Sea Urchin Egg Receptor for Sperm: Sequence Similarity of Binding Domain and hsp70

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Fertilization depends on cell surface recognition proteins that interact and thereby mediate binding and subsequent fusion of the sperm and egg. Overlapping complementary DNA's encoding the egg plasma membrane receptor for sperm from the sea urchin *Strongylocentrotus purpuratus* were cloned and sequenced. Analysis of the deduced primary structure suggests that the receptor is a transmembrane protein with a short cytoplasmic domain. This domain showed no sequence similarity to known protein sequences. In contrast, the extracellular, sperm binding domain of the receptor did show sequence similarity to the heat shock protein 70 (hsp70) family of proteins. Recombinant protein representing this portion of the receptor bound to the sperm protein, bindin, and also inhibited fertilization in a species-specific manner; beads coated with the protein became specifically bound to acrosome-reacted sperm. These data provide a basis for detailed investigations of molecular interactions that occur in gamete recognition and egg activation.

A key step at fertilization in both vertebrates and invertebrates is the species-specific binding of sperm and egg, mediated by molecules on the surface of the gametes (*1*, *2*). In sea urchins, bindin is the sperm protein that interacts with an egg surface receptor (*2*, *3*). Upon binding of sperm that have undergone the acrosome reaction to the egg, there is immediate membrane depolarization and gamete fusion (*4*). A wave of Ca^{2+} release is triggered (*5*), with subsequent exocytosis of cortical granules and elevation of the fertilization envelope (*6*). Release of Ca^{2+} and propagation of Ca^{2+} waves can be induced by inositol trisphosphate (IP_3) (*7*) or Ca^{2+} (*8*), but it is not known whether the sperm delivers an activator that initiates the Ca^{2+} release, or if its binding induces a signal transduction event that leads to production of IP_3 and subsequent Ca^{2+} release (*7*–*9*). In either case, fertilization appears to require a receptor on the egg surface that binds acrosome-reacted sperm (*10*). A 70-kD proteolytic fragment of the receptor has been purified (*11*), and antibody to it has been shown to inhibit fertilization (*12*). The Fab fragments of this immunoglobulin G (IgG) can cause fertilization envelope elevation, raising the possibility that the receptor is involved in signal transduction (*12*).

Cloning of the receptor. We used affinity-purified antibodies to the receptor (*12*) to screen a λ ZAP cDNA expression library made from polyadenylated RNA of *Strongylocentrotus purpuratus* immature ovaries. One of the four clones initially isolated (clone 45A) was characterized extensively. Experiments described in Fig. 1 using a fusion protein of glutathione S-transferase (GST) and the protein encoded by clone 45A provided evidence that the 45A cDNA encodes a 51-kD fragment of the receptor; this result was confirmed by observing identity between regions of the deduced amino acid sequence with that obtained from direct protein sequencing of two peptides isolated from the 70-kD receptor fragment (see below). To obtain the entire sequence of the receptor, clone 45A was used to probe for overlapping cDNA's and these cDNA clones were then used as probes to obtain other cDNA's (Fig. 2A). The polymerase chain reaction–rapid amplification of cDNA ends (PCR-RACE) method was used to extend the 3′ end of the cDNA. Ten PCR product subclones were analyzed to confirm the sequence of the 3′ end, which contained a polyadenylation signal (AATAAA) as part of a stop codon for translation (*13*) located 13 bp upstream from the start of the polyadenylated tail. The nucleic acid sequence was recorded in the GenBank (accession number L04969). The size of the receptor mRNA was estimated to be approximately 7 kb by Northern blot hybridization analyses (*14*). The cDNA's encode a total of 4297 bp, an indication that the receptor mRNA may have a longer 5′ untranslated region than that obtained and sequenced thus far.

Deduced sequence of the receptor. The deduced amino acid sequence of the cDNA's revealed a single, uninterrupted open reading frame of 1278 amino acids. The sequence of two peptides isolated after ArgC treatment of the 70-kD receptor fragment were found in

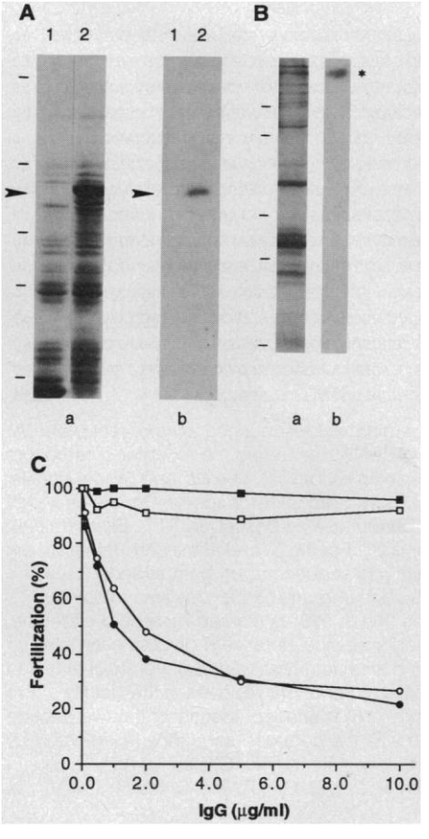

Fig. 1. Evidence that the 45A cDNA encodes a portion of the sperm receptor. (**A**) Antibody to receptor IgG's recognize the GST45A fusion protein. *Escherichia coli* expressing glutathione-S-transferase (pGEX vector alone, lanes 1 and 3) or GST45A (the cDNA in the pGEX vector, lanes 2 and 4) were induced and harvested (*32*); the cell lysates were analyzed by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) (*33*). Duplicate gels were stained with Coomassie brilliant blue (lanes 1 and 2) or transferred to nitrocellulose (*34*) and probed with antibody to the 70-kD receptor fragment IgG (*12*) and ^{125}I-labeled secondary antibody (lane 3). The arrow indicates the GST45A fusion protein. (**B**) Antibodies purified on the basis of their affinity to GST45A recognize the intact receptor. The GST45A recombinant protein was used to purify (*35*) the anti–70-kD fragment of the polyclonal IgG's. The affinity-purified IgG recognized the 350-kD receptor in plasma membranes (*36*). Proteins were fractionated by SDS-PAGE, and then stained (lane 1) or transferred to nitrocellulose (lane 2). The asterisk indicates the intact receptor. Molecular size standards are indicated in kilodaltons. (**C**) Affinity-purified IgGs inhibit fertilization. Antibodies to receptor (IgG's) affinity-purified with the 70-kD receptor fragment (○), the GST45A fusion protein (●), or GST (□) were tested for their ability to inhibit fertilization (*12*). Serum prior to immunization (■) was also was tested. Data were normalized to the percentage of fertilization in the absence of any antibody.

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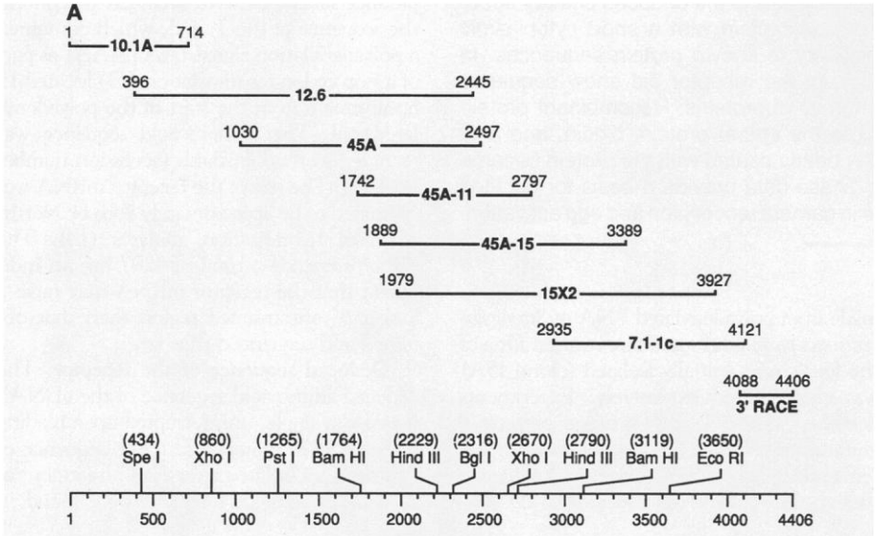
this reading frame (Fig. 2B). There are two potential initiating methionines (*15*). The methionine shown, located three residues 5′ to a predicted signal sequence (*16*), was assumed to represent the first translated codon; the actual start site is not yet known. If the glutamic acid at position +24 is the first amino acid residue of the mature protein, the predicted size of the protein is 133,784 daltons, which is much smaller than the size expected from SDS–polyacrylamide gel electrophoresis (SDS-PAGE) of the intact receptor molecule (~350 kD); (*12*). However, the receptor is heavily glycosylated; there are multiple, sulfated O-linked oligosaccharide chains that could contribute to an increase in its apparent size after SDS-PAGE (*10–12*). Attempts to remove the carbohydrate moieties without cleaving the polypeptide backbone have thus far been unsuccessful, and therefore the molecular size of the aglycoprotein has not been determined.

The receptor appears to be tightly associated with the plasma membrane (*12*). The deduced primary structure of the receptor predicts that it is an integral membrane protein containing at least one and possibly three hydrophobic transmembrane segments in the COOH-terminal domain (Fig. 2B). Because the sequences of two segments closest to the COOH-terminus have relatively high peripheral-integral values (*17*), our working model is that the protein contains a single transmembrane domain spanning residues 909 to 925. Either arrangement (one or three transmembrane sequences) is consistent with the extracellular location of the two ArgC-generated peptides from the 70-kD proteolytic fragment of the receptor that were sequenced (Fig. 2B). A diagrammatic representation of the receptor is shown in Fig. 3.

Sequence similarity to heat shock proteins. The sequence of residues 65 to 484 of the receptor (Fig. 3) bears similarity to proteins of the heat shock protein 70 (hsp70) family (*18*); the sequence identity is 26 to 31 percent and the similarity is 45 to 52 percent, depending on the species being compared (Fig. 4). The only known sea urchin heat shock protein sequence is a partial sequence derived from a genomic clone of the Mediterranean sea urchin *Paracentrotus lividus* (*19*); it exhibits 82 percent identity to human hsp70. The *P. lividus*

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VISVPQYYTDLERRGVIHAAEIAGLNCLRVISDTTAVALAYGIYKQDLPTPEEKPRNVVFVDCGHSSLQVSVCAFNKGKL 240
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ISRADFEALAAELLKRVEVPLKSVLEQTKLKPEDIHSIEIVGGSSRIPSIKETIKKVFKKECSTTLNQDEAVARGCALQC 400
AILSPTFKVRDFTVTDLTPYPIELEWKGTEGEDGSMEVSSKNHQAPFSKMLTFYRKAPFELVARYADPNLPIPERRIGRF 480
KINGVFPTTEGESSKIKVKVRVDGHGIFNVASASLIEKLPVQAEDAMEDGSPEENGPSKEEGSGASQAENDAPMDQSPVQ 560
GGAGEGEASADKEEQADNGSKETSKDSKDQTSESSKSDKESKDQNSEGSKSDNSSTETDAKAAKKTKKTIKTHELSITAT 640
TDELSITEVNNFFEKEGKLIAHDRLEKEKNDAKNAVEEYVYEMREKLCDKFEQYISEKERGSFSKLLEETENWLYEDGED 720
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DSKVNAQNQKAPHQDPVVTAAQIRSEIQSMKFVCDPIINKPKPKPKEEPPKDNGPTPEEAAKDGGPALRPRRGARRRWTP 880
VTKHLREKQAKKVKPNQMKPNQMWRWTRLTLNICSAILLFFVLYARHDCDGNCTLVLILSLNHSQRSNAMHIIVVICLLN 960
SSCELVSRLNSCRYQNLHFPASSANINFPIDILDVTNSLKIVVMITPKQSHAYEQSELLVWLSSLLVDHQSKAICDEKNT1040
FVFSLKKSFKLFAHDVKSLRMLSLRLFCCNFCCNRSCILCFPQKYFFYCELSLWSSHLFTAQVKKEDKFSTLISALFPDP1120
SSYITDACDCILHSLNLAVNFVLLTMAITTDVVMEWKMLLPSPRFFSHRLHSPHILRIDAFDVA                1184
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Fig. 2. Restriction map of cDNA's and deduced amino acid sequence of the receptor. (**A**) Sequencing strategy, restriction map, and summary of cDNA's encoding the receptor. The bottom scale indicates the common restriction sites and the size (in kilobases). See (*37* and *38*) for details. (**B**) Deduced amino acid sequence of the *S. purpuratus* egg receptor for sperm. Double-stranded dideoxynucleotide sequencing was carried out with Sequenase as described (U.S. Biochemical). Both strands were sequenced at least twice in their entirety. For the 3′ end, the insert DNA from ten transformants containing the PCR-RACE (*39*) product was sequenced on both strands to ensure against PCR error. The ORF is shown. Three putative transmembrane sequences are indicated (*17*); the most likely transmembrane sequence, at residues 909 to 925, is marked by a bold underline, whereas the other two are indicated by a single, dashed underline. Putative N-glycosylation sites on the basis of the NXS/T consensus (where X is any amino acid) for N-linked carbohydrate chains and are denoted by (●). Putative O-linked glycosylation sites at Ser or Thr residues in the vicinity of Pro residues (*27*) are denoted by (—). The signal sequence (*16*) is shaded. Identity of the two peptide sequences determined from the 70-kD fragment (*40*) with the deduced sequence is indicated by single underlining. Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

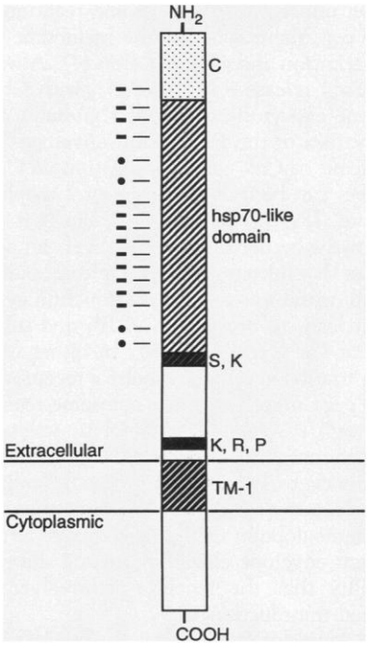

Fig. 3. Schematic diagram of the mature sperm receptor oriented in the egg plasma membrane. Only a single transmembrane domain (TM-1) (Fig. 2B), is shown. The NH_2-terminal, Cys-rich domain (C), the hsp70-like domain, and the two charged sequences—the domain rich in Ser and Lys (S and K) and the domain rich in Pro, Arg, and Lys (P, R, and K) are denoted. Putative N-linked (●) and O-linked (—) glycosylation sites are indicated.

sequence bears no greater similarity to the *S. purpuratus* receptor hsp70 domain than do the other heat shock sequences. The hsp70's are intracellular proteins that participate in the binding, folding, and translocation of peptides (*20*). The NH_2-terminal portion of the protein is required for adenosine triphosphatase activity, whereas the COOH-terminal domain is thought to form a peptide binding groove (*20, 21*).

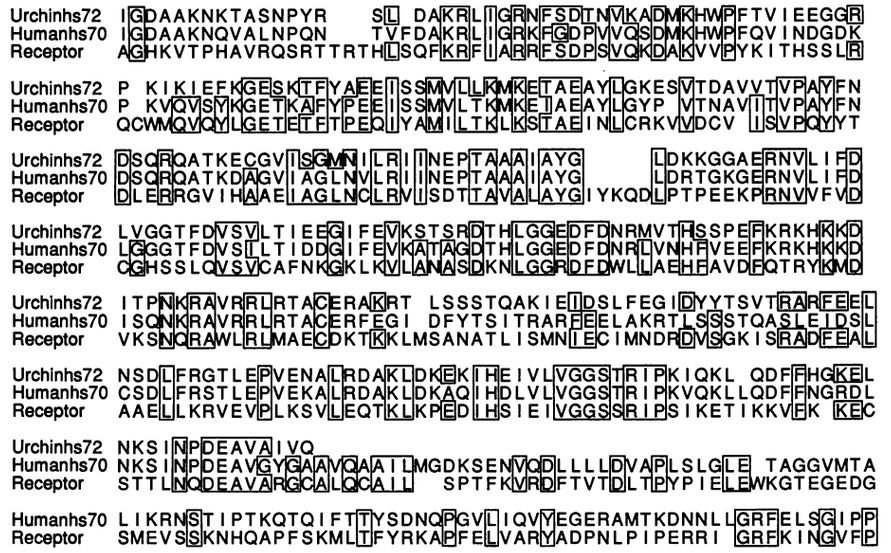

Fig. 4. Comparison of human hsp70, sea urchin hsp72, and sperm receptor sequences. Identical residues are boxed. Conserved substitutions are not denoted. The two hsp70 sequences starting at residue 47 are aligned with the receptor sequence starting at residue 65. The highly conserved NH_2-terminal domain spans residues 1 to 384 of hsp70; the presumed peptide binding domain of hsp70 is found at residues 384 to 470 (*18–22*).

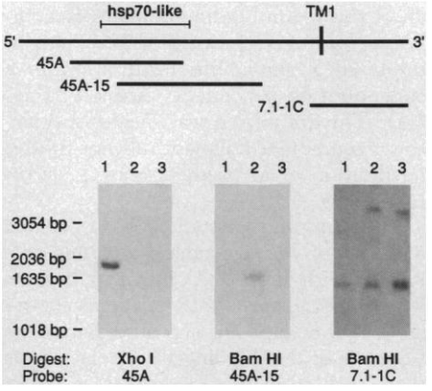

Fig. 5. Species-specific and conserved domains of the receptor. The line diagram indicates the cDNA encoding the sperm receptor; the hsp70-like external domain is indicated, as is the first putative transmembrane sequence (TM-1). The location of the three probes are indicated relative to the full-length cDNA. The three panels are autoradiograms of genomic Southern blots. To maximize sensitivity, in each panel lane 1 contained only 10 μg of *S. purpuratus* DNA; whereas, lanes 2 and 3 contained 20 μg of *S. drobachiensis* and 20 μg of *L. pictus* DNA, respectively, all of which had been digested overnight with the indicated enzyme, transferred to nitrocellulose and probed with the cDNA indicated below each panel (*38*). The stringency used in all three experiments was: hybridization at 60°C, and washes at 60°C in 2× SSC, and then 1× SSC with 0.1 percent SDS. Exposure was for 32 hours or 48 hours (far right) at −70°C with an intensifying screen. A β-tubulin probe (*41*) was used as a positive control for hybridization in all of these experiments.

Table 1. A quantitative study of sperm binding to beads coated with receptor fragments.

Bead coating	Sperm observed (no.)	Sperm bound to beads
GST45A fragment	103	84 (75%)
GST45A fragment + anti–70 kD	137	5 (4%)
70-kD fragment*	247	199 (81%)
70-kD fragment + anti–70 kD	169	12 (7%)

*Percent *S. purpuratus* sperm that had not been acrosome-reacted that bound to 70-kD–coated beads was 0%; the percent of acrosome-reacted *L. pictus* sperm that bound to 70-kD–coated beads was 0%.

Although the extracellular domain of the receptor has sequence similarity with hsp70's over both of these domains, it lacks a consensus sequence for adenosine triphosphate binding. The major histocompatibility class I proteins, which are located on the cell surface, are thought to contain the same peptide binding motif as the hsp70 proteins (*22*). The hsp70-like domain of the sperm receptor has multiple sites for N- and O-linked glycosylation, and a number of cysteine residues that are not present in the hsp70 proteins (Figs. 2B and 4). A model for the evolution of the hsp70 protein family has been proposed (*23*) and may allow the hsp70-like domain of the receptor to be viewed in an evolutionary context. Aside from the sequence similarity with the hsp70's, no similarity to other proteins was detected when the receptor sequence was compared with those in the international data banks (EMBL, PIR, and GenBank).

Other features of the primary structure. Our finding that bivalent antibody to this receptor causes patching and capping (*12*) suggests an interaction with the actin-based cortical cytoskeleton (*24*). A putative actin binding site (residues 1103 to 1109; -VKKEDKF-) could mediate this interaction (*25*). Alternatively, cytoskeletal association could occur through a linker protein (*24, 26*). Two other regions of the sequence are notable: a sequence rich in serine and lysine (residues 580 to 631) found at the end of the hsp70-like domain, and a second region (residues 840 to 880) directly preceding the transmembrane sequence that contains an abundance of lysine, arginine, and proline residues. There are four potential sites for N-glycosylation, ten sites that have a high probability for O-glycosylation (*27*), and a cysteine-rich region (*28*) at the NH_2-terminus (Fig. 3).

Southern (DNA) blot analyses of fragments of the genomic DNA of one closely (*Strongylocentrotus drobachiensis*) and one distantly (*Lytechinus pictus*) related species revealed that there was no cross hybridization with the cDNA corresponding to the extracellular sperm binding domain, even under low stringency conditions (Fig. 5). In contrast, cDNA fragments representing the more COOH-terminal portion of the receptor cross-hybridized with genomic DNA from these two other sea urchin species (Fig. 5), as well as DNA from other sea urchin species (*14*), suggesting that this is a conserved region. The specificity of the NH_2-terminal domain and the commonality of the COOH-terminal domain are consistent with postulated roles of these domains in species-specific sperm binding and signal transduction, respectively. Southern blot analysis of other marine invertebrates, yeast, *Drosophila*, and mouse did not reveal cross-hybridization with any of the cDNA

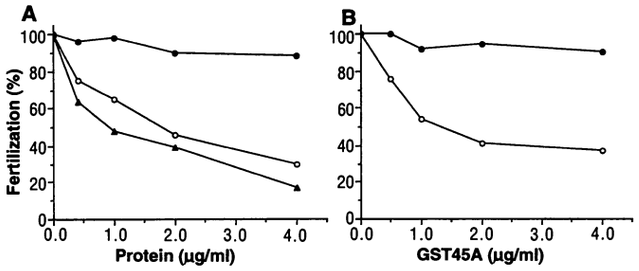

Fig. 6. Inhibition of fertilization by GST45A recombinant protein. (**A**) Various amounts of GST45A (○), GST (●), or purified 70-kD receptor fragment (▲) were used to compete for sperm binding to eggs (*12*). (**B**) Various amounts of GST45A were used in competition fertilization assays of *S. purpuratus* (○) gametes or *L. pictus* (●) gametes. The GST45A recombinant protein contains the domain of hsp70 homology (amino acid residues 210 to 700) fused to GST in the pGEX3 vector. The fusion proteins were purified (*32*) and dialyzed against seawater before use in the assays.

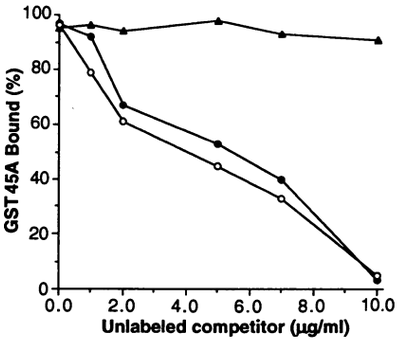

Fig. 8. Analysis of GST45A protein binding to purified bindin particles. Cosedimentation assays (*43*) were used to assess binding of the recombinant protein to *S. purpuratus* bindin. The ^{35}S-labeled GST45A protein was incubated with bindin particles in the presence of increasing amounts of unlabeled GST45A (●), purified 70-kD fragment (○), or GST15 Bam (▲). Only the 70-kD fragment and the GST45A protein blocked binding of the labeled GST45A protein.

probes under the conditions used (*14*).

The molecular characterization of the PH30 protein from guinea pig sperm, which was recently described (*29*), reveals that it is a fusogenic protein and may act as a disintegrin; it has been predicted that the egg surface component should be integrin-like. The sea urchin egg receptor for sperm bears no sequence similarity to either the integrin family of proteins or to the sperm binding proteins of the mammalian egg zona pellucida ZP2 and ZP3 (*30*). A mammalian egg plasma membrane receptor for sperm has not yet been identified.

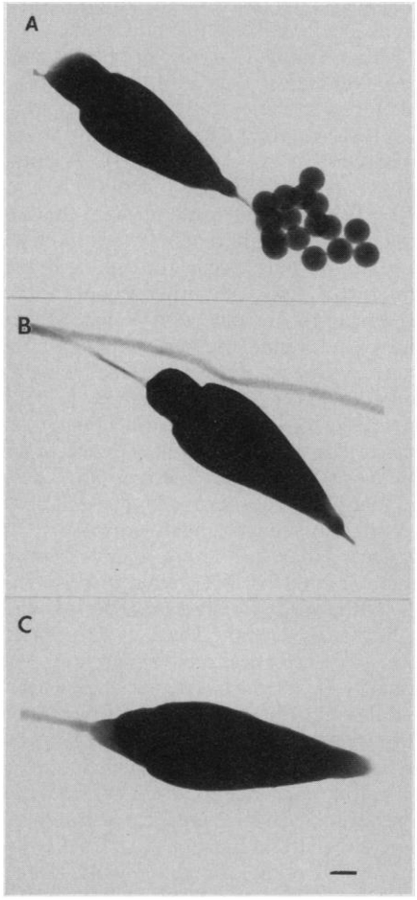

Fig. 7. Binding of *S. purpuratus* sperm to polystyrene beads coated with recombinant receptor protein. (**A**) Sperm that have undergone the acrosome reaction bind to beads coated with the GST45A fusion protein. (**B**) Treatment of beads coated with GST45A protein with IgG against the 70-kD receptor fragment blocks binding of acrosome-reacted sperm to the beads. (**C**) Sperm that have not been acrosome-reacted do not bind to beads coated with GST45A protein. Marker = 0.5 μm. Preparation and treatment of beads and microscopy are described in (*42*).

Expression and properties of the sperm binding domain. Because the partial amino acid sequence derived from the 70-kD fragment was contained in the sequence of cDNA clone 45A and the antibody to the 70-kD fragment inhibited fertilization (*11, 12*) (Fig. 1), we reasoned that clone 45A might encode a portion or all of the sperm binding domain. Recombinant protein representing this domain inhibited *S. purpuratus* fertilization in a concentration-dependent manner (Fig. 6A). This inhibition was species-specific; the recombinant fragment did not inhibit fertilization of *L. pictus* eggs by *L. pictus* sperm (Fig. 6B).

In addition, polystyrene beads coated with the GST45A recombinant fusion protein or with the 70-kD fragment became bound species-specifically to acrosome-reacted sperm (Fig. 7). The quantitative assessment of this binding (Table 1), shows that the antibodies to receptor IgG's blocked the binding mediated by coating the beads with either the GST45A protein or the 70-kD receptor fragment. Finally, we prepared ^{35}S-labeled GST45A and analyzed its ability to associate with bindin particles. Results of competitive binding assays with GST45A and control proteins indicated specific binding of the fusion protein to bindin (Fig. 8). All of these data (Figs. 6 to 8) establish that the sperm binding domain of the receptor recognizes bindin on the tip of sperm that have undergone the acrosome reaction.

The carbohydrate moieties of the receptor are thought to function in sperm recognition and binding; glycopeptides derived from the receptor by digestion with Pronase inhibit sperm binding, but they are not species-specific (*11, 31*). These results led to the hypothesis that species specificity was conferred by the polypeptide backbone and that the carbohydrate side chains were the adhesive element (*1, 31*). In addition to carbohydrate-protein interactions, the results of this study indicate that the recognition and binding of the egg and sperm involve species-specific protein-protein interactions (between the sperm receptor and bindin).

The deduced primary structure of the sea urchin egg receptor is consistent with the earlier observations that it is an integral membrane glycoprotein that species-specifically binds to the acrosome-reacted sperm (*10–12*). However, its role in egg activation has not been determined. With the availability of the cloned cDNA, the ability to express the receptor in heterologous systems, and the possibility of reconstituting the native receptor into membranes, it should be possible to test various models of sperm recognition and binding, as well as models of egg activation.

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37. Antibody to the 70-kD fragment was used to screen an expression library of *S. purpuratus* ovary and oocyte polyadenylated [poly(A)$^+$] RNA in ZAP (constructed by G. Wessel, Brown University). Clone 45A was isolated and used to rescreen the library (*38*); clones 45A-11 and 45A-15 were isolated. Clone 45A-15 was used as a probe to isolate clone 15X2. The 800-bp Bam HI fragment of 15X2 was used as a probe to isolate clone 7.1-1c. The PCR-RACE method (*39*) was used to obtain the 3′ sequence. An oligonucleotide spanning base pairs 4088 to 4115 (GenBank sequence) on the 7.1-1c cDNA was used as the sequence-specific primer to amplify single-stranded cDNA synthesized from ovary and oocyte poly(A)$^+$ RNA. A single product of 318 bp was isolated and ligated into the pBluescript II SK vector (Stratagene). To obtain the 5′ cDNA's, a probe was synthesized by PCR with the use of oligomers containing bp 1030 to 1047 and bp 1343 to 1360. The PCR product was subcloned into the Eco RV site of the pBluescript vector. For use as a probe, the construct was digested with Pst I and the resulting 289-bp fragment was used to screen the library, which resulted in the isolation of clone 12.6. This cDNA was then used to rescreen the library, and clone 10.1A was isolated.
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40. P. T. Matsudaira, *A Practical Guide to Protein and Peptide Purification for Microsequencing* (Academic Press, New York, 1989), pp. 1–131; To confirm that the cDNAs encode the receptor, the 70-kD fragment was digested with ArgC protease. The fragments were fractionated by electrophoresis and two peptides were transferred to a nylon membrane, stained with Coomassie blue, and excised. Sequencing of these two peptides was done at the SUNY–Stony Brook Center for Analysis and Synthesis of Macromolecules.
41. Supplied by D. Asai, Purdue University.
42. The 70-kD fragment or the GST45A fusion protein (250 μg) was adsorbed to 0.5-μm polystyrene beads (Polysciences) by overnight incubation in borate buffer. After being washed, the beads were resuspended (1:100) in filtered sea water. This suspension (500 μl) was then incubated at room temperature with 500 μl of the antibody (IgG) to the 70-kD fragment (2 μg/ml) or with sea water. Dry sperm were either acrosome-reacted with jelly water or suspended in sea water (non-acrosome–reacted). The sperm suspension (50 μl) was mixed with coated beads or with coated beads neutralized with IgG. After 5 minutes the specimens were fixed in glutaraldehyde and negatively stained with ammonium molybdate for transmission electron microscopy [G. L. Decker and W. J. Lennarz, *J. Cell Biol.* **81**, 92 (1979)]. During this procedure all beads not bound to the sperm were washed off of the grid.
43. Bindin was purified as described [V. D. Vacquier and G. W. Moy, *Proc. Natl. Acad. Sci U.S.A.* **74**, 2456 (1977)]. Initially, the binding assays (*11*) were done with ^{35}S-labeled fusion proteins obtained from growing *E. coli* in media supplemented with ^{35}S-labeled sodium sulfate (*32*). Neither [^{35}S]GST nor [^{35}S]GST15 Bam (the portion of the receptor encoded by the 15X2 Bam HI fragment); (Fig. 2A) bound to bindin while [^{35}S]GST45A proteins exhibited quantitative binding. For the experiment shown in Fig. 8, unlabeled GST45A, GST15 Bam, and the 70-kD receptor fragment were used as competitors. Binding was quantitated by determining the percentage of total ^{35}S-labeled GST45A protein that sedimented with the bindin.

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