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Egg surface glycoprotein receptor for sea urchin sperm bindin

(fertilization/sperm receptor/cell surface/intercellular adhesion/cell recognition)

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ABSTRACT **Bindin is an insoluble protein coating the sperm acrosome process and mediating the adhesion of sperm to sea urchin eggs. Milligrams of bindin have been isolated. Here we report the identification, isolation, and partial characterization of a high molecular weight, trypsin-sensitive glycoprotein fraction from the sea urchin egg surface having species-specific affinity for bindin. This glycoprotein may be the egg surface receptor for bindin. The bindin receptor was released from ^{125}I-labeled eggs by parthenogenetic activation of eggs with ionophore A23187 in the presence of soybean trypsin inhibitor. The receptor has an isoelectric point of 4.02 and a molecular weight in sea water $\geq 5 \times 10^6$, suggesting that it is an aggregate. It contains 34% neutral sugars, which are galactose and mannose.**

Attachment of spermatozoa to the egg surface occurs in many animal species, including mammals (1). In sea urchins, gamete adhesion is between the sperm acrosome process and the egg vitelline layer (2). An insoluble protein, named bindin, has been isolated from acrosome granules of sea urchin sperm. Peroxidase-conjugated antibody to bindin localizes bindin on the acrosome process membrane and on the vitelline layer adjacent to the point of adhesion of the acrosome process (3). Bindin is a species-specific agglutinin of unfertilized eggs (4, 5). Trypsin-generated glycopeptides from egg surfaces block the egg-agglutinating property of bindin and periodate oxidation of eggs renders them nonagglutinable by bindin. These data are evidence that bindin mediates sperm adhesion to eggs.

The presence of species-specific glycoprotein "sperm receptors" on sea urchin eggs has been inferred for several years (6–12). Here we present the direct demonstration and isolation of a large, protease-sensitive egg surface glycoprotein having specific affinity for bindin.

MATERIALS AND METHODS

Gametes and Bindin Isolation. Gametes of *Strongylocentrotus purpuratus* were obtained by pouring 0.5 M KCl into opened body cavities. Egg jelly coats were dissolved by 2-min exposure to pH 4.7 sea water, followed by settling and resuspension in pH 8.0 sea water. The pH 4.7 treatment was repeated and the washed eggs were stored at 4°. Bindin was isolated as described (4). After the final sea water wash the insoluble bindin pellet was resuspended in sea water by mild sonication, the protein concentration was determined (13), and 1-ml portions were stored at −70°.

^{125}I Labeling of Egg Surfaces. Twenty milliliters of 50% (vol/vol) suspension of dejellied eggs in sea water containing 10 mM Tris (pH 8.0) was placed in a 50-ml conical tube. Two-tenths milliliter of ^{125}I (carrier-free, 2.0 mCi/ml in 10 mM NaOH, Amersham) was added, followed by 0.1 ml of chloramine-T (10 mg/ml in sea water) at 15-sec intervals for 5 min. The reaction was stopped by adding 2.0 ml of sodium metabisulfite (10 mg/ml in sea water). The labeled eggs were washed a minimum of five times by settling through fresh sea water. ^{125}I-Labeled eggs developed to the gastrula stage.

Isolation of ^{125}I-Labeled Bindin Receptor Activity. Ten milliliters of gently sedimented ^{125}I-labeled eggs were collected in a 50-ml tube and the overlying sea water was removed. The eggs were then suspended in 20 ml of sea water containing 10 mM Tris (pH 8.0), 0.2 mg of soybean trypsin inhibitor per ml (Sigma), and 0.2 ml of dimethyl sulfoxide containing 2 mg of ionophore A23187 per ml (Eli Lilly, final concentration 38 μM), a known parthenogenetic activator of sea urchin eggs (14). The eggs were stirred gently by hand for 1 min at 5-min intervals for 30 min at 23° and then pelleted by hand centrifugation. Microscopic observations of the eggs were made during the 30-min incubation period to be certain that egg lysis was not occurring. The supernate was then removed and centrifuged for 20 min at 8000 × *g*. The resultant supernate was stored at −70°. Radioactivity was determined by liquid scintillation counting of 0.1 ml of supernate in 7 ml of Tritosol scintillation fluid (15). Usually about 10% of the total radioactivity of labeled eggs was released into the 8000 × *g* supernate; the specific activity of the supernate was $4–10 \times 10^5$ cpm/mg of protein. This preparation will be referred to as "crude receptor." To determine the amount of free ^{125}I associated with the preparation, we spotted 25-μl portions on 1-cm squares of Whatmann 3 MM filter paper, dried the squares, and either directly measured radioactivity in 10 ml of PCS scintillation fluid (Amersham) or washed the samples in several changes of 10% trichloroacetic acid and then measured the radioactivity. Approximately 22–56% of the radioactivity was acid soluble and believed to be free ^{125}I. Unlabeled crude receptor used in competition experiments and for chemical analysis was prepared by the above method and stored at −70°.

Egg surface ^{125}I-labeled material was also prepared in the absence of soybean trypsin inhibitor and will be referred to as "degraded receptor." After preparation of the 8000 × *g* supernate of degraded receptor, the inhibitor (0.2 mg/ml) was added to inhibit endogenous protease activity. Active fertilization protease was obtained by activation of unlabeled eggs by A23187 in the absence of the inhibitor.

Assay for Bindin Receptor Activity. Sea water suspensions of particulate bindin (1–2.5 mg/ml) were mixed with ^{125}I-labeled crude receptor in various proportions in a 12 × 75 mm, 6-ml tube at 23°. After the desired time of incubation (usually 10 min) the tube was filled with sea water and the contents were filtered through a Whatmann GF/C filter disk (2.4-cm diameter). The tube was filled twice more and the sample was poured into the filter cone. The filter was dried and placed in 7 ml of Tritosol (15). Radioactivity was determined by scintillation counting. Controls showed that essentially 100% of the bindin was caught by the filter. In the absence of bindin approximately 1.2–9.6% (average 3.6%) of the acid-insoluble radioactivity of the crude receptor was trapped on the filter. These "background" counts were subtracted from the amount of radioactivity retained on the filter when bindin was present.

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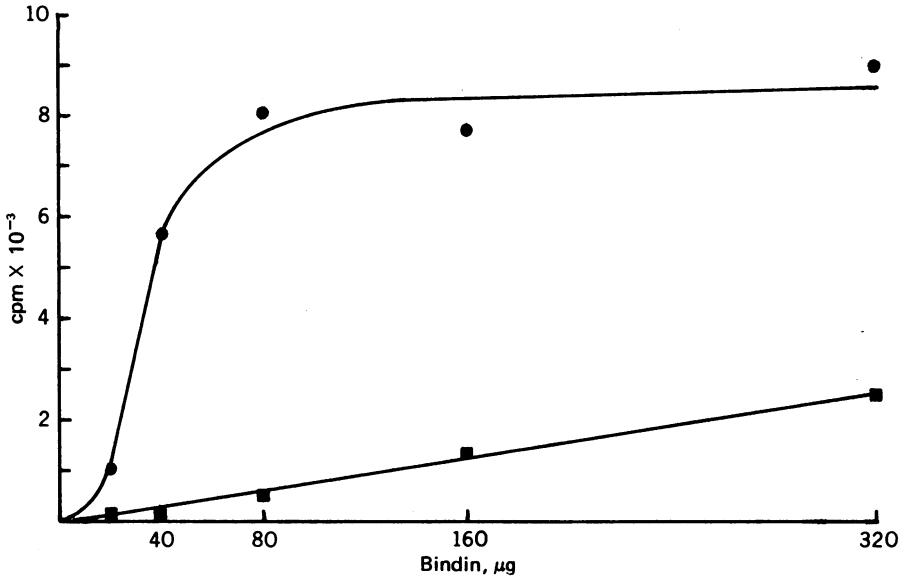

FIG. 1. Binding of ^{125}I-labeled egg surface material released during activation to bindin. One-half milliliter of crude receptor preparation (0.175 mg of protein, 817,000 cpm/mg) or 0.1 ml of degraded receptor preparation (0.24 mg of protein, 625,000 cpm/mg) was mixed with varying amounts of bindin, incubated for 10 min, and then filtered. The crude receptor (●), obtained by activating ^{125}I-labeled eggs in 0.2 mg of soybean trypsin inhibitor per ml, exhibits greater binding activity than does the degraded receptor (■) prepared in the absence of the inhibitor.

Experiments in which bindin was prefiltered and the crude receptor filtered afterward showed that the accumulation of radioactivity on the filter was not caused by the trapping of ^{125}I-labeled crude receptor by the bindin-containing filter. Data are presented as cpm of ^{125}I per filter.

Other Procedures. A 25 × 40-cm column of Bio-Gel A5m in sea water (Bio-Rad Laboratories, Richmond, CA) was used to fractionate the crude receptor at 10 ml/hr. The radioactivity of each 4-ml fraction was determined on 0.1-ml aliquots in 7 ml of Tritosol. Isoelectric focusing was done with an LKB 110-ml column containing 1% pH 3.5–5.0 Ampholines. The pH of 3-ml fractions was measured with a Corning model 12 expanded scale pH meter, and the radioactivity was determined with a gamma counter. Soluble protein from fertilized eggs was prepared by blending the eggs for 5 min in sea water. The 20,000 × *g* supernate fraction was made 0.2 mg/ml in soybean trypsin inhibitor and stored at −70°. Neutral sugars were determined by the phenol/sulfuric method (16), with galactose as standard. Neutral sugars were identified by gas chromatography of the alditol acetate derivatives (17). Sialic acid was determined by the thiobarbituric acid assay (18), and sulfate by the method of Terho and Hartiala (19).

RESULTS

Removal of Bindin Receptor from Egg Surfaces. Sea urchin (20) and mammalian (21) eggs release trypsin-like protease activity shortly after fusion with sperm. The protease is believed to aid in prevention of polyspermic fertilization by digesting hypothetical sperm receptors, thus rendering the egg surface incapable of sperm adhesion (11, 12, 21). Unfertilized eggs treated with fertilization protease rapidly lose their ability to bind sperm and to be fertilized (11, 12, 21), although they can still be activated by A23187 (11). The protease is inhibited by natural and synthetic inhibitors of pancreatic trypsin (11, 12, 20, 21).

The protease-sensitive component of the egg surface involved in sperm binding might be a receptor for bindin. If fertilization protease cleaves off the receptor (11), one way to attempt to isolate the receptor would be to activate eggs with A23187 in low concentrations of trypsin inhibitors, hoping that limited proteolysis of the vitelline layer would release the receptor in an active form. Fig. 1 shows the affinity for bindin of ^{125}I-labeled material released from eggs in the presence (crude receptor) or absence (degraded receptor) of soybean trypsin inhibitor. The binding of crude receptor is saturable. At saturation (80 μg of bindin), roughly 15 times as much crude receptor radioactivity binds to bindin as compared to degraded receptor. When bindin is present in excess, approximately 15–20% of the macromolecular ^{125}I binds to bindin. These results suggest that the crude receptor preparation contains a receptor for bindin which, in the absence of soybean trypsin inhibitor, is degraded by fertilization protease. Some affinity of the degraded receptor is apparent (Fig. 1), and we already know that trypsin-generated egg surface glycopeptides block bindin as an agglutinin of eggs (4), thus confirming this affinity.

Binding of ^{125}I-Labeled Receptor to Bindin. Excess crude receptor also saturates bindin (Fig. 2), and formation of bindin–receptor complexes is time dependent, with 50% maximum binding being achieved by 18 sec (Fig. 3). The binding kinetics of receptor to bindin are additional evidence that the radioactivity accumulated on filters does not result from trapping of receptor, but represents formation of bindin–receptor complexes.

Specificity. Specificity of affinity of receptor for bindin was investigated by competition assays. ^{125}I-Labeled crude receptor and bindin were mixed together for 10 min in proportions in which approximately 70% of the receptor sites on bindin were occupied (Fig. 2). Portions of this mixture were then added to tubes containing various amounts of unlabeled crude receptor from either *S. purpuratus* or *S. franciscanus*, whole fertilized egg protein, bovine serum albumin, or soybean trypsin inhibitor. The mixtures were incubated for 10 min, the contents of the tubes were filtered, and the radioactivity was determined. Only unlabeled crude receptor (Fig. 4) from *S. purpuratus* competed effectively for complexes of *S. purpuratus* bindin and ^{125}I-labeled receptor. At 320 μg of added unlabeled protein, 87% of the bindin–^{125}I-labeled receptor complexes were disassociated by unlabeled *S. purpuratus* receptor, while only 27% was observed for *S. franciscanus* crude receptor. Fertilized *S. purpuratus* egg protein was as ineffective a competitor as

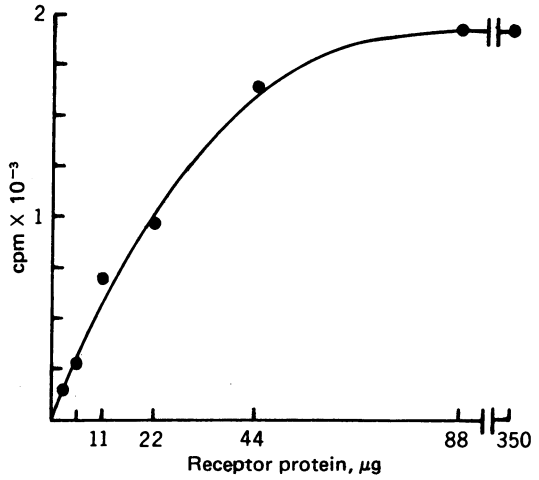

FIG. 2. Binding of ^{125}I-labeled crude receptor to bindin with receptor in excess. One milliliter of crude receptor (0.8 mg; 620,000 cpm/mg) was serially diluted in sea water 1:2 per dilution, mixed with 0.05 ml of bindin suspension (1 mg/ml), and then incubated for 10 min before filtration. Bindin can be saturated with crude receptor.

S. franciscanus crude receptor. If bovine serum albumin or soybean trypsin inhibitor was mixed with bindin either before or after addition of ^{125}I-labeled receptor, no competition for the bindin–receptor complex was observed. The successful competition by unlabeled, homologous receptor indicates that an equilibrium exists between free receptor and bindin–receptor complexes.

Fractionation of Bindin Receptor Activity. Gel filtration of ^{125}I-labeled crude receptor on Biogel A5m fractionates the radioactivity into three peaks (Fig. 5). Peak I is the void volume, molecular weight $\geq 5 \times 10^6$, peak II is the included material of undetermined size, and peak III is free ^{125}I. A portion of each fraction was assayed for bindin receptor activity, which was found only in the peak I fractions (Fig. 5). Polyacrylamide gel electrophoresis of this material dissolved in 5% sodium dodecyl sulfate/2% mercaptoethanol yielded a protein band that did not enter the running gel (6).

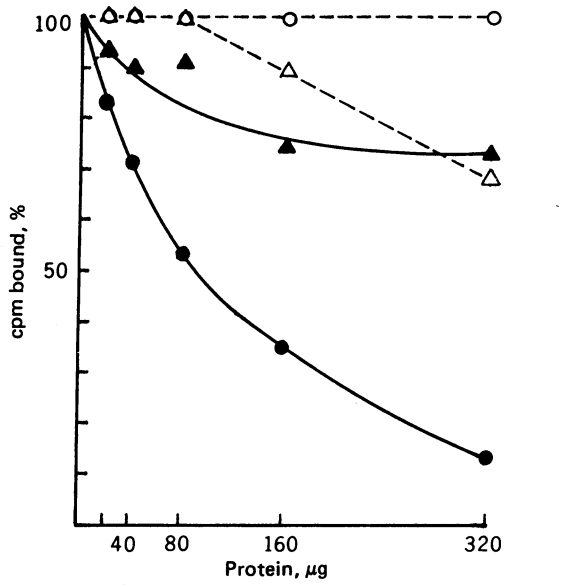

FIG. 4. Species-specific affinity of bindin for crude receptor, as shown by competition assays. ^{125}I-Labeled crude receptor (0.35 ml, 0.9 mg of protein per ml, 1.5×10^6 cpm/mg) from *S. purpuratus* was mixed with 0.14 ml of bindin suspension (2.5 mg/ml) from *S. purpuratus* and incubated for 10 min. Aliquots of 0.07 ml of this mixture were placed in tubes containing various amounts of the following unlabeled materials (1 mg of protein/ml): ●, crude receptor from *S. purpuratus*; ▲, crude receptor from *S. franciscanus*; △, soluble fertilized egg protein from *S. purpuratus*; ○, bovine serum albumin or soybean trypsin inhibitor. The data show that only *S. purpuratus* crude receptor is an effective competitor for bindin–^{125}I-labeled receptor complexes.

To test if receptor activity was sensitive to fertilization protease we treated 8 ml of peak I material for 18 hr with 2 ml of fertilization protease. The protease activity was inhibited by

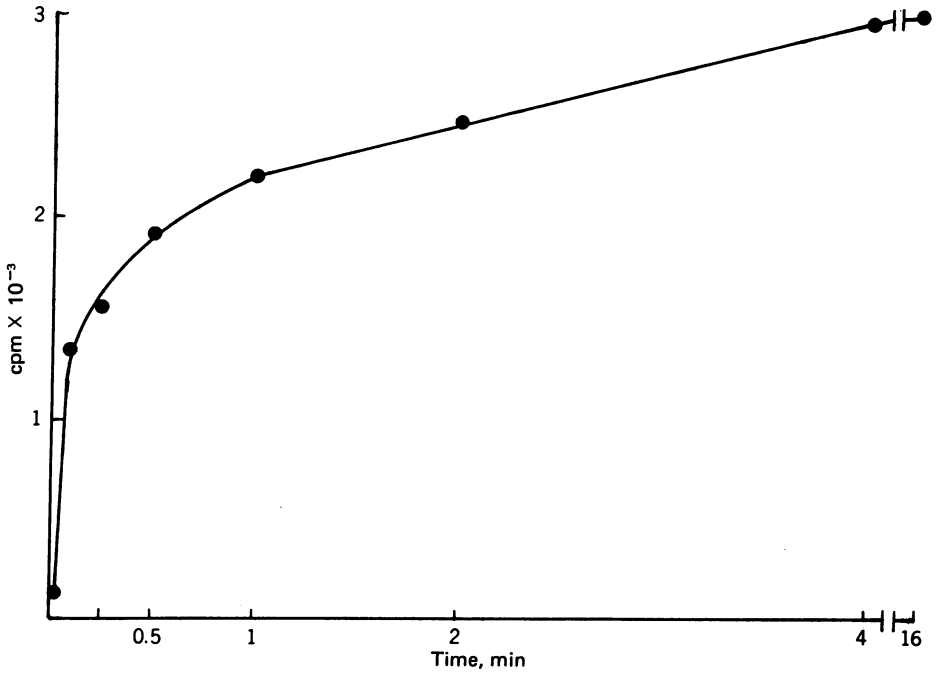

FIG. 3. Kinetics of formation of bindin–receptor complexes. One-quarter milliliter of ^{125}I-labeled crude receptor (0.09 mg of protein, 817,000 cpm/mg) was mixed with 0.05 ml of bindin (1 mg/ml) and incubated for varying times before filtration. Zero time was obtained by first filtering the bindin and then filtering the ^{125}I-labeled crude receptor. Fifty percent maximum binding occurs in 18 sec.

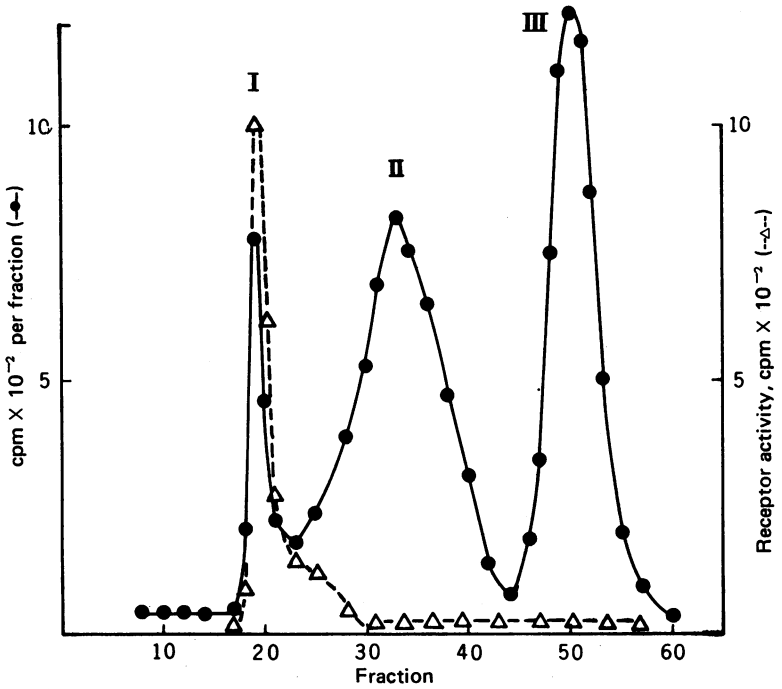

FIG. 5. Gel filtration of ^{125}I-labeled crude receptor on Bio-Gel A5m. Two milliliters of crude receptor (0.35 mg of protein per ml, 817,000 cpm/mg) was fractionated in sea water. Three peaks of radioactivity were obtained (●). Peak I is the void volume as determined by blue dextran, peak II is included material of undetermined size, and peak III is the included volume as determined by mercaptoethanol. One half milliliter of each fraction was mixed with 0.05 ml of bindin (2 mg/ml), incubated for 10 min, and then filtered. The bindin receptor activity (△) is found only in the peak I fractions, indicating a molecular weight $\geq 5 \times 10^6$.

addition of 0.2 mg of soybean trypsin inhibitor per ml and the preparation was tested for bindin receptor activity. All receptor activity was lost after treatment with fertilization protease. Refractionation of the inactive peak I on A5m (data not presented) showed that the major portion of radioactivity was now of smaller size, with one major peak at fraction 40.

Isoelectric Focusing. Isoelectric focusing of peak I (Fig. 5) in a pH 3.5–5.0 gradient separated the radioactivity into two components (Fig. 6), of average pI 4.02 (range 4.13–3.95) and pI 2.5. ^{125}I-Labeled soluble egg jelly also isoelectric focuses to pI 2.5 so we presume this peak to be egg jelly. Before isoelectric focusing, 41% of the radioactivity of peak I (Fig. 5) bound to bindin with bindin in excess. After isoelectric focusing and dialysis of the pI 4.02 peak fractions (Fig. 6) into sea water, 64% of the radioactivity had affinity for bindin. The pI 2.5 peak is precipitated and cannot be assayed for receptor activity by the filter technique. The enrichment of receptor activity in the pI 4.02 fraction is evidence that this peak is bindin receptor, which is distinct from egg jelly. Analysis of the receptor peak shows that it contains 4% sulfate compared to 17% for egg jelly (B. Brandriff and V. D. Vacquier, unpublished). The material is 34% neutral sugars, which are mannose and galactose; sialic acid was not detected.

DISCUSSION

In studying sperm–egg adhesion we have focused on isolating the interacting surface macromolecules. Bindin has been isolated and shown to be the sperm surface protein mediating gamete adhesion (3–5). An egg surface glycoprotein fraction has now been identified having specific affinity (receptor activity) for bindin. The claim that the competition experiments (Fig. 4) demonstrate the species specificity of the bindin receptor glycoprotein rests on the assumption that the receptor is released in a similar manner and concentration from eggs of both species.

After isoelectric focusing of peak I from the A5m column (Fig. 5), the enrichment of receptor activity in the pH 4.02 peak (Fig. 6) is evidence that it represents the receptor. Isoelectric point and chemical composition show that this material is certainly not egg jelly. Egg jelly has an isoelectric point of 2.5 (Fig. 6), is 17% sulfate, and contains fucose and sialic acid (B. Brandriff and C. G. Glabe, unpublished). The pI 4.02 peak is 4% sulfate and contains mannose, galactose, and no sialic acid.

During normal fertilization, in the absence of trypsin inhibitors, the fertilization protease destroys the bindin receptor (Fig. 1), digesting it to a major component of apparent molecular weight 32,500 (data not shown), which may be a basic structural unit of the receptor. The loss of receptor activity resulting from the fertilization protease correlates well with previous work demonstrating loss of sperm binding and ability of eggs to be fertilized after exposure to the protease (11, 12).

Using Japanese sea urchins, Aketa and coworkers have described a glycoprotein solubilized in 1 M urea that they believe to be the sperm receptor. Evidence of its involvement in sperm adhesion is as follows: sperm adhere to bubbles made in a solution of this factor, antibodies against the factor prevent fertilization, and the factor decreases the ability of homologous sperm to fertilize eggs (7, 8). The evidence that this factor is involved in sperm adhesion is largely indirect and circumstantial (see discussion of ref. 9). However, our finding of a molecular weight of several million for the active bindin receptor is similar to their estimated size for the urea-soluble sperm receptor factor (8).

Schmell *et al.* (9) have also identified a trypsin-sensitive glycoprotein with the characteristics of a sperm receptor from eggs of the sea urchin *Arbacia punctulata*. This glycoprotein inhibits fertilization by binding to sperm, and the inhibition is species-specific. The bindin-coated acrosome process is only exposed after the acrosome reaction (22, 23). If the sperm receptor glycoprotein identified by the above authors (7–9) interacts with bindin, it is difficult to explain why it binds to

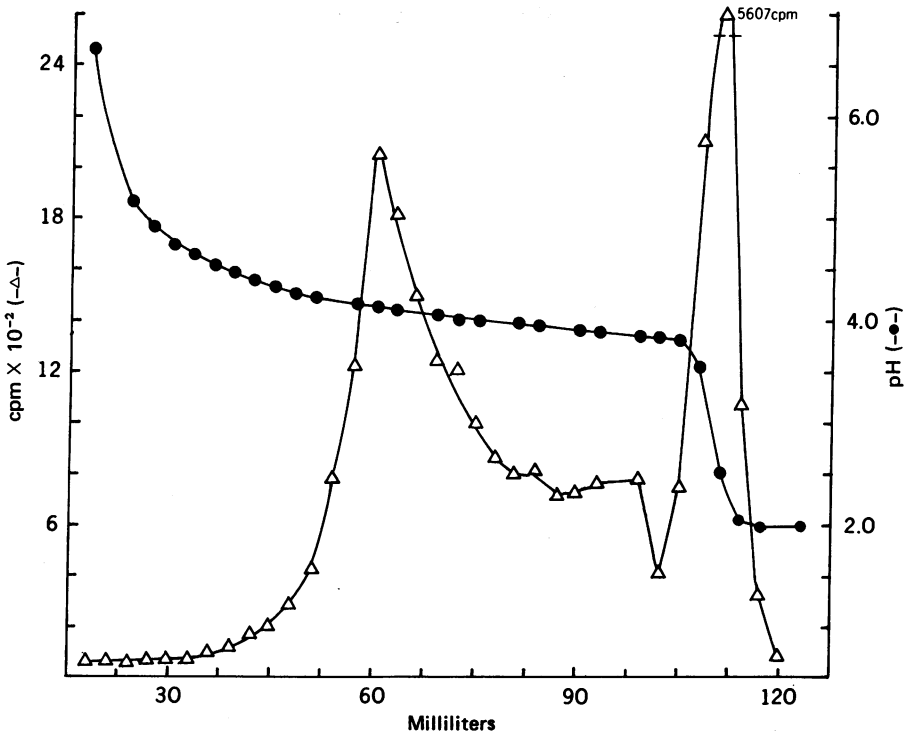

FIG. 6. Isoelectric focusing of peak I from the A5m column. Peak I fractions (Fig. 5) were dialyzed into distilled water and focused in a pH 3.5–5.0 gradient. The material separated into two components of pI 4.02 and 2.5. The pI 2.5 peak is egg jelly. The pI 4.02 peak is believed to be the bindin receptor. Before isoelectric focusing, 41% of the cpm bound to bindin. After isoelectric focusing, the pI 4.02 peak fractions were dialyzed into sea water and concentrated by further dialysis against polyvinylpyrrolidone; affinity to bindin was then determined. Sixty-four percent of this material bound bindin when bindin was present in excess. The pI 2.5 peak was irreversibly precipitated and, consequently, its bindin receptor activity could not be determined by the filtration assay.

sperm that presumably have not undergone the acrosome reaction. The inhibition of fertilization they observe could occur by one of two seemingly opposite effects. The glycoprotein might bind the sperm plasma membrane and inhibit the acrosome reaction or it might cause premature induction of the acrosome reaction. Neither of these groups has demonstrated the effect that their glycoprotein "sperm receptors" would have on the acrosome reaction, nor have they shown them to participate directly in sperm–egg adhesion (7–9).

The above-mentioned results of others on the possible identification of "sperm receptors" (7–9) and our identification of a receptor for bindin may be reconciled by proposing the existence of two recognition events between sperm and egg. The first could be the interaction of the sperm plasma membrane with a component of the egg surface that induces the acrosome reaction (e.g., egg jelly; refs. 24 and 25). The second, which would lead to sperm adhesion, would be the interaction of bindin with its receptor. The same egg surface component may mediate both interactions. All groups of workers agree that the egg surface macromolecules mediating sperm recognition and adhesion are large, trypsin-sensitive glycoproteins carried on the egg vitelline layer (4, 6–9).

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