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Dephosphorylation of Autophosphorylated Regulatory Subunit of Sea Urchin Sperm cAMP-Dependent Histone Kinase by an Endogenous Protein Phosphatase

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ABSTRACT—Seven protein phosphatases were isolated from extracts of spermatozoa and sperm tails of the sea urchin *Hemicentrotus pulcherrimus* by ion exchange and gel filtration chromatographies using [³²P]-histone and/or p-nitrophenyl phosphate (pNPP) as substrates and characterized for their enzymatic and molecular nature. Two of them isolated from the particulate fraction correspond to the mammalian type 1 and 2B protein phosphatases. Another two obtained from the soluble fraction were similar to the mammalian protein phosphatases, type 2A and 2C. A protein phosphatase corresponding to the mammalian type 1 enzyme (molecular mass of 43 kDa) dephosphorylated the [³²P]-autophosphorylated regulatory subunit of *H. pulcherrimus* sperm cAMP-dependent histone kinase.

INTRODUCTION

Cyclic nucleotide-dependent phosphorylation is one of the major signal transduction pathway in cells. The level of protein phosphorylation of any protein depends on the relative activities of protein kinases and protein phosphatases (Cohen, 1988). In sea urchin fertilization, before contacting the egg surface a spermatozoon must pass through the jelly coat which surrounds the egg. A fucose sulfate glycoconjugate (FSG), one of the high molecular weight glycoconjugates in the jelly coat, has profound effects on sea urchin spermatozoa such as induction of the acrosome reaction (SeGall and Lennarz, 1979, 1981), activation of adenylyl cyclase (Watkins *et al.*, 1978) and subsequent elevation of cAMP concentrations (Garbers and Kopf, 1980), activation of cAMP-dependent protein kinase (A-kinase) (Garbers *et al.*, 1980), and initiation of phosphorylation of sperm histone H1 (Porter and Vacquier, 1986; Porter *et al.*, 1988).

In previous studies (Harumi *et al.*, 1994), we isolated an autophosphorylated protein from the extracts of spermatozoa of the sea urchin *Hemicentrotus pulcherrimus*, and demonstrated that the protein was a cAMP-dependent histone kinase. The kinase consists of two different subunits, 39 kDa catalytic and 48 kDa regulatory; the latter subunit had been shown to be autophosphorylated. In the present study, we characterized seven protein phosphatases in *H. pulcherrimus* spermatozoa and show that one of them, which corresponds to the mammalian type 1 protein phosphatase, dephosphorylates the [³²P]-autophosphorylated 48 kDa regulatory subunit.

MATERIALS AND METHODS

Materials

Sea urchins (*H. pulcherrimus*) were collected along the coast of Toyama Bay near Noto Marine Laboratory, Kanazawa University. Spermatozoa were obtained by intracoelomic injection of 0.5 M KCl and collected as "dry sperm" at room temperature and suspended in artificial sea water containing 10 mM benzamidinium-HCl and 10 mM MES (pH 5.6) (Harumi *et al.*, 1992). The sperm suspension was centrifuged at 10,000 ×g for 10 min and the sperm pellet was stored at -40°C. [³²P]ATP (111 TBq/mmol) was purchased from Du Pont/New England Nuclear (Boston, MA, USA). 3-[(3-cholamidopropyl) dimethylammonio] propanesulfonic acid (CHAPS) was purchased from Dojindo Laboratories (Kumamoto, Japan). Catalytic subunit of bovine heart A-kinase, p-nitrophenyl phosphate (pNPP) and calf thymus histone (Type II-A) were from Sigma (St. Louis, MO, USA). Okadaic acid and calyculin A were obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Other chemicals used were of the highest purity available.

Preparation of *H. pulcherrimus* sperm cell extracts

All procedures were carried out at 0–4°C unless otherwise specified. Frozen sperm cells (1.97 g) were suspended in 10 volumes of ice-cold extraction buffer [60 mM KCl, 2 mM dithiothreitol (DTT) and 0.1 mM phenylmethanesulfonyl fluoride (PMSF), 10 mM benzamidinium-HCl and 40 mM Tris-HCl, pH 7.4], and homogenized for 10 strokes with a Teflon-glass homogenizer, followed by homogenization 3 times for 10 sec with ULTRA-TURRAX T25 (IKA-LABORTECHNIK, Staufen, Germany) and sonication 3 times for 10 sec with ULTRASONIC DISRUPTOR UD-201 (TOMY, Tokyo, Japan). The homogenate was centrifuged at 10,000 ×g for 30 min. The resulting supernatant was centrifuged at 100,000 ×g for 1 hr, and the supernatant was saved and the precipitate was suspended in 5 ml of extraction buffer and centrifuged again at 100,000 ×g for 1 hr. The supernatant was combined with the supernatant obtained from the first centrifugation at 100,000 ×g, and used for further experiments as

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the 100,000 $\times$ g supernatant fraction (21.5 ml). The precipitate obtained from the second centrifugation at 100,000 $\times$ g was suspended in 8 ml of extraction buffer containing 1% (w/v) CHAPS and homogenized for 10 strokes with a Teflon-glass homogenizer, followed by sonication 3 times for 10 sec. The homogenate was centrifuged at 10,000 $\times$ g for 15 min and the supernatant was used for further experiments as the 100,000 $\times$ g precipitate fraction (8 ml). Alternatively, the precipitate obtained by the first centrifugation at 10,000 $\times$ g was suspended in 19.7 ml of extraction buffer containing 1% (w/v) CHAPS, and homogenized as described above. The homogenate was centrifuged at 30,000 $\times$ g for 30 min and the supernatant was used for further experiments as the 10,000 $\times$ g precipitate fraction (19.6 ml).

Isolation of protein phosphatases from H. pulcherrimus spermatozoa and sperm tails

All procedures were performed at 0–4°C unless otherwise mentioned. Frozen *H. pulcherrimus* spermatozoa (21.7 g wet weight) were suspended in 217 ml of ice-cold buffer A (5 mM DTT, 2 mM EDTA, 10 mM EGTA, 10 mM benzamidine-HCl, 1 mM PMSF, 5 mM amino caproic acid and 20 mM Tris-HCl, pH 7.5) containing 1% (w/v) CHAPS and homogenized for 10 strokes with a Teflon-glass homogenizer, followed by sonication 6 times for 10 sec and brief homogenization by ULTRA-TURRAX T25. The homogenate was centrifuged at 12,000 $\times$ g for 30 min and the supernatant was centrifuged further at 100,000 $\times$ g for 2 hr. The resulting supernatant was applied onto a DEAE-Sephacel column (2.5 $\times$ 18 cm) equilibrated with buffer A containing 0.1% (w/v) CHAPS. The column was washed with 200 ml of the equilibration buffer, and then protein phosphatases were eluted with a linear gradient of NaCl from 0 to 0.5 M in buffer A containing 0.1% (w/v) CHAPS (total volume, 500 ml) at a flow rate of 50 ml/hr. Fractions of 10 ml were collected and monitored for the protein concentration and protein phosphatase activity using [32 P]-histone or pNPP as a substrate. The active fractions were pooled and concentrated with an Amicon Diaflo membrane YM 10 in a Diaflo Cell (Amicon, Inc., Beverly, MA, USA) and chromatographed on a Sepharose 6B column (2.6 $\times$ 66 cm) in buffer B (5 mM DTT, 2 mM EDTA, 0.1% (w/v) CHAPS and 50 mM Tris-HCl, pH 7.5) containing 0.2 M NaCl at flow rate 10 ml/hr. Fractions of 5 ml were collected and monitored for protein concentration and protein phosphatase activity using [32 P]-histone and/or pNPP as substrate. Active fractions were pooled and used for further experiments. Some active fractions obtained from chromatography on the Sepharose 6B column were desalted by dialysis against 10 volumes of buffer B, and subjected to ion exchange chromatography on a Mono Q HR 5/5 column equilibrated with buffer B. The protein phosphatase was eluted with a linear gradient of NaCl from 0 to 0.5 M in buffer B (total volume, 25 ml) at a flow rate of 1 ml/min. Fractions of 1 ml were collected and monitored for the protein phosphatase activity using [32 P]-histone as a substrate. The active fractions obtained from chromatography on the Mono Q column were pooled and concentrated with an Amicon Diaflo membrane YM 10 in a Diaflo Cell. The concentrated sample was applied to a Superose 12 HR 10/30 column equilibrated with buffer B containing 0.2 M NaCl and the column was developed with buffer B containing 0.2 M NaCl at a flow rate of 0.4 ml/min. The fractions of 0.4 ml were collected and monitored for protein phosphatase activity using [32 P]-histone as a substrate. Active fractions were used for further experiments.

Protein phosphatases were also purified from sperm tails (30.3g wet weight) using essentially the same method as described above. *H. pulcherrimus* sperm tails were prepared as described previously (Suzuki *et al.*, 1987).

Autophosphorylation of histone kinase

The cAMP-dependent histone kinase used in this study was purified from frozen *H. pulcherrimus* spermatozoa (39.4 g) as reported previously (Harumi *et al.*, 1994) and stored at -20°C until use. The autophosphorylation reaction of the kinase was carried out for 10 min

in 50 μ l of the reaction mixture containing 1.2 μ g kinase in buffer C [10 mM (CH₃COO)₂Mg, 1 mM DTT, 0.1 M NaCl, 0.1% (w/v) CHAPS, 10 mM Tris-HCl, pH 7.5] and 12.5 pmol [γ - 32 P]ATP (111 TBq/mmol) at 20°C. The [32 P]-autophosphorylated histone kinase was chromatographed on a Sephadex G-50 column (0.5 $\times$ 9 cm) equilibrated with buffer C to remove free [γ - 32 P]ATP. Fractions of 4 drops were collected and measured for the radioactivity, and the first radioactive peak fraction which should contain the [32 P]-autophosphorylated kinase was used for further experiments.

Dephosphorylation of autophosphorylated histone kinase and guanylyl cyclase by H. pulcherrimus sperm protein phosphatases

Protein phosphatase-containing fraction (6 μ l) was added to 30 μ l of the divalent cation-free or divalent cation-containing reaction buffer, and the mixture was preincubated for 2 min at 30°C. Dephosphorylation reaction was started by the addition of 6 μ l of the [32 P]-autophosphorylated histone kinase (3,400 cpm), continued for 5 min at 30°C, and then 3 μ l of final 2 μ M cAMP or distilled water was added. The divalent cation-free reaction buffer contained 100 mM NaCl, 1 mM DTT, 1 mM EDTA and 100 mM Tris-HCl (pH 7.5) with or without 2 μ M cAMP. The divalent cation-containing reaction buffer consisted of 100 mM NaCl, 0.5 mM DTT, 6 mM MgCl₂ and 100 mM Tris-HCl (pH 8.0) with or without 4.2 mM MnCl₂ and 0.3 mM calmodulin. The reaction was stopped by the addition of 7.5 μ l of 5 $\times$ electrophoresis sample buffer and heating at 100°C for 3 min. Then, 22.5 μ l of sample was subjected to SDS-PAGE using a 5–15% linear gradient polyacrylamide gel. The gel was silver-stained, dried and exposed to an X-ray film or an imaging plate (Fuji imaging analyzer, Japan).

Guanylyl cyclase used for dephosphorylation experiments was purified from *H. pulcherrimus* spermatozoa according to the method described by Harumi *et al.* (1992) with slight modifications: guanylyl cyclase was eluted from concanavalin A-Sepharose column with elution buffer containing 100 nM calyculin A. Dephosphorylation reaction of guanylyl cyclase was started by the addition of protein phosphatase-containing fraction (3 μ l) to the reaction buffer containing purified guanylyl cyclase (0.8 μ g in 15 μ l) and continued for 10 min at 30°C. The divalent cation-free reaction buffer for dephosphorylation of guanylyl cyclase consisted of 100 mM NaCl, 1 mM DTT and 100 mM Tris-HCl (pH 7.5). The divalent cation-containing reaction buffer used for dephosphorylation of guanylyl cyclase was the same composition as used for dephosphorylation of the [32 P]-autophosphorylated histone kinase. The reaction was stopped by the addition of 5 $\times$ electrophoresis sample buffer and heating at 100°C for 3 min, and then 35 μ l of sample was analyzed by SDS-PAGE using a 6% polyacrylamide gel. Dephosphorylation of guanylyl cyclase was assessed by the change of electrophoretic mobility shift from 131 kDa (phosphorylated form) to 128 kDa (dephosphorylated form) (Harumi *et al.*, 1992).

Determination of protein phosphatase activity

The activity of type 1, type 2A or type 2C protein phosphatase was assayed using [32 P]-histone as a substrate as described by Harumi *et al.* (1994) with slight modifications. The reaction mixture in a total volume of 50 μ l contained 100 mM KCl, 0.5 mM DTT, 20 mM Tris-HCl (pH 7.4), 30 μ M [32 P]-histone and 5 μ l of sample. The sample was preincubated in the reaction mixture without [32 P]-histone for 5 min at 30°C, and the reaction was initiated by addition of [32 P]-histone and continued for 10 min at 30°C and stopped by the addition of 100 μ l of 10 mM silicotungstic acid in 0.01 N H₂SO₄. Type 1 protein phosphatase activity was determined with the reaction mixture containing 2 mM MnCl₂. Since the mammalian type 1 protein phosphatase activity is unaffected by 1 nM okadaic acid but inhibited by 10 μ M okadaic acid (MacKintosh, 1993), difference between the activities with 1 nM and with 10 μ M okadaic acid was considered as the activity of type 1 protein phosphatase. Type 2A protein phosphatase activity was measured with the reaction mixture containing 4 mM EGTA. The mammalian type 2A protein phosphatase activity is inhibited by 1 nM

okadaic acid (MacKintosh, 1993). Thus, the activity sensitive to 1 nM okadaic acid was considered to be due to type 2A protein phosphatase. Alternatively, because the mammalian type 2C protein phosphatase activity is unaffected by okadaic acid (Bialojan and Takai, 1988), type 2C protein phosphatase activity was determined with the reaction mixture containing 10 mM $(\text{CH}_3\text{COO})_2\text{Mg}$ and 10 μM okadaic acid.

Type 2B protein phosphatase activity was assayed using pNPP as a substrate according to the method described by Tash *et al.* (1988) with slight modifications. The reaction buffer contained 100 mM NaCl, 0.5 mM DTT, 6 mM MgCl_2 , 0.1 mg/ml bovine serum albumin (BSA), 25 μM sodium orthovanadate and 20 mM Tris-HCl (pH 8.0) with or without 0.3 mM calmodulin and 3 mM CaCl_2 . To assay pNPP phosphatase activity, the enzyme sample was mixed with the reaction buffer (1 ml) and preincubated for 4 min at 28°C. Then, the reaction was started by the addition of 50 μl of 100 mM pNPP. pNPP hydrolysis was monitored by recording the changes of the absorbance at 400 nm for 10 min at 28°C. Reaction rate was calculated from the initial linear increase of the absorbance using the excitation coefficient of $1.53 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ for p-nitrophenol. It is known that the mammalian type 2B protein phosphatase activity is dependent on Ca^{2+} /calmodulin (Cohen, 1989). Thus, the difference between the activities in the presence and absence of Ca^{2+} /calmodulin was considered as type 2B protein phosphatase activity.

The pNPP phosphatase activity in the fractions obtained from a series of column eluate was assayed in 1.0 ml of a reaction buffer containing 50 μl of 100 mM pNPP. The reaction was started by the addition of the fraction, continued for 20 min at 30°C and stopped by the addition of 100 μl of 13% K_2HPO_4 . Then, the reaction mixture was centrifuged at 2,100 $\times g$ for 5 min and the supernatant was monitored for the absorbance at 400 nm.

When histone phosphatase activity was assayed with varying concentrations of okadaic acid or calyculin A, the enzyme sample was incubated with the compound for 5 min prior to initiation of the reaction.

Preparation of [^{32}P]-phosphorylated histones

Histone was phosphorylated using the catalytic subunit of bovine heart A-kinase as described by Harumi *et al.* (1994) and the phosphorylated histone ([^{32}P]-histone) was stored at -20°C.

Other methods

Protein concentration was determined by the method of Bradford (1976) using BSA as a standard. SDS-PAGE was carried out according to Laemmli (1970). The gel was silver-stained by the method of Morrissey (1981). Radioactivity of the [^{32}P]-48 kDa regulatory subunit of *H. pulcherrimus* sperm cAMP-dependent histone kinase on a dried polyacrylamide gel was measured with a Fuji BAS 2000 image analyzer and expressed as PSL value.

RESULTS

Subcellular distribution of protein phosphatases in *H. pulcherrimus* spermatozoa

When *H. pulcherrimus* sperm homogenates were separated into three fractions by differential centrifugation, four different protein phosphatase activities were detected (Table 1). According to Cohen's classification (1989), they correspond to the mammalian type 1, 2A, 2B or 2C protein phosphatase. Type 1 protein phosphatase activity was detected only in the fraction extracted from the 10,000 $\times g$ precipitate fraction with the extraction buffer containing 1% CHAPS. Type 2A protein phosphatase activity was present mainly in the 100,000 $\times g$ supernatant fraction. Type 2B protein phosphatase activity was detected mainly in the fraction extracted from the 100,000 $\times g$ precipitate fraction with the extraction buffer containing 1% CHAPS. Type 2C protein phosphatase activity was detected in the 100,000 $\times g$ supernatant fraction. The absolute requirement for Ca^{2+} /calmodulin and Mg^{2+} distinguishes type 2B and type 2C protein phosphatases from type 1 and type 2A phosphatases. When type 2B protein phosphatase activity was assayed using [^{32}P]-histone as a substrate, Ca^{2+} /calmodulin-dependent type 2B protein phosphatase activity was not detected in the presence of 10 nM okadaic acid, of which concentration has been reported to inhibit the mammalian type 2A enzyme significantly (Cohen, 1989). However, when pNPP was used as a substrate, Ca^{2+} /calmodulin-activatable protein phosphatase (corresponding to type 2B) activity could be enhanced 4-fold by 4 mM MnCl_2 , but it was not inhibited by 10 nM okadaic acid (Pallen and Wang, 1983; Cohen, 1989). In addition to type 1 protein phosphatase activity, the 10,000 $\times g$ precipitate fraction contained three other minor protein phosphatase activities. These activities may be residual activities of these phosphatases due to incomplete extraction.

Isolation and characterization of protein phosphatases from *H. pulcherrimus* spermatozoa and sperm tails

To clarify which protein phosphatases dephosphorylate the [^{32}P]-autophosphorylated 48 kDa regulatory subunit of *H. pulcherrimus* sperm cAMP-dependent histone kinase, fractions obtained by differential centrifugation were examined for the

Table 1. Subcellular distribution of *H. pulcherrimus* sperm protein phosphatases

Fractions	Activity (pmol Pi/min/mg protein)			
	Type1	Type2A	Type2B*	Type2C
10,000 $\times g$ precipitate	5.93	0.70	0.14	0.10
100,000 $\times g$ supernatant	N.D.	1.54	N.D.	0.16
100,000 $\times g$ precipitate	N.D.	0.11	0.34	0.04

Protein phosphatase activity was assayed and expressed as described in the text. The protein concentrations of the 10,000 $\times g$ precipitate, 100,000 $\times g$ supernatant and 100,000 $\times g$ precipitate fractions were 1.14, 1.10 and 1.96 mg/ml. When assaying [^{32}P]-histone phosphatase activity, the sample was diluted 100-fold, 100-fold or 200-fold, and 2.5-fold-diluted sample was used for assaying pNPP phosphatase activity. N.D. means not detected. * Activities were expressed as nmol p-nitrophenol/min/mg protein.

ability to dephosphorylate the [32 P]-autophosphorylated 48 kDa regulatory subunit. But, results proved ambiguous. The protein phosphatases from *H. pulcherrimus* spermatozoa and/or sperm tails were purified to define more precisely their biochemical parameters.

When sperm proteins extracted with the extraction buffer containing 1% CHAPS were subjected to chromatography on a DEAE-Sephacel column, three different protein phosphatases were detected when [32 P]-histone was used as a substrate in the absence of divalent-cations (Fig. 1B) and two different protein phosphatase activities were detected when pNPP was used as a substrate in the presence of Mn^{2+} /calmodulin (Fig. 1C). A protein phosphatase eluted in fraction C (fraction numbers from 67 to 72) was active for pNPP in the

presence of Mn^{2+} /calmodulin and [32 P]-histone with or without Mg^{2+} . These protein phosphatases were purified further by gel filtration chromatography on a Sepharose 6B column.

When fraction A (fraction numbers from 57 to 60) obtained from DEAE-Sephacel chromatography was subjected to gel filtration chromatography, two protein phosphatase activities were eluted at the positions corresponding to the molecular masses of 43 kDa and 30 kDa (Fig. 2A). The former protein phosphatase was active for [32 P]-histone in the presence of Mg^{2+} and the latter was active for [32 P]-histone without Mg^{2+} . These protein phosphatases were purified further by chromatography on a Mono Q column and on a Superose 12 column (data not shown). Protein phosphatases with the molecular masses of 43 kDa and 30 kDa were obtained and designated as A-1 and A-2.

When fraction B (fractions 61 to 66) obtained from DEAE-Sephacel chromatography was subjected to gel filtration chromatography on Sepharose 6B, two protein phosphatases were eluted at positions corresponding to the molecular masses of over 200 kDa and about 43 kDa (Fig. 2B). Both protein phosphatases were active for [32 P]-histone without Mg^{2+} and designated B-1 and B-2.

Similarly, three protein phosphatases were separated by gel filtration chromatography on the Sepharose 6B column of fraction C (fractions 67 to 72) obtained from DEAE-Sephacel chromatography (Fig. 2C). Two of them, eluted at the positions corresponding to the molecular masses of about 200 kDa and 30 kDa were active for [32 P]-histone without Mg^{2+} , and one of them, eluted at the position corresponding to the molecular mass of 67 kDa was active for pNPP with Mn^{2+} /calmodulin. The protein phosphatase with the molecular mass of 200 kDa was designated as C-1 and with the molecular mass of 67 kDa was designated as C-2. A protein phosphatase was further purified by gel chromatography on the Sepharose 6B column of fraction D (fractions 73 to 79) obtained from DEAE-Sephacel chromatography (Fig. 2D). The protein phosphatase which was active for pNPP with Mn^{2+} /calmodulin and designated as D was eluted at the position corresponding to the molecular mass of 55 kDa. As a result, seven different protein phosphatases were isolated from *H. pulcherrimus* spermatozoa (Table 2).

In addition, two protein phosphatases were isolated by DEAE-Sephacel ion exchange chromatography of sperm tail proteins solubilized with the extraction buffer containing 1% CHAPS (Fig. 3A). Both phosphatases (fraction 1: fractions 63 to 71 and fraction 2: fractions 72 to 80) were active for [32 P]-histone without Mg^{2+} and were purified further by gel filtration chromatography on the Sepharose 6B column. From fraction 1, a protein phosphatase (designated as 1-1) with the molecular mass of 43 kDa was obtained (Fig. 3B), and two protein phosphatases were obtained from fraction 2 (Fig. 3C). One protein phosphatase with the molecular mass of over 200 kDa was designated as 2-1 and the other with the molecular mass of 30 kDa was designated as 2-2.

Okadaic acid and calyculin A are potent and specific inhibitors for serine/threonine protein phosphatases. As shown

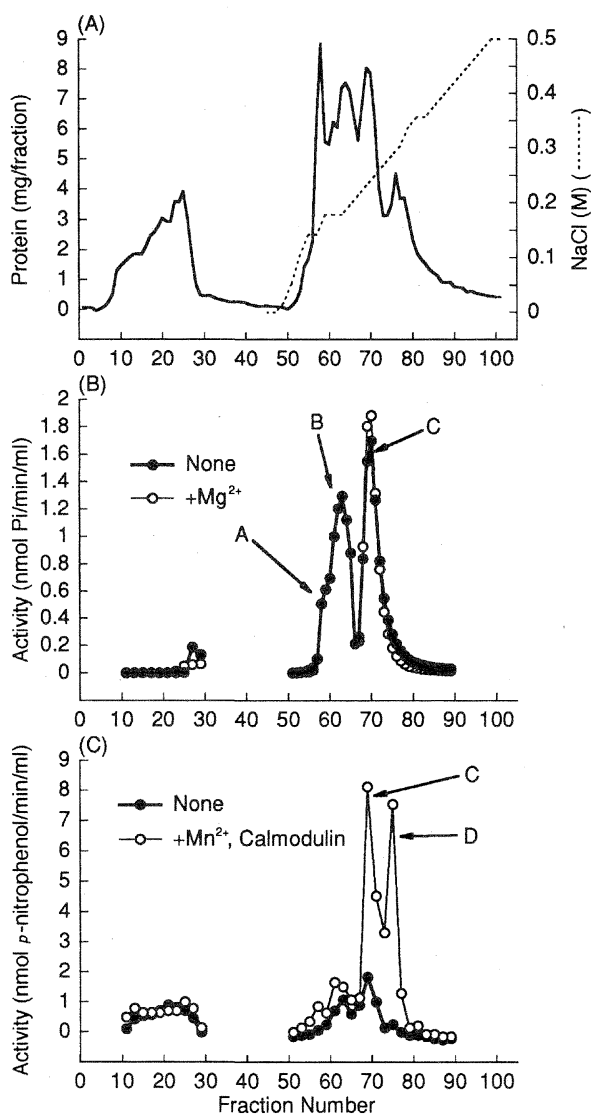


Fig. 1. Elution profiles of *H. pulcherrimus* sperm protein phosphatases on DEAE-Sephacel column chromatography. Upper panel; protein content and NaCl concentration. Mid panel; the protein phosphatase activity using [32 P]-histone as a substrate with or without Mg^{2+} . Lower panel; the protein phosphatase activity using pNPP as a substrate with or without Mn^{2+} /calmodulin.

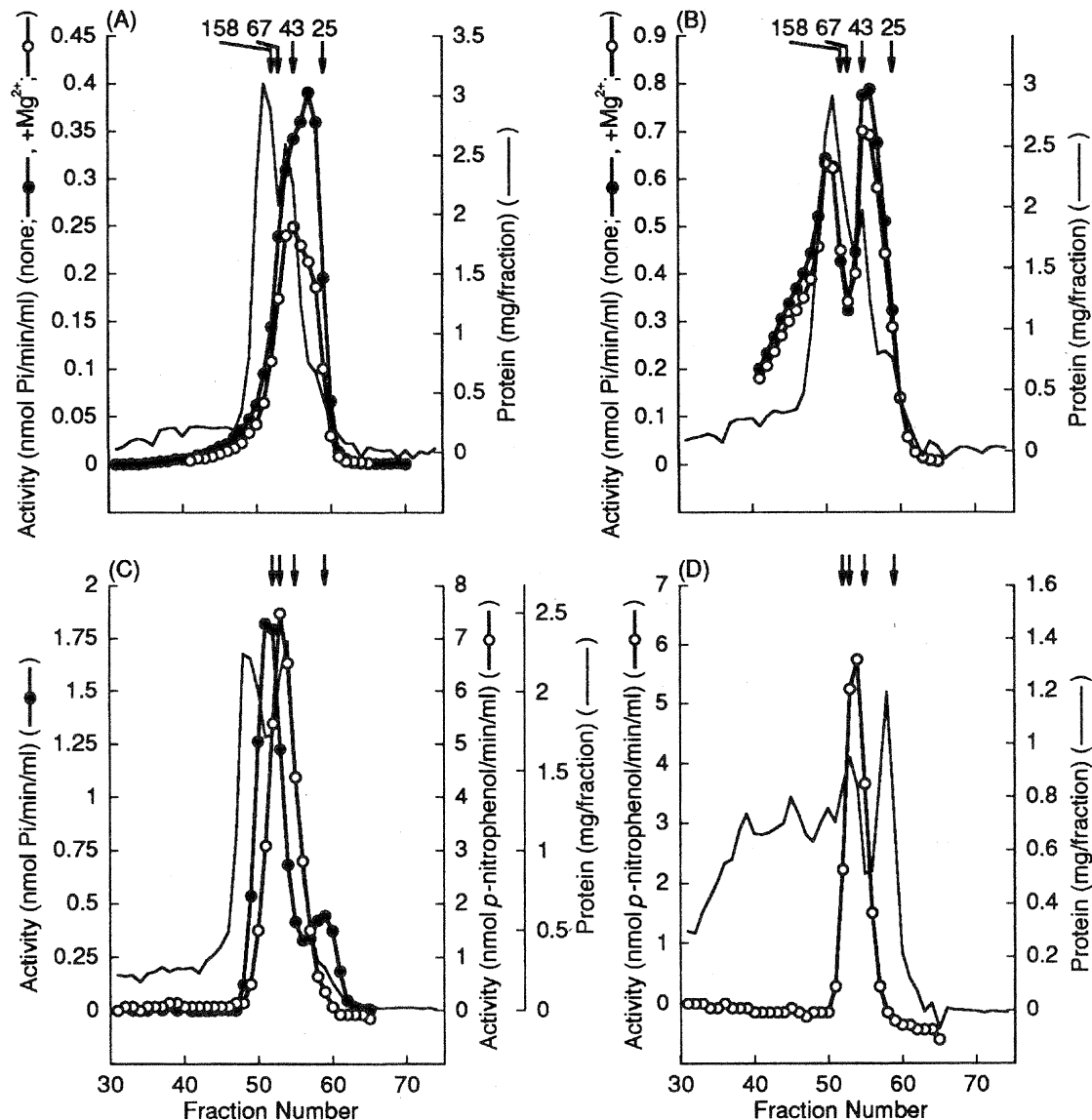


Fig. 2. Gel filtration chromatographic profiles of *H. pulcherrimus* sperm protein phosphatases on a Sepharose 6B column. Pooled fraction A, B, C or D obtained from DEAE-Sephacel chromatography shown in Fig. 1 was applied to a Sepharose 6B column. The elution profile of the sample A, B, C or D was designated as (A), (B), (C) and (D). Eluate were assayed for protein phosphatase using [32 P]-histone and/or pNPP as substrates. Numbers above the arrows denote the molecular masses of the standard proteins such as aldolase (158 kDa), BSA (67 kDa), ovalbumin (43 kDa) and chymotrypsinogen A (25 kDa).

in Fig. 4, protein phosphatase B-2 was inhibited by both inhibitors with IC_{50} of 50 nM and 3nM. Protein phosphatase 1-1 also showed similar concentration dependency for both inhibitors (data not shown).

Dephosphorylation of endogenous phosphorylated proteins by *H. pulcherrimus* sperm protein phosphatases

Seven protein phosphatases isolated from *H. pulcherrimus* spermatozoa were examined for the ability to dephosphorylate the [32 P]-autophosphorylated *H. pulcherrimus* sperm cAMP-dependent histone kinase and the phosphorylated form of *H. pulcherrimus* sperm guanylyl cyclase. As shown in Fig. 5, only the protein phosphatase designated as B-2 was active for dephosphorylation of the [32 P]-

autophosphorylated 48 kDa regulatory subunit of *H. pulcherrimus* sperm cAMP-dependent histone kinase in the absence of Mn^{2+} /calmodulin and cAMP. None of protein phosphatases caused the dephosphorylation of the phosphorylated form (131 kDa) of guanylyl cyclase (data not shown).

DISCUSSION

Serine/threonine protein phosphatases in mammalian cells are divided mainly to two groups. Protein phosphatases in one group (type 1) specifically dephosphorylate the β -subunit of phosphorylase kinase and are inhibited by the thermostable protein inhibitor-1 and inhibitor-2. Protein phosphatases in the

Table 2. Protein phosphatases isolated from *H. pulcherrimus* spermatozoa and sperm tails

Protein phosphatases from spermatozoa	Molecular mass	Substrates	Ion requirements
A-1	43 kDa	[³² P]-histone	Mg ²⁺
A-2	30 kDa	[³² P]-histone	none
B-1	>200 kDa	[³² P]-histone	none
B-2	43 kDa	[³² P]-histone	none
C-1	200 kDa	[³² P]-histone	none
C-2	67 kDa	pNPP	Ca ²⁺ or Mn ²⁺ /calmodulin
D	55 kDa	pNPP	Mn ²⁺ /calmodulin
Protein phosphatases from sperm tails	Molecular mass	Substrates	Ion requirements
1-1	43 kDa	[³² P]-histone	none
2-1	>200 kDa	[³² P]-histone	none
2-2	30 kDa	[³² P]-histone	none

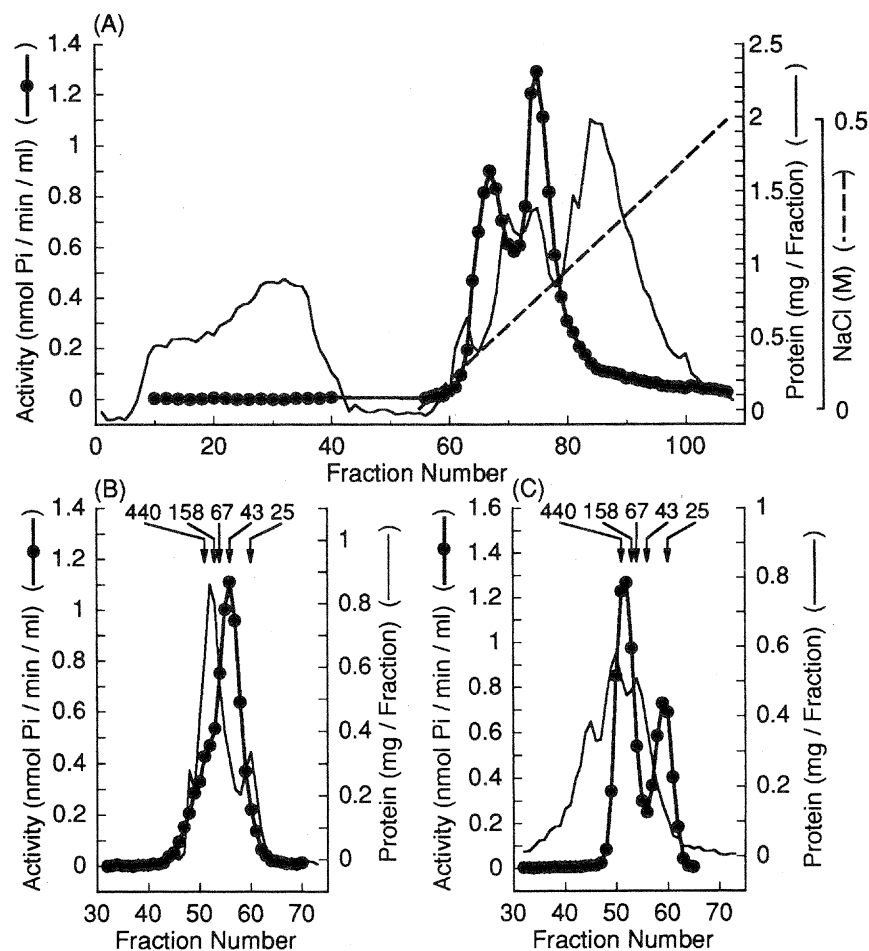


Fig. 3. Elution profiles of *H. pulcherrimus* sperm tail protein phosphatases on a DEAE-Sephacel column (A) and a Sepharose 6B column (B and C). The first (B) or second (C) active peak fractions obtained from DEAE-Sephacel column chromatography shown in (A) were further purified by gel filtration chromatography on a Sepharose 6B column. Protein phosphatase activity was determined with [³²P]-histone as a substrate without divalent cation. Numbers above the arrows denote the molecular masses of the standard proteins such as ferritin (440 kDa), aldolase (158 kDa), BSA (67 kDa), ovalbumin (43 kDa) and chymotrypsinogen A (25 kDa).

other group (type 2) preferentially dephosphorylate the α -subunit of phosphorylase kinase and are insensitive to inhibitors 1 and 2. Protein phosphatases of type 2 comprise 3 distinct enzymes, termed 2A, 2B and 2C, which are distinguished by their requirement for divalent cations. Protein phosphatase type 2A is active in the absence of divalent cations, whereas protein phosphatases type 2B and 2C require for Ca^{2+} and Mg^{2+} for their activity. Type 1, 2A and 2C have broad substrate specificity *in vitro*, whereas the substrate specificity of type 2B protein phosphatase is rather narrow (Cohen, 1989). Okadaic acid is an inhibitor against type 1 and

type 2A (but not type 2C) protein phosphatases (Bialojan and Takai, 1988; Haystead *et al.*, 1989), but 1000-fold higher concentrations are required to inhibit type 1 protein phosphatase. This makes possible the differential inhibition of type 1 and type 2A protein phosphatases using 1 nM or 1 μM of okadaic acid. Thus, our present study demonstrates that type 1 and type 2A protein phosphatases, and also type 2B and type 2C protein phosphatases are present in *H. pulcherrimus* spermatozoa. There have been several papers dealing with protein phosphatases in spermatozoa (Tang and Hoskins, 1979; Tash *et al.*, 1988; Swarup and Garbers, 1982). In spermatozoa of most animals, type 2B protein phosphatase has been suggested to be involved in flagellar motility (Tash *et al.*, 1988).

In a previous study, we demonstrated that *H. pulcherrimus* spermatozoa possess a cAMP-dependent histone kinase of which the regulatory subunit was autophosphorylated, however, it was dephosphorylated in the sperm homogenate with cAMP or cGMP (Harumi *et al.*, 1994). FSG, an inducer of the acrosome reaction, activates sperm adenylyl cyclase and cAMP-dependent protein kinase (Garbers *et al.*, 1980; Watkins *et al.*, 1978). It has also been reported that sea urchin sperm guanylyl cyclase dephosphorylates upon fertilization (Vacquier and Moy, 1986; Harumi *et al.*, 1992). Protein phosphatases of sea urchin spermatozoa must dephosphorylate these sperm proteins and play roles in fertilization. In the present study, we isolated from *H. pulcherrimus* spermatozoa five protein phos-

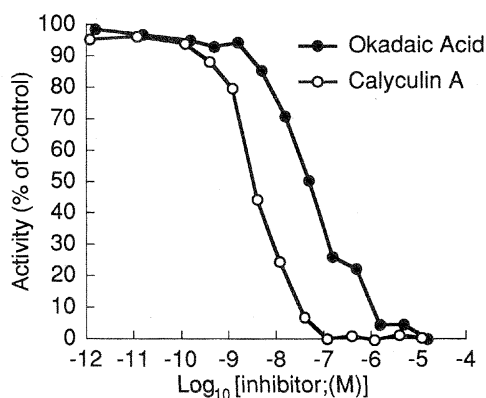


Fig. 4. Concentration-dependent effects of okadaic acid or calyculin A on an *H. pulcherrimus* protein phosphatase B-2.

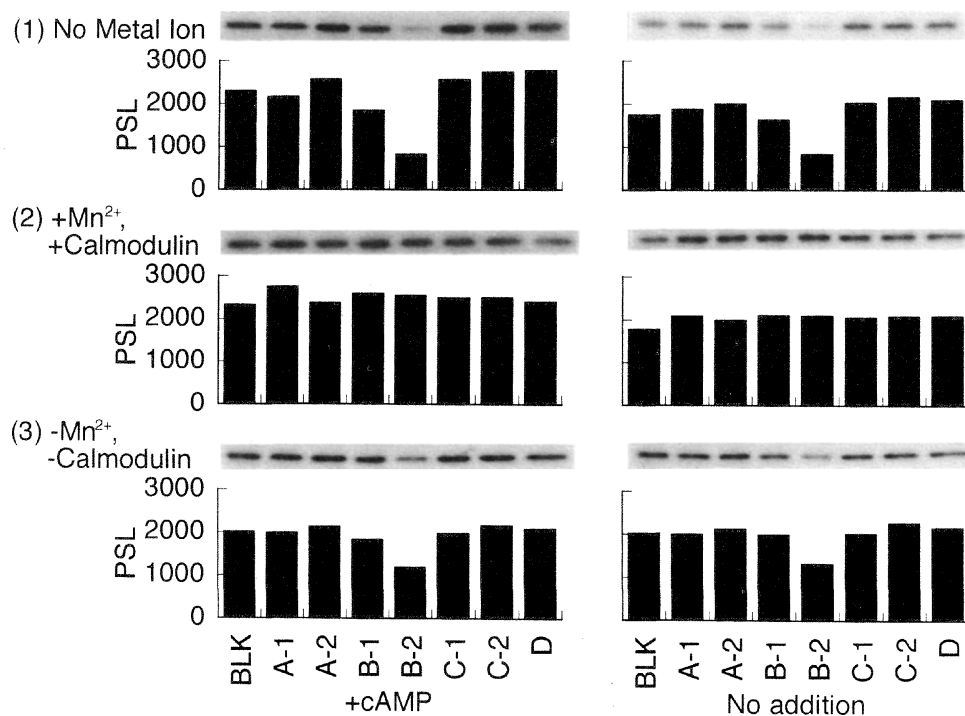


Fig. 5. Dephosphorylation of the $[^{32}\text{P}]$ -autophosphorylated *H. pulcherrimus* sperm cAMP-dependent histone kinase by various protein phosphatases. Protein phosphatases isolated from *H. pulcherrimus* spermatozoa and sperm tails were examined the ability to dephosphorylate the $[^{32}\text{P}]$ -autophosphorylated 48 kDa regulatory subunit in the presence or absence of 2 μM cAMP without divalent cation (1), with Mn^{2+} /calmodulin (2) or without Mn^{2+} /calmodulin (3).

phatases using [^{32}P]-histone as a substrate and two protein phosphatases using pNPP as a substrate. Three protein phosphatases were also isolated from *H. pulcherrimus* sperm tails using [^{32}P]-histone as a substrate. One of these protein phosphatases, designated as B-2, dephosphorylated the [^{32}P]-autophosphorylated regulatory subunit of purified *H. pulcherrimus* sperm cAMP-dependent histone kinase in the absence of Mn^{2+} /calmodulin. Judging from the sensitivity to okadaic acid and calyculin A and the molecular mass, this protein phosphatase is the same as the one designated 1-1 isolated from sperm tails and is similar to the mammalian protein phosphatase type 1. A protein phosphatase designated 1-1 was solubilized from *H. pulcherrimus* sperm tails only with CHAPS-containing solution. This suggests that the protein phosphatase is associated with sperm membranes. However, the dephosphorylation of the [^{32}P]-autophosphorylated regulatory subunit by the protein phosphatase was independent of the presence of cAMP. This is inconsistent with our previous observation with sperm homogenates (Harumi *et al.*, 1994). Therefore, we presume that there is another protein phosphatase in *H. pulcherrimus* spermatozoa which causes dephosphorylation of the regulatory subunit. In this connection, it should be mentioned that a protein phosphatase of which catalytic subunit with the molecular mass of 30 kDa has been reported to dephosphorylate the autophosphorylated regulatory subunit of bovine cardiac muscle A-kinase in a cAMP-dependent manner (Chou *et al.*, 1977).

There has been a paper showing that mammalian type 2A protein phosphatase causes *in vitro* dephosphorylation of the phosphorylated atrial natriuretic peptide receptor/guanylyl cyclase, which was over-expressed in human embryonic 293 cells (Potter and Garbers, 1992). However, none of seven protein phosphatases isolated from *H. pulcherrimus* spermatozoa caused the dephosphorylation of the phosphorylated form of *H. pulcherrimus* sperm guanylyl cyclase.

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