Identification of the Sea Urchin Egg Receptor for Sperm Using an Antiserum Raised Against a Fragment of Its Extracellular Domain

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Abstract. Sea urchin egg fertilization requires the species-specific interaction of molecules on the sperm and egg surfaces. Previously, we isolated an extracellular, 70-kD glycosylated fragment of the *S. purpuratus* egg receptor for sperm by treating the eggs with lysylendoproteinase C (Foltz, K. R., and W. J. Lennarz. 1990. *J. Cell Biol.* 111:2951–2959). To characterize the receptor further, we have generated a polyclonal antiserum (anti-70KL) against the purified 70-kD fragment. Anti-70KL was found to react with a single polypeptide of ~350 kD on Western blots, presumed to be the intact receptor, in an egg cell surface preparation. This polypeptide appeared to be tightly associated with the plasma membrane/vitelline layer complex, as it was released from these preparations only by detergent treatment. Immunofluorescence microscopy revealed that the receptor was distributed evenly over the egg surface. The anti-70KL was species specific both in its ability to recognize the egg surface protein and to inhibit sperm binding. Fab fragments generated from affinity-purified anti-70KL also bound to the egg surface and inhibited sperm binding in a concentration-dependent manner. Interestingly, treatment with Fabs caused a small percentage of eggs to undergo cortical granule exocytosis, even in the absence of external Ca^{2+}. These results confirm earlier findings indicating that the receptor is a cell surface glycoprotein of high molecular weight that species specifically binds sperm. This antiserum provides a powerful tool for further investigation of gamete interactions and the structure of the sperm receptor.

A key step in fertilization is gamete binding mediated by surface molecules of the sperm and the egg. As reviewed elsewhere, this interaction generally is species specific and involves several recognition steps (see Vacquier and Moy, 1978; Yanagimachi, 1978; Monroy, 1985; Trimmer and Vacquier, 1986; Wassarman, 1987; Ruiz-Bravo and Lennarz, 1989; Glabe et al., 1991). With respect to sea urchin fertilization, bindin (a molecule on the surface of the sperm acrosomal process) has been well-characterized and is believed to be the major sperm surface molecule involved in gamete binding (Vacquier and Moy, 1977; Glabe and Vacquier, 1977*a*; Glabe et al., 1982; Minor et al., 1989). It has been known for some time that the receptor for bindin is associated with the vitelline layer of the sea urchin egg (Summers and Hylander, 1975; Glabe and Vacquier, 1977*b*; Tsuzuki et al., 1977). The simplest hypothesis has been one that equates the bindin receptor with the actual sperm receptor; i.e., the molecule on the egg surface that species specifically recognizes acrosome-reacted sperm (Glabe and Vacquier, 1978).

Although the function of the receptor is unknown, two alternative hypotheses have dominated the thinking about sperm binding to the receptor and the consequent egg activation (see Epel, 1989). One hypothesis is that egg activation results from the introduction of a sperm component into the egg plasma membrane or egg cytoplasm. In this scenario, the receptor on the egg serves a structural function by anchoring the sperm to the egg surface; i.e. the receptor is a "sperm binding protein." The other hypothesis is that the egg receptor for sperm is linked to a signal transduction pathway such that the process of sperm binding to the receptor causes a signal to be transduced to the inside of the cell. It is possible that an activation signal is transduced through a pathway involving IP_3 production and Ca^{2+} release via a G-protein coupled mechanism (Turner and Jaffe, 1989), although there is evidence suggesting that egg activation may not involve G-proteins (Crossley et al., 1991). Until the structure of the receptor is elucidated its role in the overall activation process will not be well defined.

Several groups have reported the isolation of high molecular weight glycoconjugates from the egg surface that inhibit sperm-binding activity, and therefore have been candidates for the receptor for sperm (Metz, 1978; Schmell et al., 1977; Tsuzuki et al., 1977; Glabe and Vacquier, 1978; Rossignol et al., 1981, 1984; Yoshida and Aketa, 1983; Acevedo-Duncan and Carroll, 1986). However, none of these molecules has been further characterized. An alternative approach that was taken was the preparation of extracellular,

proteolytic fragments of putative receptors on the surface of the egg. Tryptic digestion of egg surfaces rendered the eggs unable to bind sperm and the resulting digested material was found to contain proteolytic fragments that bound to homotypic sperm and inhibited fertilization in a sensitive bioassay (Schmell et al., 1977; Ruiz-Bravo and Lennarz, 1986). In addition, such tryptic fragments were also shown to block bindin-mediated agglutination of eggs (Vacquier and Moy, 1977). However, because tryptic digests such as those isolated by Ruiz-Bravo and Lennarz (1986) are heterogeneous and unstable, this promising approach to isolation of a fragment of the receptor has not been successful.

Recently, we found that treatment of *S. purpuratus* eggs with a protease of more restrictive specificity, lysylendoproteinase C (LysC)[1], resulted in both the loss of the ability of eggs to bind sperm and in the release of stable peptide fragments (Foltz and Lennarz, 1990). A 70-kD proteolytic fragment was purified from the crude LysC digest that was shown to be a glycoprotein with sperm-binding activity. The fragment bound to sperm of *S. purpuratus* only after they had been induced to undergo the acrosome reaction; no binding to other species of sperm was observed. The fragment also bound species preferentially to isolated bindin. This evidence together provided strong support for the idea that the 70-kD fragment is a portion of the extracellular domain of the *S. purpuratus* sperm receptor. Glycopeptides generated from the 70-kD glycoprotein fragment by Pronase digestion bound to sperm from other sea urchin species. This provided additional support for the hypothesis developed earlier (Ruiz-Bravo and Lennarz, 1986) that although the oligosaccharide chains of the receptor are the adhesive element, the polypeptide chain somehow confers species specificity to the binding process.

We report here the generation and characterization of an antiserum specific for the *S. purpuratus* sperm receptor LysC 70-kD fragment. This antiserum (anti-70KL) is specific for a high molecular weight protein present on the egg cell surface. This ~350,000-M_r polypeptide is presumed to be the intact receptor and it is solubilized only by detergent treatment. Immunofluorescence microscopy has been used to localize the receptor in *S. purpuratus* eggs. The anti-70KL IgG and Fabs, which are species specific, were found to bind to the egg surface and inhibit sperm binding, but had no inhibitory effect on ionophore-mediated fertilization envelope elevation. Interestingly, Fabs prepared from the anti-70KL IgG caused fertilization envelope elevation in a small percentage of eggs, even in Ca^{2+}-free sea water. These results are discussed in the context of the function of the egg receptor for sperm.

Materials and Methods

Isolation of Gametes, Preparation of Egg Surface Digests, and Isolation of Egg Surface Complex

Adult *Strongylocentrotus purpuratus* and *Lytechinus pictus* were obtained from Marinus, Inc. (Long Beach, CA). Adult *S. drobachiensis* and *Arbacia punctulata* were from the Marine Biological Laboratory (Woods Hole, MA). Gametes were isolated by intracoelomic injection of 0.5 M KCl. Sperm were collected dry and stored on ice up to 12 h. Eggs were collected in millipore-filtered (22 μm) artificial sea water (FASW; Instant Ocean, Aquarium Systems, Mentor, OH). Eggs were washed three times in FASW at 14°C and then dejellied by passage through Nitex (Tekton, Inc., Elmsford, NY; 120 μm for *S. purpuratus*, 210 μm for *L. pictus* and 250 μm for *S. drobachiensis*) or by brief suspension in pH 5 FASW. The dejellied eggs were washed three times by settling and maintained as a 1% suspension in FASW at 14°C. Egg jelly was stored on ice up to 24 h.

Preparation of crude LysC digests of eggs was as described previously (Foltz and Lennarz, 1990). Egg cell surface complex was prepared by the method of Kinsey (1986). Briefly, dejellied eggs were suspended and homogenized in Ca^{2+}-free sea water (SWC; 29.3 g NaCl, 0.75 g KCl, 0.21 g $NaHCO_3$, 9.5 g EGTA, and 2.5 g NaOH dissolved in 1 liter of water, pH adjusted to 8.0). This, and all subsequent buffers and solutions were supplemented with protease inhibitors (PMSF, aprotonin, leupeptin, benzamidine, and antipain) to 1 mM final concentration. All procedures were carried out on ice, and all chemicals were from Sigma Chemical Co. (St. Louis, MO) unless otherwise noted. The egg homogenate was diluted fivefold in ice-cold SWC and the cell surface complexes were pelleted by centrifuging at 2,000 rpm in a refrigerated Sorvall bench top centrifuge for 1 min. The pellets were resuspended and washed three times in cold SWC. The final pellet was immediately suspended in isotonic (1 M) sucrose, mixed by inversion, and centrifuged at 2,000 rpm for 20 min at 4°C. The pellets were resuspended in 1 M sucrose by gentle homogenization and incubated on ice until the cortical granules were judged to be detached, based on microscopic observation. The membrane/vitelline layer complex was collected by centrifugation in a Sorvall SS-34 rotor at 5,000 rpm for 20 min. The pellet was washed free of sucrose by diluting in SWC and centrifuging as before.

The purified membrane/vitelline layers were then treated in SWC, with one of the following: 0.1 or 1.0% β-octyl-glucoside; 0.1 or 1.0% Triton X-100; 0.1 or 1.0% NP-40; 0.1 or 1.0% SDS; 1 or 2 M NaCl; 1 M urea; 0.5 M KI; and 0.5 M Na carbonate, pH 11. The resuspended pellets were incubated on a rocker at 4°C for 2 h, and then centrifuged at 100,000 *g* (model Ti65 rotor; Beckman Instruments, Inc., Palo Alto, CA) to separate solubilized proteins from the insoluble membrane fraction. As a control, one sample was always centrifuged in SWC alone.

The Triton X-100 insoluble vitelline layer was isolated from eggs by the method of Niman et al. (1984). The vitelline layers were either immediately dissolved in Laemmli sample buffer (Laemmli, 1970) supplemented to 1% SDS or stored in saline (0.15 M NaCl, 0.2% Na_3N, 10 mM Tris, pH 7.4, 0.1 mg/mL SBTI) for use within 24 h.

Preparation of Anti-70KL Serum, IgG, and Fabs

The 70-kD LysC fragment from *S. purpuratus* eggs was purified as described (Foltz and Lennarz, 1990). After collecting preimmune serum from a female New Zealand white rabbit, the animal was injected subcutaneously with 100 μg of DEAE-purified LysC fragment emulsified in Freund's Complete Adjuvant (Bethesda Research Laboratories, Gaithersburg, MD). After 4 wk, the animal was again injected with 100 μg of the fragment, emulsified in Freund's Incomplete Adjuvant (Bethesda Research Laboratories). At 14 d after injection, blood was collected and the serum analyzed for immunoreactivity by a plate assay (see below). The animal was boosted twice more, at 4-wk intervals, and serum was isolated and stored at −20°C in aliquots.

IgG was purified from the immune serum using a two-step procedure as described by Harlow and Lane (1988) using ammonium sulfate precipitation followed by DEAE chromatography. Purity of the IgG was monitored by SDS-PAGE. The IgG was either dialyzed against PBS, pH 7.2, or FASW, depending on the intended use.

Purified IgG was digested with papain as described (Harlow and Lane, 1988) and the resulting Fab fragments purified by chromatography on a protein A column. Purity was monitored by SDS-PAGE. Fab fragments were either dialyzed against PBS (pH 7.2) or against FASW, depending on the intended use.

Affinity-purified IgG was prepared by immobilizing the purified LysC 70-kD fragment on Immobilon P (Millipore Corp., Bedford, MA) using a dot blot template. The membrane was blocked in PBS, pH 7.4, 2% BSA and then incubated with the IgG diluted in PBS, pH 7.4, 2% BSA. Unbound IgG was washed off and the bound IgG was eluted with ice-cold 0.1 M glycine, pH 3. The eluant was immediately neutralized with 1 M Tris, pH 8. The affinity-purified IgG was dialyzed against PBS, pH 7.4 and BSA was added to 0.2% final concentration.

Protein Determination and SDS-PAGE

Protein concentration was determined by the method of Lowry et al. (1951) using BSA as a standard. SDS-PAGE was performed by the method of

1. *Abbreviations used in this paper*: FASW, filtered artificial sea water; LysC, lysylendoproteinase C; SWC, Ca^{2+}-free sea water.

Laemmli (1970) using a 3–15% polyacrylamide gradient. Gels were fixed and stained in 50% methanol, 10% acetic acid, and stained with 0.1% Coomassie blue. Molecular weight standards were from Bio-Rad (Cambridge, MA). Dynein heavy chains from *S. purpuratus* sperm were prepared as previously described (Bell et al., 1982) and served as a relative molecular mass standard of ~475 kD (Mocz et al., 1988).

Radioimmunoassay, Dot Blot, and Western Analyses

Plate immunoassays were carried out using 96-well polyvinyl microtiter plates (Falcon Labware, Oxnard, CA; Beckton Dickinson, Oxnard, CA). Various amounts (see Figures) of crude LysC digests of egg surface, egg plasma membrane/vitelline layer proteins, jelly coat material or purified LysC 70-kD fragment were applied to wells in a total volume of 50 μl and incubated at room temperature for 4 h. The wells were blocked with 150 μl of PBS (pH 7.4), 2% BSA (PBSA) for 2 h at room temperature, and the wells were rinsed with PBS (pH 7.4). Preimmune serum or anti-70KL IgG diluted in PBSA was applied in 50 μl total volume to appropriate wells and incubated at room temperature 6–18 h. Wells were rinsed thoroughly with PBS (pH 7.4), 0.5% NP-40. 50 μl of ^{125}I donkey anti-rabbit IgG (Amersham Corp. Arlington Heights, IL) diluted 1:5,000 in PBSA was added to each well and the plate was incubated for 1 h at room temperature. Wells were washed with PBS (pH 7.4), 0.5% NP-40 and then cut out and counted in a mini gamma counter (LKB Instruments Inc., Gaithersburg, MD). All plate assays were in duplicate for each experiment.

Competitive RIAs were performed using ^{125}I 70-K LysC fragment, purified and iodinated as described (Foltz and Lennarz, 1990). Purified IgG (100 μg) was bound to the wells of a polyvinyl microtiter dish. The wells were blocked with PBSA. 20 μg of ^{125}I 70-kD fragment were added to the wells along with increasing amounts of unlabeled 70-kD fragment or unlabeled Pronase glycopeptides of the fragment (prepared as described; Foltz and Lennarz, 1990). The plate was incubated for 1 h at room temperature, the wells were washed with PBS (pH 7.4), 0.5% NP-40, and then counted.

Dot blot analyses were performed essentially as the plate immunoassays. Known amounts of crude LysC digests or egg surface preparations were dotted onto Immobilon P membranes, 50 μl at a time. The membrane was then processed as described below for Western analysis. Antibody binding was detected with ^{125}I donkey anti-rabbit (Amersham Corp.) and the dots were cut out and counted in a minigamma counter (LKB Instruments, Inc., Gaithersburg, MD).

Western analysis was performed essentially as described by Towbin et al. (1979). Gels were immersed briefly in transfer buffer before electrophoretic transfer to nitrocellulose (Schleicher and Schuell Inc., Keene, NH). Membranes were blocked in PBSA for 4–12 h and then probed with anti-70KL IgG using a 1:1,000 dilution in PBSA, for 4 h at room temperature. The blot was then washed three times, 30 min each, in PBS (pH 7.4). Addition of detergent (0.5% NP-40) to the wash buffer was used for dot blots. Antibody binding was detected using ^{125}I donkey anti-rabbit IgG. Exposure to x-ray film (AR film; Eastman Kodak Co., Rochester, NY) was at −70°C with an intensifying screen for the times indicated in the figure legend. For the competition experiment shown in Fig. 4, anti-70KL IgG was preincubated with 25 μg of purified 70-kD fragment before addition to the blot.

Immunofluorescence Microscopy of Eggs

Live eggs were dejellied, washed, and incubated with antibodies as indicated in the figure legends. In all cases, the antibodies were dialyzed against FASW before their addition, and 5 μg of antibody routinely was added to a 500-μl aliquot of a 1% suspension of eggs. Eggs were washed by settling and 1 μl of FITC goat anti-rabbit (Cappel Laboratories, Malvern, PA) was used to detect antibody binding. For other experiments, dejellied eggs were lightly fixed in 1% paraformaldehyde, 0.5% glutaraldehyde (in FASW) for 30 min, washed, and then blocked in FASW, 1% BSA before antibody addition. This fixation procedure did not significantly permeabilize the eggs as evidenced by the low level of binding of an antiserum against the major yolk platelet protein (Scott and Lennarz, 1989), which was tested as a control. Observation was with an inverted microscope (Diaphot; Nikon Inc. Instrument Div., Garden City, NY) using the fluorescein channel. Magnification was as indicated in the figure legends. Exposure times and micrograph printing conditions were the same for all the examples shown.

Fertilization Assays and Sperm Binding Assays

Fertilization assays were conducted as described (Kinsey and Lennarz, 1981; Rossignol et al., 1984; Foltz and Lennarz, 1990; Ruiz-Bravo and Lennarz, 1991). All assays were in duplicate and 200–300 eggs were monitored per assay. Briefly, sperm were exposed to egg jelly to induce the acrosome reaction and were added to eggs within 10 s. The appropriate buffer controls were included in all cases. Eggs were monitored microscopically for fertilization envelope elevation and for actual sperm binding and the presence of a fertilization cone. Values were normalized to controls. Antibodies were preincubated with eggs for 10 min. The eggs were then washed by settling and 10-fold dilution in FASW four times. The washed eggs were resuspended in FASW to a final volume of 0.5 ml and acrosome-reacted sperm were added. For sperm-binding assays, the eggs were washed twice to remove excess sperm before analysis. The calcium ionophore A23187 was dissolved in DMSO and added to eggs at a final concentration of 30 μM. Gametes were monitored microscopically throughout all procedures to ensure viability and for the ability to acrosome-react or undergo exocytosis in the case of sperm or eggs, respectively.

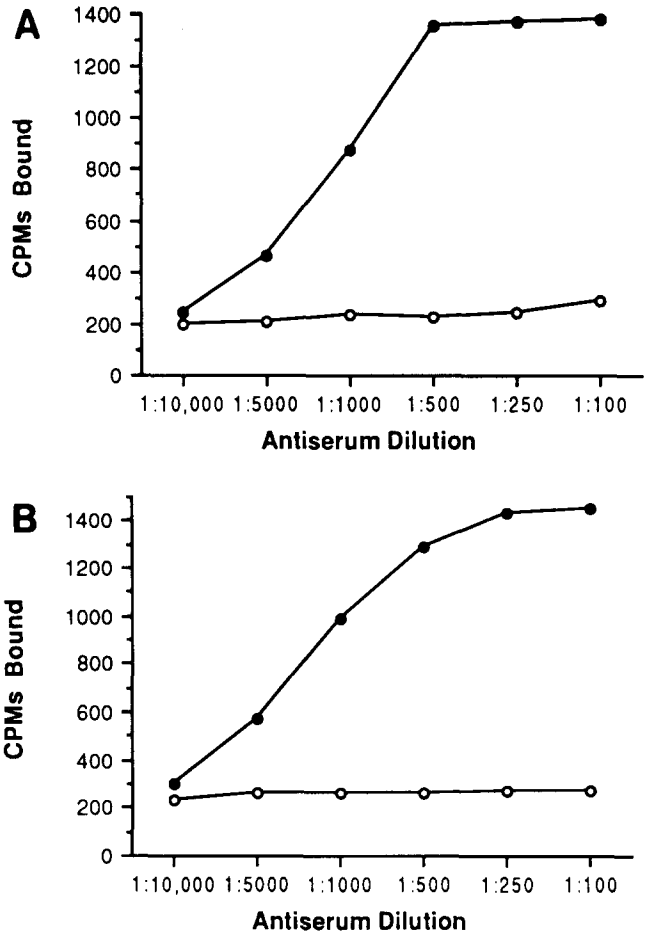
To study the effect of the anti-70KL antibody alone on eggs, we had to develop a controlled, minimally disruptive procedure. The wells of a 24-well cluster plate (Costar Data Packaging, Cambridge, MA) were coated with 2% BSA in PBS (pH 7.4) for 1 h and then rinsed with FASW. Each well contained 1.5 ml of FASW to which 0.5 ml of a 1% suspension of dejellied, washed eggs was carefully added using a wide-bore pipette tip. In some experiments, eggs were washed three times in SWC before being placed in wells that contained SWC. The appropriate amount of Fabs was added and allowed to disperse in the well (see figure legends); no mixing was done. The eggs were maintained at 15°C and all solutions were precooled to 15°C. The maximum response generally was achieved at a concentration of 30 μg/ml. A higher percentage of fertilization envelope elevation was never observed, even at 150 μg/ml. Eggs were monitored with a light microscope during the entire procedure, including the addition of antibody. Care was taken to avoid disturbing the plate during the incubation. Controls were preimmune serum and a mAb directed against an unidentified, extracellular egg surface protein (the gift of Dr. Joann Otto, Purdue University, Lafayette, IN). 46 different batches of eggs were analyzed and scored for fertilization envelope elevation. Those showing partial to full elevation were considered positive.

Results

Characterization of the Antiserum to the 70-kD Fragment of the Sperm Receptor

The 70-kD fragment of the *S. purpuratus* egg receptor for sperm that binds to homotypic sperm and inhibits sperm binding to eggs was used as an immunogen in a rabbit. IgG purified from the antiserum, designated anti-70KL, bound to crude LysC digest and egg cell surface preparations in a concentration-dependent manner in plate assays (Fig. 1, *A* and *B*, respectively). Preimmune serum did not recognize either the LysC digest (Fig. 1 *A*) or the egg surface preparations (Fig. 1 *B*). Further, the anti-70KL IgG did not recognize crude egg jelly coat (Fig. 1 *C*). As shown in Fig. 2, IgGs purified from anti-70KL serum were specific for the 70-kD fragment in competitive RIAs. Binding to the material released by LysC digestion of eggs or to egg surface preparations was inhibited specifically by the purified 70-kD fragment in a concentration-dependent fashion. In addition, the antiserum specifically immunoprecipitated the 70-kD fragment from crude LysC digests. Preincubation of the antiserum with the purified fragment neutralized the immunoreactivity in both the plate assay and immunoprecipitations (data not shown).

Previous analysis revealed that the 70-kD LysC receptor fragment was glycosylated (Foltz and Lennarz, 1990). Therefore, it was important to determine the nature of the epitope(s) recognized by anti-70KL IgG. As shown in Fig. 3, glycopeptides generated from the 70-kD fragment by extensive Pronase digestion did not compete significantly for antibody binding to the 70-kD fragment. Thus, the majority, but not necessarily all, of the epitopes recognized by the an-



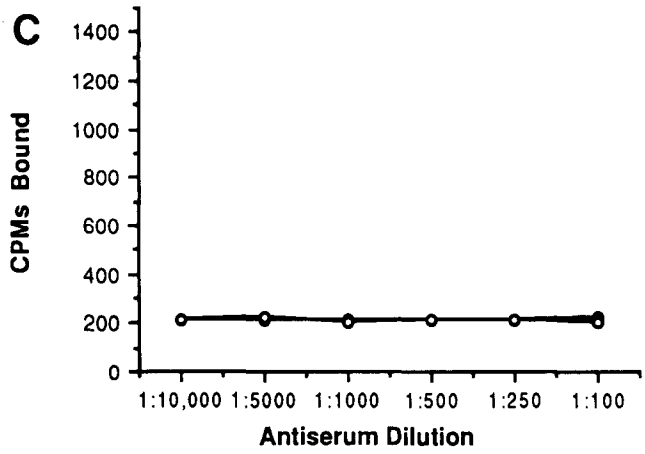
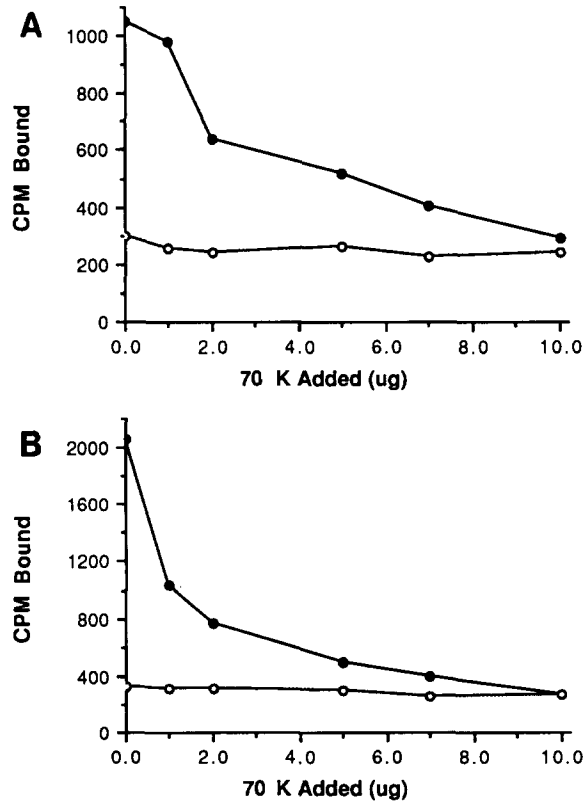


Figure 1. The anti-70KL serum IgG recognizes components of the egg surface. The wells of a microtiter dish were coated with 100 μg of *S. purpuratus* (*A*) crude LysC digest, (*B*) egg surface proteins, or (*C*) crude egg jelly and blocked. Anti-70KL (●) or preimmune IgG (○) was added to the wells in increasing concentration. Binding was detected with ^{125}I goat anti-rabbit Ig, represented as CPMs bound.



tiserum must be a part of the polypeptide backbone rather than the oligosaccharide chain(s).

The Antiserum Recognizes a High Relative Molecular Mass Polypeptide

Western analysis of egg surface preparations (containing both the plasma membrane and vitelline layer) revealed that anti-70KL IgG reacted with a single component of ~350 kD (Fig. 4 *B*, lane *3*). This approximate relative molecular mass was calculated based on the migration of the immunoreactive polypeptide relative to the migration of sperm flagellar dynein heavy chains (~475,000 M_r each) and myosin heavy chain (205 kD). The relatively wide band most likely is reflective of the fact that the protein is glycosylated. Presumably, the 70-kD fragment is derived from this high relative molecular mass polypeptide; further experiments supported this idea. For example, surface preparations prepared from eggs that had been pretreated with the LysC protease did not

Figure 2. The 70-kD receptor fragment competes with egg proteins for binding to the anti-70KL IgG. The wells of a microtiter dish were coated with 100 μg of either crude LysC digest (*A*) or egg surface proteins (*B*) and blocked. Anti-70KL IgG (●) or preimmune serum (○) was added to the wells in the presence of increasing amounts of purified 70K fragment. Wells were washed and antibody binding was detected with ^{125}I second antibody, represented as CPMs bound.

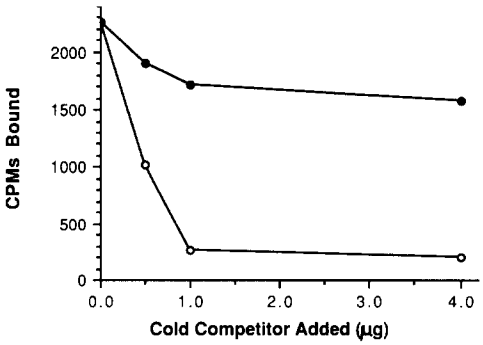


Figure 3. Glycopeptides generated by Pronase digestion of the 70-kD receptor fragment do not compete for anti-70KL binding in a competitive RIA. The wells of a microtiter dish were coated with 50 μg of anti-70KL IgG and then blocked. ^{125}I-labeled 70-kD protein (0.5 μg) was added to the wells in the presence of increasing amounts of either unlabeled, intact 70-kD fragment (○) or unlabeled, Pronase glycopeptides of the 70-kD fragment (●). Binding of the labeled fragment to the wells is represented as CPMs bound. The Pronase glycopeptides did not compete significantly for binding to the IgG.

exhibit any immunoreactivity with the antiserum (Fig. 4 *B*, lane *2*). Binding of antibody to the 350-kD polypeptide was blocked by the purified 70K LysC fragment (Fig. 4 *B*, lanes *4* and *5*). This confirms that the 70K LysC epitope on the 350-kD protein is extracellular and removed by LysC proteolysis, and provides further support for the idea that the 350-kD species is the intact receptor.

Anti-70KL Is Species Specific

The *S. purpuratus* 70-kD LysC fragment previously was shown to bind only to *S. purpuratus* sperm and had a preference for binding to purified *S. purpuratus* bindin (Foltz and Lennarz, 1990). In agreement with these previous observations, anti-70KL IgG also was found to be species specific. Immunofluorescence microscopy of *L. pictus* and *S. drobachiensis* eggs did not show any binding of the IgG to the eggs of these species (data not shown). It is interesting to note, however, that when cross-reactivity was tested in a plate assay using ^{125}I-labeled second antibody, a low level of binding above background occasionally was observed with *S. drobachiensis* (but not *L. pictus* or *A. punctulata*) egg preparations (Fig. 5). This may be significant, since this species is closely related to *S. purpuratus* and cross-fertilization can occur. However, no immunoreactivity was detected by Western analysis (data not shown).

Biochemical Properties of the Sperm Receptor Antigen

To study the nature of the association of the intact receptor with the egg surface, we treated egg cell surface preparations with various reagents and then the insoluble fraction was separated from the soluble by centrifugation. Each of the fractions was dotted onto nitrocellulose and probed with anti-70KL IgG followed by detection with ^{125}I second antibody. The results presented in Table I represent the averages of six different surface preparations. The receptor appeared to be solubilized by treatment with 1% β-octyl-glucoside, 1% Triton X-100, or 1% SDS. Treatments designed to solubilize peripheral proteins, such as high salt or urea, did not

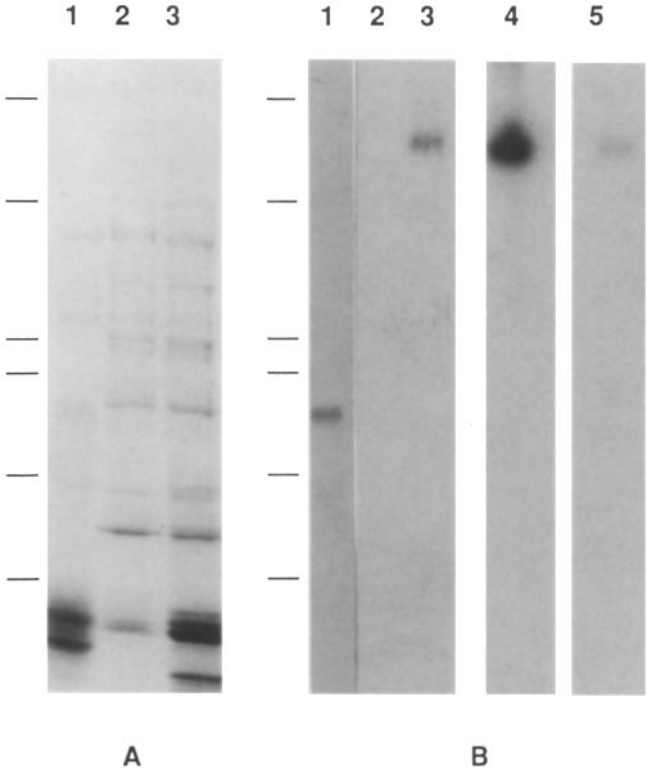


Figure 4. Immunodetection of a high relative molecular mass egg surface polypeptide. (*A*) The stained gel of 80 μg each of (lane *1*) egg crude LysC digest, (lane *2*) surface preparation from LysC-treated eggs, and (lane *3*) surface preparation of untreated eggs. (*B*) The autoradiogram of a blot probed with anti-70KL IgG and ^{125}I donkey anti-rabbit IgG. Lanes *1–3* are from the same blot; identical to *A*. Lane *1* was exposed for 36 h while lanes *2* and *3* were both exposed for 24 h. Lanes *4* and *5* are autoradiograms of two identical samples of egg surface preparations (100 μg each) blotted and probed with anti-70KL IgG (lane *4*) or anti-70KL IgG in the presence of 25 μg of purified 70-kD LysC fragment (lane *5*). Exposure for both was for 24 h. Note that the fragment competed for antibody binding. This demonstrates the specificity of the antiserum for the 70K fragment. Relative molecular mass standards are indicated by the bars and were (from top to bottom): 475 kD (dynein heavy chains), 205, 116, 94, 66, and 45 kD.

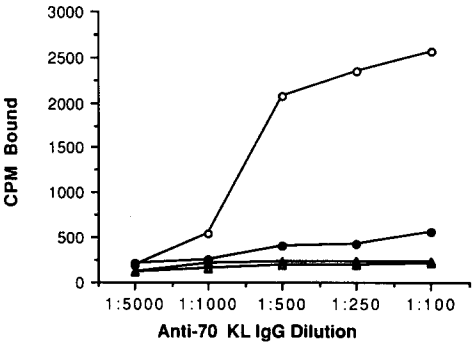


Figure 5. Antibody binding assays reveal that the anti-70KL is species specific. The wells of a microtiter dish were coated with equal amounts (100 μg) of plasma membrane/vitelline layer preparations from the eggs of *S. purpuratus* (○), *S. drobachiensis* (●), *L. pictus* (△) or *A. punctulata* (▲). After blocking the wells, increasing concentrations of anti-70KL IgG were added to the wells. Antibody binding was detected with ^{125}I goat anti-rabbit IgG, represented here as CPMs bound.

Table I. Solubilization of the Receptor

Treatment	Antibody binding to: Soluble fraction	Insoluble fraction	Percent solubilized‡
	*cpm**		
Plasma membrane/vitelline layer			
Saline	43	783	5
1.0 M NaCl	52	639	8
2.0 M Urea	103	582	15
0.5 M KI	58	746	7
Na Carbonate, pH 11	98	572	15
1.0% CHAPs	61	536	10
1.0% Lubrol	45	603	7
1.0% NP-40	69	647	10
0.1% β-Octylglucoside	102	508	17
1.0% β-Octylglucoside	486	138	78
0.1% Triton X-100	401	229	64
1.0% Triton X-100	509	98	85
1.0% SDS	849	23	97
Vitelline layer			
Saline	17	438	4
1.0 M NaCl	69	517	12
1.0% β-Octylglucoside	628	62	91
1.0% SDS	512	48	91

* Antibody binding was detected using ^{125}I donkey anti-rabbit IgG. Shown are representative values from the same preparation of averaged duplicate samples.
‡ Values normalized for loss of total antibody reaction did not differ from the values shown by >10% in any given experiment.

solubilize the receptor. Western analysis indicated that the 350-kD immunoreactive polypeptide remained in the insoluble fractions after treatment with 2 M NaCl, KI, or low levels of detergent (data not shown).

Because EM studies suggest that KI treatment removes the vitelline layer (Kinsey et al., 1980), our results with this reagent suggest that the receptor is associated with the plasma membrane. However, a major difficulty in defining the subcellular localization is that one cannot be certain that the separation of the plasma membrane and vitelline layer quantitatively is complete, as there are no established markers. When the vitelline layer was prepared by published methods (Niman et al., 1984) it was found to contain an immunoreactive 350-kD protein (Table I). However, examination of this vitelline layer preparation by electron microscopy revealed that some plasma membrane was present. Likewise, preparations of "pure" plasma membranes contained immunoreactivity, but electron microscopic analysis showed the presence of some material with the appearance of the vitelline layer (not shown). Thus, we can conclude only that the receptor is tightly associated with the egg surface, be it via anchoring in the vitelline layer, the plasma membrane, or both.

Immunofluorescence Microscopy Reveals that the Receptor Epitope(s) Is Present on the Egg Cell Surface

As shown in Fig. 6, immunofluorescence microscopy of fixed *S. purpuratus* eggs revealed that the epitope(s) reacting with the anti-70KL IgG was evenly distributed over the egg surface (Fig. 6 *d*). Eggs treated with LysC were not stained by the affinity-purified anti-70KL IgG (Fig. 6 *f*). This observation provides a strong correlation between binding of the antibody to the cell surface and the presence of the intact receptor. Preimmune serum did not exhibit any binding to the eggs (Fig. 6 *b*). If anti-70KL IgG was applied to live eggs, the immunofluorescence pattern appeared patchy, presumably because of aggregation of the receptors by crosslinking (Fig. 7, *d* and *e*). At sufficiently high antibody concentrations, (10–50 μg/ml) the eggs exhibited localized lysis and antibody staining was greatest in this local area (Fig. 7, c–f). Again, this was a species-specific effect. Neither *L. pictus* nor *A. punctulata* eggs were recognized by the anti-70KL IgG (data not shown).

Anti-70KL Fabs Inhibit Sperm Binding

As might be expected for an antibody directed against the sperm receptor, addition of anti-70KL Fabs to eggs resulted in a concentration-dependent inhibition of sperm binding (Fig. 8). Preincubation of the Fabs with the 70-kD LysC fragment neutralized their ability to inhibit sperm binding and consequent fertilization envelope elevation (Fig. 8 *B*). Preimmune serum had no effect (Fig. 8 *B*). Sperm binding without fertilization envelope elevation was never observed. Low concentrations of anti-70KL IgG inhibited sperm binding, but higher concentrations of IgG (>10 μg/ml) caused the eggs to undergo localized lysis (Fig. 7, *c–f*). This effect was never observed with Fab fragments prepared from the anti-70KL IgG. The Fabs inhibited sperm binding even at concentrations as low as 1 μg/ml. Eggs treated with Fabs elevated normal fertilization envelopes when the Ca^{2+} ionophore A23187 was added (data not shown). This finding indicates that these treated eggs did not lose the ability to be activated and that the antibody does not interfere with fertilization envelope elevation.

Anti-70KL IgG Causes Cortical Granule Exocytosis of Some Eggs

As mentioned above, treatment of living eggs with anti-70KL IgG resulted in aggregation that often led to lysis (Fig. 7 *c–f*). This effect was specific for *S. purpuratus* eggs; i.e., it did not occur in other sea urchin species and occurred only with immune IgG (Fig. 7). Preimmune serum had no effect. Treatment of live eggs with Fab fragments prepared from the anti-70KL IgG did not result in the patching phenomena, unless the Fabs were cross-linked via a bivalent second antibody (data not shown).

Of particular interest was the finding that treatment with anti-70KL Fabs caused a small percentage (2–20% in 46 different batches) of eggs from individual females to undergo fertilization envelope elevation (Fig. 9). The exact percentage exhibiting this behavior varied depending on the individual females from which the eggs were isolated. In Fig. 9 *B* the response of four different batches of eggs to increasing amounts of the Fabs is shown. At 30 μg/ml, 11–18% of these eggs showed fertilization envelope elevation. At higher concentrations, no increase in the percentage was observed. Thus, this novel effect of the Fabs was concentration dependent and saturable. This response was easily distinguished from that of the cross-linking effect of IgG. Localized lysis of the membrane resulted in vesicular debris and a "ruffling" of the membrane (Fig. 7, *c–f*). However, eggs treated with Fabs elevated a fertilization envelope, (Fig. 9 *A*). A variety

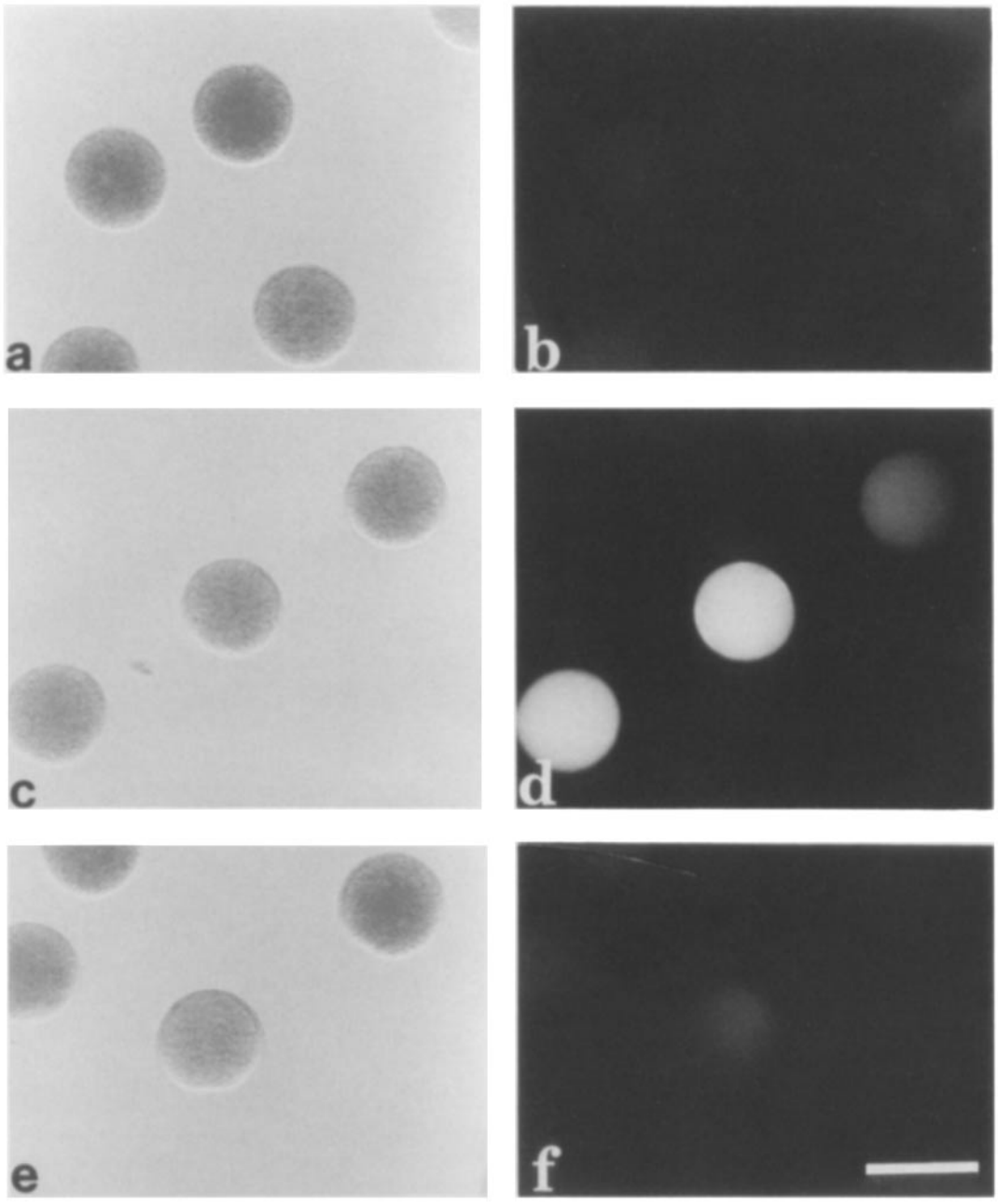


Figure 6. Immunofluorescence microscopy of fixed eggs. *S. purpuratus* eggs were washed, dejellied, and washed extensively before treatment. Eggs were then fixed, washed, and incubated with antibodies in sea water as indicated. Antibody binding was detected with FITC-conjugated goat anti-rabbit Ig. The bright field and fluorescent images, respectively, are shown in pairs. Shown are: (*a* and *b*) eggs incubated with preimmune IgG; (*c* and *d*) eggs incubated with anti-70KL IgG; (*e* and *f*) LysC-treated eggs incubated with anti-70KL IgG. Bar, 80 μm.

of control experiments were performed to verify these results. The experiments were conducted in 24-well tissue culture dishes that had been coated with BSA before addition of the eggs to the wells. This blocking step was absolutely necessary to prevent the Fabs from adhering to the sides of the wells. When wells were not coated, a number of eggs showed localized lysis as evidenced by the release of cytoplasmic granules. This presumably was because of cross-linking of the eggs by the antibody to the well, resulting in a mechanical stress on the egg surface. Blocking the wells allowed the eggs to remain in suspension and no lysis occurred. The purified Fabs were dialyzed against sea water before their addition to the eggs. A mAb that bound to the egg surface (the gift of Joann Otto) was used as a negative control, along with preimmune serum. Under the controlled assay conditions, eggs from 46 different females did not show any lysis or partial to full fertilization envelope elevation in response to these treatments nor was sperm binding blocked (data not shown). No other microscopically detectable changes to indicate activation were observed within those eggs that underwent fertilization envelope elevation (e.g., pronuclear centering or chromosome condensation). However, the same

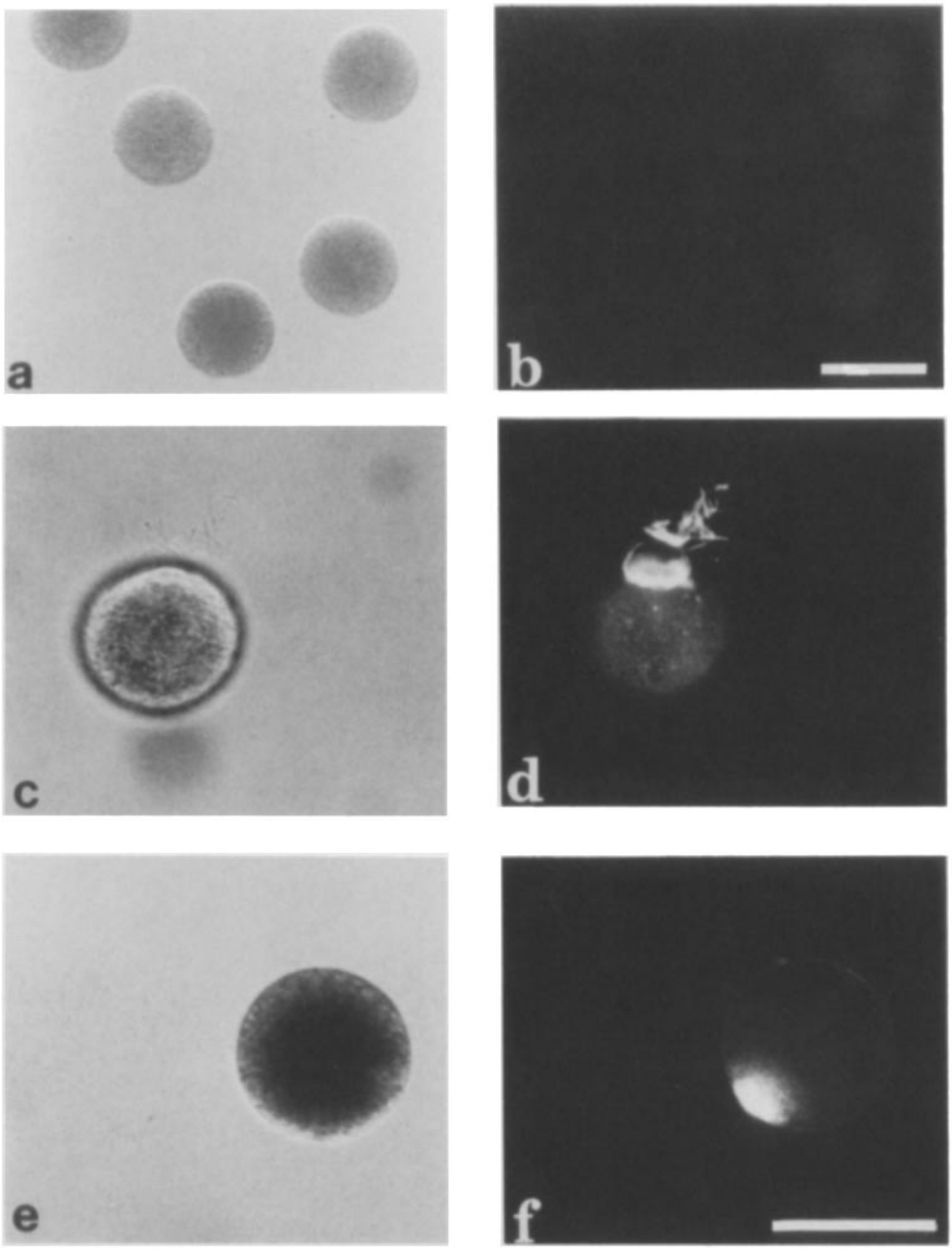


Figure 7. Immunofluorescence microscopy of live eggs. *S. purpuratus* eggs were washed, dejellied, and washed again before treatment with antibodies in sea water. Antibody binding was detected with FITC goat anti-rabbit Ig. The bright field and fluorescent images, respectively, are shown in pairs: (*a* and *b*) eggs treated with preimmune IgG; (*c* and *d*) egg treated with anti-70KL IgG; (*e* and *f*) egg treated with anti-70KL IgG. Note the localized lysis and aggregation of the receptor in *S. purpuratus* eggs treated with anti-70KL IgG. Bars, 80 μm.

result was observed when the experiment was performed in Ca^{2+}-free sea water. This suggests that the exocytic event was mediated by release of internal stores of Ca^{2+}. Thus far, efforts to increase the number of eggs that respond to antibody treatment have failed. Addition of calcium ionophore after Fab treatment resulted in the elevation of a normal fertilization envelope in all of the remaining eggs, ruling out the possibility that those eggs that could not be activated with antibody were unable to undergo normal elevation.

Discussion

The results reported here represent a significant advance in identification and characterization of the sea urchin egg receptor for sperm. Previously, we isolated and purified to homogeneity an extracellular fragment of the receptor and showed that this 70-kD glycoprotein inhibited fertilization species specifically by binding to acrosome-reacted sperm (Foltz and Lennarz, 1990). In the current study, the 70-kD fragment was used as immunogen to prepare an antibody against an extracellular region of the receptor molecule. The resulting antiserum, anti-70KL, was specific for the 70-kD fragment generated by LysC digestion of the egg surface of *S. purpuratus* eggs. Although the fragment is glycosylated (Foltz and Lennarz, 1990), the antiserum is directed primarily against polypeptide epitopes, as evidenced by the fact that glycopeptides are unable to compete for IgG binding in RIA analyses. This is significant in light of an earlier attempt to generate a polyclonal antibody against a preparation of partially purified, intact receptor. The antibody raised was

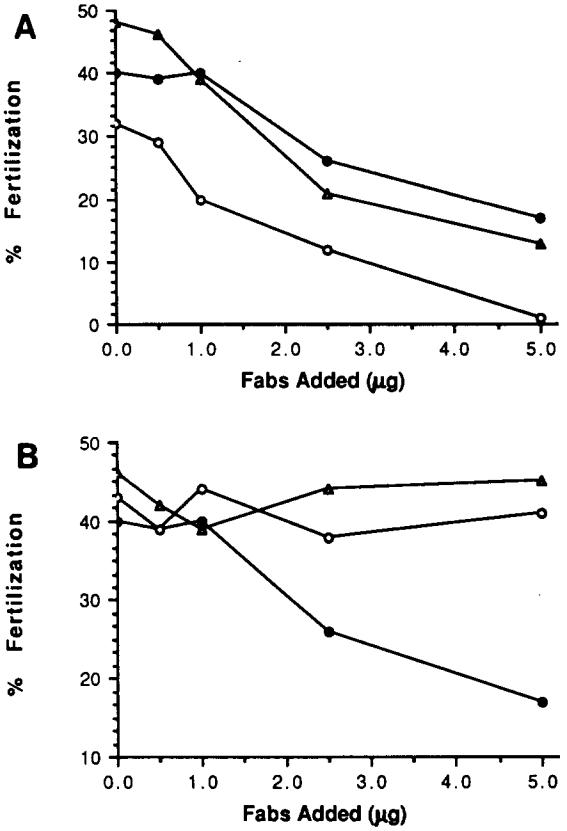


Figure 8. Anti-70KL Fabs inhibit sperm binding. Eggs were dejellied, washed, and incubated with increasing concentrations of anti-70KL Fabs for 15 min. The eggs were washed five times and acrosome-reacted sperm were added. The eggs were scored for sperm binding, and fertilization envelope elevation represented here as the percentage of fertilization. The three lines represent eggs from three different females (*A*). (*B*) The percentage of sperm binding to eggs from a single female that were treated with preimmune IgG (○), Fabs (●), and Fabs preabsorbed against the purified 70K LysC fragment (△).

directed primarily against carbohydrate epitopes and recognized several macro molecules (Ruiz-Bravo et al., 1986*a*, 1989).

Anti-70KL recognizes a single polypeptide of high relative molecular mass (~350 kD) in *S. purpuratus* egg surface preparations that we believe to be the intact form of the *S. purpuratus* egg receptor for sperm. If intact eggs were treated with the LysC protease, no immunoreactive component(s) was retained in membrane/vitelline layers prepared from these eggs. The 70K LysC fragment competed specifically with the 350-kD polypeptide for binding to anti-70KL IgG. Moreover, the antiserum was species specific; there was no immunoreactivity with polypeptides from *L. pictus* or *A. punctulata* eggs as judged by dot blot, RIA, and Western analyses of egg surface preparations or by immunofluorescence microscopy. The lack of species cross-reactivity is in good agreement with the finding that the receptor itself is species specific in its ability to inhibit fertilization and to bind to sperm (Rossignol et al., 1981; Ruiz-Bravo et al., 1987*a*; Foltz and Lennarz, 1990).

Over the past years, evidence has accumulated suggesting that the receptor is associated with the vitelline layer (Tegner and Epel, 1973, 1976; Glabe and Vacquier, 1977*b*; Kinsey et al., 1980; Ruiz-Bravo et al., 1986*b*). However, a major unresolved question has been whether this receptor is also linked to or anchored in the plasma membrane. This question has implications in terms of the receptor's function in egg activation. Models can be envisioned in which the receptor could transduce a binding signal whether or not it was directly linked to the plasma membrane. What is clear from the current work is that preparations of the vitelline layer contain immunoreactive receptor molecules, but it is not certain that these preparations are free of plasma membrane. In fact, they contain measurably less immunoreactivity than preparations of the "cell surface complex" that contain both the plasma membrane and the vitelline layer (Kinsey et al., 1980; Kinsey and Lennarz, 1981; Kinsey, 1986). Indeed, if the surface complex was isolated first and then the vitelline layer was stripped away by high salt or KI, the remaining membrane fraction retained the bulk of the immunoreactivity. Although Kinsey et al. (1980) found that these stripped plasma membranes no longer bound sperm, suggesting that the receptor had been removed, such treatment could have denatured the receptor and thereby rendered it unable to bind sperm. Given that there are no obvious markers to distinguish the membrane and the outer vitelline layer, we can only conclude that the detergent-soluble, 350-kD receptor is "tightly associated" with the egg surface. Further study, including immuno-electron microscopy, is needed to clarify this issue. It is important to note that Kinsey and his colleagues (Jiang et al., 1989, 1990, 1991) have reported the transient phosphorylation of a high relative molecular mass protein (~350 kD) at fertilization. We are investigating the possibility that the ~350-kD polypeptide detected by anti-70KL IgG is phosphorylated.

The anti-70KL IgG also proved useful in investigating the binding of the receptor fragment to sperm. It was determined previously that the purified fragment bound to acrosome-reacted sperm and to purified bindin (Foltz and Lennarz, 1990). In the current study we have shown that both the anti-70KL and Fab fragments derived from anti-70KL block the binding of sperm to eggs, inhibiting fertilization. This inhibitory effect, which is species specific, provides additional support for the premise that the 70-kD glycoprotein is a fragment of the 350-kD receptor. Interestingly, binding of the Fab fragments to the surface of the egg do not have any inhibitory effect on the ability of the egg to elevate the fertilization envelope in response to treatment with calcium ionophore, suggesting that this process is structurally independent of the receptor molecule. As expected, immunofluorescence microscopy demonstrated that the receptor is evenly distributed on the egg surface. This is in agreement with biological observations that the sperm can bind at any point on the egg surface. The production of a battery of mAbs should allow for precise determinations of epitope localization and perhaps quantitation of the receptor molecule.

As discussed earlier, Fabs prepared from affinity-purified anti-70KL IgG inhibited sperm binding to eggs. In the absence of sperm, the anti-70KL Fabs caused a small percentage of eggs (2–20%) to undergo cortical granule exocytosis and consequent fertilization envelope elevation. No other obvious effects on this subset of eggs, such as pronuclear centering or chromosome condensation, were observed. Most of the eggs, however, did not respond by elevating a fertiliza-

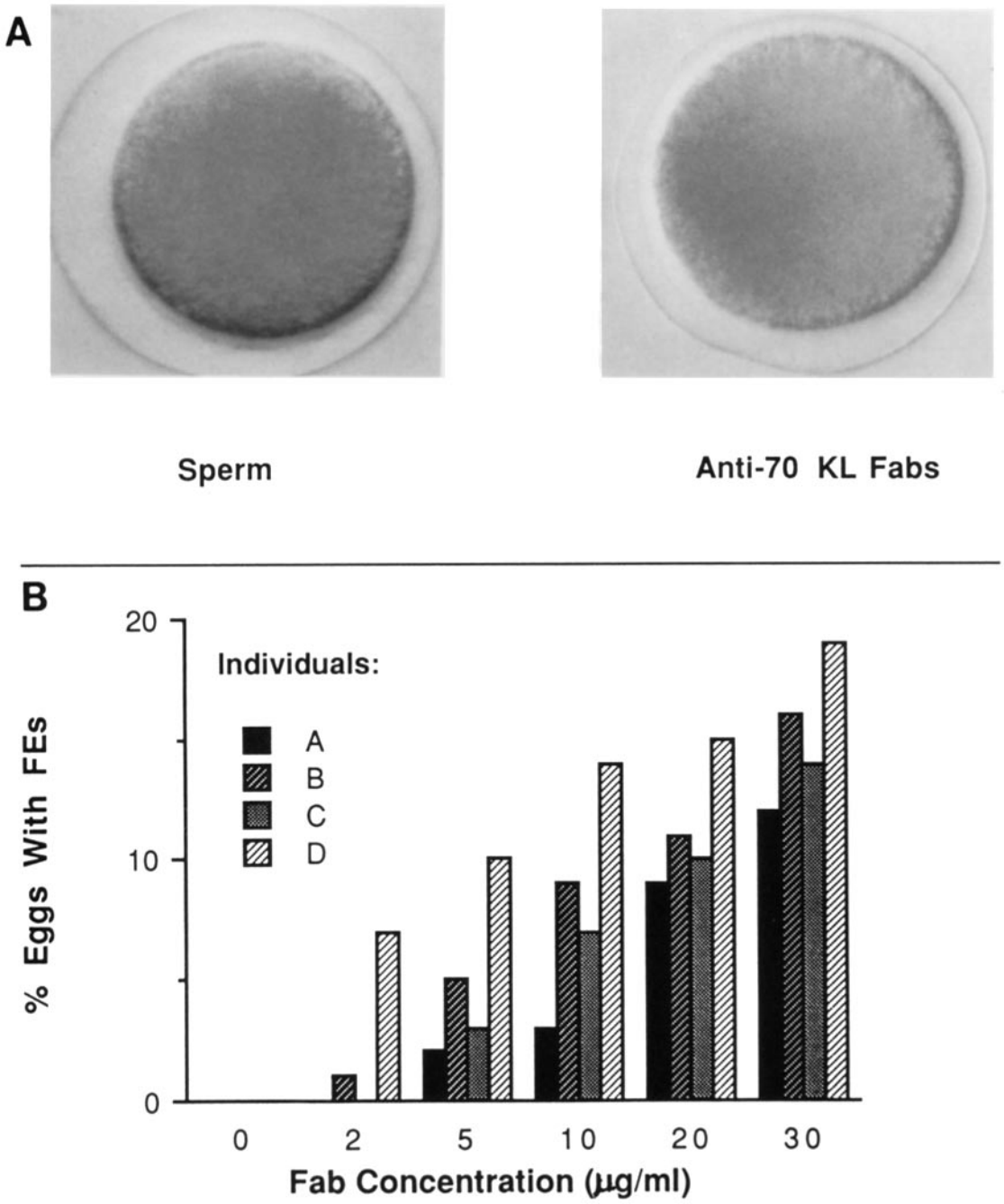


Figure 9. Anti-70KL Fabs induce fertilization envelope elevation in some eggs. Eggs were dejellied, washed, and then incubated with anti-70KL Fabs. (*A*) Two eggs that have elevated fertilization envelopes. The egg on the left was fertilized by sperm. The egg on the right was treated with 10 μg/ml of Fabs. (*B*) The variability of the response to Fab treatment depending on the batch of eggs. Eggs from four different females (*A–D*) were incubated with increasing concentrations of Fabs and then scored for fertilization envelope elevation, represented here as percent of fertilization envelope elevation, normalized to a control in the absence of Fabs. For each experiment, 136–220 eggs were scored. As noted in the text, higher concentrations of Fabs did not increase the percentage of eggs that elevated a fertilization envelope.

tion envelope; the percentage that responded was dependent on the female from which the eggs were isolated. It is possible that only a subset of the IgGs present in anti-70KL are able to induce fertilization envelope elevation. It also is possible that the sperm ligand, or in this case an antibody mimicking the sperm ligand, must be presented to the egg surface in a particular manner to achieve activation. Interestingly, coupling of the anti-70KL Fab fragments to a bead with a diameter equal to that of the sperm acrosomal tip caused dramatic lysis of all of the eggs (data not shown). Again, mAbs should help to resolve the issue of these effects of the antibody.

Others have noted that mAbs generated against isolated vitelline layers inhibit sperm binding (Gache et al., 1983; Niman et al., 1984). Rabbit polyclonal antisera against total eggs or isolated egg cortices also have been shown to affect

eggs in a variety of ways. These include wrinkling of the egg surface (Baxandall et al., 1964*a*,*b*; Perlmann, 1954, 1956; Metz, 1978; Ackerman and Metz, 1972) and parthenogenetic activation (Baxandall et al., 1964*a*,*b*; Perlmann, 1954; Perlmann and Perlmann, 1957). There are two other reports of antisera directed against putative sperm receptors. Yoshida and Aketa (1983) raised an antiserum against a 225-kD protein from *Anthocidaris crassipina* eggs. The Fab fragments from this serum bound to eggs and species specifically inhibited sperm binding. More importantly, Acevedo-Duncan and Carroll (1986) used Fab fragments against a ~305-kD protein from *S. purpuratus* eggs to demonstrate that this high relative molecular mass polypeptide was a good candidate for the sperm receptor. It is possible that the 350-kD protein we describe here is the same polypeptide, although in their study neither the Fabs nor the whole antiserum showed any "activation effects" on eggs (Acevedo-Duncan and Carroll, 1986).

In contrast to these earlier studies, the results presented here are unique in that the anti-70KL IgG is directed against a purified polypeptide known to be derived from the extracellular domain of an egg cell surface glycoprotein that has been shown directly to have sperm-binding activity. Further studies, particularly those with mAbs, will be necessary to define the mechanisms of the apparent "activation response" by occupancy of the receptor by antibody. Hopefully, these studies, coupled with current work using the anti-receptor antibody to clone the receptor cDNA, should provide new insights into the structural aspects of the receptor and how it functions.

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