Journal of Applied Biosciences 27: 1715 - 1726

ISSN 1997–5902

Biochemical characterization of the mechanism of binding of a regulatory protein to the spermatozoa- specific protein phosphatase in Swiss Albino Rat

Surabhi Nanda[1], V.P. Varshney[2] and Sanjay Mishra[1,*]

[1]Department of Biotechnology, College of Engineering and Technology, IFTM Campus, Moradabad, UP, India; [2]Physiology & Climatology Division, Indian Veterinary Research Institute, Izatnagar 243112, U.P., India.

***Author for correspondence e-mail:** sanjaymishra66@gmail.com

Original submitted in 30th November 2009. Published online at www.biosciences.elewa.org on March 8, 2010.

ABSTRACT

Objective: The germ cell/tissue- specific Protein phosphatase, PP1γ2, plays a pivotal role in sperm function. Its activity gets reduced during sperm maturation in the epididymis. Inhibition of PP1γ2 results in motility initiation and stimulation. The aim of the present study was to determine the biochemical mechanism of PP1γ2-sds22 binding in reference to motility status of Swiss albino rat spermatozoa.

Methodology and results: The enzyme from caudal and caput sperm extracts was purified by column chromatography. PP1γ2 from caudal spermatozoa was inactive, whereas in caput spermatozoa it was active. The DEAE-cellulose flow through fractions was next passed through an SP-sepharose column. Caudal sperm sds22 and PP1γ2 were co-eluted in the gradient fraction. In contrast, caput sperm sds22 and PP1γ2 were separated in the flow-through and gradient fractions, respectively. Further purification through a Superose 6 column showed that PP1γ2-sds22 complex from caudal sperm was about 80 kDa in size. Caput sperm sds22 and PP1γ2 were observed to be eluted at 65 kDa and 39 kDa, respectively. SDS-PAGE of these purified fractions revealed that in caudal sperm, the 80 kDa species is composed of sds22 (43 kDa) and PP1γ2 (39 kDa), suggesting an equal ratio complex between these two key proteins. PP1γ2 bound to sds22 in this complex was observed to be inactive.

Conclusion and application of findings: The results indicate that dissociation of sds22 from apparently 22 kDa protein is required for its binding and inactivation of PP1γ2. Further studies to determine the mechanisms responsible for development of sds22 binding to PP1γ2 during epididymal sperm maturation are in progress.

Key words: Phosphatases, signal transduction, sperm maturation, Swiss albino rat, testis.

INTRODUCTION

Studies pertaining to physiology and biochemistry of mammalian spermatozoa have shown that microsomal membrane vesicles isolated from spermatozoa contain Ca^{2+} ATPase, and exhibit Ca^{2+} transport activities that require exogenous Mg^{2+} (Girija *et al*., 1994; Shivaji *et al*., 1995, 1998). The enzyme activity is inhibited by calcium channel inhibitors, for example verapanil and diltiazen, like the well known Ca^{2+} Mg^{2+}ATPase. The uptake of calcium is ATP (energy) dependent and the accumulated Ca^{2+} can be completely released by the Ca^{2+} ionophore A23187, suggesting that a significant fraction of the vesicles are oriented inside out (Shivaji *et al*., 1995, 1998). Besides, a review

(Sharma *et al*., 2004) shows that current research in India to develop male contraceptive is directed mainly towards: (a) development of anti-spermatogenic agents to suppress sperm production; (b) prevention of sperm maturation; (c) prevention of sperm transport through the vas deferens (or rendering these sperm infertile); and (d) prevention of sperm deposition.

The testis-specific protein phosphatase, PP1γ2, is the putative protein phosphatase in spermatozoa (Smith *et al*., 1996 a, b). High PP1γ2 activity leads to low motility, and vice versa (Smith *et al*., 1996 b). This lowering in PP1γ2 activity during sperm maturation is not due to a reduction in the amount of the enzyme but to a down-fall in its catalytic activity. It has been observed that the protein phosphatase inhibitors, okadaic acid and calyculin A, initiate and stimulate motility of epididymal spermatozoa. The enzyme PP1γ2 is known to be associated with the regulation of spermatozoa from all mammalian species studied so far (Smith *et al*., a, b; Vijayaraghavan *et al*., 1996; Chakrabarti *et al*., 2007 a, b). At least four different somatic cell serine/threonine phosphatase types, namely, PP1, PP2A, PP2B, and PP2C, are known (Mumby & Walter, 1993; Wera & Hemmings, 1995; Honkanen & Golden, 2002). The four catalytic subunit isoforms of PP1, encoded by three genes, are PP1a, PP1γ1, PP1γ2, and PP1δ (Kitagawa et al., 1990; Sasaki *et al*., 1990; Wera & Hemmings, 1995). PP1γ1 and PP1γ2 are alternatively spliced isoforms generated from a single gene (Kitagawa et al., 1990; Sasaki *et al*., 1990; Wera & Hemmings, 1995). While PP1γ1 is ubiquitous, PP1γ2 is present only in germ cells and spermatozoa (Kitagawa *et al*., 1990; Varmuza *et al*., 1999). These two PP1 variants are identical in all respects except that PP1γ2 has a unique 21 amino acid carboxy terminus extension. It is noteworthy that the carboxy terminus extension of PP1γ2, which is lacking in somatic cell PP1 isoforms, is conserved in all mammalian spermatozoa studied so far, suggesting sperm-specific roles for this amino acid sequence domain.

Disruption of the PP1 γ gene in mice causes sterility in males because of arrest of spermatogenesis at the spermatid stage (Varmuza *et al*., 1999). Studies on protein phosphatases in spermatozoa, until now, have been limited (Ahmad *et al*., 1995; Aimar *et al*., 2000; Tash *et al*., 2001; Chakrabarti *et al*., 2007 a; Han *et al*., 2007). Based on the observation that protein phosphatases in the reactivation media prevent motility initiation (Han *et al*., 2007) of invertebrate spermatozoa, it has long been suspected that protein phosphatase regulates flagellar motility. This observation shows that other isoforms of PP1 can compensate for the lack of PP1γ2 in somatic cells and for the lack of PP1γ2 in germ cells until the final stages of spermatogenesis. Testis- specific PP1γ2 is indispensable in the final stages of spermatogenesis.

The earlier studies focused on understanding mechanisms regulating PP1γ2 and determination of the basis for the decline in PP1γ2 activity during epididymal sperm maturation. Protein phosphatases, in general, are regulated by their binding and targeting proteins (Oliver & Shenolikar, 1998; Cohen, 2002; Thangaraj *et al*., 2003; Boe-Hansen *et al*., 2006; Cebesoy *et al*., 2006; Andrabi, 2007; Angekopoulou *et al*., 2007; Bengum *et al*., 2007; Oliva, 2006; Marchesi & Feng, 2007; Wu & Chu, 2008; Cheng *et al*., 2009). Following identification of PP1γ2, we speculated that one or more somatic cell protein regulators of PP1 might be present in spermatozoa. The initial efforts focused on proteins I1 and I2, which are ubiquitous PP1 inhibitors found in all somatic cells (Oliver & Shenolikar, 1998). Surprisingly, sperm do not contain any detectable I1 or I2, suggesting that mechanisms regulating PP1γ2 could be unique (unpublished data). Immunoaffinity chromatography was used for the first time to isolate PP1γ2 and its associated proteins (Huang *et al*., 2002). Sds22 is a prototypic member of a family of proteins containing leucine-rich repeats (Kobe & Deisenhofer, 1995). In caudal epididymal spermatozoa, sds22-bound PP1γ2 is catalytically inactive (Huang *et al*., 2002). The co-immunoprecipitation suggested, but did not prove, that sds22 was bound to PP1γ2. Stoichiometry of PP1γ2 binding to sds22 was not known. It was also necessary to determine why PP1γ2 activity is lowering in caudal compared to caput

spermatozoa. Therefore, the present study was undertaken to purify PP1γ2 and sds22, by column chromatography, from both caput and caudal spermatozoa and to understand how sds22 may regulate PP1γ2 activity during sperm maturation and motility initiation in Swiss albino rat.

MATERIALS AND METHODS

Preparation of sperm extracts: The Swiss albino rats were procured from the Central Animal House Facility, IFTM Campus, Moradabad. Testes with intact tunica from the mature rats were excised out carefully. Spermatozoa were isolated from caudal and caput epididymis and washed as previously described (Vijayaraghavan *et al*., 1996; Mishra *et al*., 2003 a, b, c) in buffer A (10 mM Tris- HCl [pH 7.2] containing 120 mM NaCl, 10 mM KCl, 5 mM MgSO4). Sperm pellets were suspended in a homogenization buffer (buffer B) (10 mM Tris [pH 7.2] containing 1 mM EDTA, 1 mM EGTA, 10 mM benzamidine- HCl, 1 mM PMSF, 0.01 mM *N-p*-tosyl-L-phenylalanine chloromethyl ketone [TPCK], and 5 mM b-mercaptoethanol). The sperm suspension was sonicated with three 5-sec bursts of a sonicator at maximum setting. The sperm sonicate was then centrifuged at 16000 x *g* for 10 min. The 16000 x *g* supernatants were supplemented with 10% glycerol and stored at -20°C until further use for column procedures.

Column chromatography: All column procedures were conducted at 4 to 8°C. Total protein in caudal and caput sperm extracts and fractions obtained from column chromatography was measured with Coomassie brilliant blue dye reagent (Bio-Rad laboratories) as described previously (Bradford, 1976). Caudal sperm extract (50 ml prepared from 5 x 10^{10} spermatozoa in buffer B) was passed through a DEAE-cellulose (0.5cm x 15 cm) column pre-equilibrated with buffer C (buffer B with 0.05 M KCl and additional protease inhibitors-pepstatin A [1 mg/ml], aprotinin [2 mg/m], and leupeptin [0.5 mg/ml]). The column was washed with 20 ml of buffer C followed by elution with a linear gradient of 0.05–0.7 M KCl in buffer C. Flowthrough and gradient fractions (0.2–0.4 M KCl) containing PP1γ2 activity and PP1γ2 and/or sds22 immuno-reactivity were pooled separately. The pooled fractions were concentrated to 12.5 ml using Centricon-10 filter (Millipore Corp., Bedford, MA). A SP-sepharose column (5 ml prepacked, Pharmacia) was pre-equilibrated with buffer C. The DEAE-cellulose flow-through fraction was applied to this SP-sepharose column. The column was then washed with 10 ml buffer C followed by elution with a linear gradient of 0.05–0.7 M KCl in buffer C. Fractions containing sds22 and PP1γ2 were pooled, concentrated to 1 ml, and applied in five batches to Superose 6 (24 ml, prepacked high-resolution FPLC, Pharmacia) column pre-equilibrated with buffer C. The elution was performed with buffer C. Immunoreactive fractions of all five batches were pooled, concentrated to 1 ml, and applied to Mono S (1 ml, prepacked high-resolution FPLC, Pharmacia) column. The column was washed with 5 ml buffer C followed by elution with a linear gradient of 0.05–0.7 M KCl in buffer C. Immunoreactive fractions (0.15–0.20 M KCl) containing sds22-PP1γ2 complex were pooled, and concentrated to 0.5 ml. For purification of sds22 and PP1γ2 from caput epididymal spermatozoa, we followed the same protocol as described above for caudal sperm, except that 50 ml of extract derived from 1 x 10^{10} spermatozoa was used. The final step for purifying caput sds22-p22 complex through a heparin sepharose (1 ml, prepacked Pharmacia) column was similar to the protocol used for Mono S column chromatography. Purified and concentrated caudal sds22-PP1γ2 complex and caput sds22 and free PP1γ2 fractions were stored at -20°C until further use.

Protein phosphatase assay: Preparation of radiolabeled phosphorylase *a*, and its use as a substrate for measurement of PP1 activity is based on a protocol described previously (Vijayaraghavan *et al*., 1996; Mishra *et al*., 2003 a, b, c). The substrate, aliquots from sperm extracts, and fractions obtained after column chromatography were incubated (in a total volume of 40 µl) at 30°C with 1 mM Mn^{2+} and with or without inhibitors for 10 min. At the end of this incubation, the reaction was terminated by adding 180 µl of 20% trichloro acetic acid, after which the tubes were centrifuged for 5 min at 12000 x *g* at 4°C. The supernatants were analyzed for $^{32}PO4$ released from phosphorylase *a.* One unit of enzyme activity was defined as the amount of enzyme that catalyzed the release of 1 nmol of $^{32}PO4$/ min under conditions of the enzyme assay. This assay is specific for the protein phosphatases PP1 and PP2A (Vijayaraghavan *et al*., 1996; Mishra *et al*., 2003 a, b, c).

SDS-polyacrylamide gel electrophoresis: Sperm extracts (25–50 mg) and aliquots from fractions obtained from column chromatography (2–10 mg) were separated by SDS-PAGE through 12% acrylamide slab

gels based on the protocol of Laemmli (1970). One milliliter of soluble extracts (prepared from 10^9 cells) contain approximately 0.5 mg/ml and 1.5 mg/ml protein for caudal and caput sperm, respectively.

Total protein assay: Total protein was measured with Coomassie Brilliant Blue dye reagent (Bio-Rad) as described previously (Bradford, 1976). Proteins were precipitated with 10% TCA and dissolved in 0.05M NaOH. This precipitation step was necessary because of the interference in the assay by benzimidine present in the homogenization buffer.

RESULTS

Purification of PP1γ2 and sds22 From Caudal epididymal spermatozoa: Following at least three pilot purification runs, 50 ml of sperm extracts prepared from 5 x 10^{10} Swiss albino rat caudal epididymal spermatozoa were used to isolate enough PP1γ2 and sds22 for biochemical studies. Specific activity of PP1γ2 in the pooled extracts was 2.45 U/mg protein. A summary of the purification steps is shown in Table 1.

Table 1: Summary of sds22-PP1γ2 purification from caudal epididymal sperm extracts of Swiss albino rats.

Purification step	Protein (mg)	Total Activity (U)	Specific Activity (U/mg)
Caudal sperm extract	205	502	2.45
DEAE – cellulose flow through	83	Inactive	ND
DEAE cellulose gradient (0.2-0.4 M KCl) (PP γ2 without sds22)	76	396	5.21
SP Sepharose gradient (0.13-0.28 M KCl) of DEAE cellulose flow through	35	Inactive	ND
Superose 6 of SP Sepharose gradient	2.21	Inactive	ND
Mono S gradient (0.15-0.20 M KCl)	1.03	Inactive	ND

Data are purification of three batches of 50 ml each of caudal sperm extract. The total number of cells corresponding to 205 mg protein is 1.5 X 10^{11}. Activity of PP1γ2 in caudal sperm extracts and fractions obtained after column procedures was measured in the presence of 1 mM Mn^{2+}.

A portion of the enzyme was found in the flow-through fraction, and the rest was absorbed in the column. In addition to PP1γ2, the DEAE-cellulose flowthrough fraction also contained immunoreactive sds22 (data not shown). The enzyme in the DEAE-cellulose flow-through fraction was inactive whereas that released from the column by a salt gradient (0.20-0.40 M KCl) showed a specific activity of 5.21 U/mg protein.

This manuscript's focus is on the association of PP1γ2 to sds22 and details of purification of PP1γ2 released from the DEAE-cellulose column are not presented. Here PP1γ2 and sds22 were bound in the column. All the bound PP1γ2 and sds22 were released by a salt gradient between 0.13–0.28 M KCl. The occurrence of sds22 and PP1γ2 in the DEAE-cellulose and SP-sepharose column fractions was assessed by the PP1γ2 assay showing the inactivation of these fractions (Table 1). After concentrating the SP-sepharose salt gradient fractions containing both PP1γ2 and sds22 and passing them through a Superose 6 column, they were co-eluted as an 80-kDa species in fractions 27, 28, and 29 (Fig. 1). Co-elution of sds22 and PP1γ2 through these three columns, DEAE-cellulose, SP-sepharose, and Superose 6, suggested that they could be complexed.

To confirm their association, we further purified sds22 and PP1γ2 containing fractions through a Mono S column, where they were absorbed on the Mono S column and co-eluted in a 0.15–0.20 M KCl gradient. These fractions containing PP1γ2 and sds22 were pooled, concentrated and analyzed by SDS-PAGE. Coomassie blue and silver staining following SDS-PAGE (Fig. 2) showed that the complex eluting from Mono S column contains two major protein bands at 43 and 39 kDa. All column fractions containing sds22 and PP1γ2 had virtually no protein phosphatase activity (Table 1).

Purification of PP1γ2 and sds22 from Caput Epididymal Spermatozoa of Swiss Albino Rat: Subsequently, we subjected caput sperm extracts to essentially the same purification scheme used for caudal sperm extracts. Caput sperm extracts have higher PP1γ2-specific activity: 12.3 U/mg protein, compared to 2.45 U/mg protein in caudal sperm. A summary of the purification of sds22 and PP1γ2 from Swiss albino rat-caput sperm extracts is highlighted in Table 2.

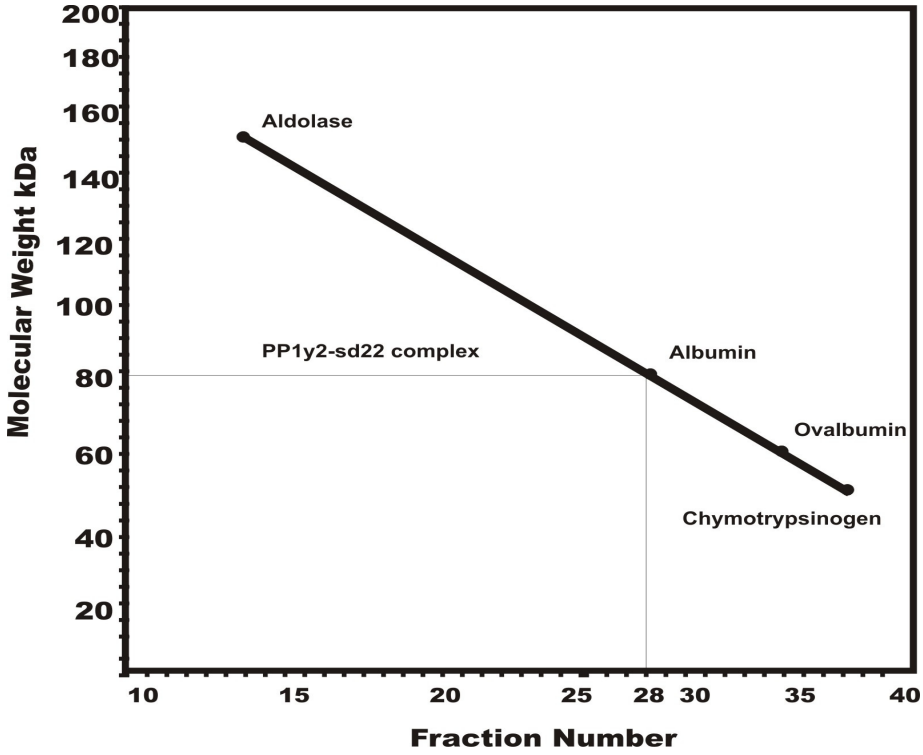

Figure 1: Molecular weight determination of the caudal sperm PP1γ2- sds22 complex. Elution profile of PP1γ2-sds22 complex from caudal sperm extracts in Superose 6. PP1γ2-sds22 complex eluted through Superose 6 at 80 kDa.

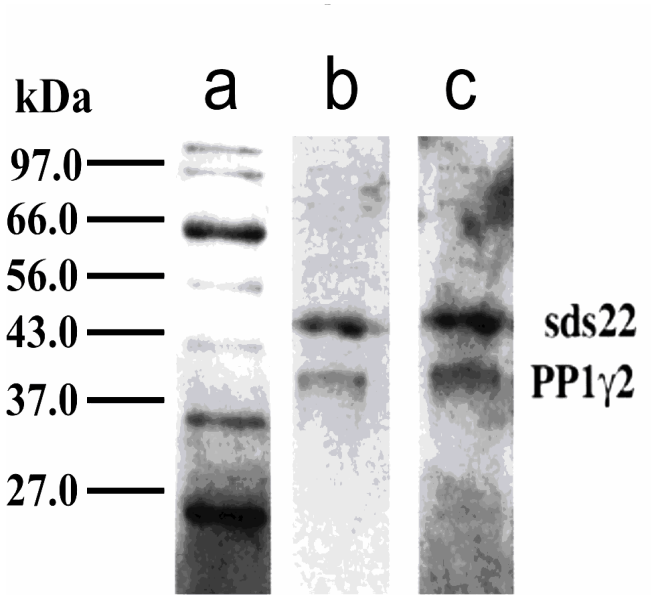

Figure 2: Caudal sperm PP1γ2 is bound to protein sds22. **Lane A** shows protein molecular weight markers. Coomassie blue (**Lane B**) and silver stained gels (**Lane C**) showing PP1γ2 and sds22 after SDS-PAGE of purified PP1γ2-sds22 complex obtained after Mono S column chromatography (2 mg). Silver staining was performed on the same gel following Coomassie blue.

Table 2. Summary of sds22 and PP1γ2 purification from caput epididymal sperm extracts of Swiss albino rats.

Purification step	Protein (mg)	Total Activity (U)	Specific Activity (U/mg)	Recovery %	Purification fold
Caput Sperm Extract	68.5	843.92	12.32	100	1
DEAE cellulose flow through (sds22 +PP1γ2)	31.7	65.71	25.81	7.8	2.1
DEAE cellulose gradient (0.2-0.3 M KCl) (PP1γ2 without sds22)	35.3	616	17.45	73	1.4
SP Sepharose flow through of DEAE cellulose flow through (free sds22)	17.6	Inactive	ND	ND	ND
Superose 6 (sds22)	1.6	Inactive	ND	ND	ND
Heparin Sepharose flow through (sds22)	0.78	Inactive	ND	ND	ND
SP Sepharose gradient (0.4-0.5 M KCl of DEAE cellulose flow through) (free PP1γ2)	6.6	241	36.75	29	3.0
Superose 6 (free PP1γ2)	0.26	217	835	26	68

NB: Data are purification of three batches of 50 ml each of caput sperm extract. The total number of cells corresponding to 68.5 mg protein is 3 X 10^{10}. Activity of PP γ2 in caput sperm extracts and fractions obtained after column procedures was measured in the presence of 1 mM Mn^{2+}.

Similar to caudal sperm extracts, PP1γ2 in caput sperm extracts also separated into flow-through and gradient fractions on DEAE-cellulose. In addition to PP1γ2, the DEAE -cellulose flow-through fraction also contained immunoreactive sds22 (data not shown). However, unlike caudal sperm (Table 1), PP1γ2 in the DEAE-cellulose flow-through fraction of caput sperm extracts was catalytically active: 25.8 U/mg protein (Table 2). The enzyme released by a salt gradient (0.20–0.30 M KCl) from the DEAE-cellulose column had a specific activity of 17.5 U/mg protein.

After passing the DEAE-cellulose flow-through fraction through an SP-sepharose column, sds22 was found in the flow through fraction, but PP1γ2 was absorbed by the matrix and was eluted by a salt gradient (0.40–0.50 M KCl). Specific activity of enzyme eluting from SP-sepharose was 36.8 U/mg protein (Table 2). The SP-sepharose column fractions containing sds22 and PP1γ2 were concentrated and passed through a Superose 6 column. Caput sperm sds22 eluted as a 65-kDa protein, fractions 30, 31 and 32 (Fig. 3). This 65-kDa protein was further passed through a heparin sepharose column, in which immunoreactive sds22 was found in the flow-through fraction (data not shown). SDS-PAGE followed by Coomassie blue and silver staining reflects that heparin Sepharose-purified 65 kDa species is composed of two major protein bands at 43 kDa and 22 kDa (p22). The SP-sepharose fractions containing PP1γ2 eluted through the Superose 6 column at about 39 kDa: a molecular size in close correspondence to the calculated size based on its amino acid sequence (39 kDa) (data not shown). Identification of the purified PP1γ2 and sds22 is underway to confirm through Western blot analysis based on the preliminary experiments carried out using ELISA.

Caput Sperm sds22-p22 Complex is Observed Not to Bind to PP1γ2: Caudal but not caput sperm sds22 is bound to PP1γ2. A logical question is whether the lack of sds22-PP1γ2 binding is due to some modification in PP1γ2 in caput spermatozoa. If this were the case, purified caput sperm sds22 should bind to recombinant PP1γ2 extracts expressed in bacteria. It

was investigated whether the purified caput sperm sds22-p22 complex can inhibit recombinant PP1γ2 *in vitro*. Variable concentrations of heparin sepharose-purified sds22-p22 complex (0 to 2 mg) were pre-incubated with PP1γ2 (Table 2) followed by measurement of protein phosphatase activity. Catalytic activity of PP1γ2 activity was not significantly inhibited at any concentration of sds22 (Fig. 4). Calyculin A (5 nM), a well known inhibitor of PP1, does inhibit catalytic activity of the recombinant enzyme (Fig. 4), reflecting that the recombinant enzyme is capable of binding to these inhibitors. Identical results to those shown in Figure 4 were also obtained when recombinant PP1α or PP1γ2 purified from caput sperm extracts (Superose 6 eluate; Fig. 3) instead of recombinant PP1γ2 was used (data not shown).

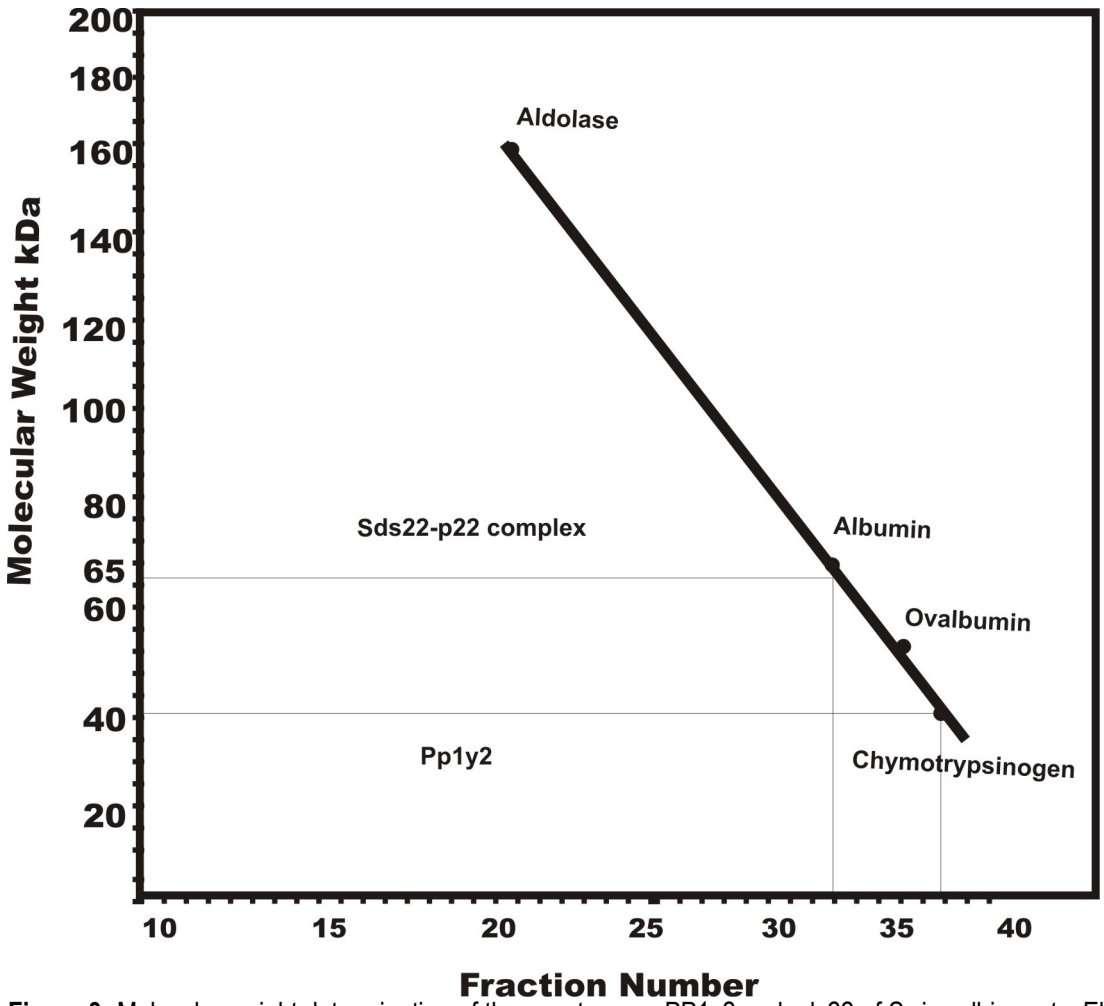

Figure 3: Molecular weight determination of the caput sperm PP1γ2 and sds22 of Swiss albino rats. Elution profile of sds22-p22 complex and PP1γ2 from caput sperm extracts in Superose 6. PP1γ2 and sds22-p22 complex eluted through Superose 6 at 39 kDa and 65 kDa, respectively.

DISCUSSION

It is well known that PP1 isoforms are regulated by several ubiquitous and cell-specific regulatory and targeting proteins in case of somatic cells (Oliver & Shenolikar, 1998). Germ cell-specific protein phosphatase PP1γ2 is the putative serine/threonine phosphatase in spermatozoa (Smith *et al*., 1996 a, b; Vijayaraghavan *et al*., 1996). The activity of this enzyme is inversely correlated with motility: low activity in vigorously motile caudal spermatozoa and high activity in immotile caput spermatozoa (Smith *et al.,* 1996 b). Inhibition of the enzyme with okadaic acid and calyculin A causes motility initiation and stimulation (Smith *et al*., 1996 a, b; Vijayaraghavan *et al*., 1996). Our focus was to understand how PP1γ2 is regulated

and how the activity of the enzyme declines during epididymal sperm maturation. Antibodies to PP1γ2 co-precipitate sds22 from sperm extracts suggesting that sds22 is bound to PP1γ2 (Huang *et al*, 2002). One of the objectives of this study was to confirm this observation by column purification techniques and determine stoichiometry of the PP1γ2-sds22 complex. Caudal sperm sds22 co-purifies with PP1γ2 through four different columns: DEAE-cellulose, SP sepharose, Superose 6, and Mono S. The fact that sds22 and PP1γ2 co-purify in column chromatography and immunoprecipitation (Huang *et al*., 2002) strongly supports the conclusion that sds22 is complexed to PP1γ2. The apparent molecular weight of the sds22 and PP1γ2 complex, determined by Superose 6 column chromatography, is 80 kDa. This is in close correspondence with a molecular weight of 82 kDa, for an equal ratio PP1γ2-sds22 complex, assessed as per their amino acid profiles.

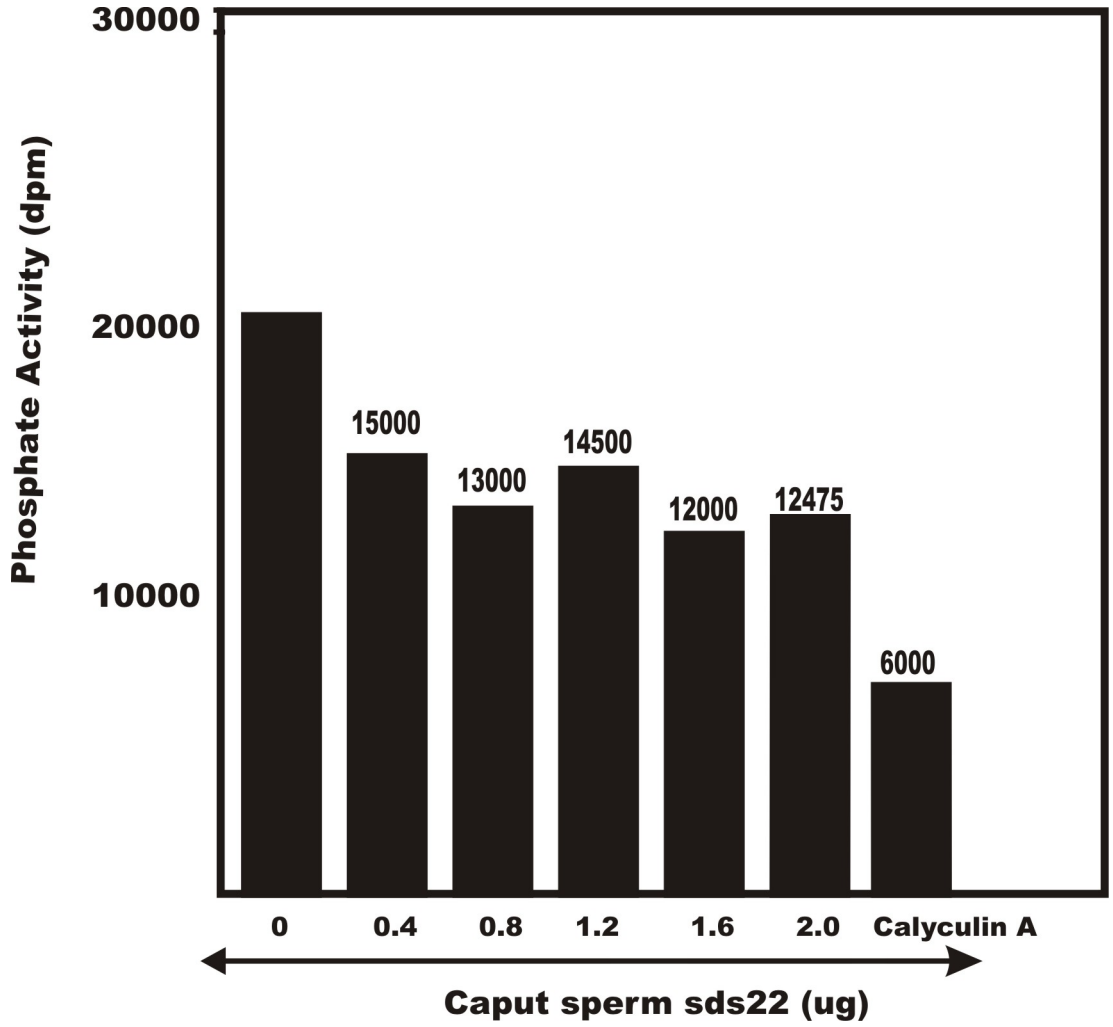

Figure 4: Effect of caput sperm sds22-p22 complex on catalytic activity of recombinant PP1γ2. The indicated amounts of sds22-p22 complex purified from caput sperm extracts, 5 nM calyculin A were added to recombinant PP1γ2 (100 mU) and incubated at 30°C for 15 minutes. After this incubation, the phosphatase assay was initiated with phosphorylase.

Our data do not rule out the possibility that some of the caudal sperm sds22 may exist as a homodimer (86 kDa). It is, however, unlikely that this sds22 will co-elute with the sds22-PP1γ2 complex through the four different column matrices. Furthermore, free sds22 (i.e., sds22 not bound to PP1γ2) exists as a 65-kDa species in caput spermatozoa (Fig. 3). It is noteworthy that immunoreactive sds22 is not detected in any other

column fraction except those also containing PP1γ2 (Table 1) suggesting that all of the sds22 present in caudal sperm extracts is bound to PP1γ2 (i.e., sds22 in sperm extracts is a PP1γ2 regulatory protein). In accordance with earlier observations made with crude caudal sperm extracts (Huang et al., 2002), we also determined that column-purified sds22-PP1γ2 complex does not also bind to microcystin (data not shown). Microcystin can bind to PP1 and PP2A catalytic subunits alone or when the catalytic subunits are associated with a variety of their regulatory proteins (Meek *et al.,* 1999). In particular, the sds22 complex with the somatic cell PP1 isoform, PP1α, can bind to microcystin (Tran *et al*., 2002). The inability of the sperm sds22-PP1γ2 complex to bind to microcystin suggests that sds22 masks the microcystin- binding site on PP1γ2. Purified PP1γ2-sds22 complex and the complex present in the different column fractions are virtually devoid of catalytic activity (Table 1).

The sds22-PP1γ2 complex isolated by immunoaffinity chromatography from crude sperm extracts was also inactive (Huang *et al*., 2002). Thus sds22 is a PP1γ2 inhibitor in spermatozoa. It is noteworthy that spermatozoa do not contain the ubiquitous somatic cell PP1 inhibitors I1 and I2 (our unpublished data). Data presented in this report provide strong evidence that in motile caudal spermatozoa PP1γ2, present as an equal ratio complex with sds22, is catalytically inactive.

The next objective of this study was to examine whether sds22 in caput epididymal spermatozoa is also bound to PP1γ2. Data presented in Table 2 and Figure 3 clearly reflect that caput sperm sds22 is not bound to PP1γ2. Unlike caudal sperm extracts, PP1γ2 from caput spermatozoa in the DEAE cellulose flow-through fraction and SP-sepharose gradient fractions is catalytically active. The specific activity of caput sperm PP1γ2, not bound to sds22, following Superose 6 column purification, is 567 U/mg protein. The enzyme at this stage of purification (68-fold purification, compared to crude extracts, Table 2) is not expected to be homogenous. Our principal motto here was not purification of the enzyme to homogeneity but to assess whether the enzyme was bound to sds22 or some other regulatory protein. The apparent molecular weight of the active subunit of the enzyme eluting through the Superose 6 column is 39 kDa in correspondence with the calculated molecular weight (39 kDa) based on its amino acid profile. In caput sperm, active PP1γ2 is not bound to sds22 or any other regulatory protein. One of the reasons for the high protein phosphatase activity in caput extracts is due to this 39-kDa free catalytic subunit form of PP1γ2. Our studies also show that a portion of PP1γ2 in both caput and caudal sperm extracts is bound to DEAE-cellulose. Specific activities of PP1γ2 released from DEAE-cellulose are 17.5 and 5.2 U/mg protein in caput and caudal sperm, respectively (Tables 1 and 2). One of the important questions emerging from this study is why sds22 from caput spermatozoa is not bound to PP1γ2. Caput sperm sds22 elutes as a 65-kDa species through the Superose 6 column (Fig. 3). This 65-kDa species consists of sds22 (43 kDa) and another 22-kDa protein (p22) (data not shown). This suggests that sds22 may exist as a complex with p22 in caput spermatozoa. It is therefore possible that p22, bound to sds22, prevents sds22 from binding to PP1γ2. Caput sperm sds22 complexed to p22 is also unable to inhibit recombinant PP1γ2 *in vitro* (Fig. 4), presumably because it cannot bind to it. Partially purified caput sperm PP1γ2 behaves like recombinant PP1γ2 with respect to inhibition by calyculin A and binding to microcystin. Thus, the reason for inability of sds22 to bind to PP1γ2 probably is not due to a modification of the PP1γ2 subunit but most likely is due to the fact that p22 bound to sds22 prevents formation of the sds22- PP1γ2 complex. Dissociation of the sds22-p22 complex and sds22-PP1γ2 complex formation may be caused by phosphorylation or some other chemical modification of sds22 or p22. Alternatively, p22 may be proteolyzed during epididymal sperm maturation (i.e., p22 may be absent in caudal spermatozoa). Molecular characterization of p22, development of antibodies against p22, and determination of the status of p22 in caudal spermatozoa will help us to explore mechanisms underlying sds22 dissociation from p22 followed by binding to PP1γ2 during epididymal sperm development.

Conclusively our data demonstrate that purified caput sds22 is free of PP1γ2. It could be argued that p22 may not be bound to sds22 but is a protein contaminant co-eluting with sds22 in the Superose 6 column (Fig. 3). Other mechanisms may be responsible for the development of sds22 binding to PP1γ2. It may be possible that sds22 phosphorylation may be necessary for its binding to PP1γ2 (i.e., sds22 may be phosphorylated in caudal but not caput spermatozoa). Insights into these questions might have been provided if we were able to immunoprecipitate sds22 from sperm extracts. Whatever the reason for the lack of binding of sds22 to PP1γ2 may be, our data clearly show that all of sds22 in caudal sperm extracts

is bound to PP1γ2, whereas in caput sperm extracts, sds22 is free of any detectable PP1γ2. A consequence of this difference is that a proportion of caput sperm PP1γ2 is in its free (39 kDa) catalytically active form, with a specific activity of 567 U/mg protein after partial purification through Superose 6 column (Table 2). Thus, one of the reasons for the higher PP1γ2 activity in caput than in caudal spermatozoa is that PP1γ2 is not bound to sds22. Further, the studies described herein clearly indicate that a fraction of PP1γ2 in motile caudal epididymal spermatozoa is inactive and present as a heterodimer with sds22. In immotile caput spermatozoa, sds22 is not bound to PP1γ2 but is either free or bound to a 22-kDa protein. Therefore, a portion of caput sperm PP1γ2 is in its catalytically active and free form. It is therefore very likely that the reason for the higher PP1γ2 activity in caput, compared to caudal spermatozoa might be due to the inability of sds22 to bind and inactivate PP1γ2. We hypothesize that the change in binding partners of sds22 from p22 to PP1γ2 and the development of the binding capacity of sds22 for PP1γ2 are key biochemical events responsible for the decline in protein phosphatase activity during epididymal sperm maturation and motility initiation.

ACKNOWLEDGEMENTS: Institutional research promotion grant to the Department of Biotechnology, College of Engineering and Technology, Moradabad, U.P., India is also acknowledged. The authors are grateful to Prof. R. M. Dubey (Managing Director) and Prof. A. Srivastav (Director), CET, IFTM Campus, Moradabad, U.P., India) for providing the necessary facilities and encouragement. The authors are also thankful to Prof. B.N. Basu (Academic Advisor) and Prof. B.N. Kaul (Dean, Administration), Prof. S.K. Maheshwari (Department of Biotechnology), College of Engineering and Technology (CET), Moradabad, U.P., India, for their generous help and suggestions during the course of experimental work.

REFERENCES

Ahmad K, Bracho GE, Wolf DP, Tash JS, 1995. Regulation of human sperm motility and hyperactivation components by calcium, calmodulin, and protein phosphatases. Archives of Andrology 35: 187-208.

Aimar C, A. Bautz, D. Durand, H. Membre, D. Chardard, P.L. Gualandris, D. Husson and C. Dournon, 2000. Microgravity and hypergravity effects on fertilization of the salamander *Pleurodeles waltl* (urodele amphibian). Biology of Reproduction, 63:551-558.

Andrabi, S.M., 2007. Mammalian sperm chromatin structure and assessment of DNA fragmentation. Journal of Assistance in Reprodive Genetics, 24: 561–569.

Angelopoulou, R., K. Plastira, and P. Msaouel, 2007. Spermatozoal sensitive biomarkers to defective protaminosis and fragmented DNA. Reproductive Biology and Endocrinology, 5**:** 36.

Boe-Hansen, G.B., J. Fedder, A.K. Ersboll and P. Christensen, 2006. The sperm chromatin structure assay as a diagnostic tool in the human fertility clinic. Human Reproduction, 21: 1576–1582.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry, 72:248–254.

Bungum, M., P. Humaidan, A. Axmon, M. Spano, L. Bungum, J. Erenpreiss, and A. Giwercman, 2007. Sperm DNA integrity assessment in prediction of assisted reproduction technology outcome. Human Reproduction, 22: 174–179.

Cebesoy, F.B., K. Aydos, and C. Unlu, 2006. Effect of sperm chromatin damage on fertilization ratio and embryo quality post-ICSI. Archives of Andrology, 52**:** 397–402.

Chakrabarti, R., L. Cheng, P. Puri, D. Soler and S. Vijayaraghavan, 2007a. Protein phosphatase PP1γ2 in sperm morphogenesis and epididymal initiation of sperm motility. Asian Journal of Andrology, 9: 445- 452.

Chakrabarti, R., D. Kline, J. Lu, J. Orth, S. Pilder and S. Vijayaraghavan, 2007b. Analysis of Ppp1cc-null Mmce suggests a role for PP1gamma2 in sperm morphogenesis. Biology of Reproduction, 76: 992-1001.

Cheng, L., S. Pilder, A.C. Nairn, S. Ramdas and S. Vijayaraghavan, 2009. PP1γ2 and PPP1R11 are parts of a multimeric complex in developing testicular germ cells in which their steady state levels are reciprocally related. PLoS One, 4(3): e 486.

Cohen, P.T., 2002. Protein phosphatase 1--targeted in many directions. Journal of Cell Science, 115: 241- 256.

Girija Devi, L., M. Bashher Ahmad and S. Shivaji, 1994. Secretory proteins of the hamster cervix, uterus and oviduct: The effects of estradiol, progesterone and testosterone on the proteins secreted into the medium. The Journal of Steroid Biochemistry and Molecular Biology, 51: 107-114.

Han, Y., C.J. Haines, and H.L. Feng, 2007. Role(s) of the Serine/Threonine Protein Phosphatase 1 on Mammalian Sperm Motility. Systems Biology In reproductive Medicine, 53: 169-177.

Honkanen, R.E. and T. Golden, 2002. Regulators of serine/threonine protein phosphatases at the dawn of a clinical era? Current Medicinal Chemistry, 9: 2055– 2075.

Huang, Z,. B. Khatra, M. Bollen, D.W. Carr and S. Vijayaraghavan, 2002. Sperm PP1 {gamma} 2 is regulated by a homologue of the yeast protein phosphatase binding protein sds22. Biology of Reproduction, 67:1936–1942.

Kitagawa, Y., K. Sasaki, H. Shima, M. Shibuya, T. Sugimura and M. Nagao, 1990. Protein phosphatases possibly involved in rat spermatogenesis Biochem Biophys Research Communications., 31: 230– 235.

Kobe, B. and J. Deisenhofer , 1995. A structural basis of the interactions between leucine-rich repeats and protein ligands. Nature, 374: 183–186.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227:680–685.

Marchesi, D. E. and H. L. Feng, 2007. Sperm DNA integrity from sperm to egg.Journal of Andrology, 28: 481-489.

Meek, S., N. Morrice and C. MacKintosh, 1999. Microcystin affinity purification of plant protein phosphatases: PP1C, PP5 and a regulatory A-subunit of PP2A. FEBS Letters, 457: 494–498.

Mishra, S., P.R. Somnath and S. Vijayaraghavan, 2003a. Distinct pools of PP1 gamma2 are regulated by sds22, protein 14-3-3 and hsp90 within spermatozoa. Experimental Biology & Translating the Genome Meeting, April 11-15: San Diego, CA, USA: Abstract number: LB270.

Mishra, S., Z. Huang and S. Vijayaraghwan, 2003b. Binding to sds22 and inactivation of catalytic activity of the protein phosphatase PP1 gamma2 occurs dutring sperm motility initiation in epididymis. 36th Annual Meeting of Society for the Study of Reproduction, July 19-22: Cincinati, Ohio, USA.

Mishra, S., P.R. Somnath, Z. Huang and S. Vijayaraghavan, 2003c. Binding and Inactivation of the Germ Cell-Specific Protein Phosphatase PP1γ2 by sds22 During Epididymal Sperm Maturation. Biology of Reproduction, 69: 1572-1579.

Mumby, M.C. and G. Walter, 1993. Protein serine/threonine phosphatases: structure, regulation, and functions in cell growth. Physiological Review, 73: 673–699.

Oliver, C.J. and S. Shenolikar, 1998. Physiologic importance of protein phosphatase inhibitors. Frontiers in Bioscience, 3: D961–D972.

Oliva, R., 2006. Protamines and male infertility. Human Reproduction Update, 12: 417-435.

Sasaki, K., H. Shima, Y. Kitagawa, S. Irino, T. Sugimura and M. Nagao, 1990. Identification of members of the protein phosphatase 1 gene family in the rat and enhanced expression of protein phosphatase 1 alpha gene in rat hepatocellular carcinomasJapanese Journal of Cancer Research, 81:1272–1280.

Sharma, R.K., T. Said and A. Agarwal, 2004. Sperm DNA damage and its clinical relevance in assessing reproductive outcome. Asian Journal of Andrology, 6: 139-148.

Shivaji, S., J. Peedicayil and L. Girija Devi, 1995. Analysis of the motility parameters of in vitro hyperactivated hamster spermatozoa. Molecular Reproduction and Development, 42: 233-247.

Shivaji, S., D. Jayaprakash and S.B. Patil, 1998. Assessment of inbreeding depression in big cats: testosterone levels and semen analysis. Current Science, 75: 923-930.

Smith, G.D., D.P. Wolf, K.C. Trautman, E.F. da Cruz e Silva, P. Greengard, and S. Vijayaraghavan, 1996a. Primate sperm contain protein phosphatase1, a biochemical mediator of motility. Biology of Reproduction, 54:719–727.

Smith, G.D., D.P. Wolf, K.C. Trautman and S. Vijayaraghavan, 1996b. Motility potential of macaque epididymal sperm: the role of protein phosphatase and glycogen synthase kinase-3 activities. Journal of Andrology, 20:47–53.

Tash, J.S., S. Kim, M. Schuber, D. Seibt and W.H. Kinsey, 2001. Fertilization of sea urchin eggs

and sperm motility are negatively impacted under low hypergravitational forces significant to space flight. Biology of Reproduction, 65 (4): 1224-1231

Thangaraj, K., M.B. Joshi, A.G. Reddy, A.A. Rasalkar and L. Singh, 2003. Sperm mitochondrial mutations as a cause of low sperm motility. Journal of Andrology, 24: 388.

Tran, H.T., D. Bridges, A. Ulke and G.B. Moorhead, 2002. Detection of multiple splice variants of the nuclear protein phosphatase one regulator sds22 in rat liver nuclei. Biochemistry and Cell Biology 80: 811–815.

Varmuza, S., A. Jurisicova, K. Okano, J. Hudson, K. Boekelheide and E.B. Shipp, 1999. Spermiogenesis is impaired in mice bearing a targeted mutation in the protein phosphatase 1cgamma gene. Developmental Biology, 205: 98–110.

Vijayaraghavan, S., D.T. Stephens, K. Trautman, G.D. Smith, B. Khatra, E.F. da Cruz e Silva, and P. Greengard, 1996. Sperm motility development in the epididymis is associated with decreased glycogen synthase kinase-3 and protein phosphatase 1 activity. Biology of Reproduction, 54:709– 718.

Wera, S. and B.A. Hemmings, 1995. Serine/threonine protein phosphatases. Biochemical Journal, 311:17– 29.

Wu, T.F. and D.S. Chu, 2008. A review: Sperm chromatin: Fertile grounds for proteomic discovery of clinical tools. Molecular & Cellular Proteomics, 7: 1876-1886.