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Identification of a novel stress regulated FERM domain containing cytosolic protein having PTP activity in *Setaria cervi*, a bovine filarial parasite

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ABSTRACT

A 67 kDa cytosolic FERM domain containing protein having significant protein tyrosine phosphatases activity (PTPL) has been purified to homogeneity from *Setaria cervi*, a bovine filarial parasite. The MALDI-MS/MS analysis of the purified protein revealed 16 peptide peaks showing nearest match to *Brugia malayi* Moesin/ezrin/radixin homolog 1 protein and one peptide showing significant similarity with a region lying in the catalytic domain of human PTPD1. PTPL showed significant cross reactivity with the human PTP1B antibody and colocalize with actin in the coelomyrian cells of hypodermis in the parasite. PTPL was stress regulated as it showed marked decrease in the expression when exposed to Aspirin, an antifilarial drug and Phenylarsine Oxide, PTP inhibitor.

1. Introduction

The protein tyrosine phosphorylation is a dynamic process controlled by the balanced actions of protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs). Though earlier mistaken as constitutively active housekeeping enzymes, PTPs have emerged as essential regulators of different cellular signalling processes like growth, differentiation and proliferation in coordination with PTKs [1]. The PTPs are classified into two broad groups, transmembrane and cytoplasmic. The transmembrane PTPs which respond to external stimuli, consist of a single transmembrane segment and two PTP domains and the cytoplasmic non-receptor PTPs, which exhibit a noncatalytic domain (apart from the catalytic PTP domain) that seems to manage its intracellular localization as well as substrate specificity [2].

One such group of cytoplasmic non-receptor PTPs possess a cytoskeletal associated domain termed as band 4.1 or FERM (band four-point-one, ezrin, radixin, moesin homology) at their N-terminal end. Human PTPs reported to belong to this family are PTP-BAS/FAP-1/PTPL1, PTPH1, PTP-MEG1, PTPD1/PTP-RL10, PTPD2/Pez. Recent reports have shown that the N-terminal domain of FAK and JAK tyrosine kinases also belongs to this band 4.1-domain family and is responsible for their association with PDGF – and EGF cytokine receptors, respectively. The main function of these proteins is to mediate linkage of the cytoskeletal protein, actin and transmembrane receptors like CD44, ICAM-1/2/3, ERM binding phosphoproteins 50 with the plasma membrane inner surface [3]. They are also known to associate the enzymatic activities of kinases and phosphatases to the membrane. Their varied role in cell–cell adhesion, cell–matrix interaction, cell morphology, metastasis, carcinogenesis as well as apoptosis via phosphatidylinositol 3-kinase/Akt pathway has been reported. These proteins are also known to control stress-response, growth and survival pathways of oncogenic importance [4].

The free living nematode, *Caenorhabditis elegans* also possess a PTPL1 protein known as PTP-FERM predominantly expressed in the neuron and peri-membrane space. The FERM domain of PTP-FERM was revealed to be sufficient and essential for ameliorating the protein in the subcellular region of the worm [3].

Regardless of the crucial significance of PTPL1/PTP-BAS, its characterization and importance in case of parasitic nematodes is still unknown. The present study describes the isolation, purification and role of a novel FERM domain containing protein with significant protein tyrosine phosphatases activity (PTPL) in *Setaria cervi*, a bovine filarial parasite.

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2. Material and methods

2.1. Parasite collection, crude extract preparation and purification

The procurement and preparation of adult female *S. cervi* extract was done as reported previously [5]. The adult female *S. cervi* crude soluble extract was subjected to purification using concanavalin A sepharose 4B affinity and gel filtration chromatography. The extract was loaded on affinity column and protein was eluted using 0.15 M NaCl and 1 M mannose. The active protein peaks were loaded further on the Sephadex G-200 column pre equilibrated with 50 mM 0.1 M Tris buffer, pH 7.0. The active protein fractions were checked for purity using SDS gel electrophoresis. The MALDI-MS/MS analysis of the purified protein band was performed at Interdisciplinary School of Life Sciences, Banaras Hindu University, Varanasi, India. ClustalW multiple alignment tool was used for the alignment studies.

2.2. Protein tyrosine phosphatase assay

Acid phosphatase and protein tyrosine phosphatase activity were measured according to the method described elsewhere [6,7] with slight modifications as described earlier [5]. The effect of different general and specific inhibitors of acid phosphatases (sodium orthovanadate, sodium fluoride, ammonium molybdate, sodium tartarate, phenylarsine oxide, okadaic acid, $ZnCl_2$) on purified PTP activity was determined as described elsewhere [5].

2.3. Western blotting

The purified protein was electrophoretically separated by a preparative 10% SDS-PAGE gel and electrotransferred onto PVDF membrane as described [8]. The monoclonal antibody (clone FG6-1G, Calbiochem, San Diego, CA) generated against the catalytic domain of a recombinant human placental PTPase 1B (1 μg/ml) as primary antibody and peroxidase conjugated rabbit anti-mouse IgG (1:5000) as secondary antibody were used as explained earlier [9]. ImageJ 7.0 software was used to quantify the western blot bands.

2.4. Tissue fixation and immunostaining

The adult female parasites were incubated at 37 °C, 5% CO2 for 4 h in Krebs-Ringer Bicarbonate (KRB) medium in the presence/absence of Aspirin and SK7, methylated chalcone. The parasites were then fixed in 4% paraformaldehyde for 20 min and dehydrated in graded series of ethanol. The parasite paraffin sections were cut at 6 μm using a Leica microtome. The sections were first rehydrated in different grades of ethanol and incubated in a blocking buffer (5% skimmed milk in phosphate buffer saline PBS) for 2 h at RT to avoid nonspecific binding. They were then incubated overnight at 4 °C with primary antibody (anti human PTP1B; 1 μg/ml) in phosphate buffer saline tween (PBST). After washing with PBST, they were incubated with secondary antibody (goat anti mouse IgG, FITC conjugated, 1:2000) in PBST for 2 h in dark to avoid photobleaching. The sections were then washed, incubated with phalloidin-TRITC (1:200) in PBS for 1 h at RT and mounted in DABCO. The images were taken using Zeiss LSM-510 Meta confocal microscope at the National confocal facility, Banaras Hindu University.

2.5. Statistical analysis

All experiments were performed in triplicate (n = 3). The statistical analysis was done using the GraphPad Prism 5.0 software. Statistical significance was determined by using two tailed Student's *t*-test. Differences were considered significant at $P < 0.05$.

3. Results

3.1. Purification and characterization of PTPL in S. cervi

We have recently reported presence of significant amount of protein tyrosine phosphatase activity in the cytosolic extract of *S. cervi* from our laboratory [5]. The enzyme was found to possess highest affinity for O-P-L-tyrosine substrate as compared to O-P-L-serine, O-P-L-threonine and pNPP as a general substrate. The enzyme was glycosylated in nature as it showed affinity for the Concavalin affinity column [5]. While characterizing tyrosine phosphatases in *S. cervi*, we discovered a FERM domain containing protein in the cytosolic extract of *S. cervi* having significant tyrosine phosphatase catalytic activity and named it PTPL. PTPL was purified to homogeneity with a specific activity of 2124 U/mg, 90-fold purification, and yield of 23.4% (Table 1) using Concavalin A affinity and Sephadex G-200 columns. The apparent molecular mass of PTPL was determined to be 67 kDa when run on a 10% SDS–PAGE (Fig. 1A). The purified protein also displayed strong cross reactivity at 67 Kda using a monoclonal anti-human placental PTPase 1B antibody (with the epitope designed against the catalytic site of the protein) (Fig. 1A). The cytosolic crude extract of *S. cervi* also showed intense cross reactivity with this antibody at the same position (Lane Ctrl of Fig. 1B).

The optimum pH and temperature for PTPL was found to be 5.9 and 37 °C, respectively (data not shown). To analyse the substrate specificity of PTPL, we checked its affinity towards different substrates like pNPP, O-phospho − L-tyrosine, O-phospho − L-serine and O-phospho-L-threonine. PTPL showed highest activity with pNPP followed by O-phospho − L-tyrosine. It exhibited almost one third of activity with O-phospho-L-threonine as compared to O-phospho − L-tyrosine but no activity could be seen using O-phospho − L-serine (Table 2). To further characterize the PTPL, we performed inhibition studies using some class specific inhibitors of protein phosphatases. Sodium orthovanadate (SOV), Phenylarsine oxide (PAO) and $ZnCl_2$ were used as protein tyrosine phosphatases specific inhibitors. Okadaic acid as serine threonine phosphatase inhibitor and ammonium molybdate, sodium fluoride, sodium tartarate as general acid phosphatase inhibitor were also used for the inhibition studies. PTPL activity was completely inhibited by SOV at 100 μM. The PAO (1 nM) also significantly inhibited the enzyme activity upto 87.36%. Although the general acid phosphatase inhibitors, ammonium molybdate and sodium fluoride showed 93% and 98% inhibition, okadaic acid did not show any inhibition in activity (Table 3). The PTPL enzyme activity was enhanced by 47.43%. in the presence of DTT.

3.2. Bioinformatics analysis

Further the purified protein band was excised from the SDS-PAGE gel for peptide mass fingerprinting analysis. The maximum

Table 1
Purification Table of PTPL from adult female *Setaria cervi*.

	Total protein (mg)	Total activity (u)[a]	Specific activity (u/mg)[b]	% Yield	Fold purification
Adult female cytosolic extract	8.1	190.5	23.5	–	–
Con A sepharose 4B affinity column	0.35	123.83	354.36	65.00	15.08
Gel filtration column G-200	0.021	44.61	2124.29	23.4	90.39

[a] One unit of enzyme activity is defined as micromoles of p-nitrophenol produced ml^{-1} min^{-1} with pNPP.
[b] Specific activity is the units of enzyme activity per milligram of protein.