MINI REVIEW
Glycobiology of sperm–egg interactions in deuterostomes

Kathryn J. Mengerink¹ and Victor D. Vacquier
Center for Marine Biotechnology and Biomedicine, Scripps Institution of Oceanography, University of California, San Diego, La Jolla CA 92093-0202, USA
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The process of fertilization begins when sperm contact the outermost egg investment and ends with fusion of the two haploid pronuclei in the egg cytoplasm. Many steps in fertilization involve carbohydrate-based molecular recognition between sperm and egg. Although there is conservation of gamete recognition molecules within vertebrates, their homologues have not yet been discovered in echinoderms and ascidians (the invertebrate deuterostomes). In echinoderms, long sulfated polysaccharides act as ligands for sperm receptors. Ascidians employ egg coat glycosides that are recognized by sperm surface glycosidases. Vertebrate egg coats contain zona pellucida (ZP) family glycoproteins, whose carbohydrates bind to sperm receptors. Several candidate sperm receptors for vertebrate ZP proteins have been identified and are discussed here. This brief review focuses on new information concerning fertilization in deuterostomes (the phylogenetic group including echinoderms, ascidians, and vertebrates) and highlights protein–carbohydrate interactions involved in this process.

Key words: acrosome reaction/fertilization/protein–carbohydrate recognition/sperm–egg interaction/zona pellucida

Introduction
Fertilization is a multistep process and a unique event that involves fusion of two haploid gametes to form a diploid zygote. Sperm must locate, adhere to, and fuse with the egg. The egg must prevent further sperm fusion to avoid pathological polyspermy, a lethal condition. Eggs are surrounded by an extracellular matrix, which varies in composition among animal groups (Figure 1). When sperm contact this matrix, there are primary binding events that, in most deuterostomes, lead to the sperm acrosome reaction (AR). The AR is triggered by increases in intracellular Ca²⁺ and pH (Darszon et al., 1999) and results in exocytosis of the acrosomal vesicle (an organelle in the sperm head). Following the AR, secondary binding events occur, and the membrane exposed by the AR fuses with the egg plasma membrane. Protein–carbohydrate interactions play a critical role in this complex process. Because there is such a vast literature, this brief review will focus only on deuterostome sperm–egg recognition events that involve carbohydrate–protein interactions.

Nonvertebrate deuterostomes

Echinoderms
Echinoderms are marine invertebrates found at the base of the deuterostome lineage. Because they spawn large quantities of gametes into sea water, they make excellent model organisms for studying molecular events involved in sperm–egg interaction.

Sea urchins
In sea urchins, protein–carbohydrate interactions take place both at the egg jelly and the vitelline layers. Sperm first contact the egg jelly layer. A 210-kDa multidomain

¹To whom correspondence should be addressed

Fig. 1. A diagram showing the various types of extracellular matrices surrounding deuterostome eggs. The ascidian extracellular matrix is composed of follicle cells (FC), a vitelline coat (VC), and a perivitelline space containing test cells (TC). The echinoderm has egg jelly (EJ) and a vitelline layer (VL). The mammalian extracellular matrix contains a cumulus matrix (CM) including cumulus cells and a zona pellucida (ZP). The teleost extracellular matrix has a dilute mucous area (DMA) and a thick chorion that has a hole through it called the micropyle. Amphibians have an EJ and VC.
Starfish There are three major components of starfish (Asterias amurenensis) egg jelly—glycoproteins, sulfated steroid saponins, and oligopeptides. The AR-inducing substance (ARIS), an enormous molecular composed of approximately 33% protein, 47% carbohydrate, and 10% sulfate. ARIS has a molecular enormous molecule composed of approximately 33% protein, amurensis et al. (DeAngelis and Glabe, 1990). However, the function of the binds to sulfated fucose polymers from the egg surface 1995; Kitazume-Kawaguchi 1995). Isolated ARIS is capable of inducing AR (Vacquier and Moy, 1997). Females of Echinometra lucunter a critical role in sperm–egg recognition. Based on studies of Ciona intestinalis and Phallusia mammillata (Hoshi et al., 1983, 1985), a glycosidase on the surface of sperm was proposed to recognize and bind to glycosides within the vitelline coat (VC) of the egg. In the case of C. intestinalis, the sperm glycosidase, α-L-fucosidase, binds to terminal L-fucose residues of the VC. For P. mammillata, N-acetylglucosaminidase recognizes terminal GlcNAc. The enzymes are thought to act as lectins because their pH optima (∼3.9) is well below that of sea water (pH 8) and the rate of hydrolysis is drastically reduced. Examination of glycans present in the vitelline coat of Halocynthia roretzi eggs, where sperm–egg binding is mediated through α-L-fucosidase on the sperm surface, indicates that the crucial glycans are O-linked and sulfated (Baginski et al., 1999). Not only do glycosidases occur on the surfaces of sperm, they are also released from the eggs at fertilization as a block to polyspermy (Lambert, 1986, 1989; Matsuura et al., 1993, 1995). The evidence suggests that a specific glycosidase on the sperm surface binds to its respective glycoside on the egg VC. This binding triggers eggs to release large quantities of a similar glycosidase, which prevents the binding of supernumerary sperm.

Carbohydrates also play a role in ascidian sperm–egg interactions at the egg plasma membrane. When vitelline envelopes are removed from the eggs of Ascidia ceratodes, application of wheat germ agglutinin (WGA; >10 μg/ml) will biochemically activate the eggs, as if they had been fertilized. Lower concentrations of WGA do not activate eggs but reduce the ability of sperm to fertilize eggs (Flannery and Epel, 1998).

Vertebrates The extracellular matric surrounding eggs of vertebrates vary between phylogenetic groups. However, they all contain members of the ZP glycoprotein family in their egg coats (called the zona pellucida [ZP] in mammals, the vitelline envelope [VE] in amphibians, and the chorion in teleost fish). No such ZP proteins have yet been described in nonvertebrate deuterostome or protostome egg envelopes. Recent reviews have appeared on the molecular basis of sperm–egg interactions in mammals (Brewis and Moore, 1997; Shalgi and Raz, 1997; Dell et al., 1999; Takasaki et al., 1999; Wassarman, 1999a,b; Evans, 2000; Prasad et al., 2000). Therefore, only the most recent findings regarding the role of carbohydrates in sperm–egg interaction will be mentioned. Because most of the work on ZP proteins concerns the mouse, this portion of the review will focus on the mouse model and include additional information from other vertebrates.

Glycoproteins of the ZP ZP3 The mouse ZP is composed of three glycoproteins, mZP1, mZP2, and mZP3 (also called ZPB, ZPA, and ZPC), that are crucial for its structural integrity. mZP2 and mZP3 dimerize to create long filaments that are cross-linked by mZP1. In addition to being structural components, ZP glycoproteins bind
to sperm receptors, causing them to cluster and induce signal transduction events leading to the sperm AR. O-linked oligosaccharides of mZP3 are the ligands for sperm that are involved in primary binding and induction of the AR (Florman and Wassarman, 1985 and reviewed by Dell et al., 1999; Shalgi and Raz, 1997; Wassarman et al., 1999; Wassarman, 1999b). Site-directed mutagenesis shows that mutating Ser-332 or Ser-334 to Ala results in complete inactivation of mZP3 (Chen et al., 1998), indicating the importance of O-linked oligosaccharides at these sites. The structures of the sperm-binding/AR-inducing components of the O-linked oligosaccharides have yet to be determined. However, several studies have begun to tackle the difficult problem of determining the crucial ZP sugars involved in sperm–egg interactions in vertebrates.

Examination of sperm–egg binding by analysis of ZP sugars and the use of neoglycoproteins, monosaccharides, and other polysaccharides have yielded conflicting results. The bioactivity of mZP3 is not dependent on sulfation, N-linked oligosaccharides, or sialic acid residues (Litscher and Wassarman, 1996; Liu et al., 1997). However, removal of sialic acid from fixed eggs increases sperm binding, suggesting that these residues may conceal sperm binding sites (Mori et al., 1997). Man-BSA, GlcNAc-BSA, and GalNAc-BSA are capable of inducing the mouse AR, whereas Glc-BSA and Gal-BSA have no effect. The same monosaccharides applied at millimolar concentrations neither induce nor block the AR (Loeser and Tulsiani, 1999), suggesting that multivalent interactions between carbohydrates of the ZP and their receptors on sperm are necessary for AR induction. Application of L-type Ca2+ channel blockers verapamil or diltiazem to sperm block the mZP3-induced AR. The same monosaccharides inhibit sperm binding to eggs to a greater extent than incubation with the trisaccharide inhibitor, pertussis toxin, which blocks the mZP3-induced AR, whereas the N-terminal peptide blocker, gp43 (ZPC or ZP3), contains several O-linked and two N-linked glycosylation sites, one of which is conserved from teleost to humans (Burkin and Miller, 2000). The pig ZP contains ZPA, ZPB, and ZPC (homologues of mZP2, mZP1, and mZP3), and unlike mouse, ZPB (ZP1) is the sperm binding protein (Yonezawa et al., 1997; Kudo et al., 1998). Analysis of ZPB carbohydrates yields conflicting results. Some studies find that O-linked oligosaccharides and not N-linked oligosaccharides are responsible for sperm–egg binding (Yurewicz et al., 1991, 1993), but other studies identify neutral N-linked oligosaccharides of ZPB as the sperm-binding components (Yonezawa et al., 1997; Kudo et al., 1998).

Among amphibians, Xenopus laevis has provided the most information about sperm–egg recognition. Egg VE glycoprotein, gp43 (ZPC or ZP3) contains several O-linked and two N-linked glycosylation sites, one of which is conserved from teleost to humans (Kubo et al., 1997; Yang and Hedrick, 1997). Proteolytic cleavage of gp69/64 (ZPA or ZP2) during fertilization results in removal of 27 amino acids from the N-terminus and loss of sperm binding. The N-terminal peptide may contain an O-linked glycan that is involved in the binding process (Tian et al., 1999). Another analysis of Xenopus ZP proteins identified complex N-linked oligosaccharides of ZPC (mZP3) as the major sperm binding ligands and that sperm binding involves GlcNAc and Fuc residues. Furthermore, mixing isolated ZPA, ZPB and ZPC in a ratio of 1:4:4 (as occurs in the ZP) results in the binding of more sperm than the sum of the separate components (Vo and Hedrick, 2000). This result suggests that instead of having one sperm-binding protein, the molecules may act synergistically to bind sperm.

The chorion surrounding eggs in teleost fish is multilayered and varies in thickness and number of layers. The zebrafish (Danio rerio) has a chorion composed of three morphologically distinct layers and contains four major proteins (116, 97, 50, and 43 kDa; Bonsignorito et al., 1996). The homologue of mZP2 was the first ZP protein identified in teleost fish (Lyons et al., 1993), and others have been identified since then. Both ZP2 and ZP3 cDNA clones have been identified in zebrafish, but the relationship of the proteins of the zebrafish chorion to the ZP protein family is unknown. In zebrafish ZP3, only one putative N-glycosylation site and no O-glycosylation sites exist (Wang and Gong, 1999). Interestingly, most teleost sperm lack an acrosome. Instead of penetrating through the chorion, they reach the egg plasma membrane by swimming directly through the follicular epithelium.

Furthermore, Gal residues localize to the inner portion of the ZP, indicating that the initial contact between the ZP and sperm does not involve Gal residues (Aviles et al., 2000). Studies of other monosaccharides have yielded similarly confusing results. For example, addition of an α-3-fucose residue to the trisaccharide Gal-α-1→3-Gal-β-1→4-GlcNAc to form Gal-α-1→3-Gal-β-1→4(Fuc-α1→3)-GlcNAc, yields a tetrasaccharide with high inhibitory activity (Johnston et al., 1998). However, there is no evidence of fucosylation of O-glycans based on structural analysis of carbohydrates from mouse eggs (Easton et al., 2000). These examples highlight the difficulties of sorting out biologically relevant carbohydrates involved in sperm–egg interactions. Understanding this process is further complicated by the fact that sperm–egg binding involves both low and high-affinity ZP binding sites on sperm (Thaler and Cardullo, 1996).
through a hole in the chorion called the micropyle, implying that AR-inducing oligosaccharides may be unnecessary. In the medaka fish (*Oryzias latipes*), two groups of glycoproteins exist in the chorion, ZI-1,2 and ZI-3, whose precursors, choriogenin H and choriogenin L, correspond to ZP2 and ZP3 (Murata et al., 1995, 1997). ZI-1,2 and ZI-3 are sparsely distributed throughout a broad dilated mucous area (DMA) on the surface of the chorion and within the micropyle (Iwamatsu et al., 1997). It is thought that the sperm bind to ZI-1,2 and ZI-3 to maneuver across the surface of the egg through the DMA until locating the micropyle.

**Sperm receptors for egg ZP glycoproteins**

The evidence for mZP3 being the ligand for mouse sperm and the inducer of the AR is well supported, but the sperm receptor for mZP3 remains controversial. Candidate ZP receptors will be discussed.

Acrosin is an acrosomal protease, originally thought to be involved in digesting a passage through the ZP. Several lines of evidence suggest that acrosin binds to sulfated polysaccharides of the ZP, as well as Fuc-BSA, Man-BSA, and non-ZP polysulfate saccharides (Jones, 1991; Urch and Patel, 1991; Jones et al., 1988). The function of acrosin in mice is questionable, because sperm of acrosin-null mice are still capable of passing through the ZP, a swella as Fuc-BSA, Man-BSA, and non-ZP carbohydrates of the ZP. Unlike mouse GalTase, GalTase on the sperm plasma membrane (Macek et al., 1991). Anti-GalTase antibodies (but not recombinant boar acrosin) bind to the ZP but do not block sperm penetration (Crosby and Barros, 1999). Recent evidence suggests that acrosin’s proteolytic activity may function in the dispersal of the acrosomal vesicle contents after the AR (Yamagata et al., 1998). Thus, the function of acrosin in the mammalian AR will require further work.

β-1→4Galactosyltransferase (GalTase) has been extensively studied as a mammalian sperm receptor involved in sperm binding to mZP3. Agents that inhibit GalTase and addition of purified GalTase inhibit sperm-zona binding in vitro (reviewed by Shur et al., 1998). GalTase specifically recognizes the oligosaccharides of mZP3 that have sperm-binding activity but does not interact with other mZP glycoproteins (Miller et al., 1992). mZP3 is thought to elicit the AR by cross-linking or aggregating the sperm receptor on the plasma membrane (Leyton and Saling, 1989). Anti-GalTase antibodies (but not their Fab fragments) will induce the AR by aggregating GalTase on the sperm plasma membrane (Macek et al., 1991). Multivalent GlcNAc-BSA is also capable of inducing the mouse sperm AR, whereas millimolar concentrations of the unconjugated sugar have no effect (Loeser and Tulsi, 1999). Structural analysis of mouse ZP glycans demonstrates that the ligand for GalTase, GlcNAc, is only present on N-linked and not O-linked oligosaccharides (Easton et al., 2000). GalTase has been localized to the anterior portion of the sperm head in several mammalian species, including guinea pig, mouse, rat, bull, pig, and rabbit (Larson and Miller, 1997). GalTase on the surface of porcine sperm binds the ZP. Unlike mouse GalTase, addition of uridine diphosphate galactose has no effect on sperm binding to the oocyte, nor does removal of zona lipids by N-acetylgallosaminidase (Rebeiz and Miller, 1999). This would argue that GalTase is not necessary for sperm–zona binding and the AR in pigs. Furthermore, GalTase-null male mice are fertile. However, in vitro studies show that the mutant sperm bind less mZP3 than wild type and do not undergo the AR in response to ZP3 or anti-GalTase antibodies (Lu and Shur, 1997). This points out the difficulty of correlating effects observed in vitro with the natural process occurring in vivo.

sp56 was identified on the basis of its affinity for mZP3 (Bleil and Wassarman, 1990). Furthermore, it was shown that sp56 localizes to the outer surface of the sperm head and that sperm binding glycopeptides of mZP3 can be crosslinked to sp56 (Cheng et al., 1994). The cDNA sequence revealed sp56 to be a peripheral membrane protein that contains seven sushi domains and a highly basic COOH-terminal domain (Bookbinder et al., 1995). AM67 is a guinea pig homologue of sp56 that localizes within the acrosome. Reexamination of the localization of sp56 in mouse sperm revealed that it was also found inside the acrosome (Foster et al., 1997). Whether sp56 is exclusively internal or external remains unresolved.

Compelling evidence exists that acrosin, GalTase, and sp56 interact with carbohydrates of the ZP and are important components of sperm–egg interaction. The difficulty lies in teasing out the exact function of each of these receptors in vivo.

**Secondary sperm receptors**

Several sperm receptors have been identified that are thought to be involved in secondary binding of acrosome-reacted sperm to egg extracellular matrices. β-N-acetylgallosaminidase is released from mouse sperm during the AR, and the inhibitor, PUGNAC, prevents sperm penetration through the ZP. The glycosidase is thought to remove terminal GlcNAc, releasing the sperm so that it can move through the ZP (Miller et al., 1992). In the toad *Bufo arenarum*, the enzyme is released from sperm and binds to the VE. Furthermore, inhibition of this enzyme results in inhibition of fertilization in vitro (Martinez et al., 2000).

Hyaluronan is a glycosaminoglycan composed of the disaccharide repeat (GlcNAcβ-1→4GlcAβ-1→3)n, and hyaluronidases selectively degrade the polymer. In mammals, hyaluronan is found in the cumulus matrix surrounding the ZP and the egg perivitelline layer surrounding the plasma membrane (Kan, 1990; Dandekar et al., 1992; Camaioni et al., 1996). PH-20 is a glycosyl phosphatidylinositol–anchored membrane protein first identified on the posterior head of guinea pig sperm. It has an N-terminal hyaluronidase domain that is used by acrosome-intact sperm to penetrate the cumulus matrix. Its C-terminal domain is thought to be involved in secondary sperm binding, but the mechanism remains unknown (Hunnicutt et al., 1996). Homologues of PH-20 have been identified and localized to the same regions in sperm from several other mammalian species, including mouse, rat, human, and macaque (Thaler and Cardullo, 1995; Sabeur et al., 1997; Yudin et al., 1999; Seaton et al., 2000).

In pigs, a ligand recognized by P-selectin is present in the ZP, and P-selectin exists on the acrosomal membrane of sperm. P-selectin is only detected by antibodies in acrosome-reacted sperm, suggesting that it plays a role in sperm-egg recognition following the AR (Geng et al., 1997). Removal of sialic acid (an important glycan of P-selectin ligands) from mZP3 does not affect binding to mouse sperm or the AR (Litscher and Wassarman, 1996), lending further support to the notion that P-selectin is involved in secondary binding. However, P-selectin deficient mice are fully fertile (Mayadas et al., 1993).
Summary
The molecules of sperm–egg recognition in echinoderms appear to be entirely different from those of ascidians and vertebrates. An intriguing possibility is that a sea urchin REJ homologue is a mammalian sperm receptor. A testis-specific mammalian homologue of REJ, PKDREJ, of unknown function, has been cloned from mouse and human (Hughes et al., 1999). The ascidian sperm–egg recognition system involves glycosidases binding to their appropriate glycodies. In vertebrates, glycosidases have also been implicated in sperm–egg binding (Martinez et al., 2000), and in cleaving glycodies so that the sperm can penetrate the ZP (Miller et al., 1992). Among vertebrates, evidence indicates that the ZP proteins are the crucial molecules responsible for the initial sperm–egg recognition events. However, the ZP protein family members serve different functions and are differentially glycosylated in the egg coats of different vertebrate groups. Furthermore, there is indirect evidence that carbohydrates play a role in species-specificity of sperm binding in vertebrates (Rankin et al., 1998; Doren et al., 1999). Although there is good support that oligosaccharides of the ZP proteins are crucial for primary sperm binding and induction of the AR in vertebrates, the identification of the ZP receptor on sperm remains uncertain. sp56, proacrosin, and GalTase are all candidate ZP receptors. The main focus of research to date has been in identifying primary binding events, but it is apparent from these data that there are many more potential factors in sperm–egg interactions leading to the fusion of two gametes. Much more work needs to be done to clearly delineate the complicated processes of sperm–egg interaction during fertilization.

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Abbreviations
AR, acrosome reaction; ARIS, acrosome reaction–inducing substance; CRDs, C-type lectin domains; DMA, diluted mucous area; FSP, fucose sulfate polymer; GalTase, β-1→4-galactosyltransferase; REJ, receptor for egg jelly; VC, vitelline coat; VE, vitelline envelope; WGA, wheat germ agglutinin; ZP, zona pellucida.

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