The Sea Urchin Egg Receptor for Sperm: Isolation and Characterization of the Intact, Biologically Active Receptor

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Abstract. The species-specific binding of sea urohin sperm to the egg is mediated by an egg cell surface receptor. Although earlier studies have resulted in the cloning and sequencing of the receptor, structure/function studies require knowledge of the structure of the mature cell surface protein. In this study, we report the purification of this glycoprotein to homogeneity from a cell surface complex of *Strongylocentrotus purpuratus* eggs using lectin and ion exchange chromatography. Based on the yield of receptor it can be calculated that each egg contains approximately 1.25×10^6 receptor molecules on its surface. The receptor, which has an apparent M_r of 350 kD, is a highly glycosylated transmembrane protein composed of ~70% carbohydrate. Because earlier studies on the partially purified receptor and on a pure, extracellular fragment of the receptor indicated that the carbohydrate chains were important in sperm binding, we undertook compositional analysis of the carbohydrate in the intact receptor. These analyses and lectin binding studies revealed that the oligosaccharide chains of the receptor are sulfated and that both N- and O-linked chains are present. Functional analyses revealed that the purified receptor retained biological activity; it inhibited fertilization in a species-specific and dose-dependent manner, and polystyrene beads coated with it bound to acrosome-reacted sperm in a species-specific manner. The availability of biochemical quantities of this novel cell recognition molecule opens new avenues to studying the interaction of complementary cell surface ligands in fertilization.

In fertilization intercellular recognition mediated by specific cell surface molecules plays a central role in the proper interaction of sperm and egg cells (as reviewed in: Yanagimachi, 1978; Wassarman, 1987; Glabe et al., 1991; Foltz and Lennarz, 1993). In the case of the sea urchin, earlier studies on fertilization revealed that interaction of the abundant sperm protein bindin (Vacquier and Moy, 1977) with a receptor molecule for sperm on the egg surface (Schmell et al., 1977) is a key step of gamete recognition. Extensive structural and functional information has been accumulated about the sperm adhesion molecule bindin, which is a 30.5-kD protein component of the acrosome granule (Vacquier and Moy, 1977; Gao et al., 1986; Minor et al., 1989). Since it is clear that bindin becomes exposed to the apical sperm surface during the acrosome reaction (Moy and Vacquier, 1979), it has been assumed that the sperm binding component of the egg surface interacts with bindin (Glabe and Vacquier, 1987). Several earlier reports described the identification of high molecular weight glycoconjugates that had the properties of a sperm receptor (Schmell et al., 1977; Rossignol et al., 1981, 1984; Ruiz-Bravo et al., 1986; Acevedo-Duncan and Carroll, 1986), but detailed characterization was thwarted by the inability to obtain the molecule in pure form.

The problems encountered in these earlier studies were overcome by an alternative approach, involving generation of proteolytic fragments of the extracellular domain of the receptor. Following initial characterization of heterogeneous tryptic fragments of the receptor (Ruiz-Bravo and Lennarz, 1986, 1989), it was found that treatment of eggs with lysylendoproteinase C released a large proteolytic fragment of the egg receptor which could be purified to homogeneity (Foltz and Lennarz, 1990). This extracellular receptor fragment of ~70 kD was found to be a glycoprotein that exhibits species specificity with respect to its ability to bind sperm and its inhibitory effect on fertilization. It was also shown to interact with purified bindin particles in a species-preferential fashion (Foltz and Lennarz, 1990). Antibodies raised against the 70-kD receptor fragment inhibit sperm binding and react with a single polypeptide of ~350 kD on immunoblots of egg cell surface preparations (Foltz and Lennarz, 1992).

Further insights into the structural features of the egg receptor came from the cloning and sequencing of the molecule using anti-receptor IgG to screen an expression library made from immature sea urchin ovary mRNAs (Foltz et al., 1993). The egg receptor for sperm was found to be a novel cell recognition molecule with at least one transmembrane

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domain and numerous potential sites for N- and O-glycosylation. Analysis of the deduced primary sequence revealed sequence similarity between the sperm-binding domain of the extracellular portion of the receptor and the hsp 70 family of proteins. A recombinant protein representing the sperm binding domain was species-specific both in binding to acrosome-reacted sperm and in inhibiting fertilization. This new finding was not consistent with the earlier hypothesis that the polypeptide backbone confers species-specificity to the binding process, whereas the oligosaccharide chains of the receptor are the adhesive element in gamete recognition (Ruiz-Bravo and Lennarz, 1986). It is now apparent that both the polypeptide backbone and the carbohydrate chains are involved in the binding process.

After identification and cloning of the receptor and the availability of the deduced amino acid sequence, it became important to define the biochemical and biological properties of the mature, intact glycoprotein. To study both the function of the carbohydrate moieties of the receptor and the possible interaction of it with other proteins that might participate in sperm binding and/or egg activation, the pure, intact molecule is a prerequisite. In this report we describe the purification of the 350-kD receptor molecule from *Strongylocentrotus purpuratus* egg surface membranes using lectin and ion exchange chromatography. Purification was monitored by immunoblot analysis with antibodies to the egg receptor. Based on the yield of purified receptor it is estimated that there are 1.25×10^6 molecules of this molecule on the egg cell surface. Analysis of the receptor revealed that, as expected, it contains ~70% carbohydrate in the form of N- and O-linked oligosaccharide chains. Several lines of evidence indicate that the isolated receptor molecule retains biological activity. First, the purified glycoprotein inhibits fertilization in a species-specific manner. Second, beads coated with the receptor bind to acrosome reacted sperm, demonstrating that it retains species-specific adhesive properties.

Materials and Methods

Preparation of Gametes and Fertilization Bioassays

Adult *S. purpuratus* (Marinus, Inc., Long Beach, CA) and *Arbacia punctulata* (Gulf Specimens, Panama City, FL) sea urchins were maintained and gametes were collected as described previously (Schmell et al., 1977). Established bioassays were employed to study the biological effect of the purified egg receptor on fertilization (Kinsey and Lennarz, 1981; Rossignol et al., 1984; Ruiz-Bravo and Lennarz, 1986). Values were normalized to parallel bioassays obtained in appropriate buffer controls to account for possible inhibitory effects of buffer components other than the biologically active component under investigation.

Preparation of Egg Surface Complex

Egg surface complex containing plasma membrane, vitelline layer and cortical granules was prepared at 0–4°C by carefully homogenizing the egg suspension with a hand operated glass homogenizer using a Teflon pestle. The formation of egg ghost membranes was monitored microscopically, as described in detail by Kinsey (1986). All buffers used were supplemented with protease inhibitors at a final concentration of 1 mM: phenylmethanesulfonyl fluoride, aprotinin, soybean trypsin inhibitor, antipain, leupeptin and benzamidine (Sigma Chemical Co., St. Louis, MO). For the purification of egg receptor for sperm, membranes were resuspended in buffer A (20 mM Tris-Cl, pH 7.4, 0.5 M NaCl) and processed immediately or stored frozen at −70°C until usage.

Purification of Egg Receptor

All purification steps were carried out at 0–4°C. Egg cell surface complex membranes were solubilized at a protein concentration of 1 mg/ml with 2% (wt/vol) β-octylglucoside (Boehringer-Mannheim Corp., Indianapolis, IN) in buffer A and incubated on ice for 60 min. Insoluble material was removed by centrifugation at 105,000 *g* (model Ti70 rotor, Beckman Instruments, Inc., Palo Alto, CA) for 60 min. The supernatant was diluted 1:4 (vol/vol) with buffer A and incubated overnight under gentle shaking with 10 ml of WGA-agarose. The lectin matrix was allowed to settle on ice at 0°C and the supernatant carefully removed. In all subsequent steps, all wash or elution buffers contained 0.1% (wt/vol) β-octylglucoside. Immobilized WGA with bound egg receptor was washed twice with 10 vol of buffer A, followed by the same amount of buffer B (20 mM Tris-Cl, pH 7.4, 0.15 M NaCl). Elution of the receptor was accomplished by incubating the WGA-agarose with 0.5 M N-acetylglucosamine in buffer B for 20 min on a shaker. The eluted fraction was immediately applied to 10 ml of DEAE-Sephacel ion-exchanger resin (Pharmacia LKB, Uppsala, Sweden) and incubated for 2 h with gentle shaking. The matrix was allowed to settle, the supernatant removed and the resin was washed twice with 10 column volumes of buffer B. The resin was then poured into a glass column and protein was eluted with a linear gradient from 0.5 M to 4 M NaCl in 20 mM Tris-Cl, pH 7.4. 15 3 ml fractions were collected and stored on ice before analysis. Peak fractions containing purified 350 kD receptor were pooled and dialyzed against distilled water before ultrafiltration using a PM-30 membrane from Amicon (Lexington, MA). The concentrated receptor sample was lyophilized and stored frozen at −20°C until use. Monitoring of the entire purification procedure and assessment of the homogeneity of the final egg receptor preparations was done by silver staining of 3–15% gradient SDS polyacrylamide gels, as well as by immunoblotting using an antibody specific to the egg receptor (see below). Protein concentration was determined by the BioRad micro protein assay (BioRad Laboratories, Richmond, CA) using type I mucin from bovine submaxillary glands (Sigma Chemical Co.) as a glycoprotein standard. Values for the protein content of the receptor determined by this method were corrected by a factor (0.86) obtained by direct determination of the amino acid composition.

Lectin Purification and Lectin Chromatography

WGA was extracted from crude wheat germ by the method of Vretblad (1976). Partially purified WGA was purified to homogeneity by affinity chromatography using N-acetylglucosamine agarose (Sigma Chemical Co.). Purification of the lectin was monitored by SDS-PAGE analysis combined with immunoblotting using a polyclonal antibody to WGA purchased from Sigma Chemical Co. Purified WGA was immobilized using cyanogen bromide-activated agarose and the affinity matrix was washed and equilibrated as described by the manufacturer (Pharmacia LKB). Before use, WGA-agarose was equilibrated twice with five column volumes of incubation buffer and re-equilibrated after N-acetylglucosamine elution by extensive washing with 1.5 M NaCl.

All other immobilized lectins were purchased from EY Labs (San Mateo, CA), except *Tetragonolobus purpureas* agglutinin which was from Sigma Chemical Co. To investigate the affinity of the purified egg receptor to different lectins, a solution containing the receptor was incubated overnight in buffer A with insolubilized lectins as described above. After extensive washing of the affinity matrix, suitable aliquots of beads were treated with SDS-PAGE sample buffer and electrophoresed. The unbound column fractions and the SDS-eluted bead fractions were analyzed by immunoblotting.

Antibodies to Egg Receptor

A female New Zealand white rabbit was immunized by the Pocono Rabbit Farm and Laboratories (Canadensis, PA) with soluble, native GST-45A fusion protein, which represents an extracellular domain of the egg receptor with sperm binding properties (Foltz et al., 1993). After primary subcutaneous injection of 200 μg of fusion protein emulsified in Freund's complete adjuvants (Bethesda Research Laboratories, Gaithersburg, MD), a 100 μg booster was given after 2 wk and a 50 μg booster was given after 4 wk, both in Freund's incomplete adjuvants. 2 wk later blood samples were taken and the immunoreactive serum isolated. Ammonium sulfate precipitation followed by DEAE chromatography was used to purify the IgG fraction from the crude antiserum (Harlow and Lane, 1988).

Gel Electrophoresis

Due to the low mobility of the receptor, SDS-PAGE analysis was performed

on 1.5 mm-thick 3–15% gradient gels (Laemmli, 1970). Under these conditions a wide molecular weight range of proteins could be monitored during the purification procedure. After fixation for 2 h in 50% methanol and 1% acetic acid, proteins were visualized by silver staining as described previously (Foltz and Lennarz, 1990).

Immunoblotting, Lectin Blotting and Glycan Detection

Proteins separated by SDS-PAGE were transferred to nitrocellulose membranes (Schleicher and Schuell, Inc., Keene, NH) by the method of Towbin et al. (1979). Prestained molecular weight standards were from Bethesda Research Laboratories. Immunoblots were blocked for 2 h in 50 mM sodium phosphate, pH 7.4, 150 mM NaCl, 5% nonfat dry milk (blotto) and subsequently incubated overnight with 1:1,500 diluted primary antibody. After extensive washing in blotto, immunoblots were incubated for 60 min with peroxidase-conjugated secondary antibody (Boehringer-Mannheim Corp., Indianapolis, IN) at a dilution of 1:2,000. The enhanced chemiluminescence (ECL) detection kit from Amersham Corp. (Arlington Heights, IL) was used to visualize and quantitate immunoreactive protein bands, as reviewed by Stott (1989). Densitometric scanning of developed films was carried out on an Ultrascan XL laser densitometer using Gelscan 2400 XL software from LKB-Bromma (Sweden).

Lectin blots carried out on nitrocellulose were blocked for 2 h in 0.05% Tween-20, 0.2 M NaCl, 50 mM Tris-Cl, pH 7.5 and incubated for 1 h with 1:2,000 diluted peroxidase-conjugated lectins (Sigma Chemical Co.) in the same buffer. After extensive washing, the membranes were incubated in a freshly prepared mixture of 0.06 ml 30% H_2O_2 in 100 ml of 0.2 M NaCl, 20 mM Tris-Cl, pH 7.5 and 0.06 g 4-chloro-1-naphthol in 20 ml ice-cold methanol. Staining was terminated by washing the membrane sheets in several changes of distilled water. Furthermore, lectin blotting studies were performed with the Digoxigenin Glycan Differentiation Kit from Boehringer Mannheim Corp. as described by the manufacturer.

Nitrocellulose-immobilized proteins were tested for the presence of glycoconjugates by mild periodate treatment followed by digoxigenin labeling and an alkaline phosphatase immunoassay as described for the Digoxigenin Glycan Detection Kit from Boehringer Mannheim Corp.

Carbohydrate Composition and Sulfate Analysis

The sugar composition was determined by anion exchange HPLC with pulsed amperometric detection (Dionex Corporation, Sunnyvale, CA). Deionized water used in sugar analysis was filtered through a Milli-Q Plus system of Millipore Corp. (Bedford, MA) and 0.45 μm Nylon 66 membrane of Alltech Associates (Deerfield, IL). A CarboPac PA-1 (250 × 4 mm i.d.) anion exchange column was used. An isocratic run of 16 mM NaOH was used throughout the analyses with a 300 mM NaOH post column addition. Sialic acids were assayed using the thiobarbituric acid (TBA) assay (Aminoff, 1961) as modified by Uchida et al. (1977) with *N*-acetylneuraminic acid (Sigma Chemical Co.) as a standard. Before the assay, 25 μg of the protein sample was hydrolyzed in 0.5 ml of 0.1 M HCl, at 80°C for 1 h to obtain free sialic acids, if present (Hardy et al., 1988). Uronic acids were assayed using meta-hydroxydiphenyl (Aldrich Chemical Co., Milwaukee, WI; Blumentkrantz and Asboe-Hansen, 1973) with galacturonic acid (Sigma Chemical Co.) as a standard.

Pronase (Sigma Chemical Co.) treatment was according to Kinsey and Lennarz (1981), using 0.2 M Tris-HCl, pH 8, instead of sea water. For release of oligosaccharides hydrazinolysis was carried out according to Patel et al. (1993). The hydrazinolyzed or Pronase-treated samples were then fractionated on Bio Gel P-2 (BioRad Laboratories, Melville, NY) column and the orcinol positive fractions (Weimer and Moshin, 1952) were pooled and lyophilized. *N*-Acetylation of the glycopeptides was done according to Finne and Krusius (1982). Acid hydrolysis of glycopeptides and oligosaccharides was as described by Hardy et al. (1988).

In an attempt to remove O-linked carbohydrate chains, purified 350 kD receptor was treated with trifluormethanesulfonic acid (Aldrich Chemical Co., Milwaukee, WI) according to Edge et al. (1981). Incubation conditions for enzymatic deglycosylation using N-glycosidase F, O-glycosidase (endo-β-*N*-acetylgalactosaminidase) (Boehringer Mannheim, Indianapolis, IN) and neuraminidases (Oxford GlycoSystems, Rosedale, NY) were as suggested by the manufacturers. Sulfate analysis of purified egg receptor and glycopeptides was carried out by Dr. G. Bousfield (Wichita State University, Wichita, KS).

Sperm Binding to Immobilized Egg Receptor

Purified receptor was adsorbed to 0.5 μm polystyrene beads (Polysciences, Warrington, PA) by overnight incubation in 0.1 M sodium borate, pH 8.5. For control purposes, an aliquot of beads containing bound purified receptor was incubated with gentle shaking for 30 min with antibody to the GST-45A fusion protein. Beads were then gently mixed for 5 min with acrosome-reacted sperm as described in detail by Foltz et al. (1993). Finally, the specimens were fixed in glutaraldehyde, placed on grids and negatively stained with ammonium molybdate for transmission electron microscopy (Foltz et al., 1993). Under these conditions beads not bound to sperm were rinsed from the grids.

Results

Purification of the Egg Receptor

Preliminary solubilization experiments led to the use of 2% β-octylglucoside, which was found to solubilize over 80% of the receptor. Initial attempts to purify the solubilized receptor by immunoaffinity chromatography with the currently available polyclonal antibodies against the extracellular domain of the receptor failed because of inefficient elution (not shown). We therefore purified the receptor by conventional biochemical techniques using lectin and ion exchange chromatography.

Initial binding and blotting experiments with egg surface membrane preparations using different lectins revealed that WGA bound to the solubilized egg receptor molecule. This finding enabled us to enrich for solubilized receptor by use of a WGA-agarose batch technique using *N*-acetylglucosamine for specific elution. As previously reported (Lotan et al., 1977), the presence of detergent resulted in selectivity in binding. Most egg surface glycoproteins that bound to peroxidase-labeled WGA in blotting experiments did not bind to the immobilized lectin in the presence of 0.5% β-octylglucoside (Fig. 1). In contrast, the receptor was the major component that bound to WGA-agarose in the presence of detergent. The silver-stained and the WGA lectin-blotted 350-kD protein band corresponds to the receptor band visualized by

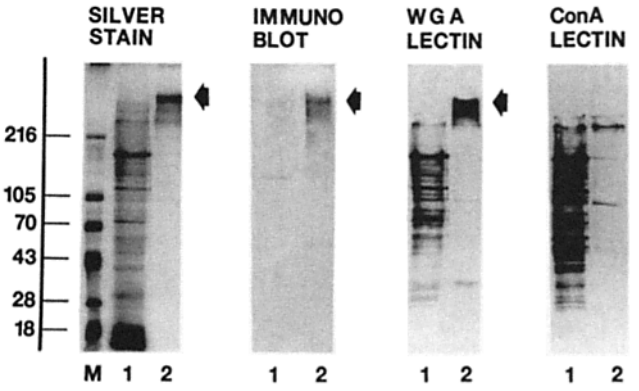

Figure 1. Analysis of the binding of receptor to WGA-agarose. After binding and elution of the receptor to WGA-agarose, fractions were subjected to SDS-PAGE and then analyzed by silver staining, immunoblotting, WGA blotting or ConA blotting. In each case, lanes *1* and *2* consisted of 100 μl of the protein fraction that did not bind to WGA-agarose and of the fraction that had bound and was subsequently eluted with *N*-acetylglucosamine, respectively. The first three detection methods show that the egg receptor with an M_r of 350 kD (*arrow*) was found in the bound and subsequently eluted fraction. ConA did not bind to the receptor. Molecular weight standards (lane *M*) ($M_r \times 10^{-3}$) are indicated on the left.

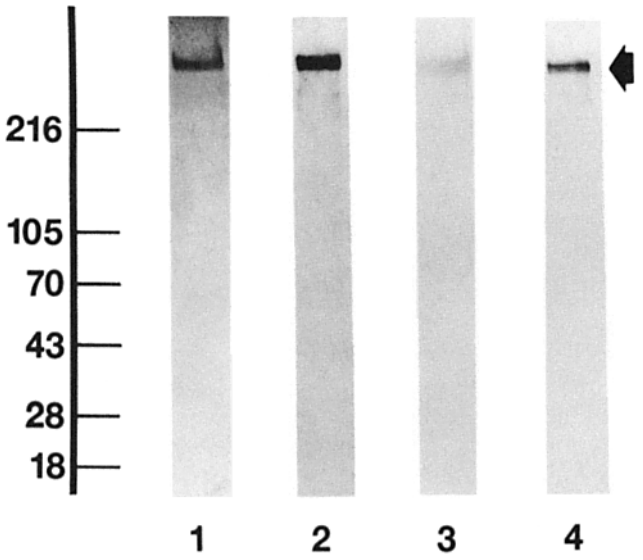

Figure 2. Analysis of purified receptor following SDS-PAGE. Shown are a silver-stained gel (lane *1*) and identical blots labeled with antibody to the extracellular receptor domain (lane *2*), or stained with the glycan detection kit from Boehringer Mannheim Corp. (lane *3*) or using peroxidase-conjugated WGA (lane *4*). Each lane contained 5 μg of the purified 350 kD receptor (*arrow*), which was prepared by ultrafiltration, dialysis and lyophilization of the DEAE-Sephacel eluted peak fractions. Molecular weight standards ($M_r \times 10^{-3}$) are indicated on the left.

immunoblotting (Fig. 1). The 70-kD receptor fragment produced by lysylendoproteinase C treatment of intact eggs also bound to immobilized WGA (not shown).

After WGA lectin chromatography proteins were bound to DEAE-Sephacel at a low concentration of salt. Elution was accomplished with a linear NaCl gradient and release of the receptor was monitored by silver staining, immunoblotting and lectin blotting (not shown). Ion exchange chromatography separated low molecular weight glycoproteins, which eluted early in the gradient, from the high molecular weight receptor, which eluted at a NaCl gradient concentration of 2 to 2.5 M. The fractions containing homogenous egg receptor were combined, dialyzed and lyophilized, and the integrity of the final preparation checked by SDS-PAGE analysis as illustrated in Fig. 2. Glycan detection blotting using mild periodate treatment followed by digoxigenin labeling and an alkaline phosphatase immunoassay revealed, as expected, the presence of glycoconjugates in the pure 350-kD preparation.

As outlined in Table I, the receptor was purified 149-fold with a yield of 61% starting with a crude surface membrane preparation. The fold purification was determined by the enhanced chemiluminescence (ECL) detection technique, under conditions in which there was a linear relationship between the amount of the antigen and the relative labeling intensity of the specific antibody. The amounts of protein analyzed were 200 μg for the crude surface complex and 2 μg for the purified 350-kD receptor. The receptor concentration in crude egg extracts was too low to be determined by immunoblotting and enhanced chemiluminescence. However, the initial subcellular fractionation step resulted in a 23-fold purification of surface membrane complex over crude egg homogenate. Therefore, if one considers the crude egg homogenate as starting material and assumes 100% recovery of the receptor during the subcellular fractionation step, the overall purification of the receptor can be calculated to be ~3,400-fold.

The Egg Receptor Contains Both N- and O-linked Carbohydrate Chains

Because earlier studies indicated that the oligosaccharide chains of the egg receptor are involved in sperm adhesion (Ruiz-Bravo and Lennarz, 1986; Foltz and Lennarz, 1990), we determined the carbohydrate composition of the purified receptor. Carbohydrate composition of the receptor was obtained after hydrolysis of the oligosaccharides released by using hydrazinolysis as well as the glycopeptides generated by Pronase digestion of the receptor. Both methods gave results that agreed within ±16%.

N-acetylation of glycopeptides and of oligosaccharides was done before the chains were hydrolyzed with 2N TFA for 6 h at 100°C. Surprisingly, omission of the acetylation step, even in the case of the Pronase treated sample, resulted in the absence of detectable glucosamine (data not shown). There are two possible explanations for this observation. First, if during acid hydrolysis the de-*N*-acetylation of amino sugars is faster than glycosidic bond cleavage, the resulting amino sugar would be resistant to cleavage (Spiro, 1966). Second, if the oligosaccharide chain contained glucosamine

Table I. Summary of Purification of Receptor

Step	Protein	Relative receptor content* units per mg protein	Total receptor content units	Fold purification	Yield
	mg				%
Crude surface complex	44.2	0.67	29.6	–	–
WGA-agarose eluted fraction	0.52	46.2	24.0	69	81
DEAE-Sephacel eluted fraction	0.18	100	18.0	149	61

* Receptor content was determined by ECL detection on immunoblots using the commercially available ECL kit from Amersham, Corp. (Arlington Heights, IL). Densitometric scanning of developed films was used to estimate quantitatively the relative antibody binding to the nitrocellulose-immobilized 350 kD antigen after various purification steps. Units are expressed as values obtained by peak integration, which were normalized with respect to the amount of protein in the various fractions separated by SDS-PAGE. Analysis of total sea urchin egg homogenates indicated that the concentration of the receptor protein in crude egg extracts is too low to be detectable by immunological analysis.

*Table II. Monosaccharide and Sulfate Content of Purified Receptor**

Moiety	Composition following treatment with: Hydrazine	Pronase	Molar Ratio‖
Galactosamine	25.2	26.3	1.0
Galactose	87.0	89.6	3.4
Glucosamine	37.7	43.9	1.6
Mannose	578.5	645.2	23.8
Fucose	28.7	30.9	1.2
SO_4^{2-}	ND	104.0‡	4.0
Glucose	(1502.0)§	(1537.2)§	

* The values shown are nmol per 150 μg of purified receptor protein. Hydrazinolysis values are the average of two independent preparations; pronase values are from one preparation.
‡ Value of sulfate content determined on the glycopeptides generated by pronase digestion.
§ Glucose is shown in parenthesis because this may be a contaminant.
ND Not determined.
‖ The ratio was calculated using an average of the values obtained using hydrazine or Pronase to release the saccharide chains. Values are normalized to galactosamine as 1.0.

rather than *N*-acetylglucosamine, the glucosamine moiety in the chain would be resistant to acid hydrolysis.

Table II shows the carbohydrate composition of the 350-kD egg receptor; galactosamine, galactose, glucosamine, mannose and fucose were detected. Glucose is a likely contaminant. Sulfate ester analysis revealed the presence of a significant amount of sulfate, all of it on the carbohydrate chains. Only trace amounts (<2% each) of uronic acids and sialic acids were detected. Based on this analysis it can be calculated that the receptor is 70% carbohydrate and 30% protein by weight. The composition and the ratio of the individual sugars is suggestive of the presence of both N- and O-linked oligosaccharide chains. It is not clear why the level of mannose relative to glucosamine is much higher than a typical N-linked high mannose chain. One possibility is that O-linked polymannose chains like those originally reported in yeast, but recently found in a higher eukaryote (Krusius et al., 1986), are present in addition to N-linked oligosaccharide chains.

The idea that both N-linked and O-linked oligosaccharide chains are present was supported by the results of differential hydrazinolysis of the receptor (data not shown). Release of galactosamine, galactose, and fucose was maximal after heating with hydrazine at 65°C for 3.75 h, conditions that favor hydrolysis of sugars on O-linked chains (Patel et al., 1993). Hydrazine treatment at 95°C, which releases both N- and O-linked sugars, showed an increase in free mannose, along with decomposition of some sugars.

Treatment of the receptor with *N*-glycosidase F or O-glycanase failed to reveal any increase in its electrophoretic mobility as judged by SDS-PAGE analysis using silver staining and immunoblotting. We also attempted to remove the O-linked carbohydrate moieties with trifluormethylsulfonic acid (TFMS) treatment. Although an increase in electrophoretic mobility was observed on SDS polyacrylamide gels, lectin and glycan detection blotting revealed that carbohydrate was still present in the smaller receptor fragments, indicating cleavage of acid-labile polypeptide bonds rather than complete removal of oligosaccharide chains.

Lectin Binding Properties of the Egg Receptor

The observed binding of the receptor to WGA must be due to lectin binding to *N*-acetylglucosamine and/or *N*-acetylgalactosamine, rather than sialic acid (Peters et al., 1979), since only trace amounts of sialic acid were detected. Consistent with this idea it was found that both *N*-acetylglucosamine and *N*-acetylgalactosamine inhibited the interaction between the receptor and WGA (not shown). Given the sugar composition of the receptor, it was of interest to examine its interaction with other lectins. Blotting experiments with labeled lectins revealed that *Wistaria floribunda* agglutinin (WFA), *Arachis hypogaea* peanut agglutinin (PNA), *Maackia amurensis* agglutinin (MAA),[1] and succinylated wheat germ agglutinin (sWGA) bound to the receptor, while other lectins like concanavalin A (ConA), *Ulex europaeus* agglutinin (UEA-I), *Tetragonolobus purpureas* agglutinin (TPA), *Phaseolus vulgaris* agglutinin E (PHA-E), *Phaseolus vulgaris* agglutinin L (PHA-L), *Galanthus nivalis* agglutinin (GNA) or *Sambucus nigra* agglutinin (SNA) showed no binding (data not shown).

In addition to providing other reagents for affinity purification of the 350-kD receptor, these results with lectins afforded further insight into the nature of the oligosaccharide chains. As shown in Fig. 3, using the immobilized forms of four of the above-mentioned lectins it was found that WFA, PNA, MAA, and sWGA beads specifically bound to the purified receptor. Binding to WFA is consistent with the presence of N-acetylgalactosamine; binding to PNA is indicative of O-linked galactose-N-acetylgalactosaminyl linkages (Lis and Sharon, 1986; Cummings, 1993). Both sugars were detected by sugar analysis. Although the binding by MAA and WGA suggested the presence of sialic acid, as noted earlier only trace amounts of this sugar were detected. Consistent with the absence of sialic acid we found that treatment of the purified receptor with a variety of neuraminidases did not abolish binding by MAA or WGA lectin (not shown), strongly suggesting that these lectins must be binding to the receptor by some other interaction. Because sWGA does not bind to sialic acid (Monsigny et al., 1980), but does bind to the receptor, we conclude that binding to unmodified WGA must be via *N*-acetylglucosamine and/or *N*-acetylgalactosamine.

1. *Abbreviation used in this paper*: MAA, *Maackia amurensis*.

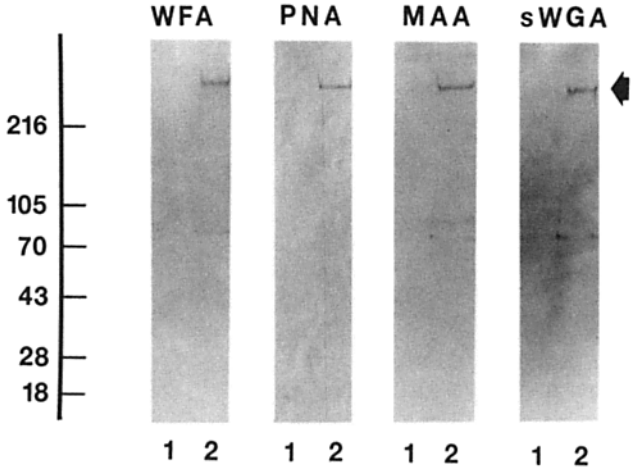

Figure 3. Lectin binding properties of purified egg receptor. Shown are identical blots labeled with antibodies to the extracellular receptor domain. Lanes *1* and *2* consist of the proteins that did not or did bind to the indicated lectin columns, respectively. The receptor band of apparent 350 kD is indicated by an arrow. Immobilized lectins used were *Wistaria floribunda* agglutinin (*WFA*), *Arachis hypogaea* peanut agglutinin (*PNA*), *Maackia amurensis* agglutinin (*MAA*), and succinylated wheat germ agglutinin (*sWGA*). The molecular weight standards ($M_r \times 10^{-3}$) are indicated on the left.

Purified Egg Receptor Is Biologically Active

Based on the original finding that the crude receptor (Ruiz-Bravo and Lennarz, 1986), as well as a fragment of the extracellular domain of the receptor (Foltz and Lennarz, 1990) inhibited fertilization in a competitive fertilization bioassay, we tested the homogeneous intact receptor for activity. As shown in Fig. 4, the lyophilized receptor after removal of detergent by dialysis, inhibited fertilization of *S. purpuratus* eggs in a dose-dependent manner. Furthermore, fertilization of eggs from another sea urchin species, *A. punctulata*, was not affected by the *S. purpuratus* receptor, thereby demonstrating the species specificity of the inhibitory effect. Inhibition of fertilization due to the egg receptor could be overcome by adding excess sperm (not shown). This finding is consistent with the assumption that the purified, exogenous receptor is functional because it competes with the endogenous, egg-bound receptor for binding to sperm and thereby inhibits fertilization.

To provide more direct evidence that the purified receptor has the ability to bind to acrosome-reacted sperm, experiments were carried out using the 350-kD protein bound to beads. The intact glycoprotein was adsorbed to polystyrene microspheres; the resulting coated beads were then incubated with acrosome-reacted sperm from *S. purpuratus* and bead-sperm interaction was analyzed by electron microscopy. In Fig. 5 *a* is shown a representative example of sperm binding to receptor-conjugated microspheres. Beads bind exclusively to the acrosomal process of sperm, not to the main body or the tail of the sperm cell, indicating direct interaction between a component of the apical tip of sperm (presumably bindin) with the immobilized egg receptor. When the receptor-coated beads were treated with antibody to the extracellular domains of the receptor no binding was observed (Fig. 5 *b*). The observed bead binding was quantitated microscopically by examining a total of 370 sperm.

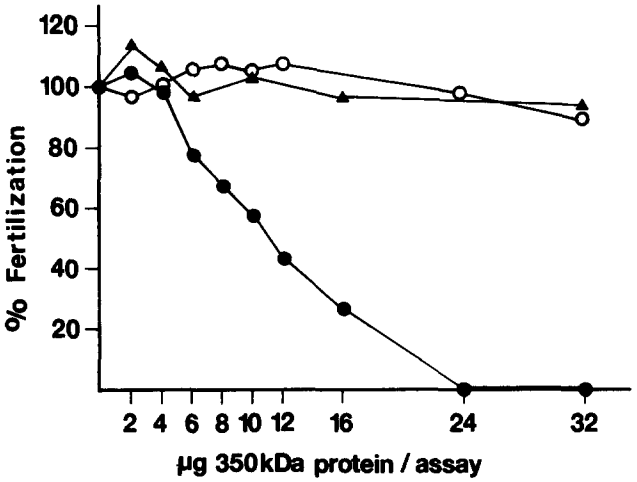

Figure 4. The purified egg receptor inhibits fertilization. Acrosome-reacted *S. purpuratus* sperm were added to dejellied, washed eggs of *S. purpuratus* (●) in the presence of increasing amounts of the purified intact 350 kD receptor from *S. purpuratus*. Eggs from *S. purpuratus* were also treated with control buffer containing no egg receptor (▲). An experiment with *A. punctulata* gametes (○) showed that the observed inhibition was species-specific.

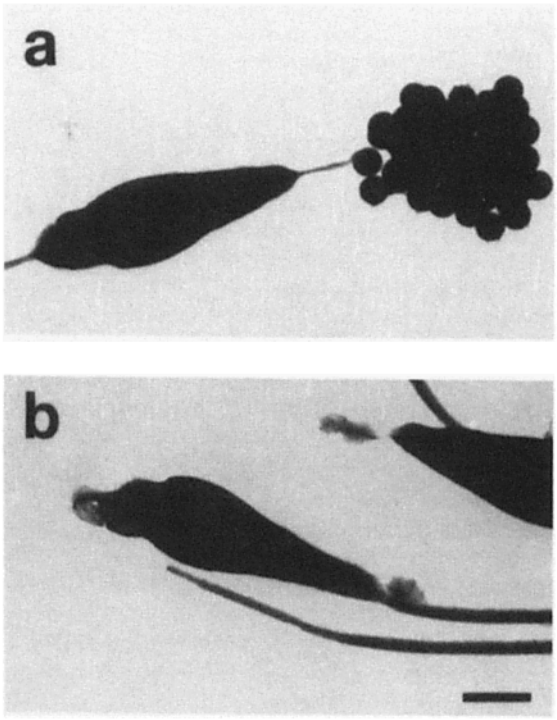

Figure 5. Receptor-coated microspheres bind to acrosome-reacted sperm. Shown is a representative electron micrograph of acrosome-reacted *S. purpuratus* sperm binding to 0.5 µm polystyrene beads that had been coated with the purified receptor (*a*). Acrosome-reacted sperm did not bind to beads that were first coated with receptor and then incubated with antibody to the sperm-binding domain of the egg receptor (*b*). Aggregation of protein-coated beads is not only observed with the purified egg receptor, but with a variety of unrelated proteins such as BSA, lactalbumin and glutathione transferase. Bar, 1 µm.

56% of the sperm were bound to receptor-coated beads. In contrast, in the sample containing beads first coated with receptor and then incubated with antibodies only 4% of the sperm were bound to beads. Control experiments with sperm that were not induced to undergo the acrosome reaction showed that receptor-coated beads did not bind nonspecifically to sperm cells. Furthermore, acrosome-reacted sperm from another sea urchin species, *L. pictus*, did not interact with the receptor-coated beads (not shown).

Discussion

The recent cloning, sequencing and expression of domains of the egg receptor for sperm (Foltz et al., 1993) has been an important prerequisite for the elucidation of the role of this molecule in sperm binding and egg activation. However, another essential requirement to investigate structure-function relationships in the receptor molecule, including the role of the carbohydrate chains of the receptor, is the availability of the intact, mature receptor molecule. This objective has been accomplished in the current study.

The receptor purification scheme using lectin and ion exchange chromatography was based on the findings that the previously characterized receptor fragments were highly glycosylated and strongly negatively charged (Ruiz-Bravo and Lennarz, 1986; Foltz and Lennarz, 1990). Difficulties in previous studies in monitoring possible steps during the purification of the egg receptor were overcome by use of a highly specific antibody to a fusion protein which represents a portion of the extracellular sperm-binding domain (Foltz et al., 1993). The entire procedure yields 180 μg of homogenous egg receptor starting with 50 ml of packed, dejellied eggs. Based on a yield of 61% for the receptor, and knowing that 50% of it is located on the cell surface (J. S. Partin and W. J. Lennarz, unpublished results), one can calculate the number of receptor molecules on the surface of each egg to be 1.25×10^6. Sperm binding to *S. purpuratus* eggs has been experimentally determined to vary from 1,098 to 3,160 sperm per egg with a mean value of 1,744 (Vacquier and Payne, 1973). Consequently, 400–1,100 receptor molecules are available to bind to each sperm cell, assuming that the receptors can laterally migrate to each site of sperm binding. Given that upon binding on the surface of the egg, sperm can be seen to have an actively moving flagellum, the need for an average valency of 700 to keep the two cells together does not seem unreasonable. It is of interest that theoretically 24,400 spermatozoa could bind to an egg, taking into account that the surface area of the unfertilized egg is 22,000 μm^2 and that the cross sectional area of the body of a sperm is 0.9 μm^2 (Vacquier, 1975). Even under these circumstances there would still be 50 receptors available to bind each sperm cell.

Characterization of the isolated receptor revealed that the purified protein has an apparent M_r of 350 kD on SDS-PAGE and is a highly glycosylated molecule containing sulfated oligosaccharide chains. This estimated molecular weight by SDS-PAGE analysis is much greater than that calculated from the deduced amino acid sequence, which indicates a mass of 131 kD (Foltz et al., 1993). Taking into account the high carbohydrate (70% by weight) and sulfate content, a mass of approximately 300 kD can be calculated. However, this value is still measurably lower than the observed M_r of 350 kD. One possibility is that the receptor has an abnormally low electrophoretic mobility because it is sulfated and highly glycosylated. Both the presence of carbohydrate and charge repulsion between SDS and the highly negatively charged receptor molecule might result in anomalous electrophoretic properties. Alternatively, the receptor could actually be a multi-subunit complex; the receptor with a mobility of a 350-kD protein band may contain another covalently linked subunit that was not detected by the cloning and sequencing strategy. If this is the case, chemical or proteolytic fragmentation of the receptor followed by sequencing should reveal sequences of these putative subunits that were not detected by the cloning approach. Such an analysis is now possible because sufficient quantities of the pure, intact receptor are available.

Analysis of oligosaccharide chains released by hydrazinolysis or of the glycopeptides generated by Pronase digestion of the receptor revealed the presence of five monosaccharides (plus glucose, which we assume to be a contaminant). The carbohydrate composition agrees with the earlier sugar analysis of the 70-kD fragment of the receptor (Foltz and Lennarz, 1990), except that in the current study we detected glucosamine after *N*-acetylation. As discussed in the Results, this suggests that either glucosamine as such (rather than *N*-acetylglucosamine) is present in the oligosaccharide chains or that it is preferentially generated by hydrolysis of *N*-acetylglucosamine prior to acid cleavage of the sugars in the oligosaccharide chains (Spiro, 1966). The finding of this amino sugar plus mannose strongly indicates the presence of *N*-linked oligosaccharide chains, although the high content of mannose is unusual.

The finding of galactosamine and galactose suggest the presence of O-linked oligosaccharide chains. These results are supported by the finding that the receptor bound to *Arachis hypogaea* peanut agglutinin, which recognizes the core disaccharide galactose β(1-3) *N*-acetylgalactosamine. The receptor also bound to *Wistaria floribunda* agglutinin and WGA, which is specific for terminal, β-linked galactosamine and *N*-acetylglucosamine, respectively. In contrast, lectins specific for fucose (*Ulex europaeus* and *Tetragonolobus purpureas* agglutinin) and mannose (concanavalin A) did not interact with the receptor, although these sugars are present. This suggests that the fucose and/or the mannose found in the intact receptor may be present in modified, possibly sulfated forms which is not recognized by these lectins. It is noteworthy that sulfated fucose residues have been reported in egg jelly glycoconjugates of various sea urchin species (SeGall and Lennarz, 1979; Shimizu et al., 1991). It is of particular interest that sulfated oligosaccharides bind to bindin, the sperm protein (Glabe et al., 1982). Both, fucoidin and egg jelly fucan, which are sulfated, fucose-containing polysaccharides, are potent inhibitors of bindin-mediated egg agglutination (Glabe et al., 1982). With respect to sulfate, analysis of the intact protein and the glycopeptides revealed that it was all linked to the carbohydrate chains, and that it was present in a ratio of three sulfates to one fucose. This high value suggests that sulfate esters are present on other sugars as well.

It was clear that the 350-kD glycoprotein purified by the protocol described in this study was the receptor because it reacted with an antibody that (*a*) inhibits fertilization and (*b*) binds to a proteolytic fragment that inhibits fertilization.

However, given that after purification the receptor was no longer associated with the plasma membrane, and therefore perhaps no longer in its native state, we undertook to determine if it retained biological activity. Two lines of evidence indicate that it is bioactive. First, it was found that it inhibited fertilization in a competitive fertilization bioassay where sperm were limiting; this inhibition was species specific, because the receptor had no inhibitory effect on fertilization of *A. punctulata* eggs. Second, it was shown that the receptor binds to sperm, a property first established with the 70-kD extracellular fragment of the receptor (Foltz et al., 1993). Thus, polystyrene beads coated with the purified receptor mimic the egg surface and specifically bind acrosome-reacted sperm. Because these experiments were performed with a homogenous preparation of the intact receptor it is clear that this molecule alone, with a transmembrane domain anchoring it in the egg cell surface and an extracellular binding domain, is sufficient to bind sperm to the egg cell surface.

In early studies it was established that glycopeptides generated from the receptor by Pronase digestion inhibited fertilization. Recently we reported that not only the glycosylated 70-kD fragment of the receptor, but also a recombinant protein overlapping the same region, but lacking carbohydrate chains, inhibited fertilization. These observations established that the binding to sperm is not solely dependent on the presence of the carbohydrate chains, but that the polypeptide chain also functions in the binding process. Perhaps in the intact receptor these two types of interactions occur in a stepwise or cooperative fashion. Having established the carbohydrate composition of the intact receptor it will now be important to determine the structure of the individual oligosaccharide chains. Only when this information, and the structure of the sperm binding domain of the polypeptide chain are known, will it be possible to understand in detail how the sperm binds to the receptor.

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References

Acevedo-Duncan, M., and E. J. Carroll. 1986. Immunological evidence that a 305-kilodalton vitelline envelope polypeptide isolated from sea urchin eggs is a sperm receptor. *Gamete Res.* 15:337–359.

Aminoff, D. 1961. Methods for the quantitative estimation of N-acetylneuraminic acid and their application to hydrolysates of sialomucoids. *Biochem. J.* 81:384–392.

Blumentkrantz, N., and G. Asboe-Hansen. 1973. New method for quantitative determination of uronic acids. *Anal. Biochem.* 54:484–489.

Cummings, R. D. 1993. Use of lectins in the analysis of glycoconjugates. *In* Guide to Techniques in Glycobiology. W. J. Lennarz and G. W. Hart, editors. Academic Press, Inc., Orlando, FL. In press.

Edge, A. S. B., C. R. Faltyneck, L. E. Reichert, and P. Weber. 1981. Deglycosylation of glycoproteins by tri-fluormethanesulfonic acid. *Anal. Biochem.* 118:131–137.

Finne, J., and T. Krusius. 1982. Preparation and fractionation of glycopeptides. *Methods Enzymol.* 83:269–277.

Foltz, K. R., and W. J. Lennarz. 1990. Purification and characterization of an extracellular fragment of the sea urchin egg receptor for sperm. *J. Cell Biol.* 111:2951–2959.

Foltz, K. R., and W. J. Lennarz. 1992. Identification of the sea urchin egg receptor for sperm using an antiserum raised against a fragment of its extracellular domain. *J. Cell Biol.* 116:647–658.

Foltz, K. R., and W. J. Lennarz. 1993. The molecular basis of gamete interaction. *Dev. Biol.* In press.

Foltz, K. R., J. S. Partin, and W. J. Lennarz. 1993. Sea urchin egg receptor for sperm: sequence similarity of binding domain and hsp70. *Science (Wash. DC).* 259:1421–1425.

Gao, B., L. E. Klein, R. J. Britten, and E. H. Davidson. 1986. Sequence of mRNA coding for bindin, a species-specific sea urchin sperm protein required for fertilization. *Proc. Natl. Acad. Sci. USA.* 83:8634–8638.

Glabe, C. G., L. B. Grabel, V. D. Vacquier, and S. D. Rosen. 1982. Carbohydrate specificity of sea urchin binding: a cell surface lectin mediated sperm-egg adhesion. *J. Cell Biol.* 94:123–128.

Glabe, C. G., and V. D. Vacquier. 1987. Egg surface glycoprotein receptor for sea urchin sperm bindin. *Proc. Natl. Acad. Sci. USA.* 75:881–885.

Glabe, C. G., K. Hong, and V. D. Vacquier. 1991. Fusion of sperm and plasma membranes during fertilization. *In* Membrane Fusion. J. Wilschut, and D. Hoekstra, editors. Marcel Dekker, Inc., New York. 627–646.

Hardy, M. R., R. R. Townsend, and Y. C. Lee. 1988. Monosaccharide analysis of glycoconjugates by anion exchange chromatography with pulsed amperometric detection. *Anal. Biochem.* 170:54–62.

Harlow, E., and D. Lane. 1988. Antibodies. A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York. 726 pp.

Kinsey, W. H. 1986. Purification and properties of the egg plasma membrane. *In* Methods in Cell Biology. T. E. Schroeder, editor. Academic Press, Inc., New York. 139–152.

Kinsey, W. H., and W. J. Lennarz. 1981. Isolation of a glycopeptide fraction from the surface of the sea urchin egg that inhibits sperm-egg binding and fertilization. *J. Cell Biol.* 91:325–331.

Krusius, T., J. Finne, R. K. Margolis, and R. U. Margolis. 1986. Identification of an *O*-Glycosidic mannose-linked sialylated tetrasaccharide and keratan sulfate oligosaccharides in the chondroitin sulfate proteoglycan of brain. *J. Biol. Chem.* 261:8237–8242.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 27:680–685.

Lis, H., and N. Sharon. 1986. Lectins as molecules and as tools. *Annu. Rev. Biochem.* 55:35–67.

Lotan, R., G. Beattie, W. Hubbell, and G. L. Nicolson. 1977. Activities of lectins and their immobilized derivatives in detergent solutions. Implications on the use of lectin affinity chromatography for the purification of membrane glycoproteins. *Biochemistry.* 16:1787–1794.

Minor, J. E., B. Gao, and E. H. Davidson. 1989. The molecular biology of bindin. *In* The Molecular Biology of Fertilization. H. Schatten and G. Schatten, editors. Academic Press, Inc., New York. 773–788.

Monsigny, M., A.-C. Roche, C. Sene, R. Maget-Dana, and F. Delmotte. 1980. Sugar-lectin interactions: How does wheat-germ agglutinin bind sialoglycoconjugates? *Eur. J. Biochem.* 104:147–153.

Moy, G. W., and V. D. Vacquier. 1979. Immunoperoxidase localization of bindin during sea urchin fertilization. *Curr. Top. Dev. Biol.* 13:31–44.

Patel, T., J. Bruce, A. Merry, C. Bigge, M. Wormald, A. Jaques, and R. Parekh. 1993. Use of hydrazine to release intact and unreduced form of both N- and O-linked oligosaccharides from glycoproteins. *Biochemistry.* 32:679–693.

Peters, B. P., S. Ebisu, I. J. Goldstein, and M. Flashner. 1979. Interaction of wheat germ agglutinin with sialic acid. *Biochemistry.* 18:5505–5511.

Rossignol, D. P., A. J. Roschelle, and W. J. Lennarz. 1981. Sperm-egg binding: identification of a species-specific sperm receptor from eggs of *Strongylocentrotus purpuratus. J. Supramol. Struct. Cell Biochem.* 15:347–358.

Rossignol, D. P., B. J. Earles, G. L. Decker, and W. J. Lennarz. 1984. Characterization of the sperm receptor on the surface of eggs of *Strongylocentrotus purpuratus. Dev. Biol.* 104:308–321.

Ruiz-Bravo, N., D. Earles, and W. J. Lennarz. 1986. Identification and partial characterization of sperm receptor associated with the newly formed fertilization envelope from sea urchin eggs. *Dev. Biol.* 117:204–208.

Ruiz-Bravo, N., and W. J. Lennarz. 1986. Isolation and characterization of proteolytic fragments of the sea urchin sperm receptor that retain species specificity. *Dev. Biol.* 118:202–208.

Ruiz-Bravo, N., and W. J. Lennarz. 1989. Receptors and membrane interactions during fertilization. *In* The Molecular Biology of Fertilization. H. Schatten and G. Schatten, editors. Academic Press, Inc., New York. 21–36.

Schmell, E., B. J. Earles, C. Breauz, and W. J. Lennarz. 1977. Identification of a sperm receptor on the surface of the eggs of the sea urchin *Arbachia punctulata. J. Cell Biol.* 72:35–46.

SeGall, G. K., and W. J. Lennarz. 1979. Chemical characterization of the component of the jelly coat from sea urchin eggs responsible for induction of the acrosome reaction. *Dev. Biol.* 71:33–48.

Shimizu, T., H. Kinoh, M. Yamaguchi, and N. Suzuki. 1991. Species specificity of the egg jelly molecule, a fucose sulfate glycoconjugate, in induction of the acrosome reaction of sea urchin spermatozoa. *In* Biology of Echinodermata. T. Yanagisawa, I. Yasumasa, C. Oguro, N. Suzuki, and T.

Motokawa, editors. A. A. Balkema Publishers, Rotterdam. 427–432.
Spiro, R. G. 1966. Analysis of sugars found in glycoprotein. *Methods Enzymol.* 8:3–25.
Stott, D. I. 1989. Immunoblotting and dot blotting. *J. Immunol. Meth.* 119:153–187.
Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA.* 76:4350–4354.
Uchida, Y., Y. Tsudada, and T. Sugimori. 1977. Distribution of neuraminidase in *Arthrobacter* and its purification by affinity chromatography. *J. Biochem. (Tokyo).* 82:1425–1433.
Vacquier, V. D. 1975. The isolation of intact cortical granules from sea urchin eggs: calcium ions trigger granule discharge. *Dev. Biol.* 43:62–74.
Vacquier, V. D., and J. E. Payne. 1973. Methods for quantitating sea urchin sperm-egg binding. *Exp. Cell Res.* 82:227–235.
Vacquier, V. D., and G. W. Moy. 1977. Isolation of bindin: the protein responsible for adhesion of sperm to sea urchin eggs. *Proc. Natl. Acad. Sci. USA.* 74:2456–2460.
Vretblad, P. 1976. Purification of lectins by biospecific affinity chromatography. *Biochim. Biophys. Acta.* 434:169–176.
Wassarman, P. M. 1987. Early events in mammalian fertilization. *Annu. Rev. Cell Biol.* 3:109–142.
Weimer, H. E., and J. R. Moshin. 1952. Serum glycoprotein concentrations in experimental tuberculosis of guinea pigs. *Am. Rev. Tuberc.* 68:594–602.
Yanagimachi, R. 1978. Sperm-egg fusion. *Curr. Top. Membr. Transp.* 32:3–43.