

The ascidian egg envelope in fertilization: structural and molecular features

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ABSTRACT In this report, unpublished and recent findings concerning the structure and function of the ascidian egg coat are compiled in context with fertilization. In the initial stage of ascidian fertilization, sperm interact with a complex egg investment that consists of a layer of follicle cells attached to an acellular vitelline coat. Increasing evidence exists that ascidian sperm are activated at their encounter with the follicle cells. The molecular basis of sperm-follicle cell interactions is discussed in context with sperm binding, membrane proteins and sperm bound glycosidase. The model that suggests a block to polyspermy established by glycosidase released from the follicle cells on fertilization is evaluated and compared with assured facts. Although a number of questions remain to be answered, our recent findings that a cloned β -hexosaminidase from *P. mammillata* binds exclusively to the follicle cells of unfertilized but not fertilized eggs, indicates that the follicle cells participate in the block to polyspermy. A dual function, mediating sperm activation and a block to polyspermy attributes to the ascidian follicle cells a key position in fertilization.

KEY WORDS: fertilization, ascidian, egg coat, follicle cell

The ascidian egg envelope: a survey of the structural features

Ascidian eggs are surrounded by an acellular envelope called chorion or vitelline coat (VC) to which follicle cells (FCs) adhere externally and test cells (TC) internally. Multitudinous functions have been attributed to the egg envelope as entity and to each of its structural components. In the present paper we focus on the role of the egg envelope in fertilization particularly the function of FCs in sperm egg-interaction.

The pictures of *Phallusia mammillata* eggs (Fig. 1 A,B) give a view of the egg and egg envelope representative for solitary and colonial ascidians with external fertilization. The egg of about 130 μm in diameter exhibits a relatively smooth plasma membrane with only few short microvilli. It is surrounded by an acellular VC with test cells attached to the inner and follicle cells to the outer surface. In most solitary species, the follicle cells are cubical or columnar or rarely tall cone-shaped as in *Ciona*. Test cells typically adhere to the inner matrix of the VC not to the egg surface. In some species, they form an almost closed sheet in others they are loosely arranged in groups. Although scanning electron microscopy (SEM) pictures reveal a good 3D impression,

the real dimension of the highly hydrated VC layers is never preserved and the highly vacuolated follicle cells are shriveled. This is also the case if conventional preparation is applied for transmission electron microscopy (TEM). Due to dehydration the VC appears as a relatively thin bi- or tripartite filamentous structure of about 400 to 900 nm (Honegger, 1986; Xie and Honegger, 1993; Cloney, 1995; Burighel and Cloney, 1997). Especially the filamentous structure of the inner VC layer mostly appears compressed and its different appearance in diverse species may be an artifact. Therefore, quick-freeze, deep-etch rotary-shadowing (DERS) techniques were applied to visualize the hydrated structure of the ascidian egg envelope. Two short reports (Koch *et al.*, 1993, Rosen *et al.*, 1994) presented precise descriptions but not

Abbreviations used in this paper: BSSP, biotinylated sperm surface proteins; CDL, central dense layer; CMFSW, calcium-magnesium free sea water; DERS, deep-etch rotary-shadowing; ECM, extracellular matrix; FAK, focal adhesion kinase; FCs follicle cells; FITC, fluorescein isothiocyanate; GlcNAc, N-acetylglucosamine; IFL, inner fibrous layer; MA, microannuli; OFL, outer fibrous layer; SEM, scanning electron microscopy; TC, test cells; TEM, transmission electron microscopy; VC, vitelline coat; WGA, wheat germ agglutinin.

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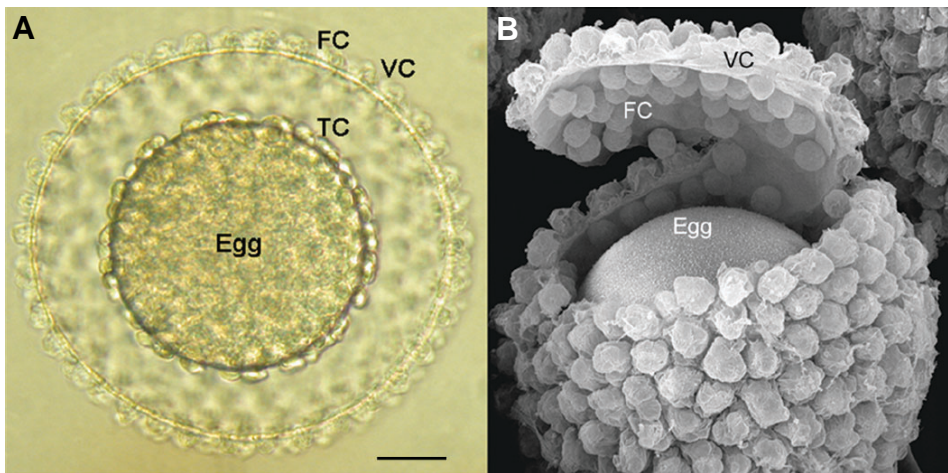


Fig. 1. The egg coat of *Phallusia mammillata*. (A,B) Light micrograph and scanning electron micrograph of an egg of *Phallusia mammillata* featuring the structure of the egg coat with follicle cells (FC), vitelline coat (VC) and test cells (TC). (A) The egg cell is kept in a central position by the transparent hydrated fibrillar matrix which fills the perivitelline space between the TC and the birefringent VC. (B) The partly lifted egg coat provides insight into the egg cell. The TCs are attached at the shrunken fibrillar inner layer of the VC. Scale bar, 35 μm .

illustrations of the *Ascidia ceratodes* egg coat prepared by these techniques. We investigated the egg coats from several phlebobranch ascidians by using quick freezing, deep etching and also freeze substitution techniques (Eisenhut and Honegger, 1997). Although shrinkage cannot be completely avoided this method gives a much more naturalistic view concerning dimension and structure of the egg coat. The most drastic difference concerns the structure of the VC and the follicle cells. The inner fibrous layer (IFL) of the VC spans 4 to 9 μm that means about ten times the dimensions observed after conventional fixation. The sperm head that measure uncoupled from the mitochondrion 4 μm to 7 μm in length and 0.5 μm in diameter has to pass this layer. Its fibrous matrix is made of fibers with diameters 15 to 85 nm and a mesh width between 0.4 to 1.2 μm . If sperm can pass by physical force solely or needs the participation of the protease used for the penetration of the central most resistant central dense layer CDL (Lambert *et al.*, 2002) is a matter of debate. The CDL is apparently only slightly hydrated and therefore features roughly the same structure and thickness independent of the preparation methods used. On the other hand, the real dimension and structure of outer VC layer (OFL) becomes visible only with freeze substitution. The OFL matrix with an overall thickness of 0.7 to 1.7 μm is formed by interconnecting fibers 15 to 30 nm in diameter that are anchored in the CDL layer. The major importance of the OFL is its suggested function in sperm binding, a transitory process which in ascidians usually lasts only few seconds before the sperm penetrates the VC. Sperm-VC binding has been illustrated in a number of studies by fairly good conventional TEM pictures (Fukumoto, 1988, Fukumoto and Numakunai, 1995) but unfortunately never with freeze substituted eggs.

Follicle cells which consist mainly of several large vacuoles arranged around a central nucleus. They appear fluid-filled in contrast to conventional preparation for TEM where the shrunken vacuoles exhibit only small clusters of coagulated mate-

rial. At their base follicle cells are attached to the OFL. Cell-cell contact between FCs is established by several filopodia which contain actin filaments (Honegger, 1986). Typical junctions cannot be recognized at contact site of the filopodia. FCs closely adjoin to each other leaving in the natural hydrated state only extremely narrow gaps between them. Even SEM preparations with slightly shrunken cells illustrate that sperm have to edge their way through and get inevitable in close contact with the surface of the FCs (Fig. 3A). However, the FCs surface they are contact during this passage is not the cell membrane proper but an extracellular matrix (ECM) secreted by the FCs themselves. In most ascidians investigated so far this ECM forms characteristic regularly spaced crater-like structures, the so called microannuli (MA) (Fig. 3 B,C). MA emerge from vesicles secreted by the FCs which then dent to adopt their particular shape (Fig. 3A). They were first described as FC products to cover the VC in *Ascidia ceratodes* (Lambert, 1982) but as it has been shown later MA occupy the whole FC surface including the filopodia but not the VC proper (Honegger and Füglistner, 2001). In *Halocynthia roretzi* a stick-like ornamentation that is most probably part of an ECM

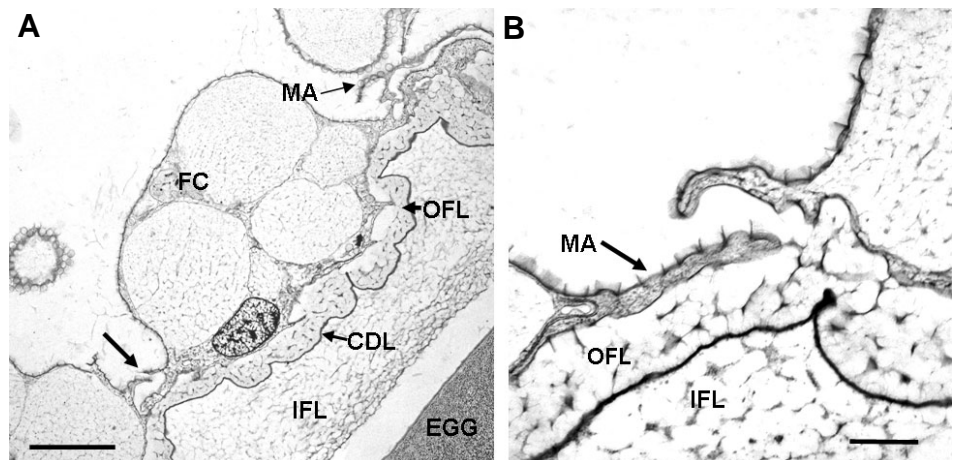


Fig. 2. Thin section of freeze substituted egg. (A) Follicle cells and vitelline coat with outer fibrous layer (OFL), central dense layer (CDL) and inner fibrous layer (IFL) of a *Phallusia mammillata* egg prepared by freeze substitution. Arrows point to filopodia with microannuli (MA). Scale bar, 5 μm . (B) Detail of filopodia with microannuli (MA). Scale bar, 2 μm .

covers the follicle cell surface (Fukumoto and Numakunai, 1995) but it has not been investigated by SEM or DERS. So far, *Ciona* is the only ascidian we investigated that lacks such an ECM (Eisenhut and Honegger, 1997). Further studies are needed to elicit if an ECM cover is a typical feature of follicle cells in most or only particular ascidian orders. This question is of particular interest because prevailing evidence indicates an important function of the ECM in fertilization as discussed below in context with sperm-egg binding and the block to polyspermy.

Test cells are found in all eggs of ascidians with indirect development. Numerous suggestions have been presented for their (still unknown) function cf. (Cloney and Hansson, 1996) but, to our knowledge, they have never been implicated in the fertilization process.

Sperm-egg coat interaction: approaching the role of the follicle cells

Sperm egg coat binding

In the fertilization process of most animals binding of sperm to the egg coat is considered as a selective step. In ascidians the prevalent model proposes that gamete recognition and discrimination is controlled by molecules localized on sperm and either on the follicle cells or on the VC or on both. However, for several reasons sperm binding is less well defined in ascidians than in other animals e.g. sea urchins or mammals. First of all, successful ascidian sperm mostly pass the egg investment within a few seconds without stopover. Therefore, compulsory transitory sperm-binding is difficult to trace. Furthermore, only part of the sperm that come in contact with FCs or the VC attach permanently and undergo visible activation that means mitochondrial translocation. In addition, possibly due to charge effects ascidian sperm exhibit firm attachment by their tip or mitochondrion to a number of substrata, especially glass. Therefore, the analysis of sperm binding in ascidians is difficult and quantifying sperm binding *in vivo* or after fixation of questionable relevance especially when distinction between adhesion to FCs or to the VC is ignored. Thus, if the term binding is used in the following we refer to a sperm egg coat interaction that is not necessarily visible as long lasting physical adhesion but implicates a recognition and signalling process necessary for sperm to get their full potential for fertilization.

Being aware of these difficulties different aspects of sperm egg-coat interactions are in the following discussed with focus on new findings concerning the role of the FCs.

Sperm egg coat interactions

First of all, as a reflection of the complexity of sperm-FC interactions, the role attributed to the FCs for self-nonsel and species-specific recognition is briefly summarized.

A number of investigations have focused on self-nonsel recognition in self-sterile ascidians attributing this function to the VC (Rosati and Santis, 1978; Kawamura *et al.*, 1991; Sawada *et al.*, 2004; Ban *et al.*, 2005).

The best characterized candidate molecule for self-nonsel recognition is a 70-kDa vitelline-coat protein from *Halocynthia roretzi* which possesses sperm receptor activity (Sawada *et al.*, 2002a, Sawada *et al.*, 2004). However, several studies presented good evidence that not only the VC but also the FCs are involved in the self-nonsel recognition process (Fuke, 1983; DeSantis, 1991; Pinto *et al.*, 1995). In *Ciona* a self sterility promoting factor released from the FCs was suggested to be deposited on the VC (Marino *et al.*, 1998).

Much less attention than to self-nonsel recognition was paid to the molecular basis of *species-specific* recognition. Pioneering experiments have shown that an intact VC is a prerequisite for the prevention of hybridization (Reverberi, 1971). Comparing *Ciona intestinalis* and *Ciona savignyi* it was suggested that the block to hybridization acts by inhibiting penetration of the vitelline coat rather than sperm binding (Byrd and Lambert, 2000). But as for self-nonsel recognition a role of the FCs in species specific recognition has been suggested (Villa and Patricolo, 1993; Patricolo and Villa, 1995). They reported that in interspecific fertilization using gametes from three closely related ascidian species foreign sperm were observed to interact with the follicle cells but never penetrate the clefts in between them. Consistent with this notion, membrane proteins from *P. mammillata* sperm were shown to possess species-specific binding activity for follicle cells (Bossi and Honegger, 1997). Admittedly, this trace was not further pursued and the molecular basis for the prevention of hybridization in ascidians remains largely enigmatic.

Sperm activation

For developmental studies the use of ascidian eggs deprived of their egg envelope can be advantageous but it is well established that such eggs are difficult to fertilize. It has been shown that this problem can be circumvented if a few intact eggs or isolated egg coats are intermixed with denuded eggs. This observation points to sperm activation by components of the egg coat.

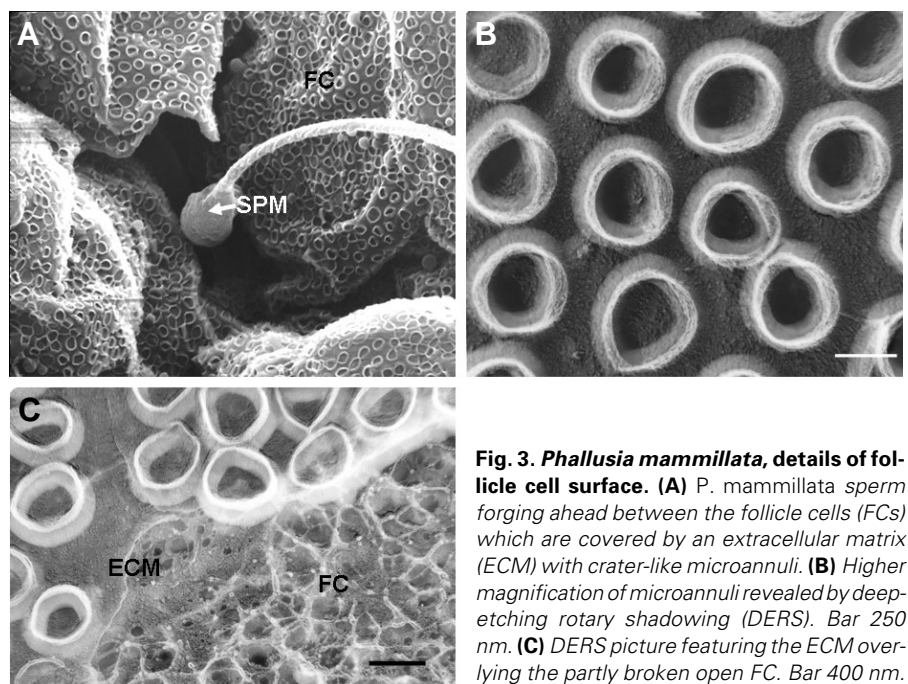


Fig. 3. *Phallusia mammillata*, details of follicle cell surface. (A) *P. mammillata* sperm forging ahead between the follicle cells (FCs) which are covered by an extracellular matrix (ECM) with crater-like microannuli. **(B)** Higher magnification of microannuli revealed by deep-etching rotary shadowing (DERS). Bar 250 nm. **(C)** DERS picture featuring the ECM overlying the partly broken open FC. Bar 400 nm.

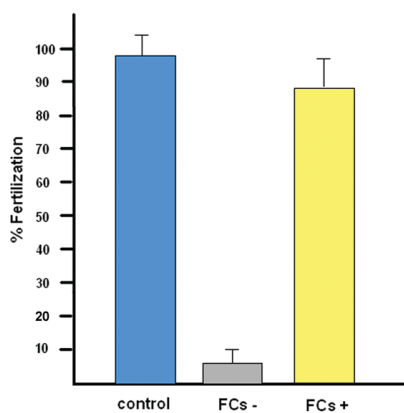


Fig. 4. Fertilization in *Phallusia mammillata* depends on the presence of follicle cells. Follicle cells (FCs) were removed by gently pipetting eggs kept in calcium-magnesium free seawater for several hours. Detached follicle cells were removed by sieving. Sperm were added to batches of 50 or 100 eggs with no visually detectable FCs. After 1 h, defolliculated eggs and the control were checked for cleavage (control, FCs-). Fifteen minutes later isolated follicle cells were added to the FC- samples and cleavage checked after 1 h.

Furthermore, the finding that in *Ascidia nigra* (Lambert and Lambert, 1981) and *P. mammillata* (Honegger, 1986) removal or reduction of the FCs slows the rate and lowers the percentage of fertilization has been attributed to sperm activation by the FCs. In *Ciona intestinalis* FCs should not only play a role in self-nonspecific recognition (see above) but also a helper function in sperm-egg interactions (Kawamura *et al.*, 1988).

We investigated the effect of FCs on fertilization in the self-fertile ascidia *P. mammillata*. FCs were removed by incubation eggs on ice in calcium-magnesium free seawater (CMFSW) for 12hrs followed by gently pipetting the eggs up and down in a glass pipette. Detached follicle cells were separated by an appropriate nylon sieve. For fertilization experiment sperm were added to batches of 50 or 100 eggs which had been carefully inspected to make sure that FCs were completely removed. This is most important since a small group of cells or even a single FC proved to be sufficient to activate sperm, a fact that was probably responsible for the low percentage of still fertilized eggs (Fig. 4). To demonstrate sperm activation we added isolated FCs to defolliculated eggs which were previously incubated with sperm but were not fertilized. As shown in Fig. 4, these eggs became now fertilized at rates only somewhat lower than intact eggs. These results proved that removing the FCs by the method used maintains the eggs capacity to be fertilized and demonstrate that primary sperm-egg interaction between sperm and FCs is an obligatory step for successful fertilization. To address the question if on the FC surface receptor molecules for sperm could be detected we labeled the surface membrane of life sperm of *P. mammillata* by biotinylation, a procedure that does not alter the fertilization capability of sperm to (Bossi and Honegger, 1997). A fraction of isolated solubilized proteins from isolated membranes were used to probe the egg coat for binding sites. The results provided circumstantial evidence for a

confined binding to the FCs (Fig. 5). In addition, using eggs or isolated egg coats as affinity matrices (Hardy and Garbers, 1994) a protein of 66kDa was identified as candidate sperm-FC binding protein (Bossi, 1997, Bossi and Honegger, 1997). The characterization of this sperm membrane protein with a number of conventional techniques proved difficult and was not tracked down. However, we propose that by now newly established techniques would allow to resolve the nature of this protein and possibly elucidate its function sperm-FC interaction.

Interesting complementary approaches to address the mechanism of adhesion-initiated sperm activation were reported by Koch and coworkers (Blackwell and Koch, 2004). They found evidence that lipid rafts play a role in signaling processes in ascidian sperm activation. This is of special interest since lipid rafts were found within mammalian sperm membranes and their involvement in sperm-ZP interactions has been proposed (Nixon *et al.*, 2007). Furthermore, they proposed that focal adhesion kinase (FAK), a key component of the signal transduction pathways triggered by integrins, plays a role in adhesion-initiated sperm activation. FAK activation induced by integrin-dependent sperm-egg adhesion and possibly by anchoring the sperm mitochondrion to follicle cell would effect cytoskeletal remodeling as required for mitochondrial translocation. At any rate, all these observations suggest sperm adhesion to follicle cells as indispensable step for sperm activation.

However, sperm-FC interactions, as possibly sperm-egg coat interactions in general, rely on multiple receptor-ligand interplays.

Follicle cells as an inhibition gate

FC have also been proposed to participate in a physical (De Santis *et al.*, 1980) or chemical block to polyspermy (Lambert *et al.*, 1997). Whereas a polyspermy block caused by a purely physical barrier is rather unlikely and not supported by in-depth studies, increasing evidence for a block due to a modification of sperm binding sites upon fertilization has been accumulated. In

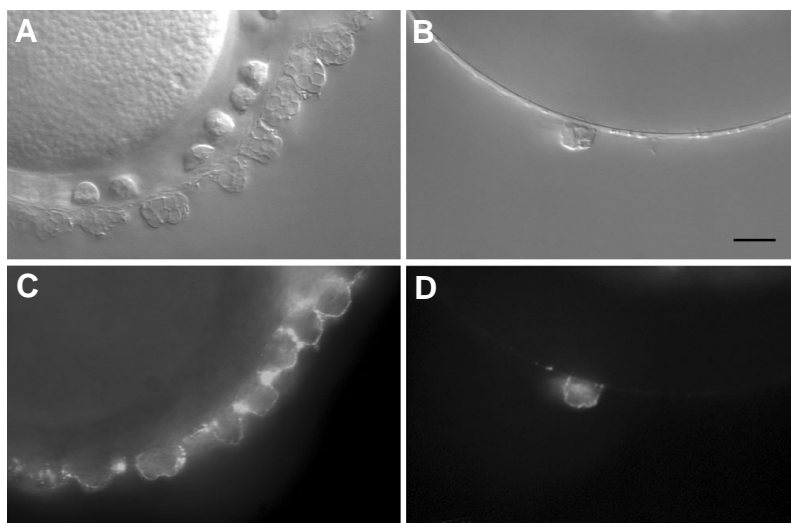


Fig. 5. Binding of biotinylated *P. mammillata* sperm surface proteins (BSSP) to live eggs. (A,C) Interference phase contrast with corresponding fluorescence micrograph showing streptavidin-FITC localization of BSSP at the FCs. (B,D) Micrographs of a largely defolliculated egg show that binding of BSSP is confined to FC. Scale bar 20 μm .

the prevailing model for ascidian sperm binding a sperm bound glycosidase recognizes and binds to a specific sugar residue of the egg coat to form an enzyme substrate complex, but hydrolysis of the substrate does not occur because the pH 8 of seawater is far above the optimal acidic value for this type of glycosidase. This model was initially proposed by Pinto *et al.*, (1981) and Hoshi (1984; 1986) (Hoshi *et al.*, 1985) and ever since supported by a wealth of findings that circumstantiate the essential function of sperm glycosidase in ascidian fertilization (Honegger, 1982; Lambert, 1986; Godknecht and Honegger, 1991; Godknecht and Honegger, 1995). Initially, in ascidians the term sperm binding was used for sperm-VC binding that was considered as the step preceding and possibly initiating the penetration of the VC. The model for the involvement of glycosidase in the block to polyspermy is based on the observation that ascidian eggs release glycosidase upon fertilization. It was suggested that the enzyme contributes to a block to polyspermy by occupying or modifying sperm receptor on the egg coat (Lambert, 1989).

This block would precede an electrically mediated block at the egg membrane which inhibits fusion with supernumerary sperm (Goudeau *et al.*, 1994, Lambert *et al.*, 1997). The hypothesis of a glycosidase mediated block to polyspermy was quickly presented as a fact although fundamental questions remained to be answered. Most important, it should be demonstrated that glycosidase release could constitute a block to polyspermy at all. Furthermore, the source of glycosidase release on fertilization, namely the egg and/or test cells and/or follicle cells has to be specified unequivocally and the receptors for the released glycosidase located.

Source of released glycosidase: in most studies which aimed at this topic ascidian species with high sperm β -hexosaminidase activity (Godknecht and Honegger, 1991) and GlcNAc on the VC (Litscher and Honegger, 1991) were used. Within seconds after fertilization an increased β -hexosaminidase activity is present in the surrounding seawater (Lambert, 1986; Lambert, 1989; Matsuura *et al.*, 1993; McDougall *et al.*, 1995). However, the situation is complicated by the fact that sperm, eggs, test cells (Koyanagi and Honegger, 2003) and the follicle cells (Honegger, 1992) contain β -hexosaminidase. The supernatant of separate batches of sperm or eggs or isolated FCs always exhibits glycosidase activity. Originally it was suggested that the glycosidase would be released on fertilization from the surface of the egg cell proper (Lambert and Goode, 1992) but later it was found that isolated follicle cell as well as defolliculated eggs release enzyme activity in response to sperm (Lambert *et al.*, 1997, Robert *et al.*, 1999). Because the β -hexosaminidases from the different sources cannot be distinguished by spectrophotometric assays with fluorogenic substrates the source and particularly the proportion of each enzyme released at fertilization have not been determined so far. Therefore, the possibility that the elevated β -hexosaminidase activity in fertilization assays could be due to a release from activated sperm could not be disclosed. A number of experiments in which glycosidase release from FC was induced by drugs, namely tyrosine kinase activators, confirmed the FCs as potential

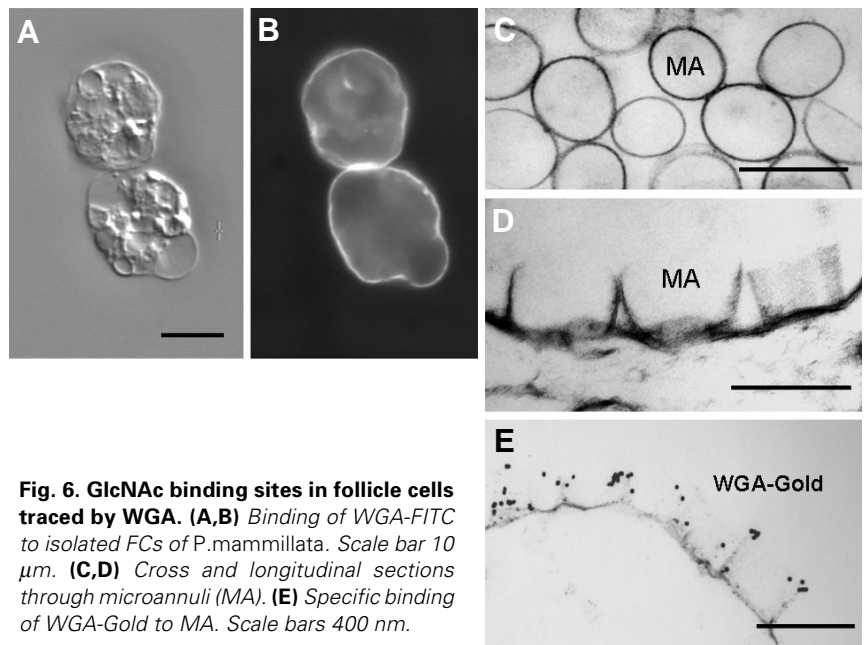


Fig. 6. GlcNAc binding sites in follicle cells traced by WGA. (A,B) Binding of WGA-FITC to isolated FCs of *P. mammillata*. Scale bar 10 μ m. **(C,D)** Cross and longitudinal sections through microannuli (MA). **(E)** Specific binding of WGA-Gold to MA. Scale bars 400 nm.

source for β -hexosaminidase but did not contribute to the understanding of the time course of the release. It has been suggested that already the first few sperm attaching to the FCs would induce glycosidase release (Lambert, 2000), but this would implicate a serious danger of self-inhibition of fertilization.

Glycosidase receptors: Potential binding sites for sperm glycosidase α -L fucosidase in *Ciona* and β -hexosaminidase in *P. mammillata* were investigated in context with sperm-egg coat binding. Fucosyl residues on the VC of *Ciona* (Rosati *et al.*, 1985), GlcNAc residues on VCs of *P. mammillata* and *Ascidia* (Honegger, 1982, Honegger, 1986, Lambert, 1986) were detected by appropriate lectins. In the latter species bright fluorescence due to WGA-FITC binding was reported to be restricted to the boundaries between the FCs. Biochemical analyses of the VC of *P. mammillata* confirmed GlcNAc as predominant sugar (Litscher and Honegger, 1991) and a component with WGA binding was isolated. In summary, these findings suggested that the receptor for glycosidase mediated sperm binding would be localized on the VC particularly at the boundaries between the FCs. One could argue that glycosidase release modifying GlcNAc residues should be visible as a decline of WGA-FITC binding. In *A. nigra* such a decline was recorded with a semi quantitative method (Lambert, 1986) but our quantitative measurements on a great number of *P. mammillata* eggs failed to confirm this result. On the other hand, we found that WGA-FITC binds to FCs (Fig. 6.), an important fact that had been overlooked so far because of the extremely bright WGA-FITC fluorescence of the VC between the FCs. This finding suggests that an essential receptor-ligand interaction between β -hexosaminidase and GlcNAc takes place during sperm-FC encounter. A block to polyspermy could be set up by β -hexosaminidase released from FCs that occupies or modifies the sperm receptors. One could argue that the supernatant of fertilized eggs should inhibit sperm binding or even fertilization by setting up a block to polyspermy. Reduced sperm binding on VCs of egg defolliculated by glycerol treatment was reported (Lambert, 1986) but effects of other compounds besides glycosidases, for in-

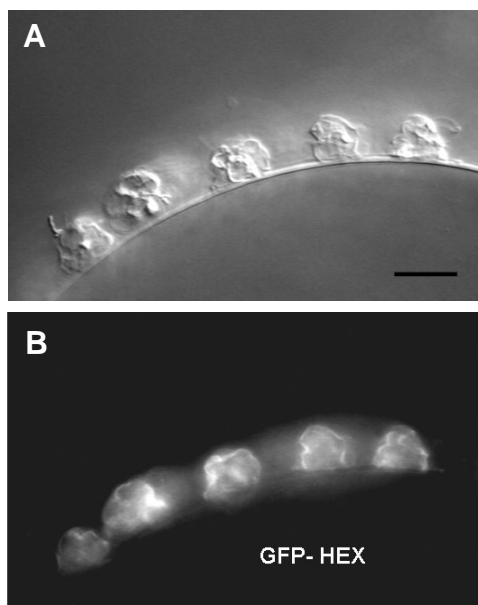


Fig. 7. Binding of fusion protein GFP-Hex. Interference phase contrast micrograph (A) and corresponding fluorescence micrograph (B) showing that the fusion protein GFP-Hex binds to follicle cells but not to the vitelline coat of unfertilized eggs. Scale bar, 20 μ m.

stance proteases, could not be ruled out. *In vivo* inhibition of β -hexosaminidase by adding GlcNAc is impracticably because due to the low affinity (Godknecht and Honegger, 1995) the high amount of the sugar needed has a damaging effect on gametes. Not surprisingly, it also proved to be impossible to set up an artificial block to polyspermy by commercially available N-acetylglucosaminidases.

The most promising way to confirm the model would be the use of purified β -hexosaminidase to localize binding and effect on fertilization. Although approaches to purify and characterize egg glycosidase were successful, isolation of an appropriate amount of enzyme for tests and the production of specific antibodies failed (Eisenhut, 2001). To circumvent this problem, we started a molecular approach by cloning β -hexosaminidase from *P. mammillata* (Koyanagi and Honegger, 2003) followed by the preparation of a recombinant GFP-fusion protein. This protein exhibits specific binding to the FCs (Fig. 7), particularly to the MA. Binding occurs on unfertilized but not fertilized eggs and is species specific (Koyanagi and Honegger, in preparation). This result confirms the modification of β -hexosaminidase binding sites on fertilization, the essential prerequisite in the suggested model for a block to polyspermy. With the successful cloning and expression of the β -hexosaminidase a promising tool for further studies of the molecular mechanism involved in glycosidase mediated block to polyspermy is now available.

Summary and conclusion

Ascidian fertilization is initiated by interactions of sperm with the follicle cells (FCs) and the vitelline coat (VC), the two outermost structures of the egg coat. The current model suggests that these primary interactions mediate species-specific and self-nonsel recognition and activation of sperm. Over the last 25 years a wealth of studies aimed at the elucidation of this cascade. The original idea that at each step a particular molecule located on the

sperm would recognize and bind to a complementary molecule located on the egg coat had to be revised by increasing evidence that at each step multiple molecules with different binding characteristics may be involved. Although, prevailing evidence for an essential intrinsic interplay between glycosidases and carbohydrates located on the surface of egg coat components of ascidians exists, it is most likely that addition molecules other than glycosidases participate at different stages in these complex interactions. Thus, the implication of proteases in ascidian sperm-VC binding as proposed by Sawada and co-workers (Sawada *et al.*, 1998, Sawada *et al.*, 2002b, Takizawa *et al.*, 1993) does not jeopardize the suggested function of glycosidases. This situation is not particular for ascidians since a growing body of evidence suggests that gamete binding in most animals involves multiple molecular interactions (Nixon *et al.*, 2007, Shur *et al.*, 2006).

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