

The evolution of sea urchin sperm bindin

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ABSTRACT Sea urchins have been model organisms for the study of fertilization for more than a century. Fertilization in sea urchins happens externally, which facilitates the study of sperm-egg attachment and fusion, and means that all of the molecules involved in gamete recognition and fusion are associated with the gametes. Sea urchin sperm bindin was the first "gamete recognition protein" to be isolated and characterized (Vacquier and Moy 1977), and bindin has since been studied by developmental biologists interested in fertilization, by biochemists interested in membrane fusion and by evolutionary biologists interested in reproductive isolation and speciation. Research on bindin was last reviewed thirteen years ago by Vacquier *et al.* (1995) in an article titled "What have we learned about sea urchin sperm bindin?" in which the authors reviewed the identification, isolation and early molecular examinations of bindin. Research since then has focused on bindin's potential role in fusing egg and sperm membranes, comparisons of bindin between distantly related species, studies within genera linking bindin evolution to reproductive isolation, and studies within species looking at fertilization effects of individual bindin alleles. In addition, the egg receptor for bindin has been cloned and sequenced. I review this recent research here.

KEY WORDS: *sea urchin, bindin, gamete recognition protein, Echinoidea, fertilization*

Overview of early research

Interested readers should refer to Vacquier *et al.* (1995) for details and references regarding the early stages of study of bindin. An overview is provided here.

Ultrastructural analyses of sea urchin sperm suggested that the contents of the acrosomal granule coated the acrosomal process after the acrosomal reaction and bound sperm to eggs (Dan 1967; Summers *et al.*, 1975). Vacquier and Moy (1977) isolated the contents of the sea urchin acrosomal granule, which was shown to be a single protein. They named this protein 'bindin' for its role in binding sea urchin sperm to eggs. Agglutination experiments showed that bindin was species-specific in its binding to eggs (Glabe and Lennarz 1979; Glabe and Vacquier, 1977). That is, bindin from one sea urchin species was more effective at binding to eggs of its own species than it was at binding to eggs of another species. The cloning and sequencing of bindin first from *Strongylocentrotus purpuratus* (Gao *et al.*, 1986) and later from three other sea urchin species (*S. franciscanus*, *Lytechinus variegatus*, and *Arbacia punctulata* (Minor *et al.*, 1991; Glabe and Clark 1991)) gave insight into the structure and evolution of the molecule.

Bindin is translated as a precursor molecule that contains a

prepro- region which is subsequently cleaved to form the mature bindin that is found in sperm (Gao *et al.*, 1986; Fig. 1). Comparisons of bindin between species indicated that mature bindin consists of a 'core' region of 55 amino acids, highly conserved even among different sea urchin orders, with more variable regions flanking the core. Repeats are present in the flanking regions in some species (Fig. 1). Agglutination experiments with modified recombinant bindin and fertilization interference experiments with peptides based on partial bindin sequences provided evidence for a role in species-specificity for the variable flanking regions (Lopez *et al.*, 1993; Minor *et al.*, 1993).

Bindin as a membrane fusogen

Bindin binds sperm to eggs, and, in agglutination experiments, binds eggs to one another in the absence of sperm. It has been less clear if bindin has a role in sperm-egg membrane fusion, another critical step in fertilization. Early research showing that bindin could induce the fusion of phospholipid vesicles suggested a role for bindin in sperm-egg membrane fusion (Glabe 1985a, b). Subsequent research has focused on an 18 amino acid peptide derived from the conserved core region of bindin (Ulrich *et al.*, 1998). This peptide ('B18', with an amino acid sequence of

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LGLLLRHLRHHSNLLANI, Fig. 1) is present and its sequence is conserved in all species from which bindin has been cloned and sequenced (Zigler and Lessios 2003a). The B18 peptide self-associates and is able to induce the fusion of lipid vesicles (Ulrich *et al.*, 1998). The current 'boomerang' model for the action of the B18 peptide is that it forms a helix-break-helix motif with a hydrophobic α -helix at each end (Afonin *et al.*, 2004). The more hydrophobic of these α -helices inserts into the lipid bilayer, disrupting the lipid membrane, while the other α -helix remains on the surface of the membrane where it replaces water molecules in the lipid headgroups. In combination, these actions favor the aggregation and fusion of lipid membranes (Afonin *et al.*, 2004).

Although experiments with B18 deal with just a portion of the bindin molecule, the fact that the amino acid sequence of the B18 region is perfectly conserved across thirteen genera and more than thirty species of sea urchins supports the idea that it plays a central and conserved role in the function of bindin. Thus, it seems probable that bindin plays two roles in sea urchin fertilization: it functions in sperm-egg attachment and it is involved in the fusion of the gamete membranes.

Taxonomic distribution of bindin

How widely is bindin distributed taxonomically? Bindin was first cloned and sequenced from species representing two sea urchin orders, the Echinoidea and the Arbacioida (Gao *et al.*, 1986; Minor *et al.*, 1991). Bindin has since been cloned and sequenced from representatives of several other distantly related orders of class Echinoidea, including the sand dollars (Clypeasteroidea), the heart urchins (Spatangoida), the diadematoids (Diadematoida), and the pencil urchins (Cidaroida) (Zigler and Lessios 2003a). The pencil urchins are the sister group to all other extant echinoids, from whom they diverged approximately 250 mya (Smith *et al.*, 2006). The presence of bindin in the pencil urchins and many other echinoid orders places a minimum bound on its origin: at least 250 mya. Since that time, bindin has been conserved in at least six orders of sea urchins (Zigler and Lessios 2003a).

Bindin is widespread within the echinoids, but is bindin present

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1  MGVHQISVIVIVIVAFVTFTRAVDDFSPRTDVPSPDCPEASSGCWCHDSFAQCWRVYDDSHVT
61  SAMGNRITQLELLYQTSEEVVITYIRGMSALRKLRISEdGVSLDCSCDI IYALDDERVTLI
121 DQNELVFGNCREHGFFLDRMTARPFINHCHILRMQDAETRRKRRDADDEDDVSKRASPRK
181 GDKPAGHKINLKDLDPSPTKVHHLVSTDDADQLKHHPASDIVNFISRHRNRNRSAATGD
241 EVSDDSERGGRQKR

      YGNYPQAMNPPMGGGNY PAPGQAPMGQLAQQGYAAPGMGGPVGGGG
301  AMASPIGGGGAMARPVGFGGAMARPVGGGGAMARPVGGGGAMARPVGGGGAMARPVGGGG
361  AGPPGYGGISQAAGGNDEDYSSSSDEEETTISAKVMDNI KAVLGATKIDLPVDINDPYDL
421  GLLLRHLRHHSNLLANIGDPEVREQVLSAMQEEE EEEEEEQDAANGVRDNVLSNLNANGP
481  YRAGFGGGGGMHAGGGGGGGGRGGMGVVGRRGGGGMMGFPGMGQGNAYNPGYRQG

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Fig. 1. Deduced amino acid sequence of full-length bindin from *Echinometra lucunter* (Genbank Accession AAR18072, McCartney and Lessios (2004)). Preprobindin extends from amino acids 1-254, ending with the splice site (in italics). Mature bindin consists of amino acids 255-539. The core region is boxed, the fusogenic 'B18' region (Ulrich *et al.*, 1998) is in bold, the repeats 5' of the core are underlined, and the location of the intron is marked with an '*'. *

outside this class of animals? Thus far, there is no evidence that it is. Molecules homologous to bindin have not been identified in any of the non-echinoid metazoan genomes that have been sequenced, indicating that bindin is not likely present outside the echinoderms. Since the genome of only one echinoderm has been sequenced (that of *Strongylocentrotus purpuratus* (Sea Urchin Genome Sequencing Consortium 2006)) it remains possible that bindin is present in other classes of echinoderms (sea stars, brittle stars, sea cucumbers, and feather stars). There is so far no evidence to support this possibility. In fact, a bindin antibody failed to react with the sperm of three species of sea stars (Moy and Vacquier 1979), and genomic probes using a sea urchin bindin sequence on a sea cucumber and a sea star were negative (Minor *et al.*, 1991). An answer to whether bindin is present outside the echinoids awaits either the identification of bindin in some non-echinoid group, or else a series of convincing negative results in the form of sequenced genomes from other echinoderms.

Bindin evolution across orders

The six echinoid orders from which bindin is known diverged from one another 200 or more mya (Smith *et al.*, 2006). How has bindin evolved in these independent lineages over this great expanse of time? First, we can note three features of the molecule that have been conserved: the core, the presence of an intron, and the splice site between the preprobindin and the mature bindin (Fig. 1). The 55 amino acid core has been remarkably conserved, including a stretch of 29 amino acids in the center of the region that is the same in each of these different orders (Zigler and Lessios 2003a). The fusogenic B18 region is included within these 29 amino acids. Second, in the three orders from which genomic sequences of bindin are available, an intron is located five amino acids 5' of the beginning of the core region (Fig. 1). Third, a motif of four basic amino acids is conserved at the presumed splice site between the preprobindin and the mature bindin portion of the molecule (Zigler and Lessios 2003a; Fig. 1). Such motifs mark the cleavage site for proprotein convertases (Seidah and Chretien 1999) and suggest that bindin in all of these orders is processed by cleavage to form mature bindin.

How does bindin differ between species based on these examples from six different orders? Two major differences are evident. First, mature bindin differs greatly in length among these orders, ranging from 193 amino acids in *Encope (Mellitella) stokesii* (Order Clypeasteroidea) to 418 amino acids in *Diadema antillarum* (Order Diadematoida). A second major difference is that bindins from some orders contain loosely repeated motifs, whereas bindins from other orders do not. These repeats may be located 5' or 3' of the core region, and are typically glycine-rich. Repeats are present in bindin sequences of members of the order Echinoidea (e.g. in *Echinometra lucunter* 5' of the core

TABLE 1

SUMMARY OF STUDIES EXAMINING BINDIN EVOLUTION WITHIN SEA URCHIN GENERA

Genus	Species examined	Distribution of examined species	Positive selection?	Form of selection	Reference
<i>Arbacia</i>	4	All allopatric	No		Metz <i>et al.</i> 1998
<i>Echinometra</i> (Neotropical)	3	One allopatric, two sympatric	Yes	along the lineage leading to <i>E. lucunter</i>	McCartney and Lessios 2004
<i>Echinometra</i> (Indo-Pacific)	4	All sympatric	Yes	between species for region 5' of the core	Metz and Palumbi 1996
<i>Heliocidaris</i>	2	All sympatric	Yes	along the lineage leading to <i>H. erythrogramma</i>	Zigler <i>et al.</i> 2003
<i>Lytechinus</i>	6	Four allopatric, two sympatric	No		Zigler and Lessios 2004
<i>Strongylocentrotus</i>	7	All sympatric	Yes	between species for regions 5' and 3' of core	Biermann 1998
<i>Tripneustes</i>	3	All allopatric	No		Zigler and Lessios 2003

GMGGPVGGGG-AMASPIGGGG-AMARPVGFGG-AMARPVGGGG-AMARPVGGGG-AMARPVGGGG-AMARPVGGGR, Fig. 1) and in *Moiratropos*, the single representative of the Spatangoida that has been studied, but are not present in bindins of the studied species from the Cidaroida, Diadematoidea, Clypeasteroidea and Arbacioidea (Zigler and Lessios 2003a).

Bindin evolution within genera

In contrast to the situation when Vacquier *et al.* (1995) reviewed sea urchin bindin, where the only intrageneric comparison possible was between *Strongylocentrotus purpuratus* and *S. franciscanus*, there now exists considerable information about how bindin has evolved within different genera of sea urchins. Studies on six genera of sea urchins have been conducted (Table 1), and studies on several more genera are underway. Perhaps the most remarkable recent discovery about bindin has come from these studies: from one genus to another, patterns of bindin evolution differ greatly. Gamete recognition proteins from a wide range of taxa have been shown to evolve rapidly, often under positive selection (reviewed in Swanson and Vacquier 2002). This is the case in the echinoid genera *Echinometra*, *Heliocidaris*, and *Strongylocentrotus*. In each of these genera there is evidence of positive selection for change on bindin (Metz and Palumbi 1996; Biermann 1998; Zigler *et al.*, 2004; Table 1). However, in contrast to results from those genera, there is no evidence for positive selection in the genera *Arbacia*, *Lytechinus*, and *Tripneustes* (Metz *et al.* 1998; Zigler and Lessios 2004; Zigler and Lessios 2003b).

Consistent with its central role in sea urchin fertilization, bindin divergence is correlated with gamete incompatibility; species with bindins that are highly similar are gametically compatible, whereas those with bindins that have diverged from one another are gametically incompatible. In fact, bindin divergence better predicts gamete compatibility between species than the length of time since two species diverged (as estimated from mitochondrial DNA divergence) (Zigler *et al.*, 2005). In some cases patterns of bindin evolution give clear insight into the evolution of reproductive isolation. For example, among the neotropical species of *Echinometra*, positive selection and rapid change has occurred in bindin on the branch leading to *E. lucunter* (McCartney and Lessios 2004). Consistent with this burst of bindin evolution, the eggs of *E. lucunter* are gametically incompatible with the sperm of its close relatives *E. viridis* and *E. vanbrunti* (McCartney and Lessios 2002). Similarly, there has been a burst of changes in bindin along the lineage leading to *Heliocidaris erythrogramma*.

The eggs of *H. erythrogramma* are gametically incompatible with sperm from *H. tuberculata* (Zigler *et al.*, 2003).

What explains a history of positive selection on bindin in some, but not all, sea urchin genera? Does a single evolutionary force explain all observed selection on bindin, or might different forces be acting in different genera? Though these questions have been discussed recently, the answers are not clear (Palumbi and Lessios 2005; Lessios 2007). If selection on bindin is occurring due to interactions between species, reinforcement – selection to prevent the formation of unfit hybrids between closely related species – is one possible answer. Positive selection on bindin has only been observed in genera that have sympatrically distributed species (Table 1), which is consistent with the hypothesis that the observed pattern may be the result of reinforcement. Bindin evolution driven by reinforcement would increase interspecific bindin divergence, while minimizing intraspecific bindin divergence, as is observed in *H. erythrogramma* and *E. lucunter* (McCartney and Lessios 2004; Zigler *et al.*, 2005).

In addition, Geyer and Palumbi (2003) found reproductive character displacement for bindin alleles between the closely related Indo-Pacific species *E. oblonga* and *E. sp. C*. *E. oblonga* is found in the central and western Pacific, and exhibits four bindin types, that differ by nonsynonymous differences and small insertions, with allelic variation within each type. Two of these bindin types are found in *E. sp. C*, and two are not. In the central Pacific, where *E. oblonga* is present but *E. sp. C* is not, *E. oblonga* populations exhibit all four bindin types. In the western Pacific, where the species have overlapping ranges, the only *E. oblonga* bindin types observed are those that are not found in *E. sp. C*. Geyer and Palumbi (2005) have also shown that these allelic differences influence gamete compatibility. This pattern of distinct bindin types present in areas where the two species overlap is consistent with reinforcement between these two species. Thus, in both *Heliocidaris* and the neotropical *Echinometra*, reinforcement may explain the observed selection on bindin, and reinforcement may explain the distribution of bindin alleles within the ranges of *E. oblonga* and *E. sp. C*.

Bindin evolution within species

Reinforcement, however, is not sufficient to explain the observed patterns of bindin diversity within the Indo-Pacific *Echinometra* and the eastern Pacific *Strongylocentrotus*. These species display a large amount of allelic diversity and, in the Indo-Pacific *Echinometra* species, an excess of nonsynonymous changes to synonymous changes in intraspecific comparisons (Metz and Palumbi 1996; Debenham *et al.*, 2000). In addition, two

studies have examined the relationship between male bindin genotype and female bindin genotype with remarkable results. The first of these studies examined the Indo-Pacific species *E. mathaei*. *E. mathaei* has a large amount of intraspecific bindin diversity; on a gene genealogy for bindin from *E. mathaei*, four clades are evident: A, B, C, and D (after Palumbi 1999). These bindin clades differ by short insertions and deletions and nonsynonymous changes. Palumbi (1999) fertilized eggs from females with known bindin genotype with a mixture of sperm from two males: one homozygous for 'A' type bindin alleles, and one homozygous for 'B' type bindin alleles. Individual larvae were subsequently genotyped. He found that eggs from 'AA' females were more likely to be fertilized by sperm from 'AA' males, and that eggs from 'BB' females were more likely to be fertilized by sperm from 'BB' males. Thus, *E. mathaei* individuals exhibit assortative mating based on bindin genotype, at least in the particular cross examined.

Levitan and Ferrell (2006) studied male and female reproductive success in induced field spawnings of groups of *Strongylocentrotus franciscanus*. *S. franciscanus* is another species with substantial intraspecific bindin diversity; in the population studied here, fifteen alleles were identified. In addition to finding the expected effects relating to distance between individuals, Levitan and Ferrell (2006) uncovered the remarkable pattern that at low densities, individuals that shared their bindin genotype with a high frequency of potential mates had the greatest reproductive success, whereas at high density, the reverse was true. At low densities, this is similar to Palumbi's (1999) result with *E. mathaei*. At high densities, it may be disadvantageous to match bindin genotypes with (and thus fertilize well with) many potential mates due to the danger of polyspermy, which prevents normal development and decreases reproductive success at high sperm concentrations. Thus, an interaction of sperm density with bindin allele frequency is likely to have significant effects on bindin evolution within this species. In a high density population, the persistence and spread of rare bindin alleles will be favored, as females with rare alleles are in less danger of losing eggs to polyspermy. In a low density population, the persistence and spread of rare bindin alleles could be suppressed if females with these alleles have reduced reproductive success. Whether or not this density dependent pattern is present in species other than *S. franciscanus* is not known.

Why is mating success influenced by the bindin genotype of females at all? Since there is no evidence that bindin is expressed in ovaries (thus ruling out some sort of a bindin-bindin interaction between sperm and egg at fertilization) (Gao *et al.*, 1986), the most likely explanation for the results in *Echinometra mathaei* and *Strongylocentrotus franciscanus* is that linkage disequilibrium exists between bindin and some other locus involved with fertilization. Such a linkage is expected, because offspring of a female with a particular preference for a male trait will inherit both the mother's preference and the father's trait. The most likely candidate for the other half of this possible relationship, the egg receptor for bindin, is discussed below.

Bindin receptor

Progress towards identifying the egg receptor stalled until Kamei and Glabe (2003) cloned and sequenced the egg receptor

for bindin (EBR1) from *Strongylocentrotus franciscanus* and *S. purpuratus*. In contrast to bindin, EBR1 is a massive molecule – 4595 amino acids in *S. franciscanus*, 3713 amino acids in *S. purpuratus* (Kamei and Glabe 2003). EBR1 contains a variety of repeats, and the number and structure of these repeats differs between the two species. EBR1 has not yet been cloned and sequenced in any other species, and assessing intraspecific variation of such a large molecule is daunting. Doing so, however, should answer the question as to whether linkage disequilibrium between bindin and EBR1 explains the intraspecific fertilization results from *Echinometra mathaei* and *S. franciscanus*. Additionally, though the sequencing of the *S. purpuratus* genome is complete, contigs have not yet been mapped onto chromosomes (as of October 2007). Once this has been done, we will know if there is physical linkage between bindin and EBR1, at least in *S. purpuratus*.

Future directions

Vacquier *et al.* (1995) proposed three lines of future research: studying bindin evolution in the neotropical *Echinometra*, conducting fertilization experiments to determine the effects of intraspecific variation on fertilization success, and solving the crystallographic structure of bindin. The first of these lines of research has been completed, the second has seen promising beginnings, and there has been no progress on the third. McCartney and Lessios (2004) examined bindin evolution in the neotropical *Echinometra* and identified an episode of positive selection on the *E. lucunter* lineage that is correlated with the evolution of gametic incompatibility. Studies by Palumbi (1999) and Levitan and Ferrell (2006) on intraspecific variation have opened the door to potential explanations of how bindin evolves within species. Unfortunately, the crystal structure of bindin has not been solved, which has limited the ability of researchers to interpret their cell biological and evolutionary results.

What are promising questions for researchers to pursue in the future? As suggested by Vacquier *et al.* (1995), obtaining the crystal structure of bindin would be invaluable. Combining the atomic structure of bindin with amino acid sequences known from more than thirty species would allow for the clearest picture yet of how bindin functions and evolves. Mapping amino acid changes between closely related species onto a crystal structure would clarify how changes in bindin are related to gamete compatibility. Another useful line of research would be to clarify how the complete bindin molecule, not just the B18 region, interacts with membranes. Research along these lines would support or reject the idea that bindin functions as a membrane fusogen. Finally, further research clarifying how bindin and the egg receptor for bindin interact would be valuable. Sea urchins are one of the few systems where the egg and sperm participants in sperm-egg attachment have been characterized. Research on the interactions of these molecules promises to provide insight into gamete interactions in general.

Studies of bindin evolution of distantly related echinoids would be informative. Though the echinoids are a diverse group, the vast majority of research on bindin evolution has focused on members of a single order (the Echinoida) of sea urchins. Does bindin evolve differently in sand dollars, heart urchins or pencil urchins? At present, we have no idea. One particularly interesting genus for

study is *Moira*, a heart urchin. *Moira* consists of five species that are allopatrically distributed. Bindin has been cloned from *Moira clotha*, and it contains repeats 5' of its core (Zigler and Lessios 2003a). To date, positive selection on bindin has only been observed in genera that have repeats, and only in genera that are members of the order Echinoidea. Studying *Moira* gives us a chance to separate those two features: is the presence of repeats correlated with positive selection, or is positive selection correlated observed only in the order Echinoidea? Studies of bindin in other species of the Spatangoida would also help clarify whether the repeats seen in *Moira* are specific to this genus, or are a shared feature of all spatangoids.

Last, we need to know if bindin is in linkage disequilibrium with its receptor (or some other egg component), particularly in *Echinometra mathaei* and *Strongylocentrotus franciscanus*, where current evidence suggests such linkage disequilibrium exists (Palumbi 1999; Levitan and Ferrell 2006). Should this be true in these species, it will then be important to determine how broadly this is true; again, the echinoids are a diverse group. If linkage disequilibrium is present, then several questions can be addressed: Does the presence of linkage disequilibrium explain patterns of intraspecific bindin diversity across the echinoids? Does the presence of linkage disequilibrium explain interspecific patterns of bindin evolution, or do other selective forces, such as reinforcement, play a role? Finally, do echinoid groups that exhibit linkage disequilibrium speciate more rapidly than groups that do not?

What have we learned about sea urchin bindin over the past thirteen years? We now have a clearer picture of how bindin functions, how it is distributed taxonomically, how it evolves, and with what it interacts with on the egg surface. Many promising avenues of research are open, and hopefully the next thirteen years will see today's mysteries solved, and new questions raised.

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