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Sperm-egg interaction at fertilization: glycans as recognition signals

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ABSTRACT This article first examines the events occurring in male and female genital tracts, which prepare human sperm to encounter the egg. Central is a glycoprotein, gp20, homologous to the leukocyte antigen CD52. This protein is secreted in the epididymal cells, inserted in the sperm plasma membrane and exposed in the equatorial region of the head at the end of the capacitation process. The mechanisms and molecules of the first interaction event between gametes in the mollusk bivalve *Unio elongatulus* and the current state of our knowledge of the same interaction in other species is then considered. The egg of *Unio* is very peculiar because it is highly polarized. Similar to other well-known egg models, the ligand for recognition is located on the egg coat which is a sort of fibrous network made up of very few glycoproteins, while the receptor is on the sperm surface. The difference is that in this egg, the ligand molecules are not uniformly distributed but are restricted to an area of the egg coat at the vegetal pole, the crater area. The role of carbohydrates in ligand function and of a specific type of oligosaccharide chain in particular, is discussed in the wider context of glycans acting as recognition signals.

KEY WORDS: *fertilization, sperm-egg interaction, glycoproteins, recognition, oligosaccharide chains.*

Introduction

Fertilization is the process by which two gametes belonging to the same species encounter one other and fuse to create a new individual. Species use different pathways to ensure that their gametes arrive correctly at the meeting point. In species with external fertilization, this goal can be achieved by contemporary spawning of the gametes and/or release of chemoattractants from eggs, which also increase sperm motility and respiration (Garbers, 1989; Ward and Kopf, 1993; Hardy *et al.,* 1994). Species with internal fertilization utilize higher organizational levels; gametes only meet under very special circumstances and under highly controlled conditions. This generally includes complex mechanisms that delay the fertilization ability of the male gamete until it encounters the egg. In mammals, sperm become ready to fertilize the egg only after a maturation step in the male genital tract and a subsequent capacitation process in the female. Even though first contact is achieved and optimized differently in different species, once the gametes come in close contact, a precise sequence of interacting events takes place that, in spite of the great differences in the morphology of the gametes between species, is very similar in the invertebrate and vertebrate species so far studied. Recognition and binding between the extracellular coat of the egg and components of the sperm plasma membrane, generally termed "primary binding", is the first of these sequential events and a prerequisite for the others. The binding triggers an acrosome reaction in the sperm, a form of signal transduced exocytosis. It is widely accepted that in invertebrates and vertebrates the primary binding is mediated by complex carbohydrates of the egg coat that are decoded by complementary sites of carbohydrate-binding proteins located on the sperm surface (see Rosati, 1985; Litscher and Wassarman, 1993; Rosati, 1995; Foltz, 1995; Töpfer-Petersen *et al.,* 1997; McLeskey *et al.,* 1998; Benoff, 1997, 1998; Zara and Naz, 1998; Sinowatz *et al.,* 1998; Shur, 1998). A secondary binding probably takes place after the initiation of the acrosome reaction involving sperm components present in the acrosome and egg coat components (Bleil *et al.,* 1988; Garbers, 1989). This binding helps the spermatozoon to cross the egg coat (Bleil *et al.,* 1988). The acrosome reaction is also instrumental in releasing enzymes that dissolve the coat by means of enzymatic (Hoshi, 1985) or non-enzymatic (Vacquier *et al.,* 1990) mechanisms, and in exposing the

Abbreviations used in this paper: ZP, zona pellucida; gp, glycoprotein; MALDI MS, Matrix Assisted Laser Desorption/Ionization Mass Spectrometry; VC, vitelline coat; GPI, glycosylphosphatidyl inositol; SB, sepharose bead.

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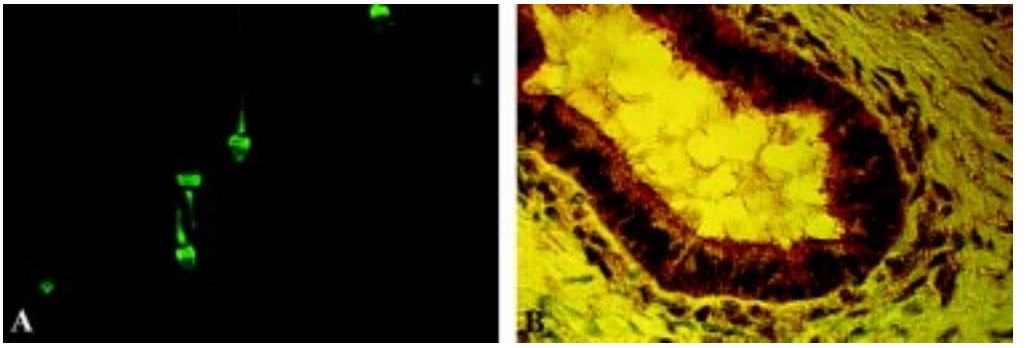

Fig. 1. Localization of the gp20-CD52 antigen on (A) the capacitated human sperm surface and (B) on epididymal cells. *Samples were treated with anti-gp20 serum followed by a goat anti-rabbit IgG, conjugated with* **(A)** *fluorescein or* **(B)** *peroxidase.*

internal sperm membrane so that it can fuse with the egg plasma membrane. Sperm-egg plasma membrane interaction is the last interaction event, after which fusion takes place resulting in an array of physiological changes within the egg collectively termed "egg activation". Generally, these include calcium release, polyspermy block and the resumption of the cell cycle (Jaffe, 1985; Whitaker and Swann, 1993; Miyazaki *et al.,* 1993). The fundamental mechanisms underlying these interaction events are conserved in cells independently from their somatic or germ line. Fertilization is unique in that the specialized cells, the gametes, use biological themes to drive reproduction and in particular to ensure the species-specificity of the process. In the last decade, considerable efforts have been made to identify molecules and pathways utilized during gamete interaction, and the details of the process are beginning to be understood. In this review, we will report our work on human sperm maturation and capacitation and on molecules and mechanisms of the primary binding in a mollusk bivalve, in the context of current ideas about this binding in other animal species.

Epididymal human sperm maturation and capacitation

Mammalian spermatozoa released from the seminiferous tubules and those present on the proximal regions of epididymis are unable to perform the primary binding and fertilization of the egg. They acquire progressive motility and fertilizing ability during a maturation passage through the other epididymal regions. Maturation involves several intracellular and above all, many extracellular modifications that change the architecture of the sperm surface (Cameo *et al.,* 1990; Cooper, 1995). Testicular proteins may be gradually removed or rearranged (Dacheux *et al.*, 1990), whereas proteins secreted by the epididymal epithelium may attach to or be inserted into the sperm surface (Hermo *et al.*, 1994). This is well documented in various species from mice to primates by means of monoclonal and polyclonal antibodies against the sperm surface (Shabanowitz and Killian, 1987; Holland and Orgebin-Crist, 1988; Ghyselinck *et al.,* 1989; Isahakia, 1989; Boué *et al.*, 1996). A mechanism of "cell-to-cell" transfer mediated by prostasomes, small vesicles derived from the epididymal cells, has also been suggested for GPI (glycosylphosphatidylinositol)-anchored proteins (Kirchhoff and Hale, 1996). Some of the acquired proteins are thought to be directly involved in sperm binding to the zona pellucida and oocyte plasma membrane (Boué *et al.*, 1995; Boué *et al.,* 1996). Male gametes undergo other complex surface transformations following their encounter with accessory gland secretions during ejaculation, and also when they are capacitated in the female genital tract. The modifications occurring in these steps are not fully understood, but they are known to involve the coating of the sperm surface with secretion products in the first step, and then the uncoating as well as a change in membrane fluidity in the second (see Moore, 1995). For years we have studied a human sperm protein, gp20, that is related to both the maturation and capacitation processes and is homologous to the leukocyte antigen CD52. Our work on human sperm first concerned modifications of surface sialylglycoconjugates as a consequence of the capacitation process *in vitro* (Focarelli *et al.,* 1990b). Sialylglycoconjugates were identified by radiolabeling the terminal sialic acid residues on freshly ejaculated sperm. Analysis of the kinetics of their release in the capacitation medium showed that these molecules are abundant in freshly ejaculated sperm but are gradually lost during capacitation (Focarelli *et al.*, 1990b). By radiolabeling the sperm surface at the end of the capacitation we then found that only a sialylglycoprotein of about 20 kDa (gp20) is present on the capacitated sperm (Focarelli *et al.*, 1995a). Using several sperm samples from healthy donors, we purified the protein to raise the relative antiserum. The antiserum was highly specific for gp20 since it recognized only this protein in an immunoblot of the SDS-PAGE separated polypeptides from the whole sperm plasma membrane (Focarelli *et al.*, 1998). No similar antigens were

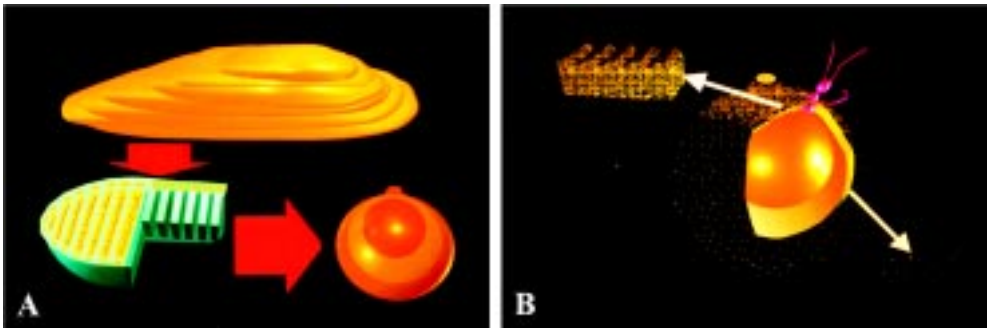

Fig. 2. Three dimensional graphic representation of Unio elongatulus and its egg. **(A)** *The shell: the branchial chamber where sperm and eggs meet and the highly polarized egg.* **(B)** *The egg is represented with bound sperm in the crater region, with details of the vitelline coat and with an inside view showing its relationship with the oocyte.*

found in murine sperm, in rat sperm or in any other murine tissues. We then used the serum to immunolocalize the antigen on the sperm surface. When applied to freshly ejaculated spermatozoa, the gp20 antigen was present everywhere on the head and on the tail (Focarelli *et al.*, 1998). In contrast, a very specific localization was detected on capacitated sperm. In this case the antigen appeared restricted to the equatorial region of the head in almost all spermatozoa with a variable faint presence in mid-piece (Fig. 1A). This result was in line with our previous results using lectins specific for the sialic acid residues (Focarelli *et al.*, 1995a). The localization of the antigen in the region indicated by some authors as involved in sperm-oocyte fusion (Bedford, 1994), suggested a specific role in the fertilizing ability of sperm. We confirmed this role by analyzing the effect of anti-gp20 on sperm penetration of hamster eggs (Focarelli *et al.*, 1995a). Blocking the gp20 antigen strongly interfered with this process. Using anti-gp20 we discovered that the antigen is a product of epididymal cells (Fig. 1B) and not of testicular cells and that it is also present in the seminal plasma (Focarelli *et al.*, 1995a). The N-terminal analysis of gp20 demonstrated that the protein was homologous to CD52 (Focarelli *et al.,* 1999b), a GPI-anchored antigen abundantly expressed on the surface of almost all human leukocytes (Hale *et al.,* 1990). The structure of CD52 is unusual but well known. It consists of a short peptide (12 amino acids) linked to a large, sialylated, polylactosamine-containing, core-fucosylated tetra-antennary oligosaccharide and to a simple GPI membrane anchor (Xia *et al.*, 1993; Treumann *et al.*, 1995). The monoclonal antibody family against CD52, comprehensively called CAMPATH-1, is reported to recognize an epitope including the GPI-anchor plus a C-terminal tripeptide (Xia *et al.*, 1993). The generic presence of CD52 in the reproductive system had already been indicated in a routine screening of different tissues with CAMPATH-1 (Hale *et al.*, 1993) and confirmed by cDNA analysis (Kirchhoff *et al.,* 1993; Kirchhoff, 1996; Krull *et al.*, 1993). The parallel use of CAMPATH-1 and anti-gp20 on the same sperm and leukocyte samples and on the antigen purified from the seminal plasma confirmed that both the antibodies recognized the same antigen, but it also suggested that the recognized epitopes were different. First of all, the appearance itself of the antigen in the immunoblot was different. The antigen revealed by CAMPATH-1 was reported to run as a broad smear between about 18-25 kDa (Hale *et al.,* 1993), whereas anti-gp20 gave rise to a very well defined band at 20 kDa, appearing as a clear doublet in the sperm samples (Focarelli *et al.*, 1998). Immunoblot analysis after one and bi-dimensional electrophoresis of several different samples of sperm and related seminal plasma, together with different leukocyte samples, confirmed the presence of two subcomponents on sperm and seminal plasma samples but not in leukocytes (Focarelli *et al.,* 1999b). Both the subcomponents appeared heterogeneous, with an isoelectric point ranging from about 3 to 6. The isoelectric point of the leukocyte antigen was less heterogeneous, with an isoelectric point of about 3 (Focarelli *et al.,* 1999a). After purification, the seminal plasma antigen was also revealed by staining with Coomassie blue. Purified leukocyte antigen has never been detected by this stain (Hale *et al.,* 1996). In this case its doublet nature was also evident. Matrix Assisted Laser Desoprtion Ionization Mass Spectrometry (MALDI) analysis of the purified antigen definitively confirmed the presence of two components on the seminal plasma antigen and also indicated their real masses (Focarelli *et al.,* 1999a). They turned out to be completely different from those indicated by electrophoretic mobility but similar to the mass calculated on the basis of the leukocyte antigen structure. The two ions found by MALDI had an Mr of 8243 and 10908 respectively (Focarelli *et al.,* 1999a). Comparison of the sperm and leukocyte antigen with anti-gp20 and CAMPATH-1 after enzymatic deglycosylation, definitively established that the epitopes recognized by our antibody were different from that recognized by CAMPATH-1. In fact, after this treatment the antigen of all samples appeared completely converted in a form still CAMPATH-1 positive and running at about 7 kDa, but now anti-gp20 negative. The overall results obtained with anti-gp20 were very intriguing but did not lead to a conclusive result about the epitopes recognized by the antibody (Focarelli *et al.,* 1999a). Experiments of anion exchange FPLC fractionation have recently suggested a further diversification of the antigen of the reproductive system. In this system, two differently negatively charged forms of the antigen could be distinguished. The first one is probably less sialylated and is present in the seminal plasma free of prostasomes and in the freshly ejaculated sperm. The second is present in the prostasomal seminal plasma fraction and in the plasma membrane of incapacitated and capacitated sperm (Focarelli *et al.,* unpublished results). Using this method the leukocyte was found to contain only the more sialylated form. The heterogeneity of the seminal plasma antigen has recently been confirmed by Schroter *et al.,* (1999). Considered together, these results indicate that the CD52 homologue of the reproductive system is a very complex and important antigen whose modulation on the sperm surface is directly related to the

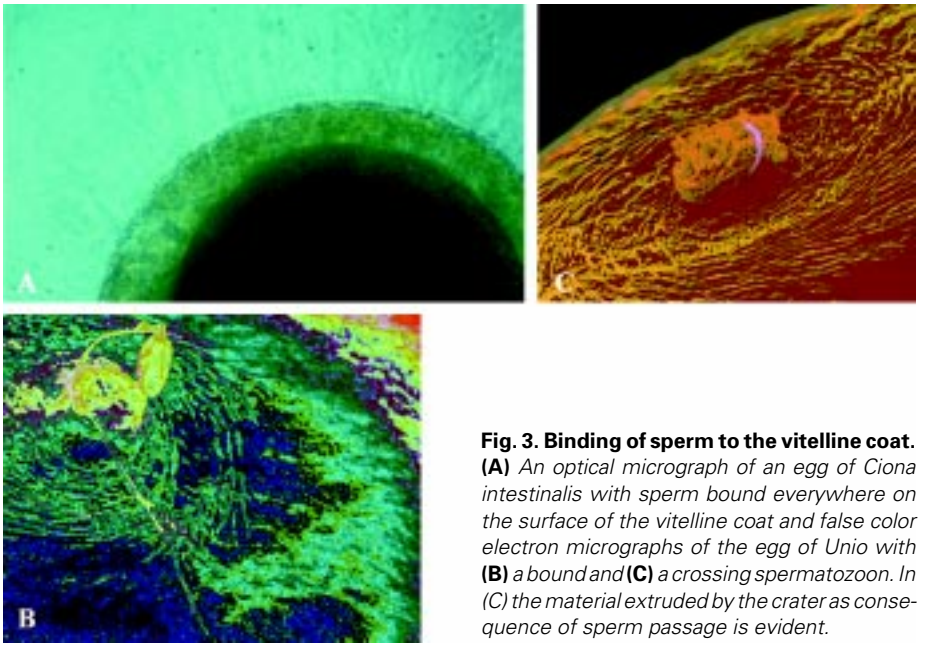

Fig. 3. Binding of sperm to the vitelline coat. **(A)** *An optical micrograph of an egg of Ciona intestinalis with sperm bound everywhere on the surface of the vitelline coat and false color electron micrographs of the egg of Unio with* **(B)** *a bound and* **(C)** *a crossing spermatozoon. In (C) the material extruded by the crater as consequence of sperm passage is evident.*

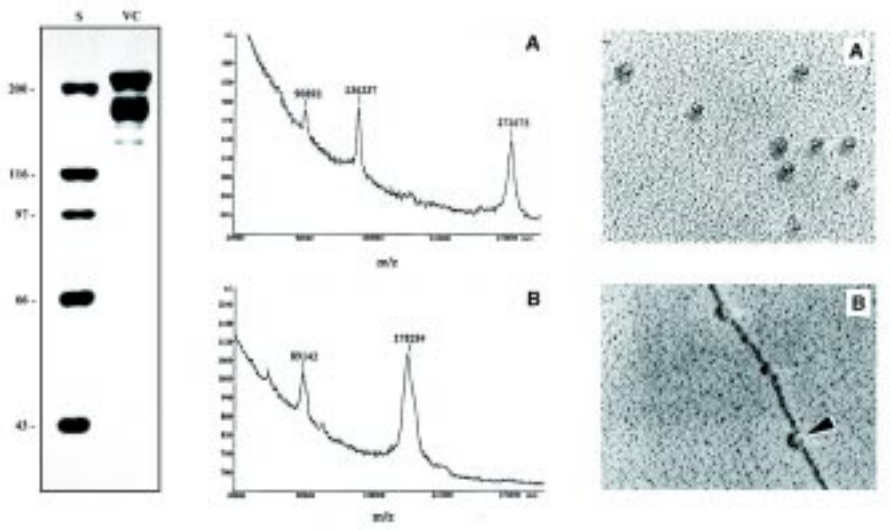

Fig. 4. Vitelline coat glycoproteins in Unio. *On the left, SDS-PAGE of the material solubilized from the coat showing that it only contains two glycoproteins with apparent Mr values of 220 and 180 kDa. To the right, the MALDI analysis and rotary shadowing of the purified glycoproteins showing that the higher Mr glycoprotein* **(A)** *has a mass of 273 kDa and a rosette-like structure whereas the other* **(B)** *has a mass of 180 kDa, a C-shaped structure (arrowhead) and forms fibrils. The m/z values for singly, doubly and triply protonated intact protein are indicated in the MALDI analysis figures.*

mechanisms of the progressive acquisition of the fertilizing ability of the male gamete.

Structure, function and synthesis of the egg coat

The egg surface with the property to first recognize and bind sperm is the outer surface of the glycoprotein coat surrounding the egg. In both invertebrates and vertebrates, the coat consists of a fibrous network whose supramolecular organization and thickness vary slightly from species to species. It can be arranged in a homogeneous single layer, as in the ascidian *Ciona intestinalis,* or be differentiated in two or three layers of differently packed fibrils as in other ascidians, mollusks and decapods (see Rosati, 1995). In mammals the zona pellucida also consists of a fibrous meshwork whose thickness varies from 2 to 25 μm and can be arranged in one or multiple layers (see Dunbar, 1991). The coat surface is generally uniform with nothing to aid the identification of an animal and a vegetal pole. The only exception is the peculiar egg of the mollusk bivalve, *Unio elongatulus. Unio* belongs to the group of Unionacea having a peculiar type of internal fertilization with sperm entering through the inhalant siphons and meeting the eggs in the suprabranchial chamber (Fig. 2A). The *Unio* egg is markedly polarized; at the vegetal pole the vitelline coat protrudes to form a truncated cone that looks like a crater and is surrounded by a wrinkled area (Fig. 2A,B). This area, whose differentiation begins early in the growing oocyte (Focarelli *et al.,* 1990a), becomes at the end of the oocyte maturation, morphologically and functionally different from the rest of the vitelline coat and also remains the only region still attached to the oocyte plasma membrane (Fig. 2B). Whereas in other invertebrate and vertebrate species, binding of the sperm generally occurs everywhere on the coat surface (Fig. 3A), the egg coat of *Unio* restricts the ability to bind sperm to this crater area (Figs. 2B, 3B) (Focarelli *et al.,* 1988). The area changes completely as the first sperm crosses the egg coat and this activates a form of polyspermy prevention (Focarelli *et al.,* 1988). In fact, oocyte material lying just below the crater becomes extruded (Fig. 3C), the oocyte detaches from the coat and the wrinkled area spiralizes becoming unable to further bind sperm (Rosati and Focarelli, 1996).

The coat is generally composed of a very few glycoproteins. The mammalian zona pellucida is composed of the transcripts of three highly conserved gene families named ZPA, ZPB and ZPC (Harris *et al.,* 1994). In mice, the mature products of the genes have been completely characterized. They are named ZP1, ZP2 and ZP3, have apparent molecular weights of 200, 120 and 83 kDa respectively (Wassarman, 1988), and contribute to the spatial organization of the coat by forming long filaments made up of ZP2-ZP3 heterodimers with ZP1 dimers serving as links between the filaments (Wassarman and Mortillo, 1991). Ancestors of the ZP gene families have also been found in amphibia (Tian *et al.,* 1999) and fishes (Epifano *et al*., 1995a; Chang *et al.,* 1996); no molecular analysis has been performed up to now in any species of invertebrates. However, although the amino acid sequence of the glycoproteins of several vertebrate species have been derived by cDNA cloning and proved to be highly conserved, the molecular details of the mature products have not yet been unraveled in any species. Among invertebrates, *Unio elongatulus* is the only species in which complete biochemical characterization of the egg coat has been obtained (Focarelli and Rosati, 1993; Focarelli *et al.,* 1995 b,c). In this egg, two main glycoproteins have been shown to account for about 90% of the material dissolved from the vitelline coat. Electrophoretic analysis showed them to have molecular weights of 220 and 180 kDa (Fig. 4) but when purified and examined by Matrix Assisted Laser Desorption Ionization (MALDI) Mass Spectrometry (Focarelli *et al.,* 1997a) they revealed masses of 273 (gp273) and 180 kDa (gp180) (Fig. 4). The two glycoproteins have a different shape: gp273 is globular and rosette like, and gp180 filamentous and has the ability to form long filaments (Fig. 4). The crater region was predominantly formed by rosette-like molecules in a network of filaments, whereas the rest of the coat was prevalently made up of interconnected filaments.

In view of the role played by the egg coat, there has long been a question of whether it is a product of the oocyte itself or of related somatic cells. Experimental evidence from the last few years suggests that the coat is a product of the oocyte itself. Morphological observations on differentiation of the coat in invertebrates such as *Astropecten* (Santella *et al.,* 1983), *Unio* (Focarelli *et al.,* 1990a), *Ciona* (Cotelli *et al.,* 1981), and in vertebrates (Pinto *et al.,* 1985) including mammals, have shown that the fibrous material of the coat is secreted by subcortical vesicles of the growing oocyte. In *Ciona*, this observation has been confirmed by following the synthesis of components of the coat with a radioactive precursor (Rosati *et al.,* 1982). In mice, the exclusive origin of the coat components from the oocyte has been conclusively demonstrated by means of molecular biology (Epifano *et al.,* 1995b). However, the advent of cDNA technology has also demonstrated that in some fishes ZP-like genes are expressed in liver and then transported to the oocyte (Lyons *et al.,* 1993).

Egg coat glycoproteins acting as ligands

The biochemical intractability of the coat components has been a continual problem. The coat glycoproteins are generally heavily glycosylated and insoluble. Furthermore, many invertebrate eggs such as those of the sea urchin have an additional external coat which is difficult to separate. These problems have made difficult the search for the ligand molecule responsible for the primary binding. Partial solubilization of the coat and purification of protein complexes with ligand activity has been performed in echinoderms (Shimizu *et al.,* 1990; Hoshi *et al.,* 1991; Keller and Vacquier, 1994), ascidians (De Santis *et al.,* 1983; Litscher and Honneger, 1991), fish (Cherr and Clark, 1985), amphibians (Tian *et al.,* 1997) and pig (Kudo *et al.,* 1998; Yurewicz *et al.,* 1998). The purification and characterization of all the glycoproteins of the egg coat and the resulting identification of the ligand molecule has only been achieved in mice (see Wassarman, 1990) and among invertebrates in *Unio elongatulus* (Focarelli and Rosati, 1995). In mice the ligand molecule is ZP3. In fact only ZP3 is able to (i) bind to the plasma membrane of the sperm head and to (ii) trigger the acrosomal reaction in the sperm; neither ZP1 nor ZP2 has these functions. Only 4 of the known 11 members of the ZPC family have been evaluated for their role in the primary binding. In contrast to the exclusive role of mouse ZP3, in the porcine system heterocomplexes formed by ZPB and ZPC components have been shown to possess sperm-binding capacity (Kudo *et al.,* 1998; Yurewicz *et al.,* 1998). In *Unio*, the ligand molecule has been clearly demonstrated to be gp273. In fact this molecule, not the other coat component (gp180), binds to the sperm (Fig. 6A) with binding taking place on the plasma membrane of the head (Focarelli and Rosati, 1995). By means of the polyclonal antibodies raised against the two egg components we also demonstrated that whereas gp180 is uniformly distributed in the coat (Fig. 5A), gp273 is prevalently present or exposed in the crater region, the egg coat area to which binding of sperm is restricted (Focarelli and Rosati, 1995), both in the mature egg (Fig. 5B) and in growing oocytes (Fig. 5C).

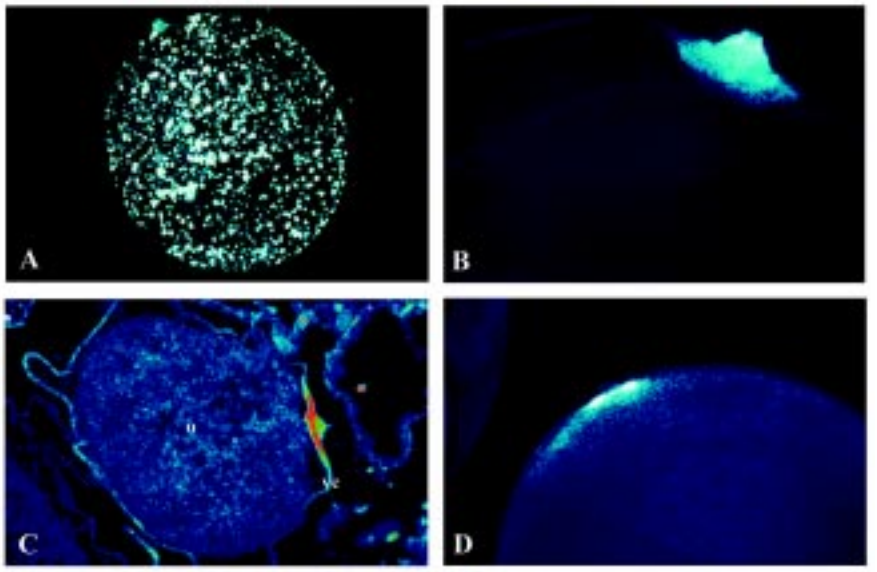

Fig. 5. Differential distribution of gp273, gp180 and fucose residues on the vitelline coat of a Unio oocyte. *Confocal scanning laser micrographs of mature (A, B, D) and differentiating oocytes (C) after treatment with* **(A)** *anti-gp180,* **(B,C)** *anti-gp273 and* **(D)** *the lectin from Lotus tetragonolobus. Gp180 is uniformly distributed on the vitelline coat whereas gp273 and fucose residues are restricted to the crater region. o, oocyte; vc, vitelline coat.*

The role of carbohydrates in ligand function

The first evidence that carbohydrates on the extracellular egg coat provide the molecular basis for sperm recognition and adhesion was obtained in mammals using lectins (Oikawa *et al.,* 1973) and in tunicates using carbohydrates (Rosati and De Santis, 1980) as competitors of the process *in vitro*. In the tunicate *Ciona intestinalis* "glycerol treated eggs" that maintained the capacity to specifically recognize *Ciona* sperm but not to trigger the acrosome reaction in them, were used as models to inhibit the sperm-egg binding by different carbohydrates. It was found that L-fucose was the only sugar interfering with binding; other sugars tested, including D-fucose, were proved to be completely ineffective. Complete inhibition of the fertilization process was also obtained, using living eggs, at the maximum fucose concentration (50 mM) used in the binding experiments. The ability of monosaccharides to effectively block sperm binding has since been proved in a number of invertebrate and vertebrate species including man. The use of lectins and removal of sugar moieties by means of glycosidases have also confirmed the importance of carbohydrates in sperm-egg interaction. In the last ten years major advances in understanding this role has come from a variety of methodologies used to purify and study the structure and function of carbohydrate chains (glycans). N- and O-glycans are generally present in the coat glycoproteins. Their selective removal from ZP3 first demonstrated that the sperm binding ability of this molecule resides within the O-glycosylation (Florman and Wassarman, 1985). Removal and use of the purified O-glycans later confirmed this observation, indicating that these chains bind sperm and interfere with sperm binding to zona pellucida. By mutating five Ser residues located in the C-terminus of mouse ZP3 to either Gly, Val, or Ala, followed by the secretion of the inactive recombinant mouse ZP3 from transfected embryonal carcinoma cells, Kinloch *et al.,* (1995) confirmed that O-linked carbohydrates, located within the C-terminal portion of the ZP3 polypeptide chain, are vital for the ligand activity. However, the identity of the terminal sugar is still controversial. Galactose (Bleil and Wassarman, 1988), N-acetylglucosamine (Miller *et al.,* 1992) and fucose (Johnston *et al.,* 1998) have all been indicated as relevant in primary binding in mice. Data concerning the involvement of either N- or O-linked glycosylation in mammals other than mice where the ligand molecule has not yet been identified and purified are equivocal (see Töpfer-Petersen *et al.,* 1997; Benoff, 1997; McLeskey *et al.,* 1998). O-linked oligosaccharide chains have been indicated as essential for ligand activity of gp273 in *Unio* (Focarelli and Rosati, 1995). The role of oligosaccharides of gp273 was investigated by removing the N- and O-linked oligosaccharides from gp273 (Fig. 6 B,C) and using them in competition binding analysis (Fig. 6D). The N-linked carbohydrate chains were split off with PNGase-F and the O-linked ones were released by alkaline borohydride treatment. The free oligosaccharides were desalted

and separated by gel permeation chromatography on Bio-Gel P4 (Fig. 6 B,C). Pooled material obtained after each treatment, was lyophilized and used in a competition binding assay. When sperm were incubated with increasing concentrations of gp273-derived O-linked oligosaccharides, concentration-dependent inhibition of the binding of gp273 to sperm was observed (Fig. 6D). In these experiments binding was reduced to about 60% of control values. In contrast gp273-derived N-linked oligosaccharides had no effect at all on the binding of gp273 to sperm (Fig. 6D). Very recently it has been shown that after cyanogen bromide fragmentation of gp273, the ability to bind sperm is retained by a large glycopeptide to which the O-linked glycans are restricted, thus confirming the role of these chains in the gp273 ligand activity. In *Unio,* fucose has been indicated as essential in the ligand function of gp273 since it (i) interferes with its binding to sperm (Focarelli and Rosati, 1995), (ii) its presence is restricted to the glycopeptide with the ligand ability (Capone *et al.,* 1999) and (iii) it is prevalently present in the crater area (Fig. 4D) (Focarelli *et al.,* 1988). Fucose is indeed the sugar residue more frequently indicated as playing a role in the sperm-egg interaction. Its effect in inhibiting the process has been reported for several animal species from invertebrates, such the sea urchin (Vacquier and Moy, 1997), *Asteria* (Okinaga *et al.,* 1992), *Ciona* (Rosati and De Santis, 1980), and *Limulus* (Ruttemberg-Barnum and Brown, 1983), to vertebrates such as mice (Johnston *et al.,* 1998), guinea pig (Huang *et al.,* 1982) and humans (Oehninger *et al.,* 1998). Interestingly, in the sea urchin, where the acrosome reaction is induced by a gelatinous layer external to the egg coat, the ligand function does not involve polypeptides but is directly related to a sulphate polysaccharide (Alves *et al.,* 1997; Vacquier and Moy, 1997). In many species the polymer is a linear sulphated α-L-fucan with the proportion of 2-O- and 4-O sulphation varying in different species and determinative in sperm recognition (Alves *et al.,* 1998; Vilela-Silva *et al.,* 1999).

Carbohydrate binding proteins on the sperm surface

The complexity of the sperm membrane architecture along with the difficulty of isolating and purifying it, have made the identification of the spermatozoon counterpart of the egg coat ligand a difficult task. A galactosyl-transferase (see Shur, 1998), and two sperm membrane proteins called sp56 (Bleil and Wassarman, 1990) and sp95 (Leyton and Saling, 1989) are the main candidates indicated as sperm receptors of mouse ZP3. In *Ciona intestinalis* (Hoshi *et al.,* 1985) and *Unio elongatulus* (Focarelli *et al.,* 1997b) α-L-fucosidase has been indicated as the best candidate for this role. In *Ciona* the activity of this enzyme is much higher than any other glycosidases assayed; the sperm-egg binding is inhibited by synthetic substrates of α-L-fucosidase and the bound spermatozoa can be detached at low pH. *Unio* expresses a fucosidase isoform bound to the sperm plasma membrane, which is completely different to that present in the surrounding fluid. Since the ligand for sperm-egg interaction in this species is known and available in sufficient quantities for biochemical studies, the *Unio* model is valuable for a detailed investigation of the mechanism of glycosidase-substrate interaction during fertilization.

Species specificity of primary binding

Fertilization is a species-specific process. Geographic isolation and mating behaviour are the highest and the strictest levels at which such specificity is ensured. Whether and how species-specificity also depends on the molecules and mechanisms at work when gametes interact is a matter of debate (O' Rand, 1988; Dunbar, 1991; Foltz, 1995; Töpfer-Petersen *et al.,* 1997; Benoff, 1997, 1998). Previous reports have indicated that the egg coat plays a role in both invertebrates (see Rosati, 1985; Osanai, 1973,1974) and vertebrates (Hanada and Chang, 1972, 1978; Yanagimachi, 1972) in ensuring species-specificity. In fact, its removal often gives rise to heterologous fertilization. However, although the presence of the coat is a prerequisite to specificity it is often not a sufficient condition for gamete incompatibility (Adams, 1974; O'Rand, 1988; Hamel and Mercier, 1994). The evidence acquired in the last few years that sperm-egg coat interaction is, at a molecular level, a multicomponent and multistep process, has more recently shifted the specificity question towards the sperm-egg molecular dyads at work in each step. A concerted evolution of the dyad lysin-receptor of the secondary binding has been clearly demonstrated in broadcasters sea urchin species with overlapping habitats and

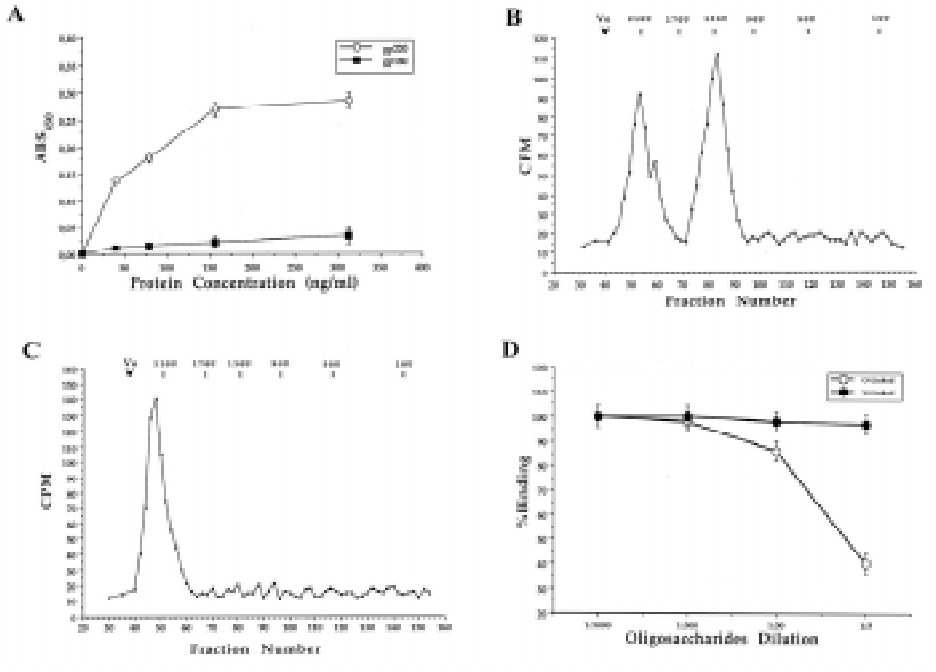

Fig. 6. The ligand molecule for sperm-egg interaction is gp273 and its O-linked chains play a key role in this interaction. (A) *Binding of HRPO-conjugated coat glycoproteins to immobilized Unio sperm.* **(D)** *Differential effect of Biogel P4 purified gp273 N-linked* **(B)** *and O-linked* **(C)** *oligosaccharides on sperm binding to gp273.*

breeding seasons (Shaw *et al.,* 1994; Vacquier, 1998). The carbohydrate-mediated primary binding, the first and indeed the most important of the gamete interactions, has been frequently indicated as highly species-specific, despite the fact that the molecular basis of such specificity is completely unknown. Indeed, since carbohydrate recognition is a "shape" problem to understand whether this specificity exists it would be necessary to explore the three-dimensional distribution of the oligosaccharide chains in the ligand polypeptide chains. Unfortunately current knowledge about the structure of the oligosaccharides of the coat components is very limited, and the spatial organization of the carbohydrate chains in the egg coat molecules is still in its infancy. We recently tested the possibility of comparing the conformation of ligand molecules by using the antibody which recognizes the ligand conformation in the mollusk *Unio elongatulus*. First we investigated the egg coat of humans (Rosati *et al.,* unpublished results), the rationale being that parameters such as O-linked chains, fucose and sulphation are common to the binding process in *Unio* (Focarelli and Rosati, 1995; Capone *et al.,* 1999) and mammals (see Benoff, 1997) including humans (Oehninger *et al.,* 1992). Isolated human zonae pellucidae were used in immunogold and immunoblot analysis with the antibody against gp273. The results showed that epitopes recognized by anti-gp273 were uniformly distributed on the outer surface of the zona pellucida but not on the inner surface or inside the zona, and that the zona pellucida glycoprotein recognized by the antibody was the lowest molecular weight human ZP component, namely hZP3. We then tested whether the exposed epitopes of hZP3 were actually functional in recognition and binding by challenging human sperm with Sepharose beads (SB) conjugated with gp273. As a control we also challenged sperm with SB, conjugated with the other *Unio* coat component (gp180). The time course obtained with gp273 SB revealed an increase in binding of sperm for the first 10-15 min of incubation with a maximum of about 1 sperm/SB at 15 min. Sperm only bound at a background level in the control with SB-derivatized with gp180. These results allowed us to identify for the first time that hZP3 epitopes are exposed on the zona pellucida and functional in binding sperm. Concerning species specificity, these results indicate that the ligand molecules of evolutively distant species may share structural and functional motives for sperm recognition. Glycosylation on proteins is now acknowledged as an important posttranslational modification for many recognition processes. The suggestion has been made that glycans on proteins establish an alphabetical system, adding to those of nucleic acids and peptides. However, whereas only the primary sequence of monomeric building blocks is responsible for variability in these last two oligomers, oligosaccharides by their unique multi-linkage monomers and branching structure can reach an unprecedented level of information storage capacity. This enormous potential for variation, along with the fact that oligosaccharides are the product of a non-template directed synthesis makes the sugar language versatile but also extremely elusive. However, oligosaccharides are not primary gene products and therefore the number of the theoretically possible structures is limited since it is necessary to provide a gene for every new structure of carbohydrates. In a receptor/ligand pair, the constraint on variation is probably increased because a change in the oligosaccharide must include the evolution of a binding site on the protein to recognize the new structure (Laine, 1997). High complexity in recognition has possibly evolved through formation of patterns in sets of carbohydrates recognized by sets of binding proteins, and conserved sets of recognition motifs may therefore take part of these systems. It is not yet possible to say whether the recognition motif(s) of human and *Unio* ligand belong to such conserved sets. Useful information in this direction will be derived from comparing other ligand molecules with the same antibody, and sequencing and analyzing conformation of the oligosaccharides of the gp273 derived O-linked oligosaccharides, that is now underway in our laboratory.

Conclusions

The study of fertilization has a rich history and a large literature. The first theory suggesting the involvement of complementary interacting molecules at the surface of the gametes dates back to Lillie (1919). From that moment outstanding progress has been made in the identification of these molecules. Today the study is becoming much more fascinating as the structure of the molecules is being revealed, and it is clear that glycosylation is determinative for their function. Glycoconjugate research was until recently considered an unexplored continent. Currently, the enormous work indicating the role of glycans in recognition has greatly increased the interest in this area of research (Sharon and Lis, 1993; Drickamer and Taylor, 1998; Gagneux and Varki, 1999; Reuter and Gabius, 1999). Some years ago it was suggested that the cell-cell recognition system is phylogenetically linked to the appearance of sexuality (Monroy and Rosati, 1979). In these last years, a relationship between the interacting molecules of primary binding and the P-selectin-ligand system has been demonstrated in pig (Geng *et al.,* 1997) and humans (Oehninger *et al.,* 1998). It is tempting to speculate that primary binding will become a preferential field in the near future to investigate the fascinating secrets of the sugar code in recognition.

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