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REVIEW

Structure, Function and Biosynthesis of Sperm-Activating Peptides and Fucose Sulfate Glycoconjugate in the Extracellular Coat of Sea Urchin Eggs

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ABSTRACT—A decapeptide (GFDLNGGGVG) isolated from the solubilized jelly layer of the sea urchin *Hemicentrotus pulcherrimus* stimulates the respiration and motility of *H. pulcherrimus* spermatozoa and, in addition, produces a number of biological effects on *H. pulcherrimus* spermatozoa including increases in cAMP and cGMP levels, activation of a Na^+/H^+ exchange system, and increases in intracellular pH (pH_i) and $[Ca^{2+}]$ ($[Ca^{2+}]^i$). The peptide activates the metabolism of endogenous phosphatidylcholine and promotes the acrosome reaction as a specific co-factor of a major acrosome reaction-inducing substance, fucose sulfate glycoconjugate. The peptide also induces an electrophoretic mobility change in the guanylate cyclase of the sperm plasma membrane with concomitant dephosphorylation and inactivation of the enzyme. Seventy-four peptides producing similar biological effects, named sperm-activating peptide (SAP), have since been purified from the solubilized jelly layer of seventeen species of sea urchins distributed over five taxonomic orders. These peptides show essentially the same biological effects on sea urchin spermatozoa although their activity and structures are specific at the ordinal level. Equilibrium binding experiments using a radioiodinated SAP-I analogue [GGGY(^{125}I)GFDLNGGGVG] to *H. pulcherrimus* spermatozoa suggests the presence of two classes of receptors (high affinity and low affinity) specific for SAP-I binding. Based on the Kd values and EC_{50}'s for SAP-I's biological activity, we presume that the high affinity receptor is associated with respiration-stimulating activity and elevations in pH_i, while the low affinity receptor is coupled to elevations in cGMP and $[Ca^{2+}]_i$. The radioiodinated SAP-I analogue crosslinks to a 71 kDa protein which contains a single membrane-spanning domain at almost near C-terminus. A SAP-I precursor which is synthesized in the accessory cells contains five SAP-I and seven SAP-I-like decapeptides, each separated by a single lysine residue.

INTRODUCTION

Fertilization occurs as a result of the interaction between egg and spermatozoa, beginning with spermatozoa reaching the egg and binding to it, and ending with the fusion of the sperm pronucleus and the egg nucleus. In the process, the egg communicates with the spermatozoa using molecules in the extracellular matrix (Fig. 1). Sea urchin gametes have long been used for analyzing these processes in fertilization [28]. Particularly in the past decade, our knowledge of the biochemistry of sea urchin fertilization has increased enormously [for reviews see refs. 22, 75]. A better understanding of the substances involved in sea urchin gamete interaction would not only lead to an understanding of fertilization in mammals and other animals, but would serve as a valuable guide to mechanisms which might be anticipated in cells other than gametes. Until the discovery of sperm-activating peptides [65, 66] and mammalian atrial natriuretic peptides [15], specific endogenous activators of the plasma membrane form of guanylate cyclase which produces the second messenger cGMP had not been identified. The natriuretic peptides are responsible for maintaining body fluid and electrolyte homeostasis through interaction with receptors on a variety of mammalian cell types. However, the mechanism has yet to be described in detail. Sea urchin spermatozoa contain extremely large amount of the plasma membrane form of guanylate cyclase. Elucidation of the biochemical mechanism of interaction between the spermatozoa and sperm-activating peptides would be helpful for understanding of the mechanism of the action of the natriuretic peptides, and also the mechanism of signal transduction by the membrane form of guanylate cyclase/receptor.

Sea urchin eggs are surrounded by a transparent, gelatinous extracellular matrix called the jelly layer. Sea urchin spermatozoa must pass through the jelly layer before contacting the egg surface. The jelly layer has been shown to induce the sperm acrosome reaction, which was first described in detail by Dan [10]. The acrosome reaction in spermatozoa is an essential requirement for fertilization of eggs in many animals [6, 80]. In sea urchins, the acrosome reaction occurs within seconds after spermatozoa have come into contact with the jelly layer [14] or the egg surface component, a sperm-binding protein on the vitelline membrane [4, 19]. The acrosome reaction which consists of the exocytosis of the acrosomal granule and extension of the acrosomal process by the polymerization of actin (G-actin to F-actin formation) [for reviews see refs. 11, 75] requires the net efflux of H^+, Ca^{2+} and K^+ [52].

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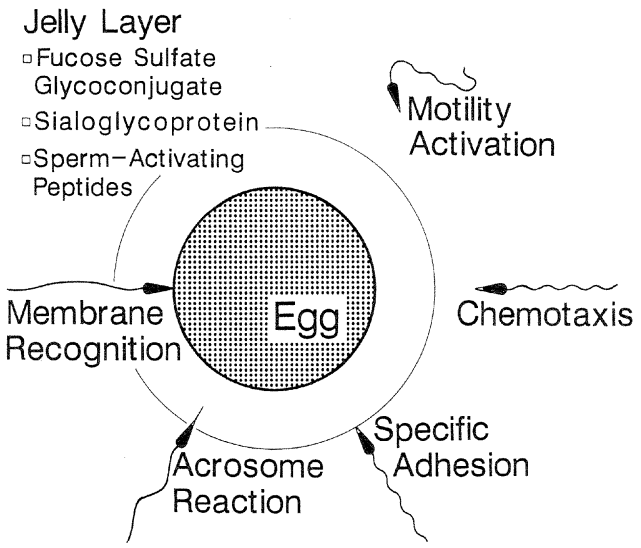

FIG. 1. Diagrammatic drawing of the major steps of sea urchin sperm egg interaction.

In the early 1900s, it was shown that a soluble factor associated with the eggs of certain species of sea urchins stimulated the respiration and motility of sea urchin spermatozoa [38]. About fifty years later the factor was shown to be diffusible in dialysis, heat stable, alcohol soluble, and non-volatile [32]. However, until Ohtake [42] demonstrated that the respiration of sea urchin spermatozoa could be reproducibly stimulated by solubilized jelly layer of the sea urchin *Pseudocentrotus depressus* when the extracellular pH is maintained at slightly acidic values, such stimulation was difficult to reproduce. The respiration and motility of sea urchin spermatozoa as well as spermatozoa of other animals are highly dependent on the pH value of the suspension medium. At pH 6.6 or 6.8, the respiration rate and motility of sea urchin spermatozoa are markedly reduced, but this effect can be reversed upon addition of solubilized jelly layer. The solubilized jelly layer also produces increases in sperm cAMP and cGMP levels, and activation of cAMP-dependent protein kinase [36]. In 1981, using acidified sea water for bioassaying the respiration-stimulating ability of the factor and sequential chromatography on Sephadex columns, we purified a factor from the solubilized jelly layer of the sea urchin *Hemicentrotus pulcherrimus* and demonstrated that the factor is a decapeptide [65]. This article describes recent work which focuses on the factors in the jelly layer responsible for these profound effects on sea urchin spermatozoa and the signal transduction mechanism that turns these signals into metabolic changes.

2. ISOLATION AND STRUCTURE OF MOLECULES FROM JELLY LAYER

2.1. *Sperm-activating peptides*

The jelly layer of sea urchin eggs contains highly viscous large molecular weight polysaccharide-protein complexes, making the use of many common biochemical methods somewhat difficult. In preparation for analysis, jelly layer must first be dissolved from the cells by treatment with acidified sea water (pH 5.0), although even with extensive washing, jelly molecules remain bound to the egg vitelline layer [75]. The solubilized jelly layer can be divided into two fractions by ethanol precipitation: a precipitate containing a fucose sulfate glycoconjugate and a sialoglycoprotein, and a soluble fraction of peptides.

In the last decade, we have purified seventy-four peptides from the ethanol-soluble fraction of the solubilized jelly layer of seventeen species of sea urchins distributed over five taxonomic orders (Echinoida, Arbacioida, Clypeasteroida, Diadematoida and Spatangoida) [for review see ref. 64] (Table 1). These peptides show essentially the same biological effects on sea urchin spermatozoa although the biological effects and structures of the peptides are specific at the ordinal level (Table 2). Therefore, we proposed a generalized name and an abbreviated name for these peptides, *i.e.*, sperm-activating peptide as general name and SAP as the abbreviation. Using this nomenclature, peptides from species in the order Echinoida would be called SAP-I (GFDLNGGGVG) and its derivatives [65]. Similarly, SAP-II would be from species in the order Arbacioida, which is divided into subgroups A and B (SAP-IIA: CVTGAPGCVGGGRL-NH_2 and SAP-IIB: KLCPGGNCV) [68, 73]. SAP-III (DSDSAQNLIG) would be from species in the order Clypeasteroida [70], SAP-IV (GCPWGGAVC) from species in the order Diadematoida [89], and SAP-V (GCEGLFHGMGNC) from species in the order Spatangoida [91]. According to a review of Shigei [56], recent species of sea urchins are classified into ten taxonomic orders (Cidaroida, Echinothurioida, Diadematoida, Arbacioida, Echinoida, Holectypoida, Clypeasteroida, Cassiduloida and Spatangoida). We predict that new specific peptides will be iso-

TABLE 1. Sperm-activating peptides purified from the egg jelly of various species of sea urchins*

Subclass Regularia
Order Diadematoida
Suborder Diademina
Family Diadematidae
Diadema setosum
GCPWGGAVC (SAP-IV)
GXPXGGAV
Order Arbacioida
Suborder Phymosomatina
Family Phymosomatidae
Glyptocidaris crenularis
SAKLCPGGNCV (Ser-Ala-SAP-IIB)
KLCPGGNCV (SAP-IIB)
LCPGGNCV (Des-Lys^{1}-SAP-IIB)
SFKLCPGGQCV (Ser-Phe-[Gln^{7}]SAP-IIB)
KLCPGGQCV ([Gln^{7}]SAP-IIB)
LCPGGQCV (Des-Lys^{1}-[Gln^{7}]SAP-IIB)
Family Stomopneustidae
Stomopneustes variolaris
KFCPEGKCV ([Phe^{2}, Glu^{5}, Lys^{7}]SAP-IIB)
Suborder Arbacina
Family Arbaciidae
Arbacia punctulata
CVTGAPGCVGGGRL-NH_2 (SAP-IIA)
Order Echinoida
Suborder Temnopleurina
Family Toxopneustidae
Lytechinus pictus
GFDLTGGGVQ ([Thr^{5},Gln^{10}]SAP-I)
FDLTGGGVQ (Des-Gly^{1}-[Thr^{5}]SAP-I)
Tripneustes gratilla
GFDLNGGGVG (SAP-I)
GFNLNGGGVG ([Asn^{3}]SAP-I)
GFSIGGGGVG ([Ser^{3},Ile^{4},Gly^{5}]SAP-I)
GFDLGGGGVG ([Gly^{5}]SAP-I)
GFSLGGGGVG ([Ser^{3},Gly^{5}]SAP-I)
GFGLGGGGVG ([Gly^{3,5}]SAP-I)
GF(o-Br)DLNGGGVG ([o-Br-Phe^{2}]SAP-I)
GF(o-Br)NLNGGGVG ([o-Br-Phe^{2},Asn^{3}]SAP-I)
GF(o-Br)DLDGGGVG ([o-Br-Phe^{2},Asp^{5}]SAP-I)
GF(o-Br)DLGGGGVG ([o-Br-Phe^{2},Gly^{5}]SAP-I)
GF(o-Br)SLSGGGVG ([o-Br-Phe^{2},Ser^{3,5}]SAP-I)
GF(o-Br)GLGGGGVG ([o-Br-Phe^{2},Gly^{3,5}]SAP-I)
GF(o-Br)SLGGGGVG ([o-Br-Phe^{2},Ser^{3},Gly^{5}]SAP-I)
GF(o-Br)SIGGGGVG ([o-Br-Phe^{2},Ser^{3},Ile^{4},Gly^{5}]SAP-I)
GF(m-Br)DLNGGGVG ([m-Br-Phe^{2}]SAP-I)
GF(p-Br)NLNGGGVG ([p-Br-Phe^{2},Asn^{3}]SAP-I)
Psudoboletia maculata
GFALDG VN (Des-Gly^{7,8}-[Ala^{3},Asp^{5},Asn^{10}]SAP-I)
GFALDG VG (Des-Gly^{7,8}-[Ala^{3},Asp^{5}]SAP-I)
GFALDG VT (Des-Gly^{7,8}-[Ala^{3},Asp^{5}, Thr^{10}]SAP-I)
Pseudocentrotus depressus
GFDLNGGGVG (SAP-I)
GFDLTGGGVG ([Thr^{5}]SAP-I)
GFALGGGGVG ([Ala^{3}, Gly^{5}]SAP-I)

Suborder Echinina
Family Strongylocentrotidae
Strongylocentrotus nudus
GFDLNGGGVG (SAP-I)
GFSLSGGGVG ([Ser^{3,5}]SAP-I)
GFALGGGGVG ([Ala^{3}, Gly^{5}]SAP-I)
GFSLGGGGVG ([Ser^{3}, Gly^{5}]SAP-I)
GFDLTGGGVG ([Thr^{5}]SAP-I)
Strongylocentrotus purpuratus
GFDLNGGGVG (SAP-I)
GFALGGGVG ([Ala^{3}, Gly^{5}]SAP-I)
GFSLTGGGVG ([Ser^{3}, Thr^{5}]SAP-I)
Hemicentrotus pulcherrimus
GFDLNGGGVG (SAP-I)
GFDLTGGGVG ([Thr^{5}]SAP-I)
GFDLNGGGVS ([Ser^{10}]SAP-I)
SFALGGGVG ([Ser^{1}, Ala^{3}, Gly^{5}]SAP-I)
GFSLTGGSVD ([Ser^{3,6}, Asp^{10}]SAP-I)
Family Echinometridae
Echinometra mathaei (type A)
GYSLSGGAVD ([Tyr^{2}, Ser^{3,5}, Ala^{8}, Asp^{10}]SAP-I)
GFALSGGGVG ([Ala^{3}, Ser^{5}]SAP-I)
GFSLSGGGVG ([Ser^{3,5}]SAP-I)
GFDLTGGGVG ([Thr^{5}]SAP-I)
Echinometra mathaei (type B)
GYSLSGGAVD (Tyr^{2}, Ser^{3,5}, Ala^{8}, Asp^{10}]SAP-I)
GYNLNGDRID ([Tyr^{2}, Asn^{3}, Asp^{7,10}, Arg^{8}, Ile^{9}]SAP-I)
GFSLSGGGVG ([Ser^{3,5}]SAP-I)
GFDLTGGGVG ([Thr^{5}]SAP-I)
Anthocidaris crassispina
GFDLTGGGVG ([Thr^{5}]SAP-I)
GFDLSGGGVG ([Ser^{5}]SAP-I)
GFSLSGSGVG ([Ser^{3,5,7}]SAP-I)
Heterocentrotus mammillatus
GTLPTGSGVS ([Thr^{2,5}, Leu^{3}, Pro^{4}, Ser^{7,10}]SAP-I)
GFEMGGTGVG ([Glu^{3}, Met^{4}, Gly^{5}, Thr^{7}]SAP-I)
GYNLGGGGID ([Tyr^{2}, Asn^{3}, Gly^{5}, Ile^{9}, Asp^{10}]SAP-I)
GFGLSGGGIG ([Gly^{3}, Ser^{5}, Ile^{9}]SAP-I)
Subclass Irregularia
Order Clypeasteroida
Suborder Clypeasterina
Family Clypeasteridae
Clypeaster japonicus
DSDSAQNLIG (SAP-III)
GTDSAQNLIG ([Gly^{1}, Thr^{2}]SAP-III)
SDSAQNLIG(Des-Asp^{1}-SAP-III)
DSDSAH LIG (Des-Asn^{7}-[His^{6}]SAP-III)
DTDSAH LIG (Des-Asn^{7}-[Thr^{2}, His^{6}]SAP-III)
NTDSAH LIG (Des-Asn^{7}-[Asn^{1}, Thr^{2}, His^{6}]SAP-III)
GTDSAH LIG (Des-Asn^{7}-[Gly^{1}, Thr^{2}, His^{6}]SAP-III)
SDSAH LIG (Des-Asp^{1}, Asn^{7}-[His^{6}]SAP-III)
DSDSAH (Br) LIG (Des-Asn^{7}-[Br-His^{6}]SAP-III)
Suborder Laganina
Family Astriclypeidae
Astriclypeus manni
DSDSAH LIG (Des-Asn^{7}-[His^{6}]SAP-III)

DTDSAH LIG (Des-Asn^7-[Thr^2, His^6]SAP-III)
TDSAH LIG (Des-Asp^1, Asn^7-[Thr^2, His^6]SAP-III)

Order Spatangoida
Suborder Spatangina
Family Brissidae
Brissus agassizii
GCEGLFHGMGNC (SAP-V)

* Amino acids in the sequences are given a one-letter abbreviation: asparagine (N), aspartic acid (D), alanine (A), arginine (R), isoleucine (I), glycine (G), glutamine (Q), glutamic acid (E), cystine (C C), cysteine (C), serine (S), tyrosine (Y), tryptophan (W), valine (V), histidine (H), phenylalanine (F), proline (P), methionine (M), lysine (K) and leucine (L).

TABLE 2. Specificity of sperm-activating peptides

	Spermatozoa used				
	Diadema setosum	*Glyptocidaris crenularis*	*Hemicentrotus pulcherrimus*	*Clypeaster japonicus*	*Brissus assizii*
SAP-IV	+	−	−	−	−
SAP-IIB	−	+	−	−	−
SAP-IIA	−	±	−	−	−
SAP-I	−	−	+	−	−
SAP-III	−	−	−	+	−
SAP-V	−	−	−	−	+

The respiratory stimulation activity of a sperm-activating peptide is expressed as a plus sign (+) when the peptide stimulated sperm respiration one half-maximally at a concentration less than 5 nM. When the half-maximal respiratory stimulation was induced by a peptide concentration between 5×10^{-9} M and 5×10^{-7} M, a plus-minus (±) was used. Practically no respiratory stimulation was depicted by a minus sign (−).

lated from species in the orders Cidaroida, Echinothurioida, Holectypoida and Cassiduloida. When new specific peptides are obtained, they will be in turns named SAP-VI, -VII and so on.

2.2. *Bromophenylalanine-containing sperm-activating peptides*

Some of the SAP-I derivatives isolated from the solubilized jelly layer of the sea urchin *Tripneustes gratilla* contained o-, m- and p-bromophenylalanine [89]. Although bromination of phenylalanine in these peptides does not appear to interfere with its biological effect on spermatozoa, it raises a question concerning the generally accepted idea of the strict specificity of phenylalanyl tRNA synthetase. Considering that the enzymatic electrophilic bromination of the phenylalanyl aromatic ring is in the ortho-para orientation, o- or p-bromophenylalanine could be generated by post-translational modification of phenylalanine with a bromide and specific peroxidase. However, there is little possibility of post-translational generation of m-bromophenylalanine, suggesting pre-translational generation of the amino acid, in other words, phenylalanyl tRNA synthetase may mistake m-bromophenylalanine for phenylalanine.

2.3. *Fucose sulfate glycoconjugate (FSG)*

For purification of highly viscous large molecular weight polysaccharide-protein complexes of the jelly layer, solubilized jelly layer of the sea urchin *H. pulcherrimus* was dialyzed against 0.1 M NaCl at 4°C. Fucose-containing material was eluted earlier than the material containing sialic acid [83–84]. FSG has been purified from the fractions containing fucose by sequential gel chromatography on Sepharose CL-2B and TSK G-3000 PW columns [57]. FSG consisted of polysaccharide-containing protein(s) and several other protein constituents (258 kDa, 237 kDa and 120 kDa) which associated with each other by disulfide bonds (Fig. 2). The polysaccharide consisting of about 39% of FSG by weight comprised fucose sulfate (87%, w/w), N-acetylglucosamine (4.7%, w/w), mannose (3.7%, w/w), N-acetylgalactosamine (2.8%, w/w), galactose (1.0%, w/w) and glucose (0.7%, w/w) (Nishimura and Suzuki, unpublished data).

2.4. *Sialoglycoprotein*

The sialic acid-containing material obtained from chromatography of solubilized jelly layer on a Sepharose 2B column was further purified by chromatography on a DEAE-Sephadex A-25 column, followed by CsCl density gradient ultracentrifugation [57]. The purified sialic acid-containing material (sialoglycoprotein) mainly consisted of sialic acid (77%, w/w) and protein (10%, w/w), although it contained several other sugars such as N-acetylgalactosamine (4.6%, w/w), N-acetylglucosamine (3.1%, w/w), galactose (2%, w/w), mannose (1%, w/w) and glucose (0.7%, w/w) (Nishimura and Suzuki, unpublished data).

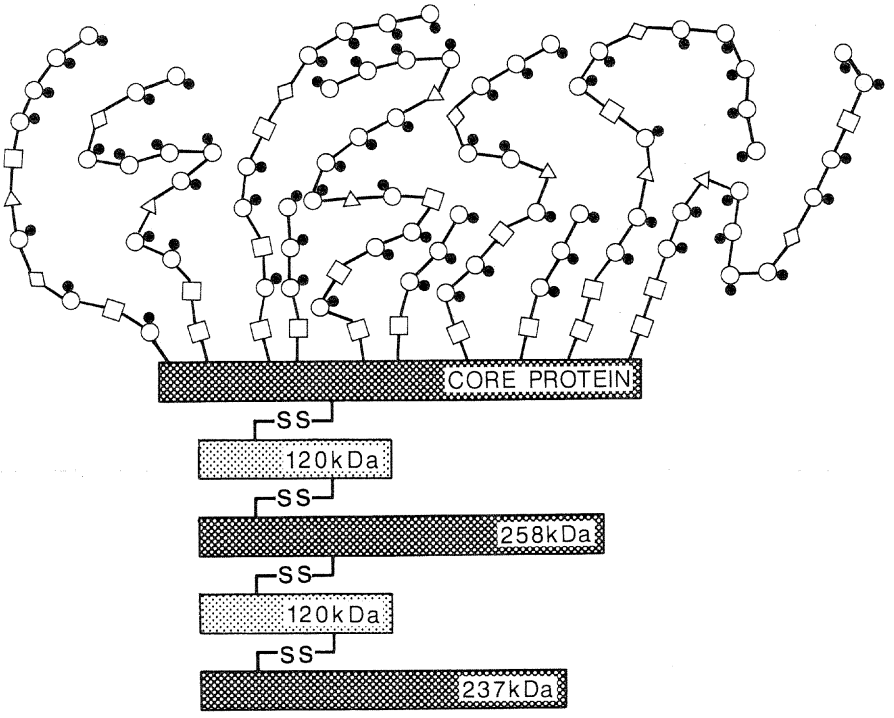

FIG. 2. Highly speculative model for fucose sulfate glycoconjugate. Fucose sulfate glycoconjugate consists of a sugar-containing core protein, and two major (258 kDa and 237 kDa) and one minor (120 kDa) proteins.

3. INTERACTION OF SPERMATOZOA WITH SPERM-ACTIVATING PEPTIDES

3.1. Respiratory stimulation

Sea urchin egg jelly molecules such as sperm-activating peptides and fucose sulfate glycoconjugate show various effects on sea urchin sperm metabolism. These are summarized in Table 3. Sperm-activating peptides stimulate the respiration rates of sea urchin spermatozoa maintained in acidified sea water, back to normal levels [69]. The stimulated respiration rate does not exceed that of spermatozoa in normal sea water (pH 8.2) even if large amounts of peptides are added to the sperm suspension medium. In an exceptional case with we encountered once in over ten years of

TABLE 3. Effects of sea urchin egg jelly molecules, fucose sulfate glycoconjugate (FSG) and sperm-activating peptide (SAP), on sea urchin spermatozoa

	FSG	SAP
Respiration	none	stimulation
Motility	none	stimulation
Acrosome reaction	inducible (major factor)	none (itself) (co-factor)
Histone phosphorylation	inducible	not known
Effects on guanylate cyclase		
Activation	none	yes
Dephosphorylation	none	yes
Activation of adenylate cyclase	yes	yes
Activation of cAMP-dependent protein kinase	yes	not known
Increase in		
intracellular pH	yes	yes
intracellular Ca^{2+}	yes	yes
intracellular cAMP	yes	yes
intracellular cGMP	none	yes
Chemoattraction	none	yes (only SAP-IIA)

research on sperm-activating peptides, SAP-IIA stimulated respiration rates of spermatozoa from a given individual of *Arbacia punctulata* in any pH tested. This may explain why sometimes in the early phase of research on egg-associated factor(s) some investigators obtained positive effects of egg water on stimulation of sperm respiration rates even when they tested the effects in sea water (pH 8.2). To obtain reproducible results, however, we recommend the use of slightly acidic sea water (pH 6.8 or 6.6) for assaying respiratory stimulation effects of sperm-activating peptides on sea urchin spermatozoa. Half-maximal stimulation of the respiration rate by these peptides is 10–100 pM. The respiratory stimulation effect is dependent upon the concentration of external Na^+ [48, 69]. The ability of SAP-I to stimulate H^+ efflux and hence increase intracellular pH correlates well with the stimulation of respiration rates [33]. Thus, induction of respiratory stimulation by sperm-activating peptides can be explained by the hypothesis that the peptides trigger Na^+/H^+ exchange across the sperm plasma membrane and raise the intracellular pH. SAP-I increases intracellular pH and Ca^{2+} in both acidic (pH 6.6) and normal (pH 8.0) sea water. The extent of the increase in intracellular pH in normal sea water is smaller than that in acidic sea water, but the extent of the increase in intracellular Ca^{2+} in normal sea water is much larger than that in acidic sea water. In sea water containing high concentration of K^+, the respiration rates and motility of sea urchin spermatozoa are lower than those in normal sea water. Under these conditions, SAP-I increases neither the lowered respiration rate and motility nor the intracellular pH and Ca^{2+}, although the peptide binds to the spermatozoa as it does in normal sea water [31, 33].

3.2. *Activation of guanylate cyclase*

Sperm-activating peptides cause increases in sea urchin sperm cGMP levels as well as cAMP levels in both acidic and normal sea water [73]. Half-maximal elevations of cGMP occurs at about 20 nM peptide versus 2 nM for cAMP elevation. SAP-I elevates the sperm cGMP level in 100 mM K^+ sea water (from 0.37 to 4.81 pmol/mg wet weight spermatozoa) more than in normal sea water (from 0.21 to 0.93 pmol/mg wet weight spermatozoa) [31]. A phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine (IBMX) and SAP-I synergistically elevate the cGMP level from 0.35 to 33.08 pmol/mg wet weight spermatozoa in 100 mM K^+ sea water. However, in high concentration K^+ sea water, SAP-I does not increase cAMP levels even in the presence of IBMX [31].

When *A. punctulata* spermatozoa were incubated with [γ-^{32}P]-ATP, a major membrane protein of 160 kDa was constitutively labeled. When spermatozoa were treated with the solubilized jelly layer of *A. punctulata* this protein lost its ^{32}P-label and changed its relative mobility on SDS gels to 150 kDa [76]. Reaction with specific antibodies and purification studies have shown that the phosphoprotein is the enzyme guanylate cyclase [77]. The increased mobility is thought to result from increased binding of SDS to the dephosphorylated form of the enzyme. The factor responsible for the change has been identified as SAP-IIA [68]. Similar changes are commonly observed in sea urchin spermatozoa treated with a specific sperm-activating peptide [7, 60, 69, 72-73, 86–88, 91] (Table 4).

Loss of phosphate from guanylate cyclase and a change in the molecular form which is dependent on external Na^+ and Ca^{2+} concentrations result in a large decrease in the specific activity of the enzyme [79]. The decrease in the

TABLE 4. Apparent molecular mass change of a major sperm protein induced by a specific sperm-activating peptide

	Species	Change in molecular mass
SAP-I	*Lytechinus pictus*[1)]	150→140
	Tripneustes gratilla[2)]	131→123
	Pseudoboletia maculata[2)]	128→120
	Pseudocentrotus nudus[3)]	132→128
	Strongylocentrotus depressus[3)]	132→127
	Strongylocentrotus purpuratus[4)]	150→140
	Hemicentrotus pulcherrimus[3)]	131→128
	Echinometra methaei type A[2)]	130→123
	Echinometra methaei type B[2)]	130→123
	Anthocidaris crassispina[3)]	136→131
	Heterocentrotus mammillatus[2)]	130→124
SAP-IIA	*Arbacia punctulata*[5)]	160→150
SAP-IIB	*Glyptocidaris crenularis*[6)]	195→200
SAP-III	*Clypeaster japonicus*[7),8)]	126→123
	Astriclypeus manni[8)]	128→122
SAP-IV	*Diadema setosum*[9)]	134→128
SAP-V	*Brissus agassizii*[10)]	133→129

1) Shimomura *et al.* [56]; 2) Yoshino *et al.* [82]; 3) Suzuki *et al.* [68]; 4) Bentley *et al.* [6]; 5) Suzuki *et al.* [64]; 6) Suzuki *et al.* [69]; 7) Suzuki *et al.* [66]; 8) Yoshino *et al.* [84]; 9) Yoshino *et al.* [83]; 10) Yoshino *et al.* [86].

specific activity could be repressed by protein phosphatse inhibitors like calyculin A to a great extent and microcystin-LR or okadaic acid to some extent (Hoshino and Suzuki, unpublished data). These suggest that a specific protein phosphatase sensitive to calyculin A dephosphorylates guanylate cyclase. On the other hand, in 100 mM K^+ sea water SAP-I did not induce the electrophoretic mobility change of guanylate cyclase [30]. Thus, we consider that the sperm-activating peptide-induced elevation of the cGMP level in sea urchin spermatozoa occurs before or independently of membrane hyperpolarization induced by the opening of K^+ channels.

3.3. *Chemoattraction*

The chemotaxis of animal spermatozoa to eggs or egg secretions from the female reproductive system is a widespread phenomenon [40]. However, the chemical nature of animal sperm chemoattractants have remained unknown. In this regard, it is important to mention that when 1 nl of 10 nM SAP-IIA was injected into a drop of randomly dispersed *A. punctulata* spermatozoa, a striking chemotactic response occurred; they changed their swimming behaviour and formed a cluster in the area of microinjected SAP-IIA [78]. The chemoattraction to SAP-IIA was species specific and dependent on concentration of the peptide and the presence of Ca^{2+}. SAP-IIA is the first egg-derived molecule of known structure that has been shown to be a chemoattractant for animal spermatozoa.

4. INTERACTION OF SPERMATOZOA WITH POLYSACCHARIDE- PROTEIN COMPLEXES IN THE JELLY LAYER

4.1. *Intact FSG*

The macromolecular fraction of the sea urchin jelly layer consists of 20% sialoglycoprotein and 80% FSG by weight [53]. At the present time, nothing is known about the effects of isolated sialoglycoprotein on sea urchin spermatozoa, although earlier studies suggested that it caused sperm-isoagglutination. The isolated FSG, which exhibits some degree of species specificity [58], induces the acrosome reaction [36, 53, 57], activates adenylate cyclase up to 50-fold [81], increases cAMP levels 400-fold [23–24, 26, 36], increases the activity of cAMP-dependent protein kinase [25] and elevates intracellular Ca^{2+} and pH in spermatozoa [33, 52]. The elevations in intracellular Ca^{2+} and pH by FSG appear to occur through different Ca^{2+} and H^+ transport systems than those for SAP-I [33]. The effects of FSG on cAMP metabolism and the acrosome reaction are blocked by a lectin wheat-germ agglutinin and the Ca^{2+} channel antagonists verapamil and D-600 [51].

Isolated FSG contains twice as much protein to fucose sulfate by weight and induces the acrosome reaction in a concentration-dependent manner [57], however, induction of the acrosome reaction by FSG is fully achieved only in Ca^{2+}-enriched sea water [53–54]. In normal sea water, isolated FSG shows only about half the ability of the original unfractionated jelly layer to induce the acrosome reaction. SAP-I, which has no acrosome reacton-inducing ability by itself, increases the rate of the acrosome reaction induced by FSG; the maximal rate of the acrosome reaction with FSG and SAP-I is that of the unfractionated jelly layer [57, 83–84]. The half-maximal increase in induction of the acrosome reaction by SAP-I with FSG occurs at 400 pM SAP-I, approximately the same concentration as that producing half-maximal stimulation of sperm respiration. These results indicate that SAP-I promotes induction of the acrosome reaction by acting as a specific co-factor of FSG.

4.2. *Modified FSG*

When 70% of the constituent proteins were removed from FSG by pronase digestion, the resulting fucose sulfate-rich glycoconjugate lost more than 50% of the acrosome reaction-inducing ability of the untreated FSG [57]. Complete carboxymethylation of cysteine residues in FSG results in the release of 258 kDa, 237 kDa and 120 kDa proteins from FSG and the resulting fucose sulfate-rich glycoconjugate shows only falf the ability of the intact molecule to induce the acrosome reaction. A similar fucose sulfate-rich glycoconjugate preparation can be obtained by HPLC of FSG on a TSK G-6000 PW column connected to a TSK G-4000 SW column in the presence of SDS and 2-mercaptoethanol (Shimizu and Suzuki, unpublished data). The resulting fucose sulfate-rich glycoconjugate also shows approximately half the ability of FSG to induce the acrosome reaction. The decreased potency of these fucose sulfate-rich glycoconjugates to induce the acrosome reaction can be partially restored by the addition of SAP-I. Fucoidan, a polymer of L-fucose-4-sulfate, which is a major sugar component in fucan sulfate isolated from the solubilized jelly layer of *H. pulcherrimus*, does not induce the acrosome reaction of *H. pulcherrimus* spermatozoa appreciably. Fucose sulfate-free protein constituents (258 kDa, 237 kDa and 120 kDa proteins) do not show any significant ability at inducing the acrosome reaction. When spermatozoa are first treated with these protein constituents, the addition of FSG induces a further increase in the rates of the acrosome reaction. The addition of FSG does not produce a further increase if spermatozoa are first treated with the fucose sulfate-rich glycoconjugate. These results suggest that the protein moiety of FSG, particularly when associated with the sugar moiety, is involved in induction of the acrosome reaction, although the mechanism remains unclear.

5. RECEPTORS ON SPERMATOZOA FOR MOLECULES OF THE EGG JELLY LAYER

5.1. *Equilibrium binding of SAP to spermatozoa*

We do not know much about the receptor for FSG because it is quite difficult to perform binding experiments using radioiodinated FSG. The radioiodinated FSG binds non-specifically to spermatozoa, test tubes and even the

glass-filters used in the experiments. However, the differential effects of FSG and SAP-I on the elevation of intracellular Ca^{2+} and pH in sea urchin spermatozoa suggest that FSG and SAP-I bind different receptors [33]. Using radioiodinated synthetic SAP-I, SAP-IIA or SAP-III analogue (which exhibit the same respiratory stimulating activities as SAP-I, SAP-IIA or SAP-III) receptors specific for these peptides have been characterized. *S. purpuratus* spermatozoa possess approximately 6,000–8,000 receptors/cell specific for SAP-I [63]. These receptors exclusively localize on the sperm tail [70]. Analyses of the data obtained from the equilibrium binding of a ^{125}I-SAP-III analogue to spermatozoa of the sand dollar *Clypeaster japonicus*, using Klotz, Schatchard and Hill plots, showed the presence of two classes of receptors specific for SAP-III [90]. One of the receptors (high affinity) has a Kd of 3.4 nM and 3.4×10^4 binding sites/spermatozoon. The other receptor (low affinity) has a Kd of 48 nM, with 6.1×10^4 binding sites/spermatozoon. The Kd of the high-affinity receptor is comparable to the half-maximal effective concentration of the intracellular-pH-increasing activity of SAP-III and the Kd of the low-affinity receptor is comparable to the half-maximal effective concentration of the cellular-cGMP-elevating activity of the peptide. Similar results are also obtained from a binding experiment using *H. pulcherrimus* spermatozoa and an ^{125}I-SAP-I analogue [59]. These binding sites (receptors) localize on sperm plasma membranes.

5.2. *SAP-crosslinking proteins*

It has been reported that SAP-IIA, natriuretic peptides and heat-stable enterotoxins are extracellular peptide ligands for which a membrane form of guanylate cyclase serves as a

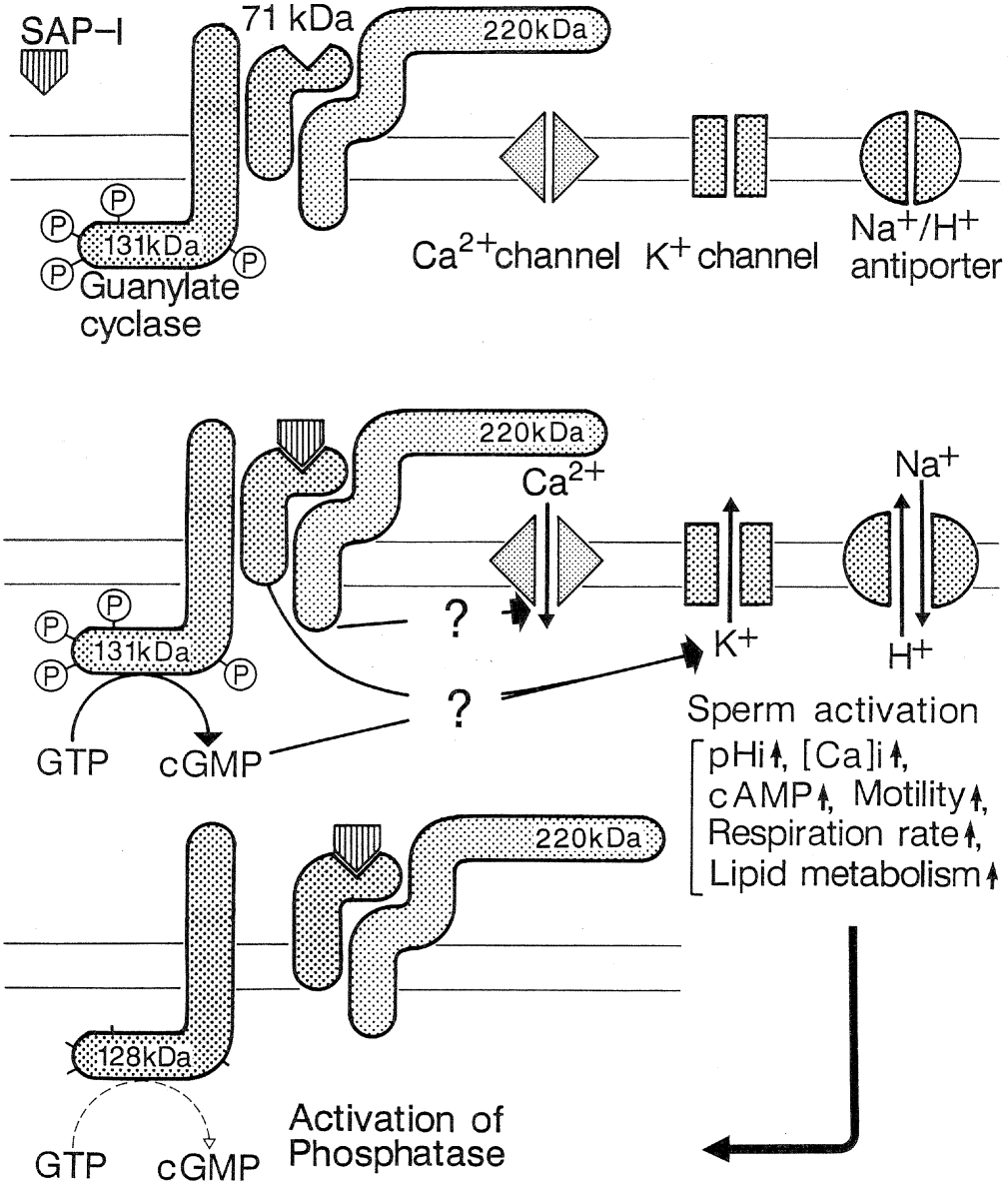

FIG. 3. Model of the signaling machanism of SAP-I in *H. pulcherrimus* spermatozoa.

cell-surface receptor [22]. These peptide ligands crosslink to the guanylate cyclase in the respective target tissue in the presence of a chemical crosslinking reagent, disuccinimidyl suberate the ^{125}I-SAP-I analogue is specifically crosslinked to a 77 kDa protein in *S. purpuratus* spermatozoa [12] and to a 71 kDa protein in *H. pulcherrimus* spermatozoa [29], although these protein themselves do not show any guanylate cyclase activity. In purification of the 71 kDa protein from *H. pulcherrimus* sperm tails, the protein was always co-purified with a 220 kDa wheat germ agglutinin (WGA)-binding protein by gel filtration, ion exchange and affinity chromatography even in the presence of detergent (CHAPS). The 71 kDa protein was separated from the 220 kDa protein only after treatment with 2% SDS at 100°C. 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 [44, 55]. SAP-I has also been reported to participate in the induction of the acrosome reaction [83]. Therefore, 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 jelly layer to the WGA-binding protein (Fig. 3).

A cDNA encoding the 71 kDa protein was isolated from a *H. pulcherrimus* testis cDNA library. An open reading frame of the cDNA predicted a protein of 532 amino acids containing a 30-residue amino-terminal signal peptide, followed by a sequence which corresponds to the N-terminal sequence of the purified 71 kDa protein. The amino acid sequence of the mature 71 kDa protein is quite similar to that of the 77 kDa protein of *S. purpuratus* (95.5% identical) and has a maximum 48% identity to a bovine and human type-I-specific scavenger receptor cysteine-rich domain [13, 20, 39, 59] (Fig. 4).

The ^{125}I-SAP-IIA analogue crosslinks to a 160 kDa protein in *A. punctulata* spermatozoa [61], which has been identified as membrane-bound guanylate cyclase [62, 77]. However, the ^{125}I-SAP-IIB analogue crosslinks to 172 kDa, 62 kDa and 58 kDa proteins in sperm heads, and 157 kDa and 62 kDa proteins in sperm tails of *Glyptocidaris crenularis* [29]. The ^{125}I-SAP-III analogue crosslinks to 126 kDa, 87 kDa and 64 kDa proteins in *C. japonicus* spermatozoa [90]. In these cases as well as the case of SAP-I, however, binding of the sperm-activating peptides to these proteins in spermatozoa resultes in a transient activation of the membrane-bound guanylate cyclase, followed by a large decrease in the activity of the enzyme. The activity is proportional to the amount of enzyme in the phosphorylated form [45–46, 77]. The dephosphorylation, which is accompanied by a change in the electrophoretic mobility of the enzyme on SDS gels, depends on the pH of the surrounding sea water, and shows an absolute requirement for Na^+ [77].

6. SYNTHESIS OF MOLECULES IN JELLY LAYER

6.1. *Oocyte growth*

It is well known that the oocytes of most groups of animals are surrounded by extracellular coats that are glycoprotein in nature and are variously designated the vitelline coat or the vitelline envelope, as well as the jelly coat in some marine invertebrates and the zona pellucida in mammals. Most egg investments are laid down between the oocyte and the follicle cells during oogenesis. Whether these investments originate from the oocyte, the follicle cells, or both, seems to vary according to the investments studied. Results of electron microscopic and histoautoradiographic studies indicate that the vitelline coat in the ascidian [49–50] and both the vitelline coat and the jelly coat in the starfish [50] are

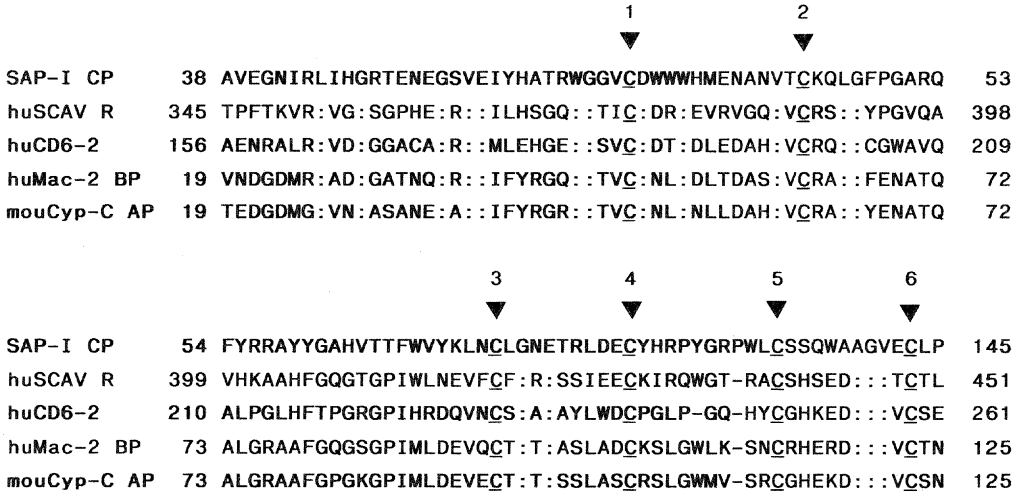

FIG. 4. Sequence alignment comparing the scavenger-receptor cysteine-rich (SRCR) domain of the 71 kDa SAP-I-crosslinking protein (SAP-I CP) with other SRCR-containing proteins. The six consensus cysteine residues underlined are indicated by numbered arrow heads. The vertical double dots show amino acid identity. The aligned sequences with SAP-I CP [59] are as follows: human macrophage scavenger receptor (huSCAV R) [39]; human lymphocyte glycoprotein CD6 (huCD6-2) [5]; human Mac-2-binding protein (Mac-2 BP) [37]; mouse cyclophilin C-associated protein (mouCyp-C AP) [21].

produced in oocytes. An immunoelectron microscopic study revealed that the envelope glycoproteins in amphibian oocytes are synthesized by the oocytes themselves [85]. In mammals, it has been shown that the zona pellucida in the mouse is synthesized in the oocytes [8, 27], whereas in the rabbit the follicle cells also participate in the formation of the zona pellucida [82]. In addition, the jelly envelopes of amphibian eggs [for review see ref. 34] and the oviductal glycoproteins of the zona pellucida of ovulated eggs in mammals appear to be secreted by the oviductal epithelial cells [1, 43]. The origin of the jelly layer molecules of sea urchins has been investigated in many ultrastructural and histochemical studies. Until recently, however, it has been unclear whether FSG and sperm-activating peptides originate from the oocyte and/or the accessory cells.

Development of oocytes in the ovary of the sea urchin *H. pulcherrimus* is divided into four apparent stages [2]. Oogonia (stage 1), identified by their small size and germinal vesicle-like nuclei, have a dense cytoplasm with little or no granularity. Early oocytes (stage 2) can be distinguished from oogonia by the presence of cytoplasmic cortical granules and yolk granules. Oocytes at stages 1 and 2 are attached to the ovarian wall. Large vitellogenic oocytes (stage 3) contain many cytoplasmic granules and possess a large germinal vesicle and nucleolus. They are usually not immediately adjacent to the ovarian wall. The most mature oocytes (stage 4) are usually located in the lumen of the ovary. They lack a germinal vesicle and nucleolus and contain many cytoplasmic granules and cortical granules. They have a distinct submembranous layer of cortical granules. Maturing oocytes are surrounded by accessory cells which contain a number of conspicuous globules.

6.2. *Immunohistochemical detection of FSG in the ovary*

Immunohistochemical examination of paraffin sections of the immature ovary of *H. pulcherrimus* using polyclonal antibodies specific to FSG in the jelly layer of *H. pulcherrimus* showed that the antibodies reacted with the accessory cells and oocytes in the ovarian lumen [2]. An intense immunohistochemical reaction was observed in many globules in the accessory cells and also frequently in the surface region of the oocytes, at a distance from the ovarian wall. At the ultrastructural level, the antibodies were found to react with the material present in the Golgi apparatus in the cytoplasm (globules) of the accessory cells [2–3]. These results strongly support our claim that FSG is produced by the accessory cells and also suggest that the Golgi apparatus plays an important role in the production of FSG. An enzyme-linked immunosorbent assay experiment using polyclonal antibodies to FSG indicated that FSG was present in the supernatants from homogenates of ovaries of the sea urchin *H. pulcherrimus* throughout ovarian development [3]. FSG was present in ovaries during both the non-breeding and the breeding seasons. However, in the non-breeding season FSG was distributed in the globules of the accessory cells, while in the breeding season FSG was present in the surface region of oocytes, and it was rarely found in the accessory cells. These results suggest that FSG is produced in the accessory cells during the non-breeding season to the early breeding season, the accessory cells showed marked ultrastructural changes and during which time FSG was detected within the Golgi apparatus and in the vacuole-like structures

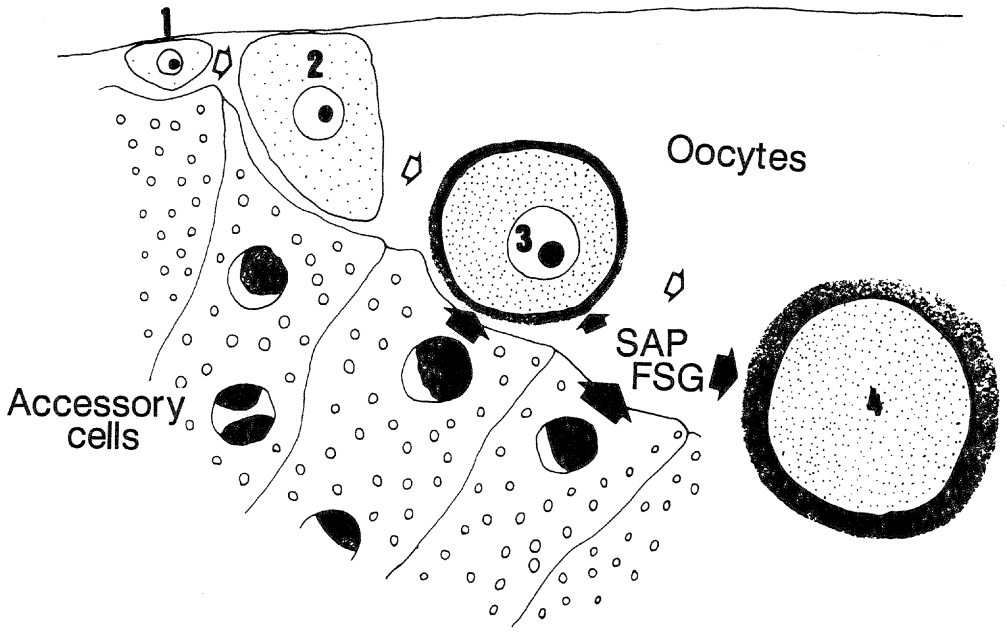

FIG. 5. Schematic drawing of formation of the *H. pulcherrimus* jelly coat during oogenesis. Sperm-activating peptides (SAP) and fucose sulfate glycoconjugate (FSG) are synthesized in the granules of the accessory cells at an early developmental stage of the ovary, and then secreted to out of the accessory cells and deposited in the surroundings of the oocyte.

of the globules in the accessory cells. This suggests that FSG is stored in these structures before it is released into the ovarian lumen. Many gold particles demonstrating specific labeling were associated with well-developed microvilli on the vitellogenic oocytes. In the mature oocytes, intense labeling was observed in the jelly layer but not in the vitelline coat. By contrast, oogonia and early oocytes were barely labeled. In all cases, neither the oocyte cytoplasm nor the subcellular organelles were labeled. These results suggest that FSG is produced by the accessory cells and is deposited initially on the surface of vitellogenic oocytes for the formation of the jelly layer (Fig. 5).

6.3. *SAP-I precursor*

A cDNA encoding the SAP-I precursor was isolated from a *H. pulcherrimus* ovary cDNA library and its nucleotide sequence was determined. An open reading frame predicted a protein of 334 amino acids containing five SAP-I and seven SAP-I-like decapeptides, each separated by a single lysine residue [35] (Fig. 6). Among them, SAP-I and four other peptides (GFDLTGGGVG, GFDLNGGGVS, GFSLTGGSVD) have been biochemically isolated from the jelly layer of *H. pulcherrimus*, and they show equivalent biological activity to SAP-I [65, 72]. The amino acid sequence of the SAP-I precursor of *H. pulcherrimus* is similar to that of the SAP-I precursor of *S. purpuratus* (92.5%, 286 out of 309 residues are identical) [35, 47]. Furthermore,

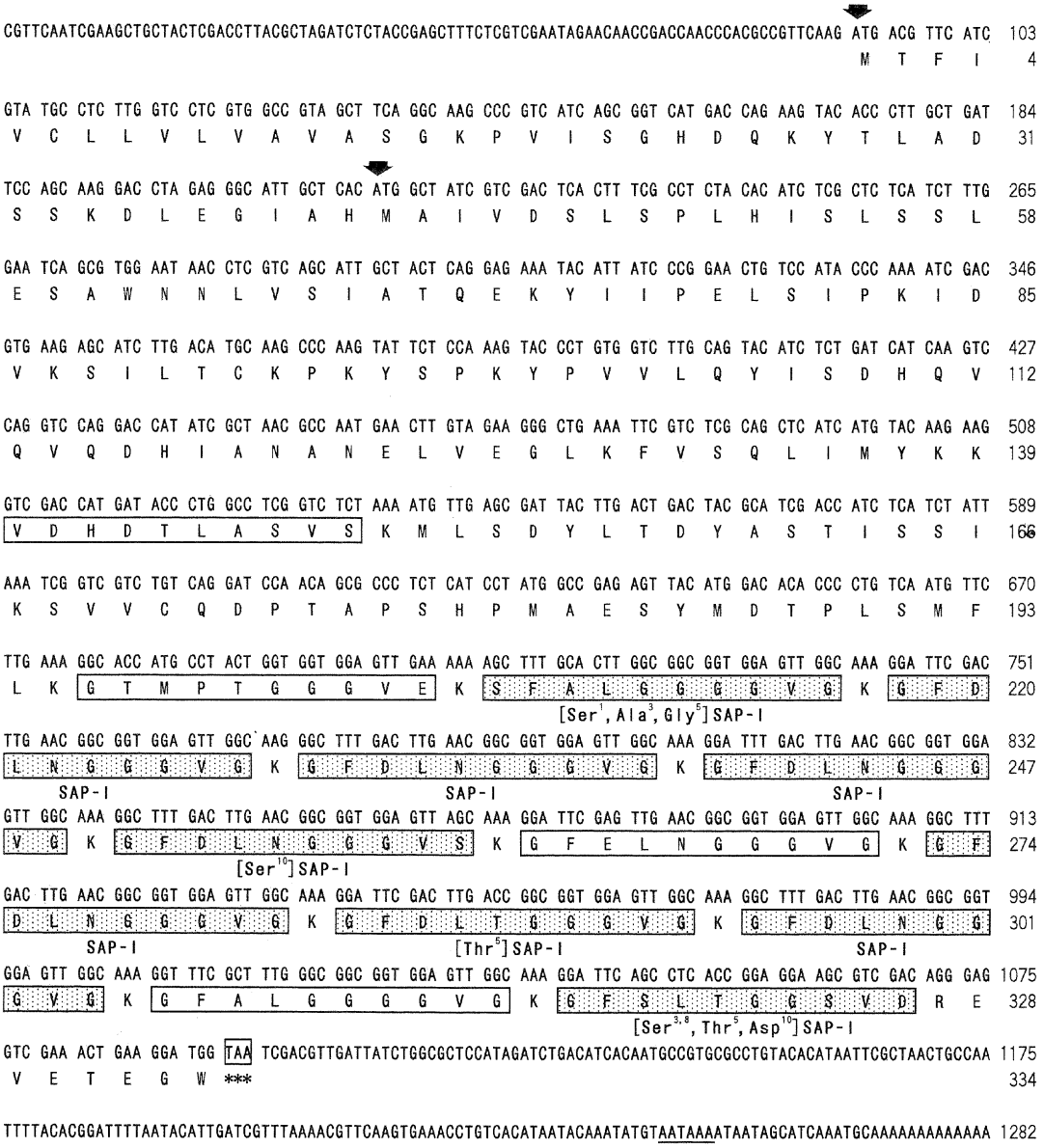

FIG. 6. Complete nucleotide sequence and deduced amino acid sequence of cDNA encoding an SAP-I precursor. The two potential start codons are indicated with arrows. SAP-I and its derivatives found in the jelly layer of *H. pulcherrimus* are indicated with stitched boxes and possible SAP-I derivatives with open boxes. The polyadenylation signal is underlined.

there is a common structural feature in the two precursors, that is, 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.

The mRNA for the *H. pulcherrimus* SAP-I precursor was detected only in the cytoplasm of the accessory cells in the pre-breeding and breeding season [35]. On the other hand, the activity of SAP-I appears in the *H. pulcherrimus* ovary containing oocytes at stage 3 [66]. Northern blot analysis showed that the cDNA encoding the SAP-I precursor of *H. pulcherrimus* does not hybridize to mRNA from the eggs of *H. pulcherrimus*. These results strongly suggest that the accessory cells of *H. pulcherrimus* serve as the site for synthesis of the *H. pulcherrimus* SAP-I precursor mRNA (Fig. 5).

7. SIGNIFICANCE OF SPERM-ACTIVATING PEPTIDES TO THE TAXONOMY AND PHYLOGENY OF ECHINOIDS

All sperm-activating peptides isolated thus far show essentially the same biological effects on sea urchin spermatozoa although these effects and the structures of the peptides are specific at the ordinal level. We have proposed a working hypothesis that sperm-activating peptides isolated from the jelly layer of sea urchin species of one taxonomic order interact with spermatozoa of other species within the same order, but not with spermatozoa within other orders [67]. This hypothesis may be extended to a further hypothesis that apparent co-evolution of peptide and receptor is due to the necessity of the resultant physiological responses. This implies that many mutations in sperm-activating peptides and their receptors could in themselves lead to the development of new orders. We have attempted to correlate specificity in the structure and biological activity of sperm-activating peptides with the taxonomic position of echinoids and have presented several changes in the taxonomy and phylogeny of echinoids [90].

Sea urchins in the order Echinoida are divided into two suborders (Temnopleurina and Echinina) but they have similar sperm-activating peptides, SAP-I and SAP-I derivatives. This is also the case in the order Clypeasteroida; *C. japonicus* and *Astriclypeus manni* belong to two different suborders, Clypeasterina and Laganina, and have SAP-III and/or SAP-III derivatives. However, in the case of the order Arbacioida, sea urchins belonging to different suborders have a structurally different sperm-activating peptide, *i.e.*, *A. punctulata* has SAP-IIA, and *Glyptocidaris crenularis* and *Stomopneustes variolaris* in the suborder Phymosomatina have SAP-IIB and/or SAP-IIB derivatives, which are quite different from SAP-IIA. Therefore, the suborders Arbacina and Phymosomatina should be placed in two different orders: Arbacioida and Phymosomatoida, respectively. This is in agreement with Durham and Melville [17] who postulated a separation of the suborder Phymosomatina from the order Arbacioida.

Disulfide linkage plays an important role in maintaining secondary and tertiary structures in proteins and peptides. SAP-IIA, SAP-IIB, SAP-IV, and SAP-V contain an intramolecular disulfide linkage, whereas SAP-I and SAP-III do not. Sea urchins in orders Arbacioida and Diadematoida are believed to be more primitive than those in the order Echinoida. Therefore, the presence of an intramolecular disulfide linkage in sperm-activating peptides could be considered a primitive characteristic of echinoids. Accordingly, *Brissus agassizii* in the order Spatangoida is more primitive than *C. japonicus* and *A. manni* in the order Clypeasteroida since SAP-V from *B. agassizii* contains an intramolecular disulfide linkage but SAP-III and SAP-III derivatives from *C. japonicus* and *A. manni* do not. This speculation is consistent with paleontological evidence that species in the order Spatangoida appeared at the beginning of the Cretaceous and species in the order Clypeasteroida appeared at the end of the Cretaceous (a time difference of about 50 million years) [16, 18]. A comparison of the amino acid sequences of SAP-V and other sperm-activating peptides shows a low degree of similarity, but SAP-IV has some similarities in its primary structure to SAP-V, such as Gly-Cys- in the N-terminal sequence and the presence of an intramolecular disulfide linkage formed by cysteine residues at the second position and C-terminus. This implies that the ancestor of echinoids in the order Spatangoida arose from a species in the order Diadematoida or that they arose from a common ancestor.

8. FUTURE PROSPECTS

Sperm-activating peptides cause many biological responses in sea urchin spermatozoa such as increases in cGMP and cAMP concentrations, induction of ionic exchanges across the plasma membrane and increases in intracellular pH. The mechanism of action of sperm-activating peptides resembles in many aspects that of the atrial natriuretic peptides, although the mechanism has yet to be described in detail. Since de Bold *et al.* [15] suggested that a factor released from the atria of the heart evokes a variety of physiological responses affecting cardiovascular homeostasis, intensive studies have been carried out in an attempt to identify the factor and a receptor(s) for the factor [41]. In this connection, it should be mentioned that the structure of a receptor for atrial natriuretic peptides was determined by Chinkers *et al.* [9] as a result of intensive research on a sperm-activating peptide receptor. This serves as an excellent demonstration that the results of research in one field can be applied to solving the problems in another fields.

The receptor for FSG, which is the major substance responsible for induction of the acrosome reaction has yet to be isolated. Study of the receptor protein appears to have great significance because questions have arisen as to whether or not this protein could also represent a calcium channel. Furthermore, FSG is a large ligand of which molecular mass may be more than 3,000 kDa. To our knowledge, there is little research on the receptor for such a ligand. Parallel to

the isolation and characterization of the receptor, the function of primary and secondary messengers will be an important future area of research, much as in other cells.

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