phosphatase activity in the IPTG induced bacterial extracts that contains genes for PP1 alone, PP1 and I-1 both and I-1 alone. Control samples are bacterial extracts without IPTG induction. Bottom: Western blot analysis in IPTG induced bacterial extracts: For I-1 in only I-1 containing bacteria and in co-transfected bacteria; For PP1 in only PP1 containing bacteria and co-transfected bacteria.

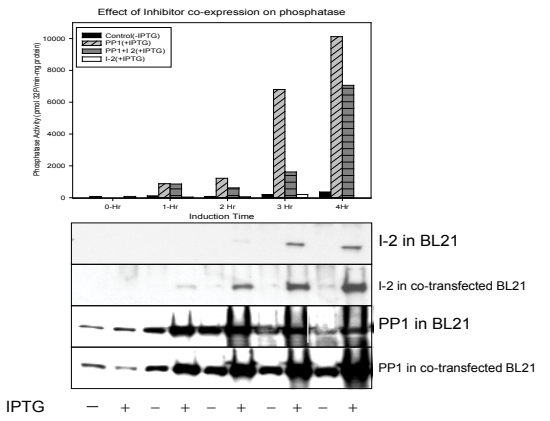

Figure 6: Co-expression of PP1 and inhibitor-2. Top: Phosphatase activity in the IPTG induced bacterial extracts that contains genes for PP1 alone, PP1 and I-2 both and I-2 alone. Control samples are bacterial extracts without IPTG induction. Bottom: Western blot analysis in IPTG induced bacterial extracts: For I-2 in only I-2 containing bacteria and in co-transfected bacteria; For PP1 in only PP1 containing bacteria and co-transfected bacteria.

expression of both genes. In our earlier study on one kidney one-clip model of hypertension, we have reported increased expression of PP1 and Inhibitor-2 [21]. This may be due to feedback mechanism proposed in the current study. One recent study on PPI-I2 complex analysis by X-ray crystallography has shown that Thr-74 phosphorylation of I-2 activates PP1 by dissociates it from metal containing active site of PP1 [22] but how they affect gene expression is not known.

In summary, phosphatase inhibition by I-1 and I-2 are very well known for long time. A detailed study about their interactions, effect on associated proteins and their role on gene expression is required. A better understanding of phosphatase-inhibitor relationship will help in better manipulation of metabolic pathways.

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