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Characterization of a serine/threonine protein phosphatase regulatory subunit in the Indian pearl mussel

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Abstract
Reversible phosphorylation is a key mechanism for regulating the biological activities of functional proteins. We have partially characterized a serine/ threonine phosphatase 6 regulatory protein from the mantle tissues of Indian freshwater pearl mussels *Lamellidens marginalis* and *Parreysia corrugata*. The characterized sequences showed more than 75% sequence identity at both the DNA and protein levels of the species. Importantly, the characterized sequences did not show similarity to any other genes in the homology search. A predicted 23 amino acid polypeptide stretch from both the species was 100% identical in all mollusc sequences available till date (across isoforms) and more than 95% similar to a wide range of species. The predicted protein folding patterns showed alpha helices separated by intervening loop regions in both the *in silico* translated polypeptides, similar to reported regulatory subunits of serine/ threonine protein phosphatases. Considering the extent of structural similarity of this polypeptide stretch among the molluscs and the fact that the sequences were isolated from the mantle tissues, it may be speculated that the genes partially characterized here could be primarily involved in shell formation and bio-mineralization process.

Keywords: *Lamellidens marginalis*, *Parreysia corrugata*, bio-mineralisation, mantle

Introduction
Bio-mineralization is a complex set of processes for the formation of inorganic solids within a living system. Formation of nacre is one such fascinating bio-mineralization process encountered in many molluscs. Central to the process of bio-mineralization are proteins that guide and regulate the formation and growth of the inorganic crystals. In mollusc shell and pearl, such proteins are collectively called shell matrix proteins (SMPs) [1].

Biological activity of key proteins is determined to a large part by the reversible phosphorylation in eukaryotic cells [2]. Reversible phosphorylation is known to affect protein function in a variety of ways such as inducing conformation changes on substrate proteins and creating docking sites for phospho-dependent protein interaction domains [3]. Indeed, erroneous phosphorylation and de-phosphorylation of SMPs have been shown to result in abnormally growing prismatic layers and nacre tablets with reduced crystallinity and possibly changed crystal orientation [1]. Therefore exploration of specific proteins involved in the reversible phosphorylation mechanism of SMPs warranted.

It is estimated that more than 98% of reversible phosphorylation in proteins occur in serine / threonine residues [2, 4] catalysed by specific classes of kinases and phosphatases that cause phosphorylation and de-phosphorylation respectively of specific target proteins. The serine threonine Protein phosphatases (PP) are classified on the basis of their substrate specificity and inhibitor sensitivity into type-1 and type 2 phosphatases [5]. One of the type-2 category phosphatases, namely PP-6, plays an important role in cell signalling and cell cycle [4]. Mutations that affected the binding of the catalytic subunit of PP6 (PP6c) to the regulatory subunits resulted in increased mitotic defects in human cells [6]. PP6 holoenzyme is thought to play an important part in DNA double strand breaks repair [7] and is reportedly indispensible for embryogenesis [8].

The evolution of highly conserved protein phosphatase enzymes has recently captured scientific attention owing to its significance in cell viability and functioning. It is now known that the diversity of functional PPPs in eukaryotic proteome is derived from the many

combinations of catalytic and regulatory subunits [9]. In other words the whole array of specific protein phosphatase complexes encountered in the eukaryotic cell system owes their specificity in function as much to the regulatory subunits as to the catalytic units.

PP6 holoenzyme is described as a heterotrimer composed on the PP6 catalytic subunit, a SAPS domain containing regulatory subunit and an ankyrin repeat-domain containing regulatory subunit (ARS) [10]. Endogenous PP6 regulatory subunit 3 (PP6R3) co-precipitated 50% of PP6 in cell extracts. In fact, PP6R3 alone was sufficient to bind to the catalytic subunit, indicating it is the major binding factor for the PP6 holoenyme [11].

In this study, an attempt was made on partial characterization of the serine/threonine protein phosphatase 6 regulatory subunit 3 from the mantle tissues of Indian freshwater pearl mussels, *Lamellidens marginalis* and *Parreysia corrugata*. Considering that the mRNA was derived from the major site of shell bio-mineralization in the species, it is possible that the characterized PP6R3 subunits isoform could have direct implications on the mussel shell matrix protein functions.

Materials and methods

RNA isolation and cDNA synthesis

Total RNA was isolated from mantle tissue samples of *Lamellidens marginalis* (Class Bivalvia; Family Unionidae) and *Parreysia corrugata* (Class Bivalvia; Family Unionidae) using RNeasy® Mini Kit (Qiagen, USA) following manufacturer's instructions. The isolated RNA was reverse transcribed using R2DFast 1st strand cDNA synthesis kit® (GCC Biotech, India). The single stranded cDNA was subjected to PCR (F: primer; R: Primer) using touchdown thermal cycling conditions of 1 cycle of 94 °C for 5 min, 35 cycle of 94 °C for 1 min/57-52 °C for 1 min/72 °C for 1 min and one cycle of 72 °C for 5 min.

Sequencing and bioinformatics analyses

The amplified products were checked on a 2% agarose gel to check size specificity of the bands. The amplicons of expected size were excised from the gel, purified, and subjected to sequencing on a Genetic Analyzer 3500xl (Applied Biosystems, USA).

The sequences were subjected to homology search on NCBI BLASTn to confirm specificity. The partial cDNA sequences were aligned with homologous mollusc sequences available in NCBI Genbank that showed more than 60% similarity. Phylogenetic trees were constructed using Phylogeny.fr platform [12, 13]. Within the online assemblage platform of tools for alignment, alignment refinement, phylogeny and tree rendering, PhyML 3.1/3.0 aLRT software was employed for phylogeny using maximum likelihood method in this study. The secondary structure prediction of predicted polypeptides was performed using Phyre 2 server [14].

Results and Discussion

Bio-mineralization is a complex set of molecular processes. Phosphorylation of shell matrix proteins is known to be a major factor that affects bio-mineralization process in mollusc shells [1]. In fact, reversible phosphorylation is one of the major post transcriptional modifications in proteins that govern their function and turn over [2]. In this study, partial characterization of serine/ threonine protein phosphatase 6 regulatory subunit 3 from two Indian freshwater pearl mussels is reported.

The single primer pair used to amplify the sequence from both the species was designed following a methodology developed by our laboratory (Unpublished data). The partial cDNA sequences of the target gene and their respective predicted amino acid sequences characterized from *Lamellidens marginalis* and *Parreysia corrugata* (NCBI Acc. No: MN518757 & MN518758 respectively) were subjected to BLASTn homology searches. The characterized sequences showed more than 75% sequence identity at both DNA and protein levels across the species. Nucleotide similarity of more than 60% satisfies the criteria for phylogenetic tree construction [15]. Fig. 1 depicts the phylogenetic tree constructed using homologous sequences available in public domain from related species.

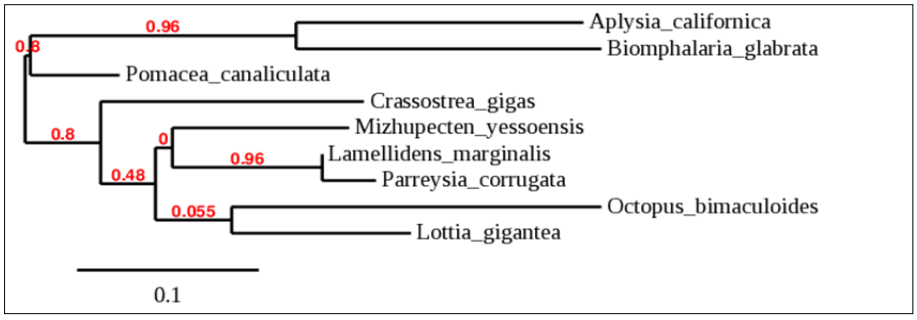
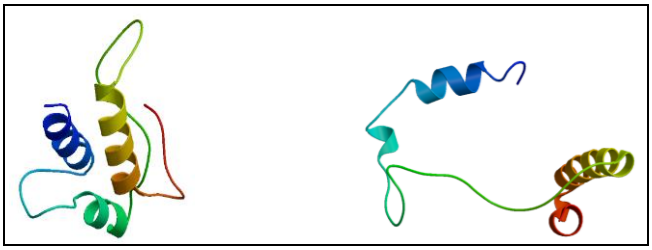


Fig 1: Neighbour joining tree for PP6R3 partial gene sequences of Indian pearl mussels

It may be observed that the clustering of the sequences followed the expected taxonomic hierarchy, and our reported sequences showed relatively higher similarity with other bivalve species. Importantly, the characterized sequence did not show similarity to any other genes in the homology search indicating that this is a highly conserved stretch.

The *in silico* translated polypeptide stretch "QQAFSDYQLQQMTSNFIDQFGFNEEEFQEQEEKADSP FGDRISSI" from *L. marginalis* was identified as SIT4 Yeast associated protein (SAP) family by Interpro software, confirming the specificity of our reported sequences. The human PP6 holoenzyme was identified to be an ortholog of the budding yeast SIT4 protein, involved in cell cycle regulation [16, 17]. The highly conserved SAPs domain held by the regulatory subunits is known to be the site of interaction between the SIT4 protein in the yeast, for the formation of the functional holoenzyme [11]. In fact, the predicted 23 amino acid polypeptide stretch from both the species "QQAFSDYQLQQMTSNFIDQFGFN" was 100% identical in all mollusc sequences available till date (across isoforms) and > 95% similar to a wide range of species.

The predicted protein folding patterns showed alpha helices separated by intervening loop regions in both the *in silico* translated polypeptides (Fig.2.a, b).



a b

Fig 2: Secondary structure simulated for the predicted polypeptide stretch of PP6R3 mRNA of *Lamellidens marginalis* (a) and *Parreysia corrugata* (b). The folding simulation was performed using Phyre 2 software, and the depicted models were trained on human protein phosphatase 1 regulatory subunit 3a as templates.

Both the simulations were modelled over human protein phosphatase 1 regulatory subunit 3a as template with 67% coverage each. Helical repeat motifs are commonly encountered in regulatory subunits of serine/ threonine protein phosphatases, and are thought to mediate protein- protein interactions [11]. However the predicted folding in this case had a significantly low confidence when trained on the reported PP6R3 subunit structures, despite the high similarity at primary structure level. In addition, the folded polypeptides were not identified with any of the reported domains by *in silico* platforms. *In silico* protein folding prediction algorithms try to envision the protein energy kinetics based on the native protein structure as well potential interactions between the molecules [18, 19, 20]. Therefore, it is possible that the secondary structure prediction could not be authoritative in the absence of the complete sequence of the polypeptide. Interestingly similar issues were also reported [21] by using standard training algorithms even when complete PP6R3 protein sequence was used.

The PP6 genes are expressed ubiquitously and are thought to be involved fundamentally in cell cycle regulation [17]. PP6R3 subunit has been found to be involved in chromosomal translocations and various cancers. Two out of five phosphorylation sites of human PP6R3 protein was found to be DNA damage induced- SQ/TQ sites [22] indicating that it is a major player in DNA damage repair processes. Damaged endogenous DNA has been shown to induce inflammatory gene expression [22] by triggering multiple signalling cascades [23, 24]. Unsurprisingly, the PP6R3 protein has also been implicated in immune related functions with high expression levels recorded in T helper cells, cytotoxic cells and monocytes in human body [25].

The processes of pearl grafting can evoke the immune system of the host mollusc [26, 27] which influences graft rejection rate, survival of the host as well the successful development of the pearl sac [28]. In other words, the successful implantation and development of pearl in a grafted mussel is heavily dependent on the host immune reactions post implantation. Considering the extend of structural similarity of this polypeptide stretch among molluscs and the fact that the sequences were isolated from the shell mantle tissues, we speculate that the genes partially characterized in our study could be primarily involved in mollusc shell bio-mineralization regulation.

Conclusion

We report the results of our study where we have partially characterized a serine/ threonine phosphatase 6 regulatory protein from the mantle tissues of Indian freshwater pearl mussels *Lamellidens marginalis* and *Parreysia corrugata*. Our characterized sequences showed significant similarity to reported homologous sequences from multiple mollusc species both at DNA and polypeptide levels. The in silico translated polypeptide stretch from the characterized sequences showed homology to the SIT4 Yeast associated protein (SAP) family proteins, which is known to be the site of interaction between the regulatory unit and the catalytic unit of the PP6 holoenzyme. Further studies to identify the role of these novel mantle tissue PP6R3 sequences in the process of shell biomineralization of the freshwater pearl mussel are warranted.

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References

1. Du J, Xu G, Liu C, Zhang R. The role of phosphorylation and dephosphorylation of shell matrix proteins in shell formation: an *in vivo* and *in vitro* study. Cryst Eng Comm, 2018. DOI: 10.1039/c8ce00755a.
2. Honkanen RE, Golden T. Regulators of Serine/ Threonone Protein phosphatases at the dawn of a clinical era? Current Medicinal Chemistry. 2002; 9:2055-2075.
3. Cohen P. The regulation of protein function by multisite phosphorylation- a 25 year update. Trends in Biochemical Sciences. 2000; 25:596-601.
4. Braughtigan DL, Shenolikar S. Protein serine/ threonine phosphatases: Keys to unlocking regulators and substrates. Annual Review of Biochemistry. 2018; 87:921-964.
5. Ingebritsen TS, Cohen P. Protein phosphatases: properties and role in cellular regulation. Science. 1983; 221:331-38.
6. Gold HL, Wengrod J, Vega-Saenz de Miera E, Wang D, Fleming N, Sikkema L *et al*. PP6C Hotspot PP6C Hotspot Mutations in Melanoma Display Sensitivity to Aurora Kinase Inhibition. Molecular Cancer Research. 2013; 12(3):433-439.
7. Doughlas P, Zhong J, Ye R, Moorhead GBG, Xu X, Lees-Miller SP. Protein phosphatase 6 interacts with the DNA dependent protein kinase catalytic subunit and dephosphorylates Ɣ-H2AX. Molecualr ans Cellular Biology. 2010; 30(6):1368-1381.
8. Ogoh H, Tanuma N, Matsui Y, Hayakawa N, Inagaki A, Sumiyoshi M *et al*. The protein phosphatase 6 catalytic subunit (Ppp6c) is indispensible for proper post-implantation embryogenesis. Mechanisms of Development. 2016; 139:1-9.
9. Shi Y. Serine/ threonine phosphatases: Mechanism through structure. Cell. 2009; 139:468-484.
10. Stefansson B, Ohama T, Daugherty AE, Brautigan DL. Protein phosphatase 6 regulatory subunits composed of ankyrin repeat domains. Biochemistry. 2008; 47:1442-1451.
11. Luke MM, Della Seta F, Di Como CJ, Sugimoto H, Kobayashi R, Arndt KT. The SAP, a new family of

proteins, associate and function positively with the SIT4 phosphatase. Molecular Cell Biology. 1996; 16:2744-2755.
12. Dereeper A, Guignon V, Blanc G, Audic S, Buffet S, Chevenet F *et al*. Phylogeny. fr: Robust phylogenetic analysis for the non-specialist. Nucleic Acids Research. 2008; 36:465-469.
13. Dereeper A, Audic S, Claverie JM, Blanc G. BLAST-Explorer helps you building datasets for phylogenetic analysis. BMC Evolutionary Biology. 2010; 10:8.
14. Kelly LA, Mezulis S, Yates CM, Wass MN, Sternberg MJ. The Phyre2 web portal for protein modelling, prediction and analysis. Nature Protocols. 2015; 10(6):845-858.
15. Salemi M, Lemey P, Vandamme AM. The Phylogenetic Handbook: A Practical Approach to Phylogenetic Analysis and Hypothesis Testing, Cambridge University Press, 2009, 750.
16. Bastians H, Ponstingl H. The novel human protein serine/ threonine phosphatase 6 is a functional homologue of budding yeast Sit4p and fission yeast ppe1, which are involved in cell cycle regulation. Journal of Cell Science. 1996; 109:2865-2874.
17. Morales- Johansoon H, Puria R, Braughtigan DL, Cardenas ME. Human protein phosphatase PP6 regulatory subunits provide Sit4-dependent and rapamycin sensitive Sap function in *Saccharomyces cerevisiae*. Plos One. 2009; 4(7):e6331.
18. Levitt M, Warshel A. Computer simulation of protein folding, Nature. 1975; 253:694-698.
19. Gsponer Caflisch. Molecular dynamics simulations of protein folding from the transition state. PNAS. 2002; 99(10):6719-6724.
20. Dagett V. Protein folding- simulation. Chemical Reviews. 2006; 106:1898-1916.
21. Guergnon J, Derewenda U, Edelson JR, Brautigan DL. Mapping of protein phosphatase-6 association with its SAPS domain regulatory subunit using a model of helical repeats. BMC Biochemistry. 2009; 10:24.
22. Ermolaeva MA, Schumacher B. Systemic DNA damage responses: Organismal adaptations to genome instability. Trends in Genetics. 2014; 30(3):95-102.
23. Nakad R, Schumacher B. DNA damage response and immune defense; links and mechanisms. Frontiers in Genetics, 2016, 7. doi.org/10.3389/fgene.2016.00147.
24. Bednarski JJ, Sleckman BP. At the intersection of DNA damage and immune responses. Nature Reviews Immunology. 2019; 19(4):231-242.
25. Ziembic MA, Bender TP, Larner JM, Braughtigan DL. Functions of protein phosphatase-6 in NF-ƙB signalling and in lymphocytes. Biochemical Society Transactions. 2017; 45:693-701.
26. Bayne CJ. Molluscan Immunobiology. In The Mollusca, Vol. 5 Physiology Pt. 2 A.S.M. Saleuddin and K.M. Wilbur, editors. Academic Press, San Diego, 1983, pp. 407-486.
27. Furuta E, Yamaguchi K. Transplant rejection in terrestrial molluscs. Invertebrate Survival Journal. 2011; 8:15-20.
28. Mariom Take S, Igarashi Y, Yoshitake K, Asakawa S, Maeyama K *et al*. Gene expression profiles at different stages for formation of pearl sac and pearl in the pearl oyster *Pinctada fucata*. BMC Genomics. 2019; 20:2240. doi.org/10.1186/s12864019-5579-3.