Structure and Function of Sperm-Activating Peptide Binding Protein

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精子活性化ペプチド結合タンパク質の構造と機能に関する研究

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博　士　論　文

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CONTENTS

CHAPTER 1 GENERAL INTRODUCTION

The union of sperm and egg to form a cell capable of developing into a new individual must be considered one of the most dramatic events in biology. Successful fusion of the gametes represents the culmination of a complex, multi-step process, fertilization, which begins with mating.

The sea urchin has served as our initial model because of the large number of gametes available and because of its general acceptance as a model system in which to study fertilization and early development. Fertilization in sea urchin is typically highly species-specific and the specificity of sperm adhesion plays an important role in ensuring that only homologous spermatozoa penetrate the egg. In the process, the egg communicates with the spermatozoa using molecules in the extracellular matrix. The jelly layer of sea urchins mainly consists of sperm-activating peptides (SAPs) and polysaccharide-protein complexes which can be separated into a fucose sulfate glycoconjugate (FSG) and a sialoglycoprotein.

Spermatozoa contain binding sites for specific molecules associated with the egg (includes the acellular matrices surrounding the egg) ; interactions with these molecules may result in substantial morphological or behavioral changes in spermatozoa. The activation of sea urchin spermatozoa at fertilization consists of the following two major events : (i) the exocytotic acrosome reaction, occurring when the spermatozoa encounters the jelly layer surrounding egg and (ii) initiation of motility and respiration resulting from an increase in pH_i.

The acrosome reaction in spermatozoa is an essential

requirements for fertilization of eggs in many animals. The acrosome reaction was first described in detail by Dan (1964) and consists of two morphologically distinct steps : (1) fusion of the outer acrosomal membrane and the plasma membrane to expose the contents of the acrosomal granule, and (2) polymerization of profilamentous actin into an acrosome rod (Tilney et al., 1973, 1978). FSG is considered to be responsible for the induction of the acrosome reaction (Decker et al., 1976; SeGall and Lennarz, 1979; Garbers et al., 1983). FSG possesses 2.0 times protein to fucose by weight (Shimizu et al., 1990). The proteins in intact FSG are separated to two major and one minor proteins by SDS-PAGE under the reduced conditions (Shimizu et al., 1990). Induction of the acrosome reaction by FSG requires extracellular Ca^{2+} (Dan, 1954; Decker et al., 1976; Collins and Epel, 1977; Tilney et al., 1978). It has been suggested that FSG acts by triggering a Ca^{2+} influx (Decker et al., 1976; Schackmann et al., 1978). The sperm acrosome reaction includes not only exocytosis of the acrosomal granules and extrusion of the acrosomal process, but also a net influx of Ca^{2+} and Na^{+}, efflux of H^{+} (Schackmann et al., 1978; Kopf and Garbers, 1980; Schackmann and Shapiro, 1981), and depolarization of the sperm plasma membrane potential (Schackmann et al., 1984). Several investigators have found evidence that Na^{+} influx and H^{+} efflux are coupled in this system (Gonzalez-Martinez and Darszon, 1987). FSG has been shown to elevate cyclic AMP concentrations in intact sperm cells (Garbers, 1981), stimulate cyclic AMP-dependent protein kinase activity in spermatozoa (Garbers et al., 1980), and increase phosphorylation of sperm histone H1 on a single site by the cyclic AMP dependent protein kinase (Porter and Vacquier, 1986).

It has been known for over 80 years that soluble factors associated

with the eggs of certain species of sea urchins enhance the respiration and motility of sea urchin spermatozoa (Lillie, 1913). The factors have been reported to be diffusible in dialysis, heat-stable, alcohol-soluble, and non-volatile (Hathaway, 1963). In 1976, Ohtake presented data to suggest that reproducible activations of spermatozoan metabolism could be observed upon addition of egg-conditioned media to sperm cells maintained under slightly acidic pH values. These observations led to the successful purification of egg peptides that could stimulate sperm respiration. Kopf et al. (1979) found that egg jelly of sea urchins *Strongylocentrotus purpuratus* and *Lytechinus pictus* contains a factor which elevates the respiratory rate and cyclic AMP and cyclic GMP concentrations of homologous spermatozoa. In 1981, Suzuki et al. first purified a substance from the solubilized jelly layer of the sea urchin *Hemicentrotus pulcherrimus* and demonstrated that the substance is a decapeptide whose structure is GFDLNGGGVG and stimulates the respiration and motility of *H. pulcherrimus* spermatozoa. This peptide was named sperm-activating peptide I (SAP-I). SAP-I initiates a variety of physiological changes in the spermatozoa, including stimulation of sperm motility and respiration (Hansbrough and Garbers 1981) and increases in potassium channel conductance (Babcok et al. 1992). SAP-I also increases cellular ion fluxes, which have been implicated in regulating intracellular cyclic GMP and cyclic AMP concentrations (Repaske and Garbers, 1981; Bentley et al., 1987). SAP-I does not appear to possess acrosome reaction-inducing activity by itself. However, there is a evidence that such peptides may act in conjunction with the fucose sulfate glycoconjugate (FSG) component of the egg jelly to alter the rate of acrosome reaction (Yamaguchi et al., 1988).

Sperm-activating peptide IIA (CVTGAPGCVGGGRL-NH_2) which was isolated from the egg jelly of the sea urchin *Arbacia punctulata* (Suzuki et al., 1984) has been identified as a potent chemoattractant (Ward et al., 1985a). This is the first egg-associated chemotactic substance identified for animal spermatozoa, although such behavior of spermatozoa in response to eggs or substances recovered from eggs has been well established (Miller, 1985). Ward et al. (1983) have shown that exposing *A. punctulata* spermatozoa to SAP-IIA results in a change in the electrophoretic mobility of an abundant sperm membrane protein which has been identified as guanylate cyclase (Ward and Vacquier, 1983; Suzuki et al., 1984; Ward et al., 1985). Several lines of evidence suggest that the mobility shift is due to a receptor-mediated dephosphorylation of the enzyme.

Using the homobifunctional, amino-reactive cross-linker disuccinimidyl-suberate, Dangott and Garbers (1984) specifically cross-linked ^{125}I-GGG[Y2]-GFDLNGGGVG to a 77 kDa glycoprotein of *S. purpuratus* sperm membranes. Using the same cross-linker, Shimomura et al. (1986) specifically cross-linked ^{125}I-GGGYG-CVTGAPGCVGGGRL-NH_2 to a 160 kDa protein from *A. punctulata* spermatozoa. In similar experiments Harumi et al. (1991) also demonstrated that a ^{125}I-GYGG-SAP-I crosslinked to two proteins of 71 kDa and 63 kDa of *H. pulcherrimus* spermatozoa and ^{125}I-GYGG-KLCPGGNCV crosslinked to three proteins of 172 kDa, 62 kDa and 52 kDa in sperm head and to two proteins of 157 kDa and 62 kDa in sperm tail of *Glyptocidaris crenularis*. Yoshino and Suzuki (1992) demonstrated that a ^{125}I-GGGY-DSDSAQNLIG crosslinked to three proteins of 126 kDa, 87 kDa and 64 kDa of *Clypeaster japonicus* spermatozoa.

Dangott et al. (1989) cloned a full length cDNA encoding the 77 kDa

SAP-I-crosslinked protein from a *S. purpuratus* testis cDNA library. The extracellular domain consisted of four cysteine-rich tandem repeats. Comparison of the cysteine-rich tandem repeats revealed that these sequences in the 77 kDa crosslinked protein had as much as 48 % identity to similar cysteine-rich repeats in the type I scavenger receptor from macrophages (Freeman et al., 1990).

Shimomura et al. (1986) immunoprecipitated the 160 kDa protein that cross-linked to SAP-IIA with a monospecific antibody to *A. punctulata* sperm guanylate cyclase, suggesting that guanylate cyclase is the receptor for SAP-IIA. Singh et al. (1988) subsequently cloned and sequenced a cDNA encoding the *A. punctulata* sperm guanylate cyclase/apparent SAP-IIA receptor. *A. punctulata* sperm guanylate cyclase is composed of a single transmembrane domain and two clearly defined intracellular regions containing a protein kinase-like and a guanylate cyclase catalytic domain. The SAP-I receptor in *S. purpuratus* and the SAP-IIA receptor in *A. punctulata* both mediate similar activation events in their respective spermatozoa. However, their structures show little similarity.

Guanylate cyclase was first purified from sea urchin spermatozoa (Garbers,1976; Randany et al., 1983; Harumi et al., 1992) probably because these cells are very rich in guanylate cyclase and the enzyme activity is located almost entirely in the particulate fraction. The sea urchin sperm plasma membrane form of guanylate cyclase appears to be regulated by its state of phosphorylation (Ward and Vacquier, 1983; Suzuki et al., 1984; Ward et al., 1985b). Bentley et al. (1986a) demonstrated that SAP-IIA activates guanylate cyclase before any loss of phosphate, and that the subsequent decrease in phosphate content is associated with a large reduction in the enzyme activity. Therefore,

dephosphorylation of the sperm guanylate cyclase appears to act as a desensitization step.

The guanylate cyclase receptor family is presently known to consist of two major classes of proteins; heterodimeric forms (soluble form) regulated by nitric oxide and cell surface receptors (particulate form) regulated by extracellular peptides (Koesling et al., 1991; Schulz et al., 1991; Yuen and Garbers, 1992). Since characteristics of particulate form of sea urchin sperm guanylate cyclase are very similar to those of mammalian tissues with respect to size, kinetic properties, immunogenic determinants and regulation by extracellular peptides (Garbers, 1989; Chinkers and Garbers, 1991), the sea urchin cDNA encoding guanylate cyclase was used to identify and isolated a mammalian cDNA encoding a guanylate cyclase (GC-A). Maximum activation of GC-A not only requires ANP but also ATP (Kurose et al., 1987; Chinkers and Garbers, 1989; Chang et al., 1990a; Chang et al.,1990b; Chinkers et al., 1991), and it has been suggested that ATP binds as a regulator to the protein kinase-like domain (Chinkers and Garbers, 1989). Deletion of this region in GC-A results in a receptor that binds ANP and continues to possess guanylate cyclase activity, but the cyclase activity of the ANP receptor (GC-A) is no longer regulated by ANP (Chinkers and Garbers, 1989), and it appears to be constitutively active, suggesting that the kinase-like region acts as a negative regulatory element.

In this study, We examined the effects of two egg jelly components, a fucose sulfate glycoconjugate (FSG) and sperm-activating peptide I (SAP-I), on the intracellular pH (pH_i) and Ca^{2+} ($[Ca^{2+}]_i$) of spermatozoa of the sea urchin *H. pulcherrimus* (CHAPTER 2). We described characterization of the SAP-I receptors and the 71 kDa SAP-I

crosslinked protein in *H. pulcherrimus* spermatozoa. We also described isolation of a cDNA encoding the 71 kDa crosslinked protein from a *H. pulcherrimus* testis cDNA library (CHAPTER 3). In CHAPTER 4, we mentioned a cDNA clone encoding the *H. pulcherrimus* sperm guanylate cyclase.

CHAPTER 2 DIFFERENTIAL EFFECTS OF THE EGG JELLY MOLECULES, FSG AND SAP-I ON ELEVATION OF INTRACELLULAR Ca^{2+} AND pH IN SEA URCHIN SPERMATOZOA

SUMMARY

We examined the effects of two egg jelly components, a fucose sulfate glycoconjugate (FSG) and sperm-activating peptide I (SAP-I: GFDLNGGGVG), on the intracellular pH (pH_i) and Ca^{2+} ($[Ca^{2+}]_i$) of spermatozoa of the sea urchin *Hemicentrotus pulcherrimus*. FSG and/or SAP-I induced elevations of $[Ca^{2+}]_i$ and pH_i in the spermatozoa at pH 8.0. At pH 8.0, a second addition of FSG did not induce further elevation of the $[Ca^{2+}]_i$ or pH_i of spermatozoa treated with FSG, but addition of FSG after SAP-I or of SAP-I after FSG induced further increases of $[Ca^{2+}]_i$ and pH_i. At pH 6.6, FSG and/or SAP-I did not induce significant elevation of the $[Ca^{2+}]_i$, although SAP-I elevated the pH_i, its half-maximal effective concentration being 10 to 100 pM. At pH 8.0, tetraethyl-ammonium, a voltage-sensitive K^+-channel blocker, inhibited induction of the acrosome reaction and elevations of $[Ca^{2+}]_i$ and pH_i by FSG, but did not affect those by SAP-I. These results suggest that FSG and SAP-I activate different Ca^{2+} and H^+ transport systems.

INTRODUCTION

Sea urchin eggs are surrounded by a gelatinous matrix that had been shown to induce an acrosome reaction in spermatozoa (Dan, 1952; Collins and Epel, 1977). The acrosome reaction is accompanied by influxes of Ca^{2+} and Na^{+}, and effluxes of K^{+} and H^{+} (Schackmann et al., 1978; Schackmann and Shapiro, 1981). These ionic movements lead to intracellular increases in both pH (pH_i) (Schackmann et al., 1981) and $[Ca^{2+}]$ ($[Ca^{2+}]_i$) (Yamaguchi et al., 1988), and to depolarization of the plasma membrane (Schackmann et al., 1981). All these changes as well as the acrosome reaction are inhibited by Ca^{2+}-channel antagonists such as verapamil, dihydropyridines such as nitrendipine and nisoldipine (Garcia-Soto et al., 1985; Kazazoglou et al., 1985), the K^{+}-channel blocker tetraethyl-ammonium chloride (TEA) (Schackmann et al., 1978), and high K^{+} seawater (Garcia-Soto et al., 1985). The extracellular matrix of sea urchin eggs is mainly composed of a fucose sulfate glycoconjugate (FSG), a sialoglycoprotein and sperm-activating peptides (SAPs) (Isaka et al., 1970; Ishihara et al., 1973; SeGall and Lennarz, 1979; Suzuki et al., 1981; Garbers et al., 1983; Shimizu et al., 1990; Suzuki, 1990). FSG induces increases in $[Ca^{2+}]_i$ (Kopf and Garbers, 1980; SeGall and Lennarz, 1981), elevation of the intracellular cyclic AMP level, and the acrosome reaction in sea urchin spermatozoa (Garbers et al., 1983; Kopf and Garbers, 1984). SAPs have many biological effects on the spermatozoa such as stimulations of respiration and motility in slightly acidic seawater (Suzuki et al., 1981; Garbers et al., 1982), inductions of transient elevation of the cyclic GMP level (Garbers et al., 1982), increases in pH_i (Repaske and Garbers, 1983) and $[Ca^{2+}]_i$ (Schackmann and Chock, 1986), and plasma

membrane hyperpolarization through activation of Na^+/H^+ and K^+/H^+ exchanges across the plasma membrane (Lee and Garbers, 1986). SAP-I (GFDLNGGGVG) acts as a specific co-factor for induction of the acrosome reaction by FSG (Yamaguchi et al., 1988). Before SAPs induce these biochemical and physiological events in spermatozoa, they seem to bind to specific receptors on the spermatozoa. There have been several studies on the SAP receptors using radioiodinated SAP analogues (Smith and Garbers, 1983; Dangott and Garbers, 1984, 1987; Bentley et al., 1986; Dangott et al., 1989; Harumi et al., 1991). A receptor that is apparently specific for SAP-IIA (CVTGAPGCVGGGRL-NH_2) (Suzuki et al., 1984; Yoshino et al., 1991) has been identified as a guanylate cyclase in spermatozoa of the sea urchin *Arbacia punctulata* (Ward et al., 1985; Bentley et al., 1986). SAP-I is specifically cross-linked to a 77 kDa protein with no known enzyme activity in *Strongylocentrotus purpuratus* spermatozoa (Dangott and Garbers, 1984, 1987; Dangott et al., 1989). Previously, we reported that SAP-I cross-links to 63 kDa and 71 kDa proteins in *Hemicentrotus pulcherrimus* spermatozoa (Harumi et al.,1991). In both cases, the bindings of SAPs to these proteins induced transient activation of the membrane-bound guanylate cyclase (Dangott and Garbers, 1984; Bentley et al., 1986; Harumi et al., 1991). We purified FSG from *H. pulcherrimus* egg jelly (Shimizu et al., 1990) and we tried to identify the FSG binding protein on spermatozoa using radioiodinated FSG, but without success: we found that binding and cross-linking experiments with radioiodinated FSG were not possible because the radioiodinated FSG binds non-specifically to spermatozoa, test tubes and even the glass-filters used in the experiments. We imagine that the FSG of *H. pulcherrimus* is not exceptional. As far as we know, little is yet known

about the site or mechanism of binding of purified FSG to sea urchin spermatozoa of any species. In the present study, therefore, we used an indirect approach to determine whether FSG binds to the spermatozoa and whether its binding site is the same as that of SAP-I. Transiently permeant fluorescent indicators of Ca^{2+} (Grynkiewicz et al., 1985) and pH (Pink et al., 1982) have been shown to be useful to study changes of $[Ca^{2+}]_i$ and pH_i on treatment of sea urchin spermatozoa with crude sea urchin egg jelly. Using these indicators, we examined the changes of $[Ca^{2+}]_i$ and pH_i in *H. pulcherrimus* spermatozoa upon treatment with FSG and/or SAP-I. The results obtained here suggest that the binding sites of FSG and SAP-I on spermatozoa are different.

MATERIALS AND METHODS

Materials

Sea urchins, *H. pulcherrimus*, were collected along the coast near Noto Marine Laboratory. Sea urchin gametes were obtained by intracoelomic injection of 0.5 M KCl. Spermatozoa were collected as "dry sperm" at room temperature and stored on ice until use. Eggs were collected in filtered seawater. The artificial seawater (ASW) used for determination of $[Ca^{2+}]_i$ and pH_i contained 430 mM NaCl, 10 mM KCl, 23 mM $MgCl_2$, 25 mM $MgSO_4$, 10 mM $CaCl_2$, and 10 mM tris(hydroxymethyl) amino-methane (Tris) at pH 8.0 or 10 mM N-(2-acetamide)-2-aminoethane-sulfonic acid (ACES) at pH 6.6. The ASW used for determination of the rates of the acrosome reaction and cyclic nucleotide concentrations contained 454 mM NaCl, 9.7 mM KCl, 24.9 mM $MgCl_2$, 9.6 mM $CaCl_2$, 27.1 mM $MgSO_4$, 4.4 mM $NaHCO_3$ and 10 mM N-2-

hydroxyethyl-piperazine-N'-ethanesulfonic acid (HEPES) (pH 8.2). In high [K+] seawater which was buffered at pH 8.2 with 10 mM HEPES, Na+ was partially replaced by K+ keeping [Na+] plus [K+] equal to 463.7 mM. Solubilization of egg jelly and purification of FSG were carried out as reported previously (Shimizu et al., 1990). SAP-I was synthesized at the Peptide Institute Inc., Osaka, Japan. The pentaacetoxy-methylester of Fura 2 (Fura 2/AM) and 2',7'-bis(carboxyethyl)-carboxyfluorescein tetraacetoxymethylester (BCECF/AM) were obtained from Dojindo Laboratories, Japan. All other reagents used were of analytical grade.

Measurement of $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ was determined by the method of Schackmann and Chock (1986) with slight modifications using the Ca^{2+} indicator Fura 2/AM. A stock solution of Fura 2/AM (1 mM Fura 2/AM in DMSO) was added to 10-fold diluted dry sperm to give a final concentration of 3 μM in Ca^{2+}-free ASW (CaFASW), consisting of 430 mM NaCl, 10 mM KCl, 23 mM $MgCl_2$, 25 mM $MgSO_4$, 1 mM EGTA and 10 mM 2(N-morpholino) ethanesulfonic acid (MES) at pH 6.8. The sperm suspension was incubated at 4°C for 6 hrs in the dark room and then centrifuged at 3,000xg for 5 min at 4°C. The resulting sperm pellet was washed twice with CaFASW and suspended in CaFASW to give 10-fold dilution of the dry sperm. A volume of 100 μl of Fura 2/AM-loaded spermatozoa was added to 1.8 ml of ASW, and the fluorescence was monitored at 500 nm in a Shimadzu RF-5000 fluorescence spectrophotometer at 20°C with excitations at 345 nm and 380 nm. When HEPES-NaOH (pH 6.8) was used as a buffer for Fura 2/AM-loading in ASW, the spermatozoa aggregated, so, we used MES as a buffer for incubation of spermatozoa with Fura 2/AM. $[Ca^{2+}]_i$ was determined by the ratio method of

Trimmer et al. (1986). $[Ca^{2+}]_i$ was expressed as $[Ca^{2+}]_i/Kd$ where Kd is the Fura 2/Ca^{2+} dissociation constant which is reported to be 770 nM (Poenie et al., 1985).

Measurement of pH_i

pH_i was measured essentially as the procedure described by Trimmer et al. (1985). Spermatozoa (10-fold-diluted dry sperm in CaFASW) were incubated with BCECF/AM (1 mM in DMSO) at a final concentration of 3 μM at 4°C for 3 hrs. Then the spermatozoa were washed twice with CaFASW and resuspended in CaFASW at 10-fold dilution of the dry sperm. The sperm suspension was added to 1.8 ml ASW, and the fluorescence was monitored at 530 nm at 20°C with a Shimadzu RF-5000 fluorescence spectrophotometer with excitation at 500 nm. The intracellular pH was estimated from a standard curve generated by varying the extracellular pH in the presence of 50 μM nigericin and monensin, as reported by Trimmer et al. (1986).

Other methods

The rate of the acrosome reaction was determined as described previously (Yamaguchi et al., 1988). Sperm cyclic AMP and cyclic GMP concentrations were determined by radioimmunoassay using cyclic AMP and cyclic GMP assay kits (Yamasa Shoyu, Chiba, Japan) as described previously (Yamaguchi et al., 1989).

RESULTS

Effects of egg Jelly, SAP-I and FSG on pH_i and $[Ca^{2+}]_i$

As shown in Fig. 1, the solubilized egg jelly induced rapid increases in pH_i and $[Ca^{2+}]_i$ of spermatozoa at pH 8.0. The increased pH_i returned to the basal value within 1 min. The $[Ca^{2+}]_i$ change peaked at around 5 sec, and a large relaxation occurred within 1 min after addition of egg jelly, but the $[Ca^{2+}]_i$ did not return to the basal value within the experimental period. We found that sialoglycoprotein purified from *H. pulcherrimus* egg jelly did not affect either the pH_i or $[Ca^{2+}]_i$ (data not shown). Therefore, we next examined the effects of two other egg jelly molecules, SAP-I and FSG, on pH_i and $[Ca^{2+}]_i$ at pH 8.0 and pH 6.6. SAP-I and FSG both induced a large elevation of $[Ca^{2+}]_i$ at pH 8.0, but no significant change in $[Ca^{2+}]_i$ at pH 6.6. SAP-I induced a large increase in pH_i at pH 6.6. Both SAP-I and FSG induced a small, but significant increase in pH_i at pH 8.0. However, we did not detect any significant increase in pH_i due to FSG at pH 6.6. In addition, FSG and SAP-I did not induce elevation in $[Ca^{2+}]_i$ or pH_i in ASW containing 100 mM KCl at pH 8.0.

SAP-I dose-dependent changes in pH_i and $[Ca^{2+}]_i$

The increases in pH_i and $[Ca^{2+}]_i$ by SAP-I were dose-dependent. Fig. 2 shows the relationships of the concentrations of SAP-I, pH_i, $[Ca^{2+}]_i$ and external pH. The net increase in pH_i by SAP-I at pH 6.6 was larger than that at pH 8.0. The half-maximal increase in pH_i at pH 6.6 was observed with between 10 and 100 pM of SAP-I, which was comparable to the effective concentration range of SAP-I for stimulation of

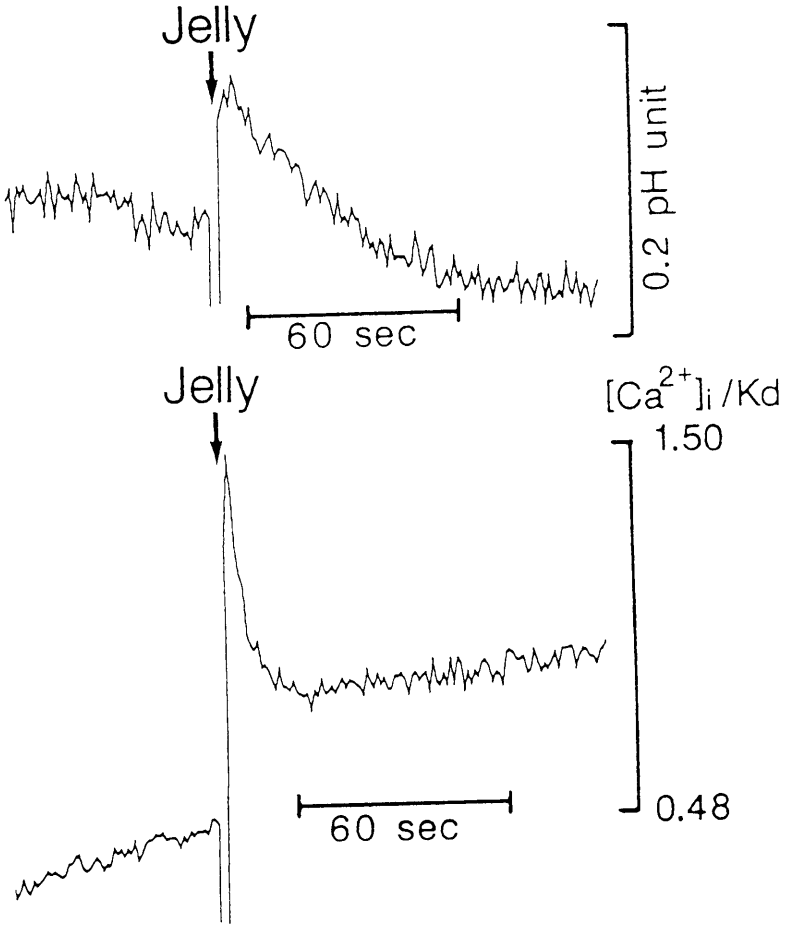

Fig.1 Effects of egg jelly on pH_i and $[Ca^{2+}]_i$. BCECF/AM (A)- or Fura 2/AM (B)-loaded spermatozoa were resuspended in ASW (pH 8.0) 1 min before the recording was started. At the times indicated by arrows, egg jelly (final, 1.24 nmol fucose/ml) was added to the sperm suspension. The record is representative of those for four different batches of spermatozoa.

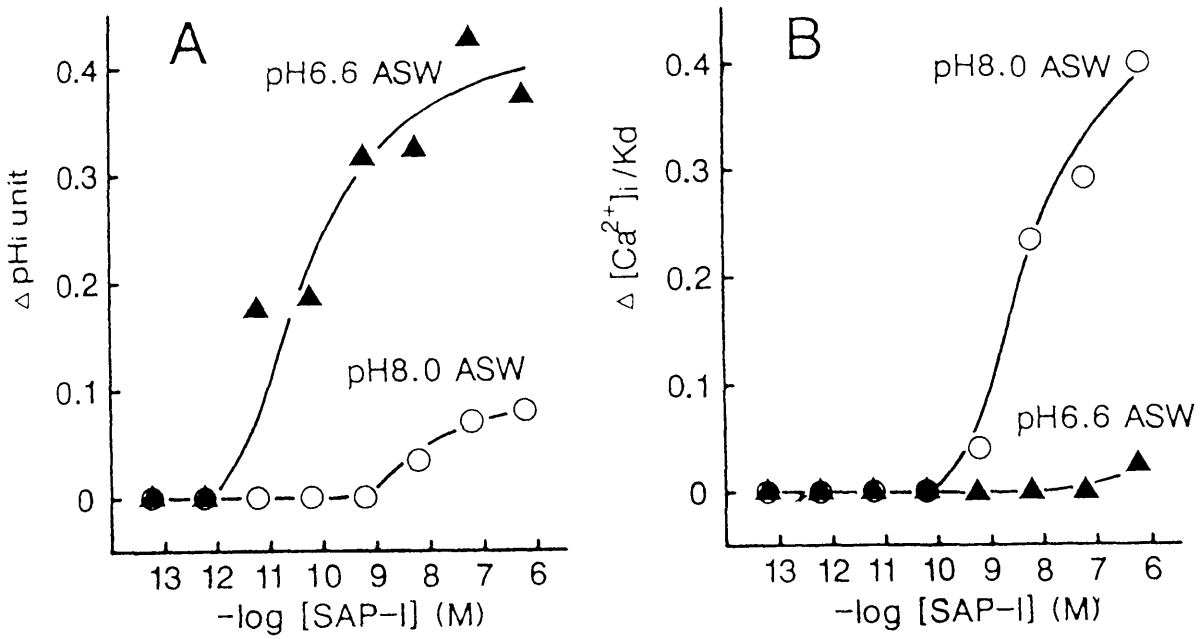

Fig.2 SAP-I dose-dependencies of net changes in pH_i and $[Ca^{2+}]_i$. SAP-I induced elevations of pH_i and $[Ca^{2+}]_i$ with peak values about 4-8 sec after its addition to BCECF/AM- or Fura 2/AM-loaded spermatozoa. All values used for calculations of ΔpH_i and $\Delta[Ca^{2+}]_i$ were taken at the peaks. The same batch of spermatozoa was used for all experiments.

respiration at pH 6.6. However, the increase in pH_i by SAP-I was less at pH 6.6 than at pH 8.0. SAP-I did not elevate $[Ca^{2+}]_i$ at pH 6.6, but at pH 8.0 it elevated $[Ca^{2+}]_i$ markedly at between 1 and 10 nM. This concentration range was similar to that for its effect in increasing cyclic GMP (Suzuki, 1990).

Three repeated addition of a low concentration of SAP-I (final, 0.59 nM) to spermatozoa at pH 6.6 repeatedly induced increases in pH_i, although the extents of these increases tended to decrease progressively (Fig. 3A). However, on repeated addition of a higher concentration of SAP-I (final, 0.59 μM) to spermatozoa, only the first addition induced an increase in pH_i (Fig. 3B).

$[Ca^{2+}]_i$-accumulation in spermatozoa induced by SAP-I and FSG

Similar results to those for pH_i were obtained for $[Ca^{2+}]_i$ on repeated additions of SAP-I. Fig. 4A shows that after sperm $[Ca^{2+}]_i$ had been increased by a first addition of SAP-I (final, 0.59 μM), a second addition of the same concentration of SAP-I did not result in further elevation of $[Ca^{2+}]_i$. Fig. 4A also demonstrates that FSG elevated the $[Ca^{2+}]_i$ further after its increase induced by SAP-I. When FSG was added to spermatozoa twice, the second addition did not induce further elevation of the $[Ca^{2+}]_i$, but subsequent addition of SAP-I did (Fig. 4B). After addition of SAP-I and then FSG or FSG and then SAP-I, egg jelly did not induce further elevation of the $[Ca^{2+}]_i$ (Fig. 5).

SAP-I- or FSG-induced $[Ca^{2+}]_i$-accumulation in the presence of 3-isobutyl-1-methylxanthine (IBMX)

IBMX, a cyclic nucleotide phosphodiesterase inhibitor, has been

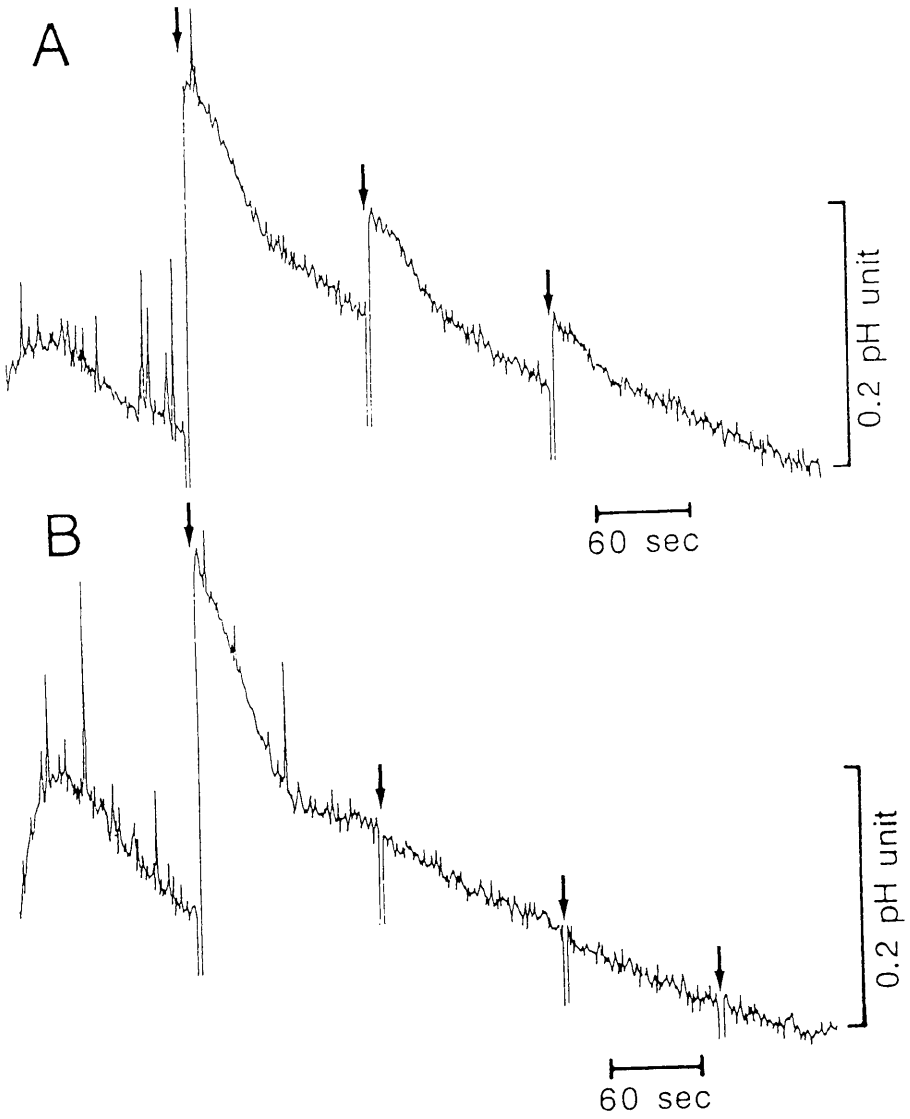

Fig.3 Effect of SAP-I on pH_i. At the times indicated by arrows, the same amounts of SAP-I were added to BCECF/AM-loaded spermatozoa at pH 6.6. The final concentration of SAP-I was 0.59 nM for A and 0.59 μM for B. The same batch of spermatozoa was used for these experiments.

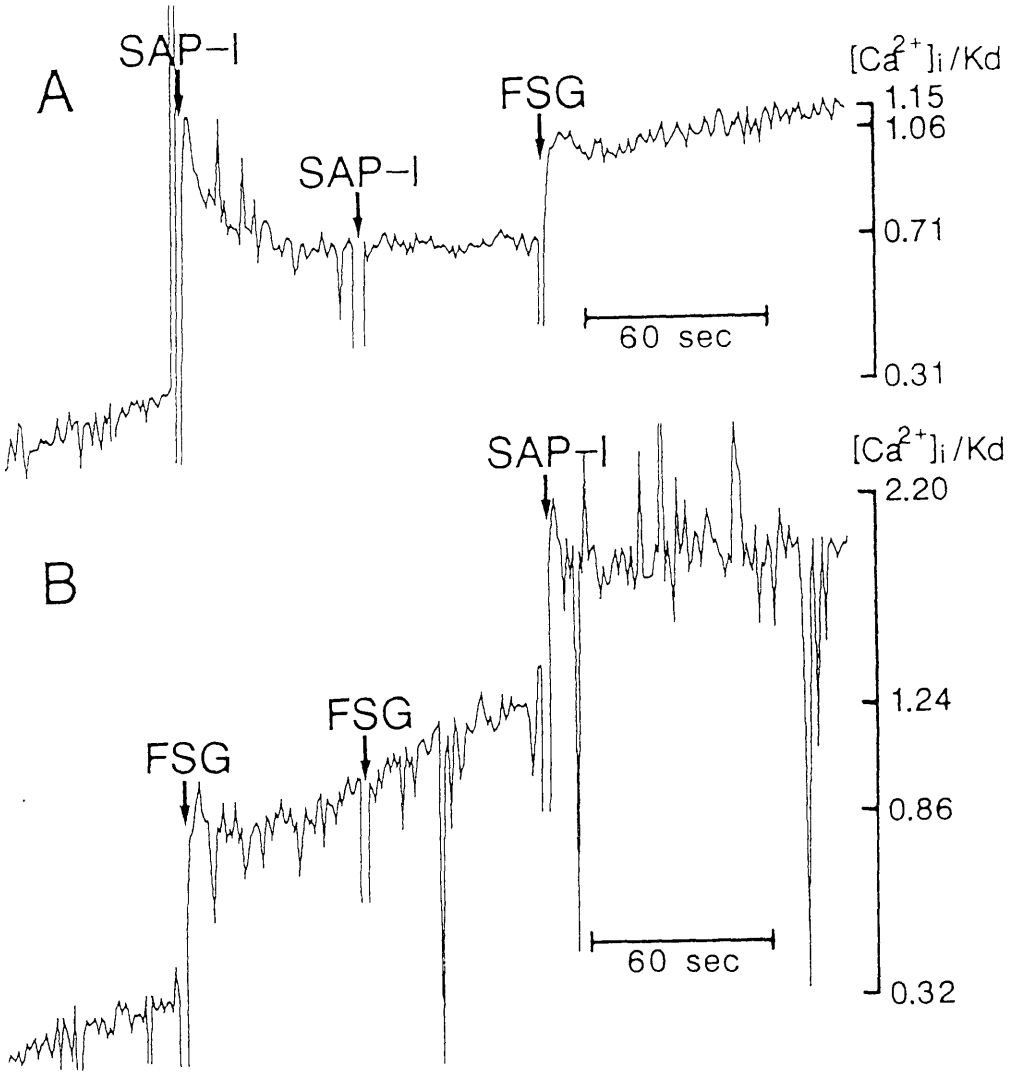

Fig.4 Ca^{2+}-accumulation induced by SAP-I or FSG. At the times indicated by arrows, SAP-I (final, 0.59 μM) or FSG (final, 50 nmol fucose/ml) was added to Fura 2/AM-loaded spermatozoa at pH 8.0. The same batch of spermatozoa was used for both experiments.

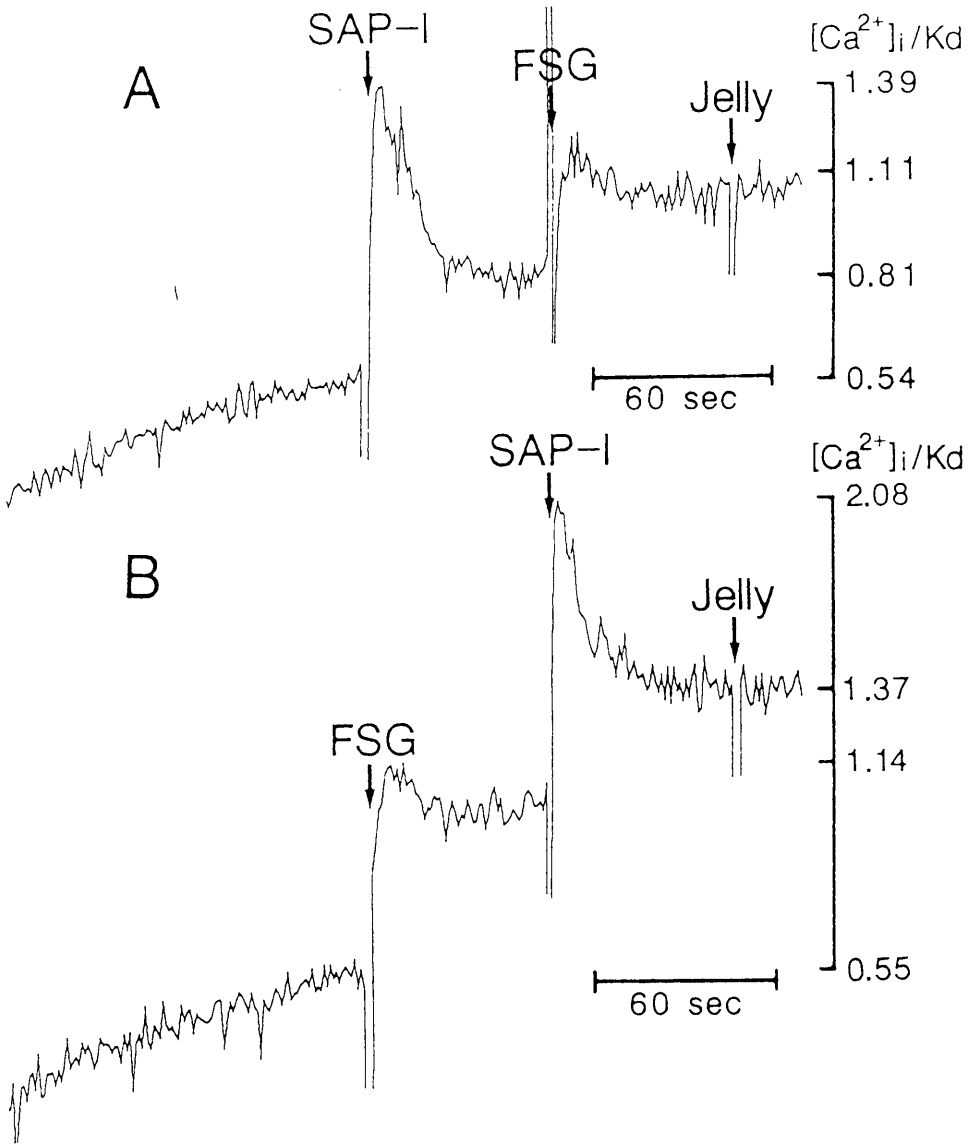

Fig.5 Elevations of $[Ca^{2+}]_i$ induced by SAP-I, FSG and egg jelly. At the times indicated by arrows, SAP-I (final, 0.59 μM), FSG (final, 50 nmol fucose/ml) or egg jelly (final, 1.24 nmol fucose/ml) was added to Fura 2/AM-loaded spermatozoa at pH 8.0.

reported to increase $^{45}Ca^{2+}$ influx and the level of cyclic AMP, and induce the acrosome reaction in abalone spermatozoa (Kopf et al., 1984). It also increases the cyclic AMP and cyclic GMP levels in spermatozoa of *S. purpuratus* and *H. pulcherrimus* in conjunction with SAP-I (Hansbrough and Garbers, 1981; Yamaguchi et al., 1989). Fig. 6 shows that addition of IBMX to *H. pulcherrimus* spermatozoa at pH 8.0 induced elevation of $[Ca^{2+}]_i$ and subsequent addition of SAP-I or FSG induced further increase of $[Ca^{2+}]_i$. The nontransient elevation in $[Ca^{2+}]_i$ by IBMX was similar to that initiated by FSG.

Effects of TEA on the elevation of $[Ca^{2+}]_i$, the acrosome reaction and cyclic nucleotide levels in spermatozoa treated with FSG or FSG plus SAP-I

TEA, a voltage-sensitive K^+-channel blocker, inhibits the inductions of both $^{45}Ca^{2+}$ influx and the acrosome reaction in *S. purpuratus* spermatozoa by egg jelly (Schackmann et al., 1978). As shown in Fig. 7, TEA partially inhibited the elevation of $[Ca^{2+}]_i$ in *H. pulcherrimus* spermatozoa treated with FSG, but did not appear to inhibit the elevation of $[Ca^{2+}]_i$ by SAP-I. TEA inhibited the increase in pH_i by FSG. It also inhibited the FSG-induced acrosome reaction and increase in the level of intracellular cyclic AMP (Table 1). However, its inhibition of induction of the acrosome reaction was partial in the presence of SAP-I (Table 1). In this connection, it should be noted that high $[K^+]$ seawater inhibited induction of the acrosome reaction by FSG plus SAP-I (Table 1).

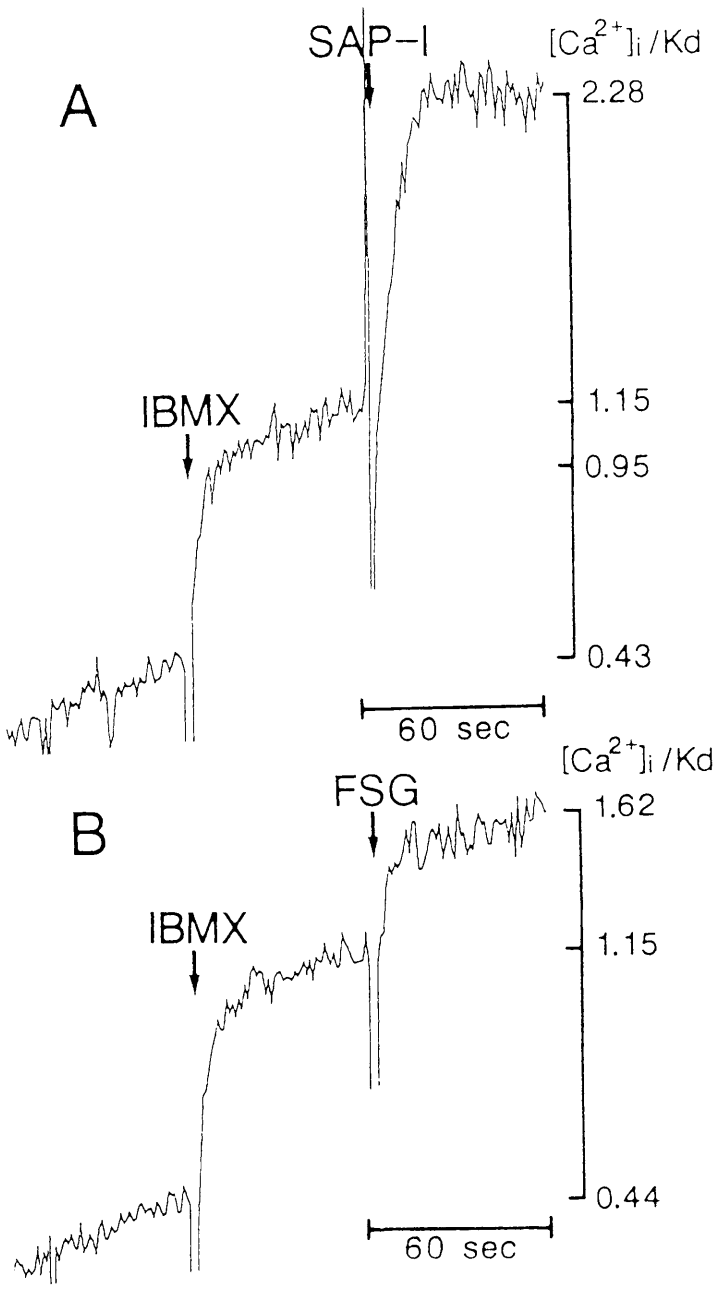

Fig.6 Ca^{2+}-accumulations induced by IBMX, SAP-I, and FSG. The final concentrations of IBMX, SAP-I, and FSG added to Fura 2/AM-loaded spermatozoa at pH 8.0 at the times indicated by arrows were 300 μM, 0.59 μM, and 50 nmol fucose/ml, respectively. The same batch of spermatozoa was used for both experiments.

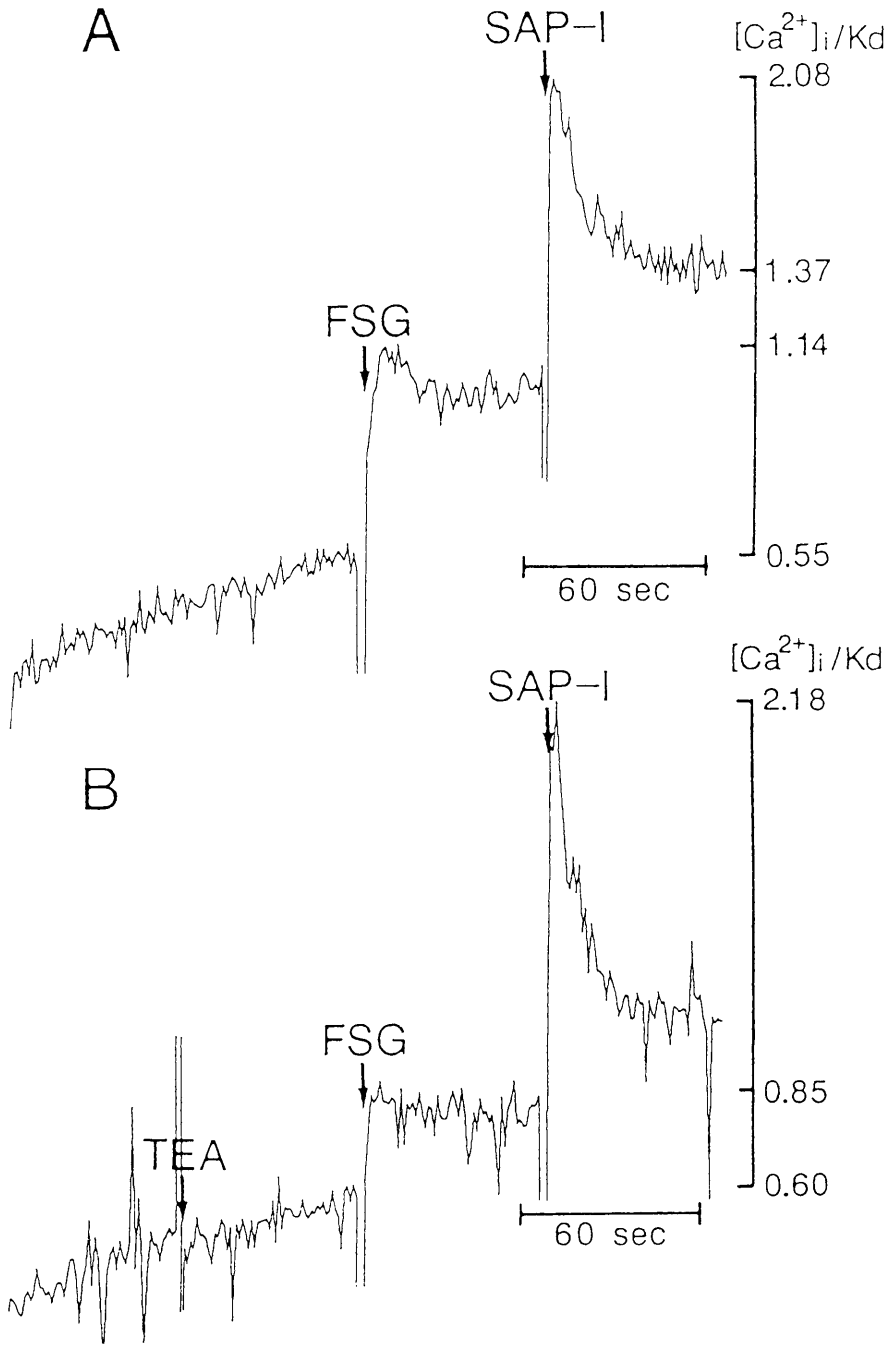

Fig.7 Effect of TEA on the elevation of $[Ca^{2+}]_i$ by FSG. At the indicated point, FSG (final concentration, 50 nmol fucose/ml) was added to Fura 2/AM-loaded spermatozoa in the presence of 10 mM TEA at pH 8.0, followed by addition of SAP-I (final, 0.59 μM). The same batch of spermatozoa was used for both experiments.

Table 1. Percentages of *H. pulcherrimus* spermatozoa showing the acrosome reaction and cyclic nucleotide concentrations of the spermatozoa after treatment with FSG or FSG plus SAP-I in ASW, ASW containing TEA or high K+-ASW

	Acrosome reaction	cyclic AMP[1)]	cyclic GMP[2)]
ASW alone	4%	2.56±0.69	60± 10
+FSG	69	14.08±2.26	80± 10
+FSG+SAP-I	93	18.38±1.21	840±110
ASW with TEA	2	1.48±0.29	59± 10
+FSG	5	1.59±0.13	60± 10
+FSG+SAP-I	47	2.74±0.66	640± 80
High K+-ASW	0	0.16±0.02	290±110
+FSG	6	0.22±0.05	110± 40
+FSG+SAP-I	2	0.24±0.03	3220±510

Spermatozoa were incubated in 0.5 ml of ASW (pH 8.2) or ASW (pH 8.2) containing 10 mM TEA or 100 mM KCl with or without FSG (final concentration, 50 nmol fucose/ml), SAP-I (0.59 μM) or both FSG (50 nmol/ml) and SAP-I (0.59 μM). 1) pmol/mg wet weight of spermatozoa; 2) fmol/mg weight of spermatozoa. Values are means± S.E. obtained for four separate experiments.

DISCUSSION

FSG is a macromolecular component of sea urchin egg jelly and is the main substance responsible for induction of the acrosome reaction of sea urchin spermatozoa. However, for full induction of the acrosome reaction in *H. pulcherrimus* spermatozoa FSG requires SAP-I as a specific co-factor (Yamaguchi et al., 1988). SAP-I is reported to cross-link to a 77 kDa protein in *S. purpuratus* spermatozoa (Dangott and Garbers, 1984, 1987). A cDNA clone for the protein was isolated from a testis cDNA library of *S. purpuratus* and sequenced. The amino acid sequence deduced from the cDNA showed no similarity to that of any reported proteins (Dangott et al., 1989). We found that SAP-I cross-links to 63 kDa and 71 kDa proteins in *H. pulcherrimus* spermatozoa, although neither protein has yet been fully characterized (Harumi et al., 1991). Little is known about the binding of FSG to sea urchin spermatozoa. The data presented here demonstrate that when $[Ca^{2+}]_i$ or pH_i was elevated by a sufficient concentration of FSG, a second addition of the same concentration of FSG did not cause further increase of the $[Ca^{2+}]_i$ or pH_i. Similarly, when the $[Ca^{2+}]_i$ or pH_i had been increased by a sufficient concentration of SAP-I, a second addition of the same concentration of SAP-I cause no further increase of either. A simple explanation of these results is that all the binding sites on the spermatozoa for FSG or SAP-I become occupied on the first addition of excess FSG or SAP-I, and so a second addition of FSG or SAP-I has no further effect on the $[Ca^{2+}]_i$ or pH_i. However, after elevations of the $[Ca^{2+}]_i$ and pH_i by SAP-I, addition of FSG induced their further increases. Similarly, SAP-I increased the $[Ca^{2+}]_i$ and pH_i levels after their increases induced by FSG. These results suggest that

FSG and SAP-I induce their effects by binding to different sites on the spermatozoa. When SAP-I was added at 1,000 lower concentration, some of its binding sites presumably remained unoccupied and so on its second addition it bound to these remaining sites and induced further elevation of pH_i. After $[Ca^{2+}]_i$ or pH_i had been increased by a sufficient concentration of FSG and then of SAP-I, egg jelly did not induce further elevation of the $[Ca^{2+}]_i$ or pH_i, so FSG and SAP-I are probably the main components in the egg jelly that induce elevations of the $[Ca^{2+}]_i$ and pH_i in the spermatozoa. Kopf et al. (1984) reported that IBMX stimulates $^{45}Ca^{2+}$ uptake by abalone spermatozoa through a carrier-mediated transport site. Furthermore, Schackmann and Chock (1986) observed that IBMX enhances SAP-I-stimulated increase of $[Ca^{2+}]_i$ in *S. purpuratus* spermatozoa in a concentration-dependent manner and that the increase in $[Ca^{2+}]_i$ by more than 10 μM IBMX in the presence of SAP-I (30 nM) is nontransient. They also found that 30-60% of *S. purpuratus* spermatozoa treated with both SAP-I (30 nM) and IBMX (300 μM) underwent the acrosome reaction, whereas spermatozoa treated with SAP-I alone did not (Schackmann and Chock, 1986). We found that IBMX at 300 μM induced the nontransient increase in $[Ca^{2+}]_i$ in *H. pulcherrimus* spermatozoa and that the $[Ca^{2+}]_i$ reached a plateau. However, subsequent addition of FSG or SAP-I to the spermatozoa induced further increase of $[Ca^{2+}]_i$. This suggests that the elevation of $[Ca^{2+}]_i$ in the spermatozoa by FSG or SAP-I treatment is induced via a different site from that for IBMX.

SAP-I has been shown to stimulate the decreased respiration rate of sea urchin spermatozoa in slightly acidic seawater (pH 6.6-6.8) at a half-maximal effective concentration of 10 to 100 pM (Suzuki et al., 1981; Garbers et al., 1982). Repaske and Garbers (1983) showed that

SAP-I stimulates H^+ efflux from *S. purpuratus* spermatozoa at a half-maximal effective concentration of ~100 pM. We found that at pH 6.6, SAP-I induced increase of pH_i at a half-maximal effective concentration of 10-100 pM, which is comparable to that for its stimulation of respiration.

Increase in the $[K^+]$ of seawater is reported to depolarize the sperm plasma membrane (Schackmann et al., 1981, 1984) and inhibit Na^+/H^+ exchange in whole spermatozoa, isolated sperm tails, and sperm membrane vesicles (Lee, 1984a, 1984b, 1985). Increase in the $[K^+]$ of seawater also blocks ion fluxes associated with the egg jelly-induced acrosome reaction (Schackmann et al., 1978). Table 1 shows that this is also the case in *H. pulcherrimus* spermatozoa. In ASW containing 100 mM KCl, neither FSG nor FSG plus SAP-I induced the acrosome reaction or increases in $[Ca^{2+}]_i$ and pH_i. However, in ASW containing TEA, FSG plus SAP-I caused slight induction of the acrosome reaction, although neither FSG nor SAP-I alone induced the acrosome reaction. In ASW, TEA decreased the elevation in $[Ca^{2+}]_i$ induced by FSG alone, but did not affect the elevation of $[Ca^{2+}]_i$ by SAP-I alone. These findings suggest that there are two K^+-channels in the spermatozoa, only one of which is a voltage-sensitive channel, and that the increase of $[Ca^{2+}]_i$ induced by SAP-I, which compensates for the smaller elevation of $[Ca^{2+}]_i$ induced by FSG alone in ASW with TEA, may be linked with opening of the non-voltage-sensitive K^+-channel. This also supports the data that the binding sites of FSG and SAP-I on the spermatozoa are different.

CHAPTER 3 IDENTIFICATION AND CHARACTERIZATION OF PUTATIVE RECEPTORS FOR SPERM-ACTIVATING PEPTIDE I (SAP-I) IN SPERMATOZOA OF THE SEA URCHIN *Hemicentrotus pulcherrimus*

SUMMARY

We characterized putative receptors specific for sperm-activating peptide I (SAP-I: GFDLNGGGVG) in spermatozoa of the sea urchin *Hemicentrotus pulcherrimus*, using both binding and crosslinking techniques. Analysis of the data obtained from the equilibrium binding of a radioiodinated SAP-I analogue [GGGY(^{125}I)-GFDLNGGGVG] to *H. pulcherrimus* spermatozoa showed the presence of two classes of receptors specific for SAP-I in the spermatozoa. The incubation of intact spermatozoa as well as sperm tails or sperm membranes prepared from *H. pulcherrimus* spermatozoa with GGGY(^{125}I)-SAP-I and a chemical crosslinking reagent, disuccinimidyl suberate, resulted in the radiolabelling of a 71 kDa protein. The protein appears to be associated with a 220 kDa wheat germ agglutinin (WGA)-binding protein. A cDNA encoding the 71 kDa protein was isolated from a *H. pulcherrimus* testis cDNA library. The cDNA was 2443 bp long and an open reading frame predicted a protein of 532 amino acids containing a 30-residue amino-terminal signal peptide, followed by the same sequence as the N-terminal sequence of the 71 kDa protein. The amino acid sequence of the matured 71 kDa protein is strikingly similar to the 77 kDa protein of *Strongylocentrotus purpuratus* (95.5% identical) and also similar to cystine rich domain of a human macrophage

scavenger receptor. Northern blot analysis demonstrated that mRNA of 2.6 kb encoding the 71 kDa protein was expressed only in the testis.

INTRODUCTION

Sperm-activating peptide I (SAP-I: GFDLNGGGVG) was originally isolated from the egg jelly of the sea urchin by measuring its respiration-stimulating ability in sea urchin spermatozoa (Suzuki et al., 1981; Garbers et al., 1982). SAP-I induces a number of biochemical events in sea urchin spermatozoa such as 1) the transient elevation of intracellular levels of cyclic AMP, cyclic GMP (Hansbrough and Garbers, 1981; Garbers et al., 1982; Suzuki et al., 1988) and $[Ca^{2+}]$ ($[Ca^{2+}]_i$) (Schackmann and Chock, 1986; Hoshino et al., 1992) the transient activation of the membrane form of guanylate cyclase (Bentley et al., 1986a). It also induces a proton efflux across the sperm plasma membrane, resulting in an increase in intracellular pH (pH_i) (Repaske and Garbers, 1983; Hoshino et al., 1992). In addition to the above, the peptide has been shown to promote an acrosome reaction in *Hemicentrotus pulcherrimus* spermatozoa as a specific co-factor of a major acrosome-reaction-inducing substance, fucose sulfate glycoconjugate (Yamaguchi et al., 1988). Since SAP-I was isolated from the egg jelly of the sea urchins *H. pulcherrimus* (Suzuki et al., 1981) and *Strongylocentrotus purpuratus* (Garbers et al., 1982), many different types of sperm-activating peptides have been isolated from the egg jelly of various sea urchin species (Suzuki and Yoshino, 1992). These peptides induce essentially the same biological events as SAP-I in their respective sea urchin spermatozoa, although the specificity is different.

Prior to the induction of these physiological and biochemical events, these sperm-activating peptides seem to bind to specific receptors on the sperm plasma membrane (Smith and Garbers, 1983; Dangott and Garbers, 1984; Bentley and Garbers, 1986; Suzuki et al., 1987; Harumi et al., 1991; Yoshino and Suzuki, 1992). SAP-IIA (CVTGAPGCVGGGRL-NH_2), isolated from the egg jelly of the sea urchin *A. punctulata*, is specifically crosslinked to a 160 kDa plasma membrane protein on *A. punctulata* spermatozoa (Bentley et al., 1986b; Shimomura et al., 1986) which has been identified as guanylate cyclase (Ward et al., 1985; Singh et al., 1988). SAP-I is specifically crosslinked to a 77 kDa protein on *S. purpuratus* spermatozoa (Dangott and Garbers, 1984, 1987; Dangott et al., 1989) or to 71 kDa and 63 kDa proteins on *H. pulcherrimus* spermatozoa (Harumi et al., 1991) while SAP-III (DSDSAQNLIG) is crosslinked to 126 kDa, 87 kDa and 64 kDa proteins on the spermatozoa of the sand dollar *Clypeaster japonicus* (Yoshino and Suzuki, 1992).

In this study, we characterized the SAP-I receptors and the 71 kDa SAP-I-crosslinked protein on *H. pulcherrimus* spermatozoa, and isolated a cDNA clone encoding the protein. Here, we report that the mature protein contains 502 amino acids with a single transmembrane domain and that the protein seems to be associated with a 220 kDa WGA-binding protein.

MATERIALS AND METHODS

Materials

The sea urchins, *H. pulcherrimus* were collected at the coast

near Noto Marine Laboratory. The testes and ovaries were dissected out from the animals as previously described (Suzuki et al., 1982). Composition of artificial sea water (ASW) was 454 mM NaCl, 9.7 mM KCl, 24.9 mM $MgCl_2$, 9.6 mM $CaCl_2$, 27.1 mM $MgSO_4$, 4.4 mM $NaHCO_3$ and 10 mM HEPES, pH 8.2. SAP-I was synthesized for us at the Peptide Institute Inc., Osaka and GGGY-GFDLNGGGVG was synthesized for us at National Institute for Basic Biology, Okazaki. $Na^{125}I$ (3.7GBq/ml, NaOH solution, pH 10) and [γ-^{32}P]ATP (111 TBq/mmol) were products of Du Pont/New England Nuclear (Boston, MA, USA). [α-^{32}P]dCTP (110 TBq/mmol) was purchased from Amersham International plc. (Amersham, UK). Disuccinimidyl suberate was obtained from Pierce Chemical Co. (Rockford, IL, USA). 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesufate (CHAPS) was purchased from Dojindo Laboratories (Kumamoto, Japan). Acetonitrile (ACN) of HPLC grade and chloramine T (sodium N-chloro-4-toluenesulfonamide trhydrate) were obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Wheat germ agglutinin (WGA) was purified from raw wheat germ (Sigma Chemical Co., St. Louis, MO, USA) by the method of Vretblad (1976) and coupled to Sepharose 4B (Pharmacia LKB Biotechnology, Uppsala, Sweden) using the cyanogen bromide method described by March et al. (1974). Restriction enzymes, T4 DNA ligase, and other enzymes were purchased from Takara Biomedicals Co. (Kyoto, Japan) or Toyobo Co. (Osaka, Japan). The cDNA synthesis kit, Hybond-N and Hybond-N+ membrane filters were obtained from Amersham International plc. (Amersham, UK). The random-primed DNA labeling kit was purchased from Boehringer-Mannheim (Indianapolis, IN, USA). The plasmid Bluescript II KS(+), Bluescript II KS(-) and M13KO7 helper phage were generously provided by Dr. Yoshitaka Nagahama at the

National Institute for Basic Biology, Okazaki, Japan.

Preparation of sperm heads and tails, and sperm membranes

Spermatozoa were obtained by intracoelomic injection of 0.5 M KCl and collected "dry sperm" at room temperature, and stored on ice until use. Dry sperm were suspended in 20 volume of ice-cold ASW and homogenized with a Teflon-homogenizer. The homogenate was centrifuged at 1,000xg for 15 min at 4°C to pellet the sperm heads. The sperm head fraction was suspended in 20 volume of ice-cold ASW and centrifuged again. This procedure was repeated several times until the supernatant was clear. The resulting pellet (sperm heads) was suspended in an appropriate volume of ice-cold ASW and used for experiments. The supernatant fraction containing sperm tails was centrifuged at 6,000xg for 30 min at 4°C to pellet sperm tails and the resulting pellet was also suspended in an appropriate volume of ice-cold ASW, and used for experiments.

To prepare sperm membranes, dry sperm were suspended in 10 volume of Solution A containing 0.5 M NaCl, 50 mM $MgCl_2$, 10 mM Na_2MoO_4, 10 mM benzamidine-HCl, 10 mM KCl, 20 mM KF and 20 mM MES, pH 6.5. The sperm suspension was centrifuged at 4,500xg for 5 min, and the resulting sperm pellet was suspended in 10 volume of Solution A. The sperm suspension, after being dispersed in the solution with a Teflon-homogenizer, was subjected to nitrogen cavitation for 20 min at 4°C and then centrifuged at 10,000xg for 30 min at 4°C. The resulting supernatant fluid was centrifuged at 100,000xg for 60 min at 4°C. The resulting pellet (sperm membranes) was resuspended in an appropriate volume of Solution B containing 0.5 M NaCl, 0.1 M NaF and 20 mM MES, pH 6.0 and was kept at -70°C until use.

Iodination of GGGY-SAP-I

GGGY-SAP-I was iodinated by the chloramine-T method using radioactive or non-radioactive sodium iodide. In general, 20 µl of 0.2 mM peptide and NaI (0.58 µg) or $Na^{125}I$ (37 MBq) were mixed with 5 µl of 3.5 mM chloramine-T in 0.15 M sodium phosphate buffer (pH 7.5) and incubated for 15 sec. The reaction was terminated by the addition of 25 µl of 7 mM sodium metabissulfite in the same buffer. Monoiodinated peptide GGGY(^{125}I or I)-SAP-I was purified by HPLC using a Shimadzu Model LC-6A chromatography system with a reverse-phase column (Cosmosil 5C18-AR, 5 µm, 4.6 x 250 mm) equilibrated with 10% ACN in 0.1% trifluoroacetic acid (TFA) in deionized and distilled water. HPLC was carried out using a linear-gradient elution of ACN (10-40% in 30 min) in 0.1% TFA at a flow rate of 1.0 ml/min. The column effluent was monitored for absorbance at 225 nm using a Shimadzu Model SPD-6V spectrophotometer.

Binding experiments

Radioactive monoiodonated peptide GGGY(^{125}I)-SAP-I was mixed with a large excess of non-radioactive monoiodinated peptide GGGY(I)-SAP-I and used for binding experiments. The specific activity of the mixture [GGGY(^{125}I/I)-SAP-I] was approximately 16.7 TBq/mol. Spermatozoa (0.4 mg wet weight) were incubated with GGGY(^{125}I/I)-SAP-I at 20°C for 10 min in 1 ml ASW, buffered with 10 mM Tris (pH 8.2). The reaction was terminated by the addition of 4 ml ice-cold ASW, followed by a rapid filtration with a glass-microfiber filter (Whatman GF/C). The filter was washed five times with 5 ml ASW, dried and measured for radioactivity with a gamma counter (Aloka Auto Well Gamma System ARC-600). Non-specific binding was determined by

incubation of spermatozoa in the presence of excess SAP-I (final 11 μM).

Cross-linking of GGGY(^{125}I)-SAP-I

Spermatozoa (16.7 mg wet weight) or sperm heads, sperm tails and sperm membranes that were prepared from spermatozoa corresponding to 16.7 mg wet weight were incubated with GGGY(^{125}I)-SAP-I (10 pmol) in 1 ml ASW buffered with 10 mM HEPES (pH 8.2) for 10 min at 20°C. Non-specific cross-linking experiments were carried out as the same as above in the presence or absence of non-radioactive SAP-I (11 μM) or SAP-III (DSDSAQNLIG) (11 μM). The reaction was stopped by the addition of ice-cold ASW (0.9 ml, 10 mM HEPES, pH 8.2), and the suspension was centrifuged at 15,000xg for 5 min at 4°C. The resulting pellet was resuspended in ASW (90 μl, 10 mM HEPES, pH 8.2) and incubated with a cross-linking reagent, 1 mM disuccinimidyl suberate in dimethylsulfoxide (10 μl) for 30 min at 20°C. The incubation was terminated by the addition of ASW (0.9 ml, 10 mM Tris, pH 8.2) and 0.5 ml of 30% (w/v) trichloroacetic acid was added.

Proteins obtained from chromatography of CHAPS-solubilized sperm tail proteins on a WGA-Sepharose 4B column were also crosslinked with GGGY(^{125}I)-SAP-I in the presence of disuccinimidyl suberate as described above. The crosslinked proteins were analyzed by SDS-PAGE according to Laemmli (1970). The gel was subsequently silver-stained by the method of Morrissey (1981). Radiolabelled protein bands were detected by autoradiography on a Kodak X-OMAT film.

Purification, characterization, and N-terminal amino acid sequence analysis of SAP-I-crosslinked protein

The 30,000xg supernatant obtained by the 10,000xg centrifugation of sperm tails solubilized in Buffer A (0.5 M NaCl, 10 mM $CaCl_2$, 10 mM $MnCl_2$, and 10 mM Tris-HCl, pH 7.5) containing 10 mM benzamidine-HCl and 1% CHAPS was applied to a WGA-Sepharose 4B column (20 x 64 mm) equilibrated with Buffer A containing 0.1% CHAPS. The column was washed with Buffer A containing 0.1% CHAPS and the proteins retained on the column were eluted with 100 mM N-acetyl-D-glucosamine in Buffer B (0.5 M NaCl, 0.1% CHAPS and 10 mM Tris-HCl, pH 7.5). Fractions containing the WGA-binding protein and the 71 kDa protein were pooled, dialyzed against Buffer C (0.1% CHAPS and 50 mM Tris-HCl, pH 7.5) and mixed with 100 ml of preswollen DEAE-Sephacel gel at 4°C. The gel was washed once with Buffer C and then twice with Buffer C containing 200 mM NaCl. The proteins retained on the gel were eluted with Buffer C containing 2 M NaCl. Fractions containing the WGA-binding protein and the 71 kDa protein were pooled, concentrated with an Amicon Diaflo Cell RK 52 and subjected to SDS-PAGE. The gel was stained with Coomassie brilliant blue R250 (Fluka Chemie AG., Buchs, Switzerland). The protein band corresponding to 71 kDa was excised and electroeluted from gel with a Max-Yield Protein Concentrator (ATTO, Tokyo, Japan). To analyze the amino acid composition and the N-terminal amino acid sequence of the 71 kDa protein, the protein in the gel was transferred to a PVDF membrane (BioRad Laboratories, Richmond, CA, USA) using a Multiphor II NovaBlot electrophoretic kit (Pharmacia LKB Biotechnology, Uppsala, Sweden) at room temperature for 1 hr at 0.8 mA/cm^2 constant current (Towbin et al., 1979). The membrane was rinsed three times with distilled water for 5 min each.

The proteins were visualized by staining briefly with Coomassie brilliant blue R250. A Coomassie-stained protein band corresponding to the 71 kDa protein was cut out. One piece of the band was subjected to amino acid analysis with an automated amino acid analyzer, Hitachi L-8500, after hydrolysis with constant-boiling HCl containing 4% thioglycolic acid at 110°C for 24 hrs and another piece of the band was submitted to automated Edman degradation an Applied Biosystems 470A gas-phase sequenator equipped with an on-line Applied Biosystems 120A PTH amino acid analyzer.

In different experiments, GGGY(^{125}I)-SAP-I-labelled sperm membrane were solubilized in Buffer A containing 10 mM benzamidine-HCl and 1% CHAPS, and centrifuged at 10,000xg. The resultant was applied to a DEAE-Sephacel column. Fractions containing radioactivity were pooled, concentrated with the Amicon Diaflo Cell RK 52 and subjected to chromatography on a Toyopearl HW55 column. Radiolabelled proteins eluted from the columns were analyzed by SDS-PAGE and autoradiography.

Fractions obtained from chromatography on a WGA-Sepharose 4B column, which mainly contained the WGA-binding protein and the 71 kDa protein, were pooled, concentrated with an Amicon Diaflo Cell RK 52, and incubated in a final concentration of 2% SDS at 100°C for 5 min. Buffer B was then added to the mixture to make the SDS final concentration of 0.1% and the sample was subjected to chromatography on the WGA-Sepharose 4B column as described above. The mixture was also subjected to HPLC on a TSK-G4000SW column (7.5 x 300 mm, TOSOH K.K., Tokyo, Japan) equilibrated with 0.1 M sodium phosphate (pH 6.8) containing 0.1% CHAPS using a Hitachi HPLC system L-6000-L-6200 equipped with a L-4200 UV-VIS detector and a D-2500 Chromato-

Integrator. The proteins were eluted with the equilibration buffer at a flow rate of 0.5 ml/min at room temperature. The column effluent was monitored for an absorbance at 280 nm and protein concentration.

Cloning and sequencing of cDNAs

A cDNA library (4.9 x 10^5 pfu) from poly(A)+ RNA isolated from growing testes of *H. pulcherrimus* was constructed in λgt10 using the cDNA synthesis system and the cDNA cloning system λgt10 (Amersham International plc., Amersham, UK).

An antisense oligonucleotide probe (45 mer; 5'-ACCAACGTCCCAGTT ATGGGTGTAGGGACACGCATGCTGATACGG-3') which corresponds to part of cDNA (nucleotides 832 to 876) for a SAP-I-crosslinked 77 kDa protein in *S. purpuratus* spermatozoa (Dangott et al., 1989), was synthesized on an Applied Biosystem Model 391 DNA synthesizer. The oligonucleotide was 5' end labeled with [γ-^{32}P]ATP (7.4 MBq), and used for screening approximately 3 x 10^5 plaques. Plaques transferred onto Hybond-N+ membranes were prehybridized at 37°C for 2 hrs in prehybridization solution containing 6xNET (1xNET; 50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, pH 7.5), 0.1% SDS, 1xDenhardt's and denatured herring sperm DNA (100 μg/ml) and then hybridized at 37 °C overnight with a 1 x 10^7 cpm probe in 10 ml of hybridization solution containing 6xNET, 0.5% SDS, 1xDenhardt's solution and denatured herring sperm DNA (100 μg/ml). The membranes were washed three times at 20°C with 6xSSC (1xSSC; 150 mM NaCl, 15 mM sodium citrate, pH 7.0) containing 0.1% SDS, and then washed at 40°C for 1 min with 5xSSC containing 0.1% SDS. Forty initial positive clones were rescreened until pure, and the phage DNA was purified. Digestion of the DNA with BamH I showed that 20 clones contained insert of 2.5 kb, and restriction

mapping showed that 16 of those were identical.

The 2.5 kb cDNA insert from a clone (λSR11-1-1) was subcloned into the plasmid vector pBluescript II KS(+) (Stratagene, La Jolla, CA, USA). EcoR I cut this insert into 2.0 kb and 0.5 kb fragments, and the 2.0 kb fragment hybridized to the probe. Serial deletion mutants of subclones were made according to Yanisch-Perron et al (1985). Nucleotide sequence were determined by the dideoxy chain termination method (Sanger et al., 1977) using the 7-DEAZA sequencing kit ver. 2.0 (Takara Biomedicals, Kyoto, Japan) and the Sequenase version 2.0 DNA sequencing kit (United States Biochemical Co., Cleveland, OH, USA) and analyzed on DNASIS software (Hitachi Software Engineering Co., Yokohama, Japan).

Northern blot analysis

Total RNA was prepared from testes, ovaries and unfertilized eggs of *H. pulcherrimus* by the LiCl method of Cathala et al. (1983). Poly(A)+ RNA was prepared by two passage of the total RNA over a column of oligo(dT)-cellulose (Pharmacia LKB Biotechnology, Uppsala, Sweden). Northern blot analysis was carried out at follows: 1.6 μg poly(A)+RNA was denatured with 2.1 M formaldehyde, electrophoresed on 1% agarose in the presence of 2.2 M formaldehyde, and transferred onto a Hybond-N membrane. Prehybridization was performed for 2 hrs at 65°C in a solution containing 6xSSC, 0.5% SDS, 5xDenhardt's solution, and denatured herring sperm DNA (100 μg/ml). The RNA on the membrane was then hybridized to the random-primed, [α-^{32}P]dCTP-labeled 1745 bp cDNA insert (base numbers from 150 to 1895 of the λSR11-1-1 cDNA insert) at 65°C for 18 hrs. The membrane was washed with 6xSSC and 0.1% SDS at room temperature for 30 min, followed by

a final wash with 0.5xSSC, 0.1% SDS at 65°C for 30 min. The size of the RNA was estimated using a 0.24-9.5 kb RNA Ladder (GIBCO BRL, Gaithersburg, MD, USA) as a marker.

Other methods

Sperm respiration rates were determined using a Yanaco PO-100A oxygraph as described previously (Suzuki et al., 1981). Intracellular cyclic GMP concentrations were determined by radioimmunoassay using a Yamasa cyclic GMP-assay kit (Yamasa Shoyu K.K., Chiba, Japan) as described previously (Yamaguchi et al., 1988). The concentration of protein was determined by the Lowry method modified by Schacterle and Pollack (1973) using bovine serum albumin as a standard.

RESULTS

Equilibrium binding of GGGY(^{125}I/I)-SAP-I to spermatozoa

Initially, we examined whether GGGY(I)-SAP-I possessed identical biological activity to SAP-I. The monoiodinated SAP-I analogue stimulated *H. pulcherrimus* sperm respiration half maximally at a concentration of 0.056 nM which was almost the same as that of SAP-I. The peptide also elevated sperm cyclic GMP concentrations with an EC_{50} of 32 nM, which was close to that of SAP-I. Therefore, we concluded that GGGY(I)-SAP-I possesses the same biological activity as SAP-I and used it in the following experiments.

Spermatozoa (0.4 mg wet weight) were incubated with various concentrations (0.6-316 nM) of GGGY(^{125}I/I)-SAP-I. The results showed

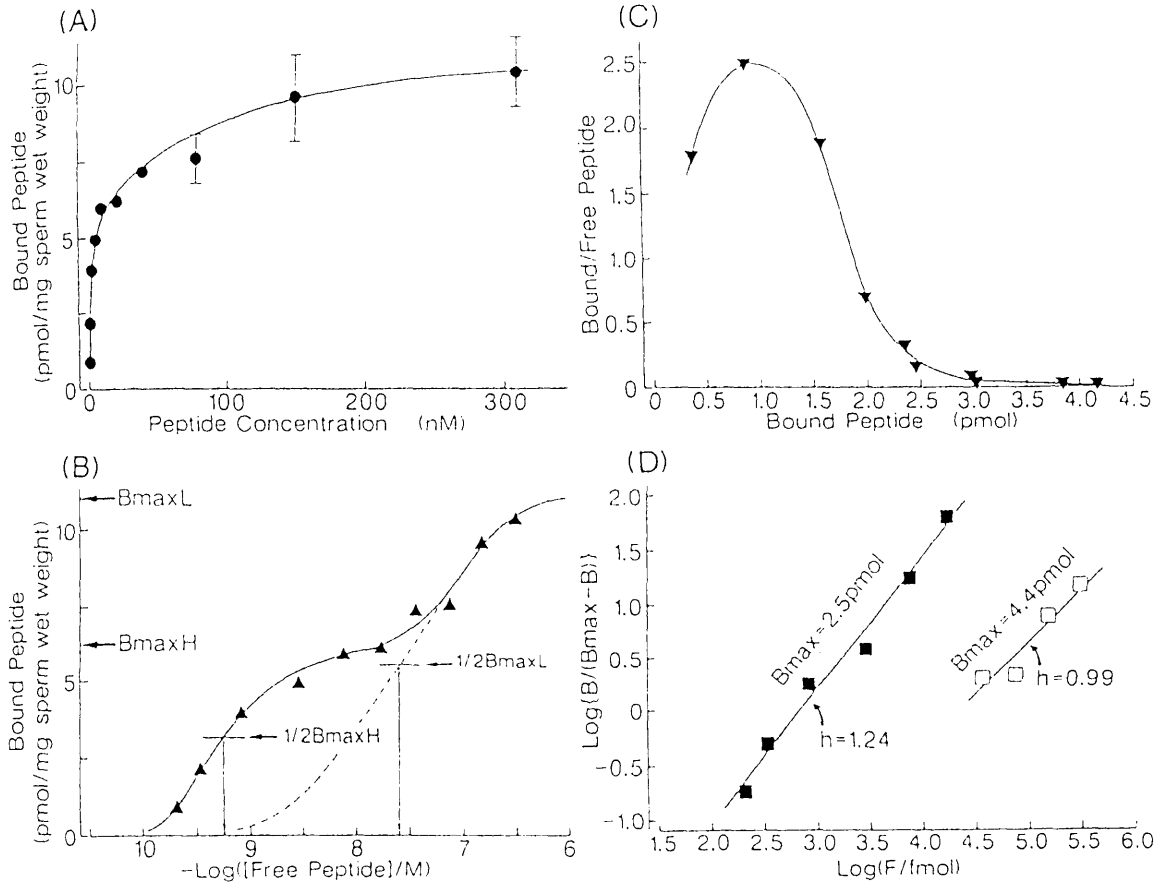

Fig. 8 Equilibrium binding of GGGY(^{125}I/I)-SAP-I to *H. pulcherrimus* spermatozoa and analysis of the data using Scatchard, Klotz and Hill plots. (A) Saturation curve of GGGY(^{125}I/I)-SAP-I binding. The specific binding estimated from the difference between GGGY(^{125}I/I)-SAP-I binding in the absence and presence of the excess SAP-I was plotted with a linear abscissa. Each point represents the mean of three independent incubations. (B) Klotz plot. The specific binding was plotted with a logarithmic abscissa according to the method of Klotz (1982). Standard error of each point was not shown because it was very small. The half-maximal binding ($1/2B_{max}H$ and $1/2B_{max}L$) provided estimates of the Kd of 0.58 nM for the high-affinity receptor and 23 nM for the low-affinity receptor. (C) Scatchard plot. The molar ratio of specifically bound peptide to free peptide was plotted against mol specifically bound GGGY(^{125}I/I)-SAP-I/0.4 mg wet H. pulcherrimus spermatozoa, according to the method of Scatchard (1949). (D) Hill plot. (■) High-affinity binding calculated with 2.6 pmol/0.4 mg wet spermatozoa as the B_{max} of the high-affinity receptor. (□) Low-affinity binding calculated with 4.4 pmol/0.4 mg wet spermatozoa as the B_{max} of the low-affinity receptor. B_{max} values were estimated from the Scatchard plot. B, the concentration of bound peptide; F, the concentration of unbound peptide.

that the binding of the peptide to spermatozoa was saturable (Fig. 8A). The data were analyzed by the method of Klotz (1982), yielding a double sigmoidal curve (Fig. 8B). This suggests the existence of two classes of receptors which are formed from low-affinity and high-affinity members. A Scatchard plot of the data yielded a downward concave curve at less than 2.6 pmol (Fig. 8C), implying that one binding sites of a single class of the high-affinity interacts positively and cooperatively with other site (Scatchard, 1949; Hart, 1965; Swillens, 1975). By extrapolating a limb of the curve, we estimated that the maximal binding capacity (B_{max}) values for the high-affinity and low-affinity receptors were 6.4 and 11 pmol/mg wet weight spermatozoa, respectively. A Hill plot produced the Hill coefficient (h) of 1.24 for the high-affinity receptors and 0.99 for the low-affinity receptors (Fig. 8D). From these plottings, the K_d values were estimated to be 0.58-0.69 nM for the high-affinity receptors and 23-25 nM for the low-affinity receptors (Table 2).

Covalent coupling of GGGY(^{125}I)-SAP-I to intact spermatozoa, sperm heads and tails, and sperm membranes

GGGY(^{125}I)-SAP-I coupled covalently to a 71 kDa protein located exclusively in sperm tails in the presence of disuccinimidyl suberate (Fig. 9). The incubation of spermatozoa, sperm tails or sperm membrane with GGGY(^{125}I)-SAP-I and disuccinimidyl suberate in excess SAP-I resulted in there being no radiolabelling of the protein, whereas the incubation of spermatozoa with GGGY(^{125}I)-SAP-I and disuccinimidyl suberate in excess SAP-III, which exhibits no biological activity toward the spermatozoa, did not affect the appearance of the radioactive protein bands (Fig. 9). When fractions containing the

Table 2. Equilibrium parameters GGGY(^{125}I/I)-SAP-I binding and half-maximal effective concentrations of GGGY(I)-SAP-I or SAP-I on spermatozoa

	Type of binding	K_d	B_{max}	h	EC_{50}
		nM			nM
Spermatozoa	high-affinity	0.58-0.65	6.4[1)]	1.24	
	low-affinity	23-25	11.0[1)]	0.99	
Respiration					0.056
Intracellular pH					0.045 [2)]
Intracellular [Ca^{2+}]					7.0 [2)]
Intracellular [cyclic GMP]					32

1) pmol/mg wet weight of spermatozoa. 2) calculated from the data of Hoshino et al. (1992).

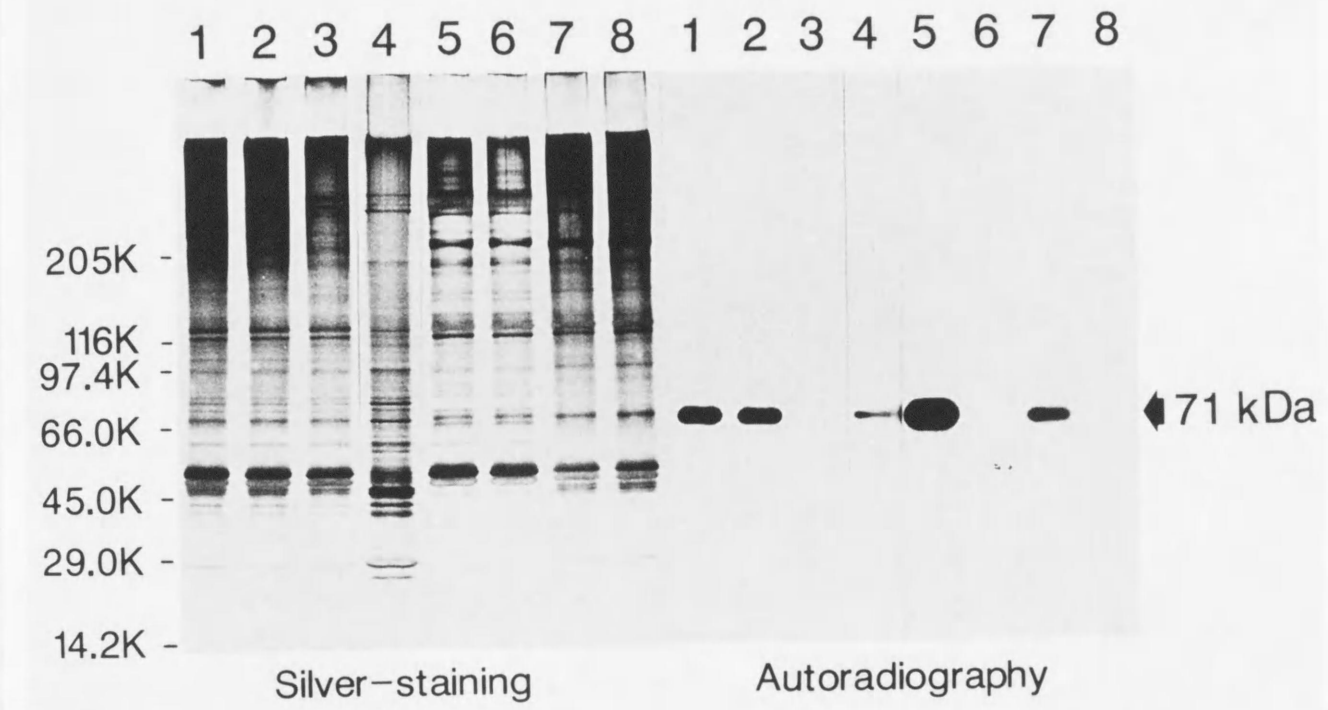

Fig. 9 SDS-PAGE of GGGY(^{125}I)-SAP-I-crosslinked *H. pulcherrimus* sperm protein. Approximately 5-10 μg of protein were electrophoresed on a 5-15% SDS-polyacrylamide gel in the presence of 2-mercaptoethanol. Intact spermatozoa (16.7 mg wet weight) or sperm heads, sperm tails and sperm membrane prepared from the spermatozoa (16.7 mg wet weight) were incubated with GGGY(^{125}I)-SAP-I in the presence of no SAP-I (lane 1), 11μM SAP-III (lane 2) or 11 μM SAP-I (lane 3). Sperm heads prepared from the spermatozoa (16.7 mg wet weight) were incubated with 10 pM GGGY(^{125}I)-SAP-I (lane 4). Sperm tails prepared from the spermatozoa (16.7 mg wet weight) were incubated with 10 pM GGGY(^{125}I)-SAP-I in the presence of no SAP (lane 5) or 11 μM SAP-I (lane 6). Sperm membrane prepared from the spermatozoa (16.7 mg wet weight) were incubated with 10 pM GGGY(^{125}I)-SAP-I in the presence of no SAP (lane 7) or 11 μM SAP-I (lane 8).

GGGY(^{125}I)-SAP-I-crosslinked protein(s) obtained from chromatography of CHAPS-solubilized GGGY(^{125}I)-SAP-I-labelled sperm membrane on a DEAE-Sephacel column and on a Toyopearl HW55 column were subjected to SDS-PAGE under the same conditions as above, two radiolabelled bands were detected at molecular masses of 75 kDa and 71 kDa, although the radioactivity of the band at 75 kDa was weaker than that of the 71 kDa band (data not shown).

Sequence of the cDNA clone

The sequence strategy for the λSR11-1-1 cDNA is shown in Fig. 10. The complete nucleotide sequence and the deduced amino acid sequence are presented in Fig. 11. The λSR11-1-1 cDNA insert was 2443 bp in length. The oligonucleotide sequence used for screening the clone was found in the sequence at nucleotide positions 847-891. We have assigned the initiation codon to the ATG at position 133 because (1) there are 4 upstream in-frame stop codons; (2) this ATG is flanked by sequences that fit Kozak's criteria for translation initiation codon (Kozak, 1981), and (3) the 30-amino acid sequence following this ATG possesses the features characteristic of signal sequences (Watson, 1984). The initiation codon is followed by an open reading frame of 1593 bp. An inframe stop codon occurs at position 1729 and the 3'-untranslated region composed of 714 bp includes polyadenylation sites (AATAAA).

The deduced amino acid sequence suggests that cleavage of the signal peptide would yield a protein of 502 amino acids with a calculated molecular weight of 54,581. The protein contains three potential N-linked glycosylation sites (NXT) at residues 48-50, 85-87, and 429-431. The protein also contains a hydrophobic region of 29

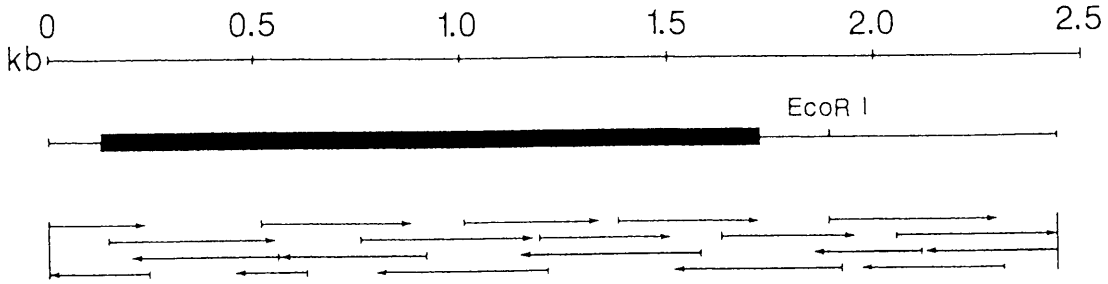

Fig. 10 The restriction endonuclease map and sequencing strategy for a λSR11-1-1 cDNA insert. The map only shows the relevant restriction site. The direction and extent of the sequence determination are indicated by arrows. The deduced open reading frame is shown by a solid box.

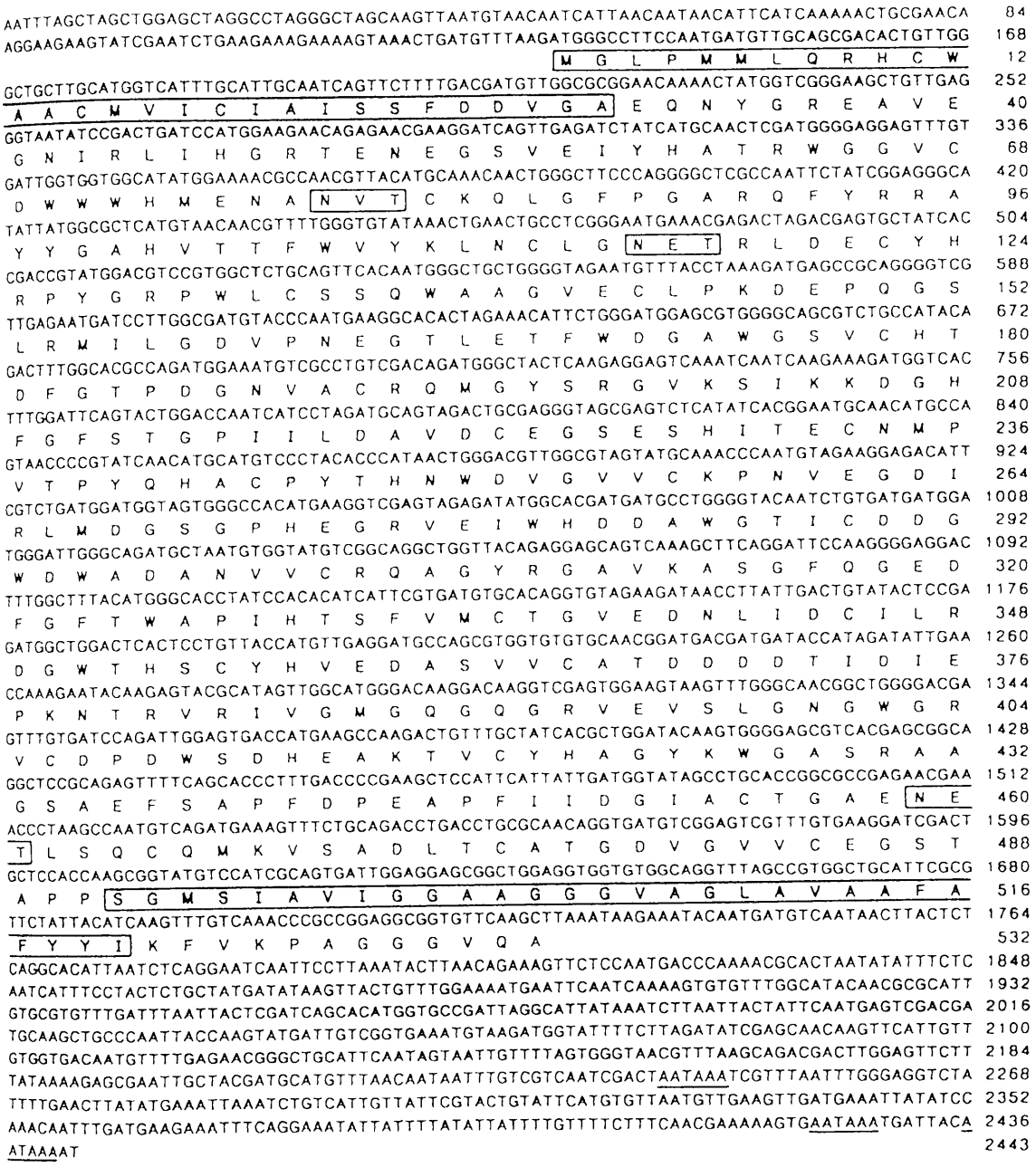

Fig. 11 Complete nucleotide sequence and deduced amino acid sequence of the λSR11-1-1 cDNA insert. Nucleotide and amino acid number are listed on the right hand side. The signal sequence and the putative transmembrane sequence are indicated by shaded-boxes. The potential N-linked carbohydrate binding sites are indicated by open boxes. Polyadenylation signal sequences at the 3'-untranslated region are underlined. The N-terminal amino acid sequence of the purified 71 kDa protein is indicated by the broken line.

amino acids at residue 462-490 that is flanked on the carboxyl-terminal side by KFVK and on the amino-terminal end by a single acidic residue (Fig. 12). These features are typical of membrane-spanning domains of membrane protein (Sabatini et al.,1982).

Northern blot analysis

To determine the size of the mRNA for the λSR11-1-1 cDNA insert and to see whether the mRNA exists in the testes, ovaries or eggs, poly(A)+ RNA prepared from tissues and eggs of *H. pulcherrimus* was analyzed by Northern blot hybridization using a part (nucleotides, 150-1895) of the λSR11-1-1 cDNA insert as a probe. A strong hybridization signal at the position corresponding to 2.6 kb was detected only with poly(A)+ RNA from a testis sample (Fig. 13).

Purification and characterization of the 71 kDa protein

The 71 kDa protein was co-purified with a 220 kDa WGA-binding protein by chromatography of CHAPS-solubilized sperm tail protein (330 mg) on a WGA-Sepharose 4B column and batchwise chromatography on the DEAE-Sephacel gel. The 71 kDa protein did not separate from the 220 kDa WGA-binding protein by gel filtration chromatography on a Toyopearl HW55 column. During the chromatography, the 71 kDa protein with the 220 kDa WGA-binding protein was eluted in the void. The 71 kDa protein (0.93 mg) was finally purified by preparative SDS-PAGE. The protein (about 2 μg) was transferred onto a PVDF membrane and was analyzed for amino acid composition and sequenced for the N-terminal amino acid sequence. As shown in Table 3, the amino acid composition of the protein deduced from nucleotide sequence of the λSR11-1-1 insert cDNA without the

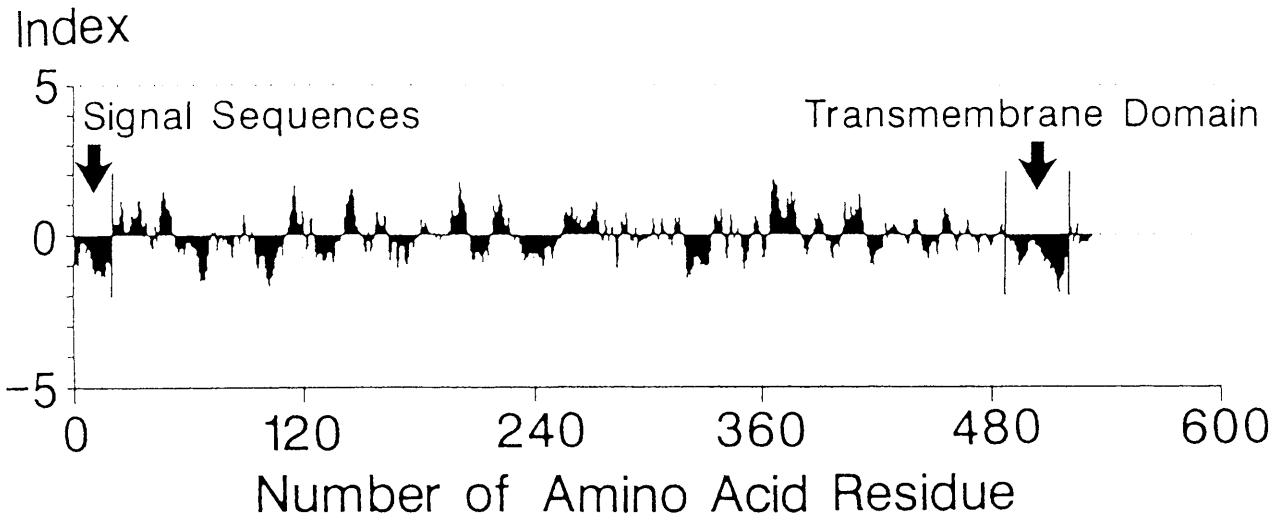

Fig. 12 Hydropathy profile of the protein with 532 amino acids. Hydropathy was calculated at a window of 12 residues, according to Kyte and Dolittle (1982). Regions with values below the midpoint line are hydrophobic.

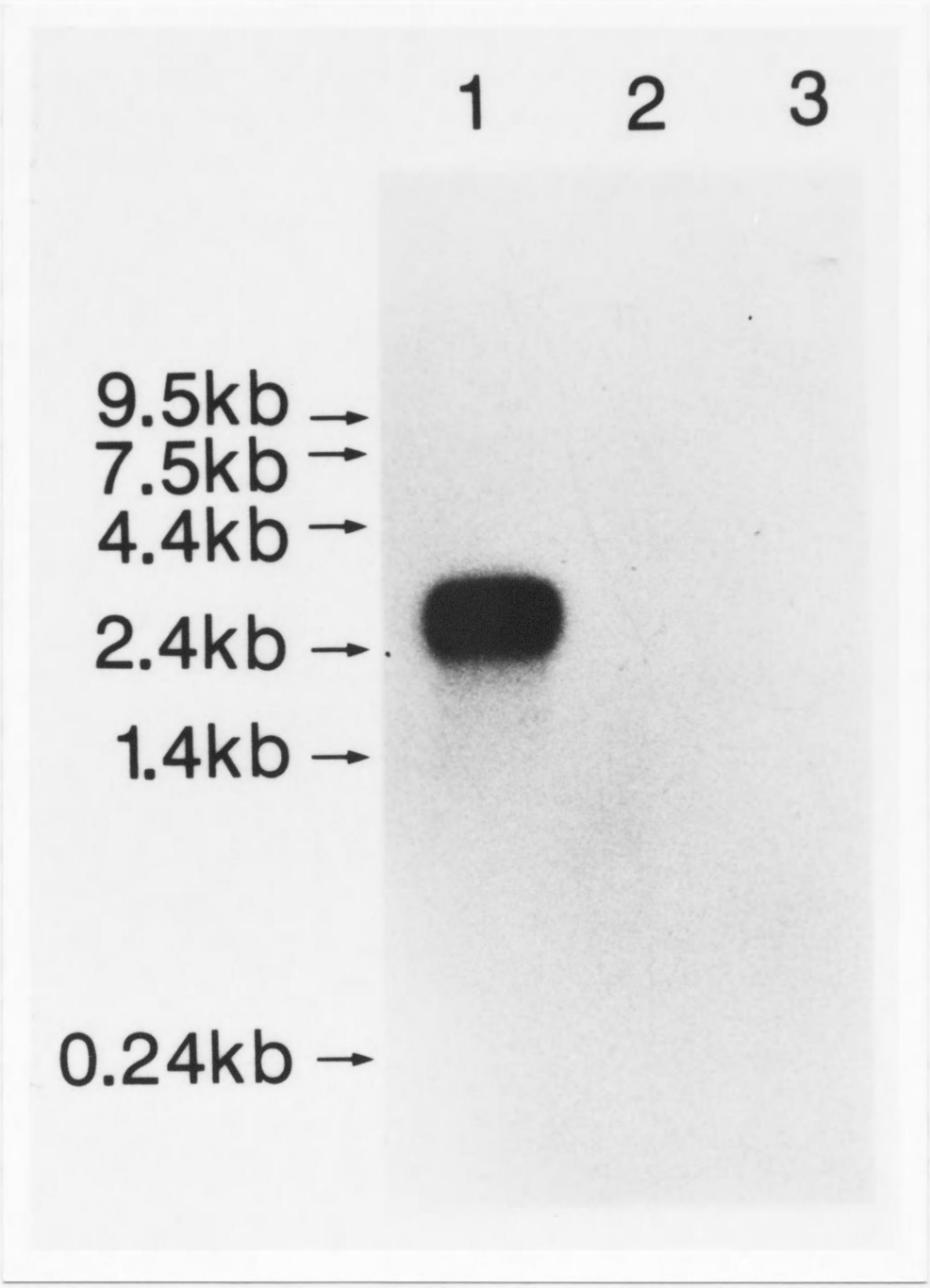

Fig. 13 Northern blot analysis. Poly(A)+RNA prepared from *H.pulcherrimus* testes (1), ovaries (2) or eggs (3) was hybridized to a part (nucleotides 150-1895) of the λSR11-1-1 cDNA insert.

Table 3. Amino acid composition of the purified 71 kDa protein was compared to a mature protein predicted by nucleotide sequence of the λSR11-1-1 cDNA insert

Residues	71 kDa protein	Mature protein predicted from nucleotide sequence of cDNA
Asp	52.5 a.a	53 a.a
Thr	27.3	28
Ser	28.8	25
Glu	48.1	44
Pro	34.4[1)]	22
Gly	61.9	64
Ala	42.6	44
Half-Cys	N.D.[2)]	24
Val	37.6	41
Met	11.2	9
Ile	23	23
Leu	24.5	18
Tyr	16.4	17
Phe	19.9	17
Lys	15.5	14
His	17.2	17
Arg	21.1	23
Trp	N.D.	19

1) Proline content was always over-estimated in the analysis of protein samples transferred to the PVDF membranes. 2) Half-cysteine was not detected, perhaps because of hydrolysis with 4% thioglycolic acid.

predicted signal peptide. The N-terminal amino acid sequence of the purified 71 kDa protein was EQNYGREAVEGNIRLIHGRTENEGS, which was the same as residue numbers 31 to 55 of the deduced amino acid sequence from nucleotide sequence of the λSR11-1-1 cDNA insert.

When the fraction containing the 71 kDa protein and the 220 kDa WGA-binding protein obtained by chromatography on a WGA-Sepharose 4B column was incubated with GGGY(^{125}I)-SAP-I in the presence of disuccinimidyl suberate and then analyzed by SDS-PAGE and autoradiography, the 71 kDa protein was radiolabelled but the 220 kDa protein was not radiolabelled (Fig. 14). In different experiments, the same fraction was incubated in 2% SDS at 100°C for 5 min, and then subjected to chromatogphy on a TSK-G4000SW column or on a WGA-Sepharose 4B column. By chromatography using the TSK column, the 71 kDa protein was separated from the 220 kDa WGA-binding protein (Fig. 15A). In chromatography using the WGA-Sepharose 4B column, the 71 kDa protein was eluted in flow-through fractions, while the 220 kDa protein was retained on the column and eluted with 100 mM N-acetyl-D-glucosamine (Fig. 15B).

DISCUSSION

In the present study, we demonstrated that *H. pulcherrimus* spermatozoa possess receptors specific for SAP-I, which are located on the tail plasma membrane. Analysis of the data obtained from the equilibrium binding of GGGY(^{125}I/I)-SAP-I to intact spermatozoa by the methods of Klotz, Scatchard and Hill suggested the presence of two classes of receptor (high-affinity and low-affinity) specific for SAP-I.

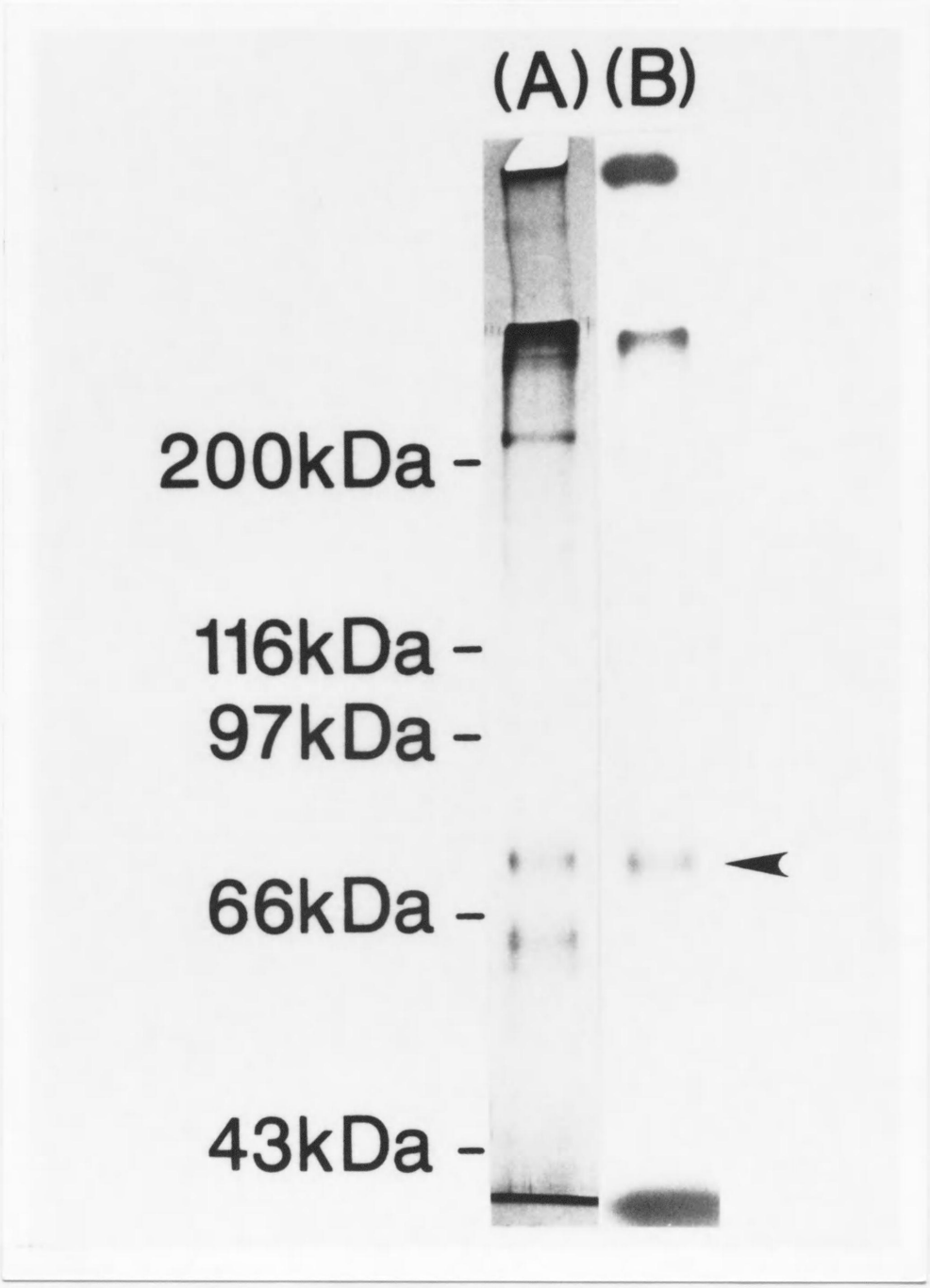

Fig. 14 GGGY(^{125}I)-SAP-I-crosslinking to the 71 kDa protein. Protein (100 μg) obtained from chromatography of CHAPS-solubilized sperm tail proteins on a WGA-Sepharose 4B column were incubated with GGGY(^{125}I)-SAP-I and disuccinimidyl suberate. The crosslinked protein (4 μg) was analyzed by SDS-PAGE using a 7.5% gel and subsequently subjected to autoradiography as described in Materials and Methods. (A) silver-staining, (B) autoradiogram. The arrow indicates the 71 kDa protein band.

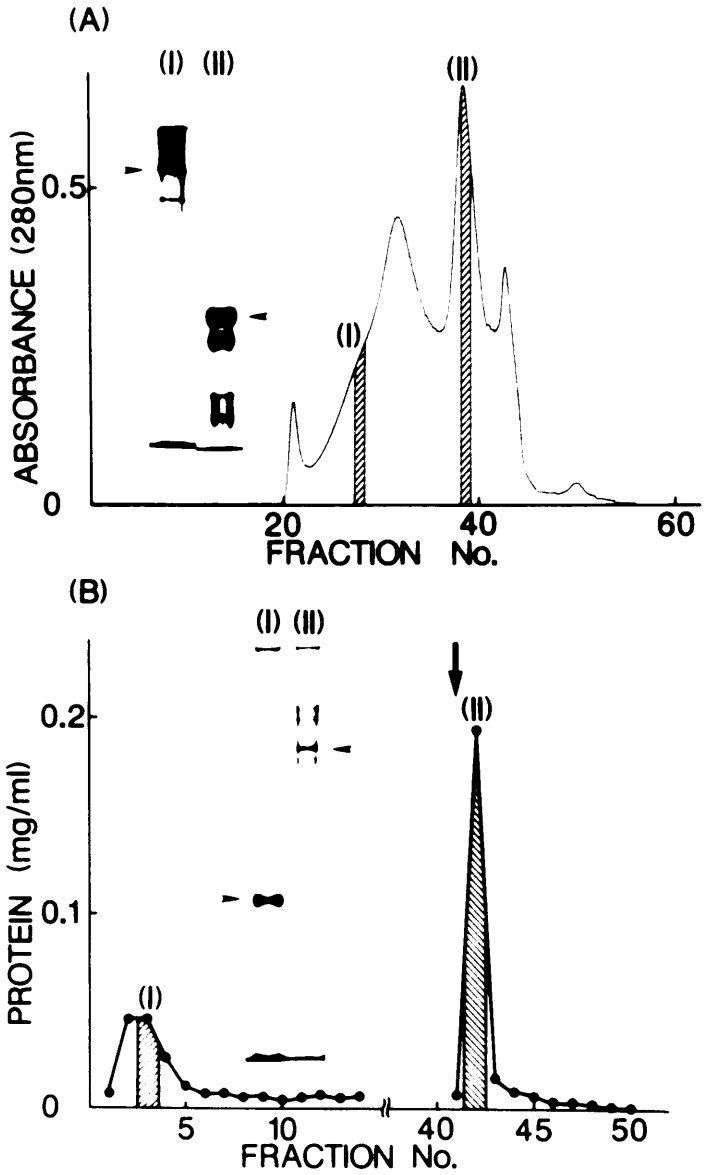

Fig. 15 Separation of the 71 kDa protein from the 220 kDa WGA-binding protein. A fraction [27.6 mg protein for (A) and 10.1 mg protein for (B)] containing the 71 kDa and 220 kDa WGA-binding proteins, was obtained from chromatography on a WGA-Sepharose 4B column. It was incubated in 2% SDS at 100°C for 5 min, and then applied to a TSK-G4000SW column (A) or a WGA-Sepharose 4B column (B). Inserts are silver-stained SDS-PAGE profiles of the fractions designated by the shaded area and Roman numeral. Arrows denote the 220 kDa WGA-binding protein or the 71 kDa protein.

The K_d of the high-affinity receptor was comparable to that of SAP-I (1.4 nM in *S. purpuratus* spermatozoa) (Smith and Garbers, 1983), SAP-IIA (1.0 nM in *A. punctulata* spermatozoa) (Bentley et al., 1986b) and SAP-III (3.4 nM in *C. japonicus* spermatozoa) (Yoshino and Suzuki, 1992). In the case of I/^{125}I-GGGY-SAP-III binding to the high-affinity receptor on *C. japonicus* spermatozoa (Yoshino and Suzuki, 1992), a positive cooperative interaction was seen among the high-affinity binding sites on *H. pulcherrimus* spermatozoa. As shown in Table 2, SAP-I has two classes of EC_{50} values with regard to activity. One class of EC_{50} values is at subnanomolar levels, which includes respiration-stimulating activity (0.056 nM) and pH_i-increasing activity (0.045 nM) (Hoshino et al., 1992). The other class of values ranges from 7-32 nM and is found in $[Ca^{2+}]_i$-increasing activity (7 nM) (Hoshino et al.,1992) and cellular cyclic GMP-elevating activity (32 nM). In the present study, we demonstrated that the high-affinity receptor had a K_d of 0.58-0.65 nM and the low-affinity receptor had a K_d of 23-25 nM. Therefore, we presume that the two different ranges of EC_{50} values found in the biological activity of SAP-I may reflect the presence of two classes of SAP-I receptors; the high-affinity receptor may be for pH_i-increasing activity as well as respiration-stimulating activity and the low-affinity receptor may be for cyclic GMP- and $[Ca^{2+}]_i$-elevating activity.

GGGY(^{125}I/I)-SAP-I was crosslinked mostly to the 71 kDa protein which was located in the sperm plasma membrane. The covalent coupling was prevented by excess SAP-I but not by excess SAP-III which did not show any biological effect on *H. pulcherrimus* spermatozoa. Thus, we think that the interaction of GGGY(^{125}I/I)-SAP-I and the 71 kDa protein is specific and that the protein is a binding

component specific for SAP-I.

The purified 71 kDa protein had the N-terminal amino acid sequence EQNYGREAVEGNIRLIHGRTENEGS which was the same as the sequence (residues 31-55) of a protein deduced from the nucleotide sequence of the λSR11-1-1 cDNA insert. The amino acid composition of the 71 kDa protein was also in close agreement with that of the protein deduced from the nucleotide sequence if amino acid residues in the predicted signal peptide were subtracted from the total amino acid residues of the protein. Thus, we concluded that the clone obtained from a *H. pulcherrimus* testis cDNA library using an oligonucleotide probe which corresponds to a part of the nucleotide sequence of a cDNA for a 77 kDa protein in *S. purpuratus* spermatozoa (Dangott et al., 1989) is of a 71 kDa protein in *H. pulcherrimus* spermatozoa and that the mature protein contains 502 amino acids. The discrepancy between the apparent molecular weight of the protein in SDS-PAGE (Mr=71,000) and the deduced molecular weight (54,581) may be due to the presence of carbohydrate on the mature protein. The presence of three potential N-linked glycosylation sites in the protein supports this speculation. The protein has a potential transmembrane domain consisting of 29-amino acids. The distance from the first basic residue following the putative transmembrane domain to the carboxyl terminus is 12 amino acids. Therefore, we can predict that the protein consists of a large N-terminal extracellular domain and a small C-terminal intracellular domain. Northern blot analysis demonstrated that mRNA of 2.6 kb encoding the 71 kDa protein is expressed only in the testis.

A homology search using NBRF-PIR and SWISS-PROT databases demonstrated that the deduced amino acid sequence of cDNA for the 71 kDa protein of *H. pulcherrimus* is strikingly similar to the 77 kDa

protein of *S. purpuratus* (95.5% identical in 532 amino acids). Both proteins have a relatively high cysteine content (about 5%), located in four cysteine-rich regions with about 100 residues. The sequences of these cysteine-rich regions have a 30-46% homology with the cysteine-rich domains of bovine and human macrophage scavenger receptor type I (Freeman et al., 1990; Kodama et al., 1990; Matumoto et al., 1990). The presence of a cysteine-rich domain has been also reported in the human T- and specialized B-lymphocyte differentiation antigen CD5 (Jones et al., 1986). At the present time, the specific function of the cysteine-rich domains in these proteins has not yet been defined. However, these disulfide crosslinked domains appear to provide stable structures that are well suited for ligand-binding and are readily juxtaposed to other types of domains to permit the construction of complex mosaic proteins (Doolittle, 1985; Krieger, 1986).

During purification of the 71 kDa protein from sperm tails, we learned that the protein was always co-purified with the 220 kDa WGA-binding protein by gel filtration and ion exchange chromatography even in the presence of a detergent like CHAPS. The 71 kDa protein was separated from the 220 kDa protein only after treatment with 2% SDS at 100°C. This suggests that the 71 kDa protein may be tightly associated with the 220 kDa protein. The WGA-binding protein is reported to be involved in the induction of the acrosome reaction through regulating ion fluxes associated with the acrosome reaction (Podell and Vacquier, 1984, 1985; Sendai et al., 1989; Sendai and Aketa, 1989, 1991). SAP-I has also been reported to participate in the induction of the acrosome reaction (Yamaguchi et al., 1988). Thus, SAP-I binding to the 71 kDa protein on a spermatozoon may affect the

regulatory system of ion fluxes induced by the binding of component(s) in the egg jelly to the WGA-binding protein. In a previous paper, we reported that the incubation of *H. pulcherrimus* sperm tails with ^{125}I-GYGG-SAP-I and disuccinimidyl suberate resulted in the radiolabelling of two proteins at 71 kDa and 63 kDa which were identified as diffuse radioactive bands and the radioactivity of the band at 63 kDa was much stronger than that of the band at 71 kDa (Harumi et al., 1991). In the present study, when radioactive fractions obtained from chromatography of CHAPS-solubilized GGGY(^{125}I)-SAP-I-labelled sperm membrane proteins on a DEAE-Sephacel column were analyzed by SDS-PAGE and subsequent autoradiography, a diffuse radioactive band was observed just on the 71 kDa radioactive protein band. Therefore, the molecular weight estimation in the previous study might have been inadequate because of the diffused radioactive bands, but these results imply that GGGY(^{125}I)-SAP-I may crosslink to another minor protein present in sperm membranes. In connection with this, it may be important to mention that the K_d values between the high- and low-affinity receptors are about 40-fold different. Therefore, it is possible that the protein component in the low-affinity receptor is barely crosslinked with the radioiodinated SAP-I analogue. The problem of determining which proteins are the real components of the low- and high-affinity receptor, however, remains to be solved. We have the isolated cDNA clone for the 71 kDa protein which binds SAP-I, so we are now in a position to pursue expression studies which should help to resolve at least part of that problem.

CHAPTER 4 CLONING OF *Hemicentrotus pulcherrimus* SPERM MEMBRANE-BOUND GUANYLATE CYCLASE

SUMMARY

A cDNA clone for the membrane form of guanylate cyclase was isolated from a *Hemicentrotus pulcherrimus* testis cDNA library. An open reading frame predicts a protein of 1125 amino acids including an apparent signal peptide of 21 residues; a single transmembrane domain of 25 amino acids which divides the mature protein into an amino-terminal, extracellular domain of 485 amino acids and a carboxyl-terminal, intracellular domain of 594 amino acids. Three potential N-linked glycosylation sites were present in the proposed extracellular domain. Analysis of poly(A)+ RNA from testes by Northern blot revealed a 4.4 kb RNA.

INTRODUCTION

Guanylate cyclase [GTP pyrophosphate-lysase (cyclizing), EC 4.6.1.2] exists in both soluble and particulate fractions of cells, its distribution varying dependent on the cell type (Mittal and Murad, 1982). Following homogenization of sea urchin spermatozoa, most or all of the enzyme activity are recovered in particulate fractions (Garbers et al., 1974; Garbers, 1976; Gray and Drummond, 1976; Radany et al., 1983). This suggest that guanylate cyclase of sea urchin

spermatozoa is the membrane-bound form. The guanylate cyclases of membrane-bound form are composed of a single transmembrane domain and two clearly defined intracellular regions, a protein kinase-like and a guanylate cyclase catalytic. The sea urchin sperm guanylate cyclase is phosphorylated on the serine residues (Ward and Vacquier, 1983; Ward et al., 1985). The binding of a sperm-activating peptide to sea urchin sperm surface receptors causes a marked and transient increase in guanylate cyclase activity, where the apparent desensitization appears closely linked to the state of phosphorylation of the enzyme. A specific sperm-activating peptide causes a rapid dephosphorylation of the enzyme along with a large decrease in enzyme activity after an initial transient increase in activity (Suzuki et al., 1984; Ramarao and Garbers, 1985). The guanylate cyclase from *Arbacia punctulata* spermatozoa contains up to 17 mol phosphate/mol enzyme, all on serine residues, but after the treatment of the spermatozoa with the specific sperm-activating peptide, SAP-IIA (CVTGAPGCVGGGRL-NH_2), the amount of phosphate associated with the guanylate cyclase falls to less than 2 mol/mol enzyme (Vacquier and Moy, 1986). The specific activity of the phosphorylated form of guanylate cyclase is 5-fold higher than that observed with the dephosphorylated form (Ramarao and Garbers, 1988). Harumi et al (1992) demonstrated that the 131 kDa and 128 kDa forms of guanylate cyclase from *Hemicentrotus pulcherrimus* spermatozoa contained 24 and 4 mol phosphate/mol enzyme, respectively, and the purified phosphorylated form of guanylate cyclase had higher activity than the dephosphorylated form. Ligand-induced dephosphorylation has been shown to be one mechanism of desensitization. These are similar to those reported for the atrial natriuretic peptide receptor/guanylate cyclase, an enzyme that is

known to desensitize in response to atrial natriuretic peptide (Potter and Garbers, 1992). Intracellular domain of the membrane forms of guanylate cyclase are highly conserved between sea urchin and mammalian (Garbers 1992; Garbers and Lowe, 1994). Cloning of another sea urchin guanylate cyclase therefore has been of importance with respect to understanding the regulation of the membrane forms of guanylate cyclase. In this chapter, We describe the guanylate cyclase on *H. pulcherrimus* spermatozoa, which contains 1104 amino acids with a single transmembrane domain.

MATERIALS AND METHODS

Materials

Sea urchins (*H. pulcherrimus*) were collected along the coast of Toyama Bay near the Noto Marine Laboratory. The testes and ovaries were dissected out from the animals as previously described (Suzuki et al., 1982). Composition of artificial sea water (ASW) was 454 mM NaCl, 9.7 mM KCl, 24.9 mM $MgCl_2$, 9.6 mM $CaCl_2$, 27.1 mM $MgSO_4$, 4.4 mM $NaHCO_3$ and 10 mM HEPES, pH 8.2. SAP-I (GFDLNGGGVG) was synthesized for us at the Peptide Institute Inc., Osaka. Sodium dodecyl sulfate (SDS) (95% grade; 69% lauryl sulfate, 26% myristyl sulfate, 5% centyl sulfate) was from Sigma Chemical Co. (St. Louis, MO, USA). 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesufate (CHAPS) was purchased from Dojindo Laboratories (Kumamoto, Japan). Lysyl endopeptidase (*Achromobacter* Protease I) and acetonitrile (ACN) of HPLC grade were obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Concanavalin A-Sepharose was obtained from Pharmacia

LKB Biotechnology (Uppsala, Sweden). Wheat germ agglutinin (WGA) was purified from raw wheat germ (Sigma Chemical Co., St. Louis, MO, USA) by the method of Vretblad (1976) and coupled to Sepharose 4B (Pharmacia LKB Biotechnology, Uppsala, Sweden) using the cyanogen bromide method described by March et al. (1974). Phenylisothiocyanate (PITC) was purchased from Pierce Chemical Co. (Rockford, IL, USA). Restriction enzymes, T4 DNA ligase, and other enzymes for molecular cloning were purchased from Takara Shuzo Co (Kyoto, Japan). The cDNA synthesis kit and Hybond-N membrane filters were obtained from Amersham International plc. (Amersham, UK). The random-primed DNA labeling kit was purchased from Boehringer Mannheim (Indianapolis, IN, USA). The plasmid pBluescript II KS(+), pBluescript II KS(-), and M13KO7 helper phage were generous gifts from Dr. Yoshitaka Nagahama at the National Institute for Basic Biology, Okazaki, Japan. Site-directed antibody against *Strongylocentrotus purpuratus* sperm guanylate cyclase (residue number from 1102 to 1125: KPPPQKLTQEAIEI AANRVIPDDV) was generously gifted by Dr. David L. Garbers at the University of Texas Southwestern Medical Center, Dallas, Texas, USA.

Preparation of RNA

Total RNA was prepared from various samples of *H. pulcherrimus* ovaries, testes, and unfertilized eggs by the LiCl method (Cathala et al., 1983). Poly(A)+RNA was then purified from the total RNA using oligo(dT) cellulose (Pharmacia) column chromatography (Davis et al., 1986).

Cloning and sequencing of cDNAs

A cDNA library (4.9 x 10^5 pfu) from poly(A)+RNA isolated from

growing testes of the sea urchin *H. pulcherrimus* was constructed in λgt10 using the cDNA synthesis system and the cDNA cloning system λgt10 (Amersham). Approximately 7.1 x 10^4 plaques were screened on replicate Hybond-N nylon membranes with ^{32}P-end-labeled, mixed antisense oligonucleotide probes which correspond to part of the extracellular domain (probe II; base numbers from 760 to 803) and the intracellular domain (probe I; base numbers from 2688 to 2732) of the membrane-bound guanylate cyclase of the sea urchin *A. punctulata* (Singh et al., 1988). Probe II (44 mer; 5'-GTCCCATGGATCGAATCCCGCGT AGTACTCCTCGTGCAGAATCT-3') and probe I (45 mer; 5'-CTCCTCCAGATT GTTGGTGTAGCGCTCCATGATGGCGATCATGTT-3') were synthesized on an Applied Biosystem Model 391 nucleotide synthesizer. Plaques transferred onto nylon membranes were hybridized at 37°C overnight with 2 x 10^7 cpm probes in 40 ml of hybridization solution containing 6xNET (1xNET; 50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, pH 7.5), 0.1% SDS, 1xDenhardt's and denatured herring sperm DNA (100 μg/ml). The membranes were washed three times at 20°C with 6xSSC (1xSSC; 150 mM NaCl, 15 mM sodium citrate, pH 7.0) containing 0.1% SDS and then washed at 51°C for 2 min with 3xSSC containing 0.1% SDS. The membranes were subjected to autoradiography. Eighteen initially positive clones were rescreened until pure. Finally six positive clones were obtained and the phage DNA was purified. Digestion of the DNA with Kpn I showed that four of them contained inserts of 4.0 kb and two them contained inserts of 4.3 kb. Restriction mapping showed that two clones with inserts of 4.3 kb were identical.

The cDNA inserts (4.3 kb) of isolated clones were subcloned into the plasmid vector pBluescript II KS(+) (Stratagene). An internal EcoR I site divided this insert into 2.3 kb and 2.0 kb fragments, and the 2.3 kb

fragment hybridized to probe II and the 2.0 kb fragment hybridized to probe I. To obtain overlapped sequence between 2.3 kb and 2.0 kb cDNA fragments, the insert of 4.3 kb in Bluescript was digested by Pst I. By this digestion a 1.5 kb cDNA fragment was cut off and a 2.8 kb fragment remained in Bluescript. The sequencing strategy is shown in Fig. 17. Serial deletion mutants of subclones were made according to Yanisch-Perron et al. (1985). Nucleotide sequences were determined with the 7-DEAZA sequencing kit ver. 2.0 (Takara Biomedicals, Kyoto) and the Sequenase version 2.0 DNA sequencing kit (United States Biochemical) using [α-^{32}P]dCTP and analyzed on DNASIS software (Hitachi Software Engineering Co.).

Northern blot analysis

A 1.6 μg of poly(A)+RNA was denatured with 2.1 M formaldehyde, electrophoresed on 1% agarose gel in the presence of 2.2 M formaldehyde, and transferred onto a Hybond-N membrane. Prehybridization was performed for 2 hrs at 65°C in a solution containing 6xSSC, 0.1% SDS, 5xDenhardt's solution, and denatured herring sperm DNA (100 μg/ml). The RNA on the membrane was then hybridized to the random-primed, [α-^{32}P]dCTP-labeled cDNA insert (base numbers from 1 to 2248 of the λGC4-7-1 cDNA insert) at 65°C for 18 hrs. The membrane was washed with 6xSSC and 0.1% SDS at room temperature for 5 min, then washed finally in 0.5xSSC and 0.1% SDS at 65°C for 30 min. The size of the RNA was estimated using 0.24-9.5 kb RNA Ladder (GIBCO BRL, Gaithersburg, MD. USA) as a marker.

Purification of guanylate cyclase from *H. pulcherrimus* spermatozoa

All manipulations were carried out at 4°C unless other mentioned.

The pellet of sperm (10 g wet weight) was suspended in 200 ml of Solution A (10 mM Tris, 10 mM benzamidine-HCl and 0.5 M NaCl, pH 7.4) containing 1% CHAPS with the aid of a Yamato Ultra Disperser LK-22. The suspension was sonicated in a TOMY Ultrasonic Disruptor UD-201 and then centrifuged at 30,000xg for 30 min. The resulting supernatant fluid was applied to a wheat germ agglutinin (WGA)-agarose column (20 ml) connected to a Concanavalin A-Sepharose column (20 ml), equilibrated with Solution A containing 0.1% CHAPS. After the column was washed with Solution A containing 0.1% CHAPS instead of benzamidine-HCl, the WGA-agarose column was disconnected from the Concanavalin A-Sepharose column. Guanylate cyclase was eluted from Concanavalin A-Sepharose column with 200 ml of Solution B (10 mM Tris, 0.5 M NaCl, 1 M methyl-α-D-mannopyranoside and 0.1 % CHAPS, pH 7.4). Fractions containing 128 kDa protein were pooled, concentrated with an Amicon Stirred Ultrafiltration Cell Model 8400, 8050 or 8010, and then incubated in a final concentration of 2% SDS at 100°C for 5 min. The mixture was subjected to HPLC on a TSK-G4000SW column (7.5 x 300 mm, TOSOH K. K., Tokyo, Japan) equilibrated with 0.1 M sodium phosphate (pH 6.8) containing 0.1% CHAPS using a Hitachi HPLC system L-6000-L-6200 equipped with a L-4200 UV-VIS detector and a D-2500 Chromato-Integrator. The proteins were eluted with the equilibration buffer at a flow rate of 0.5 ml/min at room temperature. The column effluent was monitored for an absorbance at 280 nm and protein concentration.

Proteolytic digestion of guanylate cyclase

The purified guanylate cyclase was digested for 8 hrs at 37°C with lysyl endopeptidase (*Achromobacter* Protease I) in 50 mM Tris-HCl (pH

9.0) containing 0.1% CHAPS at enzyme to substrate ratio of 1:100 (w/w). The peptides generated were separated by HPLC using a Shimadzu Model LC6A chromatography system on a reverse-phase column (Unisil Q C18, 5 μm, 6.0 x 250 mm), which was developed with a linear gradient of 5-60% ACN in 0.1% trifluoroacetic acid (TFA) and then 0-60% ACN in 5 mM sodium-phosphate (pH 5.7) at a flow rate 1 ml/min at 40°C. The column effluent was monitored for absorbance at 225 nm.

Amino acid composition analysis

Purified peptides were hydrolyzed with constant-boiling HCl at 110°C for 20 hrs. The hydrolysate was dried and dissolved 100 μl of coupling solution (ethanol : 0.1 M boric acid buffer (pH 9.0) : PITC, 79:20:1, v/v/v). The mixture incubated at room temperature (20-25°C) for 15 min. After dried, 100 μl of sample buffer (3% ACN in 50 mM sodium phosphate buffer (pH 6.5) containing 50 mM sodium perchlorate) was added and analyzed by HPLC on the reverse-phase column (TSKgel ODS 80 T_M, 5 μm, 4.6 x 150 mm), which was developed with a linear gradient of 3-37.5% ACN in 50 mM sodium phosphate buffer (pH 6.5) containing 50 mM sodium perchlorate for 30 min at a flow rate of 1 ml/min at 40°C. The column effluent was monitored for an absorbance at 254 nm.

Amino acid sequence analysis

Amino acid sequence analysis was performed on an Applied Biosystems Model 476A Pulsed-Liquid Sequencer with an on-line Model 120A Phenylthiohydantoin (PTH) Amino Acid Analyser.

Other methods

For preparation of samples for SDS-polyacrylamide gel electrophoresis (SDS-PAGE), spermatozoa were incubated in ASW (pH 8.0) with or without 2 μM SAP-I. At 5 sec, the reaction was stopped by the addition of trichloroacetic acid (final concentration of 10 %) and the mixture was centrifuged for 30 min at 10,000xg at 0°C. The resultant pellet was suspended in ice-cold 90% (v/v) acetone and then centrifuged. The pellet was resuspended in ice-cold acetone. After being centrifuged, the acetone layer was removed and the pellet was lyophilized. Then, 10% SDS was added to the residue, and the mixture was placed in a boiling water bath for 5 min and vortexed vigorously. This procedure was repeated until the solution became clear. Approximately 10 μg of sperm proteins was added per a lane. SDS-PAGE was carried out essentially as described by Laemmli (1970). The gel was stained with silver by the method of Morrissey (1981). Immunoblotting on a nitrocellulose filter was carried out as described by Towbin et al. (1979). The proteins in the gel were transferred electrophoretically to a nitrocellulose filter using a Multiphor II Nova Blot electrophoretic kit (Pharmacia LKB Biotechnology) at room temperature for 1 hr at 0.8 mA/cm^2 constant current. The filter was blocked with 5% skim milk in Tris-buffered saline (TBS) (137 mM NaCl, 20 mM Tris-HCl, pH 7.6) containing 0.1% Tween 20 for 1 hr at room temperature , soaked in anti-guanylate cyclase rabbit anti-serum diluted 1:500 with TBS containing 0.1% Tween 20, for 1 hr at room temperature and then washed with TBS containing 0.1% Tween 20. The filter was incubated with peroxidase conjugated anti-rabbit IgG (diluted 1:1000 in TBS containing 0.1% Tween 20) for 1 hr at room temperature and washed with TBS containing 0.1% Tween 20. Then

protein that reacted with antibody protein was located by addition of 1% H_2O_2 and 0.05% 3'3'-diaminobenzidine tetrahydrochloride (DAB) in 50 mM Tris-HCl (pH 7.6). Protein was determined by the method of Schacterle and Pollack (1973) using bovine serum albumin as a standard.

RESULTS

In vivo effect of SAP-I on electrophoretic mobility of *H. pulcherrimus* guanylate cyclase

When *H. pulcherrimus* spermatozoa were treated with 2 μM of SAP-I in ASW (pH 8.0), electrophoretic mobility of sperm guanylate cyclase changed from an apparent molecular mass of 131 to 128 kDa in SDS-PAGE under the reduced conditions (Fig. 16).

Sequence of guanylate cyclase

The sequencing strategy for the λGC 4-7-1 is shown in Fig. 17. The complete nucleotide sequence and the deduced amino acid sequence are presented in Fig. 18. The λGC 4-7-1 cDNA insert was 4123 bp in length. The oligonucleotide sequence used for screening the clone was found in the sequence at nucleotide positions 708-751 and 2656-2700, respectively. We have assigned the initiation codon to the ATG at the position 100 because (1) there is an upstream in-frame stop codon; (2) this ATG is flanked by sequences that fit Kozak's criteria for translation initiation codon (Kozak, 1981) and (3) the 21-amino acid sequence following this ATG possesses the features characteristic of signal sequences (Watson, 1984). The initiation codon is followed by an

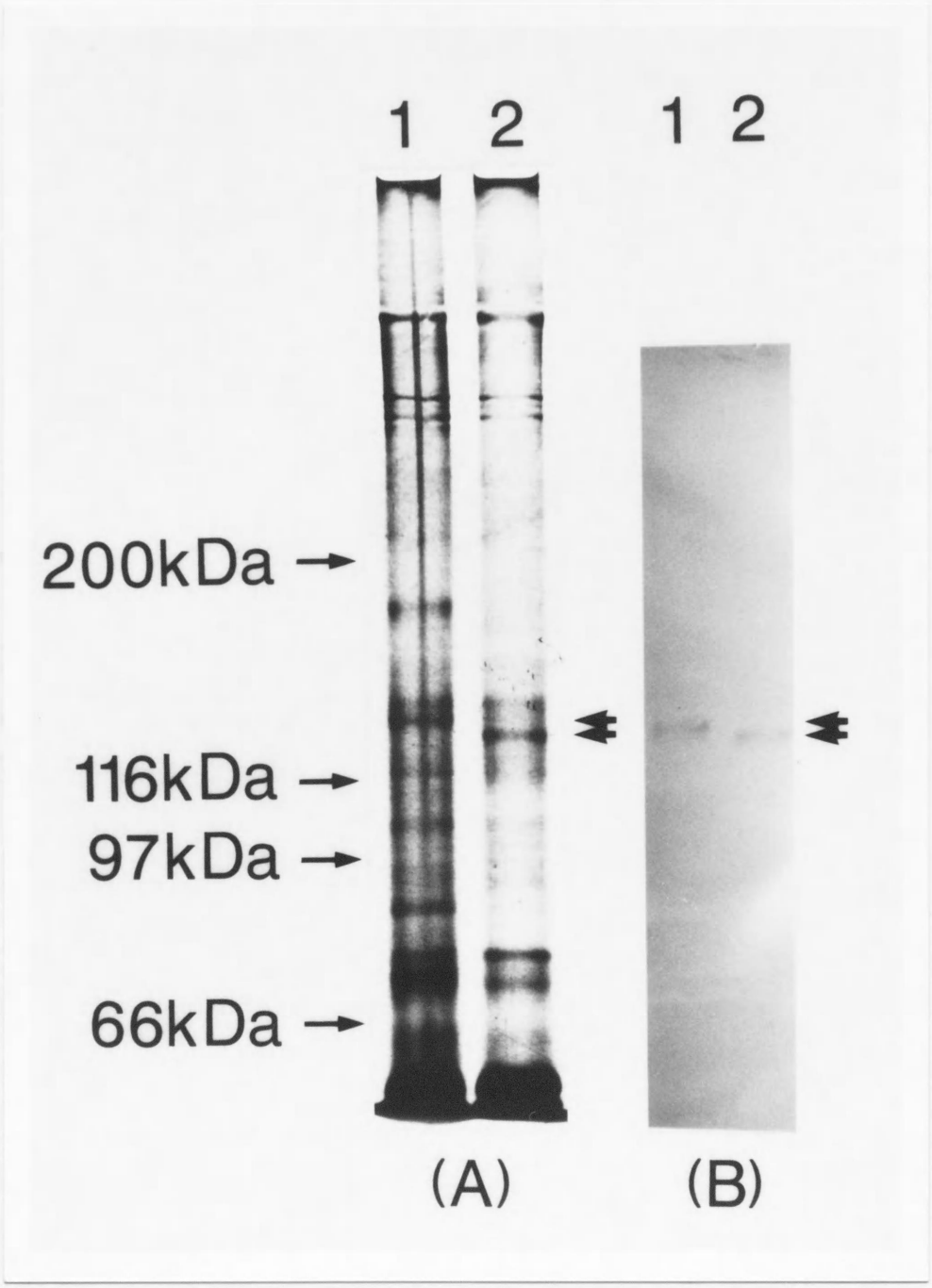

Fig. 16 Immunoblots showing cross-reaction between site-directed antibody against *S. purpuratua* spermatozoa guanylate cyclase and *H. pulcherrimus* spermatozoa (10 μg) (1) and SAP-I treated spermatozoa (10 μg) (2). (A) Silver-staining; (B) Immunoblotting.

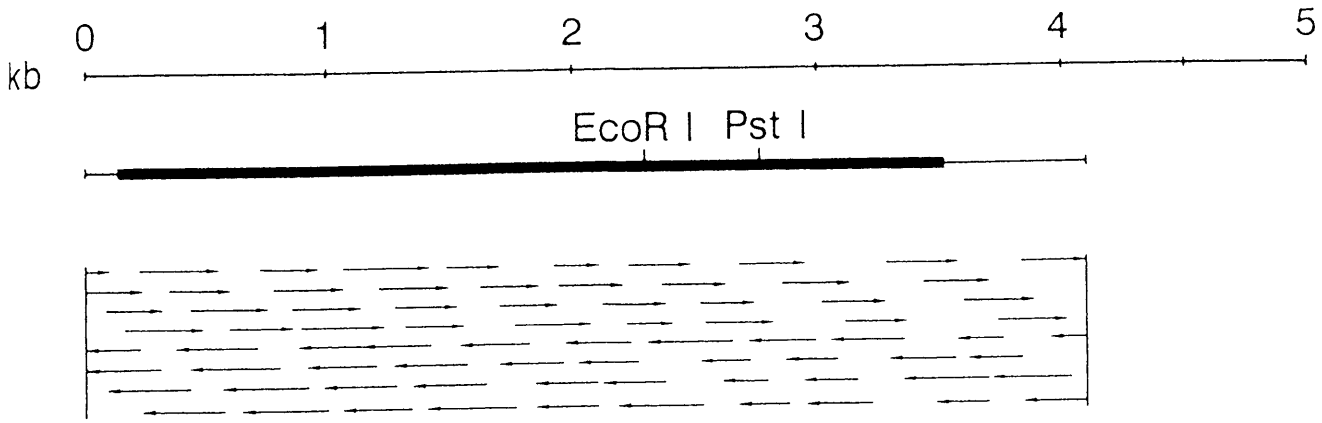

Fig. 17 The restriction endonuclease map and sequencing strategy for a λ GC4-7-1 cDNA insert. The map only shows the relevant restriction site. The direction and extent of the sequence determination are indicated by arrows. The deduced open reading frame is shown by a solid box.

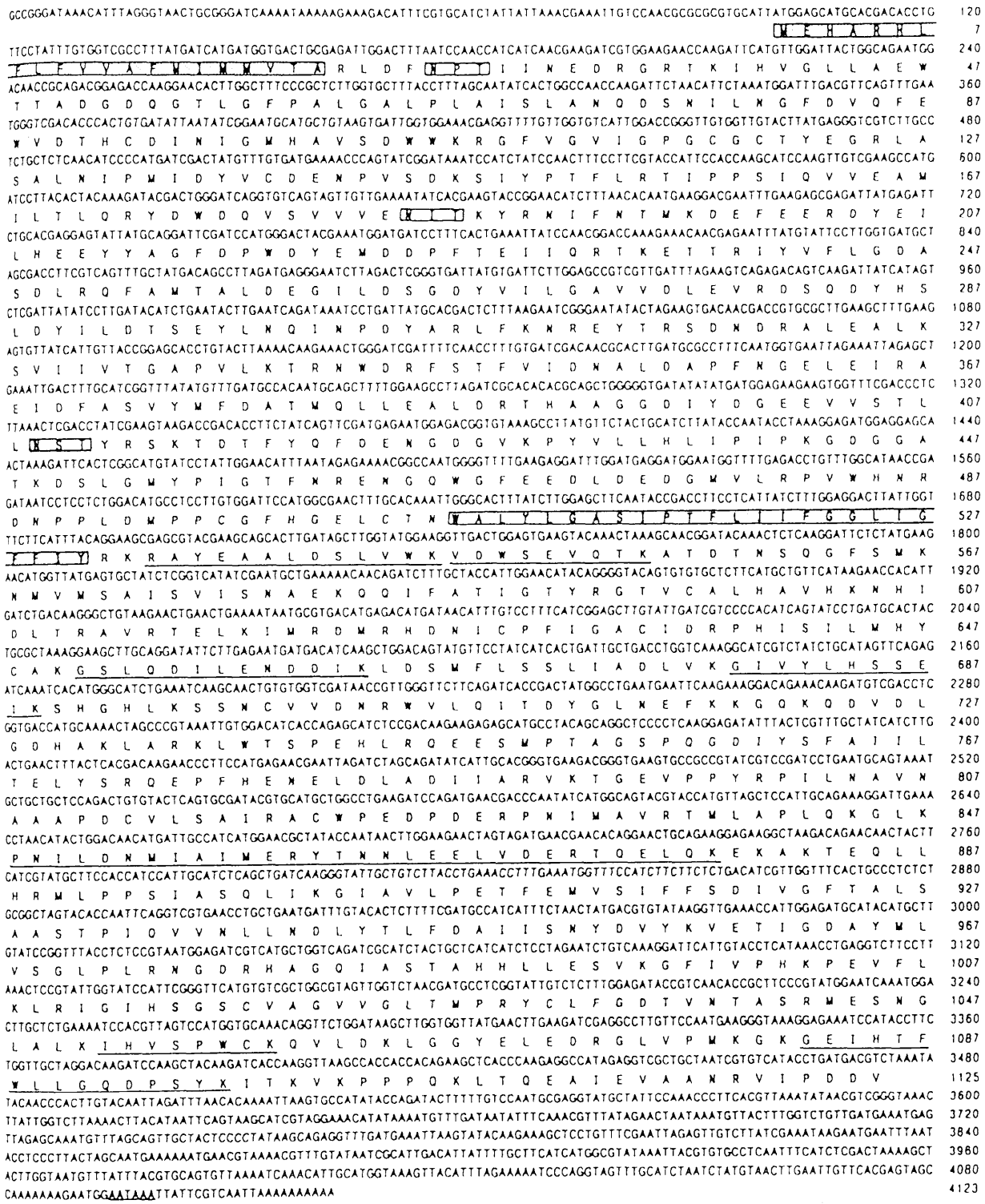

Fig. 18 Complete nucleotide sequence and deduced amino acid sequence of the λGC4-7-1 cDNA insert. Nucleotide and amino acid number are listed on the right hand side. The signal sequence and the putative transmembrane sequence are indicated by shaded-boxes. The potential N-linked carbohydrate binding sites are indicated by open boxes. Polyadenylation signal sequences at the 3'-untranslated region and the predicted amino acid sequence that corresponds to the sequence of the lysyl endopeptidase peptide are underlined.

open reading frame of 3378 bp. An in-frame stop codon occurs at position 3475 and the 3'-untranslated region composed of 646 bp includes polyadenylation sites (AATAAA). The deduced amino acid sequence suggests that cleavage of signal peptide would yield a protein of 1104 amino acids with a calculated molecular weight of 124061.54. The protein contains three potential N-linked glycosylation sites (NXT) at residues 5-7, 164-166 and 409-411 at the mature protein. The protein also contains a hydrophobic region of 25 amino acid at residue 486-510 that is flanked on the carboxyl-terminal side by RKR. These features are typical of membrane-spanning domain of many membrane proteins (Fig. 19).

Northern blot analysis

To determine the size of the mRNA for the λGC 4-7-1 cDNA insert and to see whether the mRNA exists in testes, ovaries or eggs, poly(A)+RNA prepared from these tissues and eggs of *H. pulcherrimus* was analyzed by Northern blot hybridization using a part (base numbers from 1 to 2248 of the λGC4-7-1) of the λGC 4-7-1 cDNA insert as a probe. A strong hybridization signal at the position corresponding to 4.4 kb was detected only with poly(A)+RNA from a testis sample (Fig. 20).

Purification of guanylate cyclase

We purified guanylate cyclase from *H. pulcherrimus* spermatozoa by chromatography on a WGA-Sepharose 4B column connected to a column of Concanavalin A-Sepharose and then by chromatography on a TSK-G4000SW column. Approximately 20 nmol of guanylate cyclase were obtained from an equivalent of 10 g of spermatozoa. The purified

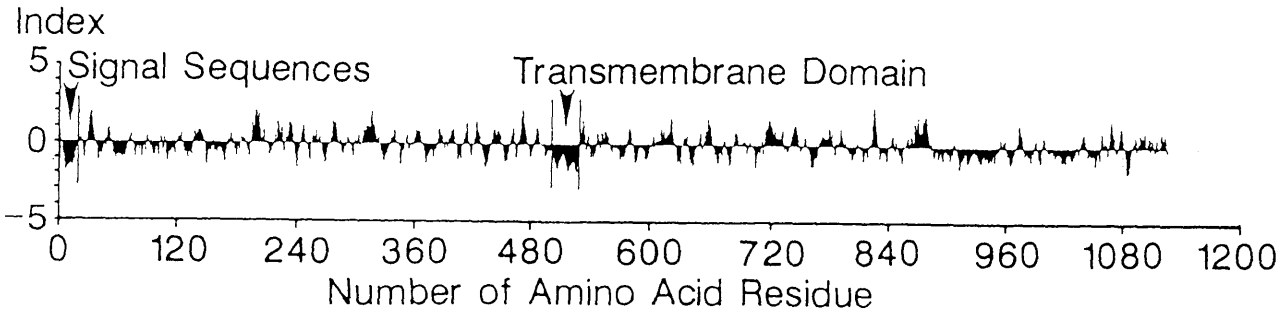

Fig. 19 Hydropathy profile of the protein with 1125 amino acids. Hydropathy was calculated at a window of 12 residues, according to Kyte and Dolittle (1982). Regions with values below the midpoint line are hydrophobic.

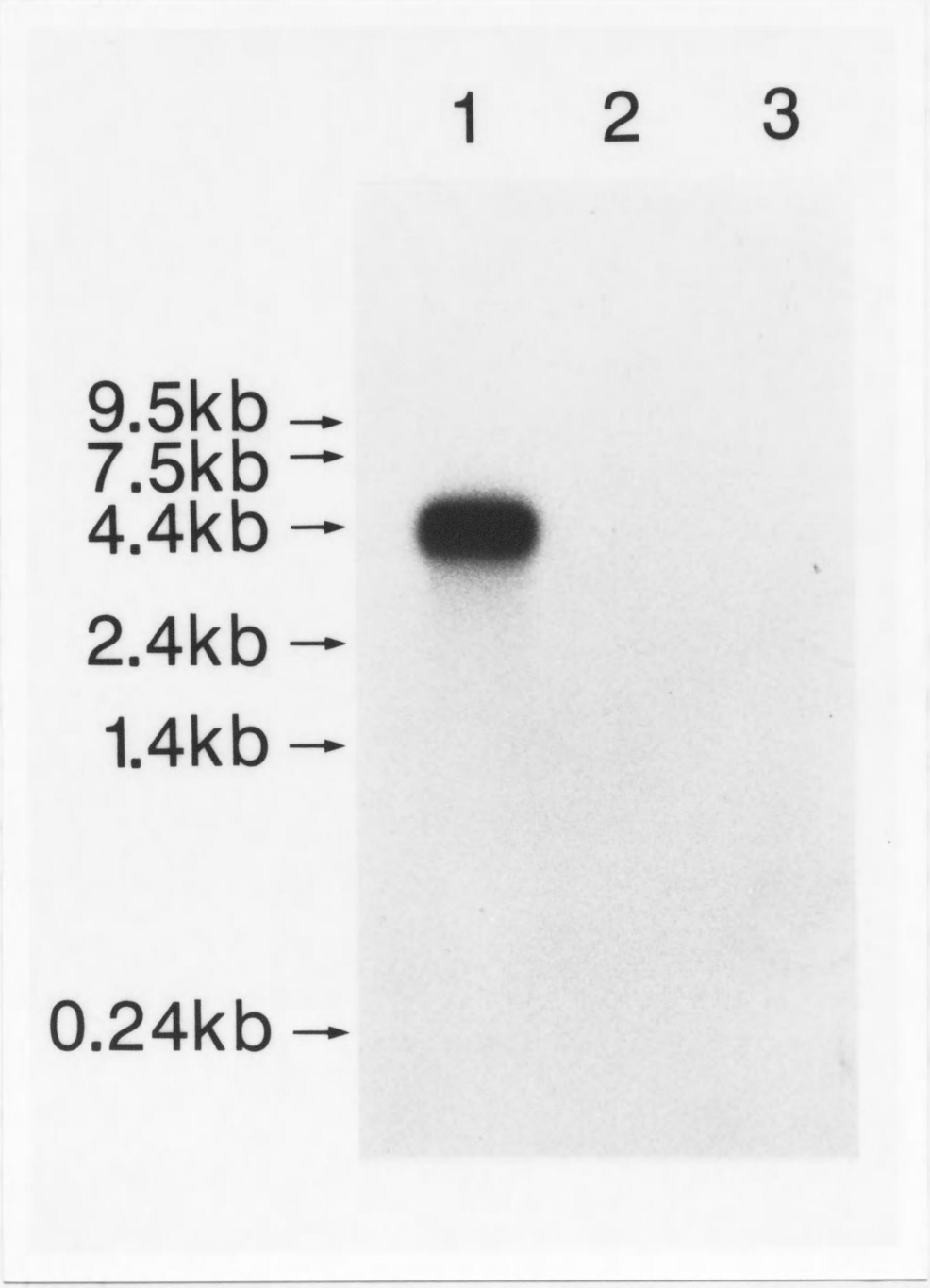

Fig. 20 Northern blot analysis. Poly(A)+RNA prepared from *H. pulcherrimus* testes (1), ovaries (2) or eggs (3) was hybridized to a part (nucleotides 1-2248) of the λGC4-7-1 cDNA insert.

guanylate cyclase was digested with lysyl endopeptidase. The peptides generated were separated by reverse phase HPLC. Seven amino-acid sequences were obtained from lysyl endopeptidase-generated peptides: IHVSPWXK (SS-6), VDWSEVQTK (14-1), GIVYLHSSEIK (20-1-1), GSLQDIL ENDDIK (29-1-1), RAYEAALDSLVWK (32-1-3), GEIHTFWLLGQDPSYK (36-1-1), PNILDNMIAIMERYTNLEELVDERTQELQK (59-1) (Fig. 18).

DISCUSSION

Site-directed antibody against *S. purpuratus* sperm guanylate cyclase recognized both phosphorylated (131 kDa) and dephosphorylated (128 kDa) form of *H. pulcherrimus* sperm guanylate cyclase when tested on immunoblots of spermatozoa from *H. pulcherrimus*. Such a large mobility shift upon dephosphorylation has been considered to be due to the effect of charged phosphate groups on the binding of SDS to protein (Ward and Vacquier, 1983). Therefore, both types of *H. pulcherrimus* guanylate cyclase being recognized by site-directed antibody against *S. purpuratus* sperm guanylate cyclase suggests that both types of *H. pulcherrimus* sperm guanylate cyclase contain the amino acid sequence which is identical or similar to KPPPQKLTQEAIEIAANRVIPDDV. Indeed, deduced amino acid sequence for *H. pulcherrimus* sperm guanylate cyclase contains KPPPQKLTQEAIEVAANRVIPDDV in the C-terminus which is identical sequence from *S. purpuratus* sperm guanylate cyclase (residue number from 1102 to 1125) except underlined valine residue (Thorpe and Garbers, 1989). As with guanylate cyclase from *H. pulcherrimus* spermatozoa, the loss of protein phosphates is correlated with a decrease in enzymatic activity and an increase in mobility on

SDS-PAGE (Harumi et al., 1992). A model for the sea urchin guanylate cyclase is that the binding of ligand activates the cyclase; either in response to a protein phosphatase activation or to a its conformational change, or both, the guanylate cyclase is then desensitized by dephosphorylation. The protein phosphatase activity of sea urchin sperm is known not to increase upon SAP-I treatment (Swarup and Garbers, 1982). However, the possibility that guanylate cyclase may be desensitized by dephosphorylation in response to activation of a protein phosphatase has been not excluded.

A homology search using NBRF-PIR and SWISS-PROT databases demonstrated that the deduced amino acid sequence of cDNA for the guanylate cyclase of *H. pulcherrimus* spermatozoa is similar to that of guanylate cyclase of *S. purpuratus* (98% identical in 1125 amino acids) (Thorpe and Garbers, 1989) and *A. punctulata* (77% identical in 926 amino acids) (Singh et al., 1988). The predicted primary structure of guanylate cyclase from *H. pulcherrimus* bears virtually no resemblance to mammalian plasma membrane forms of guanylate cyclase in the putative extracellular domain. Within intracellular regions, however, all of the membrane forms of guanylate cyclase isolated possess both a protein kinase-like domain and a cyclase catalytic domain (Garbers and Lowe, 1994; Yang et al., 1995). The protein kinase-like domain contains a majority of the conserved amino acids identified by Hanks et al. (1988) as conserved or invariant within the catalytic domain of protein kinases. The function of the protein kinase-like domain has been studied principally in mammalian guanylate cyclase where ATP is known to potentiate or be required for transduction of the ligand binding signal to activation of the cyclase catalytic domain (Chinkers et al., 1991). There are no reports of protein kinase activity associated

with membrane form of guanylate cyclases. Recently, a novel protein phosphatase that binds to the protein kinase-like domain of mammalian guanylate cyclase *in vitro* was discovered using the yeast two-hybrid system (Chinkers, 1994). These results suggest that it is possible that protein kinase-like domains of sea urchin and mammalian guanylate cyclase are phosphorylated in unbound state.

The predicted molecular weight of 124061 is comparable to the mass of 128000 for the dephosphorylated form of guanylate cyclase. The apparent difference in mass may be attributable to glycosylation; at least one of three potential N-linked glycosylation sites may be glycosylated since guanylate cyclase bound strongly to Con A-Sepharose Concanavalin A has been reported to recognize a specific glycosyl chain structure, Manα1-6(Manα1-3)Man, in which at least one hydroxyl group at the C-3 position of C-6-linked should be free (Ohyama et al., 1985).

Northern blotting of *H. pulcherrimus* testes, ovaries and eggs using the cDNA encoding *H. pulcherrimus* guanylate cyclase as a probe indicated that a 4.4 kb mRNA was expressed at high levels in only testes. This distribution parallels that of the sperm-activating peptide I (SAP-I)- crosslinked protein (Shimizu et al., 1994). The abundance of this mRNA suggests that the levels of its protein product may be equal to those of the SAP-I-crosslinked protein; if so, SAP-I-crosslinked protein may be SAP-I receptor.

CHAPTER 5 SUMMARY AND CONCLUSION

The sea urchin is surrounded by an extracellular investment known as the jelly layer. The major macromolecular component of the jelly layer is a high molecular weight fucose sulfate glycoconjugate (FSG) that induces the acrosome reaction. Sperm-activating peptides also have been isolated from the jelly layer. In present study, we examined the effect of FSG and SAP-I on the pH_i and $[Ca^{2+}]_i$ of spermatozoa of the sea urchin *Hemicentrotus pulcherrimus*. FSG and/or SAP-I induced elevations of pH_i and $[Ca^{2+}]_i$ in the spermatozoa at pH 8.0. At pH 6.6, FSG did not induce any significant change in $[Ca^{2+}]_i$ nor pH_i. At the same pH, however, SAP-I induced a large increase in pH_i. Three-times repeated addition of a low concentration of SAP-I (final, 0.59 nM) to spermatozoa at pH 6.6 induced subsequent increases in pH_i. However, addition of a high concentration of SAP-I (final, 0.59 μM) induced only one time increase in pH_i. At pH 8.0, a second addition of FSG did not induce further elevation of the $[Ca^{2+}]_i$ or pH_i of spermatozoa treated with FSG, but addition of FSG after SAP-I or of SAP-I after FSG induced further increases of $[Ca^{2+}]_i$ and pH_i. TEA, a voltage-sensitive K^+-channel blocker, partially inhibited the elevation of $[Ca^{2+}]_i$ in spermatozoa treated with FSG, but did not appear to inhibit the elevation of $[Ca^{2+}]_i$ by SAP-I. It also inhibited the FSG-induced acrosome reaction and increase in the level of intracellular cyclic AMP. These results suggest that FSG receptor and SAP-I receptor on the spermatozoa are different and at least there are two types of K^+-channel on the spermatozoa and one of them is a voltage-sensitive channel.

Analysis of the data obtained from the equilibrium binding of a radioiodinated SAP-I analogue [GGGY(^{125}I)-SAP-I] to *H. pulcherrimus* spermatozoa showed the presence of two classes of receptors specific for SAP-I in the spermatozoa. We estimated that the maximal number of binding sites for each spermatozoon was calculated to be 7.0×10^4 for the high-affinity receptor and 12.1×10^4 for the low-affinity receptor, and the Kd values were estimated to be 0.58-0.69 nM for the high-affinity receptors and 23-25 nM for the low-affinity receptors. Radioiodinated SAP-I analogue was cross-linked to a 71 kDa protein in *H. pulcherrimus* intact spermatozoa, sperm tail and sperm membrane in the presence of disuccinimidyl suberate. The covalent coupling was prevented by excess SAP-I but not by excess SAP-III which did not show any biological effect on *H. pulcherrimus* spermatozoa. This result suggests that the radioiodinated SAP-I analogue binds to the 71 kDa protein. We isolated a cDNA clone encoding the 71 kDa crosslinked protein from a *H. pulcherrimus* testis cDNA library. The cDNA insert was 2443 bp in length. An open reading frame predicts a protein of 532 amino acids including a signal peptide of 30 residues. A single transmembrane domain of 29 amino acids divided the mature protein into an amino-terminal, extracellular domain of 461 amino acids and a carboxyl domain of 12 intracellular amino acids. Three potential N-linked glycosylation sites were present in the proposed extracellular domain. The deduced protein sequence represents a domain that is very similar to protein superfamily defined by the macrophage scavenger receptor cysteine-rich domain. mRNA for the 71 kDa crosslinked protein was detected only in the testis. The protein appears to be associated with a 220 kDa wheat germ agglutinin (WGA)-binding protein.

Using site-directed antibody against *S. purpuratus* sperm guanylate cyclase, we demonstrated that SAP-I causes an electrophoretic mobility shift of *H. pulcherrimus* sperm guanylate cyclase from 131 kDa to 128 kDa. We isolated a cDNA clone encoding guanylate cyclase from a *H. pulcherrimus* testis cDNA library. The isolated guanylate cyclase cDNA is 4123 bp long and encodes a distinctive mature protein of 1104 amino acids with a predicted molecular mass of 128 kDa (dephosphorylated from). A putative transmembrane domain of 25 amino acids partitions the protein into a 485 amino acids extracellular domain, containing three consensus sequences for N-linked glycosylation and intracellular domain of 594 amino acids, consists of kinase-like domain and catalytic domain. Northern blot analysis demonstrated that mRNA of 4.4 kb encoding the guanylate cyclase was expressed only in the testis.

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Differential Effects of the Egg Jelly Molecules FSG and SAP-I on Elevation of Intracellular Ca^{2+} and pH in Sea Urchin Spermatozoa

(FSG/SAP-I/$[Ca^{2+}]_i$/pH_i)

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We examined the effects of two egg jelly components, a fucose sulfate glycoconjugate (FSG) and sperm-activating peptide I (SAP-I: Gly-Phe-Asp-Leu-Asn-Gly-Gly-Gly-Val-Gly), on the intracellular pH (pH_i) and Ca^{2+} ($[Ca^{2+}]_i$) of spermatozoa of the sea urchin *Hemicentrotus pulcherrimus*. FSG and/or SAP-I induced elevations of $[Ca^{2+}]_i$ and pH_i in the spermatozoa at pH 8.0. At pH 8.0, a second addition of FSG did not induced further elevation of the $[Ca^{2+}]_i$ or pH_i of spermatozoa treated with FSG, but addition of FSG after SAP-I or of SAP-I after FSG induced further increases of $[Ca^{2+}]_i$ and pH_i. At pH 6.6, FSG and/or SAP-I did not induce significant elevation of the $[Ca^{2+}]_i$, although SAP-I elevated the pH_i, its half-maximal effective concentration being 10 to 100 pM. At pH 8.0, tetraethyl-ammonium, a voltage-sensitive K^+-channel blocker, inhibited induction of the acrosome reaction and elevations of $[Ca^{2+}]_i$ and pH_i by FSG, but did not affect those by SAP-I. These results suggest that FSG and SAP-I activate different Ca^{2+} and H^+ transport systems.

Introduction

Sea urchin eggs are surrounded by a gelatinous matrix that had been shown to induce an acrosome reaction in spermatozoa (2, 3). The acrosome reaction is accompanied by influxes of Ca^{2+} and Na^+, and effluxes of K^+ and H^+ (25, 26). These ionic movements lead to intracellular increases in both pH (pH_i) (27) and $[Ca^{2+}]$ ($[Ca^{2+}]_i$) (40), and to depolarization of the plasma membrane (27). All these changes as well as the acrosome reaction are inhibited by Ca^{2+}-channel antagonists such as verapamil, dihydropyridines such as nitrendipine and nisoldipine (7, 15), the K^+-channel blocker tetraethyl-ammonium chloride (TEA) (25), and high K^+ seawater (7). The extracellular matrix of sea urchin eggs is mainly composed of a fucose sulfate glycoconjugate (FSG), a sialoglycoprotein and sperm-activating peptides (SAPs) (8, 13, 14, 30, 32, 34, 36). FSG induces increases in $[Ca^{2+}]_i$ (16, 31), elevation of the intra-cellular cAMP level, and the acrosome reaction in sea urchin spermatozoa (9, 16). SAPs have many biological effects on the spermatozoa such as stimulations of respiration and motility in slightly acidic seawater (8, 34), inductions of transient elevation of the cGMP level (8), increases in pH_i (23) and $[Ca^{2+}]_i$ (29), and plasma membrane hyperpolarization through activation of Na^+/H^+ and K^+/H^+ exchanges across the plasma membrane (21). SAP-I (Gly-Phe-Asp-Leu-Asn-Gly-Gly-Gly-Val-Gly) acts as a specific co-factor for induction of the acrosome reaction by FSG (40). Before SAPs induce these biochemical and physiological events in spermatozoa, they seem to bind to specific receptors on the spermatozoa. There have been several studies on the SAP receptors using radioiodinated SAP analogues (1, 4, 5, 6, 12, 33). A receptor that is apparently specific for SAP-IIa (Cys-Val-Thr-Gly-Ala-Pro-Gly-Cys-Val-Gly-Gly-Gly-Arg-Leu-NH_2) (35, 42) has been identified as a guanylate cyclase in spermatozoa of the sea urchin *Arbacia punctulata* (1, 39). SAP-I is specifically cross-linked to a 77 kDa protein with no known enzyme activity in *Strongylocentrotus purpuratus* spermatozoa (4, 5, 6). Previously, we reported that SAP-I cross-links to 63 kDa and 71 kDa proteins in *Hemicentrotus pulcherrimus* spermatozoa (12). In both cases, the bindings of SAPs to these proteins induced transient activation of the membrane-bound guanylate cyclase (1, 4, 12). We purified FSG from *H. pulcherrimus* egg jelly (32) and we tried to identify the FSG binding protein on spermatozoa using radioiodinated FSG, but without success: we found that binding and cross-linking experiments with radioiodinated FSG

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were not possible because the radioiodinated FSG binds non-specifically to spermatozoa, test tubes and even the glass-filters used in the experiments. We imagine that the FSG of *H. pulcherrimus* is not exceptional. As far as we know, little is yet known about the site or mechanism of binding of purified FSG to sea urchin spermatozoa of any species. In the present study, therefore, we used an indirect approach to determine whether FSG binds to the spermatozoa and whether its binding site is the same as that of SAP-I. Transiently permeant fluorescent indicators of Ca^{2+} (10) and pH (24) have been shown to be useful to study changes of $[Ca^{2+}]_i$ and pH_i on treatment of sea urchin spermatozoa with crude sea urchin egg jelly. Using these indicators, we examined the changes of $[Ca^{2+}]_i$ and pH_i in *H. pulcherrimus* spermatozoa upon treatment with FSG and/or SAP-I. The results obtained here suggest that the binding sites of FSG and SAP-I on spermatozoa are different.

Materials and Methods

Materials: Sea urchins, *H. pulcherrimus*, were collected along the coast near Noto Marine Laboratory. Sea urchin gametes were obtained by intracoelomic injection of 0.5 M KCl. Spermatozoa were collected as "dry sperm" at room temperature and stored on ice until use. Eggs were collected in filtered seawater. The artificial seawater (ASW) used for determination of $[Ca^{2+}]_i$ and pH_i contained 430 mM NaCl, 10 mM tris(hydroxymethyl)amino-methane (Tris) at pH 8.0 or 10 mM N-(2-acetamide)-2-aminoethane-sulfonic acid (ACES) at pH 6.6. The ASW used for determination of the rates of the acrosome reaction and cyclic nucleotide concentrations contained 454 mM NaCl, 9.7 mM KCl, 24.9 mM $MgCl_2$, 9.6 mM $CaCl_2$, 27.1 mM $MgSO_4$, 4.4 mM $NaHCO_3$ and 10 mM N-2-hydroxyethyl-piperazine-N'-ethanesulfonic acid (HEPES) (pH 8.2). In high $[K^+]$ seawater which was buffered at pH 8.2 with 10 mM HEPES, Na^+ was partially replaced by K^+ keeping $[Na^+]$ plus $[K^+]$ equal to 463.7 mM. Solubilization of egg jelly and purification of FSG were carried out as reported previously (32). SAP-I was synthesized at the Peptide Institute Inc., Osaka, Japan. The pentaacetoxymethylester of Fura 2 (Fura 2/AM) and 2',7'-bis(carboxyethyl)-carboxyfluorescein tetraacetoxymethylester (BCECF/AM) were obtained from Dojindo Laboratories, Japan. All other reagents used were of analytical grade.

Measurement of $[Ca^{2+}]_i$: $[Ca^{2+}]_i$ was determined by the method of Schackmann and Chock (29) with slight modifications using the Ca^{2+} indicator Fura 2/AM. A stock solution of Fura 2/AM (1 mM Fura 2/AM in DMSO) was added to 10-fold diluted dry sperm to give a final concentration of 3 μM in Ca^{2+}-free ASW (CaFASW), consisting of 430 mM NaCl, 10 mM KCl, 23 mM $MgCl_2$, 25 mM $MgSO_4$, 1 mM EGTA and 10 mM 2(N-morpholino)ethanesulfonic acid (MES) at pH 6.8. The sperm suspension was incubated at 4°C for 6 hr in the dark room and then centrifuged at 3,000 × g for 5 min at 4°C. The resulting sperm pellet was washed twice with CaFASW and suspended in CaFASW to give 10-fold dilution of the dry sperm. A volume of 100 μl of Fura 2/AM-loaded spermatozoa was added to 1.8 ml of ASW, and the fluorescence was monitored at 500 nm in a Shimadzu RF-5000 fluorescence spectrophotometer at 20°C with excitations at 345 nm and 380 nm. When HEPES-NaOH (pH 6.8) was used as a buffer for Fura 2/AM-loading in ASW, the spermatozoa aggregated, so, we used MES as a buffer for incubation of spermatozoa with Fura 2/AM. $[Ca^{2+}]_i$ was determined by the ratio method of Trimmer *et al.* (38). $[Ca^{2+}]_i$ was expressed as $[Ca^{2+}]_i/Kd$ where Kd is the Fura 2/Ca^{2+} dissociation constant which is reported to be 770 nM (22).

Measurement of pH_i: pH_i was measured essentially as the procedure described by Trimmer *et al.* (37). Spermatozoa (10-fold-diluted dry sperm in CaFASW) were incubated with BCECF/AM (1 mM in DMSO) at a final concentration of 3 μM at 4°C for 3 hr. Then the spermatozoa were washed twice with CaFASW and resuspended in CaFASW at 10-fold dilution of the dry sperm. The sperm suspension was added to 1.8 ml ASW, and the fluorescence was monitored at 530 nm at 20°C with a Shimadzu RF-5000 fluorescence spectrophotometer with excitation at 500 nm. The intracellular pH was estimated from a standard curve generated by varying the extracellular pH in the presence of 50 μM nigericin and monensin, as reported by Trimmer *et al.* (38).

Other methods: The rate of the acrosome reaction was determined as described previously (40). Sperm cAMP and cGMP concentrations were determined by radioimmunoassay using cAMP and cGMP assay kits (Yamasa Shoyu, Chiba, Japan) as described previously (41).

Results

Effects of egg jelly, SAP-I and FSG on pH_i and $[Ca^{2+}]_i$

As shown in Fig. 1, the solubilized egg jelly induced rapid increases in pH_i and $[Ca^{2+}]_i$ of spermatozoa at pH 8.0. The increased pH_i returned to the basal value within 1 min. The $[Ca^{2+}]_i$ change peaked at around 5 sec, and a large relaxation occurred within 1 min after addition of egg jelly, but the $[Ca^{2+}]_i$ did not return to the basal value within the experimental period. We found that sialoglycoprotein purified from *H. pulcherrimus* egg jelly did not affect either the pH_i or $[Ca^{2+}]_i$ (data not shown). Therefore, we next examined the effects of two other egg jelly molecules, SAP-I and FSG, on pH_i and $[Ca^{2+}]_i$ at pH 8.0 and pH 6.6. SAP-I and FSG both induced a large elevation of $[Ca^{2+}]_i$ at pH 8.0, but no significant change in $[Ca^{2+}]_i$ at pH 6.6. SAP-I induced a large increase in pH_i at pH 6.6. Both SAP-I and FSG induced a small, but significant increase in pH_i at pH 8.0. However, we did not detect any significant increase in pH_i due to FSG at pH 6.6. In addition, FSG and SAP-I did not induce elevation in $[Ca^{2+}]_i$ or pH_i in ASW containing 100 mM KCl at pH 8.0.

SAP-I dose-dependent changes in pH_i and $[Ca^{2+}]_i$

The increases in pH_i and $[Ca^{2+}]_i$ by SAP-I were dose-dependent. Fig. 2 shows the relationships of the concentrations of SAP-I, pH_i, $[Ca^{2+}]_i$ and external pH. The net increase in pH_i by SAP-I at pH 6.6 was larger than that at pH 8.0.

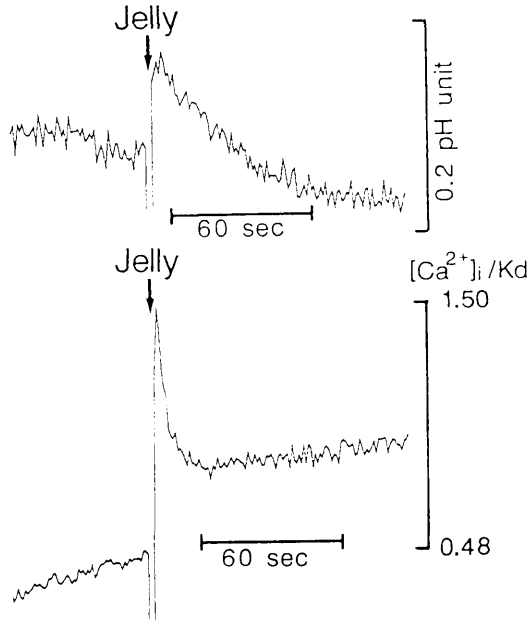

Fig. 1. Effects of egg jelly on pH_i and $[Ca^{2+}]_i$. BCECF/AM (A)- or Fura 2/AM (B)-loaded spermatozoa were resuspended in ASW (pH 8.0) 1 min before the recording was started. At the times indicated by arrows, egg jelly (final, 1.24 nmol fucose/ml) was added to the sperm suspension. The record is representative of those for four different batches of spermatozoa.

The half-maximal increase in pH_i at pH 6.6 was observed with between 10 and 100 pM of SAP-I, which was comparable to the effective concentration range of SAP-I for stimulation of respiration at pH 6.6. However, the increase in pH_i by SAP-I was less at pH 6.6 than at pH 8.0. SAP-I did not elevate $[Ca^{2+}]_i$ at pH 6.6, but at pH 8.0 it elevated

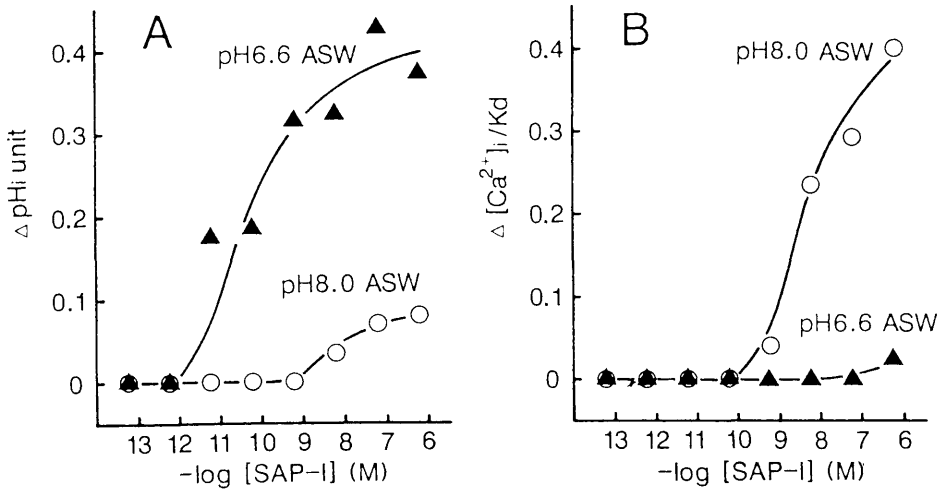

Fig. 2. SAP-I dose-dependencies of net changes in pH_i (A) and $[Ca^{2+}]_i$ (B). SAP-I induced elevations of pH_i and $[Ca^{2+}]_i$ with peak values about 4–8 sec after its addition to BCECF/AM- or Fura 2/AM-loaded spermatozoa. All values used for calculations of Δ pH_i and Δ $[Ca^{2+}]_i$ were taken at the peaks. The same batch of spermatozoa was used for all experiments.

$[Ca^{2+}]_i$ markedly at between 1 and 10 nM. This concentration range was similar to that for its effect in increasing cGMP (36).

Three repeated addition of a low concentration of SAP-I (final, 0.59 nM) to spermatozoa at pH 6.6 repeatedly induced increases in pH_i, although the extents of these increases tended to decrease progressively (Fig. 3A). However, on repeated addition of a higher concentration of SAP-I (final, 0.59 μM) to spermatozoa, only the first addition induced an increase in pH_i (Fig. 3B).

$[Ca^{2+}]_i$-accumulation in spermatozoa induced by SAP-I and FSG

Similar results to those for pH_i were obtained for $[Ca^{2+}]_i$ on repeated additions of SAP-I. Fig. 4A shows that after sperm $[Ca^{2+}]_i$ had been increased by a first addition of SAP-I (final, 0.59 μM), a second addition of the same concentration of SAP-I did not result in further elevation of $[Ca^{2+}]_i$. Fig. 4A also demonstrates that FSG elevated the $[Ca^{2+}]_i$ further after its increase induced by SAP-I. When FSG was added to spermatozoa twice, the second addition did not induce further elevation of the $[Ca^{2+}]_i$, but subsequent addition of SAP-I did (Fig. 4B). After addition of SAP-I and then FSG or FSG and then SAP-I, egg jelly did not induce further elevation of the $[Ca^{2+}]_i$ (Fig. 5).

SAP-I- or ESG-induced $[Ca^{2+}]_i$-accumulation in the presence of 3-isobutyl-1-methylxanthine (IBMX)

IBMX, a cyclic nucleotide phosphodiesterase inhibitor, has been reported to increase $^{45}Ca^{2+}$ influx and the level of cAMP, and induce the acrosome reaction in abalone spermatozoa (17). It also increases the cAMP and cGMP levels in spermatozoa of *S. purpuratus* and *H. pulcherrimus* in conjunction with SAP-I (11, 41). Fig. 6 shows that addition of IBMX to *H. pulcherrimus* spermatozoa at pH 8.0 induced elevation of $[Ca^{2+}]_i$ and subsequent addition of SAP-I or FSG induced further increase of $[Ca^{2+}]_i$. The nontransient elevation in $[Ca^{2+}]_i$ by IBMX was similar to that initiated by FSG.

Effects of TEA on the elevation of $[Ca^{2+}]_i$, the acrosome reaction and cyclic nucleotide levels in spermatozoa treated with FSG or FSG plus SAP-I

TEA, a voltage-sensitive K^+-channel blocker,

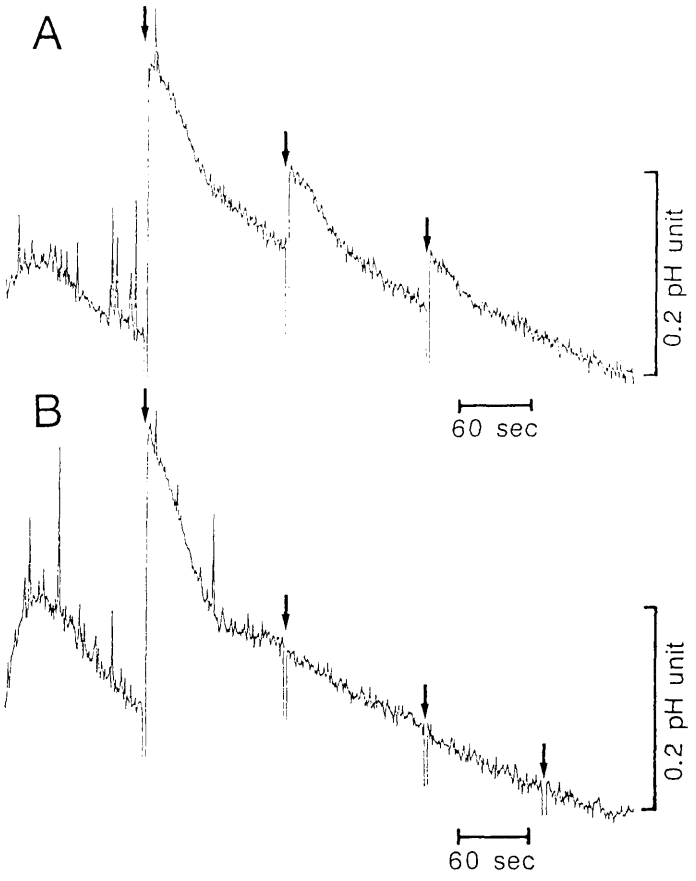

Fig. 3. Effect of SAP-I on pH_i. At the times indicated by arrows, the same amounts of SAP-I were added to BCECF/AM-loaded spermatozoa at pH 6.6. The final concentration of SAP-I was 0.59 nM for A and 0.59 μM for B. The same batch of spermatozoa was used for these experiments.

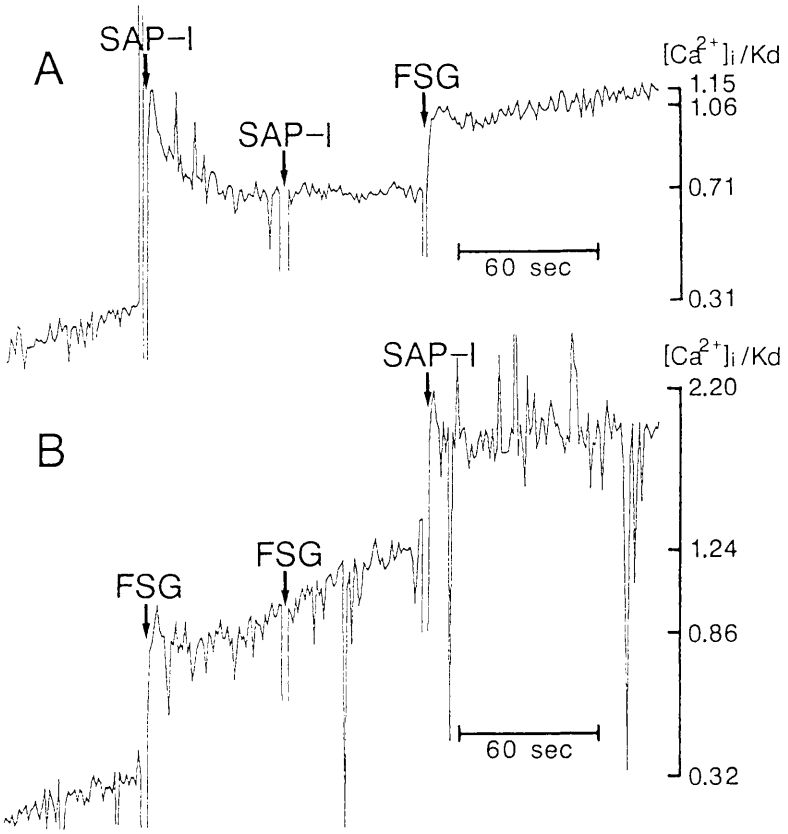

Fig. 4. Ca^{2+}-accumulation induced by SAP-I or FSG. At the times indicated by arrows, SAP-I (final, 0.59 μM) or FSG (final, 50 nmol fucose/ml) was added to Fura 2/AM-loaded spermatozoa at pH 8.0. The same batch of spermatozoa was used for both experiments.

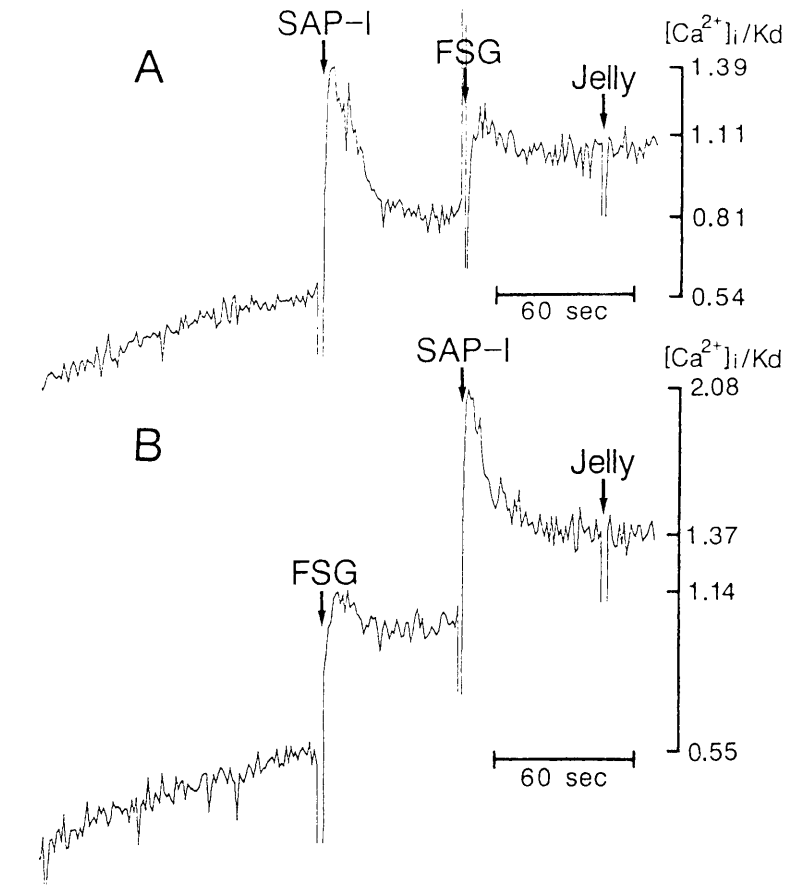

Fig. 5. Elevations of $[Ca^{2+}]_i$ induced by SAP-I, FSG and egg jelly. At the times indicated by arrows, SAP-I (final, 0.59 μM), FSG (final, 50 nmol fucose/ml) or egg jelly (final, 1.24 nmol fucose/ml) was added to Fura 2/AM-loaded spermatozoa at pH 8.0.

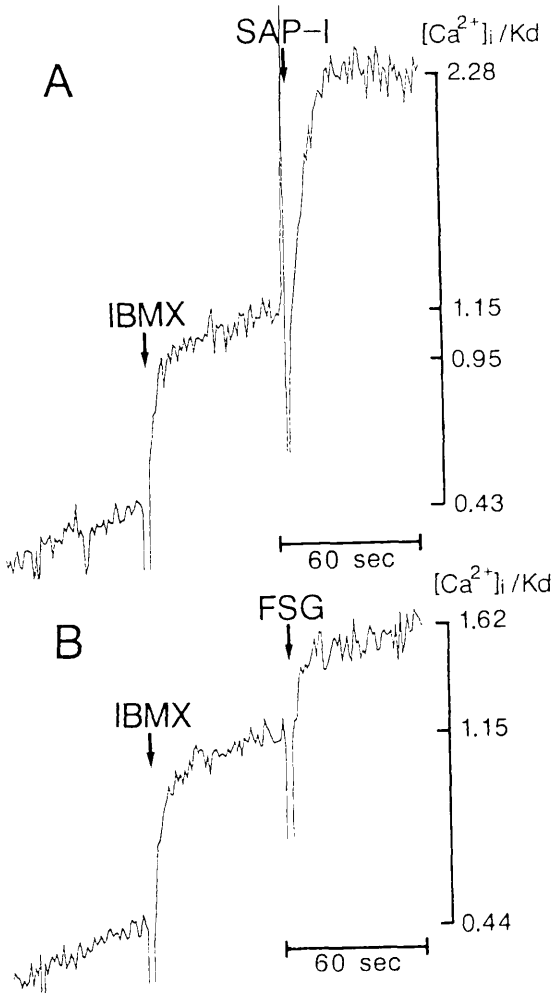

Fig. 6. Ca^{2+}-accumulations induced by IBMX, SAP-I, and FSG. The final concentrations of IBMX, SAP-I, and FSG added to Fura 2/AM-loaded spermatozoa at pH 8.0 at the times indicated by arrows were 300 μM, 0.59 μM, and 50 nmol fucose/ml, respectively. The same batch of spermatozoa was used for both experiments.

inhibits the inductions of both $^{45}Ca^{2+}$ influx and the acrosome reaction in *S. purpuratus* spermatozoa by egg jelly (25). As shown in Fig. 7, TEA partially inhibited the elevation of $[Ca^{2+}]_i$ in *H. pulcherrimus* spermatozoa treated with FSG, but did not appear to inhibit the elevation of $[Ca^{2+}]_i$ by SAP-I. TEA inhibited the increase in pH_i by FSG. It also inhibited the FSG-induced acrosome reaction and increase in the level of intracellular cAMP (Table 1). However, its inhibition of induction of the acrosome reaction was partial in the presence of SAP-I (Table 1). In this connection, it should be noted that high $[K^+]$ seawater inhibited induction of the acrosome reaction by FSG plus SAP-I (Table 1).

Table 1. Percentages of *H. pulcherrimus* spermatozoa showing the acrosome reaction and cyclic nucleotide concentrations of the spermatozoa after treatment with FSG or FSG plus SAP-I in ASW, ASW containing TEA or high K^+-ASW

	Acrosome reaction	cAMP[1]	cGMP[2]
ASW alone	4%	2.56±0.69	60± 10
+FSG	69	14.08±2.26	80± 10
+FSG+SAP-I	93	18.38±1.21	840±110
ASW with TEA	2	1.48±0.29	59± 10
+FSG	5	1.59±0.13	60± 10
+FSG+SAP-I	47	2.74±0.66	640± 80
High K^+-ASW	0	0.16±0.02	290±110
+FSG	6	0.22±0.05	110± 40
+FSG+SAP-I	2	0.24±0.03	3220±510

Spermatozoa were incubated in 0.5 ml of ASW (pH 8.2) or ASW (pH 8.2) containing 10 mM TEA or 100 mM KCl with or without FSG (final concentration, 50 nmol fucose/ml), SAP-I (0.59 μM) or both FSG (50 nmol/ml) and SAP-I (0.59 μM). 1) pmol/mg wet weight of spermatozoa; 2) fmol/mg weight of spermatozoa. Values are means±S.E. obtained for four separate experiments.

Discussion

FSG is a macromolecular component of sea urchin egg jelly and is the main substance responsible for induction of the acrosome reaction of sea urchin spermatozoa. However, for full induction of the acrosome reaction in *H. pulcherrimus* spermatozoa FSG requires SAP-I as a specific co-factor (40). SAP-I is reported to cross-link to a 77 kDa protein in *S. purpuratus* spermatozoa (4, 5). A cDNA clone for the protein was isolated from a testis cDNA library of *S. purpuratus* and sequenced. The amino acid sequence deduced from the cDNA showed no similarity to that of any reported proteins (6). We found that SAP-I cross-links to 63 kDa and 71 kDa proteins in *H. pulcherrimus* spermatozoa, although neither protein has yet been fully characterized (12). Little is known about the binding of FSG to sea urchin spermatozoa. The data presented here demonstrate that when $[Ca^{2+}]_i$ or pH_i was elevated by a sufficient concentration of FSG, a second addition of the same concentration of FSG did not cause further increase of the $[Ca^{2+}]_i$ or pH_i. Similarly, when the $[Ca^{2+}]_i$ or pH_i had been increased by a sufficient concentration of SAP-I, a second addition of the same concentration of SAP-I cause no further increase of either. A simple explanation of these results is that all the binding sites on the spermatozoa for FSG or SAP-I become occupied on the first addition of excess FSG or SAP-I, and so a second addition of FSG or SAP-I has no further effect on

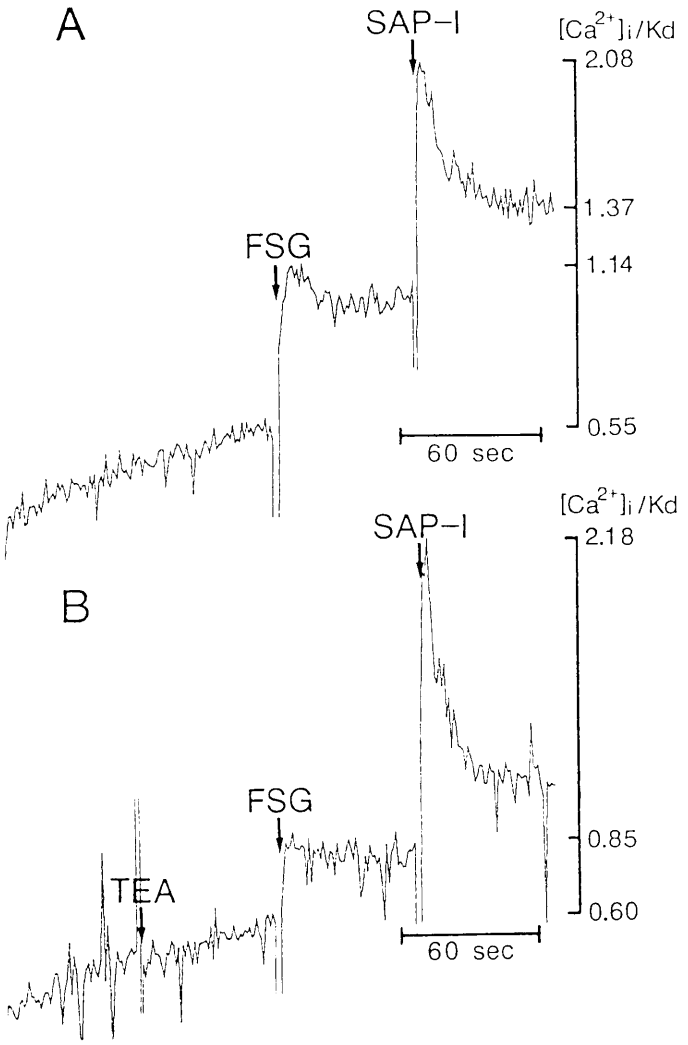

Fig. 7. Effect of TEA on the elevation of $[Ca^{2+}]_i$ by FSG. At the indicated point, FSG (final concentration, 50 nmol fucose/ml) was added to Fura 2/AM-loaded spermatozoa in the presence of 10 mM TEA at pH 8.0, followed by addition of SAP-I (final, 0.59 μM). The same batch of spermatozoa was used for both experiments.

the $[Ca^{2+}]_i$ or pH_i. However, after elevations of the $[Ca^{2+}]_i$ and pH_i by SAP-I, addition of FSG induced their further increases. Similarly, SAP-I increased the $[Ca^{2+}]_i$ and pH_i levels after their increases induced by FSG. These results suggest that FSG and SAP-I induce their effects by binding to different sites on the spermatozoa. When SAP-I was added at 1,000 lower concentration, some of its binding sites presumably remained unoccupied and so on its second addition it bound to these remaining sites and induced further elevation of pH_i. After $[Ca^{2+}]_i$ or pH_i had been increased by a sufficient concentration of FSG and then of SAP-I, egg jelly did not induce further elevation of the $[Ca^{2+}]_i$ or pH_i, so FSG and SAP-I are probably the main components in the egg jelly that induce elevations of the $[Ca^{2+}]_i$ and pH_i in the spermatozoa.

Kopf *et al.* reported that IBMX stimulates $^{45}Ca^{2+}$ uptake by abalone spermatozoa through a carrier-mediated transport site (17). Furthermore, Schackmann and Chock observed that IBMX enhances SAP-I-stimulated increase of $[Ca^{2+}]_i$ in *S. purpuratus* spermatozoa in a concentration-dependent manner and that the increase in $[Ca^{2+}]_i$ by more than 10 μM IBMX in the presence of SAP-I (30 nM) is nontransient (29). They also found that 30–60% of *S. purpuratus* spermatozoa treated with both SAP-I (30 nM) and IBMX (300 μM) underwent the acrosome reaction, whereas spermatozoa treated with SAP-I alone did not (29). We found that IBMX at 300 μM induced the nontransient increase in $[Ca^{2+}]_i$ in *H. pulcherrimus* spermatozoa and that the $[Ca^{2+}]_i$ reached a plateau. However, subsequent addition of FSG or SAP-I to the spermatozoa induced further increase of $[Ca^{2+}]_i$. This suggests that the elevation of $[Ca^{2+}]_i$ in the spermatozoa by FSG or SAP-I treat-

ment is induced via a different site from that for IBMX.

SAP-I has been shown to stimulate the decreased respiration rate of sea urchin spermatozoa in slightly acidic seawater (pH 6.6–6.8) at a half-maximal effective concentration of 10 to 100 pM (8, 34).Repaske and Garbers (23) showed that SAP-I stimulates H^+ efflux from *S. purpuratus* spermatozoa at a half-maximal effective concentration of ~100 pM. We found that at pH 6.6, SAP-I induced increase of pH_i at a half-maximal effective concentration of 10–100 pM, which is comparable to that for its stimulation of respiration.

Increase in the $[K^+]$ of seawater is reported to depolarize the sperm plasma membrane (27, 28) and inhibit Na^+/H^+ exchange in whole spermatozoa, isolated sperm tails, and sperm membrane vesicles (18, 19, 20). Increase in the $[K^+]$ of seawater also blocks ion fluxes associated with the egg jelly-induced acrosome reaction (25). Table 1 shows that this is also the case in *H. pulcherrimus* spermatozoa. In ASW containing 100 mM KCl, neither FSG nor FSG plus SAP-I induced the acrosome reaction or increases in $[Ca^{2+}]_i$ and pH_i. However, in ASW containing TEA, FSG plus SAP-I caused slight induction of the acrosome reaction, although neither FSG nor SAP-I alone induced the acrosome reaction. In ASW, TEA decreased the elevation in $[Ca^{2+}]_i$ induced by FSG alone, but did not affect the elevation of $[Ca^{2+}]_i$ by SAP-I alone. These findings suggest that there are two K^+-channels in the spermatozoa, only one of which is a voltage-sensitive channel, and that the increase of $[Ca^{2+}]_i$ induced by SAP-I, which compensates for the smaller elevation of $[Ca^{2+}]_i$ induced by FSG alone in ASW with TEA, may be linked with opening of the non-voltage-sensitive K^+-channel. This also supports the data that the binding sites of FSG and SAP-I on the spermatozoa are different.

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Identification and Characterization of Putative Receptors for Sperm-Activating Peptide I (SAP-I) in Spermatozoa of the Sea Urchin *Hemicentrotus pulcherrimus*[1]

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We characterized putative receptors specific for sperm-activating peptide I (SAP-I: GFDLNGGGVG) in spermatozoa of the sea urchin *Hemicentrotus pulcherrimus*, using both binding and crosslinking techniques. Analysis of the data obtained from the equilibrium binding of a radioiodinated SAP-I analogue [GGGY(^{125}I)-SAP-I] to *H. pulcherrimus* spermatozoa showed the presence of two classes of receptors specific for SAP-I in the spermatozoa. The incubation of intact spermatozoa as well as sperm tails or sperm membranes prepared from *H. pulcherrimus* spermatozoa with GGGY(^{125}I)-SAP-I and a chemical crosslinking reagent, disuccinimidyl suberate, resulted in the radiolabelling of a 71 kDa protein. The protein appears to be associated with a 220 kDa wheat germ agglutinin (WGA)-binding protein. A cDNA encoding the 71 kDa protein was isolated from a *H. pulcherrimus* testis cDNA library. The cDNA was 2443 bp long and an open reading frame predicted a protein of 532 amino acids containing a 30-residue amino-terminal signal peptide, followed by the same sequence as the N-terminal sequence of the 71 kDa protein. The amino acid sequence of the matured 71 kDa protein is strikingly similar to the 77 kDa protein of *Strongylocentrotus purpuratus* (95.5% identical) and also similar to cysteine rich domain of a human macrophage scavenger receptor. Northern blot analysis demonstrated that mRNA of 2.6 kb encoding the 71 kDa protein was expressed only in the testis.

Introduction

Sperm-activating peptide I (SAP-I: GFDLNGGGVG) was originally isolated from the egg jelly of the sea urchin by measuring its respiration-stimulating ability in sea urchin spermatozoa (11, 42). SAP-1 induces a number of biochemical events in sea urchin spermatozoa such as 1) the transient elvevation of intracellular levels of cAMP, cGMP (11–12, 45) and $[Ca^{2+}]$ ($[Ca^{2+}]_i$) (16, 34) the transient activation of the membrane form of guanylate cyclase (2). It also induces a proton efflux across the sperm plasma membrane, resulting in an increase in intracellular pH (pH_i) (16, 30). In addition to the above, the peptide has been shown to promote an acrosome reaction in *Hemicentrotus pulcherrimus* spermatozoa as a specific co-factor of a major acrosome-reaction-inducing substance, fucose sulfate glycoconjugate (52). Since SAP-I was isolated from the egg jelly of the sea urchins *H. pulcherrimus* (42) and *Strongylocentrotus purpuratus* (11), many different types of sperm-activating peptides have been isolated from the egg jelly of various sea urchin species (46). These peptides induce essentially the same biological events as SAP-I in their respective sea urchin spermatozoa, although the specificity is different.

Prior to the induction of these physiological and biochemical events, these sperm-activating peptides seem to bind to specific receptors on the sperm plama membrane (1, 6, 14, 41, 44, 54). SAP-IIA (CVTGAPGCVGGGRL-NH_2), isolated from the egg jelly of the sea urchin *Arbacia punctulata*, is specifically crosslinked to a 160 kDa plasma membrane protein on *A. punctulata* spermatozoa (3, 39) which has been identified as guanylate cyclase (40, 50). SAP-I is specifically crosslinked to a 77 kDa protein on *S. purpuratus* spermatozoa (6–8) or to 71 kDa and 63 kDa proteins on *H. pulcherrimus* spermatozoa (14) while SAP-III is crosslinked to 126 kDa, 87 kDa and 64 kDa proteins on the spermatozoa of the sand dollar *Clypeaster japonicus* (54).

In this study, we characterized the SAP-I receptors and the 71 kDa SAP-I-crosslinked protein on *H. pulcherrimus* spermatozoa, and isolated a cDNA clone encoding the protein. Here, we report that the mature protein contains 502 amino

[1] The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL, and GenBank Nucleotide Sequence Databases with the accession number D21100.

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acids with a single transmembrane domain and that the protein seems to be associated with a 220 kDa WGA-binding protein.

Materials and Methods

Materials: Sea urchins, *H. pulcherrimus* were collected from the coast near Noto Marine Laboratory. The testes and ovaries were dissected out from the animals as previously described (43). Spermatozoa were obtained by intracoelomic injection of 0.5 M KCl and "dry sperm" were collected at room temperature and stored on ice until use. The composition of the artificial sea water (ASW) used in this study was 454 mM NaCl, 9.7 mM KCl, 24.9 mM $MgCl_2$, 9.6 mM $CaCl_2$, 27.1 mM $MgSO_4$, 4.4 mM $NaHCO_3$ and 10 mM HEPES (pH 8.2). $Na^{125}I$ (3.7 GBq/ml, NaOH solution, pH 10) and [γ-^{32}P]ATP (111 TBq/mmol) were products of Du Pont/New England Nuclear (Boston, MA, USA). [α-^{32}P]dCTP (110 TBq/mmol) was purchased from Amersham International plc. (Amersham, UK). Disuccinimidyl suberate was obtained from Pierce Chemical Co. (Rockford, IL, USA). 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesufate (CHAPS) was purchased from Dojindo Laboratories (Kumamoto, Japan). Acetonitrile (ACN) of HPLC grade and chloramine T (sodium N-chloro-4-toluenesulfonamide trihydrate) were obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Wheat germ agglutinin (WGA) was purified from raw wheat germ (Sigma Chemical Co., St. Louis, MO, USA) by the method of Vretblad (49) and coupled to Sepharose 4B (Parmacia LKB Biotechnology, Uppsala, Sweden) using the cyanogen bromide method described by March *et al.* (25). SAP-I was synthesized for us at the Peptide Institute Inc., Osaka, Japan and GGGY-SAP-I was synthesized for us at the National Institute for Basic Biology, Okazaki, Japan. Restriction enzymes, T4 DNA ligase, and other enzymes were purchased from Takara Biomedicals Co. (Kyoto, Japan) or Toyobo Co. (Osaka, Japan). The cDNA synthesis kit, Hybond-N and Hybond-N$^+$ membrane filters were obtained from Amersham International plc. (Amercham, UK). The random-primed DNA labeling kit was purchased from Boehringer-Mannheim (Indianapolis, IN, USA). The plasmid Bluescript II KS(+). Bluescript II KS(−) and M13K07 helper phage were generously provided by Dr. Yoshitaka Nagahama at the National Institute for Basic Biology, Okazaki, Japan.

Preparation of sperm heads and tails, and sperm membranes: Dry sperm were suspended in 20 vol of ice-cold ASW and homogenized with a Teflon-homogenizer. The homogenate was centrifuged at 1,000xg for 15 min at 4°C to pellet the sperm heads. The sperm head fraction was resuspended in 20 vol of ice-cold ASW and centrifuged again. This procedure was repeated several times until the supernatant became clear. The resulting pellet (sperm heads) was resuspended in an appropriate volume of ice-cold ASW and used for experiments. The supernatant fraction containing sperm tails was centrifuged at 6,000xg for 30 min at 4°C to pellet sperm tails and the resulting pellet was also resuspended in an appropriate volume of ice-cold ASW used for experiments.

To prepare sperm membranes, dry sperm were suspended in 10 vol of Solution A containing 0.5 M NaCl, 50 mM $MgCl_2$, 10 mM Na_2MoO_4, 10 mM benzamidine-HCl, 10 mM KCl, 20 mM KF and 20 mM MES (pH 6.5). The sperm suspension was centrifuged at 4,500xg for 5 min at 4°C, and the resulting sperm pellet was resuspended in 10 vol of Solution A. The sperm suspension, after being dispersed in the solution with a Teflon-homogenizer, was subjected to nitrogen cavitation for 60 min at 1000 psi and then centrifuged at 10,000xg for 30 min at 4°C. The resulting supernatant fluid was centrifuged at 100,000xg for 60 min at 4°C. The resulting pellet (sperm membranes) was resuspended in an appropriate volume of Solution B containing 0.5 M NaCl, 0.1 M NaF and 20 mM MES (pH 6.0) and was maintained at −70°C until use.

Iodination of GGGY-SAP-I: GGGY-SAP-I was iodinated by the chloramine-T method using radioactive or non-radioactive sodium iodide. In general, 20 μl of 0.2 mM peptide and NaI (0.58 μg) or $Na^{125}I$ (37 MBq) were mixed with 5 μl of 3.5 mM chloramine-T in 0.15 M sodium phosphate buffer (pH 7.5) and incubated for 15 sec. The reaction was terminated by the addition of 25 μl of 7 mM sodium metabissulfite in the same buffer. Monoiodinated peptide GGGY(^{125}I or I)-SAP-I was purified by HPLC using a Shimadzu Model LC-6A chromatography system with a reverse-phase column (Cosmosil 5C18-AR, 5 μm, 4.6×250 mm, Nacalai Tesque, Kyoto, Japan) equilibrated with 10% ACN in 0.1% trifluroacetic acid (TFA) in deionized and distilled water. HPLC was carried out using a linear-gradient elution of ACN (10–40% in 30 min) in 0.1% TFA at a flow rate of 1.0 ml/min.

The column effluent was monitored for absorbance at 225 nm using a Shimadzu Model SPD-6V spectrophotometer.

Binding experiments: Radioactive monoiodonated peptide, GGGY(^{125}I)-SAP-I, was mixed with a large excess of non-radioactive monoiodinated peptide, GGGY(I)-SAP-I, and used for binding experiments. The specific activity of the mixture [GGGY(^{125}I/I)-SAP-I] was approximately 16.7 TBq/mol. Spermatozoa (0.4 mg wet weight) were incubated with GGGY(^{125}I/I)-SAP-I at 20°C for 10 min in 1 ml of ASW, buffered with 10 mM Tris (pH 8.2). The reaction was terminated by the addition of 4 ml ice-cold ASW, followed by rapid filtration with a glass-microfibre filter (Whatman GF/C). The filter was washed five times with 5 ml of ASW, dried and measured for radioactivity with a gamma counter (Aloka Auto Well Gamma System ARC-600, Aloka, Tokyo, Japan). Non-specific binding was determined by the incubation of spermatozoa in the presence of excess SAP-I (final 11 μM).

Crosslinking of GGGY(^{125}I)-SAP-I: Spermatozoa (16.7 mg wet weight) or sperm heads, sperm tails and sperm membranes which were prepared from spermatozoa corresponding to 16.7 mg wet weight were incubated with GGGY(^{125}I)-SAP-I (10 pmol) in 1 ml of ASW buffered with 10 mM HEPES (pH 8.2) for 10 min at 20°C. Non-specific crosslinking experiments were carried out as above in the presence or absence of non-radioactive SAP-I (11 μM) or SAP-III (11 μM). The reaction was stopped by the addition of ice-cold ASW (0.9 ml, 10 mM HEPES, pH 8.2), and the suspension was centrifuged at 15,000xg for 5 min at 4°C. The resulting pellet was resuspended in ASW (90 μl, 10 mM HEPES, pH 8.2) and incubated with a crosslinking reagent, disuccinimidyl suberate (1 mM), in dimethylsulfoxide (10 μl) for 30 min at 20°C. The incubation was terminated by the addition of ASW (0.9 ml, 10 mM Tris, pH 8.2) followed by the addition of 0.5 ml of 30% (w/v) trichloroacetic acid. Proteins obtained from chromatography of CHAPS-solubilized sperm tail proteins on a WGA-Sepharose 4B column were also crosslinked with GGGY(^{125}I)-SAP-I in the presence of disuccinimidyl suberate as described above. The crosslinked proteins were analysed by SDS-PAGE according to Laemmli (23). The gel was subsequently silver-stained by the method of Morrissey (27). Radiolabelled protein bands were detected by autoradiography on a Kodak X-OMAT film.

Purification, characterization, and N-terminal amino acid sequence analysis of SAP-I-crosslinked protein: The 30,000xg supernatant obtained by the 10,000xg centrifugation of sperm tails solubilized in Buffer A (0.5 M NaCl, 10 mM $CaCl_2$, 10 mM $MnCl_2$, and 10 mM Tris-HCl, pH 7.5) containing 10 mM benzamidine-HCl and 1% CHAPS was applied to a WGA-Speharose 4B column (20×64 mm) equilibrated with Buffer A containing 0.1% CHAPS. The column was washed with Buffer A containing 0.1% CHAPS and the proteins retained on the column were eluted with 100 mM N-acetyl-D-glucosamine in Buffer B (0.5 M NaCl, 0.1% CHAPS and 10 mM Tris-HCl, pH 7.5). Fractions containing the WGA-binding protein and the 71 kDa protein were pooled, dialyzed against Buffer C (0.1% CHAPS and 50 mM Tris-HCl, pH 7.5) and mixed with 100 ml of preswollen DEAE-Sephacel gel at 4°C. The gel was washed once with Buffer C and then twice with Buffer C containing 200 mM NaCl. The proteins retained on the gel were eluted with buffer C containing 2 M NaCl. Fractions containing the WGA-binding protein and the 71 kDa protein were pooled, concentrated with an Amicon Diaflo Cell RK 52 and subjected to SDS-PAGE. The gel was stained with Coomassie brilliant blue R250 (Fluka Chemie AG., Buchs, Switzerland). The protein band corresponding to 71 kDa was excised and electroeluted from the gel with a Max-Yield Protein Concentrator (ATTO, Tokyo, Japan). To analyse the amino acid composition and the N-terminal amino acid sequence of the 71 kDa protein, the protein in the gel was transferred to a PVDF membrane (BioRad Laboratories, Richmond, CA, USA) using a Multiphor II NovaBlot electrophoretic kit (Pharmacia LKB Biotechnology, Uppsala, Sweden) at room temperature for 1 hr at 0.8 mA/cm^2 constant current (48). The membrane was rinsed three times with distilled water for 5 min each. The proteins were visualized by staining briefly with Coomassie brilliant blue R250. A Coomassie-stained protein band corresponding to the 71 kDa protein was cut out. One piece of the band was subjected to amino acid analysis with an automated amino acid analyser, Hitachi L-8500, after hydrolysis with constant-boiling HCl containing 4% thioglycolic acid at 110°C for 24 hrs and another piece of the band was submitted to automated Edman degradationon an Applied Biosystems 470A gas-phase sequenator equipped with an on-line Applied Systems 120A PTH amino acid analyser.

In different experiments, GGGY(^{125}I)-SAP-I-labelled sperm membranes were solubilized in

Buffer A containing 10 mM benzamidine-HCl and 1% CHAPS, and centrifuged at 10,000xg. The resultant supernatant was applied to a DEAE-Sephacel column. Fractions containing radioactivity were pooled, concentrated with the Amicon Diaflo Cell RK 52 and subjected to chromatography on a Toyoperal HW55 column. Radiolabelled proteins eluted from the columns were anlaysed by SDS-PAGE and autoradiography.

Fractions obtained from chromatography on a WGA-Sepharose 4B column, which mainly contained the WGA-binding protein and the 71 kDa protein, were pooled, concentrated with an Amicon Diaflo Cell RK 52, and incubated in a final concentration of 2% SDS at 100°C for 5 min. Buffer B was then added to the mixture to make the SDS final concentration of 0.1% and the sample was subjected to chromatography on the WGA-Sepharose 4B column as described above. The mixture was also subjected to HPLC on a TSK-G4000SW column (7.5×300 mm, TOSOH K.K., Tokyo, Japan) equilibrated with 0.1 M sodium phosphate (pH 6.8) containing 0.1% CHAPS using a Hitachi HPLC system L-6000-L-6200 equipped with a L-4200 UV-VIS detector and a D-2500 Chromato-Intergator. The proteins were eluted with the equilibration buffer at a flow rate of 0.5 ml/min at room temperature. The column effluent was monitored for an absorbance at 280 nm and protein concentration.

Cloning and sequencing of cDNAs: A cDNA library (4.9×10^5 pfu) from poly(A)$^+$RNA isolated from growing testes of the sea urchin *H. pulcherrimus* was constructed in λ gt10 using the cDNA synthesis system and the cDNA cloning system λ gt10 (Amersham International plc., Amersham, UK).

An antisense oligonucleotide probe (45 mer; 5'-ACCAACGTCCCAGTTATGGGTGTAGGGACA CGCATGCTGATACGG-3') which corresponds to part of the cDNA (nucleotides 832 to 876) for a SAP-I-crosslinked 77 kDa protein in *S. purpuratus* spermatozoa (8), was synthesized on an Applied Biosystem Model 391 DNA synthesizer. The oligonucleotide was 5' end labelled with [γ-^{32}P]ATP (7.4 MBq), and used for screening approximately 3×10^5 plaques. Plaques transferred onto Hybond-N$^+$ membranes were prehybridized at 37°C for 2 hrs in prehybridization solution contianing 6×NET (1×NET; 50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, pH 7.5), 0.5% SDS, 1×Denhardt's solution and denatured herring sperm DNA (100 μg/ml) and then hybridized at 37°C overnight with a 1×10^7 cpm probe in 10 ml of hybridization solution containing 6×NET, 0.5% SDS, 1×Denhardt's solution and denatured herring sperm DNA (100 μg/ml). The membranes were washed three times at 20°C with 6×SSC (1×SSC; 150 mM NaCl, 15 mM sodium citrate, pH 7.0) containing 0.1% SDS and then washed at 40°C for 1 min with 5×SSC containing 0.1% SDS. Forty initial positive clones were rescreened until pure, and the phage DNA was purified. Digestion of the DNA with BamHI showed that 20 clones contained inserts of 2.5 kb, and restriction mapping showed that 16 of those were identical.

The 2.5 kb cDNA insert from a clone (λ SR11-1-1) was subcloned into the plasmid vector Bluescript II KS(+) (Stratagene, La Jolla, CA, USA). EcoRI cut this insert into 2.0 kb and 0.5 kb fragments, and the 2.0 kb fragment hybridized to the probe. Serial deletion mutants of subclones were made according to Yanisch-Perron *et al.* (53). Nucleotide suquences were determined by the dideoxy chain termination method (32) using the 7-DEAZA sequencing kit ver. 2.0 (Takara Biomedicals, Kyoto, Japan) and the Sequenase version 2.0 DNA sequencing kit (United States Biochemical Co., Cleaveland, OH, USA) and analysed on DNASIS software (Hitachi Software Engineering Co., Yokohama, Japan).

Northern blot analysis: Total RNA was prepared from testes, ovaries and unfertilized eggs of *H. pulcherrimus* by the LiCl method of Cathala *et al.* (5). Poly(A)$^+$ RNA was prepared by two passage of the total RNA over a column of oligo (dT)-cellulose (Pharmacia LKB Biotechnology, Uppsala, Sweden). Northern blot analysis was carried out at follows: 1.6 μg of poly(A)$^+$ RNA was denatured with 2.1 M formaldehyde, electrophoresed on a 1% agarose gel in the presence of 2.2 M formaldehyde, and transferred onto a Hybond-N membrane. Prehybridization was performed at 65°C for 2 hrs in a solution containing 6×SSC, 0.1% SDS, 5×Denhardt's solution, and denatured herring sperm DNA (100 μg/ml). The RNA on the membrane was then hybridized to the random-primed, [α-^{32}P]dCTP-labelled 1745 bp cDNA insert (base numbers from 150 to 1895 of the λ SR11-1-1 cDNA insert) at 65°C for 18 hrs. The membrane was washed with 6×SSC and 0.1% SDS at room temperature for 30 min, followed by a final wash with 0.5×SSC and 0.1% SDS at 65°C for 30 min. The size of the RNA was estimated using a 0.24–9.5 kb RNA Ladder (GIBCO BRL, Gaithersburg, MD, USA) as a marker.

Other methods: Sperm respiration rates were determined using a Yanaco PO-100A oxygraph as described previously (42). Intracellular cGMP concentrations were determined by radioimmunoassay using a Yamasa cGMP-assay kit (Yamasa Shoyu K.K., Chiba, Japan) as described previously (52). The concentration of protein was determined by the Lowry method (24) modified by Schacterle and Pollack (35) or the method of Bradford (4) using bovine serum albumin as a standard.

Results

Equilibrium binding of GGGY(^{125}I/I)-SAP-I to spermatozoa

Initially, we examined whether GGGY(I)-SAP-I possessed identical biological activity to SAP-I. The monoiodinated SAP-I analogue stimulated *H. pulcherrimus* sperm respiration half maximally at a concentration of 0.056 nM which was almost the same as that of SAP-I. The peptide also elevated sperm cGMP concentrations with an EC_{50} of 32 nM, which was close to that of SAP-I. Therefore, we concluded that GGGY(I)-SAP-I possesses the same biological activity as SAP-I and used it in the following experiments.

Spermatozoa (0.4 mg we weight) were incubated with various concentrations (0.6–316 nM) of GGGY(^{125}I/I)-SAP-I. The results showed that the binding of the peptide to spermatozoa was saturable (Fig. 1A). The data were analysed by the method of Klotz (18), yielding a double sygmoidal curve (Fig. 1B). This suggests the existence of two classes of receptors which are formed from low-affinity and high-affinity members. A Scatchard plot of the data yielded a downward concave curve at less than 2.6 pmol (Fig. 1C), implying that one binding site of a single class of the high-affinity interacts positively and cooperatively with other site (13, 33, 47). By extrapolating a limb of the curve, we estimated that the maximal binding

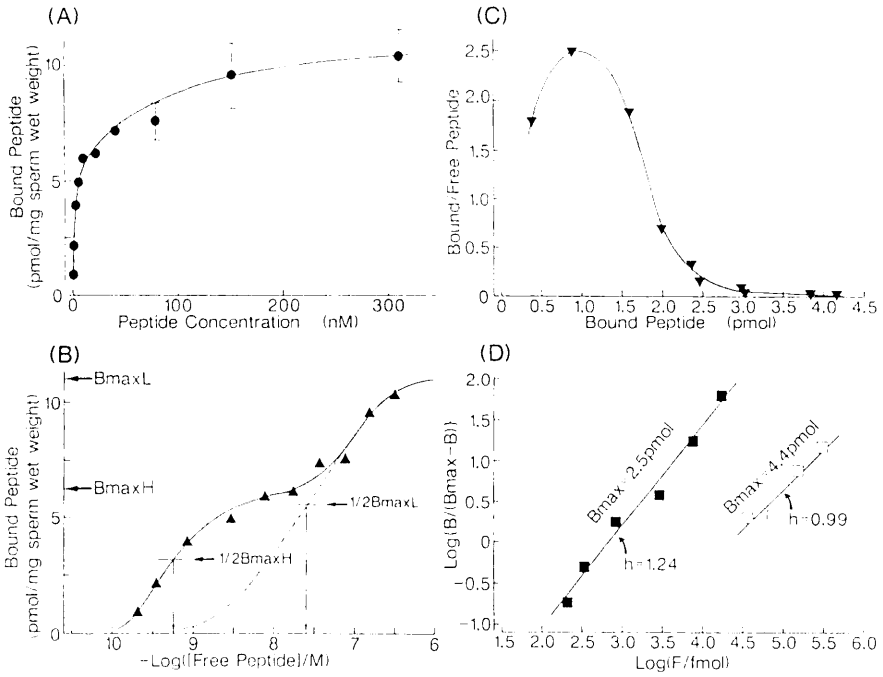

Fig. 1 Equilibrium binding of GGGY(^{125}I/I)-SAP-I to *H. pulcherrimus* spermatozoa and analysis of the data using Scatchard, Klotz and Hill plots. (A) Saturation curve of GGGY(^{125}I/I)-SAP-I binding. The specific binding estimated from the difference between GGGY(^{125}I/I)-SAP-I binding in the absence and presence of excess SAP-I was plotted with a linear abscissa. Each point represents the mean of three independent incubations. (B) Klotz plot. The specific binding was plotted with a logarithmic abscissa according to the method of Klotz (18). Standard error of each point was not shown because it was very small. The half-maximal binding (1/2 B_{max} H and 1/2 B_{max} L) provided estimates of the Kd of 0.58 nM for the high-affinity receptor and 23 nM for the low-affinity receptor. (C) Scatchard plot. The molar ratio of specifically bound peptide to free peptide was plotted against mol specifically bound GGGY(^{125}I/I)-SAP-I/0.4 mg wet *H. pulcherrimus* spermatozoa, according to the method of Scatchard (33). (D) Hill plot. (■) High-affinity binding calculated with 2.6 pmol/0.4 mg wet spermatozoa as the B_{max} of the high-affinity receptor. (□) Low-affinity binding calcuated with 4.4 pmol/0.4 mg wet spermatozoa as the B_{max} of the low-affinity receptor. B_{max} values were estimated from the Scatchard plot. B, the concentration of bound peptide; F, the concentration of unbound peptide.

capacity (B_{max}) values for the high-affinity and low-affinity receptors were 6.4 and 11 pmol/mg wet weight spermatozoa, respectively. A Hill plot produced the Hill coefficient (*h*) of 1.24 for the high-affinity receptors and 0.99 for the low-affinity receptors (Fig. 1D). From these plottings, the K_d values were estimated to be 0.58–0.69 nM for the high-affinity receptors and 23–25 nM for the low-affinity receptors (Table 1).

Covalent coupling of GGGY(^{125}I)-SAP-I to intact spermatozoa, sperm heads and tails, and sperm membranes

Table 1. Equilibrium parameters of GGGY(^{125}I/I)-SAP-I binding and half-maximal effective concentrations of GGGY(I)-SAP-I or SAP-I on spermatozoa

Type of binding	K_d	B_{max}	h	EC_{50}
	nM			nM
Spermatozoa high-affinity	0.58–0.65	6.4[1)]	1.24	
low-affinity	23–25	11.0[1)]	0.99	
Respiration				0.056
Intracellular pH				0.045[2)]
Intracellular [Ca^{2+}]				7.0[2)]
Intracellular [cGMP]				32

1) pmol/mg wet weight of spermatozoa. 2) calculated from the data of Hoshino *et al.* (16).

GGGY(^{125}I)-SAP-I coupled covalently to a 71 kDa protein located exclusively in sperm tails in the presence of disuccinimidyl suberate (Fig. 2). The incubation of spermatozoa, sperm tails or sperm membranes with GGGY(^{125}I)-SAP-I and disuccinimidyl suberate in excess SAP-I resulted in there being no radiolabelling of the protein, whereas the incubation of spermatozoa with GGGY(^{125}I)-SAP-I and disuccinimidyl suberate in excess SAP-III, which exhibits no biological activity toward the spermatozoa, did not affect the appearance of the radioactive protein bands (Fig. 2). When fractions containing the GGGY(^{125}I)-SAP-I-crosslinked protein(s) obtained from chromatography of CHAPS-solubilized GGGY(^{125}I)-SAP-I-labelled sperm membranes on a DEAE-Sephacel column and on a Toyopearl HW55 column were subjected to SDS-PAGE under the same conditions as above, two radiolabelled bands were detected at molecular masses of 75 kDa and 71 kDa, although the radioactivity of the band at 75 kDa was weaker than that of the 71 kDa band (data not shown).

Sequence of the cDNA clone

The sequencing strategy for the λ SR11-1-1 cDNA is shown in Fig. 3. The complete nucleotide sequence and the deduced amino acid sequence are presented in Fig. 4. The λ SR11-1-

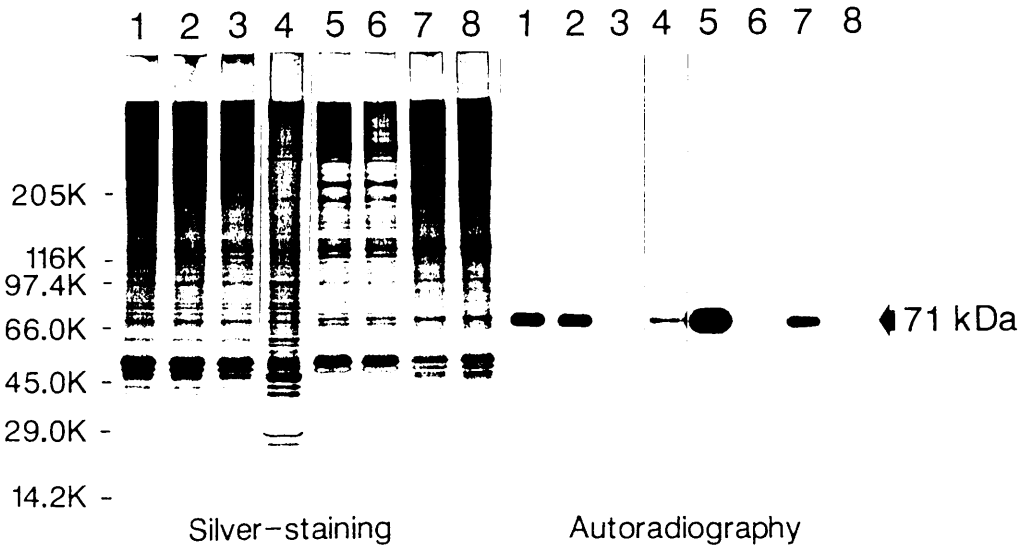

Fig. 2 SDS-PAGE of GGGY(^{125}I)-SAP-I-crosslinked *H. pulcherrimus* sperm protein. Approximately 5-10 μg of protein were electrophoresed on a 5-15% SDS-polyacrylamide gel in the presence of 2-mercaptoethanol. Intact spermatozoa (16.7 mg wet weight) or sperm heads, sperm tails and sperm membranes prepared from the spermatozoa (16.7 mg wet weight) were incubated with GGGY(^{125}I)-SAP-I in the presence of no SAP-I (lane 1), 11 μM SAP-III (lane 2) or 11 μM SAP-I (lane 3). Sperm heads prepared from the spermatozoa (16.7 mg wet weight) were incubated with 10 pM GGGY(^{125}I)-SAP-I (lane 4). Sperm tails prepared from the spermatozoa (16.7 mg we weight) were incubated with 10 pM GGGY(^{125}I)-SAP-I in the presence of no SAP (lane 5) or 11 μM SAP-I (lane 6). Sperm membranes prepared from the spermatozoa (16.7 mg wet weight) were incubated with 10 pM GGGY(^{125}I)-SAP-I in the presence of no SAP (lane 7) or 11 μM SAP-I (lane 8).

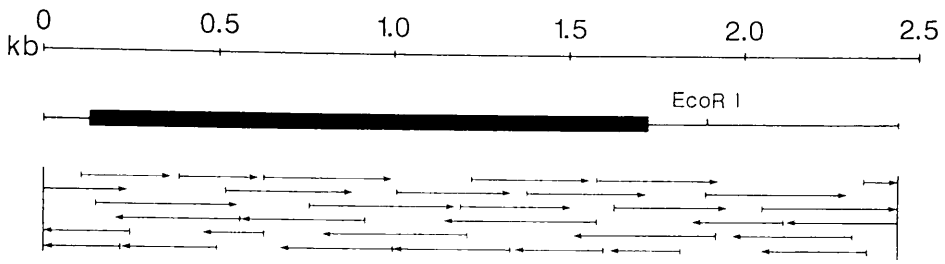

Fig. 3 The restriction endonuclease map and sequencing strategy for a λ SR11-1-1 cDNA insert. The map only shows the relevant restriction site. The direction and extent of the sequence determination are indicated by arrows. The deduced open reading frame is shown by a solid box.

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AATTTAGCTAGCTGGAGCTAGGCCTAGGGCTAGCAAGTTAATGTAACAATCATTAACAATAACATTCATCAAAAACTGCGAACA   84
AGGAAGAAGTATCGAATCTGAAGAAAGAAAAGTAAACTGATGTTTAAGATGGGCCTTCCAATGATGTTGCAGCGACACTGTTGG  168
                                  M  G  L  P  M  M  L  Q  R  H  C  W                    12
GCTGCTTGCATGGTCATTTGCATTGCAATCAGTTCTTTTGACGATGTTGGCGCGGAACAAAACTATGGTCGGGAAGCTGTTGAG  252
A  A  C  M  V  I  C  I  A  I  S  S  F  D  D  V  G  A  E  Q  N  Y  G  R  E  A  V  E     40
GGTAATATCCGACTGATCCATGGAAGAACAGAGAACGAAGGATCAGTTGAGATCTATCATGCAACTCGATGGGGAGGAGTTTGT  336
G  N  I  R  L  I  H  G  R  T  E  N  E  G  S  V  E  I  Y  H  A  T  R  W  G  G  V  C     68
GATTGGTGGTGGCATATGGAAAACGCCAACGTTACATGCAAACAACTGGGCTTCCCAGGGGCTCGCCAATTCTATCGGAGGGCA  420
D  W  W  W  H  M  E  N  A  N  V  T  C  K  Q  L  G  F  P  G  A  R  Q  F  Y  R  R  A     96
TATTATGGCGCTCATGTAACAACGTTTTGGGTGTATAAACTGAACTGCCTCGGGAATGAAACGAGACTAGACGAGTGCTATCAC  504
Y  Y  G  A  H  V  T  T  F  W  V  Y  K  L  N  C  L  G  N  E  T  R  L  D  E  C  Y  H    124
CGACCGTATGGACGTCCGTGGCTCTGCAGTTCACAATGGGCTGCTGGGGTAGAATGTTTACCTAAAGATGAGCCGCAGGGGTCG  588
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L  R  M  I  L  G  D  V  P  N  E  G  T  L  E  T  F  W  D  G  A  W  G  S  V  C  H  T    180
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V  T  P  Y  Q  H  A  C  P  Y  T  H  N  W  D  V  G  V  V  C  K  P  N  V  E  G  D  I    264
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TGGGATTGGGCAGATGCTAATGTGGTATGTCGGCAGGCTGGTTACAGAGGAGCAGTCAAAGCTTCAGGATTCCAAGGGGAGGAC 1092
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CCAAAGAATACAAGAGTACGCATAGTTGGCATGGGACAAGGACAAGGTCGAGTGGAAGTAAGTTTGGGCAACGGCTGGGGACGA 1344
P  K  N  T  R  V  R  I  V  G  M  G  Q  G  Q  G  R  V  E  V  S  L  G  N  G  W  G  R    404
GTTTGTGATCCAGATTGGAGTGACCATGAAGCCAAGACTGTTTGCTATCACGCTGGATACAAGTGGGGAGCGTCACGAGCGGCA 1428
V  C  D  P  D  W  S  D  H  E  A  K  T  V  C  Y  H  A  G  Y  K  W  G  A  S  R  A  A    432
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G  S  A  E  F  S  A  P  F  D  P  E  A  P  F  I  I  D  G  I  A  C  T  G  A  E  N  E    460
ACCCTAAGCCAATGTCAGATGAAAGTTTCTGCAGACCTGACCTGCGCAACAGGTGATGTCGGAGTCGTTTGTGAAGGATCGACT 1596
T  L  S  Q  C  Q  M  K  V  S  A  D  L  T  C  A  T  G  D  V  G  V  V  C  E  G  S  T    488
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F  Y  Y  I  K  F  V  K  P  A  G  G  G  V  Q  A                                        532
CAGGCACATTAATCTCAGGAATCAATTCCTTAAATACTTAACAGAAAGTTCTCCAATGACCCAAAACGCACTAATATATTTCTC 1848
AATCATTTCCTACTCTGCTATGATATAAGTTACTGTTTGGAAAATGAATTCAATCAAAAGTGTGTTTGGCATACAACGCGCATT 1932
GTGCGTGTTTGATTTAATTACTCGATCAGCACATGGTGCCGATTAGGCATTATAAATCTTAATTACTATTCAATGAGTCGACGA 2016
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TATAAAAGAGCGAATTGCTACGATGCATGTTTAACAATAATTTGTCGTCAATCGACTAATAAATCGTTTAATTTGGGAGGTCTA 2268
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AAACAATTTGATGAAGAAATTTCAGGAAATATTATTTTATATTATTTTGTTTTCTTTCAACGAAAAAGTGAATAAATGATTACA 2436
ATAAAAT                                                                              2443
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Fig. 4 Complete nucleotide sequence and deduced amino acid sequence of the λ SR11-1-1 cDNA insert. Nucleotide and amino acid number are listed on the right hand side. The signal sequence and the putative transmembrane sequence are indicated by shaded-boxes. The potential N-linked carbohydrate binding sites are indicated by open boxes. Polyadenylation signal sequencs at the 3'-untraslated region are underlined. The N-terminal amino acid sequence of the purified 71 kDa protein is indicated by the broken line.

1 cDNA insert was 2443 bp in length. The oligonucleotide sequence used for screening the clone was found in the sequence at nucleotide positions 847–891. We have assigned the initiation codon to the ATG at position 133 because (1) there are 4 upstream in-frame stop codons; (2) this ATG is flanked by sequences that fit Kozak's criteria for translation initiation codon (20), and (3) the 30-amino acid sequence following this ATG possesses the features characteristic of signal sequences (51). The initiation codon is followed by an open reading frame of 1593 bp. An inframe stop codon occurs at position 1729 and the 3'-untranslated region composed of 714 bp includes polyadenylation sites (AATAAA).

The deduced amino acid sequence suggests that cleavage of the signal peptide would yield a protein of 502 amino acids with a calculated molecular weight of 54,581. The protein contains three potential N-linked glycosylation sites (NXT) at residues 48–50, 85–87, and 429–431. The protein also contains a hydrophobic region of 29 amino acids at residue 462–490 that is flanked on the carboxyl-terminal side by KFVK and on the amino-terminal end by a single acidic residue (Fig. 5). These features are typical of membrane-spanning domains of membrane proteins (31).

Northern blot analysis

To determine the size of the mRNA for the λ SR11-1-1 cDNA insert and to see whether the mRNA exists in the testes, ovaries or eggs, poly(A)$^+$ RNA prepared from tissues and eggs of *H. pulcherrimus* was analysed by Northern blot hybridization using a part (nucleotides, 150–1895) of the λ SR11-1-1 cDNA insert as a probe. A strong hybridization signal at the position corresponding to 2.6 kb was detected only with poly(A)$^+$ RNA from a testis sample (Fig. 6).

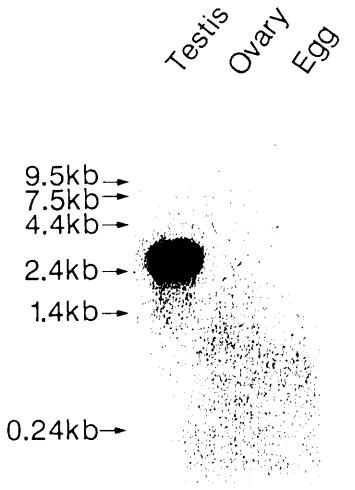

Fig. 6 Northern blot analysis. Poly(A)$^+$RNA prepared from *H. pulcherrimus* testes, ovaries and eggs was hybridized to a part (nucleotides 150-1895) of the λ SR11-1-1 cDNA insert.

Purification and characterization of the 71 kDa protein

The 71 kDa protein was co-purified with a 220 kDa WGA-binding protein by chromatography of CHAPS-solubilized sperm tail proteins (330 mg) on a WGA-Sepharose 4B column and batchwise chromatography on the DEAE-Sephacel gel. The 71 kDa protein did not separate from the 220 kDa WGA-binding protein by gel filtration chromatography on a Toyopearl HW55 column. During the chromatography, the 71 kDa protein with the 220 kDa WGA-binding protein was eluted in the void. The 71 kDa protein (0.93 mg) was finally purified by preparative SDS-PAGE. The protein (about 2 μg) was transferred onto a PVDF membrane and was analysed for amino acid composition and sequenced for the N-terminal amino acid sequence. As shown in Table 2, the amino acid composition coincided well with the amino acid composition of the protein deduced from nucleotide sequence of

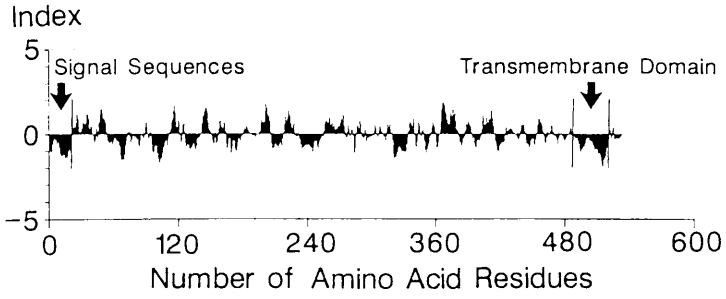

Fig. 5 Hydropathy profile of the protein with 532 amino acids. Hydropathy was calculated at a window of 12 residues, according to Kyte and Dolittle (22). Regions with values below the midpoint line are hydrophobic.

the λ SR11-1-1 insert cDNA without the predicted signal peptide. The N-terminal amino acid sequence of the purified 71 kDa protein was EQNYGREAVEGNIRLIHGRTENEGS, which was the same as residue numbers 31 to 55 of the deduced amino acid sequence from nucleotide sequence of the λ SR11-1-1 cDNA insert.

When the fraction containing the 71 kDa protein and the 220 kDa WGA-binding protein obtained by chromatography on a WGA-Sepharose 4B column was incubated with GGGY(^{125}I)-SAP-I in the presence of disuccinimidyl suberate and then analysed by SDS-PAGE and autoradiography, the 71 kDa protein was radiolabelled but the 220 kDa protein was not radiolabelled (Fig. 7). In different experiments, the same fraction was incubated in 2% SDS at 100°C for 5 min, and then subjected to chromatogaphy on a TSK-G4000SW column or on a WGA-Sepharose 4B column. By chromatography using the TSK column, the 71 kDa protein was separated from the 220 kDa WGA-binding protein (Fig. 8A). In chromatography using the WGA-Sepharose column, the 71 kDa protein was eluted in flow-through fractions, while the 220 kDa protein was retained on the column and eluted with 100 mM N-acetyl-D-glucosamine (Fig. 8B).

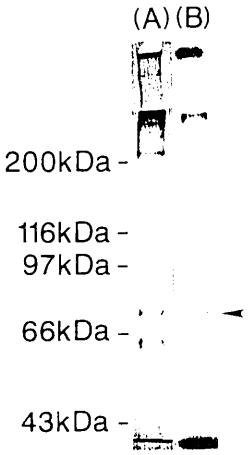

Fig. 7 GGGY(^{125}I)-SAP-I-crosslinking to the 71 kDa protein. Protein (100 μg) obtained from chromatography of CHAPS-solubilized sperm tail proteins on a WGA-Sepharose 4B column were incubated with GGGY(^{125}I)-SAP-I and disuccinimidyl suberate. The crosslinked protein (4 μg) was analysed by SDS-PAGE using a 7.5% gel and subsequently subjected to autoradiography as described in Materials and Methods. (A) silver-staining, (B) autoradiogram. The arrow indicates the 71 kDa protein band.

Discussion

In the present study, we demonstrated that *H. pulcherrimus* spermatozoa possess receptors specific for SAP-I, which are located on the tail plasma membrane. Analysis of the data obtained from the equilibrium binding of GGGY(^{125}I/I)-SAP-I to intact spermatozoa by the methods of Klotz, Scatchard and Hill suggested the presence of two classes of receptor (high-affinity and low-affinity) specific for SAP-I. The K_d of the high-affinity receptor was comparable to that of SAP-I (1.4 nM in *S. purpuratus* spermatozoa) (41), SAP-IIA (1.0 nM in *A. punctulata* spermatozoa) (3) and SAP-III

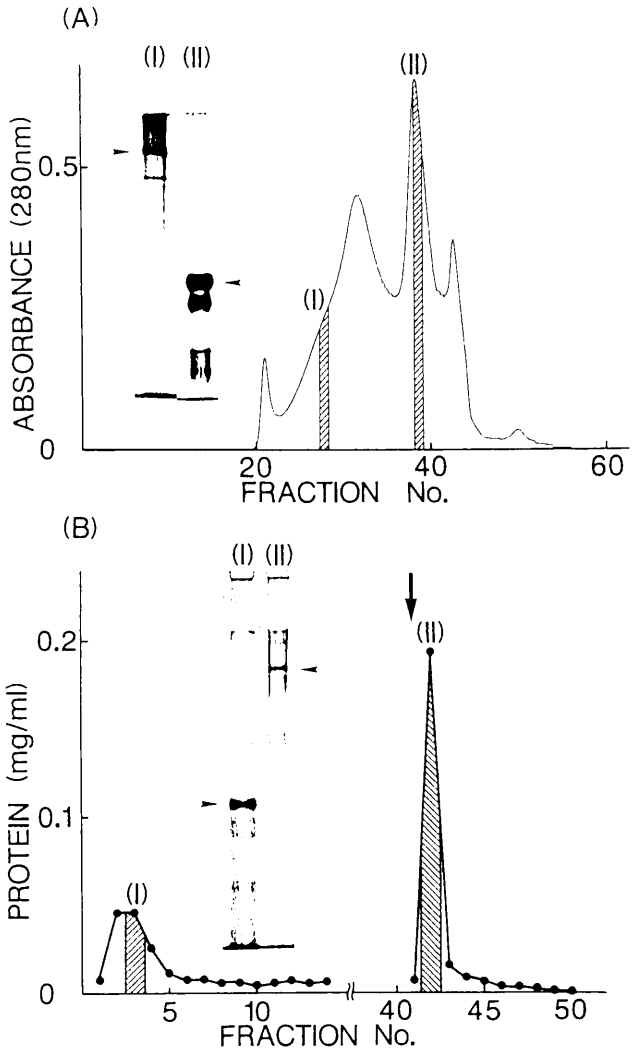

Fig. 8 Separation of the 71 kDa protein from the 220 kDa WGA-binding protein. A fraction [27.6 mg protein for (A) and 10.1 mg protein for (B)] containing the 71 kDa and 220 kDa WGA-binding proteins, was obtained from chromatography on a WGA-Sepharose 4B column. It was incubaed in 2% SDS at 100°C for 5 min, and then applied to a TSK G4000SW column (A) or a WGA-Sepharose 4B column (B). Inserts are silver-stained SDS-PAGE profiles of the fractions designated by the shaded area and Roman numeral. Arrows denote the 220 kDa WGA-binding protein or the 71 kDa protein.

Table 2. Amino acid composition of the purified 71 kDa protein was compared to a mature protein predicted by nucleotide sequence of the λ SR11-1-1 cDNA insert

Residues	71 kDa protein	Mature protein predicted from nucleotide sequence of cDNA
Asp	52.5 a.a	53 a.a
Thr	27.3	28
Ser	28.8	25
Glu	48.1	44
Pro	34.4[1)]	22
Gly	61.9	64
Ala	42.6	44
Half-Cys	N.D[2)]	24
Val	37.6	41
Met	11.2	9
Ile	23	23
Leu	24.5	18
Tyr	16.4	17
Phe	19.9	17
Lys	15.5	14
His	17.2	17
Arg	21.1	23
Trp	N.D.	19

1) Proline content was always over-estimated in the analysis of protein samples transferred to the PVDF membranes. 2) Half-cysteine was not detected, perhaps because of hydrolysis with 4% thioglycolic acid.

(3.4 nM in *C. japonicus* spermatozoa) (54). In the case of I/^{125}I-GGGY-SAP-III binding to the high-affinity receptor on *C. japonicus* spermatozoa (54), a positive cooperative interaction was seen among the high-affinity binding sites on *H. pulcherrimus* psermatozoa. As shown in Table 1, SAP-I has two classes of EC_{50} values with regard to activity. One class of EC_{50} values is at subnanomolar levels, which includes respiration-stimulating activity (0.056 nM) and pH_i-increasing activity (0.045 nM) (16). The other class of values ranges from 7–32 nM and is found in $[Ca^{2+}]_i$-increasing activity (7 nM) (16) and cellular cGMP-elevating activity (32 nM). In the present study, we demonstrated that the high-affinity receptor had a K_d of 0.58–0.65 nM and the low-affinity receptor had a K_d of 23–25 nM. Therefore, we preseume that the two different ragnes of EC_{50} values found in the biological activity of SAP-I may reflect the presence of two classes of SAP-I receptors; the high-affinity receptor may be for pH_i-increasing activity as well as respiration-stimulating activity and the low-affinity receptor may be for cGMP-and $[Ca^{2+}]_i$-elevating activity.

GGGY(^{125}I/I)-SAP-I was crosslinked mostly to the 71 kDa protein which was located in the sperm plasma membrane. The covalent coupling was prevented by excess SAP-I but not by excess SAP-III which did not show any biological effect on *H. pulcherrimus* spermatozoa. Thus, we think that the interaction of GGGY(^{125}I/I)-SAP-I and the 71 kDa protein is specific and that the protein is a binding component specific for SAP-I.

The purified 71 kDa protein had the N-terminal amino acid sequence EQNYGREAVEGNIRLIH-GRTENEGS which was the same as the sequence (residues 31–55) of a protein deduced from the nucleotide sequence of the λ SR11-1-1 cDNA insert. The amino acid composition of the 71 kDa protein was also in close agreement with that of the protein deduced from the nucleotide sequence if amino acid residues in the predicted signal peptide were subtracted from the total amino acid residues of the protein. Thus, we concluded that the clone obtained from a *H. pulcherrimus* testis cDNA library using an oligonucleotide probe which corresponds to a part of the nucleotide sequence of a cDNA for a 77 kDa protein in *S. purpuratus* spermatozoa (8) is of a 71 kDa protein in *H. pulcherrimus* spermatozoa and that the mature protein contains 502 amino acids. The discrepancy between the apparent molecular weight of the protein in SDS-PAGE (M_r=71,000) and the deduced molecular weight (54,581) may be due to the presence of carbohydrate on the mature protein. The presence of three potential N-linked glycosylation sites in the protein supports this speculation. The protein has a potential transmembrane domain consisting of 29-amino acids. The distance from the first basic residue following the putative transmembrane domain to the carboxyl terminus is 12 amino acids. Therefore, we can predict that the protein consists of a large N-terminal extracellular domain and a small C-terminal intracellular domain. Northern blot analysis demonstrated that mRNA of 2.6 kb encoding the 71 kDa protein is expressed only in the testis.

A homology search using NBRF-PIR and SWISS-PROT databases demonstrated that the deduced amino acid sequence of cDNA for the 71 kDa protein of *H. pulcherrimus* is strikingly similar to the 77 kDa protein of *S. purpuratus* (95.5% identical in 532 amino acids). Both proteins have a relatively high cysteine content (about 5%), located in four cysteine-rich regions with about 100 residues. The sequences of these cysteine-rich regions have a 30–46% homology with the cysteine-rich domains of bovine and human mac-

rophage scavenger receptor type I (10, 19, 26). The presence of a cysteine-rich domain has been also reported in the human T- and specialized B-lymphocyte differentiation antigen CD5 (17). At the present time, the specific function of the cysteine-rich domains in these proteins has not yet been defined. However, these disulfide crosslinked domains appear to provide stable structures that are well suited for ligand-binding and are readily juxtaposed to other types of domains to permit the construction of complex mosaic proteins (9, 21).

During purification of the 71 kDa protein from sperm tails, we learned that the protein was always co-purified with the 220 kDa WGA-binding protein by gel filtration and ion exchange chromatography even in the presence of a detergent like CHAPS. The 71 kDa protein was separated from the 220 kDa protein only after treatment with 2% SDS at 100°C. This suggests that the 71 kDa protein may be tightly associated with the 220 kDa protein. The WGA-binding protein is reported to be involved in the induction of the acrosome reaction through regulating ion fluxes associated with the acrosome reaction (28–29, 36–38). SAP-I has also been reported to participate in the induction of the acrosome reaction (52). Thus, SAP-I binding to the 71 kDa protein on a spermatozoon may affect the regulatory system of ion fluxes induced by the binding of component(s) in the egg jelly to the WGA-binding protein. In a previous paper, we reported that the incubation of *H. pulcherrimus* sperm tails with ^{125}I-GYGG-SAP-I and disuccinimidyl suberate resulted in the radiolabelling of two proteins at 71 kDa and 63 kDa which were identified as diffuse radioactive bands and the radioactivity of the band at 63 kDa was much stronger than that of the band at 71 kDa (14). In the present study, when radioactive fractions obtained from chromatography of CHAPS-solubilized GGGY(^{125}I)-SAP-I-labelled sperm membrane proteins on a DEAE-Sephacel column were analysed by SDS-PAGE and subsequent autoradiography, a diffuse radioactive band was observed just on the 71 kDa radioactive protein band. Therefore, the molecular weight estimation in the previous study might have been inadequate because of the diffused radioactive bands, but these results imply that GGGY(^{125}I)-SAP-I may crosslink to another minor protein present in sperm membranes. In connection with this, it may be important to mention that the K_d values between the high-and low-affinity receptors are about 40-fold different. Therefore, it is possible that the protein component in the low-affinity receptor is barely crosslinked with the radioiodinated SAP-I analogue. The problem of determining which proteins are the real components of the low- and high-affinity receptor, however, remains to be solved. We have the isolated cDNA clone for the 71 kDa protein which binds SAP-I, so we are now in a position to pursue expression studies which should help to resolve at least part of that problem.

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Purification and Characterization of the Egg Jelly Macromolecules, Sialoglycoprotein and Fucose Sulfate Glycoconjugate, of the Sea Urchin *Hemicentrotus Pulcherrimus*

(sialoglycoprotein/fucose sulfate glycoconjugate/acrosome reaction/egg jelly/sea urchin spermatozoa)

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A sialoglycoprotein and a fucose sulfate glycoconjugate (FSG) were purified from egg jelly of the sea urchin *Hemicentrotus pulcherrimus*. Sialoglycoprotein which consisted of sialic acid (90%, w/w) and protein (10%, w/w) did not cause induction of the acrosome reaction and sperm isoagglutination. FSG which contained one mol sulfate/mol fucose possessed 2.0 times protein to fucose by weight. The proteins in intact FSG were separated to two major (258 kDa and 237 kDa) and one minor (120 kDa) proteins by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in the presence of 2-mercaptoethanol (2-ME) while the proteins could not be separated by HPLC in the presence of 0.1% SDS or SDS-PAGE without 2-ME. However, after carboxymethylation of FSG, two major (260 kDa and 240 kDa) proteins and two minor (140 kDa and 135 kDa) proteins were separated from the fucose sulfate moiety by HPLC in the presence of 0.1% SDS or SDS-PAGE without 2-ME. When FSG was first carboxymethylated with non-radioactive iodoacetic acid and then reduced with 2-ME and finally carboxymethylated with ^{14}C-iodoacetic acid, the most of radioactivity was detected in 140 kDa and 135 kDa proteins. Carboxymethylted-FSG was less potent than intact FSG in induction of the acrosome reaction. Fucoidan, a fucose sulfate polymer, did not induce the acrosome reaction.

Introduction

The sea urchin egg is surrounded by a transparent extracellular matrix which is mainly composed of two large acidic glycoconjugates and oligopeptides (6, 14, 15, 18, 19, 21, 48, 52, 54). The jelly coat has been shown to activate sperm metabolism (1, 11, 36) and induce the acrosome reaction in spermatozoa (3, 4, 5, 6, 8, 23, 24, 29, 48, 50, 51, 56). The acrosome reaction of sea urchin spermatozoa is accompanied with net Na^+ and Ca^{2+} influx, and H^+ and K^+ efflux (7, 13, 17, 25, 27, 42, 43, 44, 45, 60). Activation of sperm metabolism is also accompanied with these ionic changes which cause an increase in intracellular pH and trigger plasma membrane depolarization (30, 36, 46, 51, 60). In the last nine years substances responsible for activation of sperm metabolism were intensively studied and were found to be oligopeptides. These oligopeptides are called as sperm-activating peptides. Sperm-activating peptide I (SAP-I, Gly-Phe-Asp-Leu-Asn-Gly-Gly-Gly-Val-Gly) is the first oligopeptide isolated from the egg jelly coats of *Hemicentrotus pulcherrimus* and *Strongylocentrotus purpuratus* (14, 52, 54).

Fucose sulfate-rich glycoconjugate, a large acidic glycoconjugate in the jelly coat, is reported to trigger the acrosome reaction (8, 15, 23, 24, 25, 48, 49), increase cAMP concentration (13, 15, 25), activate a cyclic AMP-dependent protein kinase (16, 39, 40) and stimulate Ca^{2+}-accumulation by sperm cells (25, 48, 49). However, it induces the acrosome reaction fully only in Ca^{2+}-enriched sea water. SeGall and Lennarz reported that fucose sulfate glycoconjugates purified from the egg jelly of four species of sea urchins contained less than 30% protein (48, 49). They implied that the acrosome reaction in spermatozoa of these sea urchins is not dependent on the protein moiety of the fucose sulfate glycoconjugates. In connection with this, it should be noted that starfish egg jelly contains a sulfated glycoprotein called acrosome

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reaction-inducing substance (ARIS), steroidal saponins (Co-ARIS) and oligopeptides, and that pronase-digested ARIS (P-ARIS) is fully effective for induction of the acrosome reaction with Co-ARIS, although P-ARIS as well as intact ARIS is not effective individually (30, 31, 34, 35, 63). This suggests that in starfish, the protein moiety of ARIS is not critical for induction of the acrosome reaction. We previously reported that SAP-I-free macromolecular fraction or SAP-I-free fucose-rich glycoconjugate (FRG) of the jelly coat of *H. pulcherrimus* eggs was less active than the crude jelly in induction of the acrosome reaction and that addition of synthetic SAP-I increased the rates of the acrosome reaction in *H. pulcherrimus* spermatozoa to those seen with the crude jelly (65, 66). FRG contained a large amount of protein, compared with fucose sulfate glycoconjugate from the egg jelly of sea urchins, *Arbacia punctulata* and *S. purpuratus*. Pronase digestion of FRG resulted in a 50% decrease in induction of the acrosome reaction and also in the elevation of cAMP concentration in *H. pulcherrimus* spermtozoa (66). Ishihara and Dan (20) reported that treatment of sea urchin egg jelly with trypsin and pronase resulted in a reduction of acrosome reaction-inducing ability to about half of that with untreated jelly. These results suggest that the protein moiety of a major acrosome reaction-inducing substance in sea urchin egg jelly, which is considered to be FRG in the case of *H. pulcherrimus*, plays an important role in induction of the acrosome reaction. The present study was performed to characterize FRG and proteins associated with FRG as well as an another macromolecule, a sialic acid-containing material of *H. pulcherrimus* egg jelly.

Materials and Methods

Preparation of egg jelly

H. pulcherrimus were collected near the Noto Marine Laboratory. Artificial sea water (ASW) was prepared as described previously (65). Eggs were collected in ASW without buffers by intracoelomic injection of 0.5 M KCl. The egg suspension was adjusted to pH 5.0 with 0.1 N HCl and centrifuged at 600×g for 10 min at room temperature. The supernatant was centrifuged at 10,000×g for 30 min at 4°C, and the resultant supernatant was used as a jelly solution.

Purification of sialoglycoprotein

A jelly solution was frozen at −70°C for over night. After thawing, the solution was centrifuged at 10,000×g for 30 min at 4°C. The resultant supernatant was dialyzed against 0.1 M NaCl and then applied to a Sepharose 2B column (5×88 cm) equilibrated with 0.1 M NaCl, followed by elution with 0.1 M NaCl at a flow rate of 50 ml/hr at 4°C. Fractions which contained sialic acid were pooled and dialyzed against deionized and distilled water (DDW) containing 0.1 M NaCl. Then, 1 M sodium acetate (pH 5.0) was added to the dialysate to give a final concentration of 10 mM and the solution was subjected to chromatography on a DEAE-Sephadex A-25 column (2.5×20 cm) with a linear gradient of NaCl from 0.1 M to 1.2 M in 10 mM sodium acetate (pH 5.0). Fractions containing sialic acid were pooled and dialyzed against DDW. Solid guanidine hydrochloride, CsCl and 1 M sodium acetate (pH 5.0) were added to the dialysate at final concentrations of 4 M, 0.47 g/ml and 10 mM, respectively. The solution was centrifuged at 100,000×g for 48 hr in a Hitachi RPS 50 rotor at 4°C. After centrifugation, fractions of 0.5 ml were collected and monitored for the density. Sialic acid, protein and fucose concentrations of the fractions, after being dialyzed against DDW, were colorimetrically determined.

Purification of fucose sulfate glycoconjugate

Fucose sulfate glycoconjugate was purified from a jelly solution by sequential chromatography on Sepharose 4B and Sepharose 2B columns according to the method of Yamaguchi *et al* (66) with a slight modification. A jelly solution was applied to a Sepharose 4B column (5×84 cm) equilibrated with ASW containing 10 mM sodium acetate buffer (pH 5.0) and eluted with the same solution at a flow rate of 60 ml/hr at 4°C. Fractions containing fucose and sialic acid were pooled (macromolecular fraction) and dialyzed against 0.1 M NaCl. The macromolecular fraction was applied to a Sepharose 2B column (5×84 cm) equilibrated with 0.1 M NaCl and eluted with 0.1 M NaCl at a flow rate of 50 ml/hr at 4°C. Fractions which contained most of fucose were pooled and solid NaCl was added to the pooled fraction at a final concentration of 1 M. The mixture was kept in a freezer at −70°C for over night and then thawed. The solution was centrifuged at 10,000×g for 30 min at 4°C. The resultant precipitate was dissolved in DDW and followed by addition of an equal volume of 0.2 M NaCl. The mixture was applied to a Sepharose 2B column (5×84 cm) equilibrated with 0.1 M NaCl and eluted with 0.1 M NaCl at a flow rate of 50 ml/hr at 4°C. Fractions which contained fucose were pooled. The pooled

fraction contained fucose, sulfate and protein and is referred to as fucose sulfate glycoconjugate (FSG) in the present study. When FSG was subjected to chromatography on a DEAE Bio-Gel A column (2.5×25 cm) and eluted with a linear gradient of NaCl from 0.1 M to 1.2 M in N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) (pH 7.8), about 30% of applied FSG was eluted with 1.0–1.2 M NaCl and about 70% of FSG did not come out of the column even with 4 M NaCl in 10 mM HEPES (pH 7.8). FSG was also purified from the precipitate fraction of frozen-thawed jelly solution by a similar procedure as described above.

Treatment of FSG with NaOH and $NaBH_4$

FSG containing 160 μg fucose was incubated in 15 ml of 0.1 N NaOH and 1 mM $NaBH_4$ at 37°C for 48 hr. After the incubation, pH of the solution was adjusted to 7.0 with 1 N acetic acid. Solid urea (biochemical grade, Nakarai Chemicals, Japan) and HEPES were added to the solution at final concentrations of 7 M and 10 mM, respectively. The mixture was adjusted to pH 7.0 with 1 N NaOH, then applied to a Sepharose CL-2B column (2.6×61.5 cm) equilibrated with 7 M urea and 10 mM HEPES (pH 7.0) and eluted with the equilibrating solution at a flow rate of 15 ml/hr at 4°C.

Carboxymethylation of sialoglycoprotein and FSG

FSG (1.2 mg protein) or sialoglycoprotein (91 μg protein) in 45 ml of 8 M urea was reduced with 2-mercaptoethanol and then carboxymethylated with iodoacetic acid according to the method of Crestfield *et al* (2). When free sulfhydryl groups in FSG were carboxymethylated, FSG was not treated with 2-mercaptoethanol before reacting with iodoacetic acid. After the reaction, the solution (30 ml) was applied to a Sepharose CL-2B column (2.6×61.5 cm) equilibrated with 7 M urea and 10 mM HEPES (pH 7.0) and eluted with the urea solution at a flow rate of 15 ml/hr at 4°C. The rest of the solution (15 ml) was dialyzed against 0.2 M acetic acid, then against DDW and lyophilized. The sample was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

[1-^{14}C]-iodoacetic acid-labeling of FSG

FSG solution (20 μg fucose/ml) was concentrated to 110 μg fucose/ml with a Amicon YM-10 membrane. To 0.5 ml of the concentrated solution, 0.5 ml of 1.73 M Tris-HCl solution (pH 8.6) containing 3 mg EDTA-2Na and 0.72 g urea were added. The mixture was flushed with N_2 gas for 20 min and followed by addition of 0.5 mg iodoacetic acid in 20 μl of 1 N NaOH in order to carboxymethylate free sulfhydryl groups in FSG. After incubation for 30 min at room temperature, 2 μl of 2-mercaptoethanol and then 0.2 ml of 1 N NaOH which contained 4.86 mg of iodoacetic acid containing 50 μCi of [1-^{14}C]iodoacetic acid (17.9 mCi/mmol, New England Nuclear) were added to the solution. The mixture was incubated for 30 min at room temperature, dialyzed against 0.2 M acetic acid, then against DDW at 4°C, and lyophilized.

High performance liquid chromatography

High performance liquid chromatography (HPLC) was carried out using a Hitachi HPLC system L-6000-L-6200 equipped with a L-4200 UV-VIS Detector and a D-2500 Chromato-Integrator. A sample in 0.1 M sodium phosphate (pH 6.8) containing 0.1% SDS was applied onto a TSK G-6000 PW column (7.5×300 mm, Toyo Soda K.K.) or a TSK G-6000 PW column connected to a TSK G-4000 SW column, and eluted with 0.1 M sodium phosphate (pH 6.8) containing 0.1% SDS at a flow rate of 0.5 ml/min (for a single column system) or 0.25 ml/min (for double column system) at room temperature. The column effluent was monitored for an absorbance at 280 nm and protein and fucose concentrations.

SDS-PAGE

SDS-PAGE was carried out according to the method of Laemmli (26). Gels were stained by the method of Morrissey (32). For fluorography gel was stained according to the method of Fairbanks *et al.* (12), soaked in 100 ml of 1 M sodium salicylate (pH 6.0) for 30 min, dried with an aspirator at 75°C and exposed to a Kodak X-omat AR film at −70°C for a desired period.

Cellulose acetate electrophoresis

FSG (0.5 μg) in DDW was applied to a cellulose acetate strip (Separax, Joko Sangyo, Japan) and electrophoresed in 0.47 M formic acid in 0.1 M pyridine (pH 3.0) for 20 min or in 0.1 M barium acetate for 90 min at a constant current of 1 mA/cm. The strip was stained with 0.1% alcian blue in 50% ethanol, 50 mM $MgCl_2$ and 1% acetic acid (pH 3.0) for detection of sulfated fucan, and with 0.01% nigrosine in 6% trichloroacetic acid (TCA) for detection of protein. Standards (hyaluronic acid, chondroitin sulfate C and dermatan sulfate) for electrophoresis were obtained from Seikagaku Kogyo, Japan. Heparin was a product of Fluka

AG (Switzerland). For fluorography, the strip stained with alcian blue or nigrosine was dipped rapidly into 1 M sodium salicylate (pH 6.0), dried in air and exposed to a Kodak X-omat AR film at −70°C for a desired period.

Acrosome reaction

Dry sperm (3.5 mg wet weight/5 μl) were suspended and incubated in 0.5 ml of ASW (pH 8.2) containing various agents at 20°C for 30 sec. The spermatozoa were fixed with 2.5% glutaraldehyde in ASW (pH 8.2) and stained with 1% erythrosin in 50% ASW (pH 8.2) as reported previously (65). Acrosomal vesicles were observed as stained spots by the oil immersion method (64). Spermatozoa without a stained spot were regarded as having reacted. Counts were made of at least 100 spermatozoa in each assay.

Cyclic nucleotide determination

Dry sperm (10 mg wet weight) were suspended and incubated in 0.5 ml of ASW (pH 8.2) containing various agents at 20°C for 5 sec. The reaction was stopped by addition of 0.5 ml of 20% (w/v) TCA. Zero time cyclic nucleotide concentrations were determined in the samples prepared by adding spermatozoa to the incubation mixture containing 10% TCA. The samples were then centrifuged at 700×g for 20 min, and the supernatant was extracted four times with an equal volume of diethyl ether to remove TCA. The resultant aqueous layer was lyophilized and the residue was kept at −20°C until use. Cyclic AMP and cGMP were determined by radioimmunoassay using cAMP and cGMP assay kits (Yamasa Shoyu K.K., Chiba, Japan) as described previously (66).

Immunological methods

Purified FSG was dialyzed against DDW. The dialysate (20 μg protein/ml) was emulsified in complete Fruend's adjuvant (1/1, v/v). The emulsion containing 2 μg of protein was injected intracutaneously in the back neck of a Balb/c AnNCrj mouse (age, ten weeks) at six different sites. Subsequently the antigen (1 μg of protein) in complete adjuvant was administered at one week and three weeks after the first injection. Three weeks after the third injection, the mouse was bled from eyes with capillary, and the serum was stored at −70°C until use. Immunoelectrophoretic blotting using a nitrocellulose filter and immunodiffusion experiments with a cellulose acetate sheet were carried out by the method described by Tjian *et al* (57) and Towbin *et al* (59). Horseradish peroxidase-conjugated goat IgG anti-mouse IgG (Organon Teknika Corp, West Chester, PA, USA) was reconstituted before use according to the manufacturer's instruction.

Other methods

Amino acids were analyzed on a Hitachi L-8500 amino acid analyzer after hydrolysis *in vacuo* in constant-boiling HCl at 110°C for 24 hr. For amino acid analysis of a 260 kDa or 240 kDa protein, reduction-carboxymethylated FSG (CmFSG) was subjected to SDS-PAGE and then blotted to a polyvinylidene difluoride (PVDF) sheet (Millipore, 0.45 μm). The sheet having the corresponding protein was cut and put into a hydrolysis tube. Protein was determined by the method described by Schacterle and Pollack using bovine serum albumin as a standard (47). Fucose was estimated by the method of Dishe and Shettles using α-L-fucose as a standard (9). Sulfate was determined by the method of Terho and Hartiala with sodium sulfate as a standard (55). Sialic acid was determined by the method of Jourdian *et al* with N-acetylneuraminic acid as a standard (22). Sperm-isoagglutination was examined under a light microscope as described by Isaka *et al* (19).

Results

Sialoglycoprotein

A jelly solution which contained 35.5 μg/ml sialic acid and 22.5 μg/ml fucose was frozen and thawed, and then centrifuged. The supernatant containing 27.0 μg/ml sialic acid and 1.8 μg/ml fucose was applied to a Sepharose 2B column (Fig. 1). By this chromatography, sialic acid-containing material was separated from low molecular weight substances. Fractions which contained sialic acid were pooled and purified by chromatography on a DEAE-Sephadex A-25 column. In the chromatography, sialic acid-containing material was eluted with 0.5 M NaCl in 10 mM sodium acetate (pH 5.0), while fucose-containing material remained in the column. Sialic acid-containing material obtained from the ion-exchange chromatography was further purified by density gradient centrifugation (Fig. 2). The clear, bottom fraction with a density of 1.47 g/ml was dialyzed exhaustively against DDW and then lyophilized. The residue was placed in a desiccator over solid NaOH and phosphorus pentoxide (P_2O_5) and weighed (470 μg), and was dissolved in 0.5 ml of DDW. The solution contained 417.5 μg/0.5 ml of sialic acid and 41.9 μg/0.5 ml of pro-

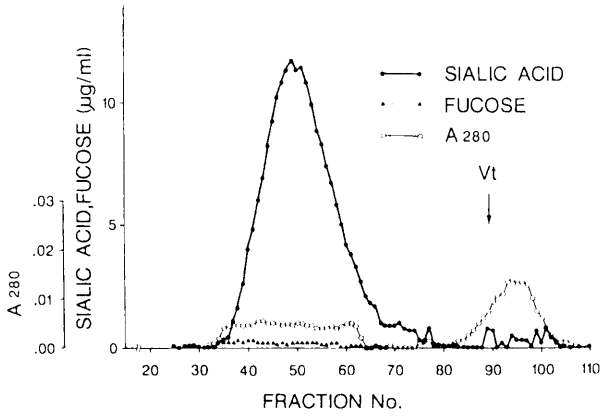

Fig. 1 Chromatography of the sialic acid-rich supernatant obtained from frozen-thawed egg jelly on a Sepharose 2B column. The column (5×88 cm) was equilibrated with 0.1 M NaCl and eluted with 0.1 M NaCl at a flow rate of 50 ml/hr at 4°C. Fractions of 20 ml were collected. The eluate was monitored for sialic acid, fucose and absorbance at 280 nm. Fractions (No. 39-64) were pooled and used for further purification.

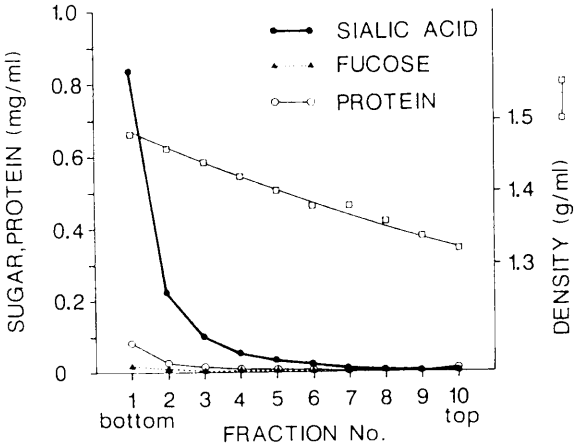

Fig. 2 CsCl density gradient centrifugation of sialoglycoprotein. See text for details.

tein. Practically no fucose was detected in the sample. Sialic acid-containing material thus purified migrated as a single, alcian blue-staining band on cellulose acetate strips during electrophoresis. These results indicate that the sialic acid-containing material is pure and consists of sialic acid (90%, w/w) and protein (10%, w/w).

Amino acid composition of the purified sialoglycoprotein, presented in Table 1, shows that Asx (Asp+Asn), Thr, Ser and Glx (Glu+Gln) predominated, followed by Pro, Gly, Ala, Ile and Leu. No cysteine or carboxymethylcysteine was detected in native and/or carboxymethylated sialoglycoprotein.

Fucose sulfate glycoconjugate

A jelly solution, which contained 68 mg pro-

Table 1. Amino acid compositions of sialoglycoprotein, carboxymethylated-sialoglycoprotein, FSG and CmFSG

	Sialoglycoprotein	Carboxymethylated-sialoglycoprotein (*)	FSG	CmFSG (*)
CmCys	0.0	0.0	0.0	2.0
Asp	11.8	10.1	14.4	14.2
Thr	11.1	9.1	10.0	9.8
Ser	15.2	16.7	7.8	7.5
Glu	14.2	15.4	9.9	9.6
Pro	7.7	6.3	2.3	2.3
Gly	6.3	10.1	8.3	8.7
Ala	5.4	6.4	6.7	6.5
Half-Cys	0.0	0.0	1.2	0.0
Val	3.2	3.2	7.9	7.9
Met	1.7	1.1	4.2	3.9
Ile	5.3	4.0	5.7	5.8
Leu	7.0	5.5	7.9	7.9
Tyr	2.4	2.0	2.1	2.5
Phe	1.3	1.6	4.8	4.9
Lys	2.2	2.5	2.9	3.0
His	3.0	3.7	2.0	2.0
Arg	2.1	2.4	2.4	2.4
Total	99.9	100.1	100.5	100.9

Amino acid compositions are expressed as mol%. Tryptophan was not determined. (*) Sialoglycoprotein and FSG were reduced with 2-ME and then carboxymethylated.

tein, 27 mg fucose and 23 mg sialic acid, was applied to a Sepharose 4B column equilibrated with ASW containing 10 mM sodium acetate buffer (pH 5.0). Fractions containing fucose, sialic acid and protein were pooled, dialyzed against 0.1 M NaCl and applied to a Sepharose 2B column equilibrated with 0.1 M NaCl. Fucose-containing material was eluted earlier than sialic acid-containing material as reported previously (65). Fractions which contained most of fucose were pooled (fucose-rich fraction). Solid NaCl was added to the fraction at a final concentration of 1 M, and the mixture was frozen and then thawed. The freezing-thawing made fucose-containing material insoluble in 1 M NaCl and about 80% of fucose in the original sample was recovered in the precipitate fraction by centrifugation at 10,000×g for 30 min. The precipitate thus obtained was easily dissolved in DDW but not in ASW or even in 4 M guanidine hydrochloride solution. The precipitate was dissolved in DDW, followed by addition of an equal volume of 0.2 M NaCl, and the mixture was submitted to gel filtration on a Sepharose 2B column. Fucose was co-eluted with materials having an absorbance at 280 nm. Fractions which contained fucose and protein were pooled (FSG) and used for further experiments. FSG possessed 2.0 times protein to fucose by weight when FSG was purified from a freshly prepared jelly solution, but it contained a little less amount of protein when it was purified from long time-stored jelly soltuion even if the jelly solution was kept at −40°C. However, FSG purified from either material contained one mol sulfate/mol fucose and practically no sialic acid. When FSG was analyzed by SDS-PAGE in the presence of 2-mercaptoethanol, two major protein bands (258 kDa and 237 kDa) and one broad protein band (120 kDa) were observed, and they were also major protein components of crude jelly. Among them a 120 kDa protein was silver-stained to be red. These three components, however, could not be detected in an electrophoretogram of SDS-PAGE of FSG without 2-mercaptoethanol (Fig. 3). An electrophoretogram of the fucose-containing fraction obtained from chromatography on a DEAE Bio-Gel A column was the same as that of FSG purified by chromatography on Sepharose CL-2B column. However, FSG which was purified from a jelly solution stored in a refrigerator or a freezer for long time without protease inhibitors showed a 210 kDa protein as a major band in addition to 258 kDa, 237 kDa and 120 kDa protein bands in SDS-PAGE, suggesting that some proteases which might come from eggs degrade

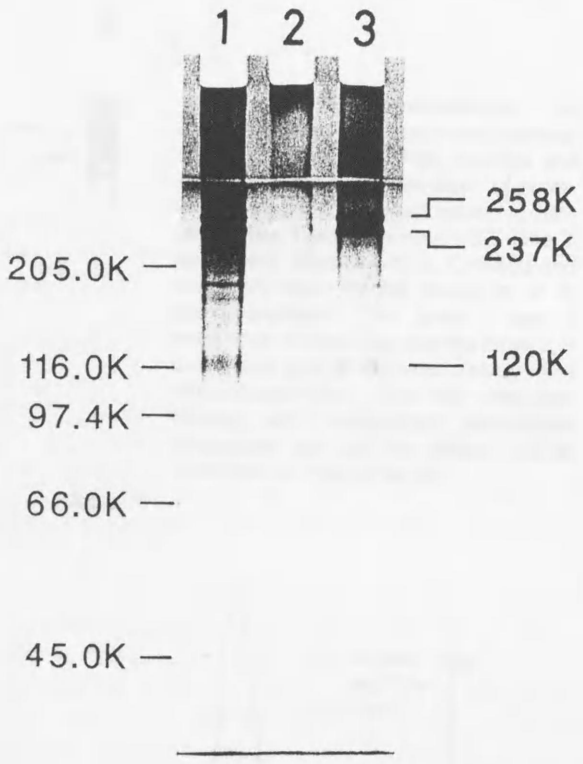

Fig. 3 SDS polyacrylamide gel electrophoresis of crude jelly and purified FSG. Approximately 2 μg of protein was loaded to each lene (lane 1: crude jelly with 2-mercaptoethanol; lane 2: FSG without 2-mercaptoethanol; lane 3: FSG with 2-mercaptoethanol). The gel was silver-stained according to the method of Morrissey (32).

proteins in FSG. However, purified FSG could be stored in 0.1 M NaCl at −70°C without any change in protein bands in SDS-PAGE for at least six months. In some cases, FSG, stored particularly in high concentration, formed precipitate but this precipitate could be completely dissolved in 0.1 M NaCl at any time.

For testing the specificity of anti-serum against FSG, crude jelly was electrophoresed on a polyacrylamide gel containing SDS and 2-mercaptoehtanol and then electrophoretically blotted to a nitrocellulose filter. The blots were reacted with mouse anti-serum against FSG, followed by treatment with horseradish peroxidase-conjugated goat IgG anti-mouse IgG and the color reaction. As shown in Fig. 4, two bands, which correspond to 258 kDa and 237 Da proteins, respectively, were detected on the filter. As expected, two colored bands, corresponding to 258 kDa and 237 kDa proteins, respectively, were detected on the filter when purified FSG was subjected to immunoelec-

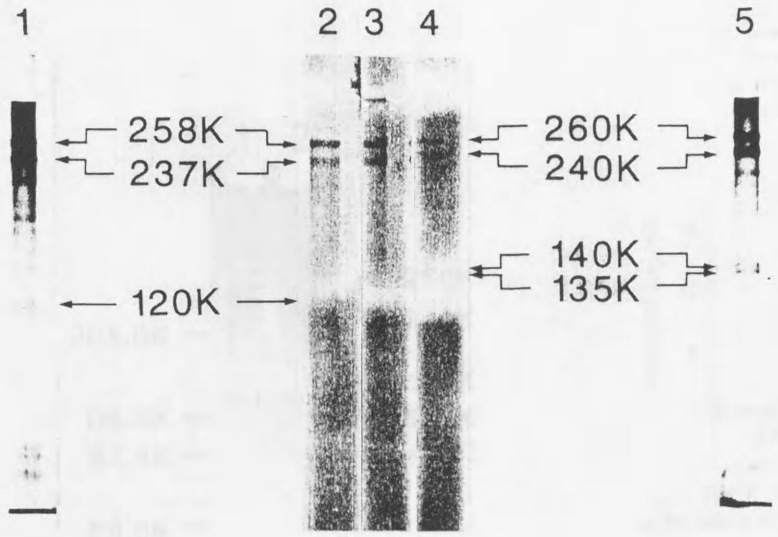

Fig. 4 SDS-polyacrylamide gel electrophoresis and immunolectrophoretic blotting of purified FSG, CmFSG and crude jelly of *H. pulcherrimus*. Approximately 2 μg of protein was loaded to each lane (lanes 1 and 2: purified FSG; lane 3: crude jelly; lanes 4 and 5: CmFSG) and electrophoresed in the presence of 2-mercaptoethanol. The lanes 1 and 5 were silver-stained (32) and the lanes 2, 3 and 4 were blotted electrophoretically to a nitrocellulose filter. The filter was then treated with horseradish peroxidase-conjugated goat IgG anti-mouse IgG as described by Tjian *et al* (57).

trophoretic blotting experiments in the same condition as above.

Earlier studies have reported that treatment of fucose sulfate complex with NaOH and $NaBH_4$ resulted in the release of most of the proteins associated with fucose sulfate polymers from the complex (48). However, it still remained to be proved whether the protein in FSG from *H. pulcherrimus* egg jelly is covalently bound to the sugar moiety of the conjugate. When FSG was applied to a Sepharose CL-2B column equilibrated with 7 M urea and 10 mM HEPES (pH 7.0) and eluted with the urea solution, most of the materials having an absorbance at 280 nm were co-eluted with fucose. FSG was also eluted with the protein as a single peack by HPLC on a TSK G-6000 PW column (Fig. 5). An electrophoretogram of SDS-PAGE of the peak fraction was the same as that of the original FSG. No protein band was detected in SDS-PAGE without 2-mercaptoethanol. When FSG treated with NaOH and $NaBH_4$ was subjected to the gel filtration on a Sepharose CL-2B column equilibrated with 7 M urea and 10 mM HEPES (pH 7.0) and eluted with the equilibration solution, fucose-containing material was eluted at almost the same fractions as intact FSG.

FSG was reduced with 2-mercaptoethanol, carboxymethylated with iodoacetic acid and then analyzed by SDS-PAGE. Four protein bands with apparent molecular masses of 260 kDa, 240 kDa, 140 kDa, and 135 kDa were detected on the gels regardless of the absence of 2-mercaptoethanol (Fig. 6). The differences of the apparent molecular masses between proteins of intact FSG and carboxymethylated FSG, estimated by SDS-PAGE

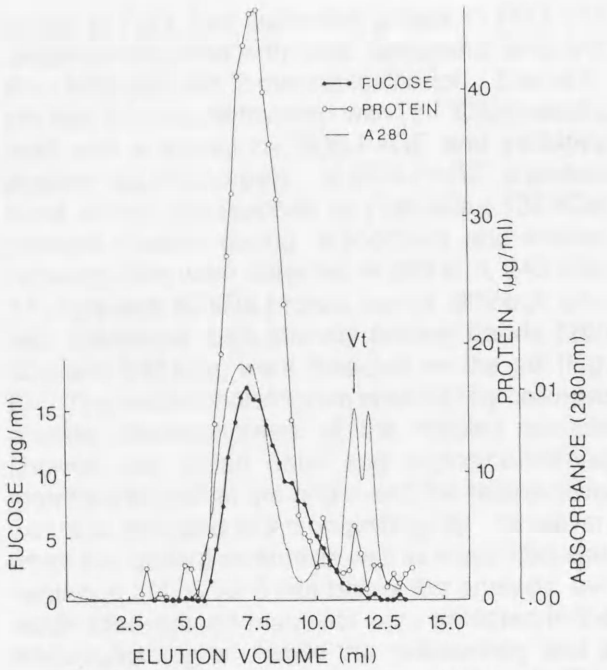

Fig. 5 HPLC profile of FSG on a TSK G-6000 PW column. The column was equilibrated with 0.1 M sodium phosphate (pH 6.8) containing 0.1% SDS and eluted with equilibration solvent at a flow rate of 0.5 ml/min at room temperature. Fractions of 0.5 ml were collected. The effluent was monitored for fucose, protein and absorbance at 280 nm.

with 2-mercaptoehtanol, is thought to result from different amount of SDS bound to these proteins. The 140 kDa and 135 kDa proteins seem to derive from the protein with 120 kDa detected in the electrophoretogram of intact FSG because of their same red staining nature as 120 kDa protein. When reduction-carboxymethylated FSG was subjected to the gel filtration on a Sepharose CL-2B

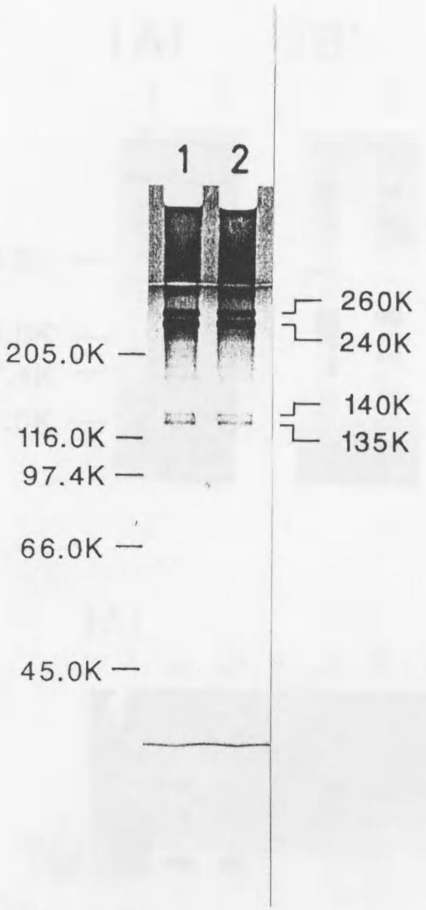

Fig. 6 SDS polyacrylamide gel electrophoresis of CmFSG. Approximately 2 μg of proteins was loaded to each lane (lane 1: without 2-mercaptoethanol; lane 2: with 2-mercaptoethanol). The gel was silver-stained by the method of Morrissey (32).

column equilibrated with 7 M urea and 10 mM HEPES (pH 7.0) and eluted with equilibration solution, fucose-containing material and most of protein originally associated with FSG were eluted together in almost the same fractions as in those obtained with intact FSG. However, when the CmFSG was subjected to HPLC, fucose-containing material, which possessed about 30% of protein originally associated with FSG, was separated from the rest of proteins (Fig. 7). The proteins separated from fucose-containing material were analyzed by SDS-PAGE. Four protein bands (260 kDa, 240 kDA, 140 kDa and 135 kDa) were deceted. These results suggest that 258 kDa, 237 kDa and 120 kDa protein components associated each other in intact FSG by disulfide bonds and reduction-alkylation released these components from FSG.

In order to confirm the presence of disulfide

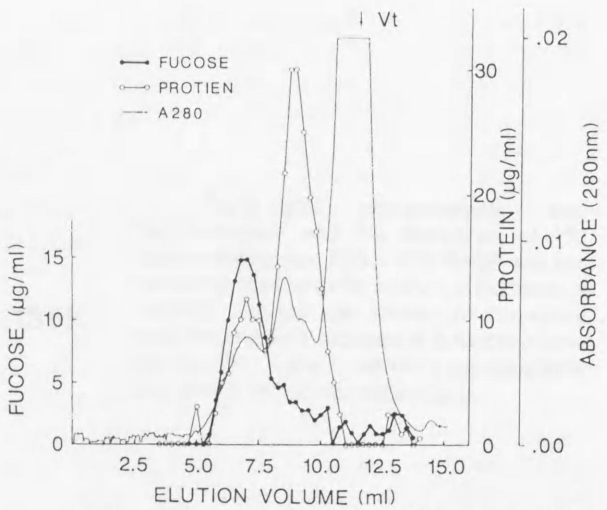

Fig. 7 HPLC profile of CmFSG. HPLC was carried out in the same condition as described in the legend for Fig. 5.

bonds in FSG, free sulfhydryl groups in FSG was carboxymethylated with 'cold' iodoacetic acid and then reduced with 2-mercaptoethanol. The sample was carboxymethylated with [1-^{14}C]iodoacetic acid and analyzed by SDS-PAGE and cellulose acetate electrophoresis. In SDS-PAGE, a protein band which corresponds to (140 kDa+135 kDa) proteins showed strong radioactivity and weaker radioactivities were detected in 260 kDa, 240 kDa, 110 kDa and 87 kDa protein bands although only two coomassie blue-stained protein bands (260 kDa and 240 kDa) were detected on the gel (Fig. 8). The electrophoretogram obtained by cellulose acetate electrophoresis of the labeled sample showed that alcian blue- and nigrosine-stained materials stayed at the origin and the radioactivity was also detected at the origin (Fig. 9). However, when the labeled sample as well as intact FSG was heated at 100°C for 5 min before the analysis, two alcian blue-stained materials were detected in the electrophoretogram, while the radioactivity and a nigrosine-stained material stayed at the origin (Fig. 9). Both an alcian blue-stained material and a nigrosine-stained material were reacted with mouse anti-serum agianst FSG.

Proteins associated with FSG were purified from CmFSG using HPLC on a TSK G-6000 PW column connected to a TSK G-4000 SW column system and/or SDS-PAGE (Fig. 10). Amino acid compositions of FSG, CmFSG, sugar-associated protein moiety of CmFSG, 260 kDa protein, 240 kDa protein, and 140 kDa plus 135 kDa proteins are shown in Tables 1 and 2. Purified FSG contained 1.2 mol% of cysteine of which value is not reliable because of breakdown during acid hyd-

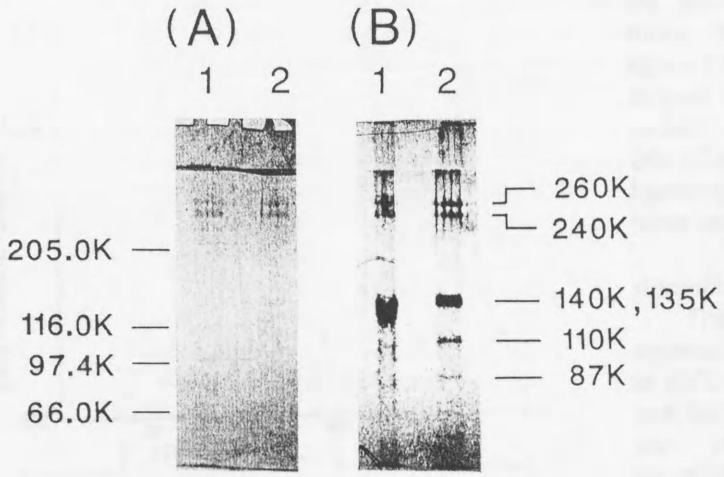

Fig. 8 SDS polyacrylamide electrophoretogram and the fluorogram of ^{14}C-carboxymethylated FSG. SDS-PAGE was performed according to the method of Fairbanks *et al* (12). The gel was stained by coomassie blue (A) and then exposed to a Kodak X-omat AR film (B). Lane 1: without 2-mercaptoethanol; lane 2: with 2-mercaptoethanol.

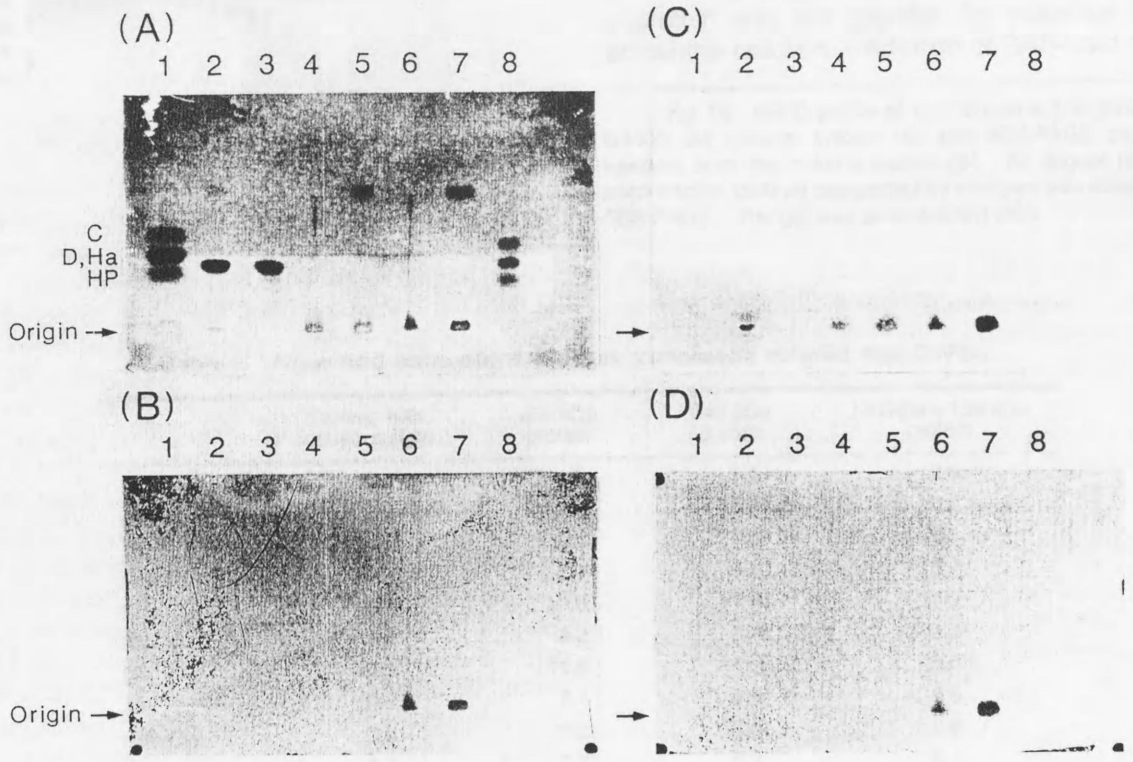

Fig. 9 Cellulose acetate electrophoretogram and the fluorogram of ^{14}C-carboxymethylated FSG. (A), Alcian blue-staining; (B), Fluorogram of the alcian blue-stained strip; (C), Nigrosine-staining; (D), Fluorogram of the nigrosine-stained strip. Lanes 1 and 8: standards, C: chondroitin sulfate C, D: dermatan sulfate, Ha: hyaluronic acid, Hp: heparin; lane 2: crude jelly; lane 3: sialoglycoprotein; lane 4: FSG; lane 5: FSG heated at 100°C for 5 min; lane 6: ^{14}C-carboxymethylated FSG; lane 7: ^{14}C-carboxymethylated FSG heated at 100°C for 5 min.

rolysis. When FSG was carboxymethylated without 2-mercaptoethanol, resultant carboxymethylated FSG contained 0.9 mol% of carboxymethylated cysteine (CmCys), while reduction-carboxymethylated FSG possesed 2.0 mol% of CmCys. However, none of major protein components (protein with fucose sulfate, 260 kDa and 240 kDa proteins) isolated from CmFSG of which cysteine residues have been completely carboxymethylated on the basis of total amino acid analy-

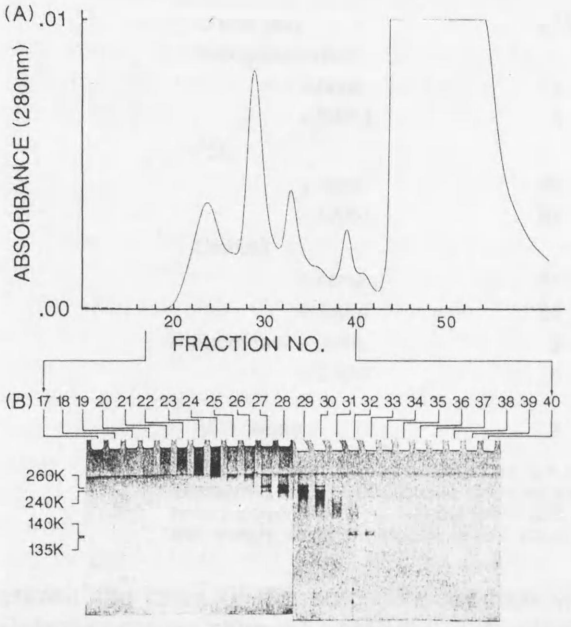

Fig. 10 HPLC profile of CmFSG on a TSK G6000 PW G4000 SW column system (A) and SDS-PAGE pattern of fractions from the column system (B). An aliquot (50 μl) of each fraction (500 μl) designated by numbers was subjected to SDS-PAGE. The gel was silver-stained (32).

sis after carboxymethylation of FSG contained more than 1.6 mol% CmCys although 140 kDa+135 kDa protein fraction contained the largest mol% of CmCys (3.6 mol%) and some of protein components (protein with fucose sulfate, 260 kDa protein and 140 kDa+135 kDa protein fraction) analyzed contained non-derivatized cysteine residues.

Acrosome reaction

The acrosome reaction of *H. pulcherrimus* spermatozoa occurred in crude jelly within 30 sec at 20°C and the number of the reacted spermatozoa did not increase significantly for further incubation. In some cases, prolonged incubation gave us difficulty to count the reacted spermatozoa because of their sticking nature. Therefore, we usually stopped the reaction at 30 sec unless mentioned. As shown in Table 3, purified sialoglycoprotein was not capable for induction of the acrosome reaction. Addition of SAP-I did not in-

Table 2. Amino acid compositions of protein components obtained from CmFSG

	Protein with fucose sulfate	260 kDa protein	240 kDa protein	140 kDa+135 kDa protein
CmCys	0.6	1.6	1.4	3.6
Asp	9.9	11.8	11.0	10.2
Thr	4.8	9.1	7.8	6.5
Ser	14.9	7.7	9.1	8.7
Glu	12.8	10.6	11.8	12.3
Pro	3.8	3.2	3.2	4.6
Gly	18.3	11.8	14.5	15.5
Ala	6.5	7.7	6.9	6.6
Half-Cys	0.3	0.2	0.0	0.6
Val	3.3	7.7	6.3	5.1
Met	1.6	2.2	1.4	1.5
Ile	2.4	5.1	4.3	3.9
Leu	4.9	7.7	6.9	6.2
Tyr	2.6	2.0	1.7	2.0
Phe	3.2	4.6	4.1	3.2
Lys	3.2	2.2	3.0	2.4
His	4.1	4.0	4.6	4.9
Arg	2.8	1.8	2.1	2.0
Total	100.0	101.0	100.1	99.8

Amino acid compositions are expressed as mol%. Tryptophan was not determined.

Table 3. The rates of the acrosome reaction and cyclic nucleotid levels in spermatozoa treated with crude jelly, FSG and CmFSG in the presence or absence of SAP-I

	Reacted sperm	cAMP[1]	cGMP[2]
Crude jelly	85%	26.7±7.3	907±66
Sialoglycoprotein			
+none	3	1.4±0.3	76±20
+SAP-I	5	4.7±0.9	1484±256
FSG			
+none	46	2.6±0.4	115±20
+SAP-I	81	12.9±1.7	1073±29
CmFSG			
+none	24	1.4±0.6	126±55
+SAP-I	54	9.3±0.9	917±200
Fucoidan +none	3	1.6±0.3	78±30
+SAP-I	6	5.6±1.7	1278±113
ASW alone	2	0.8±0.1	33±18

* Spermatozoa were incubated in 0.5 ml of ASW or ASW containing crude jelly (100 nmol fucose/ml), sialoglycoprotein (100 nmol sialic acid/ml), FSG (100 nmol fucose/ml) or CmFSG (100 nmol fucose/ml) with or without SAP-I (0.5 μM). 1. pmol/mg wet weight of spermatozoa, 2. fmol/mg wet weight of spermatozoa (Each value is mean±S.E. from four separate experiments).

crease the rates of the acrosome reaction with sialoglycoprotein although cAMP concentrations in spermatozoa increased by the addition of SAP-I. Purified sialoglycoprotein did not appear to cause sperm-isoagglutination of *H. pulcherrimus*. On the other hand, purified FSG induced the acrosome reaction although the rates were about half of that with crude jelly. The rates of the acrosome reaction induced by FSG in the presence of SAP-I were comparable values with crude jelly. FSG increased cAMP level more than about 3-fold of that without FSG, and FSG and SAP-I elevated cAMP level up to 16-fold. Fucoidan, a fucose sulfate polymer, did not cause elevation of cAMP concentration and induction of the acrosome reaction, while simultaneous addition of fucoidan and SAP-I increased cAMP concentration to the level with FSG. However, FSG with three protein bands (258 kDa, 237 kDa and 210 kDa) exhibited much less potency than FSG with two protein bands (258 kDa and 237 kDa) for induction of the acrosome reaction. Thus we always checked FSG by SDS-PAGE in the presence of 2-mercaptoethanol before starting experiments for induction of the acrosome reaction and used FSG with two protein bands.

The rates of the acrosome reaction induced in 30 sec by CmFSG was about half of those induced by intact FSG. Cyclic AMP concentration of spermatozoa treated with CmFSG was slightly lower than that with intact FSG. SAP-I increased the rates of the acrosome reaction with CmFSG two times as high as the rates with CmFSG. Addition of CmFSG and SAP-I elevated cAMP concentration to higher level than that with intact FSG alone (Table 3). However, prolonged incubation of spermatozoa with CmFSG in the presence or absence of SAP-I increased the number of the reacted spermatozoa. At 1 min, the rates of the acrosome reaction induced by CmFSG with or without SAP-I were comparable to those induced by intact FSG with or without SAP-I, respectively. In all cases,

Table 4. The rates of the acrosome reaction in spermatozoa treated with crude jelly or FSG in the presence of various agents

	Reacted sperm
Crude jelly	75%
FSG+α-L-fucose (50 nmol/ml)+SAP-I	60
FSG+α-L-fucose (200 nmol/ml)+SAP-I	64
FSG+fucoidan (50 nmol fucose/ml)+SAP-I	72
FSG+fucoidan (200 nmol fucose/ml)+SAP-I	79
FSG+D-galactosamine (200 nmol/ml)+SAP-I	71
FSG+D-mannosamine (200 nmol/ml)+SAP-I	67
FSG+D-glucosamine (200 nmol/ml)+SAP-I	59
FSG+sialoglycoprotein (50 nmol sialic acid/ml) +SAP-I	65
FSG+sialoglycoprotein (200 nmol sialic acid/ml) +SAP-I	81
ASW alone	3

Spermatozoa were incubated in 0.5 ml of ASW or ASW containing crude jelly (50 nmol fucose/ml), or FSG (50 nmol fucose/ml) with various agents in the presence of SAP-I (0.5 μM).

SAP-I elevated sperm cGMP concentrations. As shown in Table 4, α-L-fucose, fucoidan, amino sugars such as D-galactosamine, D-mannosamine and D-glucosamine and sialoglycoprotein, which were not capable for induction of the acrosome reaction by themselves, did not inhibit the ability of FSG to induce the acrosome reaction in the presence of SAP-I.

Discussion

We purified and characterized two major egg jelly macromolecules, a sialoglycoprotein and a fucose sulfate glycoconjugate, of *H. pulcherrimus* in the present study. Sialoglycoprotein seems to consist only of sialic acid and protein since dry weight of purified sialoglycoprotein was almost equal to weight of sialic acid plus protein, which were determined colorimetrically, and it did not contain measurable amount of fucose. It did not appear to cause sperm-isoagglutination of *H. pulcherrimus*. These results are not compatible with results of earlier studies (18, 19). We think that these incompatiblities might come from impurity of sialoglycoprotein used in earlier studies or use of different species of sea urchins. Sialoglycoprotein purified in the present study did not induce the acrosome reaction of *H. pulcherrimus* spermatozoa. This agrees with results of earlier studies.

Fucose sulfate glycoconjugate isolated from *H. pulcherrimus* contained a large amount of protein. The proteins associated with fucose sulfate was not removed even when FSG was dissolved in 7 M urea or 0.1% SDS and chromatographed on a Sepharose CL-2B or a TSK G-6000 PW column. Furthermore, no protein band was detected in the electrophoretogram of SDS-PAGE of intact FSG without 2-mercaptoethanol. These results suggest that the proteins may be covalently bound to the polysaccharide portion of FSG. When FSG was dissolved in SDS solution containing 2-mercaptoethanol and subjected to SDS-PAGE in the presence of 2-mercaptoethanol, two major (258 kDa and 237 kDa) and one minor (120 kDa) protein bands were appeared. Reduced and carboxymethylated FSG showed two major (260 kDa and 240 kDa) and two minor (140 kDa and 135 kDa) protein bands in the electrophoretogram of SDS-PAGE despite absence of 2-mercaptoethanol. These results indicate that FSG consists of fucose sulfate polymers and several protein components which associate each other with disulfide bonds. In this regard, it should be noted that when FSG was carboxymethylated with 'cold' iodoacetic acid and then reduced and carboxymethylated with ^{14}C-iodoacetic acid, most of radioactivity was detected in 140 kDa plus 135 kDa protein bands. Iodoacetic acid treatment of FSG without 2-mercaptoethanol resulted in carboxymethylation of about half of cysteine residues, indicating that about half of cysteine residues in FSG involved in formation of disulfide bonds. Furthermore, a protein with fucose sulfate, 260 kDa and 240 kDa proteins isolated from reduced-carboxymethylated FSG contained much less mol% of carboxymethylated cysteine than that of 140 kDa plus 135 kDa proteins. These results suggest that 140 kDa and 135 kDa proteins, which are probably the same protein(s) as detected in a 120 kDa protein band dected in SDS-PAGE of intact FSG with 2-mercaptoethanol have more sulfhydryl groups than 258 kDa and 237 kDa proteins available for disulfide bonds and may serve to make bridges between the polysaccharide-containing protein and other protein components.

As we reported previously (66), purified FSG induced the acrosome reaction, but the rates were about half of that with crude jelly. SAP-I promoted the rates of the acrosome reaction with FSG to values comparable with those induced by crude jelly. In the paper, we have shown that pronase-digestion of fucose-rich glycoconjugate which should be the same molecule as FSG in the present study resulted in a large reduction of the acrosome reaction-inducing capacity (66). Complete carboxymethylation of cysteine residues in FSG resulted in release of the major constituent proteins from FSG and an about 50% decrease in acrosome reaction-inducing capacity of FSG. This does not agree with the results of SeGall and Lennarz demonstrating that the acrosome reaction-inducing capacity of the egg jelly of four species of sea urchins resides solely in the fucose sulfate polysaccharide (48, 49), and suggests that at least in FSG of *H. pulcherrimus* the association of these proteins with fucose sulfate polymers is important for induction of the acrosome reaction. However, pronase-digested ARIS, a major acrosome reaction-inducing substance in starfish, is reported to be fully effective for induction of the acrosome reaction with Co-ARIS (31, 34, 35). At the present time, it is not clear whether or not the differences in the role of proteins associated with a major acrosome reaction-inducing substance are due to species variations.

Ishihara *et al* reported that the fucan sulfate isolated from *H. pulcherrimus* and *Pseudocentrotus depressus* egg jelly contained L-fucose-4-

sulfate (21). Fucoidan which possesses L-fucose-4-sulfate did not appreciably induce the acrosome reaction of *H. pulcherrimus* spermatozoa despite increase in cAMP concentration induced by SAP-I. L-Fucose and amino sugars showed almost no effect on induction of the acrosome reaction by FSG with SAP-I. These results suggest that the fucose sulfate and amino sugars in FSG may not play an important role in induction of the acrosome reaction and that cAMP is not involved in the acrosome reaction.

Sperm-activating peptides stimulate net H^+ efflux and enhance sperm cAMP and cGMP concentrations (14, 28, 41, 46, 54). A fucose sulfate-rich glycoconjugate causes Ca^{2+}-accumulation, elevates cAMP and induces the acrosome reaction (13, 25). In connection with this, it should be noted that monoclonal antibodies to a 210 kDa glycoprotein of *S. purpuratus* sperm plasma membrane cause an increase in intracellular Ca^{2+} of *S. purpuratus* spermatozoa and induce the acrosome reaction when the intracellular pH is increased with NH_4Cl (29, 37, 38, 60, 61, 62). The antibodies induce the cAMP-dependent phosphorylation of sperm histone H1 as occurs upon treatment of spermatozoa with egg jelly (16, 39, 40). However, ionophore A23187 which is known to induce the acrosome reaction and acts as a co-factor of pronase-digested FSG for induction of the acrosome reaction neither induces H1 phosphorylation nor increases cAMP concentration in sea urchin spermatozoa (8, 39, 66). These support the idea suggesting that cAMP is not directly involved in the acrosome reaction. Recently, Domino and Garbers reported that FSG induces the elevation of inositol trisphosphate (10). The elevation is dependent on external Ca^{2+}. The Ca^{2+} channel blockers, verapamil and nifedipine, inhibited increases in both inositol trisphosphate and cAMP. The products generated from stimulated PI turnover in sea urchin spermatozoa may be potential second messengers in induction of the acrosome reaction.

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Species specificity of the egg jelly molecule, a fucose sulfate glycoconjugate, in induction of the acrosome reaction of sea urchin spermatozoa

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ABSTRACT: Sea urchin egg jelly consists of a fucose sulfate glycoconjugate (FSG), a sialoglycoprotein and sperm-activating peptides (SAPs). FSG induces the acrosome reaction of sea urchin spermatozoa, and sperm-activating peptide I (SAP-I: Gly- Phe-Asp-Leu-Asn-Gly-Gly-Gly-Val-Gly) promotes the acrosome reaction as a specific co-factor of FSG. We isolated FSG from the egg jelly of four species of sea urchins, Hemicentrotus pulcherrimus, Pseudocentrotus depressus, Strongylocentrotus nudus and Anthocidaris crassispina. FSG from H. pulcherrimus possessed protein about two times more than fucose by weight. The protein/fucose ratio was about 0.5, 0.5, and 1.0 for FSG from P. depressus, S. nudus and A. crassispina, respectively. When FSGs from four species were analyzed by SDS-polyacrylamide gel electrophoresis under reduced conditions, they showed two major similar protein bands. However, polyclonal rabbit or mouse antibody raised against H. pulcherrimus FSG did not react with FSGs from other three species when they were examined by double diffusion test (Ouchterlony) and electroimmunoblotting. Among FSGs obtained from P. depress, S. nudus and A. crassispina tested, only FSG from P. depressus induced partially the acrosome reaction of H. pulcherrimus spermatozoa although it required about five times higher concentration of FSG than that of H. pulcherrimus. SAP-I promoted the rates of the acrosome reaction of H. pulcherrimus with FSG from P. depressus. FSGs from P. depressus, S. nudus or A. crassispina did not elevate cAMP levels in H. pulcherrimus spermatozoa.

1 INTRODUCTION

Sea urchin eggs are surrounded by a gelatinous matrix called the jelly coat. The jelly coat has been shown to induce the acrosome reaction of sea urchin spermatozoa, which was first described in detail by Dan (1952), and to activate sperm metabolisms. The jelly coat contains a fucose sulfate glycoconjugate (FSG), a sialoglycoprotein and sperm-activating peptides (SAPs)(Isaka et al. 1970, Ishihara et al. 1973, SeGall and Lennarz 1979, Suzuki et al. 1981, Suzuki 1989 & 1990, Garbers et al. 1982). FSG has been considered to be a major substance responsible for induction of the acrosome reaction of sea urchin spermatozoa. It increases intracellular calcium ion concentrations and elevates cAMP levels in sperm cells (Kopf & Garbers 1980). SAPs activate sperm lipid metabolism (Mita et al. 1990), stimulate net proton efflux (Repaske & Garbers 1983) and elevates cAMP and cGMP levels in sperm cells (Hansbrough & Garbers 1981). However, purified FSG was less active than the solubilized crude egg jelly in induction of the acrosome reaction, and addition of synthetic SAP-I to the reaction mixture of H. pulcherrimus spermatozoa with H. pulcherrimus FSG increased the rates of the acrosome reaction (Yamaguchi et al. 1988 & 1989). FSG of H. pulcherrimus is composed of a

sugar-containing core protein, a 258 kDa protein, a 237 kDa protein and a 120 kDa protein which may be associated with one another through disulfide bonds (Shimizu et al. 1990). Complete carboxymethylation of cysteine residues in H. pulcherrimus FSG resulted in the release of most of the constituent proteins from FSG and about a 50% decrease in the acrosome reaction-inducing capacity of FSG (Shimizu et al. 1990). This contribution describes species specificity of FSGs isolated from the egg jellys of four species of sea urchins in the order Echinoida.

2 MATERIALS AND METHODS

2.1 Materials

Artificial sea water (ASW) containing 454 mM NaCl, 9.7 mM KCl, 24.9 mM $MgCl_2$, 9.6 mM $CaCl_2$, 27.1 mM $MgSO_4$ and 4.4mM $NaHCO_3$ was prepared in the laboratory. SAP-I was synthesized for us at the Peptide Institute Inc., Osaka, Japan.

2.2 Preparation of solubilized egg jelly and purification of FSG

Sea urchins, H. pulcherrimus, P. depressus, S. nudus and A. crassispina, were collected at the coast near Noto Marine Laboratory. Eggs were collected in ASW without buffer after intracoelomic injection of 0.5M KCl. The egg suspension was adjusted to pH 5.0 with 0.1N HCl to solubilize the egg jelly layer and centrifuged at 600xg for 10 min at room temperature. The supernatant fluid was centrifuged at 10,000xg for 30 min at 4°C. The resulting supernatant fluid was dialyzed against 0.1M NaCl and then applied to a Sepharose 2B column (5x78 cm) equilibrated with 0.1M NaCl at 4°C. FSG was eluted earlier than both sialoglycoprotein and SAPs. Fractions containing FSG were pooled and used for experiments.

2.3 Acrosome reaction

Dry sperm (5 μ l) were added to 0.5 ml of ASW containing FSG and incubated at 20°C. After 30 sec of incubation, spermatozoa were fixed by addition of 0.5 ml of 5% glutaraldehyde in ASW and stained with 1% erythrosin in deionized and distilled water. The acrosomal vesicle was detected as a stained spot. Spermatozoa without the spot were regarded as having reacted. One hundred spermatozoa were counted in each assay.

2.4 Determination of cAMP concentrations

Five microliters of dry sperm were added to 0.5 ml of ASW containing FSG. The mixture was incubated for 5 sec at 20°C. The reaction was stopped by addition of 0.5 ml of 10%(w/v) trichloroacetic acid (TCA). The sample was then centrifuged at 700xg for 20 min, and the resulting supernatant was extracted four times with an equal volume of diethyl ether to remove TCA. The aqueous layer was lyophilized. The residue was kept at -70°C until use. Cyclic AMP was determined by radioimmunoassay using a cAMP assay kit (Yamasa Shoyu K.K., Chiba, Japan).

2.5 Other methods

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the method of Laemmli (1970). Gels were stained by the method of Morrissey (1981). Fucose content in samples was estimated by the method of Dische and Shettles (1951) using L-fucose as a standard. Sialic acid was determined according to the method of Jourdian et al.(1972) with N-acetylneuraminic acid as a standard. Protein was determined by the method of Schacterle and Pollack (1973). The concentration of SAP-I was estimated by the respiration-stimulating activity toward H. pulcherrimus spermatozoa using synthetic SAP-I as a standard as described previously (Yamaguchi et al. 1988). Polyclonal antibody against purified FSG of H. pulcherrimus was prepared as described previously (Shimizu et al. 1990).

3 RESULTS AND DISCUSSION

In previous study, we demonstrated that the egg jelly of H. pulcherrimus in a high ionic strength solution such as 1M NaCl raised precipitates upon freezing-thawing. The precipitates contained most of fucose but little sialic acid present in the original unfractionated solubilized egg jelly. The precipitates could be dissolved in 0.1M NaCl. Therefore, we sometimes used the precipitates for purification of FSG. However, the solubilized egg jellys from P. depressus, S. nudus and A. crassispina were not precipitated by freezing-thawing.

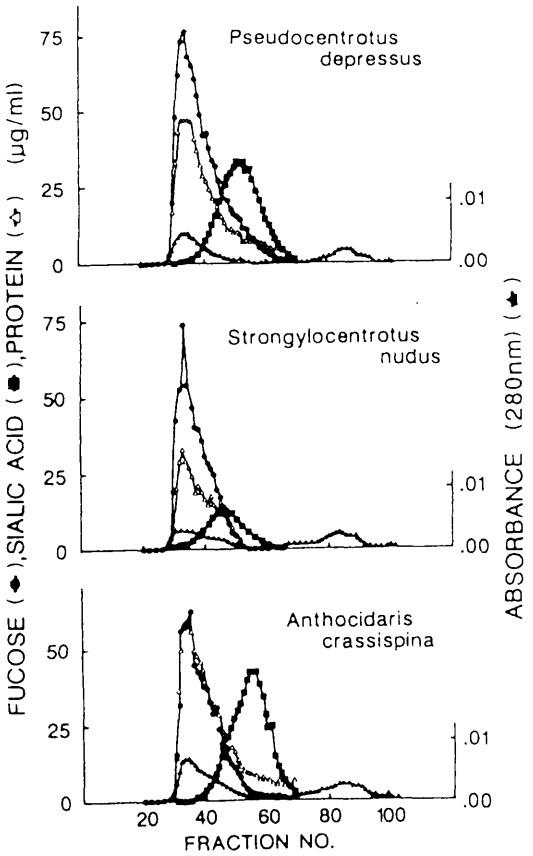

Fig.1 Gel filtration profiles of the solubilized egg jellys from P. depressus, S. nudus and A. crassispina on a Sepharose 2B column (5x79 cm) equilibrated and eluted with 0.1 M NaCl at a flow rate of 50 ml/hr at 4°C. Fractions of 20 ml were collected.

The solubilized egg jelly of P. depressus contained 100 μ g/ml fucose, 50 μ g/ml sialic acid and 70 μ g/ml protein, that of S. nudus possessed 65 μ g/ml fucose, 21.5 μ g/ml sialic acid and 39 μ g/ml protein, and solubilized A. crassispina egg jelly composed of 65 μ g/ml fucose, 65 μ g/ml sialic acid and 100 μ g/ml protein. To purify FSG from the egg jelly of each species, the solubilized egg jelly (200ml) was applied to a Sepharose 2B column (Fig.1). Fractions containing fucose and protein were pooled and used for experiments. The protein/fucose ratio of purified FSG was about 0.5 for P. depressus, 0.5 for S. nudus and 1.0 for A. crassispina. These values were lower than the value for FSG from H. pulcherrimus, that was 2.0 (Shimizu et al. 1990). When FSGs from P. depressus, S. nudus or A. crassispina were analyzed by SDS-PAGE in the presence 2-mercaptoethanol, two major proteins (225 kDa and 240 kDa for P. depressus, 230 kDa and 245 kDa for S. nudus, 220 kDa and 235 kDa for A. crassispina) were detected on the gels (Fig.2). They were also major protein components of respective unfractionated solubilized egg jelly (Fig.2).

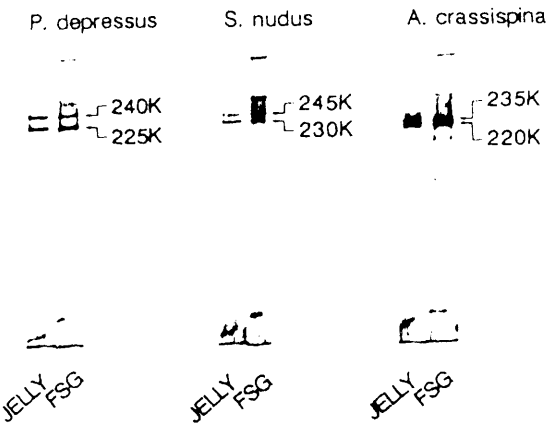

Fig.2 SDS-polyacrylamide gel electrophoresis of the solubilized egg jellys and FSGs purified from the solubilized egg jellys of sea urchins, P. depressus, S. nudus and A. crassispina. Approximately 2 μ g of proteins were loaded to each lane.

However, these proteins were not detected on the gels when SDS-PAGE was carried out without 2-mercaptoethanol. FSG isolated from the egg jelly of P. depressus, S. nudus or A. crassispina did not react with polyclonal antibody raised against H. pulcherrimus FSG when tested by Ouchterlony method (Fig.3) and electroimmunoblotting, suggesting some species specific differences in the structure of FSG.

As shown in Table 1, FSG purified from the solubilized egg jelly of H. pulcherrimus induced the acrosome reaction of H. pulcherrimus spermatozoa and SAP-I promoted the rates of the acrosome reaction as previously reported (Yamaguchi et al. 1988, Shimizu et al. 1990). When FSG isolated from the solubilized egg jelly of other sea urchin species was examined the capability for induction of the acrosome reaction of H. pulcherrimus spermatozoa, only P. depressus FSG induced the acrosome reaction although it required five times higher concentration of FSG (based on fucose concentration) than that of H. pulcherrimus FSG. SAP-I also promoted the rates of the acrosome reaction of H. pulcherrimus spermatozoa with P. depressus FSG. However, P. depressus FSG did not elevate cAMP levels in H. pulcherrimus spermatozoa. This suggests that the

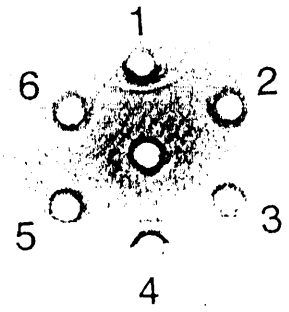

Fig.3 Immunodiffusion test of FSGs isolated from the solubilized egg jellys of H. pulcherrimus, P. depressus, S.nudus and A. crassispina. A mouse antiserum against H. pulcherrimus FSG was placed in the center well.
1,4: H. pulcherrimus FSG
2,5: P. depressus FSG
3: S.nudus FSG
6: A.crassispina FSG

Table 1. The rates of the acrosome reaction and cAMP concentrations in H. pulcherrimus spermatozoa treated with FSGs of various species of sea urchins with or without SAP-I.

	Reacted spermatozoa	cAMP (pmol/mg wet weight)
H. pulcherrimus FSG		
+ none	63%	17.7
+ SAP-I	90	34.4
P. depressus FSG		
+ none	32	1.31
+ SAP-I	61	2.51
S. nudus FSG		
+ none	2	1.05
+ SAP-I	7	4.79
A. crassispina FSG		
+ none	11	2.58
+ SAP-I	7	5.51
ASW alone	1	0.56

H. pulcherrimus spermatozoa were incubated in 0.5 ml of ASW containing FSG (50 nmol fucose/ml for H. pulcherrimus, 526 nmol fucose/ml for P. depressus, 517 nmol fucose/ml for S. nudus and 435 nmol fucose/ml for A. crassispina) with or without SAP-I (0.5 μ M).

induction of the acrosome reaction of H. pulcherrimus spermatozoa with P. depressus FSG may not be connected with elevation of cAMP levels in the cells.

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Expression of a putative precursor mRNA for sperm-activating peptide I in accessory cells of the ovary in the sea urchin *Hemicentrotus pulcherrimus*

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Abstract. A cDNA clone encoding egg-jelly peptide, SAP-I (Gly-Phe-Asp-Leu-Asn-Gly-Gly-Gly-Val-Gly), was isolated from a *Hemicentrotus pulcherrimus* ovary cDNA library and its nucleotide sequence was determined. The cDNA was 1282 bp long and an open reading frame predicted a protein of 334 amino acids containing 5 SAP-I and 7 SAP-I-like decapeptides, each separated by a single lysine residue. The cDNA hybridized to two species of mRNA (1.3 kb and 2.0 kb) from *H. pulcherrimus* ovaries. Northern blot analysis showed that the 1.3 kb transcripts appeared in ovaries collected from November to April and the 2.0 kb transcripts were detected only in ovaries collected in January. An expression study of the SAP-I precursor gene, by in situ hybridization with a non-radioactive RNA probe synthesized using the 1.3 kb cDNA as template, demonstrated that abundant SAP-I precursor transcripts were expressed in the accessory cells, but not in the growing oocytes.

Key words: Sea urchin – Sperm-activating peptide – Accessory cell – Oogenesis – In situ hybridization

Introduction

Sea urchin eggs are surrounded by a transparent extracellular matrix called the jelly coat, through which a spermatozoon must pass before coming into contact with the plasma membrane of the egg during fertilization. The jelly coat contains sperm-activating peptides (SAPs) that stimulate sperm respiration and motility under slightly acidic conditions. In the last decade, we have isolated 74 SAPs from the egg jelly of 17 species of sea urchins over 5 taxonomic orders, and have demonstrated that the biological effects of these peptides are essentially the same, although the biological effects and structures are specific to taxonomic order (for review see Suzuki and Yoshino 1992). Sperm-activating peptide I (SAP-I, Gly-Phe-Asp-Leu-Asn-Gly-Gly-Gly-Val-Gly) was the first oligopeptide to be isolated from the jelly coats of *Hemicentrotus pulcherrimus* and *Strongylocentrotus purpuratus* (Suzuki et al. 1981; Garbers et al. 1982). In addition to stimulation of respiration and motility, SAP-I has other biological effects on sea urchin spermatozoa such as increasing cyclic AMP and cyclic GMP levels (Garbers et al. 1982), activation in a Na^+/H^+ exchange system, and increasing intracellular pH (Repaske and Garbers 1983; Lee and Garbers 1986) and Ca^{2+} levels (Schackmann and Chock 1986). These facts suggest that the peptide plays an important role in the process of sea urchin fertilization.

In this study, we isolated cDNA clones encoding SAP-I and its derivatives from a *H. pulcherrimus* ovary cDNA library, and showed that the mRNA for SAP-I and its derivatives was exclusively detected in the accessory cells of the ovary.

Materials and methods

Materials. Sea urchins (*H. pulcherrimus*) were collected monthly along the coast of Toyama Bay near the Noto Marine Laboratory. The ovaries were dissected out from the animals as described previously (Suzuki et al. 1982). Restriction enzymes, T4 DNA ligase, and other enzymes were purchased from Takara Shuzo Co.; the cDNA Synthesis Kit and Hybond-N membrane filters were obtained from Amersham; the Random-primed DNA labelling Kit, Dig-RNA Labelling Kit, and Dig-detection Kit were purchased from Boehringer Mannheim; peroxidase conjugated anti-mouse IgG was purchased from Cappel (West Chester, Pa.); a putative *S. purpuratus* SAP-I precursor cDNA (2.3 kb) was a generous gift from Dr. Chodavarapu S. Ramarao of Vanderbilt University (Nashville, Tenn.); the plasmid pBluescript II Ks(+), pBluescript II KS(−), and M13K07 helper phage were generously provided by Dr. Yoshitaka Nagahama at the National Institute for Basic Biology, Okazaki, Japan.

Preparation of RNA and construction of a H. pulcherrimus cDNA library. Total RNA was prepared from various samples of ovaries, testes and embryos by the LiCl method (Cathala et al. 1983). Poly(A)$^+$ RNA was then purified from the total RNA using oligo(dT) cellulose (Pharmacia) column chromatography (Davis et al. 1986).

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Using poly(A)$^+$ RNA from *H. pulcherrimus* ovaries, cDNA was synthesized using oligo (dT) primed and random hexanucleotide-primed cDNA. The cDNA library was constructed in λ gt11 and contained 70% recombinants that represented approximately 1.0×10^6 independent clones.

Isolation of cDNA clones and DNA sequencing. Approximately 3.1×10^4 clones of the amplified cDNA library were screened with a ^{32}P-labelled random-primed cDNA for the putative *S. purpuratus* SAP-I precursor. Forty-four initially positive clones were purified through tertiary screening. DNA from positive recombinant phages was isolated and the cDNA inserts were subcloned into pBluescript II KS(+). DNA sequencing was performed using an Exo III/Mung Bean Nuclease Deletion System (Takara Shuzo Co.; Henikoff 1984), sequenced in both directions by the dideoxy chain termination method (Sanger et al. 1977) using a Sequenase Kit (U.S. Biolabs) and 7-DEAZA Sequence Kit (Takara Shuzo Co.), and analysed on DANASIS software (Hitachi Softoware Engineering Co.).

Northern blot analysis. A 2 µg sample of poly(A)$^+$ RNA was denatured with formaldehyde, electrophoresed on 1% agarose gel in the presence of formaldehyde, and transferred onto a Hybond-N membrane. The RNA on the membrane was then hybridized to the random-primed [α-^{32}P]dCTP-labelled cDNA insert at 37° C for 16 h. The filter was washed three times with 2 × standard saline citrate (SSC) and 0.1% sodium dodecyl sulphate (SDS) at room temperature (RT) for 20 min each, followed by a final wash in 0.1 × SSC, 0.1% SDS at 50° C for 15 min.

Histological observation of ovaries. The ovaries were fixed in Bouin's fluid, dehydrated in an alcohol series and embedded in paraffin. Sections (4 µm thick) were cut and stained with hematoxylin and eosin. The diameter of oocytes and mature ova were measured on sections per 1 mm^2 in five ovaries.

Respiration-stimulating activity. All samples of ovaries collected monthly were dissected out and stored at −80° C. A 0.1 g sample of ovary was homogenized in 1 ml 70% ethanol using a Teflon-glass homogenizer and then centrifuged at 10000 *g* for 30 min at 4° C. The respiration-stimulating activity of the sample was polarographically determined with *H. pulcherrimus* spermatozoa using an oxygraph (Yanagimoto PO-100A) equipped with a 3 ml capacity chamber fitted with a Clark-type electrode, as reported previously (Suzuki et al. 1981). The oxygen concentration in artificial sea water (ASW) was taken as 234 nmol O_2/ml ASW at 20° C.

Expression of glutathione S-transferase fusion proteins. Fresh overnight cultures of *Escherichia coli* (NM522) transformed with pGEX-2T recombinants and pGEX-2T were diluted 1:10 in Luria-Bertani medium containing ampicillin (50 µg/ml) and incubated for 5 h at 37° C with shaking. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM. After 7 h incubation at 37° C, the bacterial culture was pelleted by centrifugation at 5000 rpm for 10 min at 4° C, resuspended in 4% SDS and electrophoresed. The gel was stained with Coomassie brilliant blue. The 60 kDa fusion protein and 26 kDa glutathione S-transferase (GST) bands were then cut out and eluted electrophoretically with a Max-Yield Protein Concentrator (Atto Co.), dialysed against distilled water, and lyophilized. The residue was dissolved in a minimum volume of distilled water.

Immunological methods. The 60 kDa protein (500 µg/ml) was emulsified in complete Freund's adjuvant (1/1, v/v), and an emulsion containing 50 µg of protein was injected intracutaneously into the back of a Balb/c AnNCrj mouse (age, 9 weeks). Subsequently, the antigen (25 µg) in complete adjuvant was administered 2 and 3 weeks after the first injection. One week after the third injection, the mouse was bled. The antiserum specific to the 34 kDa SAP-I precursor protein was prepared as follows: a mixture of 100 µl antiserum (1:10 dilution) and 100 µg 26 kDa protein (GST) was incubated overnight at 4° C and centrifuged. The supernatant was used as the absorbed antiserum.

Immunoblotting was carried out as described by Towbin et al. (1979). Proteins in the gel were transferred electrophoretically to a nitrocellulose filter using the Pharmacia Multiphor II electrophoresis system for 1 h at 200 mA constant current. Protein reacting with antibody was detected by the ECL Western Blotting Detection System (Amersham). An enzyme-linked immunosorbent assay (ELISA) was carried out according to the procedures of Voller et al. (1976). Protein measurements were determined by the method of Peterson (1977).

In situ hybridization. The 1.3 kb cDNA insert was used as a template to synthesize a single strand digoxigenin deoxyuridine triphosphate-(dUTP)-labelled RNA probe (Angerer et al. 1987). *H. pulcherrimus* ovaries at different stages were fixed for 2 h in 2% glutaraldehyde in phosphate-buffered saline (PBS), dehydrated, cleared in xylene, and embedded in paraffin. Sections were cut and mounted on a glass microslide coated with 3-aminopropyltriethoxysilane (Shinetu Chemical. Co.). In situ hybridization was performed according to a modified version of Tautz and Pfeifle (1989). Digoxigenin-labelled RNA probe (0.1 to 0.5 µg/ml) was added to the hybridization buffer [300 mM NaCl, 5 mM ethylenediaminetetraacetic acid (EDTA), 1 × Denhardt's solution, 10% dextran sulphate, 1 mg/ml yeast tRNA, 50% formamide and 10 mM TRIS HCl, pH 8.0]. Hybridization was carried out overnight (18 h) in a humidified chamber at 50° C with a baked coverslip on the slide.

The coverslip was removed by shaking slowly in 6 × SSC and sections were washed for 1 h at 45° C in solution A (50% formamide/2 × SSC). This was followed by two 5 min-washings in solution B (500 mM NaCl, 10 mM TRIS HCl, pH 8.0) at RT. The sections were subsequently incubated in 20 µg/ml RNase A in solution B for 30 min at 37° C, washed for 1 h in solution A and for another 1 h in solution C (50% formamide/1 × SSC) at 45° C.

Detection of in situ hybridized mRNA was performed using a Dig Detection Kit (Boehringer Mannheim). Following post-hybridization washes, the slide was incubated in buffer 1 (150 mM NaCl, 100 mM TRIS HCl, pH 7.5) and buffer 2 (0.5% Boehringer blocking reagent/buffer 1) for 1 h each. The slide was then incubated in a diluted (1:1000) anti-digoxigenin antibody conjugated to alkaline phosphatase for 1 h at RT. The slide was washed three times in buffer 1 for 15 min and once in buffer 3 (100 mM NaCl, 50 mM $MgCl_2$, 100 mM TRIS HCl, pH 9.5). Colour development was initiated by adding 150 µl of a solution containing X-phosphate and nitroblue tetrazolium salt and allowed to proceed for 10 h in a humidified chamber. The reaction was terminated by adding buffer 4 (1 mM EDTA, 10 mM TRIS HCl, pH 8.0) and the slide was mounted with a coverslip using glycerol.

Results

Isolation of cDNA clones and characterization of the 1.3 kb insert

Using random-primed *S. purpuratus* SAP-I precursor cDNA as a probe, 17 positive clones were obtained from about 3.1×10^4 recombinants. The clones were digested with Eco RI to yield a 1.3 kb insert in 13 clones, a 1.1 kb insert in 3 clones and a 2.0 kb insert in 1 clone. Restriction mapping showed that the 13 clones with an insert of 1.3 kb were identical and the 3 clones with an insert of 1.1 kb were truncated versions of the 1.3 kb insert (data not shown).

The nucleotide sequence and the deduced amino acid sequence of the 1.3 kb insert are shown in Fig. 1. The 1.3 kb insert was 1282 bp long, and open reading frames beginning with methionine at nucleotide position 92 or 215 and extending through to a termination signal at

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CGTTCAATCGAAGCTGCTACTCGACCTTACGCTAGATCTCTACCGAGCTTTCTCGTCGAATAGAACAACCGACCAACCCACGCCGTTCAAG ATG ACG TTC ATC  103
                                                                                                 Met Thr Phe Ile    4

GTA TGC CTC TTG GTC CTC GTG GCC GTA GCT TCA GGC AAG CCC GTC ATC AGC GGT CAT GAC CAG AAG TAC ACC CTT GCT GAT  184
Val Cys Leu Leu Val Leu Val Ala Val Ala Ser Gly Lys Pro Val Ile Ser Gly His Asp Gln Lys Tyr Thr Leu Ala Asp   31

TCC AGC AAG GAC CTA GAG GGC ATT GCT CAC ATG GCT ATC GTC GAC TCA CTT TCG CCT CTA CAC ATC TCG CTC TCA TCT TTG  265
Ser Ser Lys Asp Leu Glu Gly Ile Ala His Met Ala Ile Val Asp Ser Leu Ser Pro Leu His Ile Ser Leu Ser Ser Leu   58

GAA TCA GCG TGG AAT AAC CTC GTC AGC ATT GCT ACT CAG GAG AAA TAC ATT ATC CCG GAA CTG TCC ATA CCC AAA ATC GAC  346
Glu Ser Ala Trp Asn Asn Leu Val Ser Ile Ala Thr Gln Glu Lys Tyr Ile Ile Pro Glu Leu Ser Ile Pro Lys Ile Asp   85

GTG AAG AGC ATC TTG ACA TGC AAG CCC AAG TAT TCT CCA AAG TAC CCT GTG GTC TTG CAG TAC ATC TCT GAT CAT CAA GTC  427
Val Lys Ser Ile Leu Thr Cys Lys Pro Lys Tyr Ser Pro Lys Tyr Pro Val Val Leu Gln Tyr Ile Ser Asp His Gln Val  112

CAG GTC CAG GAC CAT ATC GCT AAC GCC AAT GAA CTT GTA GAA GGG CTG AAA TTC GTC TCG CAG CTC ATC ATG TAC AAG AAG  508
Gln Val Gln Asp His Ile Ala Asn Ala Asn Glu Leu Val Glu Gly Leu Lys Phe Val Ser Gln Leu Ile Met Tyr Lys Lys  139

GTC GAC CAT GAT ACC CTG GCC TCG GTC TCT AAA ATG TTG AGC GAT TAC TTG ACT GAC TAC GCA TCG ACC ATC TCA TCT ATT  589
Val Asp His Asp Thr Leu Ala Ser Val Ser Lys Met Leu Ser Asp Tyr Leu Thr Asp Tyr Ala Ser Thr Ile Ser Ser Ile  166

AAA TCG GTC GTC TGT CAG GAT CCA ACA GCG CCC TCT CAT CCT ATG GCC GAG AGT TAC ATG GAC ACA CCC CTG TCA ATG TTC  670
Lys Ser Val Val Cys Gln Asp Pro Thr Ala Pro Ser His Pro Met Ala Glu Ser Tyr Met Asp Thr Pro Leu Ser Met Phe  193

TTG AAA GGC ACC ATG CCT ACT GGT GGT GGA GTT GAA AAA AGC TTT GCA CTT GGC GGC GGT GGA GTT GGC AAA GGA TTC GAC  751
Leu Lys Gly Thr Met Pro Thr Gly Gly Gly Val Glu Lys Ser Phe Ala Leu Gly Gly Gly Gly Val Gly Lys Gly Phe Asp  220

TTG AAC GGC GGT GGA GTT GGC AAG GGC TTT GAC TTG AAC GGC GGT GGA GTT GGC AAA GGA TTT GAC TTG AAC GGC GGT GGA  832
Leu Asn Gly Gly Gly Val Gly Lys Gly Phe Asp Leu Asn Gly Gly Gly Val Gly Lys Gly Phe Asp Leu Asn Gly Gly Gly  247

GTT GGC AAA GGC TTT GAC TTG AAC GGC GGT GGA GTT AGC AAA GGA TTC GAG TTG AAC GGC GGT GGA GTT GGC AAA GGC TTT  913
Val Gly Lys Gly Phe Asp Leu Asn Gly Gly Gly Val Ser Lys Gly Phe Glu Leu Asn Gly Gly Gly Val Gly Lys Gly Phe  274

GAC TTG AAC GGC GGT GGA GTT GGC AAA GGA TTC GAC TTG ACC GGC GGT GGA GTT GGC AAA GGC TTT GAC TTG AAC GGC GGT  994
Asp Leu Asn Gly Gly Gly Val Gly Lys Gly Phe Asp Leu Thr Gly Gly Gly Val Gly Lys Gly Phe Asp Leu Asn Gly Gly  301

GGA GTT GGC AAA GGT TTC GCT TTG GGC GGC GGT GGA GTT GGC AAA GGA TTC AGC CTC ACC GGA GGA AGC GTC GAC AGG GAG 1075
Gly Val Gly Lys Gly Phe Ala Leu Gly Gly Gly Gly Val Gly Lys Gly Phe Ser Leu Thr Gly Gly Ser Val Asp Arg Glu  328

GTC GAA ACT GAA GGA TGG TAA TCGACGTTGATTATCTGGCGCTCCATAGATCTGACATCACAATGCCGTGCGCCTGTACACATAATTCGCTAACTGCCAA 1175
Val Glu Thr Glu Gly Trp •••                                                                                  334

TTTTACACGGATTTTAATACATTGATCGTTTAAAACGTTCAAGTGAAACCTGTCACATAATACAAATATGTAATAAAATAATAGCATCAAATGCAAAAAAAAAAAAA 1282
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Fig. 1. Complete nucleotide sequence and deduced amino acid sequence of the 1.3 kb insert. Amino acid sequence deduced from an open reading frame is shown *below* the nucleotide sequence. The two potential start codons are marked with *arrows*. Sperm-activating peptide I (SAP-I) and other possible sperm-activating peptides are boxed. The 'AATAAA' polyadenylation signal is *underlined* and the termination codon is *boxed*

1094–1096 represented an apparent precursor polypeptide which contained five decapeptides with a sequence corresponding to SAP-I. Seven other decapeptides with the sequence (Gly-Phe-Asp-Leu-Thr-Gly-Gly-Gly-Val-Gly, Gly-Phe-Asp-Leu-Asn-Gly-Gly-Gly-Val-Ser, Ser-Phe-Ala-Leu-Gly-Gly-Gly-Gly-Val-Gly, Gly-Phe-Ser-Leu-Thr-Gly-Gly-Ser-Val-Asp, Gly-Phe-Ala-Leu-Gly-Gly-Gly-Gly-Val-Gly, Gly-Phe-Glu-Leu-Asn-Gly-Gly-Gly-Val-Gly, Gly-Thr-Met-Pro-Thr-Gly-Gly-Gly-Val-Glu) were also present in the apparent precursor. Each decapeptide was separated by a single lysine residue. The deduced amino acid sequence also contained another decapeptide (Val-Asp-His-Asp-Thr-Leu-Ala-Ser-Val-Ser) bordered by lysine but having little similarity to SAP-I. One polyadenylation signal sequence AATAAA was present at nucleotide position 1247–1252.

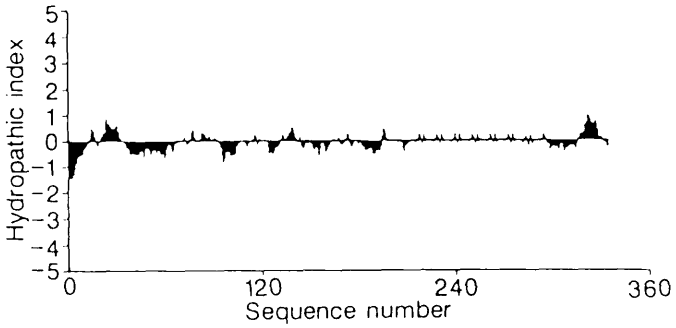

Fig. 2. Hydropathicity of the SAP-I precursor protein. Hydropathy was calculated at a span of 12 residues according to Kyte and Doolittle (1982). Regions with values below the midpoint line are hydrophobic

Assignment of the start codon

The 5′ region of the 1.3 kb insert contained two possible in-frame translation start sites at nucleotide positions 92 and 215: CAAGATGA, TCACATGG (see Fig. 1). The initiation codon was assigned to the ATG at position 92 for several reasons. This codon was the first in-frame ATG from the 5′ end. Most 5′ end untranslated regions of eukaryotic genes range from 40–80

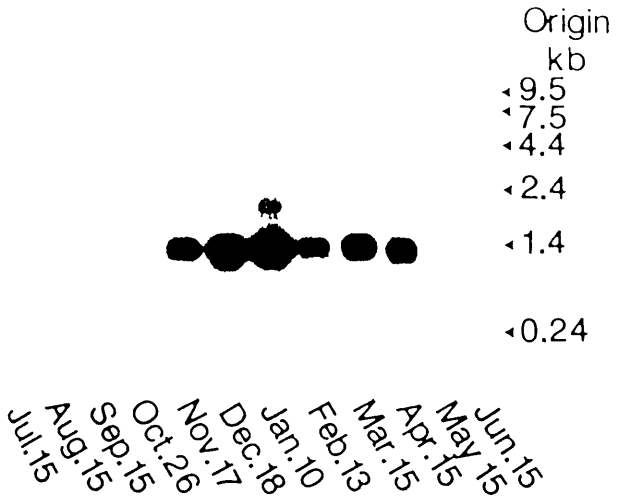

Fig. 3. Annual cycle of SAP-I precursor mRNA accumulation in the ovaries during maturation. Northern blot analysis was carried out with poly(A)$^+$ RNA (2 μg) prepared from the ovary samples collected throughout the year and with the 1.3 kb insert as probe

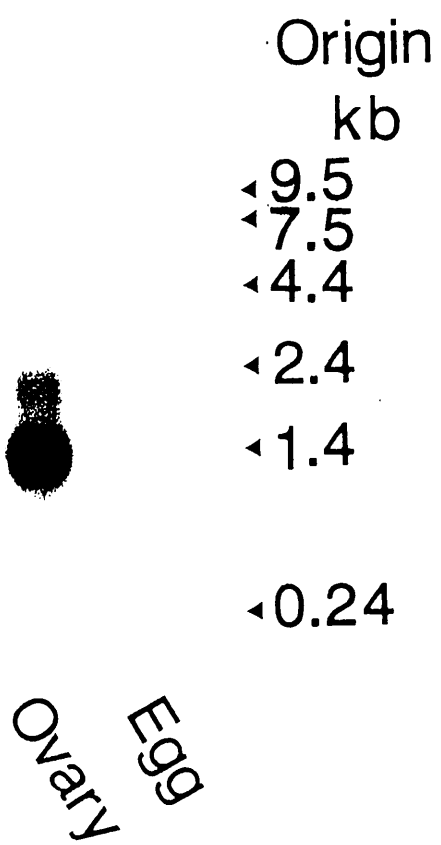

Fig. 4. Analysis of *Hemicentrotus pulcherrimus* ovary and egg mRNA by Northern blot hybridization with the 1.3 kb insert as probe

nucleotides (Kozak 1983). The sequence CAAGATGA was similar to the initiation consensus sequence $^{C}/_{A}AA^{A}/_{C}ATG$ described by Carvener (1987) for *Drosophila*. The protein resulting from initiation at this site would have a molecular weight of 34273. The initiation codon was followed by a sequence coding for a putative signal peptide (von Heijne 1986) with a central hydrophobic core as indicated by the hydropathy analysis (Fig. 2). The first 14 residues of the amino terminus of the full-length protein were quite hydrophobic. According to the (−3, −1)-rule method of von Heijne (1986), we identified a potential peptidase cut site after the 22nd amino acid.

Northern blot analysis

To determine the size of the mRNA for SAP-I and to know when the mRNA was synthesized in the ovary, poly(A)$^+$ RNA obtained from ovary samples collected throughout the year was analysed by Northern blot hybridization using the 1.3 kb insert as a probe (Fig. 3). Among poly(A)$^+$ RNA prepared from ovaries collected from November to April, only 1.3 kb mRNA was detected, and a strong hybridization signal was observed with the poly(A)$^+$ RNA from ovaries collected in December and January. A weak hybridization signal with the 2.0 kb mRNA was additionally observed with poly(A)$^+$ RNA from an ovary collected in January. SAP-I precursor cDNA (1.3 kb) did not detectably hybridize mRNA from eggs (Fig. 4), embryos or testes (data not shown) of *H. pulcherrimus*, or the *Clypeaster japonicus* ovary (data not shown).

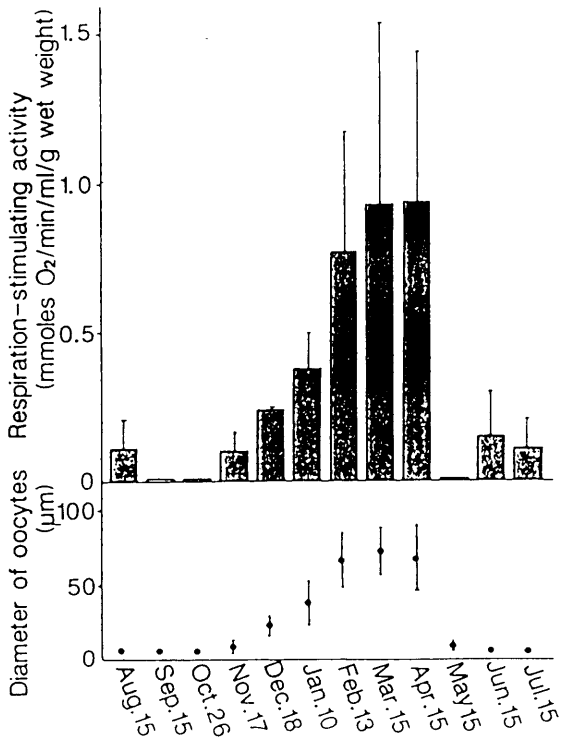

Fig. 5. Annual cycle of diameter of oocytes and respiration-stimulating activity in extracts from ovaries of *H. pulcherrimus*. Five female individuals were collected each month and their ovary samples were tested for respiratory stimulation. *Each bar* represents the mean and standard error of five tests

Diameter of oocyte and respiration-stimulating activity

Figure 5 shows the mean size of oocytes and respiration-stimulating activity using extracts from ovarian samples taken throughout the year to determine when the final bioactive peptides appear. Extracts prepared from ovaries in May, September and October showed no significant respiration-stimulating activity. The activity increased from November (pre-breeding season) and reached a peak between February and April (breeding season). After spawning, the activity fell down to zero in May. Accessory cells began to grow in August and oocytes started to grow in November when the ovaries were filled with accessory cells. In December and January, numerous large oocytes (40–80 μm in diameter) projected markedly into the acinous lumen from the ovarian wall. Ovaries were filled with mature ova between February and April. These results indicate that respiration-stimulating activity increases in the ovary with the maturation of oocytes.

Expression of the 1.3 kb clone in E. coli

In order to ascertain the stimulation of spermatozoa by 1.3 kb cDNA-expressed products, an expression vector was constructed as described in "Materials and methods". The synthesis of an abundunt GST fusion protein was observed (data not shown). The molecular mass of this recombinant protein was about 60 kDa which is in reasonable agreement with the value calculated for the recombinant vector (34 kDa 1.3 kb cDNA expressed-products plus 26 kDa GST protein). The fusion protein was cut out and electroeluted from a polyacrylamide gel to bioassay the respiration-stimulating activity. The synthetic SAP-I (3.5×10^{-9} M) stimulated sperm respiration up to the level obtained at pH 8.2 (3489 nmol O_2/min/ml·g wet weight). The 60 kDa fusion protein (2.4×10^{-7} M) stimulated respiration to about 20% of the level obtained at pH 8.2 (668 nmol O_2/min/ml·g wet weight spermatozoa). The 26 kDa GST protein (3.2×10^{-7} M) also stimulated sperm respiration slightly (219 nmol O_2/min/ml·g wet weight spermatozoa), however, the respiration-stimulating activity of the two proteins was signif-

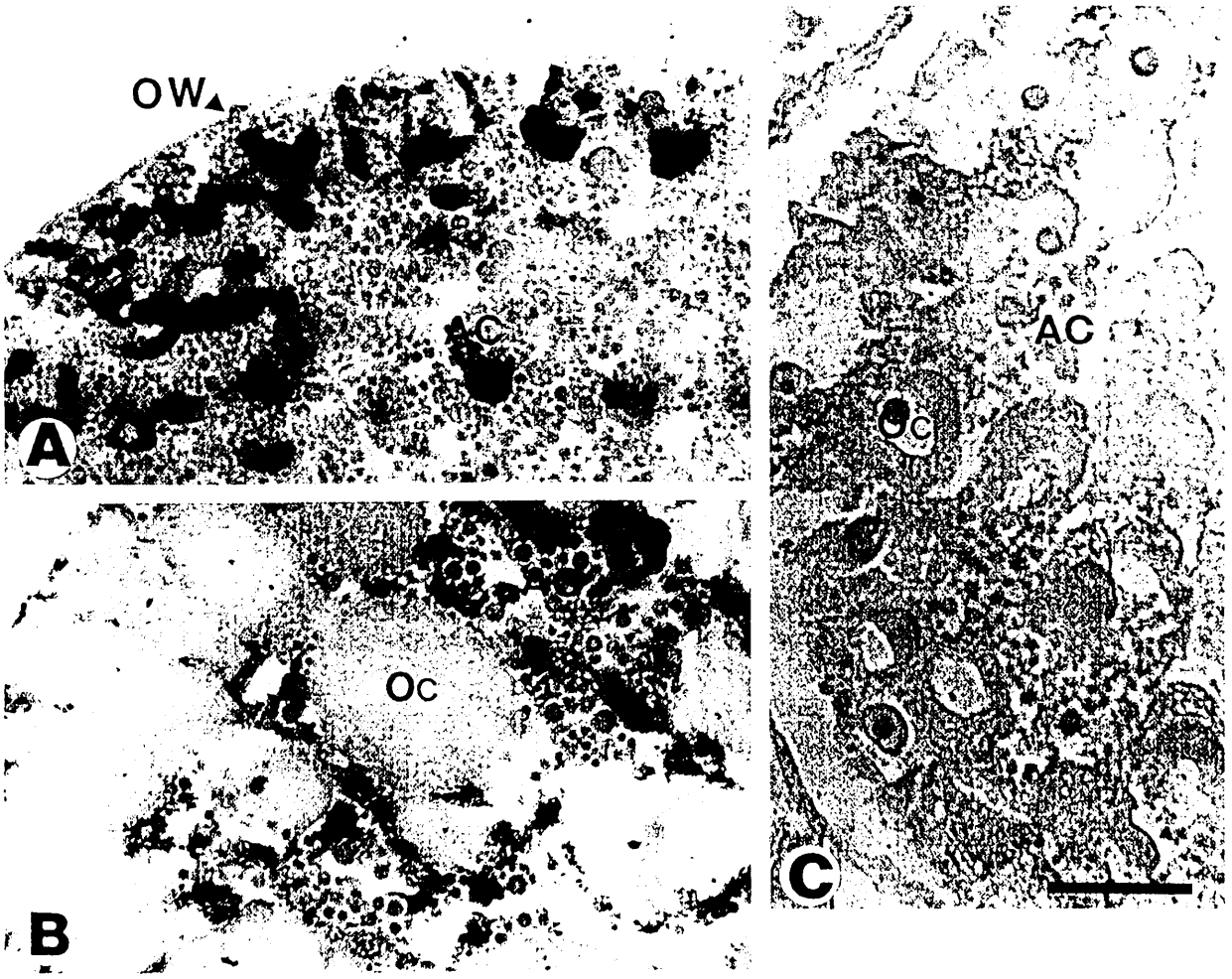

Fig. 6A–C. Localization of the SAP-I precursor mRNA by in situ hybridization with Dig-RNA probes. The ovaries were hybridized with the antisense (A, B) or sense probes (C) and processed with an alkaline phosphatase-conjugated antibody. Transcripts are present in cytoplasm of accessory cells (*AC*). However, they are not present in the oocytes (*Oc*) or ovarian wall (*OW*). A Prebreeding season (December), B, C breeding season (February). *Bars*, 100 μm

icantly different (for the 60 kDa protein, 668 ± 120 nmol O_2/min/ml·g wet weight, $n=7$ and for the 26 kDa protein, 219 ± 59 nmol O_2/min/ml·g wet weight, $n=5$; $t=2.95$, $df=10$, $P<0.05$). The untreated antiserum against the 60 kDa fusion protein, which had a half-maximum absorbance titer of 9.1×10^3 dilution as measured by an ELISA, reacted specifically to the 60 kDa fusion protein. However, the absorbed antiserum which had a half-maximum absorbance titer of 4.8×10^3 dilution, did not react specifically to an expected SAP-I precursor (34 kDa) in *H. pulcherrimus* ovaries collected in December and February.

In situ hybridization with SAP-I precursor RNA probes

Using a plasmid containing the 1.3 kb cDNA for the putative SAP-I precursor, we made digoxigenin-labelled RNA transcripts in both sense and antisense orientations. Glutaraldehyde-fixed paraffin-embedded ovaries collected in December and February were sectioned and hybridized with each probe. The sense strand showed no cell-specific hybridization (Fig. 6C). In marked contrast, the antisense probe hybridized strongly to the cytoplasm of the accessory cells (Fig. 6A, B). Staining was not significant in the cytoplasm of oocytes or in the ovarian epithelial and smooth muscle cells.

Discussion

In this paper we described the isolation and sequence of a cDNA clone encoding a putative SAP-I precursor from a cDNA library for *H. pulcherrimus* ovary, and the site of accumulation of SAP-I precursor mRNA during *H. pulcherrimus* oogenesis using in situ hybridization. Our results indicate that the SAP-I gene is transcribed in the accessory cells, but not in the growing oocytes. In our knowledge this is the first report which demonstrates accessory cell-specific gene expression in the sea urchin ovary.

The jelly envelopes of amphibian eggs (for review see Katagiri 1987) and the oviductal glycoprotein of the zona pellucida of ovulated eggs in mammals (Oikawa et al. 1988; Abe and Oikawa 1990, 1991) appear to be secreted by the oviductal epithelial cells. Concerning the sea urchin, there has been a paper dealing with the origin of the jelly coat through ultrastructural and histochemical studies. In this paper, the authors concluded that the egg jelly components were synthesized in the oocytes (Verhey and Moyer 1967). Recently, we purified a fucose sulphate glycoconjugate (FSG) which is a major component of egg jelly in the sea urchin *H. pulcherrimus* (Shimizu et al. 1990) and demonstrated that the antiserum raised against purified FSG strongly reacted with the globules in the accessory cells (Abe et al. 1992). In the present study we demonstrated that mRNA for the putative SAP-I precursor was detected only in the cytoplasm of the accessory cells in the pre-breeding (Fig. 6A) and breeding season (Fig. 6B). Northern blot analysis

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H.p. 1.3 kb  MTFIVCLLVLVAVASGKPVISGHDQKYTLADSSKDLEGIAHMAIVDSLSP   50
S.p. 2.3 kb        MPPGPRGVASGKPVISGQDQKYTLADSSKDLEGIAHMAIVDSLSP   45
                   *******           *

H.p. 1.3 kb  LHISLSSLESAWNNLVSIATQEKYIIPELSIPKIDVKSILTCKPKYSPKY  100
S.p. 2.3 kb  LHISLSSLESAWNNLLNIASQEDYSIPELSIPKIDVKSILSCKPKYSPKY   95
                             **  *  **              *

H.p. 1.3 kb  PVVLQYISDHQVQVQDHIANANELVEGLKFVSQLIMYKKVDHDTLASVSK  150
S.p. 2.3 kb  PVVLQYISDHYVQVQDHIANAKELTEGLKFVSQLIMYKKIDHDTLASVSK  145
                         *           *           *

H.p. 1.3 kb  MLSDYLTDYASTISSIKSVVCQDPTAPSHPMAESYMDTPLSMFLKGTMPT  200
S.p. 2.3 kb  MLSNYLTDYASTISSLKSVVCQDQTAPSHPMDESYMDTPLSMILKGTMPT  195
               *           *           *           *           *

H.p. 1.3 kb  GGGVGKGFALGGGGVGKGFDLNGGGVGKGFDLNGGGVGKGFDLNGGGVGK  250
S.p. 2.3 kb  GGGVDKGFALGGGGVGKGFNLNGGGVGKGFDLNGGGVGKGFDLNGGGVGK  245
                 * *           *

H.p. 1.3 kb  GFDLNGGGVSKGFELNGGGVGKGFDLNGGGVGKGFDLTGGGVGKGFDLNG  300
S.p. 2.3 kb  ----------------------GFDLNGGGVGKGFDLNGGGVGK------  267
                                                 *

H.p. 1.3 kb  GGVGKGFALGGGGVGKGFSLTGGSVDREVETEGW   334
S.p. 2.3 kb  -----GFALGGGGVGKGFSLTGGGVGREVEIKDW   296
                           *     ***
```

Fig. 7. Comparison of deduced amino acid sequence of the 1.3 kb clone in *H. pulcherrimus* (*H.p.*) and the 2.3 kb clone in *Strongylocentrotus purpuratus* (*S.p.*). The 2.3 kb clone is devoid of 3 decapeptides. *Asterisks* denote differences in the amino acid sequence

Table 1. Amino acid sequences of potential sperm-activating peptides predicted from the nucleotide sequence of cDNA isolated from *H. pulcherrimus* (*H. p*) and *S. purpuratus* (*S. p*) cDNA libraries

Sequence	Number of copies	
	H. p 1.3 kb	S. p 2.3 kb
Gly-Phe-Asp-Leu-Asn-Gly-Gly-Gly-Val-Gly	5	4
Gly-Phe-Asp-Leu-Thr-Gly-Gly-Gly-Val-Gly	1	
Gly-Phe-Asp-Leu-Asn-Gly-Gly-Gly-Val-Ser	1	
Ser-Phe-Ala-Leu-Gly-Gly-Gly-Gly-Val-Gly	1	
Gly-Phe-Ser-Leu-Thr-Gly-Gly-Ser-Val-Asp	1	
Gly-Phe-Ala-Leu-Gly-Gly-Gly-Gly-Val-Gly	1	
Gly-Phe-Glu-Leu-Asn-Gly-Gly-Gly-Val-Gly	1	
Gly-Thr-Met-Pro-Thr-Gly-Gly-Gly-Val-Gly	1	
Val-Asp-His-Asp-Thr-Leu-Ala-Ser-Val-Ser	1	
Gly-Phe-Asn-Leu-Asn-Gly-Gly-Gly-Val-Gly		1
Gly-Phe-Ser-Leu-Thr-Gly-Gly-Gly-Val-Gly		1
Gly-Thr-Met-Pro-Thr-Gly-Ala-Gly-Val-Asp		1

showed that the 1.3 kb cDNA did not hybridize to mRNA from the eggs of *H. pulcherrimus* (Fig. 4). These results strongly suggest that the accessory cells serve as the site for accumulation of the SAP-I precursor mRNA.

The deduced amino acid sequence of the putative SAP-I precursor contained multiple copies of SAP-I and SAP-I derivatives (Table 1). Among them, SAP-I and four other peptides (Gly-Phe-Asp-Leu-Thr-Gly-Gly-Gly-Val-Gly, Gly-Phe-Asp-Leu-Asn-Gly-Gly-Gly-Val-Ser, Ser-Phe-Ala-Leu-Gly-Gly-Gly-Val-Gly, Gly-Phe-Ser-Leu-Thr-Gly-Gly-Ser-Val-Asp) have been biochemically isolated from the jelly coat of *H. pulcherrimus*, and they have been reported to possess equivalent biological activity to SAP-I (Suzuki et al. 1981, 1988). As shown in Fig. 7, we found a considerable similarity (92.5%, 286 out of 309 residues are identical) between the amino acid sequence deduced from the 1.3 kb clone from *H. pulcherrimus*, and the 2.3 kb clone from *S. purpuratus* (Ramarao et al. 1990). The difference was that the 1.3 kb clone contained three additional SAP-I and SAP-I derivatives and lacked 1159 bp from the 3′-untranslated region. Furthermore, there is a common structural feature in these two precursors, each decapeptide is separated by a single lysine residue. This suggests that these precursors may be cleaved by a specific endoprotease at these sites to produce biologically active decapeptides. It has been reported that many peptide hormones and neuropeptides are initially synthesized as larger precursor molecules, with proteolytic cleavage at two basic amino acids resulting in the release of active hormones or neuropeptides (Steiner et al. 1967). However, processing of a precursor molecule at the position of a single basic amino acid has also been described (Schwartz 1986). Processing after a single lysine residue has been documented in two cases. In the polyvalent precursor for FMRF-amide, which contains at least 19 copies of this peptide, it is assumed that monobasic cleavage takes place in at least 15 places, followed by removal of lysine and transformation of the exposed COOH-terminal glycine to the amide group (Schaefer et al. 1985). The cholecystokinin precursor may also be cleaved after a single lysine, giving rise to cholecystokinin-22 (Zhou et al. 1985). A similar processing mechnism may be postulated for the SAP-I precursor. At the present time, however, we do not know the mechanism by which the SAP-I precursor is processed, nor how SAP-I and its derivatives are secreted.

By Northern blot analysis, we detected the 1.3 kb and the 2.0 kb mRNA in *H. pulcherrimus* ovary but could not detect the hybridized mRNA in *Clypeaster japonicus* ovary. Ramarao et al. (1990) also detected two hybridized bands (2.3 kb, 1.2 kb) of mRNA in three species of sea urchin (*S. purpuratus*, *Lytechinus pictus*, *S. franciscanus*) using the 2.3 kb insert as a probe. An explanation for the existence of two transcripts may be genetic variation among sea urchin individuals or alternative mRNA splicing. The egg jelly of *C. japonicus* does not contain SAP-I and SAP-I derivatives but it does contain SAP-III (Asp-Ser-Asp-Ser-Ala-Gln-Asn-Leu-Ile-Gly) and SAP-III derivatives (Suzuki et al. 1987; Yoshino et al. 1990). This may provide some insight as to why the 1.3 kb probe did not hybridize to mRNA from the *C. japonicus* ovary.

The 60 kDa recombinant protein which consisted of a 34 kDa SAP-I precursor and a 26 kDa GST stimulated *H. pulcherrimus* sperm respiration significantly but not maximally. This implies the possible need for correct processing of the precursor molecule. Antiserum to the 60 kDa fusion protein reacted with the protein, but did not react with an expected 34 kDa SAP-I precursor in the ovary. In this connection, it may be important to mention that in pulse-chase experiments of proinsulin with insulin-producing cells, the radioactivity incorporated into the prohormone form initially is almost all converted into fragments after a 60–90 min chase period (Schwartz 1986). Considering this fact, failure to detect the SAP-I precursor in ovary proteins of *H. pulcherrimus* may be due to very fast turnover of the SAP-I precursor to SAP-I.

Evaluating previous histochemical studies and this in situ hybridization study, we conclude that the accessory cell-specific accumulation of the egg jelly component or its precursor can serve as a useful system for further studies to elucidate the function of accessory cells.

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[RAPID COMMUNICATION]

Nucleotide Sequence of the Proton ATPase Beta-Subunit Homologue of the Sea Urchin *Hemicentrotus pulcherrimus*[1]

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ABSTRACT—A cDNA with 2.3 kb encoding F_1-F_0 ATP synthase (proton ATPase) beta-subunit homologue was isolated from a testis cDNA library of the sea urchin, *Hemicentrotus pulcherrimus*. The deduced amino acid sequence consisted of 523 residues which contained a 19-residue amino-terminal signal peptide and a 8-residue glycine-rich consensus sequences. Analysis of poly(A)$^+$ RNA and/or total RNA from *H. pulcherrimus* testis, ovary, unfertilized eggs, and embryos by Northern blot revealed a 2.4 kb RNA.

INTRODUCTION

A sperm-activating peptide (SAP-I: GLy-Phe-Asp-Leu-Asn-Gly-Gly-Gly-Val-Gly), isolated from the egg jelly of sea urchins, *Hemicentrotus pulcherrimus* [13] and *Strongylocentrotus purpuratus* [3], increases sea urchin sperm respiration rate and motility. It induces a Na^+-dependent net proton efflux and raises the intracellular pH [10]. As the result SAP-I stimulates sperm energy metabolism which depends on the oxidation of endogenous phosphatidylcholine [8]. ATP synthesis by oxidative phosphorylation is a multistep membrane-located process that occurs in the inner membranes of mitochondria. F_0-F_1 ATP synthase (proton ATPase) in membranes of mitochondria synthesizes ATP coupled with an electrochemical gradient of protons generated by the electron transfer chain. The enzyme from many different sources have been studied extensively at the molecular biological level [2]. However, no molecular biological study has been made on the enzyme from spermatozoa of any kind of animals.

In this study, we screened a *H. pulcherrimus* testis cDNA library with oligonucleotide probes synthesized based on the amino acid sequence of peptide obtained from the protease V8 digest of wheat germ agglutinin (WGA)-binding protein of *H. pulcherrimus* spermatozoa and isolated a cDNA encoding the beta-subunit homologue of mitochondrial F_1-F_0 ATP synthase. Here, we report that the cDNA is 2259 bp long and an open reading frame predicts a protein 523 amino acids.

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[1] The nucleotide sequence data reported in this paper will appear in the DDBJ, GenBank and EMBL Nucleotide Sequence Databases with the following accession number D17361.

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MATERIALS AND METHODS

Cloning and sequencing of cDNA

A cDNA library (4.9×10^5 pfu) from poly(A)$^+$ RNA isolated from growing testes of the sea urchin *H. pulcherrimus* was constructed in λ gt10 using the cDNA Synthesis System and the cDNA Cloning System λ gt10 (Amersham International plc., Amersham, UK). A 220 kDa WGA-binding protein was purified from *H. pulcherrimus* spermatozoa by affinity chromatography on a WGA-Sepharose 4B column as described previously [12], and digested by protease V8. The partial amino acid sequence of a peptide purified from the digest by preparative SDS-gel electrophoresis was determined to be V-S-S-*I-D-N-I-F-R*-V. The sequence indicated by italics was the same as the conserved sequence found in F_1-F_0 ATP synthase beta-subunit from various sources. Based on the sequence of the decapeptide, the mixed oligonucleotides (5′-GACACGGAAGATGTTGTCGATGCTGCTGAC-3′/5′-GACACGGAAGATGTTGTCGATAGAGGAGAC-3′) were synthesized and used to screen. Forty-six positive hybridizing clones were isolated from approximately 6×10^4 recombinants. Restriction endonuclease mapping of the inserts indicated that five different types of clones had been isolated. The insert of 2.3 kb from one member of the largest group in which fifteen clones belong was subcloned into the plasmid vector Bluescript II KS(+) (Stratagene, La Jolla, CA, USA) for further analysis. Serial deletion mutants of subclones were made according to Yanisch-Perron *et al* [16]. Nucleotide sequences were determined by the dideoxy chain termination method [11] using the Sequenase Kit (United States Biochemical Co., Cleaveland, OH, USA) and the 7-DEAZA Sequencing Kit (Takara Shuzo Co., Kyoto, Japan) analyzed on DANASIS software (Hitachi Software Engineering Co., Yokohama, Japan).

Northern blot analysis

Total RNA was prepared from testes, ovaryies, unfertilized eggs, and embryos of *H. pulcherrimus* by the LiCl method of Cathala *et al* [1]. Poly(A)$^+$ RNA was prep ared by two passage of the total RNA over a column of oligo(dT)-cellulose (Pharmacia LKB Biotechnology, Uppsala, Sweden). Northern blot analysis was carried out as follows: 2–5 μg of poly(A)$^+$ RNA or total RNA was denatured

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5'CGTGACCCCTGGAAGAATTTCACATCGCCATGTTTAGCAGGGTTGCAAAGACGAGTTTTTCGGCCGTAAGGGCTGCAAAATCACAATTT   89
    *                  M  F  S  R  V  A  K  T  S  F  S  A  V  R  A  V  A  K  S  Q  F          20

TCACACTCATTATCACAACAGACGAGTAAAACATGGGTACCAGCAGCAACTTGTAGCAAAAGATCATATGCTGCTGAGGCAAAGACGTCG  179
 S  H  S  L  S  Q  Q  T  S  K  T  W  V  P  A  A  T  C  S  K  R  S  Y  A  A  E  A  K  T  S    50

GCAGCCCCAGTTTCGGGTCAGATCGTAGCTGTCATTGGAGCTGTCGTCGACGTTCAGTTCGAGGATGACCTCCCACCCATTCTCAATGCC  269
 A  A  P  V  S  G  Q  I  V  A  V  I  G  A  V  V  D  V  Q  F  E  D  D  L  P  P  I  L  N  A    80

TTGGAGGTTCAGGGAAGGACATCCAGGCTGGTGTTGGAAGTTGCACAGCATCTTGGTGAGAACACAGTCAGGACAATTGCCATGGACGGT  359
 L  E  V  Q  G  R  T  S  R  L  V  L  E  V  A  Q  H  L  G  E  N  T  V  R  T  I  A  M  D  G    110

ACAGAAGGTCTGATCCGAGGCCAGAAGTGCGTTGACACTGGCTCCCCCATCAGCATCCCCGTCGGCCCCGAGACGCTGGGACGCATCATC  449
 T  E  G  L  I  R  G  Q  K  C  V  D  T  G  S  P  I  S  I  P  V  G  P  E  T  L  G  R  I  I    140

AATGTCATTGGTGAACCCATTGACGAGAGAGGACCAATTGGAACAGACAGGAGATCAGCAATCCATGCAGAAGCTCCAGAGTTTGTAGAG  539
 N  V  I  G  E  P  I  D  E  R  G  P  I  G  T  D  R  R  S  A  I  H  A  E  A  P  E  F  V  E    170

ATGAGTGTAAACCAGGAAATCCTTGTTACTGGAATCAAGGTTGTAGATCTACTCGCCCCATACGCCAAGGGAGGAAAGATTGGTCTGTTT  629
 M  S  V  N  Q  E  I  L  V  T  G  I  K  V  V  D  L  L  A  P  Y  A  K  G  G  K  I  G  L  F    200

GGCGGTGCTGGTGTAGGAAAGACTGTACTCATCATGGAGCTGATTAACAACGTAGCCAAGGCCCACGGAGGTTACTCTGTGTTTGCCGGT  719
[G  G  A  G  V  G  K  T] V  L  I  M  E  L  I  N  N  V  A  K  A  H  G  G  Y  S  V  F  A  G    230

GTAGGAGAGAGGACCCGTGAGGGTAACGATCTTTACCATGAGATGATTGAAGGAGGTGTCATCTCCCTCAAGGATGACACATCAAAGGTA  809
 V  G  E  R  T  R  E  G  N  D  L  Y  H  E  M  I  E  G  G  V  I  S  L  K  D  D  T  S  K  V    260

GCGTTGGTGTACGGACAGATGAACGAGCCTCCCGGCGCCCGTGCCCGTGTCGCCTTGACCGGACTGACCGTTGCCGAATACTTCCGTGAC  899
 A  L  V  Y  G  Q  M  N  E  P  P  G  A  R  A  R  V  A  L  T  G  L  T  V  A  E  Y  F  R  D    290

CAAGAGGGACAGGATGTGCTGCTCTTCATTGACAACATCTTCCGCTTCACACAGGCTGGATCAGAGGTATCTGCTCTGCTGGGACGTATC  989
 Q  E  G  Q  D  V  L  L  F  I  D  N  I  F  R  F  T  Q  A  G  S  E  V  S  A  L  L  G  R  I    320

CCATCTGCCGTAGGATACCAGCCAACCCTGGCCACTGACATGGGTACTATGCAGGAGCGTATTACCACCACCAAGAAGGGATCCATCACT 1079
 P  S  A  V  G  Y  Q  P  T  L  A  T  D  M  G  T  M  Q  E  R  I  T  T  T  K  K  G  S  I  T    350

TCCGTACAGGCCATCTACGTGCCTGCTGACGATCTCACTGACCCTGCCCCTGCCACCACCTTCGCCCATTTGGACGCCACCACTGTGCTG 1169
 S  V  Q  A  I  Y  V  P  A  D  D  L  T  D  P  A  P  A  T  T  F  A  H  L  D  A  T  T  V  L    380

TCCCGTGGTATCGCTGAGCTGGGTATCTACCCTGCTGTGGATCCTCTGGATTCCTCCTCCCGTATCATGGACCCCAACGTCGTCGGAGAG 1259
 S  R  G  I  A  E  L  G  I  Y  P  A  V  D  P  L  D  S  S  S  R  I  M  D  P  N  V  V  G  E    410

CGTCACTACAGCATCGCTCGTGGAGTACAGAAAATCCTTCAGGACAACAAGACCCTGCAGGACATCATCGCCATCTTGGGTATGGACGAG 1349
 R  H  Y  S  I  A  R  G  V  Q  K  I  L  Q  D  N  K  T  L  Q  D  I  I  A  I  L  G  M  D  E    440

TTGTCTGAGGACGACAAACTGACCGTGTCCCGAGCCAGGAAGATCCAGAGGTTCTTGTCCCAACCCTTCCAGGTTGCCGAGGTCTTCACC 1439
 L  S  E  D  D  K  L  T  V  S  R  A  R  K  I  Q  R  F  L  S  Q  P  F  Q  V  A  E  V  F  T    470

GGCAGTCCAGGCAAGCTCGTCTCAATGGCGGAGACCATCGATGGATTCGAGTCCATTATCAAGGGCGAGTGCGACCATCTACCAGAGATT 1529
 G  S  P  G  K  L  V  S  M  A  E  T  I  D  G  F  E  S  I  I  K  G  E  C  D  H  L  P  E  I    500

GCTTTCTACATGGTAGGCAACATTCAAGATGTCAAGGATAAGGCCGACAGGCTCGCAGAAGAACTATCATAAATTATCCCCCCTCTCCCA 1619
 A  F  Y  M  V  G  N  I  Q  D  V  K  D  K  A  D  R  L  A  E  E  L  S  *                       523

AACAATGAAGTTTAGAGCTGGCATGGCTACGGGTCAGAGACACCCCTCTTGATTGTTGTTATTCAGGGCTAGTTGTCTAACACTACCCGT 1709
GCCTGGGCCCAAAGAATTTATGTTCAGAGTTATAACTTATATCAAGATTGTTTTCTAAATTGTAATTGTGAAAAATTGAGAGCAAGGGAA 1799
TTCCAACCTAGCGTACTTTTGTCATATGAATCTGTCGTTTTCCTTCTTTTTTTTGCTTGTTATCCACCATAGATTGTAAATGCACAAACA 1889
GCTTGGCAAAGTTTGTAAATTTGATCATAACCAATTATCCCAATTTAAGGCAGTACCTTTAGCACATTGGTGTGTCACCGATGCCTGATT 1979
TCATGTTTATTGTCTGATCTGATCTTACAAGAAATTGGCCGATGTCCAAACATTTCCAATGTAGATATAGACATATATCTTCACTTGATT 2069
TCTGTGTAGAGCCGTTCACGTATGACAGATGATTGGCATTTATTTTGAATGGATGTTTTAGAGCTTTACTGAACCCAGTTGCGATTGTGA 2159
TTTCCTTGTGTGAACAGAATCGCAACTGGCCTTGAAAAAGAAAAACAAGTGTATTAAAAATTATTGGAAGGTTCAAGAACCAAAAAAAAA 2249
AAAAAAAAA 3'                                                                                  2259
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FIG. 1. Nucleotide sequence and deduced amino acid sequence of the 2.3 kb insert. The shadowed box indicates predicted signal peptide sequence and the open box denotes glycine-rich consensus sequence. The amino acid sequence designated by an underline is the same as partial sequence of the decapeptide used for synthesis of oligonucleotide probes. * denotes start or stop codon.

with 2.1 M formaldehyde, electrophoresed on a 1% agarose gel in the presence of 2.2 M formaldehyde, and transferred onto a Hybond-N-membrane. The RNA on the membrane was hybridized to the random-primed ECL labelled (Amersham International plc., Amersham, UK) or random-primed [α-^{32}P]dCTP-labelled 2.3 kb cDNA insert at 65°C for 18 hr. The membrane was washed with 0.5×SSC and 0.1% SDS at 65°C for 30 min. The size of the RNA was estimated using a 0.24–9.5 kb RNA Ladder (GIBCO BRL, Baithersburg, MD, USA) as a marker.

RESULTS AND DISCUSSION

The 2.3 kb insert contained DNA sequences encoding an open reading frame of 523 amino acids including I-D-N-I-F-R which is the same as the partial sequence of the peptide used for synthesis of oligonucleotide probes (Fig. 1). The deduced amino acid sequence suggests that the protein contains a 19-residue amito terminal signal peptide which has the potential to form amphipathic helix being characteristic of mitochondrial signal peptide sequence [5] and a 8-residue (residues 201-208) glycine-rich consensus sequence (G-X-X-X-G-K-T/S) found in the F_1-F_0 ATP synthase beta-subunit, adenylate kinase, p21 *ras* protein, and other nucleotide-binding proteins [14]. The deduced amino acid sequence has 68% homology with those of chloroplast F_1-F_0 ATP synthase beta-subunits and 85% with those of mitochondrial F_1-F_0 ATP synthase beta-subunits from various

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                                  10         20         30         40         50         60
Spermatozoa (sea urchin)   MFSRVAKTSFSAVRAAKSQFSHSLSQQTSKTWVPAATCSKRSYAAEAKTSA--APVSGQIVAVIGAVVDV
Mitochondria (human)     MLGFVG...AAPA.GALRRLTPSASLPPA.LLLRAA.T.VHPV.D...QTSP.PKAGAAT.R..........
Mitochondria (rat)       MLSLVG...SA.A.GALRGLNPLAALPQAHLLLRTA..GVHPA.D...QSSAAPKAGTAT.............
Chloroplast (potato)                 .                                  MRINPTTSGS.VS.VE--KKNL.R..KI..P.L..
Chloroplast (spinach)                                                   MRINPTTSDPGVS.LE--KKNL.R.AQI..P.LN.

     70         80                  90         100        110        120        130        140        150
QF-EDDLPPILNALEVQGR----TS---RLVLEVAQHLGENTVRTIAMDGTEGLIRGQKCVDTGSPISIPVGPETLGRIINVIGEPIDER
..-DEG..............----ET---.............S.............V....VL.S.A..K...........M..........
..-DEG..............----E.---.............S.............V....VL.S.A..K...........M..........
A.PPGKM.N.Y...V....----GNEQTNVTC..Q.L..N.R..AV..SD.D..M..MEVI...A...V...GS.....F..L.Q.V.NL
A.PPGKM.N.Y...I.K..DTAGQPM--NVTC..Q.L..N.R..AV..SA.D..T..MEVI...A.L.V...GP.....F..L...V.NL

       160        170        180        190        200        210        220        230        240
GPIGTDRRSAIHAEAPEFVEMSVNQEILVTGIKVVDLLAPYAKGGKIGLFGGAGVGKTVLIMELINNVAKAHGGYSVFAGVGERTREGND
...K.KQFAP........M....E.....................................................................
...K.KQFAP........I....E.....................................................................
..VD.NTT.P..RS..A.IQLDTKLS.FE.............RR.........................I......V...G...........
R.VD.RTT.P..RS..A.TQLDTKLS.FE......N.....RR.........................I......V...G...........

       250        260        270        280        290        300        310        320        330
LYHEMIEGGVISLKD-DTSKVALVYGQMNEPPGARARVALTGLTVAEYFRDQEGQDVLLFIDNIFRFTQAGSEVSALLGRIPSAVGYQPT
.......S...N...-A.....................................................................
.......S...N...-A.....................................................................
..L..K.S...NEENIPE.........-.......M..G..A..M......VNE.............V............M.........
..M..K.S...NEQNIAE.................M..G..A..M......VNE.............V............M.........

       340        350        360        370        380        390        400        410        420
LATDMGTMQERITTTKKGSITSVQAIYVPADDLTDPAPATTFAHLDATTVLSRGIAELGIYPAVDPLDSSSRIMDPNVVGERHYSIARGV
.....................................................A..............T.......I..SE..DV....
.....................................................A..............T.......I..SE..DV....
.....................................................L.AK...........T.TMLQ.RI...E..ET....
.S.E..YL.....S..E.....I..V...........................L.AK...........T.TMLQ.RI...E..E..QR.

       430        440        450        460        470        480        490        500        510
QKILQDNKTLQDIIAILGMDELSEDDKLTVSRARKIQRFLSQPFQVAEVFTGSPGKLVSMAETIDGFESIIKGECDHLPEIAFYMVGNIQ
......Y.S.............E.....................HM....PLK...K..QQ.LA..Y.....Q......P.E
......Y.S.............E.....................HM....PLK...K..QQ.LA.DY.....Q......P.E
KQT..RY.E.........L.....E.R...A.....E.......F...........Y.GL....R..QL.LS..L.G...Q...L....D
KET..RY.E.........L.....E.R...A.....E.......F...........Y.GL....R..QL.LS..L.S...Q...L....D

       520                 Homology
DVKDKADRLAEELS             100%
EAVA...K....H.S            85%
EAVA...K....HGS            85%
EATA..MN.KT                68%
EATA..MN.EM.SKLKK          68%
```

FIG. 2. Comparison of deduced amino acid sequence of the sea urchin homologue and mitochondrial (human [9], rat [4]) and chloroplast (potato [7], spinach [17]) F_1-F_0 ATP synthase beta-subunits. Dots indicate the same amino acid residues as sea urchin homologue and positions where gap have been introduced for maximum homology are indicated by a dash.

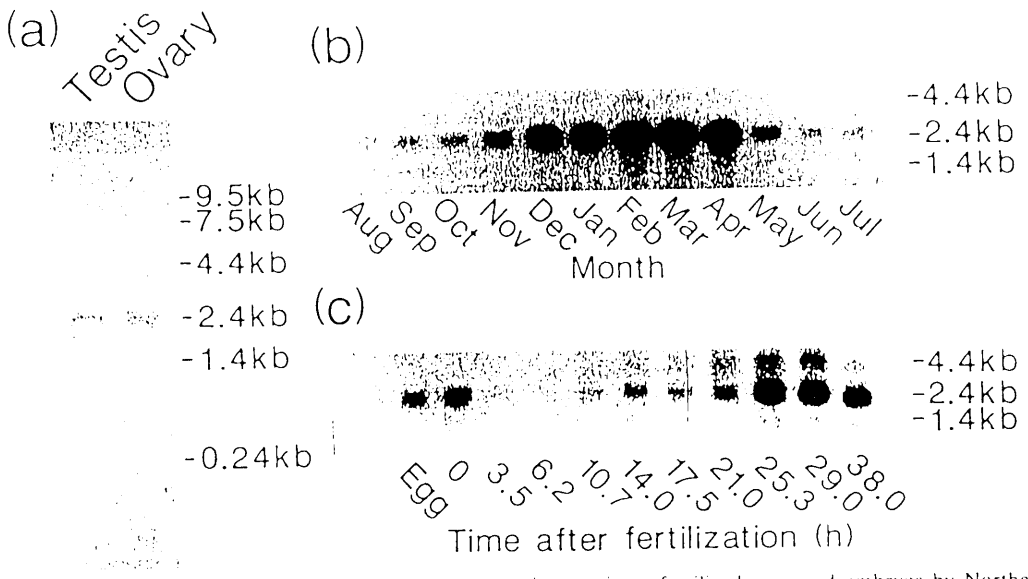

FIG. 3. Analysis of RNA prepared from *H. pulcherrimus* ovaies, testis, unfertilized eggs and embryos by Northern blot hybridization. (a): poly(A)$^+$ RNA (2 μg) prepared from ovaries and testis samples collected in March, detected by ECL; (b): total RNA (5 μg) from the testis samples collected throughout the year, detected by autoradiography; (c); total RNA (5 μg) from unfertilized eggs and embryos cultured at 20°C, detected by autoradiography.

sources (Fig. 2) [4, 7, 9, 17]. This suggests that the cDNA clone isolated from the *H. pulcherrimus* testis cDNA library codes for the beta-subunit of mitochondrial F_1-F_0 ATP synthase and the primary strucutres of the beta-subunits are highly conserved in very different species.

Norhtern blot analysis using the 2.3 kb insert as a probe indicated that the mRNA of 2.4 kb presents both in the ovary and testis of the sea urchin (Fig. 3a). In previous study, we demonstrated that *H. pulcherrimus* spermatozoa contained a large amount of membrane-bound guanylate cyclase and creatine kinase and the activities of both enzymes increased during the testis development [6]. As shown in Figure 3b, the mRNA encoding F_1-F_0 ATP synthase beta-subunit began to the accumulated in the testis collected in November when spermatogenic cells appeared along the wall of testicular lobes, suggesting that F_1-F_0 ATP synthase is also synthesized in the testis with formation of mature spermatozoa. The mRNA was also identified in unfertilized eggs and developing embryos, while the signal of hybridizing RNA from the unfertillized eggs was weaker than that from the developing embryos (Fig. 3c). This may be due to imcomplete polyadenylation of the stored mRNA in unfertilized eggs [15]. Additional polyadenylation reaction appears to begin rapidly upon fertilization (Fig. 3c). The mRNA was not appreciably detected in the embryos during early cleavage stage and became detectable in the embryos of the gastrula stage (Fig. 3c).

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