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The quest for the sea urchin egg receptor for sperm ☆

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ABSTRACT

This review discusses identification, isolation and characterization of proteins mediating species-selective sperm-to-egg adhesion during sea urchin fertilization. Bindin is the only sea urchin sperm protein known to mediate species-selective sperm attachment to eggs. Two completely different egg surface proteins, 350-kDa and EBR1, have affinity for bindin and each one meets all the criteria to be a species-selective sperm receptor. Experiments suggest that sperm bindin recognizes both the sulfated O-linked oligosaccharides on the egg 350-kDa glycoprotein, and also the repeated protein sequence modules of EBR1.

1. Introduction: the state of fertilization research in 1977

Fertilization, the process by which sperm and egg interact and then fuse to form a zygote, has been studied for approximately 140 years. However, by 1977 animal fertilization still ranked as one of the least understood fundamental biological processes. No proteins had yet been isolated that mediated sperm-to-egg binding in any animal. At that time, the major animals in which fertilization was studied were: ascidians, starfish, sea urchins, polychaetes, fish, amphibians, rabbits, hamsters, rats and mice. The majority of fertilization research utilized sea urchins for the following reasons: the sexes are separate, sperm and eggs can be easily obtained in large quantities, fertilization occurs in seawater external to the adult body, many species are available at marine laboratories throughout the world and the cost of obtaining and maintaining sea urchins is low.

There are five sequential events in the process of sea urchin fertilization, but all five might not be demonstrable in a single species. Generally speaking, the five events are: the chemo-attraction of sperm to egg-released peptides, the induction of the exocytotic acrosome reaction of sperm by egg jelly, the binding (adherence) of sperm to the egg vitelline layer, the fusion of the plasma membranes of sperm and egg, and lastly, the fusion of haploid egg and sperm pronuclei in the egg cytoplasm that restores the diploid genome. Each of these events can exhibit species-selectivity [1].

2. Species-selective fertilization

One striking phenomenon, easily demonstrated in sea urchins and of utmost importance to this review, is the species-selectivity of the binding of acrosome-reacted sperm to eggs. Usually, sperm of species "A" will not bind to eggs of species "B". This is not true in every case of cross-species insemination, but it is a general phenomenon that is almost always true. This cross-species incompatibility in binding could be due to failures in the egg jelly of species "B" to induce acrosomal exocytosis in species "A" sperm, or it could be that the species "A" sperm adhesive protein, bindin, exposed by the acrosome reaction, has low affinity for its egg vitelline layer sperm receptor [2–5]. By the mid 1970s researchers studying sea urchin fertilization saw the possibility of discovering the protein basis for species-selective binding of sperm to eggs. This review discusses bindin and the sea urchin egg surface receptor for sperm bindin. Because of length restrictions not all relevant publications can be included. The following reviews contain the detailed background information [6–9].

3. Isolating bindin, the sperm half of the sea urchin sperm–egg bond

Electron micrographs showed that when sea urchin sperm contact egg jelly, the acrosome vesicle undergoes exocytosis exposing the protein content of the vesicle. At the same time the globular actin behind the acrosomal vesicle polymerizes to create the F-actin cored, plasma membrane covered, 1 μm long "acrosomal process" that pushes through the vesicle, the membrane of the process becoming coated with vesicle protein. These electron micrographs showed the insoluble protein content of the acrosomal vesicle to be bonding the sperm to the egg as if it were a glue [10]. To understand species-selective sperm–egg binding, the contents of sea urchin sperm acrosomal vesicles would have to be isolated and biochemically characterized.

A method was developed where the insoluble acrosomal vesicle content was released from *Strongylocentrotus purpuratus* (Sp) sperm by agitating the cells in calcium free artificial seawater con-

☆ This review is dedicated to Professor William J. Lennarz.

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taining EGTA, 1.25% Triton X-100, and trypsin inhibitor, at pH 5.8. After being freed from the cells, the circular, particulate masses of acrosomal vesicle content were purified by differential centrifugation and particle sieving. SDS–PAGE showed that the acrosomal particles were composed of a single protein of ~30-kDa that was named "bindin". Bindin particles agglutinated unfertilized eggs and isolated egg vitelline layers. The agglutination was blocked by preincubation of bindin particles with a trypsin-generated glycopeptide digest of isolated egg vitelline layers and trypsinized unfertilized eggs were not agglutinated by bindin particles. The agglutination of glutaraldehyde-fixed eggs did not occur if the eggs were treated with metaperiodate, suggesting that bindin has affinity for a carbohydrate of the egg surface [11,12]. Lastly, isolated Sp bindin particles agglutinated unfertilized Sp eggs, but not eggs of the sea urchins *Strongylocentrotus franciscanus* (Sf) or *Arbacia punctulata* (Ap) [13,14]. Thus, at least one sperm component of the sperm–egg bond had been isolated and the quest for its egg receptor began.

4. Bindin may also mediate fusion of sperm and egg plasma membranes

Bindin coats the membrane of the sperm acrosomal process and that is where fusion with the egg plasma membrane occurs. Experiments suggest that bindin may also mediate membrane fusion between gametes. Bindin particles aggregated gel-phase phospholipid vesicles and the aggregation was lost above the transition temperature [15]. Bindin particles fused mixed-phase liposomes, as shown by a 5-fold increase in vesicle diameter, and fusion was enhanced 4-fold by 2 mM calcium [16]. All sea urchin bindins have a conserved core of ~60 residues [17]. Within this core is an 18 residue sequence named "B18" (LGLLLRHLRHHSNLLANI) that has the same liposome fusion potency as the mature Sp bindin protein of 236 residues. CD spectra show that in solution B18 is mainly random chain. Depending on the composition of the liposomes, B18 will either aggregate or fuse the vesicles and both these events are enhanced by Zn^{2+}. The conformational change induced by Zn^{2+}, detergents and lipids increases the α-helical content of B18 and that is the reason for the enhancement of fusion [18].

Sea stars and sea urchins separated roughly 450 million years ago, yet the sea star bindin B18 differs from the sea urchin by only one residue, position 17 being N in the sea urchin and D in the sea star [19]. Assuming that B18 mediates fusion of gamete membranes, this extreme sequence conservation suggests that in all this evolutionary time the B18 sequence remained critical to the reproductive success of these echinoderms.

5. Bindin, molecular evolution and speciation

Bindin amino acid sequences are now known for many species of several sea urchin genera. Evolutionary analysis shows that parts of the bindin protein have been subjected to rapid change and "positive selection" (adaptive protein evolution), meaning there is increased survival potential for the species to alter the bindin sequence. Among evolutionary biologists the idea arose that the divergence of bindin sequences could lead to gamete incompatibility and the evolution of reproductive barriers within a population and in time new species. Thus, the evolutionary divergence of bindin could be a component in the speciation process in free spawning marine invertebrates. The molecular evolution of bindin has become a hot topic in the field of speciation of marine invertebrates [17,20–25].

6. The sea urchin egg surface receptor for sperm

In the quest for the sea urchin egg receptor for sperm the species-selective agglutination of eggs by isolated, insoluble, bindin was a breakthrough because it showed that bindin's receptor must be on the egg vitelline layer [11]. When bindin agglutinates eggs, agitation does not break up the aggregates, showing that bindin has high affinity for its egg receptor and also that the receptor is firmly bonded to the egg vitelline layer. The vitelline layer is a lacy meshwork of glycoproteins intimately attached to the egg plasma membrane [26]. It can be degraded with dithiothreitol or trypsinization, both treatments being employed to bypass species-selective barriers to fertilization in the production of inter-species hybrid embryos [4,27,28]. Several laboratories began to pursue the isolation of the sea urchin egg receptor for sperm, the Lennarz laboratory being a major contributor. Because the isolation of bindin particles from sperm was straightforward, most of us thought that isolation of the egg's bindin receptor could also be accomplished with ease in little time. No one in the field would have guessed that the identification and isolation of the sperm receptor would be so difficult and take so long.

7. Potential problems in assaying for the egg sperm receptor

There are three major methods to assay for the egg receptor for sperm: the first is to use isolated bindin particles in egg agglutination tests, the second is to use live sperm in sperm binding-to-eggs and fertilization assays, and the third is to coat beads with egg surface proteins and then study sperm binding to the beads. The potential problem is that these three assays might not be comparable. Isolated bindin particles have been extracted at pH 5.8 in calcium free medium with EGTA and 1.25% Triton X-100. Such insoluble bindin particles have no lipid or carbohydrate [11]. Other acrosomal proteins could be involved in sperm-to-egg binding that are extracted and washed away during bindin particle isolation. If there are non-bindin acrosomal proteins involved in sperm binding to eggs they might have different egg surface receptors. In other words, sperm-to-egg binding might be mechanistically redundant where a second or third cognate pair of sperm–egg proteins is also involved, but any single pair can mediate successful fertilization. Such "backup" systems are known for the control of the increase in free Ca^{2+} during the metabolic activation of sea urchin eggs, where inositol trisphosphate (IP_3), cyclic ADP ribose, and nicotinic acid adenine dinucleotide phosphate (NAADP) are all involved in calcium release during egg activation [29,30]. The insoluble bindin particles, which appear as demembranated acrosomal vesicle content, might be the most simple assay system for the presence of an egg receptor for sperm, but one must be careful in drawing conclusions from the outcomes of experiments using different assays.

8. Early attempts to isolate a sperm receptor

Anthocidaris crassispina eggs were stripped of their vitelline layers by treatment with urea and the extract fractionated into major glycoprotein peaks of 225-, 87- and 80-kDa. Whole rabbit IgG and corresponding Fab were prepared against each fraction. Eggs were exposed to the Fab fractions and their ability to bind sperm and fertilize was assessed. Only the 225-kDa Fab blocked sperm binding and fertilization, and this inhibition did not occur with eggs of five other sea urchin species. The 225-kDa antigen disappeared from the egg surface after elevation of the fertilization envelope [31]. Similar experiments with Sp eggs showed that 305- and 225-kDa glycoconjugates could be extracted from the fertilization envelopes of Sp eggs fertilized in soybean trypsin inhibitor (SBTI).

Rabbit IgG and Fab against these two glycoconjugates were prepared and found to be species-selective inhibitors of fertilization [32].

In other experiments egg membranes were isolated from two sea urchin species (Sp and Ap). The membranes inhibited fertilization species-selectively by competing with eggs for sperm. Eggs pretreated with trypsin before membrane isolation did not show this inhibitory effect. A soluble fraction of egg membranes was prepared that had the same inhibitory activity. The soluble sperm receptor bound concanavalin-A, which inhibited fertilization, indicating the egg's sperm receptor was a glycoprotein [33].

Sea urchin eggs fail to bind sperm after exposure to proteases. When a sperm fuses with the egg, the cortical granules undergo exocytosis, and the vitelline layer elevates and transforms into the fertilization envelope. Sperm binding sites are digested by a trypsin-like protease released from the egg cortical granules and the non-fertilizing sperm detach. If eggs are fertilized in the presence of soybean trypsin inhibitor (SBTI), supernumerary sperm remain bound to the elevated fertilization envelope [32,34]. In one study sea urchin eggs were surface radioiodinated and then activated with ionophore A23187 in the presence and absence of SBTI. The egg-released radioactive molecules, prepared with or without SBTI, were mixed with bindin particles and a filtration assay performed. Much more surface released radio labeled "crude receptor" bound to the bindin particles than did the degraded receptor prepared without SBTI. Binding of crude receptor saturated bindin and competition assays showed the bindin particles exhibited species-selectivity in binding crude receptor. The crude receptor had an apparent mass greater than 5 million Da and isoelectric focusing showed the active fraction had an isoelectric point of 4.02. Chemical analysis of this fraction showed it was not egg jelly because egg jelly is 17% sulfate. The crude bindin receptor fraction was 4% sulfate and contained mannose, galactose and did not contain sialic acid. This was the first study to use isolated bindin particles in attempts to identify a sperm receptor from the egg [35].

Continuing the quest for the sperm receptor, eggs of two sea urchin species (Sp and Ap) were surface radioiodinated and extracted for retention of large radiolabeled molecules. Isolated sperm bindin particles were mixed with this fraction, which was then subjected to density gradient centrifugation. One class of giant macromolecule bound to the bindin particles. This material bound species-selectively to sperm that had undergone the acrosome reaction and inhibited fertilization. Protease digestion of this egg surface fraction yielded a large molecular mass glycopeptide that bound to bindin [36].

9. What moieties of the egg receptor glycoprotein does bindin recognize?

Bindin was solubilized by sonication and shown to have lectin-like activity in the agglutination of trypsinized, fixed, rabbit erythrocytes. The best inhibitor of bindin-mediated red cell agglutination was a pronase digest of the egg surface, followed by fucoidin, egg jelly, xylan, dextran sulfate and yeast mannan; these polymers all contain sulfated sugars [37]. Other experiments showed that bindin has affinity for sulfated fucose polysaccharides larger than 15-kDa. Removal of the sulfate group destroys bindin affinity, which can be restored by resulfation. The sulfated polysaccharide fucoidin has affinity for bindin that is salt insensitive, indicating hydrogen bonding may also be involved in bindin's affinity. Polyvinyl sulfate also inhibits bindin-mediated egg agglutination [38]. Additional studies showed that bindin is highly specific for binding sulfate polymers as opposed to phosphate or carboxyl polymers. Such specificity for sulfate polymers is unknown for other proteins [39].

Continuing this story, a 350-kDa, highly glycosylated (70% carbohydrate) glycoconjugate was purified to homogeneity from sea urchin egg surfaces. It contained both N- and O-linked oligosaccharide chains with sulfated sugars. This purified glycoprotein inhibited fertilization species-selectively and beads coated with this molecule bound acrosome-reacted sperm species-selectively [40]. Experiments showed that this potential sperm receptor is a S–S bonded, multimeric complex. Dithiothreitol treatment of eggs blocked fertilization suggesting that the S–S bonding pattern of the complex is functionally important. The reduced, or alkylated receptor did not inhibit fertilization. Beads coated with receptor bound acrosome-reacted sperm, but reduced and alkylated receptor beads did not [41]. Additional work on the 350-kDa sperm receptor showed that the isolated N-linked oligosaccharide chains did not inhibit fertilization, but the O-linked chains were potent fertilization inhibitors. The O-linked chains did not show species-selectivity as inhibitors of fertilization. Oligosaccharides with the highest amount of sulfate were the most potent inhibitors [42]. Oligosaccharide chains of the receptor covalently linked to bovine serum albumin had much more fertilization inhibitory activity than did free oligosaccharides, indicating that multiple valency of oligosaccharides attached to larger backbone molecules was important for biological activity [43]. The active O-linked chains of the 350-kDa sperm receptor had the structure (SO_4^-)-9Neu5Gcα2(→5-O_{glycolyl}Neu5GGcα2→)n [44]. From this section it is reasonable to conclude that bindin is lectin-like and recognizes a 350-kDa glycoprotein that is an S-S bonded homo-multimeric complex of the egg surface. O-linked sulfated oligosaccharides are important in the high affinity binding between sperm (bindin) and its 350-kDa egg receptor.

10. Cloning the first sea urchin egg sperm receptor for sperm

Protease treatment of sea urchin eggs destroys their ability to bind sperm. Experiments showed that the protease lysylendoproteinase C (LysC) released a 70-kDa glycoprotein fragment from the surface of unfertilized Sp eggs. This material, purified to homogeneity, possessed all the attributes of a sperm receptor. It was an inhibitor of fertilization, it bound to the bindin-coated acrosome process, and it bound to bindin particles. All these binding experiments showed species-selectivity. This glycoprotein was sulfated and contained fucose, mannose, galactose and glucosamine, but it did not contain sialic acid. Chemical removal of O-linked oligosaccharides shifted the relative mass to 41-kDa [45]. Antiserum was prepared against the 70-kDa LysC fragment and western blots showed a single reaction with the egg surface glycoprotein of 350-kDa, which was evenly distributed on the egg surface. Fab of this antibody inhibited fertilization species-selectively. This 70-kDa LysC fragment was later found within the 350-kDa egg surface glycoprotein discussed in the preceding paragraph [40–44].

Antibody to the 70-kDa fragment was used for screening an expression library of Sp ovary cDNA and the deduced sequence published [46]. Later work showed that the original sequence contained errors, some of which probably resulted from chimeric cDNAs generated from the ligation of two or more unrelated cDNAs. (Using pBluescript as a vector we sequenced a 5.3-kb insert composed of four completely different Sp cDNAs; Moy and Vacquier, unpublished). The corrected full-length sequence of the 70-kDa mature protein is 889 amino acids. It does not have a signal sequence or a transmembrane segment, and has high sequence identity to the HSP 110 subfamily of chaperone (heat shock) proteins [47,48]. However, new antibodies to this sequence continued to show its location to be the egg surface [49,50]. The predicted protein mass of 105-kDa is much smaller than the 350-kDa intact gly-

coprotein, the anomalous migration attributed to the 350-kDa being 70% carbohydrate [47].

11. Further experiments with the 350-kDa egg surface sperm receptor

A new set of experiments involved the expression of different parts of the corrected 889 residue Sp sperm receptor sequence as GST-fusion proteins and then coupling the recombinant proteins to glutathione agarose beads. Two domains in the N-terminal half of the receptor had sperm binding activity. One was 247 residues and was not species-selective in its inhibition of fertilization. The other domain was 32 residues and was genus-selective, but not species-selective [51]. It was a surprise to find that bacterial-expressed, and thus carbohydrate lacking, protein showed fertilization inhibition or sperm binding activity, because all previous work had demonstrated the importance of sugars, especially sulfated sugars, in sperm-to-egg binding. Additional work showed that sperm gangliosides have affinity for the native form of the 350-kDa egg receptor for sperm. The bacterial-expressed N-terminal HSP 110 portion of the sperm receptor was required for sperm ganglioside affinity. Treatment of the sperm gangliosides with sialidase resulted in a loss of affinity. The intact 350-kDa receptor appears to bind sialic acid residues in the sperm lipid rafts and is thought to be a new form of sialic binding lectin. Liposomes made with the membrane rafts inhibit fertilization [52].

12. EBR1, a second, unrelated sea urchin egg surface bindin receptor

The two sea urchin species, Sp and Sf, occur together on the California coast and show species-selective fertilization. To attempt to isolate species-specific mRNAs from Sf ovaries the technique of "representation difference analysis" (cDNA subtraction) was used to search for cDNA fragments specific to the Sf species. One clone, named SfH2, was found that was large enough to code for the 350-kDa sperm receptor. Bacterial recombinant protein and antibody were prepared to SfH2. Western blots showed a reaction at 350-kDa for Sf, but not for Sp egg vitelline layers. Immunofluorescence showed the antibody reacted only with Sf VEs. Antibody to SfH2 inhibited fertilization of Sf eggs, but not of Sp eggs. Sf sperm bound to beads coated with SfH2 recombinant protein. Sf H2 protein inhibited Sf, but not Sp, egg agglutination. Radiolabeled SfH2 bound to isolated Sf bindin, but not to Sp bindin. Antibody to SfH2 does not react with the elevated Sf fertilization envelope. These experiments showed that the SfH2 protein meets all the criteria expected of a species-selective egg receptor for sperm bindin, and this sequence was therefore named egg bindin receptor 1 (EBR1). These experiments with recombinant SfH2 also showed that carbohydrate is not involved in EBR1's interaction with bindin, or acrosome-reacted sperm [53]. (Previous work had shown that bacterial recombinant bindin would agglutinate eggs species-selectively [54].)

The full-length cDNAs for Sf and Sp EBR1 were sequenced and translated into protein. Sp EBR1 is 3713 and Sf EBR1 4595 amino acids. Both proteins have a signal sequence, but neither has a transmembrane domain and both proteins are thought to be secreted into the extracellular matrix of growing oocytes. When aligned, the N-terminal halves of the two proteins are fairly similar. Sf has 19 "EBR repeats", each repeat being made of a CUB domain (Complement C1s/C1r, Uegf, BMP1) contiguous to a TSP domain (Thrombospondin-1), the two together being 171 residues. The last 9 Sf-EBR repeats are quite different in sequence from the first 10. The C-terminal half of Sp-EBR1 is made of 8.5 EBR repeats followed by 11 hyalin (HYR) repeats, each being 81 residues. Thus, the Sf and Sp C-terminal regions of the two EBR1 proteins are completely different from each other and are hypothesized to be responsible for species-selective affinity for bindin. DNA probes to these two species-unique regions do not cross hybridize on Southern blots, but probes to the 3′UTR sequences do cross hybridize to fragments of similar size [53]. The sea urchin egg EBR1 protein is not related to the 350-kDa egg surface sperm receptor glycoprotein.

13. Conclusions

13.1. Bindin

This sperm protein is one of the few so far isolated from sperm of any animal that clearly mediates sperm–egg binding and probably also fusion between gametes. The continued biochemical study of bindin will require a high resolution crystal structure. The only high resolution crystal structure known for a sperm acrosomal protein that fuses membranes is that of abalone 18 K, which shares no primary or secondary structural features with bindin [55]. Every major animal taxon may have evolved its own gamete fusion protein. The study of bindin sequences remains a boon to evolutionary population biologists [20–25], and such studies are now extending to EBR1 [20,23].

13.2. 350-kDa and EBR1 as the bindin (sperm) receptors of sea urchin eggs

The biochemical study of these two proteins has been difficult because of their size and aggregation in the sea urchin egg extracellular matrix. Both these egg surface molecules exhibit all the characteristics of species-selective bindin (sperm) receptors. With their discovery, the quest for the "egg surface sperm receptor" may have been satisfied. We were probably naive to think there would only be one sperm receptor on eggs. Fertilization bridges successive generations of animals by the fusion of single cells. Excusing the teleology, but evolution may have been prudent in providing a backup system to increase the probability of zygote formation. The recognition between egg 350-kDa sulfated glycosylation sites and sperm bindin might provide one adhesive system, and bindin's recognition of the protein backbone of EBR1 might provide another. Having a fail-safe system for important biological phenomena is well known, and it has been argued that such "degeneracy" is an inevitable consequence of natural selection [56]. The 350-kDa has been criticized because as an HSP homolog it should be intracellular. However, there are now many examples of extracellular HSPs that function immunologically and in cell signaling [57]. We will learn how gamete recognition occurs at the atomic level by the solution of the crystal structures of these gamete recognition proteins.

Dedication

During the past 25 years the Lennarz laboratory made many significant advances to our understanding of the proteins involved in sea urchin fertilization. This review is dedicated to Professor William J. Lennarz as thanks for his tenacity to have had the patience to stick with this difficult problem and use it to train many young scientists who went on to successful careers.

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