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Sperm–Egg Binding: Identification of a Species-Specific Sperm Receptor From Eggs of Strongylocentrotus purpuratus

Daniel P. Rossignol, Aimee J. Roschelle, and William J. Lennarz

Department of Physiological Chemistry, Johns Hopkins University School of Medicine, Baltimore, Maryland 21201

We have attempted to identify a surface component of echinoderm eggs that is involved in the species-specific binding of sperm. Cell surface membranes from eggs of the sea urchins Strongylocentrotus purpuratus or Arbacia punctulata were radioiodinated, detergent-treated, and subjected to density-gradient centrifugation. In the presence of bindin, the complementary binding protein isolated from sperm, one component of the membranes sedimented to a different density. This membrane component bound species specifically to sperm that had undergone the acrosome reaction. This binding led to an inhibition of the ability of treated sperm to fertilize eggs. Exhaustive proteolytic digestion of this receptor fraction yields a high molecular weight glycopeptide that can also bind to bindin. It therefore appears that this egg surface membrane fraction contains a functionally intact, species-specific receptor for sperm.

Key words: egg receptor for sperm, cell surface glycoconjugate, sea urchin fertilization, sperm–egg binding, bindin–receptor interaction

Earlier work from this laboratory [1] and from the laboratory of Vacquier and co-workers [2] has provided evidence for the existence of a species-specific, egg surface receptor involved in the binding of sperm. Because earlier efforts to obtain a stable, soluble form of the receptor were unsuccessful, we have attempted to isolate a proteolytic fragment of the receptor that would be soluble and stable in solution. Studies on such a fragment, isolated from the surface of eggs of Stronglyocentrotus purpuratus, are reported in the accompanying paper [5].

Following the isolation of bindin [6], and the demonstration that it was the species-specific protein in sperm involved in binding to eggs [7], it became apparent that this protein would be a useful tool to identify the receptor for it on the egg surface. In this paper we report experiments using bindin as a probe, and present results indicating that a species-specific egg surface receptor has been partially purified.

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EXPERIMENTAL PROCEDURES

Materials

A punctulata and S purpuratus were maintained as previous described [3]. Gametes were obtained, and eggs were dejellied as described [3]. Bindin was isolated from S purpuratus and A punctulata sperm by the method of Vacquier and Moy [6], but with the addition of 0.3 M sucrose to the buffer used to dissociate the acrosomal vesicle. A punctulata bindin was further purified by centrifugation as described in the Results section. The purity of bindin preparations was assessed by SDS-PAGE. Typically, such preparations were more than 90% pure by this criterion [3]. Artificial seawater (ASW) was prepared as described [3], and filtered through a Millipore filter (0.45 μm pore size) before use.

Aprotonin, diisopropylfluorophosphate, and soybean typsin inhibitor were from Sigma Chemical Co. Iodogen was from Pierce Chemical Co. 3-(p-hydroxyphenyl) Propionic acid N-hydroxysuccinimide ester was from Aldrich Chemical Co., and Na ^{125}I was from Amersham Radiochemicals. Pronase was from Calbiochem Behring Corp., and cesium chloride was from Beckman. All other chemicals were reagent grade or better.

Purification of Receptor Fraction

Membranes from S purpuratus and A punctulata eggs were isolated by the method of Schmell et al [1] in the presence of 0.1 mM diisopropylfluorophosphate, Aprotonin 1,000 units/ml, and soybean trypsin inhibitor, 0.01 mg/ml. Approximately 75 μg of membrane protein was obtained per mg egg protein. Egg membranes (1 mg/ml) were treated with 0.2 mg/ml 3-(p-hydroxyphenyl) propionic acid N-hydroxysuccinimide ester in 10 mM sodium borate in ASW for 10 min at 0°C. The reaction was then quenched with a final concentration of 0.33 M glycine, and the membranes were washed twice by resuspension and centrifugation (37,000g, 10 min) in iodine-free seawater prepared as described [8], and the pellet was resuspended to a final concentration of 15 mg protein/ml in the same buffer. Iodination of 1-ml aliquots was performed by the method of Fraker and Speck [9] with 4 μg Iodogen and carrier-free ^{125}I (17.26 m Ci/μg) at 0°C for 10 min. The membranes were washed twice in ASW buffered to pH 8.0 with 10 mM Tris-HCl (TBASW), resuspended by sonication, and exhaustively dialyzed against TBASW. Ninety-nine percent of the ^{125}I label in the retentate was precipitable in 5% (w/v) trichloroacetic acid. Modification of membranes with 3-(p-hydroxyphenyl) propionic acid N-hydroxysuccinimide ester prior to ^{125}I labeling resulted in at least a 10% increase in specific activity of the membranes, and had no effect on the ability of these membranes to inhibit the bindin-mediated agglutination of eggs [3].

Isolation of Crude Receptor Fraction

^{125}I-Labeled membranes were detergent-extracted by incubation in 1.5% Triton X-100 in ASW for 30 min. The mixture was then diluted to a final detergent concentration of 0.15%, and loaded on preparative gradients consisting of 5 ml CsCl (1.07 g/ml), 8 ml 60% (w/w) sucrose and 15 ml 28% (w/w) sucrose prepared in TBASW. The gradients were centrifuged for 16 hours at 24,000 rpm in a Beckman SW27 rotor at 4°C. Fractions containing the receptor fraction were pooled and diluted in TBASW,

pelleted by high speed centrifugation (100,000g), washed twice in TBASW by centrifugation, and resuspended by sonication in TBASW. Over 99% of the ^{125}I label in the receptor fraction was precipitable in 5% (w/v) trichloroacetic acid, and less than 1% was soluble in the lipid extraction solvent of Bligh and Dyer [10]. One gram of total membrane protein yielded approximately 350 mg of crude receptor fraction. An average preparation of the receptor fraction contained 1.2 μmole hexose and 0.185 μmole hexosamine, and less than 1 μg lipid phosphorous/mg protein. It showed no loss in receptor activity when stored for several months at −20°C.

Binding of Receptor Fraction to Sperm

Binding of ^{125}I-labeled receptor fraction was assayed with unreacted and acrosome-reacted sperm. The acrosome reaction was induced by incubation of sperm for 20 sec at room temperature with 200 μl crude jelly coat (55 μmoles fucose/ml). This amount of jelly coat has been shown nearly quantitatively to induce the acrosomal reaction [11]. Following this incubation, ^{125}I-labeled receptor fraction was added as indicated to a constant final volume, and the mixture was incubated an additional 20 sec. A sample of 125 μl was then removed and layered onto 200 μl of a 5% (w/v) sucrose solution and centrifuged at 9,000g for 40 sec in a Beckman microfuge. The tube was cut in two just above the pellet of sperm, and the radioactivity in the portion containing the pellet was counted. This centrifugation technique pelleted more than 95% of the added sperm, as determined by measurement of the turbidity of the remaining supernatant.

Other Methods

Electron microscopy of sperm on substrate films and quantitation of the acrosome reaction, by counting a minimum of 100 sperm, were performed as previously described [12]. Hexose content was determined by the phenol-sulfuric acid assay [13], and amino sugars were detected by the method of Svennerholm [14]. Protein was determined by the method of Lowry [15].

RESULTS

Partial Purification of Receptor Fraction

Using ^{125}I-labeled egg surface membranes, which have been shown to retain species-specific sperm binding [1], we have attempted to identify an ^{125}I labeled protein(s) that binds to bindin. Since purified bindin forms insoluble aggregates in seawater [3, 6], we developed a centrifugation assay that measured the alteration in density of the egg receptor that results from its interaction with bindin particles. When ^{125}I-labeled membranes from S purpuratus were treated with detergent and analyzed by density-gradient centrifugation, a fraction of high density was found to be well separated from the majority of the radioactivity (Fig. 1A). When membranes were incubated with bindin and then detergent-treated, this high density fraction was found to sediment to a position corresponding to the density of bindin. This indicates that bindin interacts with an ^{125}I-labeled component of the membrane preparation, resulting in an increase in the density of it. It should be noted that measurement of this interaction by this technique requires detergent treatment of membranes. Without detergent pretreatment, all of the ^{125}I-la-

beled material sedimented to a density intermediate to the two major peaks seen in Figure 1A, regardless of whether or not bindin had been added. This indicates that, in the absence of detergent, the receptor fraction is firmly bound to the bulk of the labeled material, suggesting that the receptor fraction is an integral part of the egg plasma membrane–vitelline layer preparation.

As shown in Figure 1B, when crude receptor fraction is isolated from mem-

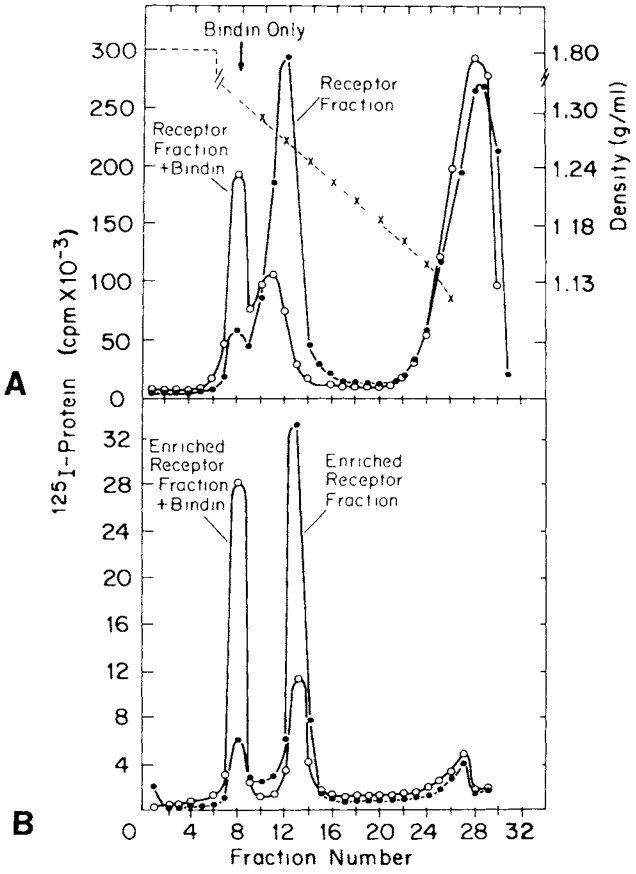

Fig. 1. A. Interaction of bindin and components of Triton X-100-treated membranes from S purpuratus. Membranes were isolated and radioiodinated as described in Experimental Procedures. Twenty μg of membrane protein were incubated with (○——○) or without (●——●) 15 μg bindin for 20 min with gentle shaking. After this incubation, Triton X-100 in ASW was added to a final concentration of 1.5%, and the mixture was again incubated with shaking for 5 min. Samples were then diluted to a final detergent concentration of 0.15% and loaded onto gradients consisting of a 3-ml pad of CsCl (1.07 g/ml) in TBASW overlaid with a continuous sucrose density gradient of 60% (w/w) sucrose to 28% (w/w) sucrose in TBASW. After centrifugation for 16 hours at 76,000g (average), 0.5 ml fractions were collected and assayed for radioactivity and refractive index. The arrow indicates the density to which bindin alone sediments. B. Interaction of isolated receptor fraction with bindin. Receptor fraction was separated from detergent treated membranes (without bindin) as described in Figure 1A. Fractions 10–14, containing ^{125}I-labeled receptor, were combined and diluted with TBASW, and then centrifuged at 100,000g for 1 hour. The pellet was washed one time in TBASW and then resuspended by sonication. The recovery of ^{125}I label was greater than 95% by this procedure. This suspension was incubated in the absence (●——●) or presence (○——○) of bindin (100 μg), and centrifuged as described in Experimental Procedures.

branes by density-gradient centrifugation, it retains the capacity to interact with bindin. The results shown in Figure 2 indicate that this interaction is dependent on the amount of bindin added; with increasing amounts of bindin, there is an increasing amount of bindin–receptor complex formed (Fig. 1; fractions 7–9) and a corresponding decrease in the amount of receptor fraction (Fig. 1; fractions 12–15).

In an attempt to determine the species specificity of this interaction, similar experiments were carried out utilizing membranes and bindin isolated from the gametes of A punctulata. Centrifugation of A punctulata bindin alone indicated that it sediments to a density of 1.23 g/ml compared to greater than 1.3 g/ml for bindin from S purpuratus. This is consistent with the finding that A punctulata bindin contains bound lipid [3], which may decrease its density. As shown in Figure 3, the receptor fraction from A. punctulata sedimented to a higher density than that observed for S purpuratus. When bindin was added, the density of the A punctulata receptor fraction was decreased to a value near that of bindin alone. Therefore, binding activity of A punctulata fractions was measurable by this technique, although both the receptor fraction and bindin from A punctulata exhibit different densities from those prepared from S purpuratus gametes. However, attempts to measure binding of the receptor fraction to heterologous bindin by this technique were unsuccessful. This appeared to be due to the similarities in density of the receptor and bindin preparations in the heterologous cross.

Although it is apparent on the basis of the distribution of ^{125}I label in the membrane proteins that the procedure described above results in considerable purification of

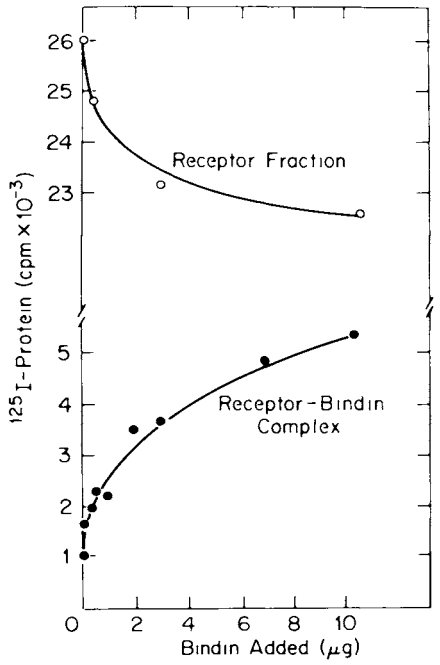

Fig. 2. Dependence of crude receptor interaction on bindin concentrations. Receptor fraction (1.3 μg; 27,000 cpm ^{125}I-labeled protein) was incubated with varying amounts of bindin and assayed by density-gradient centrifugation as described in the legend to Figure 1B. The amounts of radioactivity disappearing from fractions 12–15, and the amount appearing in fraction 7–9 were measured.

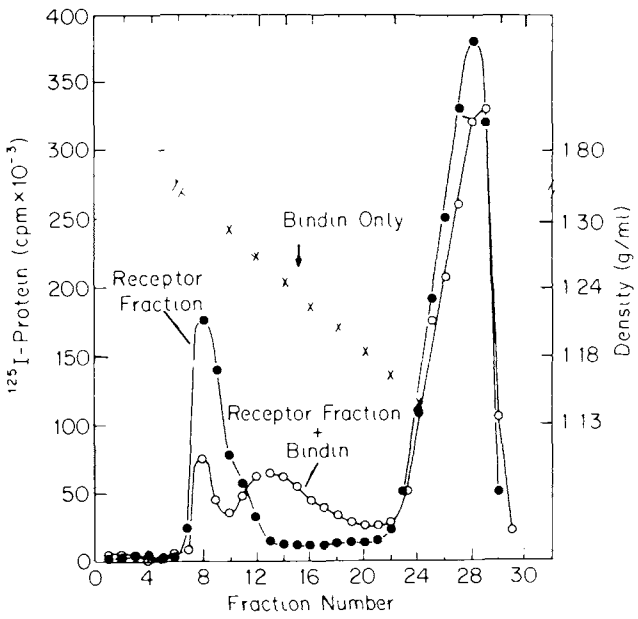

Fig. 3. Interaction of bindin with membranes from A punctulata. Membranes were isolated and radioiodinated as described in Experimental Procedures. Membranes (2.5 μg protein) were incubated in the absence (●——●) or presence (○——○) of 15 μg bindin, solubilized, and assayed as described in Figure 1. The arrow indicates the position to which A punctulata bindin alone sediments.

the receptor, the material recovered from the gradients is still particulate, since it can be sedimented at 100,000g. Furthermore, in preliminary experiments, it has been found that over 85% of the receptor fraction remained insoluble after treatment with a wide variety of detergents, organic solvents, or chaotrophic agents.

Binding of Receptor Fraction to Sperm

As an alternative approach to measure the species specificity of receptor binding, we measured the ability of the density-gradient purified ^{125}I-labeled receptor to bind to sperm. Sperm were preincubated with or without jelly coat to induce the acrosome reaction, and then binding of the ^{125}I-receptor fraction was measured. It is clear from the results shown in Figure 4 that, if sperm were preincubated with sufficient egg jelly to induce the acrosome reaction [11], ^{125}I receptor bound to the sperm in a saturable manner. In contrast, sperm incubated in the absence of egg jelly bound much lower levels of ^{125}I receptor. When the amount of receptor was varied at a constant sperm concentration, receptor binding was dependent on the amount of receptor added, and was also saturable (data not shown).

As shown in Figure 4, when sperm from A punctulata were incubated with receptor from S purpuratus, no specific binding was observed, either in the absence or presence of homologous jelly coat. In other experiements (data not shown), the receptor fraction isolated from A punctulata egg membranes showed saturable binding to A punctulata sperm. At saturation, 10% of the receptor was bound. These data argue that

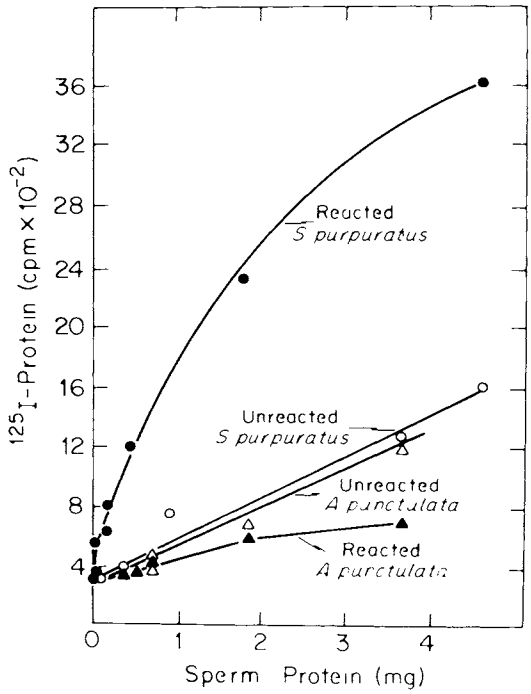

Fig. 4. Binding S purpuratus receptor fraction to sperm. Sperm from S purpuratus (circles) or A punctulata (triangles) were incubated in either TBASW (open symbols) or with homologous jelly coat in TBASW (closed symbols) for 20 sec, followed by the addition of 27,000 cpm ^{125}I-labeled receptor fraction, and binding was assayed as described in Experimental Procedures.

the receptor fraction derived from S purpuratus eggs binds preferentially to acrosome-reacted sperm, and that this binding occurs in a species-specific manner.

Inhibition of Fertilization by the Receptor Fraction

If the observed acrosome-dependent binding is physiologically similar to that that occurs in sperm–egg binding, it would be expected that the receptor fraction could compete with eggs in sperm binding and therefore inhibit fertilization. As shown in Figure 5, nearly complete inhibition of fertilization of S purpuratus eggs could be achieved by increasing the amount of homologous receptor fraction present during induction of the acrosome reaction. In contrast, similar concentrations of receptor fraction derived from A punctulata had little effect on the ability of acrosome reacted S purpuratus sperm to fertilize homologous eggs.

When sperm treated as described in the fertilization assay above were examined by electron microscopy on substrate films, several points became apparent. First, the presence of large amounts of receptor fraction (160 μg) had very little effect on the number of sperm that were induced to undergo the acrosome reaction with jelly coat (70% vs 80% reacted in controls lacking the receptor fraction). Second, in the absence of jelly coat, incubation of sperm with receptor fraction resulted in induction of the acrosome reaction in less than 1% of the sperm. These data indicate that inhibition of

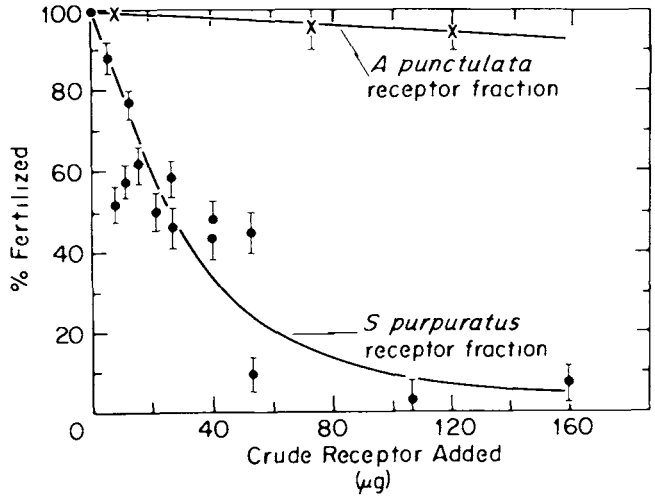

Fig. 5. Inhibition of fertilization of S purpuratus eggs by receptor fraction. S purpuratus sperm (75 μg protein/ml) were incubated for 20 sec with homologous jelly coat (44 μmoles fucose/ml) in the presence of varying amounts of receptor fraction derived from S purpuratus (●——●) or A punctulata (x——x), and then their ability to fertilize eggs was assayed as described in [1].

fertilization caused by preincubation of sperm with the receptor fraction does not occur by inhibition of induction of the acrosome reaction per se, or by premature induction of the acrosome reaction, leading to a loss in sperm viability [16]. Third, as shown in Figure 6, acrosome-reacted sperm bound to the aggregated receptor fraction, with approximately 75% of the reacted sperm being bound via their acrosomal process. Fewer than 20% of the unreacted sperm were found in any type of association with these aggregates. In addition, very few (< 20%) acrosome reacted S purpuratus sperm were found associated with the receptor fraction derived form A punctulata eggs. These data indicate that the particulate receptor fraction species specifically binds to the acrosomal process of sperm and inhibits fertilization, presumably by competing for sperm binding sites on the egg surface.

Generation of a Glycopeptide by Pronase Digestion of the Receptor Fraction

It was of interest to determine if protease treatment of the receptor fraction produced a soluble glycopeptide similar to that shown in the accompanying paper [5] that is released from intact eggs. Exhaustive Pronase digestion of the crude receptor (32 mg protein) followed by gel filtration revealed that approximately 4 mg of hexose-containing material could be recovered in the void fraction of a Biogel A5m column (Fig. 7). Less than 18% of the ^{125}I label was found in this fraction. These results indicated that extensive proteolysis had occurred. Preliminary data indicate that this soluble glycopeptide, as well as that released from the egg cell surface by Pronase treatment [5], can compete with the receptor fraction for binding to bindin particles in the centrifugation assay described above. At present, we are attempting to identify the active component(s) of this glycopeptide fraction.

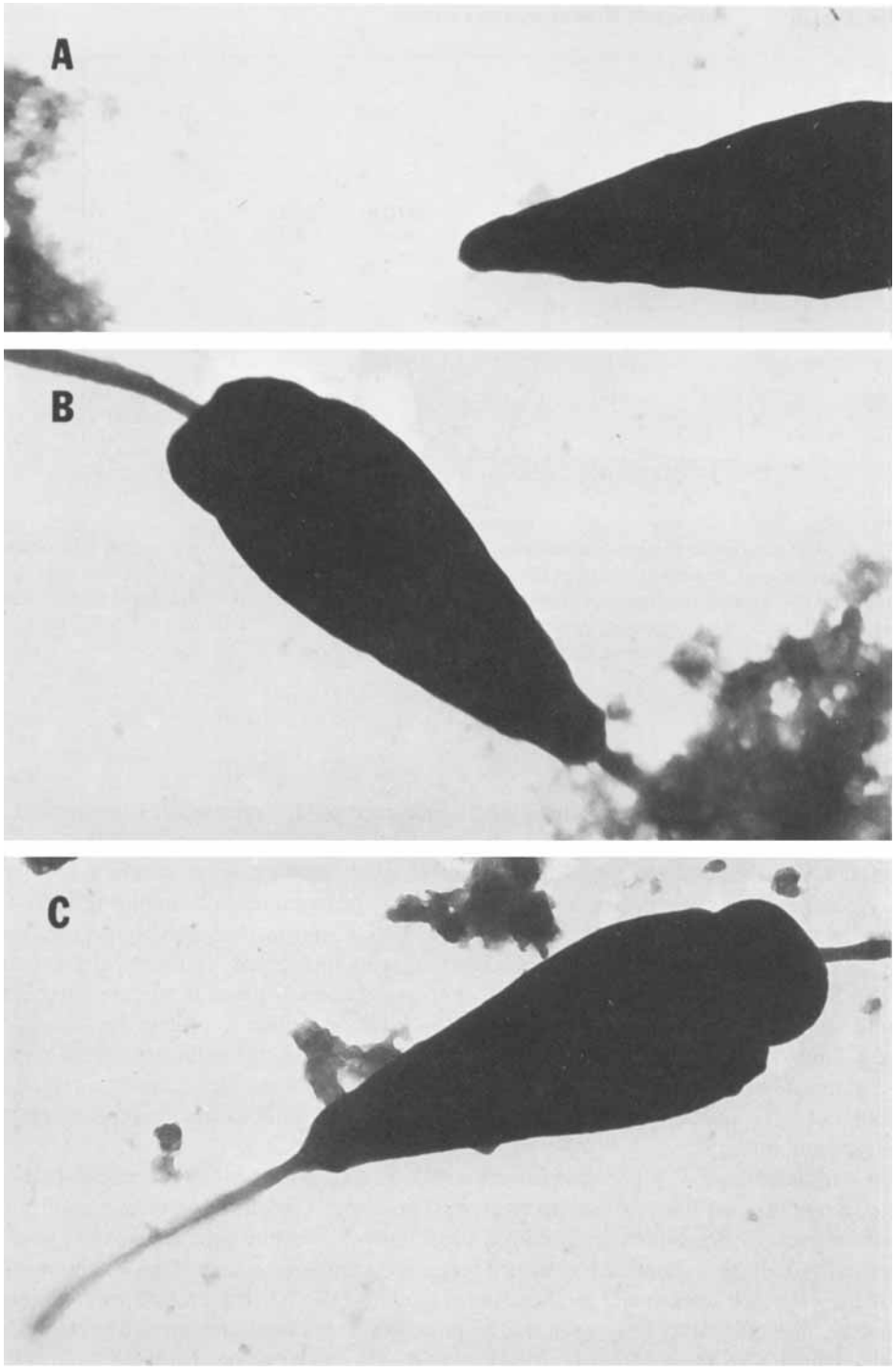

Fig 6 Electron microscopic examination of sperm–receptor fraction interactions Samples of sperm that were used in the experiments described in Figure 5 were examined on substrate films as described by Decker et al [12] A) S purpuratus sperm incubated in the presence of receptor fraction (160 μg) only B) S purpuratus sperm incubated with homologous jelly coat and receptor fraction (160 μg) protein from S purpuratus C) S purpuratus sperm incubated with jelly coat and receptor fraction (120 μg protein) from A punctulata Mag ×10,000

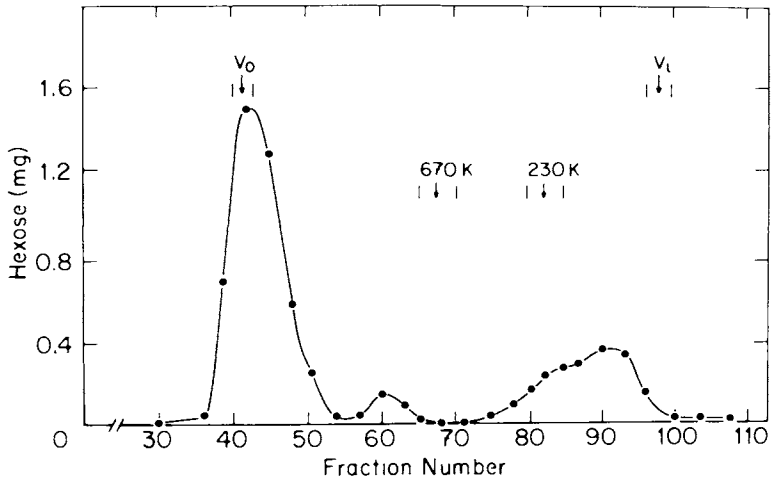

Fig. 7. Gel filtration of Pronase-digested receptor fraction. Receptor fraction (32 mg protein) was exhaustively digested with Pronase as described [5] and chromatographed on a 1 × 75 cm column of Biogel A 5 m in TBASW. One-ml fractions were collected and assayed for hexose content by the phenol-sulfuric acid method. The positions of elution of standards, blue dextran (v_o), thyroglobulin (M.W. = 670 K), catalase (M.W. = 230 K), and cytochrome C (v_t) are indicated.

DISCUSSION

Several lines of evidence indicate that the receptor for sperm that is associated with S purpuratus eggs is a glyconconjugate of high molecular weight, and that it is associated with the plasma membrane–vitelline layer complex. First, receptor activity (assessed either by sperm binding or by the ability of membranes to inhibit fertilization) is present in isolated egg membranes [1, 17] as well as in the highly purified cell surface complex consisting of vitelline layer, plasma membrane, and cortical granules [18]. Second, receptor activity is absent from membranes isolated from eggs pretreated with proteases [1]. The receptor appears to be highly sensitive to proteolysis, since very limited treatment of eggs with trypsin results in a loss of bindin-dependent agglutination [3]. Finally, one of the fragments released by protease treatment is a carbohydrate-rich glycopeptide of high molecular weight, which inhibits bindin-dependent egg agglutination [3, 5].

In an extension of previous efforts to obtain a soluble form of the receptor, it was found that the receptor activity was very unstable. The elevation of the fertilization envelope following fertilization has been shown to require protease activity, and several groups have identified a "sperm receptor hydrolase," which removes the receptor from the egg surface and inhibits sperm binding [19–21]. It therefore seems likely that the instability may have been due to proteolysis. Furthermore, limited proteolysis may explain why the receptor preparations of Glabe and Vacquier [2] were "soluble," and had lost the species-specific binding exhibited by the intact receptor. Indeed, the parthenogenically activated egg membranes, which were used as the starting material for the preparations, could not have been formed in the absence of proteolysis [21] (unpublished results from this lab).

For these reasons, in the approach taken in the current study membranes from eggs were prepared in the presense of inhibitors of proteolysis. The membranes, which contained stable receptor activity, were then treated with Triton X-100 in an effort to solubilize the receptor. In order to identify the receptor in detergent-treated membranes, we took advantage of the fact that S purpuratus bindin exists in seawater as high density aggregates. For this reason we anticipated that addition of bindin particles to Triton-treated membranes would result in a density shift in proteins (such as the receptor) that have an affinity for bindin. Such a density shift was observed, and it was possible to isolate a receptor-enriched fraction. This receptor fraction retains the species specificity seen in the crude membrane preparation. It binds not only to bindin but also to acrosome-reacted sperm. By fertilization bioassay it was found that this interaction with sperm competes for their binding to the egg surface receptor, and results in inhibition of fertilization.

Unfortunately, the receptor fraction isolated after detergent treatment of membranes is not truly soluble, since it can be sedimented at 100,000g. While it is possible that the receptor, after extraction, simply exists in an aggregated form, this seems unlikely, based on its insolubility in a wide range of detergents, chaotrophic agents, and organic solvents. It is also possible that the receptor fraction may be cross-linked by the tyrosine cross-linking reaction that occurs at the egg surface during hardening of the fertilization envelope after fertilization [22]. In this respect, preliminary data indicate that the crude membrane preparation used as the starting material in this study contains nearly as much dityrosine as total membranes isolated from fertilized eggs containing the fertilization envelope. Currently we are carrying out studies using membranes obtained from eggs pretreated with inhibitors of the cross-linking reaction in an effort to see if this explains the insolubility of the receptor fraction.

In the accompanying paper [5] we report that a carbohydrate-rich glycopeptide can be realeased from the cell surface of intact S purpuratus eggs by protease treatment. The glycopeptide inhibits bindin-dependent egg agglutination, although without species specificity. The fact that a similar, if not identical, glycopeptide can be isolated from the partially purified receptor fraction indicates that in fact it may be a component of the receptor. Future studies with a soluble, more highly purified form of the intact receptor should define the relationship between the glycopeptide and the intact receptor.

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