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Characterization of the Sperm Receptor for Acrosome Reaction-Inducing Substance of the Starfish, *Asterias amurensis*

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ABSTRACT—Acrosome reaction-inducing substance (ARIS) in the jelly coat of starfish eggs is a highly sulfated proteoglycan-like molecule of an apparent molecular size over 10^4kDa and plays a pivotal role in the induction of acrosome reaction in homologous spermatozoa. It is known in *Asterias amurensis* that ARIS binds to a restricted area of the anterior portion of sperm head, and that a glycan fragment of ARIS, named Fragment 1, consisting of 10 repeats or so of a pentasaccharide unit retains the biological activity of ARIS to an appreciable extent. In this report, we have shown the binding of Fragment 1, a relatively small pure glycan fragment of ARIS, to the putative ARIS receptor on the sperm surface by three independent methods. First, the specific binding of P-ARIS to isolated sperm membranes was monitored in real-time by using a surface plasmon resonance detector, namely a Biacore sensor system. The specific and quantitative binding of Fragment 1 to the intact sperm and to isolated sperm membranes was similarly monitored. Secondly, the binding of ^{125}I-labeled Fragment 1 to the intact sperm was stoichiometrically measured, for which we had developed a unique procedure for radioiodination of saccharide chains. It is found that Fragment 1 competes with P-ARIS for the binding to ARIS-receptor, suggesting that Fragment 1 is a useful ligand in the search for ARIS receptor protein(s). Thirdly, the putative receptor molecules were specifically labeled by using Fragment 1 as a ligand for photoaffinity crosslink technique. Taking these results into account, we conclude that starfish sperm have the ARIS receptor, which consists most probably of 50 to 60 kDa proteins, of reasonably high affinity (for Fragment 1, K_d=15 μM, B_{max}=8.4×10^4 per cell).

Key words: acrosome reaction, ARIS, receptor, photoaffinity crosslink, starfish sperm

INTRODUCTION

The acrosome reaction of sperm is an essential event for fertilization in most animals (Dan, 1967). It involves the exocytosis of the acrosomal vesicle to expose the devices for penetration through the egg coats and for subsequent fusion with the egg plasma membrane (Longo, 1997). Although it is well established that the egg coat contains signal molecules for the induction of acrosome reaction, our knowledge on the chemical structure of such molecules is much limited except for some echinoderms (for reviews see Miller and Ax, 1990; Litscher and Wassarman, 1993; Mengerink and Vacquier, 2001). In sea urchins, egg jelly polysaccharides such as the fucose sulfate polymer (FSP) (Vacquier and Moy, 1997; Alves *et al*, 1997, 1998; Vilela-Silva *et al*., 1999) plays a key role for the induction of acrosome reaction though some other jelly components are involved in the induction (Yamaguchi *et al*., 1987; Hirohashi and Vacquier, 2002). A 210-kDa protein named REJ (receptor for egg jelly) is thought to be the receptor for FSP (Moy *et al*., 1996; Vacquier and Moy, 1997). Although the study of REJ has provided us a new insight into the human polycystic kidney disease (Mengerink *et al*., 2000), stoichiometric character of REJ as the FSP receptor remains unknown.

In the starfish, *Asterias amurensis*, the actions of three egg jelly components in concert are responsible for the induction of acrosome reaction (Hoshi *et al*., 1994, 1999). Namely, a highly sulfated proteoglycan-like molecule named acrosome reaction-inducing substance (ARIS) (Uno and Hoshi, 1978; Ikadai and Hoshi, 1981a, b), sulfated steroid

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saponins named Co-ARIS (Nishiyama *et al.*, 1987a, b), and sperm-activating peptides named asterosap (Nishigaki *et al.*, 1996). ARIS alone but no others can induce the acrosome reaction in high calcium or high pH sea water, whereas either Co-ARIS or asterosap is required besides ARIS for the induction in normal sea water (Ikadai and Hoshi, 1981a, b; Matsui *et al.*, 1986). ARIS has an apparent molecular size over 10^4kDa and retains its activity even after extensive digestion with Pronase. The Pronase digest of ARIS (P-ARIS) still has an apparent molecular size of 10^4kDa or so (Ikadai and Hoshi, 1981a).

Although it is reported from our laboratory that a small number of high affinity receptor for ARIS locates in a restricted area of the anterior portion of sperm heads (Ushiyama *et al.*, 1993; Longo *et al.*, 1995), the receptor remains to be isolated and characterized. An extremely large molecular size of ARIS even after Pronase digestion interfered with our trials to characterize and identify the receptor. Poor information on the chemical structure of ARIS also limited our search for the receptor. Thus we tried to disintegrate ARIS into a much smaller fragment with the biological activity. We have recently found that sugar chain fragments liberated from ARIS or P-ARIS by ultra-sonication retains the biological activity of ARIS to an appreciable extent. The fragments are composed of 10 repeats or so of the following pentasaccharide unit and collectively named Fragment 1 (Koyota *et al.*, 1997): [4-β-D-Xyl*p*-1-3-α-D-Gal*p*1-3-α-L-Fuc*p*-4(SO_3^-)-1-3-α-L-Fuc*p*-4(SO_3^-)-1-4-α-L-Fuc*p*-1-]. Because we know the structure of Fragment 1 precisely and its size is not too large to handle, Fragment 1 seems a proper ligand for characterization and identification of the ARIS receptor even though its activity was much lower than that of ARIS or P-ARIS.

In this paper, first we show the specific binding of Fragment 1 in real time to the intact sperm and isolated sperm membranes by using a Biacore sensor system, a surface plasmon resonance detector. Then we quantitatively measure the binding of ^{125}I-Fragment 1 to the intact sperm to obtain kinetic parameters of the receptor. Finally, we use photoaffinity crosslinkers to detect the receptor molecules.

MATERIALS AND METHODS

Animals and Gametes

The starfish, *Asterias amurensis*, were collected in the breeding season from Tokyo Bay and Otsuchi Bay on the Pacific coast of Honshu, Japan, and from the coast of Tasmania, Australia. The Tasmanian population is known to be the offspring of animals that were accidentally introduced from Tokyo Bay recently. Spermatozoa of the two populations did not distinguish the egg jelly of domestic animals from that of foreign ones.

Sperm was collected as “dry sperm” by cutting the sperm ducts of the testes. When the acrosome reaction was scored 60% or less with egg jelly, the sperm was not used for experiments. Mature eggs were collected by treating the ovaries with 10 μM 1-methyladenine (Sigma Chemicals, St. Louis, MO) and washed with artificial sea water (ASW) consisting of 430mM NaCl, 9mM $CaCl_2$, 9mM KCl, 23mM $MgCl_2$, 25mM $MgSO_4$, 10mM EPPS (*N*-2-hydroxyethylpiperazine-*N*′-3-propanesulfonic acid; Dojindo Lab., Kumamoto, Japan) buffer, pH 8.2, in MQ water (water purified with a Milli-Q system from Millipore Corp., Bedford, MA). Throughout the present study, MQ water was used unless otherwise specified.

Preparation of Egg Jelly, P-ARIS and Fragment 1

Egg jelly solution was prepared according to Ikadai and Hoshi (1981a). The egg suspension was gradually acidified to pH 5.5 with 0.1N HCl to dissolve the jelly coat, then returned to pH 8.2 with 0.1N NaOH, and centrifuged at 2,000 g for 5min to remove the eggs. The supernatant was centrifuged at 10,000 g for 30min to remove cell debris and the clear solution of egg jelly was stored at –20ºC until use. P-ARIS was prepared according to Matsui *et al.* (1986) with slight modifications; briefly, ethanol precipitation of the egg jelly solution, digestion of the precipitate with Pronase (Sigma Chemicals, St. Louis, MO), gel-filtration of the digest on Sepharose CL-4B (Amersham Pharmacia Biotech AB, Uppsala, Sweden), ion-exchange chromatography of the digest on DEAE Toyopearl 650M (Tosoh Corp., Tokyo), dialysis against MQ water, and lyophilization. Fragment 1 was prepared from purified P-ARIS according to Koyota *et al.* (1997) with slight modifications; briefly dissolution of 30mg P-ARIS in 10ml of MQ water, sonication for 30min by using a Branson Sonifier Cell Disruptor 2000 (Branson Ultrasonics Corp., Danbury, CT), ion-exchange chromatography on DEAE Toyopearl 650M with a linear gradient elution program starting from water to 1.0M NaCl, dialysis of the fraction eluted about 0.9M NaCl against water, and lyophilization.

Preparation of Biotinyl Fragment 1

Fragment 1 was biotinylated according to Shinohara *et al.* (1995) with slight modifications. A 100μl-aliquot of 10mg (ca. 1μmol)/ml Fragment 1 was mixed with the same volume of 10mM EZ-link biotin-LC-hydrazide (Pierce Chemical Co., Rockford, IL) in 30% acetonitrile, and the mixture was incubated at 90°C for 2hr. The mixture was directly used for the Biacore system as described later.

^{125}I-Labeling of P-ARIS and Fragment 1

^{125}I-P-ARIS was prepared according to a modification using iodo-beads (Pierce Chemical Co., Rockford, IL) of the chloramine-T method (Hunter and Greenwood, 1962) as described previously (Ushiyama *et al.*, 1993). Prior to radioiodination, Fragment 1 was coupled with 4-methoxyphenethylamine. Fragment 1 (1.1mg; ca. 0.11μmol) was dissolved in 40μl of 3.1M 4-methoxyphenethylamine (Sigma-Aldrich Japan Corp., Tokyo) in acetic acid and incubated at 90°C for 1hr. Then, 70μl of 22.6M borane dimethylamine complex (Sigma-Aldrich Japan Corp., Tokyo) in 61.5% acetic acid was added and the mixture was stirred at 80°C for 35min. 4-Methoxyphenethylamine- conjugated Fragment 1 was purified with a Bio-Gel P-4 column (1.0×12.7cm; Bio-Rad Labs., Hercules, CA) and lyophilized. 4-Methoxyphenethylamine-conjugated Fragment 1 (1.2mg) was radioiodinated at C3 and C5 of the benzene ring by the iodo-beads/chloramine-T method as described above, and the product was purified with a Bio-Gel P-4 column (1.0×10cm). ^{125}I-Fragment 1 (11μg as fucose, 1.44×10^7cpm) thus prepared was stored below –20°C.

Conjugation of Fragment 1 with Sulfo-SBED

For photoaffinity crosslink of ARIS receptor, we developed a procedure to conjugate the partially oxidized Fragment 1 with an amino-reactive and biotin-containing photoaffinity crosslinker, sulfosuccinimidyl-2-[6-(biotinimido)-2-(*p*-azidobenzamido)-hexanoamido]ethyl-1,3′-dithiopropionate (Sulfo-SBED; Pierce Chemical Co., Rockford, IL). The reducing terminal of Fragment 1 was first reduced to prevent the “over-oxidation” of sugars from the reducing terminal during modification. Fragment 1 (10mg, ca. 1.0μmol) was dissolved in 200μl of 50mM sodium borate buffer, pH 8.3 containing

5mg (0.13mmol) sodium borohydride. After incubation at room temperature for 4hr, pH was adjusted to 4.0 with glacial acetic acid to destroy excess borohydride. The reaction mixture was left at room temperature for 14hr. The reduced Fragment 1 was purified by chromatography on a Bio-Gel P-4 column (1.0×10cm) equilibrated and eluted with water, and lyophilized. It was then dissolved in 230µl of 40mM imidazole buffer, pH 6.5 and partially oxidized with 2.5mg (12µmol) sodium periodate for 2hr at 0°C. The reaction mixture was directly applied to a Bio-Gel P-4 column (1.0×10cm) equilibrated and eluted with water, and the product was lyophilized. The partially oxidized Fragment 1 was dissolved in 100µl water and 240µl of 2M 1,6-hexanediamine (Wako Pure Chemicals, Osaka) in water was added to convert most if not all aldehyde residues into corresponding Schiff base structures. After incubation for 15min at room temperature, 20mg (0.32mmol) of sodium cyanoborohydride was added to convert the Schiff bases into more stable secondary amines. After incubation for 4hr at room temperature, 3.0mg (79µmol) of sodium borohydride was added, and incubated another 4 hr at room temperature to complete the reduction. The product, namely modified Fragment 1 having primary amine residues, was purified with a Bio-Gel P-4 column (1.0×10cm) as described, and lyophilized.

This product (5.0mg, ca. 0.5µmol) was dissolved in 250µl of 0.1M phosphate-buffered saline (PBS), pH 7.2 and mixed with 20µl of 50mM Sulfo-SBED in dimethylsulfoxide. After incubation for 1hr at room temperature, the mixture was applied to a Bio-Gel P-4 column (1.0×10cm) equilibrated and eluted with water, and the sugar-positive fractions were pooled and lyophilized. Then, the lyophilizate was dissolved in 500µl of 0.1M PBS, pH 7.2 and applied slowly to a monomeric avidin affinity column (2ml; Pierce Chemical Co., Rockford, IL). The column was thoroughly washed with 0.1M PBS, pH 7.2 to remove contaminants. The biotin-labeled, thus most probably the Sulfo-SBED-conjugated, product was eluted out of the column with 2mM D-biotin in 0.1M PBS, pH 7.2. The eluted fractions were pooled, concentrated into 400µl by a centrifuge evaporator, and applied to a Bio-Gel P-4 column (1.0×10cm) equilibrated with water. Then the product was eluted with water to be free of contaminants such as biotin and Sulfo-SBED, and lyophilized. It was estimated for sugar content by resorcinol-sulfuric acid method (Monsigny *et al.*, 1988) and for conjugated Sulfo-SBED moiety by estimation of biotin with 4´-hydroxyazobenzene-2-carboxylic acid (HABA) according to Green (1965).

Preparation of Sperm Membrane Fraction

Dry sperm was diluted 5-fold with a cavitation buffer containing 40mM KCl, 1mM $MgCl_2$, 10mM piperazine-*N,N*´-bis (2-ethanesulfonic acid) (PIPES; Dojindo Lab., Kumamoto, Japan), pH 6.5. The sperm suspension was subjected to nitrogen cavitation in a Parr nitrogen cavitation bomb (Parr Instrument Co., Moline, IL) for 30min at 1000psi with stirring on ice. The resultant suspension was fractionated by differential centrifugation at 10,000 g for 30min and 100,000 g for 1hr. The microsome fraction was re-suspended in a storage buffer containing 40mM KCl, 1mM $MgCl_2$, 20mM $MgSO_4$ and 10mM PIPES, pH 6.7, and stored at –80°C.

Bioassay of Acrosome Reaction

The acrosome reaction was assayed according to Matsui *et al.* (1986) with slight modifications. Dry sperm was diluted 100-fold in ASW containing a test sample like egg jelly, and the mixture was incubated for 5min at room temperature. The spermatozoa were fixed with glutaraldehyde at the final concentration of 0.83% and stained with erythrosine. More than 100 spermatozoa were scored in each experiment under a Nomarski microscope for the extrusion of acrosomal process. For the activity assessment of ARIS, P-ARIS and Fragment 1, high Ca^{2+} ASW (430mM NaCl, 50mM $CaCl_2$, 9mM KCl, 23mM $MgCl_2$, 25mM $MgSO_4$, 10mM EPPS buffer, pH 8.2) was used instead of ASW.

Binding Assay with Biacore System

The basic principle of the binding assay using a Biacore X (Biacore Inc., Uppsala, Sweden) has been documented previously (Johnsson *et al.*, 1991). P-ARIS was immobilized on the sensor chip surface (C1, research grade) by amino-coupling; briefly, acti-

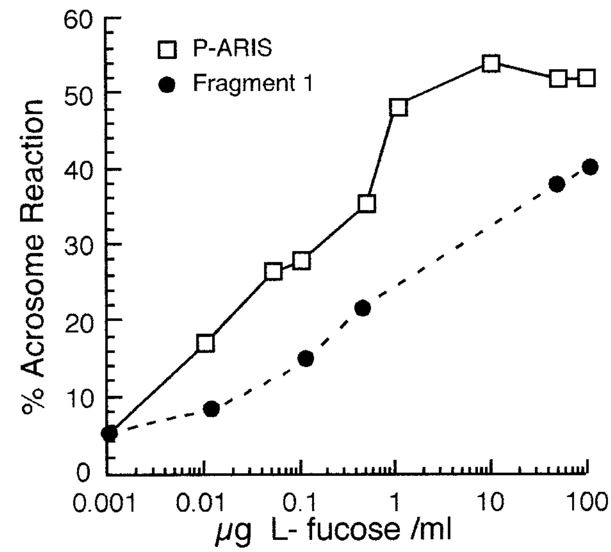

Fig. 1. Acrosome reaction-inducing activity of Fragment 1 in high calcium sea water. The capacity of Fragment 1 (●) to induce the acrosome reaction in 50mM Ca^{2+}-sea water was estimated. P-ARIS (□) was used as the positive control. Concentrations of Fragment 1 and P-ARIS are expressed as L-fucose.

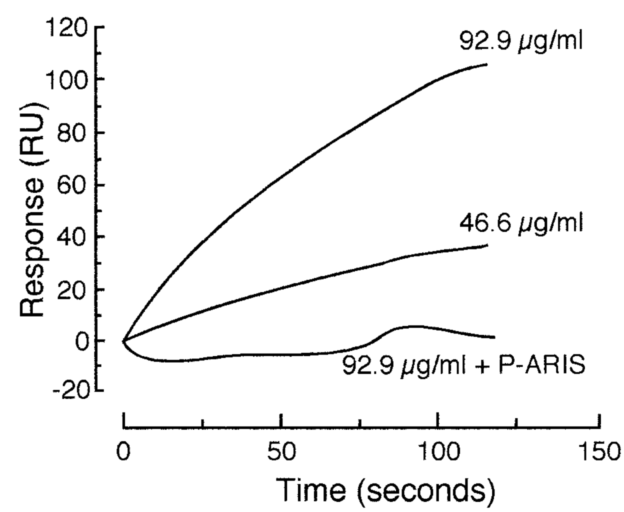

Fig. 2. Real-time assay of the binding of isolated sperm membrane to immobilized P-ARIS by Biacore system. About 0.2ng of P-ARIS was immobilized on the sensor chip surface. At the flow rate of 20µl/min, 40µl of sperm membrane suspension of the protein concentration as expressed on the corresponding sensogram was introduced to the chip surface. The bottom sensogram represents the competition of the binding by the addition of 0.4 mg P-ARIS to 40µl of the membrane suspension. Values of the ordinate, expressed in resonance units (RU), represent the increments in mass concentration of the sensor surface. The increment of 1000 RU corresponds to the binding of ca. 1ng membrane proteins. The sensograms show relative responses in RU after background subtraction versus time in seconds.

vation of carboxyl groups on the chip surface with *N*-hydroxysuccinimide (Wako Pure Chemicals, Osaka) and *N*-ethyl-*N*′- (3-dimethylaminopropyl) carbodiimide hydrochloride (Tokyo Kasei Kogyo Co., Tokyo), coupling of 0.1ml of 1mg/ml P-ARIS in 10mM sodium acetate buffer pH 4.0, blocking of excess carboxyl groups by 1.0M ethanolamine (Sigma Chemicals, St. Louis, MO). In this way, about 0.2ng of P-ARIS was immobilized. For immobilization of Fragment 1, streptavidin was immobilized on the sensor chip surface (C1, research grade) by amino-coupling. When biotinyl Fragment 1 was introduced to the streptavidin tip, about 70pg of it was immobilized. For the mock-coupled sensor chips, the immobilization procedure was followed except for the addition of the ligand, namely P-ARIS or biotinyl Fragment 1.

Samples were injected over the sensor chip surface at a flow rate of 20μl/min at 20°C. The buffer used for sample dilution was 10mM sodium acetate buffer, pH 4.0, and the running buffer was *N*-2-hydroxyethylpiperazine-*N*′- 2-ethanesulfonic acid (HEPES; Dojindo Lab., Kumamoto, Japan) buffered saline (HBS) containing 0.15M NaCl, 3.4mM EDTA (Dojindo Lab., Kumamoto, Japan), 0.005 % Tween 20 and 10mM HEPES, pH 7.4. After every run, the sensor tip was washed with 20μl of 0.05% Nonidet P-40 (Sigma Chemicals, St. Louis, MO). To make sure that the binding is a specific one, it was monitored also in the presence of a large excess of ligand in the running buffer. In each experiment, the sample was simultaneously injected over the mock-coupled sensor chip surface as a blank run. All data were corrected for the background by subtracting the blank run, using BIA evaluation 3.0 software.

Binding Assay of ^{125}I-P-ARIS and ^{125}I-Fragment 1

A 20μl aliquot of sperm suspension in ASW (1.4×10^{7}cells/μl) was mixed with 30 μl of ASW containing ^{125}I-P-ARIS (0.22 μg as fucose, 2.5×10^4 cpm) or ^{125}I-Fragment 1 (0.33μg as fucose, 4.8×10^{3}cpm). After incubation for 30min at room temperature, the reaction mixture was layered onto the top of 0.35ml of isotonic, 20% sucrose in buffered saline (138mM NaCl, 9.0mM KCl, 9.3mM $CaCl_2$, 23.3mM $MgCl_2$, 25.5mM $MgSO_4$, 10mM EPPS, pH 8.3 prepared in a 0.4ml test tube (4mm in diameter). Spermatozoa were spun down by centrifugation at 15,000 g for 5min. After removal of the supernatant, the radioactivity bound to the spermatozoa was measured with a gamma counter (Aloka Co. Ltd., Tokyo). For competition assays, the cold ligand of an adequate amount was mixed with the radioiodinated ligand before incubation with sperm.

Kinetic parameters for the binding of ^{125}I-Fragment 1 were estimated by Scatchard analysis (Scatchard, 1949).

Photoaffinity Crosslink Experiments

Dry sperm was diluted 5-fold with ASW, and 50μl of the sperm suspension was mixed with 50μl of ASW with or without 1 mg Fragment 1 (ca. 100nmol). After incubation for 10min on ice, 2μg (ca. 2nmol) of Sulfo-SBED-conjugated Fragment 1 in 20μl ASW was added to the mixture and incubated in the dark for another 10min on ice. After the mixture was photoactivated with UV light (365μm) for 15min on ice, the spermatozoa were precipitated by centrifugation at 2,700 g for 1min. The precipitate was washed three times with 1ml ASW, and finally mixed with 50μl of double concentrated sample buffer for SDS-PAGE and boiled for 4min with mixing by a pipette to destroy DNA. Each sample of 5μl was subjected to 10% SDS-PAGE, and protein bands were detected with Western Blot with anti-biotin goat IgG (1mg/ml solution from Pierce Chemical Co., Rockford, IL; 1:1000) or normal goat IgG (0.5mg/ml solution from Santa Cruz Biotechnology Inc., Santa Cruz, CA; 1:500) as the 1st antibody, and anti-goat IgG donkey IgG conjugated with horseradish peroxidase (HRP) as the 2nd. The activity of HRP was detected by an ECL-plus system (Amersham Pharmacia Biotech AB, Uppsala, Sweden).

Chemical Analysis

Sugar content was determined by phenol-sulfuric acid method (Dubois *et al.*, 1956) or resorcinol-sulfuric acid method (Monsigny *et al.*, 1988), with L-fucose as a standard. The amount of protein was determined according to Bradford (1976) using bovine serum albumin as a standard.

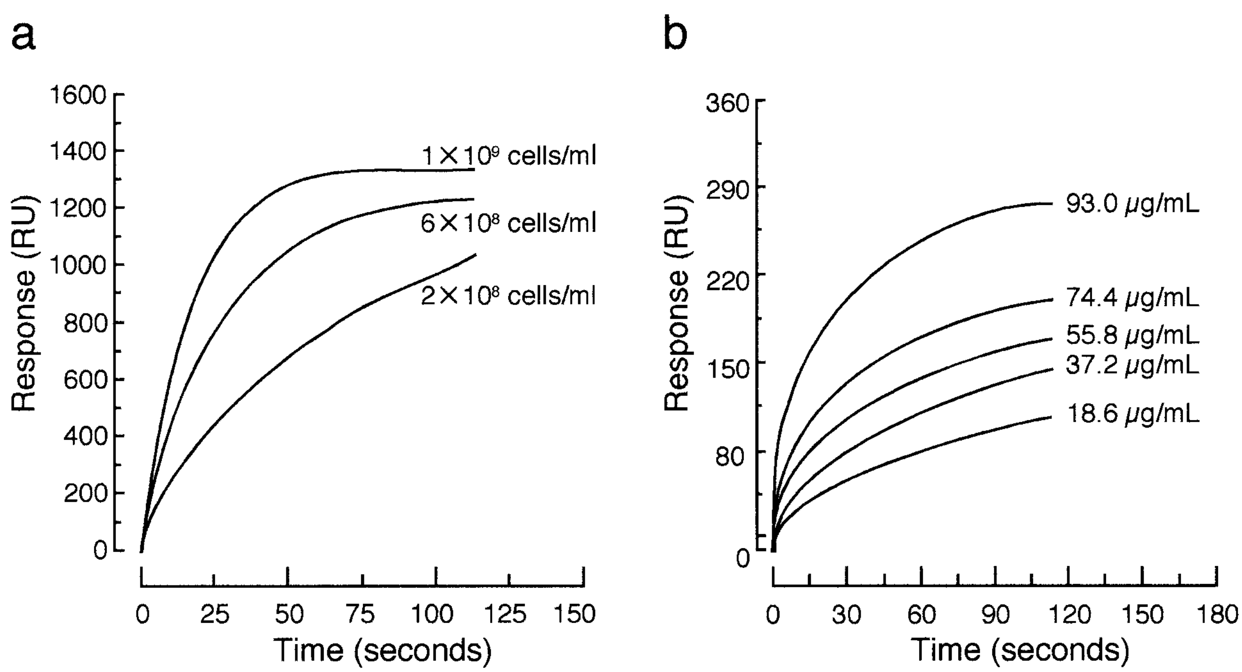

Fig. 3. Real-time assay of the binding of intact sperm (a) and sperm membrane (b) to immobilized Fragment 1 by Biacore system. About 70pg of biotinyl Fragment 1 was immobilized by streptavidin fixed to the sensor chip surface. At the flow rate of 20μl/min, 40μl suspension of sperm (a) or sperm membrane (b) of the concentration as expressed on the corresponding sensogram was introduced to the chip surface.

RESULTS

Biological Activity of Fragment 1

The capacity to induce the acrosome reaction in high Ca^{2+} (50mM) sea water was estimated with Fragment 1 and P-ARIS. Fig. 1 shows that Fragment 1 alone induces the acrosome reaction significantly in high Ca^{2+} sea water though the specific activity is almost two-order lower than P-ARIS.

Real-Time Monitoring of Ligand Binding to ARIS Receptor with Biacore System

Direct immobilization of about 0.2ng P-ARIS to the sensor tip surface caused the shift of almost 210RU (resonance units), whereas indirect immobilization of about 70pg biotinyl Fragment 1 resulted in the shift of 70RU. These values fit well with those of the standard Biacore assays, suggesting that the system is applicable to the study of ARIS receptor. As shown in Fig. 2, the binding of sperm membrane to immobilized P-ARIS is blocked by an excess of free P-ARIS, confirming the suitability of the Biacore system for the study of ARIS receptor. Fig. 3 shows that Fragment 1 indeed binds to the intact sperm and to the isolated membrane. The binding reaches a plateau within one to two minutes after the injection. Fig. 3 also shows that binding of intact sperm to the immobilized Fragment 1 causes much larger RU shifts than that of sperm membrane, which further supports the methodological validity.

Stoichiometric Analysis of Ligand Binding to ARIS Receptor

Binding of ^{125}I-P-ARIS to sperm was inhibited by a large excess of cold P-ARIS and Fragment 1 as shown in Fig. 4. Here again, Fragment 1 is less potent than P-ARIS in the inhibition of ^{125}I-P-ARIS binding to sperm. Since the cold ligands inhibit P-ARIS binding greatly but not completely, the binding to sperm should consist of two components at least, specific binding and non-specific one.

Since we cannot figure out the molecular size of P-ARIS, we have to use Fragment 1 for stoichiometric analysis despite its capacity to trigger the acrosome reaction and

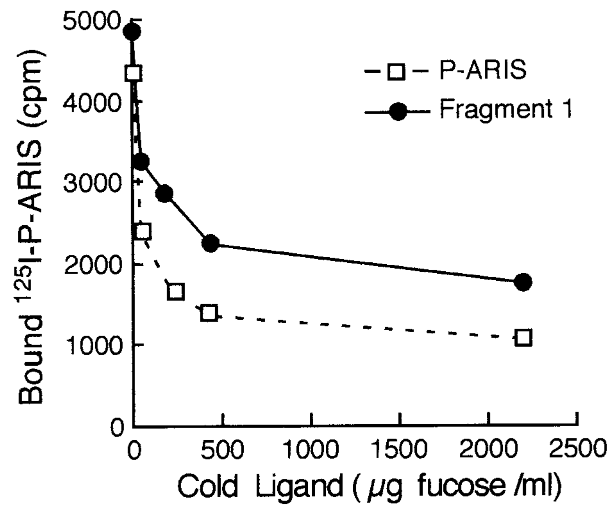

Fig. 4. Competitive inhibition of ^{125}I-P-ARIS binding to sperm with Fragment 1 and cold P-ARIS. Sperm (2.8×10^9 cells) was incubated with ^{125}I-P-ARIS (0.22μg as L-fucose, 2.5×10^4 cpm) in the presence or absence of Fragment 1 (●) or cold P-ARIS (□) in 50μl of ASW for 30min. The radioactivity bound to sperm was plotted against the concentration of competitors.

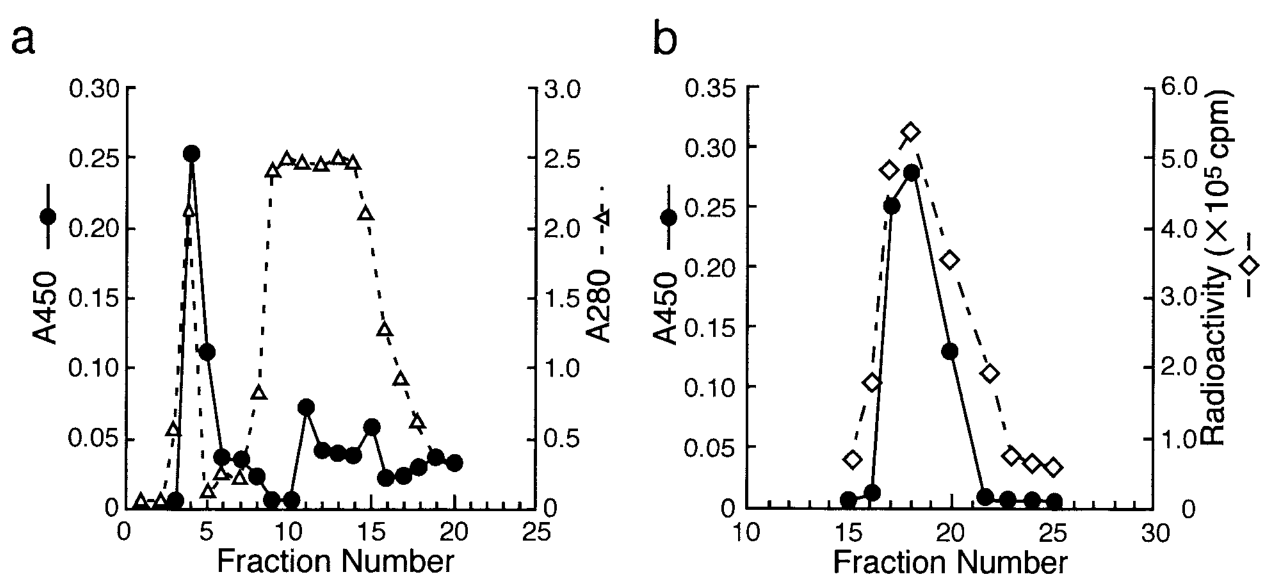

Fig. 5. Purification by gel-filtration of 4-methoxyphenetylamine-conjugated Fragment 1 before (a) and after (b) radioiodination. Bio-Gel P-4 columns were used for purification. The size of column was 1.0×12.7cm (a) and 1.0×10cm (b). Fragment 1 represented by sugar content (–●–), 4-methoxyphenetylaminyl residue by UV absorption at 280nm (--△--), and radioactivity (–◇–) were monitored. Sugar content was estimated by resorcinol-sulfuric acid method and expressed by the absorption at 450nm. See Materials and Methods for the details of conjugation and radioiodination.

bind to the receptor is significantly lower than that of P-ARIS. Fragment 1 has one aldehyde residue at the reducing terminal. Thus it seems reasonable to label the molecule at the reducing terminal, since such modification may not affect the biological activity very much. We developed an easy method of radioiodination of sugar chains by using 4-methoxy-phenethylamine as summarized in Materials and Methods and Fig. 5. The first step is conjugation of Fragment 1 at the reducing terminal with 4-methoxy-phenethylamine. As shown in Fig. 5a, this compound was easily separated from Fragment 1 or its derivatives. The final product, 4-methoxy-3,4-diiodo(^{125}I)-phenethylamine derivative of Fragment 1, was easily purified also by a simple chromatography as shown in Fig. 5b.

By using the ^{125}I-Fragment 1 derivative, we estimated the specific binding of Fragment 1 to the intact sperm as shown in Fig. 6a. Kinetic parameters for the ARIS receptor were calculated from the Scatchard plot shown in Fig. 6b; K_d=15 μM, B_{max}=8.4×10^4 per cell.

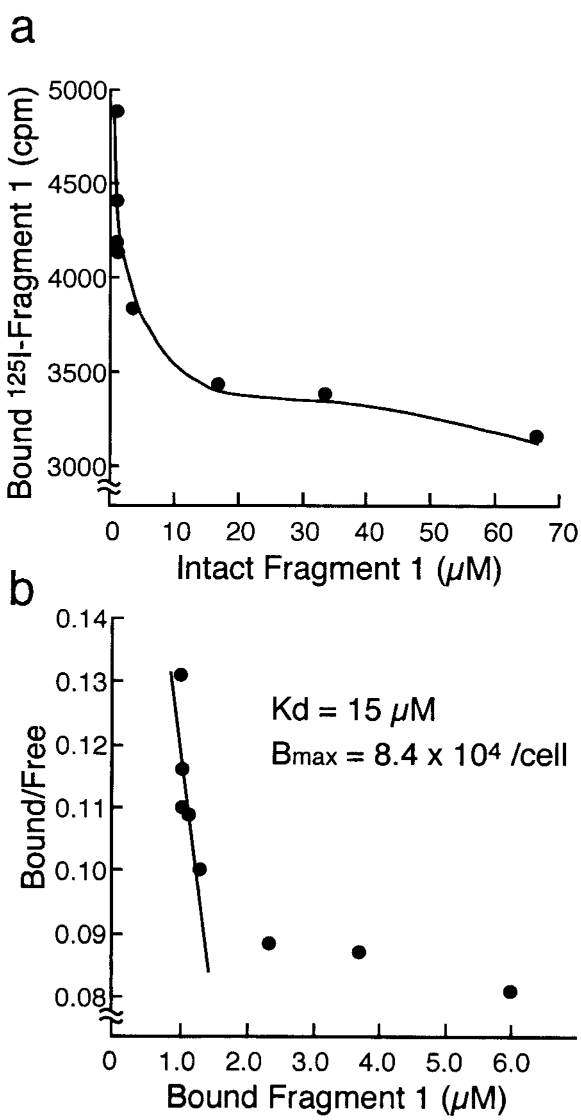

Fig. 6. Scatchard analysis of quantitative and specific binding of Fragment 1 to sperm. Sperm (2.8×10^9 cells) was incubated for 30 min in 50μl of ASW with ^{125}I-Fragment 1 (0.33μg as L-fucose, 4.8×10^3 cpm) in the presence of cold Fragment 1 at various concentrations. (a) The radioactivity bound to sperm was plotted against the concentration of cold Fragment 1. (b) Kinetic parameters for the binding of Fragment 1 to sperm were estimated by Scatchard plot analysis of the data presented in (a).

Detection of the Putative ARIS Receptor Proteins by Photoaffinity Crosslink

To identify ARIS receptor proteins, we tested several crosslinker reagents without success such as *N*-(β-maleimidopropionic acid) hydrazide, *N*-(ε-maleimidocaproic acid) hydrazide, *N*-(κ-maleimidoundecanoic acid) hydrazide and *p*-azidobenzoyl hydrazide from Pierce Chemical Co., Rockford, IL, and Biotin-*N*-BOC-phenylaminodeazirin (Seikagaku Corp., Tokyo). However, we found that Sulfo-SBED conjugate seemed useful to detect the ARIS receptor proteins. We first tried to conjugate the intact Fragment 1 at the reducing terminal with Sulfo-SBED via 1,6-hexanediamine, but the signal was not strong enough. If Fragment 1 is oxidized with periodate, the number of aldehyde residues to which Sulfo-SBED can be conjugated via 1,6-hexanedi-

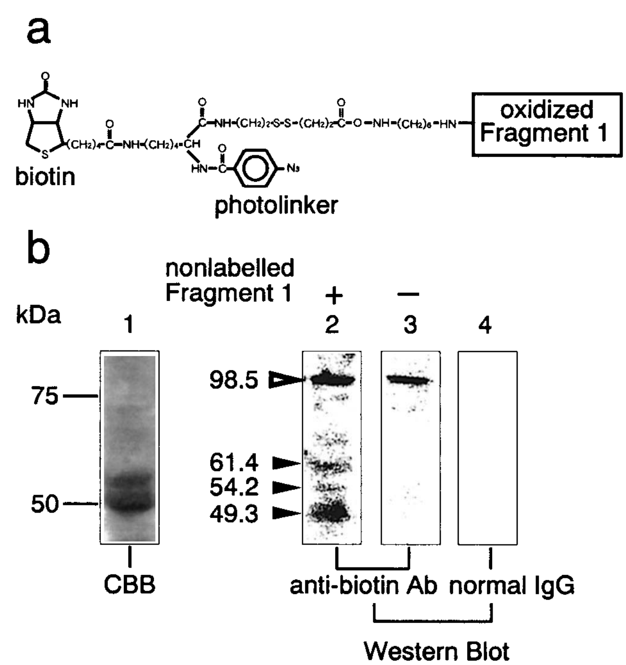

Fig. 7. Detection of putative ARIS receptor proteins by photoaffinity crosslink of Fragment 1. Partially oxidized Fragment 1 was conjugated with a photoaffinity, amino-reactive, biotin-labeled crosslinker, Sulfo-SBED (a). The conjugate was incubated with sperm in the presence (lanes 2 and 4) or absence (lane 3) of an excess of Fragment 1 and photoactivated. Sperm proteins were subjected to SDS-PAGE (10%) and detected with Coomassie Brilliant Blue (CBB) (lane 1). The cross-linked biotin was detected by Western Blot with anti-biotin goat IgG (lanes 2 and 3) or normal goat IgG (lane 4) as the 1st antibody, and anti-goat IgG donkey IgG conjugated with HRP as the 2nd.

amine increases. However, the biological activity of Fragment 1 decreases as the oxidation proceeds (Koyota *et al.*, 1997). Taking the results of pilot experiments into account, we chose the procedure summarized in Materials and Methods to make the Sulfo-SBED-conjugate of partially oxidized Fragment 1 (Fig. 7a). Biacore assay showed that this derivative retained enough capacity to bind to the sperm (data not shown).

Fig 7b shows that three sperm proteins of 50-60kDa are cross-linked with the Sulfo-SBED conjugate. The crosslink was specifically blocked by an excess of intact Fragment 1. The band near 100kDa is not sensitive at all to the presence of intact Fragment 1. This band seems to be a sperm protein that has a structure partially at least similar to biotin because similar bands appear whenever avidin is used. The positive bands of 50-60kDa are not distinct, suggesting that they are glycoproteins.

DISCUSSION

It is well established that saccharide chains of the egg coats play pivotal roles in the induction of acrosome reaction, but their structures remain to be determined except for some echinoderms (for reviews see Miller and Ax, 1990; Litscher and Wassarman, 1993; Mengerink and Vacquier, 2001). In these animals, a very long sulfated linear polysaccharide chain plays a key role in the induction of acrosome reaction and the sperm seem to recognize the highly ordered spatial arrangement of the sulfate moieties (Hoshi *et al*, 1999). Despite of recent progress in structural analysis of these polysaccharide chains (Koyota *et al.*, 1997; Alves *et al*, 1997, 1998; Vilela-Silva *et al.*, 1999), our understanding of the sperm receptors for them is not much improved. Indeed, no information is available on the kinetic parameters for FSP binding to REJ in sea urchins. Although the binding of FSP to REJ is reported (Vacquier and Moy, 1997), there is no direct evidence that the binding is really a specific one. Such parameters published for ARIS binding to starfish sperm were calculated by assuming the molecular size of ARIS to be 1×10^4kDa (Ushiyama *et al.*, 1993). This assumption seems not very bad, yet we do not know the real size even now.

In this paper, we have characterized the ARIS receptor by using Fragment 1, which is chemically well characterized, as the ligand. Although the biological activity of Fragment 1 is much lower than ARIS/P-ARIS, our data provide the rationale of using it as the ligand. Firstly, specific and quantitative binding of Fragment 1 to the intact sperm and isolated sperm membranes is shown by two methods of different principles (Figs. 3 and 4). Secondly, it competes quantitatively with P-ARIS for the specific binding to intact sperm (Fig. 4). Thirdly, and most importantly, specific labeling of some sperm proteins of 50-60kDa is achieved by photoaffinity crosslink technique using a Fragment 1 derivative. It is most likely that these proteins are the components of ARIS receptor, or at least those closely related to the receptor. Analysis of these proteins is now under progress in our laboratory.

The kinetic parameters obtained in this study (K_d=15μM, $B_{max}=8.4\times10^4$ per cell) appear quite different from those that we published previously (K_d=2nM, $B_{max}=1\times10^2$ per cell) on the assumption that the molecular size of ARIS is 1×10^4kDa. However, the apparent difference may not be irrational if the clustering effect, which is generally observed with protein-carbohydrate interactions (for a review see Lee, 1992), is taken into account; Fragment 1 itself has a repetitive structure (Koyota *et al.*, 1997) and ARIS seems to have many, extremely long saccharide chains ending with 200 repeats or so of Fragment 1 (Hoshi *et al.*, 1994, 1999). In many cases, multivalent binding of low affinity single sites generates high affinity and specificity of protein-carbohydrate interactions. Thus, very high affinity of intact ARIS and P-ARIS may be generated by its extreme polyvalence. Indeed, the activity of ARIS fragments correlates to their size, and the minimal functional size of ARIS is known to be almost the size of Fragment 1 (Ushiyama *et al.*, 1995).

This paper will contribute also to solve some technical problems in glycobiology. Firstly, we developed a procedure to conjugate sulfated sugar chains such as Fragment 1 with the photoaffinity crosslinker, Sulfo-SBED. This method has advantages over those we tried but failed previously; the most important two are the multiplicity of crosslink sites in the derivative and easiness in purification and detection of the derivative due to the presence of biotin. Needless to say, it is essential for any method of ligand modification for specific binding assays that the derivative is free of the original ligand. When we designed the methods described above, this point was carefully considered. Secondly, we developed a simple, reproducible and efficient procedure of radioiodination of sulfated saccharide chains by 4-methoxy-phenetylamination of the reducing terminal followed by iodination at C3 and C5 of the benzene ring. Thirdly, we showed that the Biacore system is useful for the study of sperm-egg glycoconjugate interactions.

Identification of ARIS receptor protein(s) using the procedure developed in the present work will expose a new cutting-edge in the study of the molecular mechanism of the induction of acrosome reaction.

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