Surface Localization of the Sea Urchin Egg Receptor for Sperm

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The sea urchin egg receptor for sperm is thought to be involved in species-specific sperm-egg interactions at the egg surface. Recent revisions in the deduced amino acid sequence of the cloned cDNAs indicate that the protein encoded does not possess the common structural hallmarks of a membrane protein. Thus, investigation of the localization and association of the protein with the egg surface is crucial. We describe and characterize a new monoclonal antibody raised against recombinant sperm receptor protein. This antibody, in conjunction with several polyclonal antibodies, was used to study the receptor protein in eggs. Immunoprecipitation studies indicated that the antibodies recognize the high M₉ (ca. 350 K) sperm receptor protein which copurified with egg plasma membrane-vitelline layer complexes. The sperm receptor protein was solubilized only by detergents and not by treatments designed to solubilize peripherally associated or lipid-anchored membrane proteins, suggesting a tight association with the membrane fraction. Confocal immunofluorescence microscopy of live eggs indicated surface staining. Finally, lysylendoproteinase C treatment of live eggs resulted in a loss of the high M₉, receptor protein epitopes, and the concomitant release of a 70-kDa proteolytic fragment, which correlated with a reduced ability of the eggs to be fertilized. Taken together, these data indicate that at least some fraction of the sperm receptor protein is present on the egg surface, a requisite locale for a sperm binding protein.

INTRODUCTION

Fertilization in sea urchins and many other organisms involves a series of interactions between the sperm and egg and is generally thought to involve gamete surface glycoproteins. Identification of some of these gamete interaction proteins, particularly in the mouse and the sea urchin, has provided insight into the molecular mechanisms of fertilization and egg activation (for recent reviews, see Litscher and Wassarman, 1993; Ohlendieck and Lennarz, 1995; Snell and White, 1996).

We have been studying the molecular mechanism of fertilization in the common purple sea urchin, Strongylocentrotus purpuratus. Treatment of intact eggs with lysylendoproteinase C (LysC) reduced the ability of eggs to bind sperm and released a 70-kDa fragment which bound to sperm species-specifically and acted as a competitive inhibitor for egg binding (Foltz and Lennarz, 1990). A polyclonal antibody generated against the purified fragment (anti-70KL) blocked sperm binding to eggs species-specifically and was used to identify the parent protein, of calculated M₉, 350 K (Foltz and Lennarz, 1992; Ohlendieck et al., 1993).

Several biochemical criteria support the hypothesis that the 350-kDa protein is associated with the egg surface, a requisite location for a sperm binding protein. In addition to accessibility of the protein to LysC digestion on intact eggs, the protein copurified with plasma membrane fractions and was solubilized only under detergent conditions (Foltz and Lennarz, 1992; Ohlendieck et al., 1993, 1994a). Further investigations revealed that the native sperm receptor is a heavily glycosylated, disulfide-bonded multimer (Ohlendieck et al., 1994a; Dhume and Lennarz, 1995). These data are consistent with a cell surface localization. Subsequent studies provided evidence that this 350-kDa protein functions as a “sperm receptor” (reviewed in Ohlendieck and Lennarz, 1995).

Expression cloning using the anti-70KL IgGs to screen an urchin ovary/oocyte library identified a partial cDNA (designated 45A). The identity of this clone was independently confirmed by amino acid sequence analysis of two peptides derived from the 70-kDa LysC fragment (Foltz et al., 1993). Recombinant protein encoded by the 45A cDNA inhibited sperm-egg binding and bound directly to sperm species-specifically (Foltz et al., 1993). Using recombinant...
protein, Stears and Lennarz (personal communication) have shown recently that the species-specific sperm binding activity maps to a 30-residue region. Cameron et al. (1996) have demonstrated a specific interaction between recombinant bindin protein and the recombinant 45A protein. Further, a polyclonal antibody raised against the 45A recombinant protein reacted with and immunoprecipitated the 350-kDa native egg protein (Ohlendieck et al., 1994a,b).

The 45A cDNA was used to rescreen the expression library and a series of overlapping cDNA clones was isolated (Foltz et al., 1993). Initial sequence analysis indicated an N-terminal signal sequence and the presence of hydrophobic stretches near the C terminus, suggesting that the protein was transmembranous. However, two recent developments have prompted us to study the topology and localization of the receptor protein itself in more detail. First, the originally reported sequence (Foltz et al., 1993) of the sperm receptor cDNAs has been revised in light of cloning and sequencing errors, as outlined by Just and Lennarz (1997). We have also resequenced the cloned cDNAs and have confirmed the revised 889 residue open reading frame encoded by these cDNAs (Just and Lennarz, 1997; GenBank Accession No. L04969). Although the original 45A cDNA (see above) corresponding to the sperm-binding domain is not affected by these revisions, there is no obvious cleavable, N-terminal signal sequence and there is a frame shift and an in-frame stop codon immediately 5' of the original putative transmembrane domain region. Thus, this revised sequence analysis indicates that the overlapping, cloned cDNAs do not predict that the receptor is a typical cell surface protein. A second reason for studying the receptor localization is the identification of a new family of heat shock-related proteins, called the HSP110 family. The deduced sequence of the sperm receptor places it within this family (Lee-Yoon et al., 1995; and see also Yasuda et al., 1995). Although relatively little is known about the function of these proteins, it is assumed that the HSP110 family members generally are not surface proteins. Although heat shock proteins have been reported to be expressed on the surface of some cells (Hightower and Guidon, 1989; Heufelder et al., 1992), the mechanism of surface expression and function is unknown. Thus, this sequence homology between a presumed surface recognition protein and a heat shock protein family is intriguing and bears further investigation.

The experiments presented in this paper were designed to address two basic questions. First, do antibodies raised against the native sperm receptor protein and antibodies raised against recombinant protein derived from the overlapping series of cloned cDNAs have common reactivity? Second, do these antibodies recognize epitopes on the egg surface (a requisite locale for a “sperm receptor”)? The data indicate that the native and recombinant proteins do share epitopes and that these epitopes are located on the egg surface. Further, the epitopes recognized by these antibodies are sensitive to protease treatment of intact eggs and their loss correlates with a reduction in sperm binding, loss of the high M, surface form of the sperm receptor, and release of a soluble 70-kDa LysC fragment. Finally, these antibodies, particularly a well-defined monoclonal IgG, will be especially useful in addressing experimentally the possibility that alternative forms of the protein exist and in assessing the actual manner in which the receptor is placed on the egg surface.

**MATERIALS AND METHODS**

**Maintenance of Animals and Gamete Collection**

Adult S. purpuratus were collected from the Santa Barbara Channel and maintained in open system aquaria at 10°C. Macrocystis pyrifera served as the main food source. Gametes were collected by intracoelomic injection of ~1 mL of 0.55 M KCl. Eggs were collected by inverting the female over a beaker of filtered seawater (FSW; natural seawater was passed through glass fiber filters and then through 0.22-μm filters and stored at 10°C). Sperm were precipitated “dry” into Eppendorf tubes and stored on ice for up to 6 hr. For all experiments, gametes were used within several hours of collection. Typically, eggs were washed by gravity settling in large volumes of FSW, dejellied by 10 passages through 120-μm mesh Nitex, and then washed three times more by gravity settling in FSW. A constant temperature of 10°C was maintained. For every experiment, a sample of eggs was assessed for general health and maturity. The sample of eggs was fertilized and development was monitored over several days. Only data from experiments using gametes which were healthy, mature, and which fertilized (and developed) normally were evaluated. For all experiments, only bio-cleaned (Lutz and Inoué, 1986) glassware was used.

**Antibodies**

The sperm receptor antibodies used are listed in Table 1. The stock concentration of the purified pAb96-586 IgG was 3 mg/mL. This polyclonal antibody was formerly referred to as anti-EXO (Abassi and Foltz, 1994). To avoid confusion regarding the topology issue, we have renamed this antibody based upon the recombinant protein (residues 96–586; revised sequence) used as the immunogen. The anti-70KL (Foltz and Lennarz, 1990) and anti-350KL (Ohlendieck et al., 1994a) polyclonal (purified IgGs) were the gift of Dr. William Lennarz, SUNY–Stony Brook. The anti-sea urchin profilin polyclonal antiserum (Smith et al., 1992) was the gift of Dr. C. Smith, Georgetown University. IgGs were purified from antisera using standard procedures on protein A–agarose (Harlow and Lane, 1988).

The monoclonal antibody 7.3 was generated at the Purdue University monoclonal facility (Dr. D. Asai, Director). BALB/c mice were immunized with purified recombinant N-terminal glutathione-S-transferase (GST) fusion protein representing amino acid residues 413–889 of the sperm receptor deduced sequence (see Fig. 1). Premimmune sera from these mice did not react with sea urchin egg proteins or recombinant sperm receptor proteins (data not shown). Hybridomas were produced, selected, cloned, and expanded by standard methods (Asai and Wilder, 1993). ELISA and Western analyses using GST and the GST fusion protein were used to screen the hybridomas. Of 141 positive clones, 7 were characterized further. Here we describe one of these (mAb7.3). This antibody was determined to be an IgG1 using an isotype determination kit (Boehringer-Mannheim, Indianapolis, IN.). Ascites fluid was produced in
BALB/c mice were primed with pristane. Ascites serum was routinely used at a dilution of 1:1000 except where indicated.

Confocal Immunofluorescence Microscopy

Staining of live eggs was carried out at 10°C in disposable 75 × 12-mm borosilicate glass tubes which had been rinsed extensively in artificial seawater (ASW; 484 mM NaCl, 10 mM KCl, 11 mM CaCl₂, 29 mM MgSO₄, 27 mM MgCl₂, 2.4 mM NaHCO₃, pH 8.0) after precoating with 1% BSA (Sigma) in ASW for 1 hr. One milliliter of a 1% (v/v) suspension of washed, dejellied eggs was transferred to the tube and the eggs were allowed to settle. The eggs were gently resuspended and washed in 1 mL of ASW three times and then the volume was reduced to approximately 0.1 mL. Primary antibody was added and the eggs were resuspended gently every 5 min. The monoclonal antibodies mAb 7.3 and mAb4.1 were used at a dilution of 1:250 while the polyclonal antibodies (anti-70KL, Poly(A) etc.) were used at a dilution of 1:250. The egg envelopes in response to the calcium ionophore A23187. The other antibodies were used at a dilution of 1:2000.

Northern Blot Analysis of Uchinn RNA

Total RNA was isolated from ovary/oocyte or eggs of individual females by extraction with guanidinium (TriZol; Gibco BRL). Poly(A)+ RNA was isolated from total RNA using Oligotex beads (Qiagen). RNA samples were electrophoresed on a 0.9% agarose formaldehyde gel and transferred to positively charged nylon membrane (Amersham). Riboprobes were synthesized using the Maxiscript In-Vitro Transcription kit (Ambion, Inc.), labeled with [α-32P]UTP (DuPont NEN). Blots were probed and washed under high stringency (68°C; final washes in 0.2× SSPE, 0.1% SDS; Sambrook et al., 1989) and exposed to film for 72 hr with intensifying screens. For the experiments shown in Fig. 1B, two identical blots were probed first with probes representing nucleotides 1–445 and 477–1944. After exposure, the blots were stripped (Sambrook et al., 1989) and exposed to film to ensure that the probe was removed, and then rehybridized with probes representing nucleotides 2072–2375 and 2382–3459.

PMVL Preparation and Solubility Studies

Egg plasma membrane/vitelline layers were prepared essentially as described (Kinsey, 1986). Washed, dejellied eggs from a single female were suspended in 50 vol ice-cold PMVL buffer (PMVLB; 500 mM NaCl, 10 mM KCl, 25 mM NaHCO₃, 25 mM EGTA, 6.25 mM NaOH, pH 8.0) spiked with 0.1 mg/mL soybean trypsin inhibitor (SBI) and protease inhibitor cocktail (PIC; 1 µM final concentration of each of leupeptin, aprotinin, and benzamidine). Eggs were lysed mechanically by hand using a Teflon pestle fitted in a Dounce homogenizer. After ~95% of eggs were lysed, the homogenate was diluted 100-fold and centrifuged at 750g at 4°C for 5 min. The supernatant (S1) containing small pieces of membrane, organelles, and cytosol was carefully removed. The pellet of plasma membrane/vitelline layers with intact cortical granules was washed extensively in PMVLB. Cortical granules were detached by resuspending the preparation in 1 M sucrose (with protease inhibitors present) and incubating on ice for 30–60 min. The PMVLs were then harvested by centrifuging at 2500g at 4°C for 20 min. The pellet was washed once in PMVLB, resuspended to approximately 1–2 mg/mL in PMVLB, aliquoted, and stored at –70°C. Failure to remove the cortical granules resulted in severe proteolysis of the sperm receptor over time and upon freeze-thawing. This was also found to be true for total homogenates of eggs. Western analysis of frozen egg homogenates revealed proteolysis of the sperm receptor and often resulted in the appearance of an immunoreactive band at ca. M, 120 K that was not detectable in freshly prepared samples (16 preparations).

For the solubilization studies, purified PMVLs (50 µg) were harvested by centrifuging 5 min at 4°C at top speed (16,000g) in a
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microfuge. The pellets were resuspended in 50 μL of the indicated buffer (containing PIC), incubated on ice for 1 hr, and then centrifuged at 16,000g at 4°C for 30 min. The supernatants (soluble fraction) and pellets (insoluble fraction) were separated and immediately electrophoresed on SDS–polyacrylamide gels (see below). The buffers used in these experiments were as follows: 0.5 M Na carbonate, pH 11, 150 mM NaCl, 1 mM EDTA; 0.5 M KI; 50 mM Tris, pH 8.0, 1 M NaCl, 5 mM EDTA; 50 mM Tris, pH 8.0, 150 mM NaCl, 5 mM EDTA, 10 mM dithiothreitol; 50 mM Tris, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% β-oglycerocephosphate; 50 mM Tris, pH 8.0, 150 mM NaCl, 5 mM EDTA, 1% SDS. For one set of experiments, duplicate samples of PMVs were resuspended in 70 mM triethanolamine (TEA), 1 mM EDTA, 0.1% TX-100. Phosphoinositide-specific phospholipase C (0.6 Units, PI-PLC; Boehringer-Mannheim) was added to one sample and both were incubated at 18°C for 1 hr followed by centrifugation as described above.

Immunoprecipitations

PMVs (25–50 μg, depending on the experiment) were resuspended in IPB (50 mM Tris, pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.5% TX-100, 0.5% NP-40, 2% β-oglycerocephosphate) supplemented with 1% SDS in a volume that maintained the protein concentration at 1.0 mg/mL. After incubation for 30 min on ice, the sample was centrifuged at top speed in a microfuge at 4°C for 30 min. The soluble fraction was transferred to a new Eppendorf tube and diluted 10-fold with IPB to reduce the SDS concentration to 0.1%. Primary antibody was added (1–3 μg/mL final concentration) and the samples were incubated on a rocker at 4°C for 90 min to 2 hr. Protein A-Sepharose (20 μL of a 90% slurry) was then added and the samples were incubated on a rocker at 4°C for 2 hr. Immunoprecipitates were harvested by centrifugation in a microfuge at 4°C, washed four times with the immunoprecipitation buffer, and prepared for gel electrophoresis immediately.

For immunoprecipitations from the in vitro transcription and translation reactions, 10 μL of a 50-μL reaction was diluted into 490 μL of IPB. Incubation with the precipitation (primary) antibody was for 2 hr at 4°C with rocking followed by 8–10 hr with protein A-Sepharose. Harvesting, washing, and preparation of the immune complexes for electrophoresis was the same as described above.

Protein Determination, SDS-PAGE, and Western Blots

Protein concentration was determined by the BCA method (Pierce). Samples were prepared for electrophoresis by suspension in freshly prepared Laemmli SDS sample buffer (Laemmli, 1970) and heated for 10 min in a boiling water bath. Following brief centrifugation in a microfuge, samples were loaded immediately on either 4–20% polyacrylamide gradient gels (BioRad) or on regular (8% or 10%) polyacrylamide–SDS gels. For experiments in which the native sperm receptor was being assessed, the 4–20% gradient gels were electrophoresed at 130 V and electrophoresis continued for an additional 30–45 min after the dye front had run off of the gel to ensure that the protein entered the gel. M, markers were from BioRad. After electrophoresis, gels were either stained in 0.1% Coomassie blue R250 or processed for Western blotting. Gels of the in vitro transcription and translation reaction products were stained, destained, and then treated with EnHance (DuPont NEN) for 60 min. Gels were dried and exposed to Fuji RX film.

For Westerns, gels were equilibrated in transfer buffer (WTB; 0.2 M glycine, 25 mM Tris, pH 8.8, 0.1% SDS, 25% methanol; Towbin et al., 1979) for 15 min. Proteins were transferred to nitrocellulose (0.45 μm NitroBind, MSI) for a total of 1.2 A-hr in the case of gradient gels or for 1 A-hr for the regular gels. Following transfer, membranes were blocked in wash buffer (WB; 50 mM Tris, pH 7.6, 150 mM NaCl) containing 3% dry milk for 1.5–2 hr at room temperature. Primary antibody incubation was in the same buffer (1–3 μg/mL) at room temperature for 2–8 hr. Blots were washed 15 min in WB, 10 min in WB supplemented with 0.05% NP-40, and 15 min in WB. Secondary antibodies conjugated to HRP were diluted in the blocking buffer according to the manufacturer’s recommendations (the goat anti-rabbit IgG HRP conjugate was from Transduction Laboratories; the sheep anti-mouse IgG HRP conjugate was from Cappel, Inc.). After 45–60 min incubation at room temperature, the blots were washed as described above with an additional 5 min wash in WB. Antibody binding was detected using the Super Signal chemiluminescence kit from Pierce. Blots were exposed immediately to Fuji RX film. For every blot, several exposures were obtained.

Purification of GST Fusion Proteins and in Vitro Transcription and Translation

GST fusion proteins were purified from Escherichia coli DH5α harboring the various pGEX constructs as described (Gish et al., 1995). Induction was for 30–60 min with 0.1 mM IPTG. Glutathione–agarose beads were from Sigma. In vitro transcription and translation was performed using the TNT T3 transcription–translation-coupled rabbit reticulocyte lysate kit from Promega. Qiagen-purified plasmid DNA (1.0 μg) was used in each reaction (50 μL total volume). For radiolabeling of the products, [3S]methionine (1000 Ci/mmol; DuPont NEN) was used. For gel samples, 10 μL of each reaction per lane was loaded.

Protease Treatment of Eggs and Fertilization Assays

Aliquots of 18 mL of a 10% suspension (v/v) in ASW of washed, dejellied eggs were carefully placed into biowashed beakers at 14–16°C. One sample served as a mock control; lysoyslendoproteinase C (LysC; Promega) was added to the other sample at a final concentration of 0.15 units/mL. The suspensions were gently resuspended over a period of 25 min. At various times, 6-mL aliquots were transferred to small beakers and allowed to settle and the digest (supernatant) was collected, spiked with protease inhibitors (including soybean trypsin inhibitor, SBTI, at 0.7 mg/mL), and placed on ice. Eggs were washed in a large volume of ASW containing 0.1 mg/mL SBTI and two times with FSW. For fertilization assays, 1 mL of a 1% suspension of the washed eggs was transferred to 75 × 12-mm borosilicate tubes washed twice by gravity settling in ASW and sperm (diluted in ASW/jellywater to induce the acrosome reaction) was added. The sperm dilution was determined empirically for each experiment, generally in the range of 1.10–10 in order to achieve 90–95% fertilization. Exactly 3 min after adding sperm to each sample of eggs, an equal volume of 3% paraformaldehyde in ASW was added. After fixation for 15 min, the eggs were washed three times in ASW and the samples were coded and scored for fertilization envelope (FE) elevation. Partial elevation was scored as positive. Approximately 600 eggs were scored per coded sample by three different persons. As a control for viability and ability to elevate fertilization envelopes, a small sample of unfixed
eggs was tested for ability to elevate FEs in response to 10 μM A23187 in DMSO in a given amount of time.

After allowing the eggs to settle by gravity, the digested material (supernatant) was collected by carefully pipetting and placed on ice and PIC and SBTI were added to stop proteolysis. The digests were concentrated approximately 20-fold using Centricon 10 concentrators (Amicon, Inc.). Concentrated, crude digest material was diluted 10-fold in dilution buffer (50 mM Tris, pH 8.0, 50 mM NaCl, 5 mM EDTA, 0.5% NP-40) such that the final salt concentration was ~150–200 mM. Immunoprecipitation of this material with anti-70KL or pAb96-586 IgGs was as described above. Following protease treatment, eggs were washed in a large volume of ASW/0.1 mg/mL SBTI, twice in ASW, and twice in PMVL buffer by gravity settling at 14°C. The eggs were collected by centrifugation at 900g for 5 min, the supernatant was carefully removed, and the eggs were homogenized in PMVL buffer/PIC. Both the digest material and the egg homogenates were prepared for electrophoresis immediately.

RESULTS

Cross-Reactivity of Antibodies with Native and Recombinant Sperm Receptor Protein

In order to establish that the antibodies raised against recombinant protein and against native protein recognize common epitopes, a monoclonal antibody against recombinant protein was produced and characterized. This antibody was used along with previously described polyclonal antibodies in a series of experiments designed to characterize the specificity of the antibodies and to investigate the localization of the native protein in the egg. Table 1 lists the antibodies used in this study, along with their antigen source and specificity. Figure 1A depicts the cDNA constructs as well as the GST fusion protein constructs used. Note that the cDNAs overlap extensively and the 3' end truncations are derived from the same physical fragment of cDNA (see Materials and Methods). The original 45A cDNA, which corresponds to the portion of the protein exhibiting sperm binding activity (Foltz et al., 1993), encompasses amino acid residues 96–586. Northern blots of total and poly(A)⁺ RNA probed with the extreme ends of these cloned cDNAs as well as two internal probes revealed a large (ca. 6.5–7 kb) RNA which hybridized to all of the probes (Fig. 1B and see also Just and Lennarz, 1997). These observations are consistent with the conclusion that the cloned, overlapping cDNAs represent a contiguous mRNA present in the egg, although the large size indicates a significant amount of untranslated region.

As shown in Fig. 2, the five cDNA constructs representing C-terminal truncations of the sperm receptor can be transcribed and translated in vitro. Interestingly, the resulting protein products consistently migrate on SDS–polyacrylamide gels with an apparent M, larger than the predicted M, (see Fig. 1A). The degree of abberation appears to vary somewhat depending on the percentage of acrylamide used in the gel. This appears to be a general phenomenon for the products of the cloned cDNAs. The minor, lower M, products (Fig. 2) are typical of these in vitro transcription–translation systems and most likely represent truncated products. Note that the protein products of both the pSR(1–3459) and the pSR(1–2832) constructs migrate approximately the same on these gels, indicating that the stop codon at nt position 2855 is functional. Both the polyclonal anti-70KL IgGs and the pAb96-586 IgGs recognized all five of the in vitro synthesized products based on immunoprecipitation studies (Fig. 2). As controls, both protein A alone (data not shown) and preimmune serum (Fig. 2) were incubated with the lysates; neither precipitated any of the 35S-labeled proteins. Since the immunogen for both of the polyclonals corresponds to a region that is retained in all of these constructs, represented by the original 45A construct (Fig. 1A, Table 1), it is not surprising that all are recognized and able to be precipitated by the antibodies.

The monoclonal antibody (mAb7.3) appears to recognize an epitope encoded by the cDNAs 3' of nucleotide position 1901, as shown in Fig. 3; truncation at position 1901 resulted in the loss of immunoreactivity (Fig. 3A). Since the recombinant protein encoded by the pSR(1–2120) product was recognized by the monoclonal antibody, this suggests that the epitope lies between nucleotide positions 1901 and 2120. Neither protein A alone (data not shown) nor another monoclonal antibody of the same isotype (Fig. 3A) precipitated these protein products. The immunogen used for the monoclonal antibody was a GST fusion protein encompassing nucleotides 1426–3459; thus, the epitope assignment is reasonable.

To further map the epitope recognized by the monoclonal

<table>
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<tr>
<th>Antibody</th>
<th>Immunogen</th>
<th>Specificity</th>
<th>Reference</th>
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<tr>
<td>Anti-70KL IgG (polyclonal IgG)</td>
<td>70-kDa LysC fragment</td>
<td>High M, egg protein, recombinant protein (residues 96–586)</td>
<td>Foltz and Lennarz, 1992</td>
</tr>
<tr>
<td>Anti-350K (polyclonal IgG)</td>
<td>Purified 350-kDa protein</td>
<td>High M, egg protein</td>
<td>Ohlendieck et al., 1993</td>
</tr>
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<td>mAb7.3 (monoclonal IgG)</td>
<td>Recombinant protein, residues 413–889</td>
<td>High M, egg protein, recombinant protein (residues 587–644)</td>
<td>This work</td>
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FIG. 1. Schematic diagram of the cDNA constructs and Northern blot analyses. (A) The nucleotide positions are given and the presumed start codon (ATG) at nucleotide position 188 is shown for the cDNA constructs used in this work. The stop codon is at nucleotide position 2855 (codon 890). The predicted Mr values are based on the start site at nucleotide 188 (amino acid residue 1) and the stop at position 2855 or the indicated truncated position. In-frame GST fusions at the 5′-end (black boxes) are shown. For the GST fusion protein constructs, the predicted Mr of the protein product includes the ~26kDa provided by the GST protein. Note that the cDNA clone (45A) isolated in the original library screen (Foltz et al., 1993) corresponds to nucleotide positions 477–1944. For Northern blot analyses (B), total (T) RNA (10 μg) and poly(A)+RNA (2.5 μg) isolated from ovary/oocyte (ov/oo) or eggs of an individual female were electrophoresed on a 0.9% agarose formaldehyde gel and transferred to positively charged nylon membrane. Riboprobes (spanning the base pairs indicated) were labeled with [α-32P]UTP and blots were probed and washed under high stringency (see Materials and Methods) and exposed to film for 72 hr. The GenBank Accession No. for the sequence is L04969.
Having established the specificity of the antibodies using recombinant protein, we sought to investigate the specificity of these antibodies for sea urchin egg protein. To this end, a series of immunoprecipitation experiments were conducted. The basic strategy was immunoprecipitation of egg plasma membrane/vitelline layer (PMVL; see Materials and Methods) proteins with the various antibodies followed by Western analyses of the immunoprecipitates using each of the antibodies. To establish the immunoprecipitation conditions and confirm the specificity of the interaction, several control experiments were conducted. To demonstrate that the antibodies were not forming high Mₙ complexes under the buffer conditions used, mock immunoprecipitations were performed. These mock samples contained only the monoclonal antibody and the protein A-Sepharose in the immunoprecipitation buffer but were treated exactly the same as the experimental samples. The mock immunoprecipitates did not exhibit any complex formation under the conditions used (Fig. 4A, lanes 1 and 2). PMVLs contained a high Mₙ protein recognized by the monoclonal antibody (Fig. 4A, lanes 3 and 4) that was partially solubilized in the nonionic detergent buffer. Likewise, a high Mₙ protein in the immunoprecipitates was recognized by the monoclonal antibody (Fig. 4A, lane 6), but not by the control monoclonal antibody (Fig. 4A, lane 8). This experiment was repeated at least twice for each of seven PMVL preparations with the same results. For every set of immunoprecipitation experiments, the mock, negative antibody and the secondary antibody alone controls were performed.

The high Mₙ protein in these monoclonal antibody immunoprecipitates was also recognized by each of the polyclonal antibodies (Fig. 4B, lanes 1–3) but not by preimmune IgG (Fig. 4B, lane 4). Likewise, when the pAb96-586 or the anti-350K IgGs were used to immunoprecipitate PMVL protein, a high Mₙ protein was detected in each of the precipitates by the other antibodies, including mAb7.3, but not by preimmune or control antibodies (data not shown). These cross-immunoprecipitation experiments were repeated on five different PMVL preparations with the same results. Based on these results, we conclude that the antibodies raised against recombinant protein recognize epitopes shared with the native protein and that all of the antibodies used here (Table 1) recognize the ca. Mₙ 350-kDa sperm receptor protein described by Ohlendieck et al. (1993).

Characterization of the Sperm Receptor in Egg Membrane (PMVL) Preparations

To confirm that the antibodies raised against recombinant protein recognize a high Mₙ protein in PMVLs, we characterized the cofractionation of this high Mₙ protein with the plasma membrane. These preparations have been described and characterized extensively (Kinsey, 1986) and consist of the egg plasma membrane as well as the vitelline layer (egg coat). To prepare the egg surfaces, washed, dejellied eggs were mechanically lysed in a calcium-free seawa-
FIG. 3. Analysis of immunoreactivity of the monoclonal antibody. (A) Fluorographs of in vitro synthesized proteins. The total reaction (total) and products immunoprecipitated by mAb7.3 (mAb7.3 IPs) or a control antibody of the same isotype (mAb4.1 IPs) were electrophoresed on 8% polyacrylamide-SDS gels. Gels were Enhanced and exposed to film for 10 hr. The cDNA templates (top labels) and Mr markers (left; \( \times 1000 \) Da) are indicated. (B) Purified, recombinant GST fusion proteins were electrophoresed on 10% polyacrylamide-SDS gels. One gel was stained (Gel) and duplicates were transferred to nitrocellulose and probed with mAb7.3 and, as a negative control, mAb4.1. Antibody binding was detected with HRP-conjugated secondary antibody and enhanced chemiluminescence. The blots shown were treated exactly the same and exposed to film for 2 min. Longer exposure times did not reveal any additional immunoreactivity. Probing of the same blots with anti-GST revealed that the fusion proteins were present and corresponded to the purified proteins as visualized by Coomassie staining.

ter buffer. This was followed by differential centrifugation to obtain the plasma membrane/vitelline layer complex from which the cortical granules were subsequently detached. Figure 5 shows duplicate Western blots of equal mass of freshly prepared samples of the total (T), first supernatant (S1, which contains the cytosol, yolk platelets, cortical granules, and small fragments of membrane), and the final PMVL pellet (P) probed with mAb7.3 or the control mAb. The high Mr protein recognized by the monoclonal antibody is enriched in the surface fraction (P). No other immunoreactive proteins were detected in any of the fractions. The same result was obtained for five different PMVL preparations. The high Mr protein in preparations of total egg lysates appeared to be susceptible to degradation. Freeze–thawing of the total lysate occasionally resulted in the appearance of lower Mr bands in addition to the consistent 350-kDa band that were recognized by the monoclonal antibody (data not shown). On occasion, the pAb96-586 polyclonal detected lower Mr, immunoreactive bands, even in freshly prepared material. We currently are assessing these observations in more detail.

To investigate the association of the high Mr protein with the PMVLs, the preparation was subjected to various treatments designed to test the solubility of the proteins. These
As described under Materials and Methods, 40 \( \mu \)g of freshly prepared samples of total egg homogenate (T), the first supernatant (S1), and the final PMVL (P) were electrophoresed on a 4-20% polyacrylamide-SDS gradient gel and transferred to nitrocellulose. Blots were probed with mAb7.3 and the negative control antibody mAb4.1. Antibody binding was detected with an HRP-conjugated secondary antibody and enhanced chemiluminescence. The blots were treated identically (shown is a 10-min exposure). Mr markers (\( \times 1000 \) Da) are shown on the left.

experiments are an extension of those conducted previously using the anti-70KL IgG as a probe to assess solubilization of the intact sperm receptor (Foltz and Lennarz, 1992). Here, the monoclonal antibody (raised against recombinant protein) was used to determine if the native protein was soluble or insoluble under a given condition. Figure 6 is a Western blot showing the soluble (S) and insoluble (I) fractions of PMVLs following a variety of treatments. Treatments designed to strip away proteins that are associated with membranes but not transmembranous, included incubation in 0.5 M KI or 1 M NaCl. The PMVLs also were treated with 0.1 M sodium carbonate at pH 11, which should disrupt most protein-protein interactions. None of these treatments resulted in the solubilization of detectable amount of sperm receptor protein (Fig. 6, lanes 1-6). To assess the possibility that the receptor protein may be associated with an unidentified transmembrane protein via disulfide bridges, preparations were incubated with dithiothreitol. DTT has been found to disrupt sperm-egg interactions (Ohlendieck et al., 1994a) and is also thought to strip away some of the vitelline layer proteins (Epel et al., 1970). However, no detectable receptor protein was released (Fig. 6, lanes 7 and 8). Finally, the possibility that the receptor protein may be associated with the membrane via a glycosylphosphatidylinositol anchor was assessed. The membranes were incubated with phosphoinositide-specific phospholipase C (PI-PLC); no detectable receptor protein was solubilized by this enzymatic treatment (Fig. 6, lanes 13-16). However, treatment with detergents such as \( \beta \)-octylglucoside or SDS did result in solubilization (Fig. 6, lanes 9-12). It is difficult to...
Surface Localization of the Sperm Receptor

FIG. 6. Western blot of soluble and insoluble fractions of surface proteins probed with mAb7.3. Purified plasma membrane/vitelline layers (25 μg per experiment) were treated with various solubilization buffers and then centrifuged to separate the soluble (S) and insoluble (I) fractions, as described under Materials and Methods. Total (T) PMVL starting material was loaded as well. The insoluble material was dissolved in sample buffer and the entire sample was loaded on the gel. For the soluble fractions, one-half of the solubilized material was loaded (due to limitations of well size). Following electrophoresis of 4–20% polyacrylamide gradient-SDS gels, the proteins were transferred to nitrocellulose and probed with mAb7.3. Antibody binding was detected with an HRP-conjugated secondary antibody and enhanced chemiluminescence. A 10-min exposure is shown. The various treatments (indicated at top) are described under Materials and Methods. For the PI-PLC treatment, membranes were resuspended in triethanolamine (TEA) buffer in the absence (lanes 13 and 14) or presence (lanes 15 and 16) of PI-PLC. Mr markers (±1000 Da) are shown on the left.

interpret the negative results of solubilization with DTT and PI-PLC, particularly because the sea urchin egg vitelline layer–plasma membrane complex may not be amenable to the same type of solubilization studies commonly used on tissue or cultured cells. Nonetheless, these data indicate that the sperm receptor protein is tightly associated with the PMVL surface complex.

The Antibodies Recognize Epitopes on the Surface of Live Unfertilized Sea Urchin Eggs

Both the anti-70KL and the anti-350K IgGs have been used in standard indirect immunofluorescence and EM–immunogold studies to localize the sperm receptor in sea urchin eggs to both the egg surface and the cortical granules (Ohlendieck et al., 1994b; Partin et al., 1996). The optical sectioning capability of confocal microscopy provides a more refined analysis of whether or not the sperm receptor exhibits surface-exposed epitopes. The pAb96-586 and anti-70KL IgGs as well as the monoclonal antibody were used to stain live (and presumably impermeable) unfertilized sea urchin eggs (Fig. 7). As shown previously (Foltz and Lennarz, 1992; Partin et al., 1996), the anti-70KL IgG exhibited patchy staining that is most likely due to crosslinking of the epitopes. Approximately 90% of all of the eggs examined by confocal microscopy exhibited some form of staining with this polyclonal, about 50% of those stained were of a patchy nature while the remainder exhibited an unbroken pattern. With increased incubation time, the patchy staining became more pronounced (data not shown). Figure 7 shows eggs that were incubated with antibody for 20 min prior to viewing. The pAb96-586 IgGs showed some degree of variability in staining. On average, 94% of all eggs in a given batch were stained with pAb96-586; about 20% of these eggs showed a patchy stain and the remainder exhibited the unbroken pattern (Fig. 7). The monoclonal antibody stained >95% of all eggs in a given sample and showed a consistent unbroken pattern as viewed by confocal immunofluorescence microscopy (Fig. 7); no patchy staining was observed. Preimmune antibodies did not show any staining, nor did antibodies against known intracellular egg proteins, such as profilin (Fig. 7).

The Epitope(s) Is Removed by Protease Treatment of Intact Eggs

As a further comparison of the IgGs raised against native versus recombinant protein and as another means to verify the surface location of the protein, we returned to assays involving lysylendoproteinase C (LysC) digestion of eggs. It was shown previously that eggs released a 70-kDa fragment in response to this protease and that this fragment bound sperm species-specifically (Foltz and Lennarz, 1990). Antibody raised against this fragment (Foltz and Lennarz, 1992) and amino acid sequence derived from peptides of this 70-kDa fragment ultimately resulted in the cloning of the 45A cDNA (Foltz et al., 1993). The goal of the experiments described here was to determine if antibodies raised against recombinant protein recognized the 70-kDa LysC fragment and to determine if protease treatment of eggs affected the 350-kDa sperm receptor protein.

To confirm the validity of the assay, unfertilized eggs
Giusti, Hoang, and Foltz were treated with LysC and then were assessed for their ability to bind sperm. As previously determined (Ruiz-Bravo and Lennarz, 1986; Foltz and Lennarz, 1990), treatment with LysC resulted in a reduction in fertilization (Fig. 8A). Although the actual percentage of eggs able to be fertilized (elevate an FE) in a given time varied from experiment to experiment (possibly reflecting variation from clutch to clutch of gametes; cf. Levitan, 1996), a dramatic reduction in fertilization was observed in response to protease treatment over time in 15 of 16 different experiments. In these 15 experiments, treatment with LysC for 25 min resulted in a reduction of fertilization over a range of 89–98%. Because sperm binding was scored by assessing fertilization envelope elevation, eggs were tested for the ability to elevate visible FEs in response to calcium ionophore after protease treatment. LysC-treated eggs were capable of elevating fertilization envelopes at the same rate as mock-treated eggs in all cases (Fig. 8A).

As expected, the anti-70KL IgGs immunoprecipitated a 70-kDa fragment released from the egg surfaces in response to LysC (Fig. 8B, lane 4). This fragment also was immunoprecipitated by the pAb96-586 IgGs (Fig. 8B, lane 3). A number of smaller immunoreactive bands, migrating with and below the IgG heavy chains, were also precipitated in these experiments (Fig. 8B, lanes 3 and 4) and probably represent further-digested material. The “digested material” from mock-treated eggs did not contain the 70-kDa fragment or the smaller fragments (Fig. 8B, lanes 1 and 2). The monoclonal antibody did not recognize the 70-kDa fragment (data not shown), as expected, since the epitope mapping experiments described above suggested that this epitope maps outside of this region.

Concomitant with the release of the 70-kDa proteolytic fragment from the surface, the amount of the high M₂ sperm receptor decreased, as detected with mAb7.3 on Western blots of the treated eggs (Fig. 8C). Thus, the appearance of the 70-kDa LysC fragment coincides with the loss of the 350-kDa protein and with a decrease in the ability of eggs to bind to sperm. Further, since the eggs remained intact throughout the LysC treatment, the loss of the epitope and the release of the 70-kDa fragment from the eggs indicate that the sperm receptor protein is exposed on the egg surface.

DISCUSSION

This study addresses the questions of whether the recombinant proteins encoded by the cloned cDNAs encode epi-
topes shared with the native egg receptor for sperm protein and whether this protein is localized to the egg surface. These questions have been assessed using a collection of antibodies, including a newly characterized monoclonal, for confocal immunofluorescence microscopy and biochemical studies. With regard to the first question, multiple antibodies (Table 1) raised against native or recombinant proteins all appear to recognize the same egg protein, of ∼350 K ca. M,. Cross-immunoprecipitation experiments were conducted in which the monoclonal antibody, raised against recombinant protein, was used to immunoprecipitate protein from egg PMVs followed by electrophoresis and Western analysis of the immune complex using the other antibodies. In all cases, the antibodies specifically recognized the high M, sperm receptor protein. Further, antibodies raised against native sperm receptor protein recognized recombinant proteins. The conclusion from these data is that the cloned cDNAs encode epitopes which are present in the native, high M, egg protein, which has been shown to bind sperm (Ohlendieck et al., 1993).

With respect to the question of localization, the data indicate that at least a subset of the protein is located on the egg surface, which is a requisite locale for sperm binding at fertilization. First, confocal immunofluorescence microscopy using the anti-sperm receptor antibodies showed distinct surface localization of the epitopes. To distinguish between binding to the extracellular surface or to the cytoplasmic face of the egg surface (e.g., in the cortical granules), these studies were conducted on live eggs. Two of the polyclonal antibodies exhibited a patchy staining pattern in some cases, indicative perhaps of a patch-cap response (Bourguignon and Singer, 1977; Toh and Hard, 1977). The monoclonal antibody also showed distinct surface localization, although no patching was observed. These data do not rule out the possibility that the protein is localized intracellularly as well. Previous work by others has suggested that the cortical granules also contain immunoreactive protein (Partin et al., 1996).

In addition to the confocal immunofluorescence microscopy experiments, complementary biochemical investigations confirmed the surface localization of the protein. Preparations of egg plasma membranes were found to be enriched for the high M, glycoprotein as assessed by immunoblotting with the monoclonal antibody. When eggs were treated with LysC, this high M, protein was reduced in abundance. Further, the material released from these eggs (which remained intact throughout the protease treatment) contained a ca. M, 70-kDa fragment which was recognized by the original anti-70KL polyclonal antibody and a polyclonal antibody raised against recombinant protein. Thus, this epitope was susceptible to proteolysis, indicating a surface exposure. Eggs which were treated with LysC exhibited a decreased ability to bind sperm and this also was dependent on the time of protease treatment. Thus, a loss in ability to bind sperm correlated with: (i) the release of a proteolytic fragment recognized by the antibodies against both native and recombinant protein; and (ii) the loss of the high M, sperm receptor protein.

Additional biochemical strategies were undertaken to assess the association of the high M, protein with the egg surface. The protein generally is not very soluble (Ohlendieck and Lennarz, 1995) and this taken together with the fact that the egg vitelline layer is not well-characterized biochemically makes assessment of the receptor protein’s membrane association by standard means difficult to interpret. Treatment of isolated egg plasma membranes with agents which generally cause release of peripherally associated proteins (such as high pH, high salt, and 0.5 M potassium iodide) did not solubilize the high M, protein. However, detergents such as SDS and β-octyl glucoside did result in solubilization, suggesting a strong association with the membrane, either as an integral membrane protein, a lipid-anchored protein or via an unusually tight association with a distinct transmembrane protein (Hjelmeland, 1990; Neugebauer, 1990). Examples of cell surface proteins which exhibit tight membrane association but which are not themselves transmembranous have been described (Jing et al., 1996; Treanor et al., 1996). Some of the most common examples are extracellular proteins which are tethered via a glycosyl phosphatidylinositol anchor (Englund, 1993). In a few documented cases, these GPI-anchored proteins are part of large, detergent-resistant complexes (Cinek and Horesi, 1992; Fra et al., 1994). Although the deduced sequence of the cDNAs in hand do not indicate a consensus for a GPI anchor (Englund, 1993), the possibility that the high M, sperm receptor was anchored to the membrane via a GPI anchor was assessed. Eggs were treated with phosphoinositol-specific phospholipase C. No detectable sperm receptor protein was released (Fig. 7). Admittedly, these are negative data and the possibility of a GPI or other type of lipid anchor cannot be ruled out.

Another possibility is that the high M, sperm receptor could be associated with the egg surface via a mechanism involving disulfide bridges to a neighboring transmembrane protein, analogous to the α-chains of the insulin receptor, for example (van der Geer et al., 1994). Ohlendieck et al. (1994a) have shown that the sperm receptor multimeric structure is maintained by disulfide bonds and that a reduction in these bonds prevents sperm binding, suggesting that this may be a possibility. However, treatment of egg surface preparations with 10 mM dithiothreitol (which is known to modify the surface sufficiently to preclude sperm binding; Ohlendieck et al., 1994a) did not result in solubilization of the high M, protein (Fig. 6). Again, these are negative data and thus this model remains a formal possibility. Finally, we have considered the possibility that the heavily glycosylated, ca. M, 350 K protein recognized by the antibodies is actually a complex of proteins that is not amenable to dissociation. This could account for the discrepancy between the predicted M, based on the cDNA sequence and the apparent M, based on migration in SDS–polyacrylamide gels. However, despite harsh treatments in SDS, DTT, or β-mercaptoethanol and boiling, the high M, form was always observed.
Protease treatment reduces the ability of eggs to be fertilized and correlates with the release of a 70-kDa fragment and loss of the high Mr sperm receptor. (A) Eggs were mock treated (dark boxes) or treated with LysC protease (striped boxes) for 15, 20, and 25 min. Following extensive washing, sperm were added to the eggs and the samples were fixed exactly 3 min after sperm addition. The eggs were scored for fertilization envelope (FE) elevation as an indicator of successful fertilization. Partial as well as complete envelope elevation was counted as positive. As a control to ensure that protease-treated eggs were capable of elevating FEs at the same rate as mock-treated eggs, the calcium ionophore A23187 was added to a sample of eggs from the same experiment. Eggs were fixed after exactly 3 min and scored for FE elevation; only fully formed fertilization envelopes were scored as positive. (B) Immunoprecipitation of the 70-kDa fragment from the released material. Eggs were mock treated or treated with LysC protease for 25 min. The digested material was concentrated and 160 μg of total digest from the mock (lanes 1 and 2) and LysC (lanes 3 and 4)-treated eggs was immunoprecipitated with pAb96-586 (lanes 1 and 3) or anti-70KL (lanes 2 and 4) under identical conditions. The immunoprecipitates were electrophoresed on an 8% polyacrylamide–SDS gel, transferred to nitrocellulose, and probed with pAb96-586 under identical conditions. Note the presence of the 70-kDa fragment in the immunoprecipitates from the LysC crude digest (arrow). Smaller, immunoreactive fragments were observed as well, migrating with and faster than the IgG heavy chains and protein A in the precipitates from the LysC-treated samples (lanes 3 and 4). Antibody heavy chains are indicated by an asterisk (*). Preimmune IgG/protein A (PA; lane 5) served as a control and did not precipitate any detectable proteins from the LysC crude digest. (C) Mock or LysC-treated eggs were washed extensively, homogenized and 40 μg of total protein was electrophoresed on a 4–20% polyacrylamide gradient–SDS gel, transferred to nitrocellulose, and probed with mAb7.3. Antibody binding was detected with an HRP-conjugated secondary antibody and enhanced chemiluminescence. For (B), exposure was 10 min; for (C), exposure was 7 min.

We also tested the possibility that the protein was crosslinked via dityrosine crosslinking, since the egg contains ovoperoxidase which is released at the time of granule exocytosis as part of envelope hardening (Kay and Shapiro, 1987). However, addition of ovoperoxidase inhibitors to isolation buffers did not affect the solubility or apparent Mr of the 350-kDa protein (data not shown), making this an unlikely explanation.

Taken together with the data indicating that the recombinant protein exhibits properties of a sperm binding protein (reviewed in Ohlendieck and Lennarz, 1995), the biochemical and immunological data presented here support the hypothesis that the high Mr, sperm binding protein is associated with the extracellular face of the egg plasma membrane. These data also demonstrate that the cloned cDNAs encode epitopes shared by the native egg protein.

Previously, we observed a rapid, transient tyrosine phosphorylation of the sperm receptor at fertilization (Abassi and Foltz, 1994), which would imply that this protein spans the plasma membrane. In that study, the sperm receptor protein was immunoprecipitated using pAb96-586 and tyrosine phosphorylation was demonstrated using a phosphotyrosine-specific antibody. A third antibody (referred to as anti-CYTO) was used to quantify the amount of sperm receptor.
protein immunoprecipitated (Abassi and Foltz, 1994). However, the sequence revision of the cloned cDNAs invalidates this polyclonal antibody. Based on the revised sequence, the recombinant protein used to make this antibody represents a region of the cDNA downstream of the stop codon, in the 3'UTR (Fig. 1A) and therefore cannot represent a part of the receptor protein. Nevertheless, the anti-CYTO polyclonal IgG specifically recognizes recombinant protein encoded by the cloned sperm receptor cDNAs as well as the 350-kDa egg protein (Abassi and Foltz, 1994). There are at least two possible explanations. First, the immunogen may encode an ORF which shares epitopes with the sperm receptor. Second, a different immunogen encoding an upstream region of the sperm receptor sequence may have been injected into the rabbit at some point. We can neither confirm nor rule out either possibility at this time. In light of this, we have repeated the tyrosine phosphorylation experiments originally described in Abassi and Foltz (1994) using mAb7.3 instead of anti-CYTO (data not shown) and have confirmed the observation that the 350-kDa protein is tyrosine phosphorylated at fertilization.

The data regarding surface-exposed epitopes and tyrosine phosphorylation present a paradox with regard to the cloned cDNAs in hand, which do not predict an obvious transmembrane topology. Several explanations are possible. First, it could be that in the tyrosine phosphorylation studies (Abassi and Foltz, 1994) the protein which is being phosphorylated is actually a protein that is transmembranous and tightly associated with the sperm receptor. Second, the cDNAs may indeed encode a surface protein with a cryptic transmembrane domain (von Hejne, 1995; Reithmeier, 1995). A third possibility is that alternative forms of the sperm receptor protein may exist, possibly generated by alternative splicing (McKeown, 1992) or RNA editing (Bass, 1995; Scott, 1996; Seeburg, 1996). Previous work using EM–immunogold studies has suggested that a form of the sperm receptor exists in the cortical granules (Ruiz-Bravo et al., 1999; Ohlendieck et al., 1994b; Partin et al., 1996), but this has not been assessed in biochemical detail. Since the monoclonal antibody (mAb7.3) maps to a region that does not have much homology with the mammalian HSP110 proteins (see Just and Lennarz, 1997) we are investigating the possibility that other, perhaps cryptic cytoplasmic forms exist by using the polyclonal antibodies of the vitelline membrane of sea urchin eggs.

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Foltz, K. R., Partin, J. S., and Lennarz, W. J. (1993). Sea urchin egg protein (Abassi and Foltz, 1994). There are at least two possible explanations. First, the immunogen may encode an ORF which shares epitopes with the sperm receptor. Second, a different immunogen encoding an upstream region of the sperm receptor sequence may have been injected into the rabbit at some point. We can neither confirm nor rule out either possibility at this time. In light of this, we have repeated the tyrosine phosphorylation experiments originally described in Abassi and Foltz (1994) using mAb7.3 instead of anti-CYTO (data not shown) and have confirmed the observation that the 350-kDa protein is tyrosine phosphorylated at fertilization.

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