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New insights into sperm-zona pellucida interaction: involvement of sperm lipid rafts

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TABLE OF CONTENTS

1. ABSTRACT

Sperm-zona pellucida (ZP) binding is the first step of gamete interaction. This binding occurs in two sequential steps, starting with the primary binding of acrosome-intact sperm to the ZP followed by the secondary ZP binding of acrosome reacting/reacted sperm. While there are only a few ZP sulfoglycoproteins involved in these binding events, a large number of sperm surface molecules have been shown to possess ZP affinity. In this review, we have given explanations to the existence of these many ZP binding molecules. We have also summarized their origin and the mechanisms of how they are targeted to the sperm surface and acrosome. Recently, we have shown that sperm lipid rafts have affinity for the ZP. A number of ZP binding molecules are also present in sperm lipid rafts. In this review, we have provided an argument that sperm lipid rafts may be the platforms on the sperm surface for ZP interaction.

2. INTRODUCTION

The fertilizing sperm encounters its target, the mature egg, in the isthmic ampullary junction of the oviduct in most mammals (1,2). In some species such as the mouse, this mature egg is enclosed in a cumulus matrix consisting of cumulus cells interlinked with hyaluronic acid/chondroitin sulfate proteoglycan networks. The sperm penetrate this matrix through their motility force and the enzymatic action of their surface hyaluronidase (2). In other species such as the bovine, the cumulus matrix is rapidly removed from the egg after ovulation, presumably by oviductal hyaluronidase (3). Motile sperm then bind to the zona(e) pellucida(e) (ZP), an extracellular glycoprotein matrix surrounding the egg, in a species-specific manner (4,5). Specific interaction including initial binding of sperm to the zona pellucida was recognized by Hartmann and colleagues (6). This was subsequently revealed as a receptor-ligand interaction (7,8). In many species

Table 1. Nomenclature of Mouse, Human and Pig ZP Glycoproteins and Their Homology among Species

ZP sulfoglycoprotein family (based on the mouse ZP nomenclature)	Species	Nomenclature	Homology between mouse and human ZP glycoproteins	Homology between human and pig ZP glycoproteins	Homology between mouse and pig ZP glycoproteins
ZP1	Mouse	ZP1	67.4%	45.7%	50.3%
	Human	ZP1			
	Pig	ZP3alpha or ZPB[1]			
ZP2	Mouse	ZP2	58.7%	64.7%	54.7%
	Human	ZP2			
	Pig	ZP1 or ZPA			
ZP3	Mouse	ZP3	68.8%	75.5%	65.6%
	Human	ZP3			
	Pig	ZP3beta or ZPC[1]			

[1]A hetero-oligomer of pig ZP3alpha and pig ZP3beta is called ZP3 or ZP(B + C)

including mouse, acrosome-intact sperm bind to the ZP, and this leads to the induction of acrosomal exocytosis (acrosome reaction). However, in a number of other species including guinea pig, sperm may undergo an acrosome reaction prior to ZP binding (2,9-14). Subsequent sperm penetration of the zona pellucida is sometimes described as mechanical, enzymatic, or as a ratcheted binding through the ZP or a combination of these methods. Following sperm penetration of the ZP, the acrosome-reacted sperm attaches at its apex to the egg plasma membrane, rapidly reorients to bind to the egg plasma membrane at the equatorial segment, and subsequently fuses with the egg plasma membrane (2).

The zona pellucida is comprised of 3-4 families of sulfoglycoproteins, each of which shows peptide sequence homology across marsupial and placental mammals (15-17). The differences in the carbohydrate moieties are considered the main factor governing species specificity in sperm binding. In mice and humans, ZP3 is the primary sperm receptor for acrosome-intact sperm, whereas ZP2 is the secondary receptor for acrosome-reacted sperm (see more details in (17) and Table 1)). Nonetheless, recent observations suggest that primary mouse sperm binding to the homologous ZP can occur through a ZP3-independent mechanism, and oviduct-derived ligand(s), deposited onto the ZP during egg transit through the oviduct, may be important for this alternative binding machinery (18,19). In porcine, pig ZP3 (or ZP(B+C)), the hetero-oligomer of ZPB (or ZP3alpha) and ZPC (or ZP3beta) is essential for interaction with acrosome-intact sperm, although ZPB is more important for this sperm binding (20,21). To date, it is still unclear which pig ZP glycoprotein is involved in secondary binding to acrosome-reacted sperm. Although Yonezawa et al. (22) reported that ZPB bound to partially acrosome-reacted sperm and to proacrosin/acrosin, it did not bind to fully acrosome-reacted sperm. Table 1 lists these ZP glycoproteins of mice, humans and pigs based on their peptide homology.

3. BASIS FOR MULTIPLE SPERM MOLECULES WITH AFFINITY FOR THE ZONA PELLUCIDA

A large number of sperm molecules have been demonstrated to have affinity for the ZP and to be involved in sperm-ZP binding (Table 2). Sperm interaction with the zona pellucida includes attachment, binding, induction of the acrosome reaction, and penetration of the ZP matrix. Initial attachment is described as readily disrupted by a mild physical force. This attachment is apparently not particularly species- or order-specific, e.g., between human sperm and mouse oocytes as observed by Bedford (6,23). This loose attachment is followed by a tight binding between sperm and the ZP of the homologous species. Binding is differentiated from attachment by the resistance of sperm to being removed from the ZP by a physical force such as repeated pipetting through a small bore pipet or centrifugation through a density gradient (7,24). Some sperm surface molecules involved in the initial ZP binding are expected to be species- or order-specific as sperm from a species in one order generally do not bind to eggs from a species in a different order. Other sperm surface molecules found in common among various species are also likely involved in this initial sperm-ZP binding. While some of the ZP binding molecules are strictly engaged in the adhesion mechanisms in the initial step of sperm-ZP interaction, others are responsible for the activation of ZP-induced sperm signaling events that culminate in the acrosome reaction. In mice, this activation is a consequence of the aggregation of ZP3 receptors on the sperm surface, as induced by ZP3 multivalent oligosaccharides (25,26). Shur and colleagues have shown that the cytoplasmic domain of sperm transmembrane beta-1,4-galactosyltransferase (GALT), a mouse ZP3 receptor (27,28), interacts with the alpha subunit of G_i protein, and the acrosome reaction is initiated following ZP-induced aggregation of GALT (29). As expected, sperm from *Galt*-null mice do not bind to purified ZP3. However, *Galt*-null sperm can bind to intact ovulated ZP and can fertilize eggs although at only 7% of the wild-type sperm capacity (30). This result confirms that there exists more than one molecule on the sperm surface with ZP affinity and they can act as backups for one another. Specifically for *Galt*-null sperm, Shur et al. have shown that SED-1, a sperm surface protein normally involved in the initial ZP binding, is still functioning, thus allowing gamete interaction (31). Through a low rate of spontaneous acrosome reaction, *Galt*-null sperm can then fertilize the egg (30).

The interaction between sperm and the ZP leads to the activation of sperm signaling events and consequently the acrosome reaction, which initiates with the fusion between the plasma membrane and the outer

Table 2. ZP-Binding Proteins

Name	Site on mature sperm	Origin	Remarks
Glycoenzymes			
Beta-1,4-galactosyltransferase (GALT) (27,79,132)	Plasma membrane overlying the acrosome region (27,78,133,134)	Primary spermatocytes (134,135)	As a transmembrane protein (136), GALT is aggregated following binding to ZP3, leading to activation of G_i–dependent sperm signaling events with the final outcome of the acrosome reaction (29,137) Galt-null male mice have been generated; they can sire offspring but their fertility is reduced. Sperm from null males can fertilize eggs only at 7% compared to wild-type sperm (30).
Alpha-D-mannosidase (138,139)	Plasma membrane overlying the acrosome (140,141)	Spermatocytes, round spermatids and condensing spermatids (primarily) (140)	Alpha-D-mannosidase is an integral plasma membrane protein (140).
PH-20 (Spam1) (142,143)	Guinea Pig: Plasma membrane overlying the postacrosomal region and inner acrosomal membrane in acrosome-intact sperm and inner acrosomal membrane in acrosome-reacted sperm (37,143) Mouse: Anterior head plasma membrane in acrosome-intact sperm (68,71,144) Human and monkey: Sperm head plasma membrane in acrosome-intact sperm (71,145,146) and inner acrosomal membrane in acrosome-reacted sperm (145,146)	Round spermatids (65,68) Epididymal epithelia (69,70) Uterine/ Oviductal epithelia (73)	PH-20 in the inner acrosomal membrane of acrosome-reacted sperm is involved in secondary ZP binding (12,38,72,143,147) PH-20 possesses hyaluronidase activity, used by sperm, to disperse the cumulus mass for sperm movement towards the egg ZP (38,71,148). Interaction between hyaluronan and sperm surface PH-20 leads to activation of sperm signaling events and acceleration of induced-acrosomal exocytosis (149-151) Ph-20-null male mice are still fertile and their sperm can disperse cumulus masses although with a lower efficiency than wild-type sperm (152). Hyal5, another sperm hyaluronidase, may also contribute to sperm-induced cumulus mass dispersion (153). PH-20 is a GPI-linked protein and this may be the basis of how PH-20 in the epididymal fluid is incorporated into the transit sperm plasma membrane, as well as how it moves from the plasma membrane in the postacrosomal region to the inner acrosomal membrane during the acrosome reaction (66,70)
"Lectins" and "glycosaminoglycan binding proteins			
Proacrosin (36,154-156)	Acrosome and inner acrosomal membrane (33,154,156-159)	Primary spermatocytes and spermatids (primarily) (160-163)	It is involved in the binding of acrosome reacting/ reacted sperm to the ZP. Direct binding of proacrosin to mouse ZP2 (secondary sperm receptor) has been demonstrated (36) and the binding is dependent on the sulfate group of the sugar residues of ZP carbohydrate moieties (36,154,155). Acrosin knockout mice have been generated (164,165). Although the null males can sire offspring, their sperm have compromised fertilizing ability, specifically in ZP penetration (165,166)
Sp38 (167-169)	Inner acrosomal membrane (168,169)	Spermatogenic cells (169)	Sp38 is involved in secondary ZP binding presumably to sulfated sugar residues of the ZP glycans, a similar mechanism to proacrosin-ZP binding. Furthermore, the ZP binding motif of Sp38 is also present in proacrosin (167-169).
Zonadhesin (170-172)	The acrosome (172,173), associated with the luminal aspect of the outer acrosomal membrane and adjacent acrosomal matrix (173)	Round spermatids (170,172)	The mature form with ZP binding activity of zonadhesin contains two covalently associated polypeptides possessing D domains of prepro-von Willebrand factor. The precursor form of zonadhesin also contains a MAM domain and a mucin-like domain in its N-terminal region (170,173). Zonadhesin is the major sperm membrane protein that has ZP binding ability (170). Zonadhesin binds to glycosaminoglycans (e.g., heparin and fucoidan) (Hardy, D. personal communication)
Sp17 (174,175)	Acrosome (174)	Primary spermatocytes and spermatids (abundantly) (176)	Sp17 is highly antigenic (177,178). It contains 3 domains: RII alpha subunit of protein kinase A in the N-terminal domain (enabling it to bind to A-kinase anchoring protein (AKAP)); a central sulfated carbohydrate binding domain; and a C-terminal Ca^{2+}/calmodulin (CaM) binding domain (179)
sp56 (AM67) (34)	Acrosome (32,180,181), sperm head plasma membrane (34,182)	Primary spermatocytes and spermatids (primarily) (35,180)	sp56 is a rodent specific protein. In mice, it has been shown for its binding ability to ZP3 O-linked oligosaccharides (34). Detection of sp56 on the sperm surface might be an artifact due to the exposure of the sperm acrosomal content during the early phase of the acrosomal exocytosis (180).

Spermadhesin (AWN, AQN-1 and AQN-3) (183-186)	Sperm head plasma membrane (187,188)	Male accessory glands (189) and epididymal/uterine/oviductal epithelia (186,190)	Deposition of spermadhesins onto sperm is via their interaction with sperm phospholipids (186)
Others			
SED-1 (P47) (31,191)	Plasma membrane overlying the sperm head (31,191,192)	Spermatogenic cells and epididymal epithelial cells (31,191,192)	SED-1 is a mosaic peripheral plasma membrane protein with a high structural homology to milk fat globule-EGF factor 8 (MFG-E8) (191). It consists of 2 Notch like-EGF repeats and 2 discoidin/F5/8 C domains (with known adhesion functions). Its ubiquitous expression suggests that SED-1 may be a generic adhesion molecule in the body (193). SED-1 has been shown to have direct binding ability to both ZP3 and ZP2 of unfertilized eggs. The discoidin/C domains are important for SED-1-ZP binding. However, the interaction between SED-1 and oviduct-derived ligand(s) on the ZP of unfertilized ovulated eggs has also been suggested (18,19). The significance of SED-1 in initial sperm-ZP binding has been confirmed by the subfertility observed in SED-1 null males (31).
Gluthathione S-transferase (GST) (49,194,195)	Plasma membrane at the sperm head anterior, the postacrosomal region and the principal piece (GST Pi isoform); plasma membrane overlying the acrosome and the postacrosomal region (GST Mu isoform) (194,195).	Sertoli cells; GST is secreted into the lumen of seminiferous tubules (194)	GST (both Pi and Mu isoforms) attach to the sperm plasma membrane peripherally (49). Both isoforms bind to solubilized as well as intact ZP of unfertilized eggs in a ZP3-dependent manner (49). The roles of sperm surface GSTs in fertilization are independent of their enzymatic activities (194,195).
Carbonyl reductase (P26h/P34H/P31m) (196-198)	Plasma membrane overlying the acrosome (197,199,200).	Hamster: Primarily spermatogenic cells (spermatocytes and round and elongated spermatids) (201,202) Human and monkey: corpus epididymal epithelia (196,203)	Carbonyl reductase is involved in primary ZP binding (197); the activity of the enzyme is important for this binding (48) It is a GPI-linked protein and a component of epididymosomes, which are important for the transfer of carbonic hydrase to the transit sperm plasma membrane (204,205).
Basigin/MC31/CE9 (60)	Plasma membrane overlying the acrosome (60)	Primary spermatocytes and spermatids (206)	Basigin is a protein in the immunoglobulin superfamily (206). Basigin first exists on the tail of elongated spermatids and immature sperm, but is then relocalized to the convex ridge of capacitated sperm (60,206). Basigin null male mice are sterile due to spermatogenesis arrest (207).
FA-1 (208-210)	Postacrosomal region (210)	Secondary spermatocytes (211)	It is expressed specifically in testes (208) and its immunocontraceptive effect has been shown in mice (212). Antibodies against FA-1 present in infertile men (213) can be preadsorbed by FA-1, thus rendering sperm with higher fertilizing ability (214)
Arylsulfatase A (AS-A) (44,45)	Plasma membrane overlying the acrosome ((44,45) and the acrosome (46))	Pachytene spermatocytes for the AS-A in the acrosomal processes and epididymal epithelia for sperm surface AS-A (46)	Although AS-A is known for its desulfation activity on sulfoglycolipids, it also interacts with sulfated glycoconjugates (including SGG) at its molecular surface (distinct from the active site pocket) (43). Presumably, AS-A from the epididymal fluid deposits onto the sperm surface via its interaction with SGG (46) As-a null male mice can sire offspring (215), although they might be subfertile.
SGG (47,216,217)	Mouse and human: Plasma membrane overlying the acrosome and postacrosomal region (216,217) Pig: Anterior head plasma membrane (44)	Pachytene spermatocytes (218)	Its expression is restricted to mammalian male germ cells and it exists at 10 mole % of total sperm lipids (42). The majority of SGG (70 %) is present in capacitated sperm lipid rafts, possessing ZP affinity (47). Male mice transgenetically deficient in SGG are infertile due to spermatogenesis disruption (219,220)

acrosomal membrane (2). The acrosomal matrix of acrosome-reacting sperm is then exposed for the interaction with the ZP. A number of reports indicate that acrosomal proteins, such as proacrosin, sp56, Sp38, have ZP affinity (see Table 2 with references therein) and this would be the basis of how acrosome reacting sperm remain bound to the ZP (32). In fact, the acrosomal exocytosis occurs in a gradual manner with transient changes of the ZP binding partners in the acrosome (32,33), and both ZP3 and ZP2 have been shown to interact with acrosomal proteins (34-36). Once the acrosome reaction is complete, sperm are left with the inner acrosomal membrane in the head

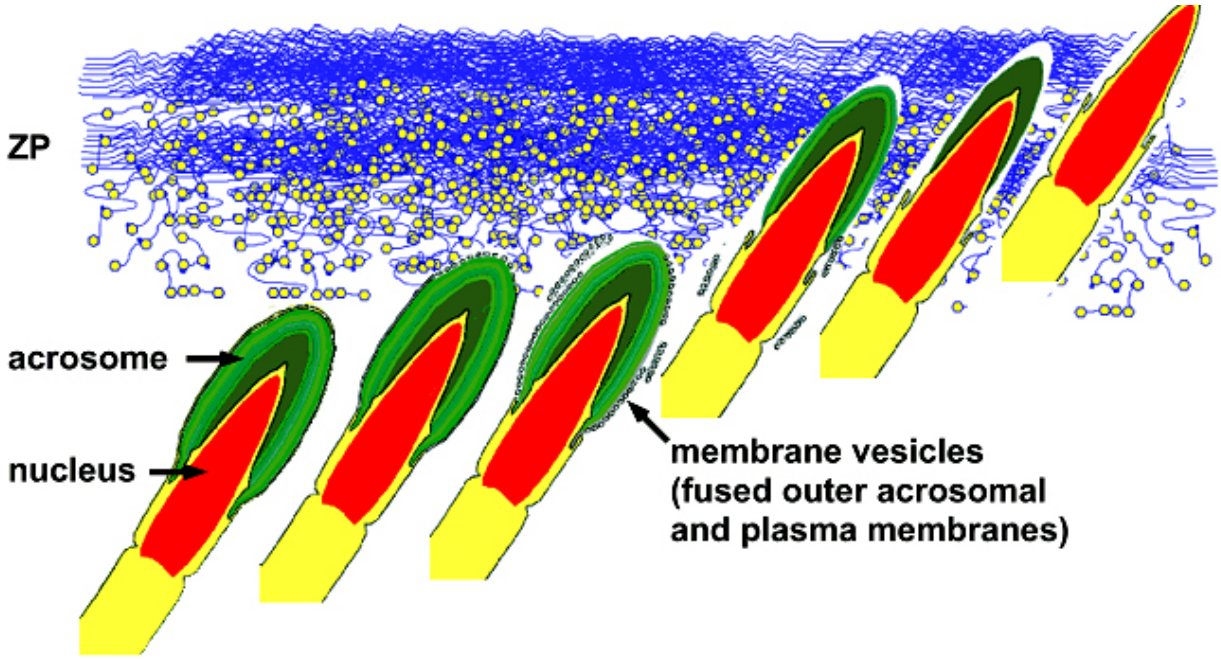

Figure 1. Diagram of acrosomal exocytosis and the penetration of the zona pellucida. A series of images showing the head region of a mammalian sperm and representing successive stages of acrosomal exocytosis and zona penetration (left to right). The various parts of the sperm are: cytoplasm (yellow); nucleus (red); acrosome (green). The zona is composed of glycoproteins (blue lines: protein backbones; yellow hexagons: carbohydate moieties). The acrosome is portrayed in various shades of green to illustrate the subcompartments that are present in the sperm acrosomes of several species. The acrosomal matrix is perceived to be gradually released from the sperm following exposure to the external milieu by fusion of the outer acrosomal membrane with the plasma membrane overlying the acrosome. The matrix may serve as a scaffold for enzymes/proteins that are required for zona penetration. As a result, a narrow penetration slit through the zona is created. Binding of the sperm to the zona via plasma membrane and/or internal acrosomal components may be considered as a two-step process (primary and secondary binding) or as a continuum.

anterior, and the binding of acrosome-reacted sperm to the ZP is dependent on the molecules in the inner acrosomal membrane (e.g., PH-20) (37,38). In short, a number of molecules in the acrosome and the inner acrosomal membrane are temporally involved in the binding between acrosome reacting/reacted sperm and the ZP. In most cases, these sperm acrosomal molecules are distinct from those on the sperm head surface that are involved in the initial binding of acrosome-intact sperm to the ZP. The continuum of the interaction between acrosome intact/acrosome reacting/acrosome reacted sperm and the ZP is pictorially shown in Figure 1.

Listed in Tables 2 are three categories of ZP binding molecules based on their biochemical properties. These include glycoenzymes, lectins and others (those that do not fit into the first two categories). The fact that a number of ZP binding molecules are glycoenzymes and lectins are consistent with the results demonstrating that the carbohydrate moieties of the ZP are important for sperm binding (17,39). Other proteins contain domains known to be involved in extracellular matrix/cell adhesion; these include SED-1 having discoidin domains (19) and basigin being in the immunoglobulin superfamily (40,41). Of particular interest to our research is sulfogalactosylglycerolipid (SGG, also known as seminolipid), a male germ cell-specific sulfoglycolipid. SGG and its structural analog, sulfogalactosylceramide (SGC, also known as cerebroside sulfate and sulfatide) have been shown to bind to several extracellular proteins (fibronectin, laminin, selectin, von Willebrand factor and gp120) (42). Besides its adhesion to the ZP, SGG has high affinity for arylsulfatase A (AS-A) (43), another ZP binding protein (44,45), and this is the basis of how AS-A in the epididymal fluid is peripherally deposited onto the sperm head plasma membrane during sperm transit through the epididymis (46). It is likely that AS-A and SGG function together in ZP binding (see further description on this in Section 5). The co-operative action of ZP binding molecules in sperm-ZP interaction may be another explanation of the existence of multiple molecules with ZP affinity.

A number of ZP binding proteins in the "Others" category listed in Table 2 are enzymes. While the enzymatic activity of carbonic reductase is essential for its role in sperm-ZP binding (48), the ZP binding property of gluthathione S-transferase (GST) appears to be independent of its enzymatic activity (49). It is still unknown whether the sulfatase activity of AS-A is important for sperm

binding to the ZP as well as their penetration through the ZP layer. Since the binding of sperm surface SGG to AS-A does not result in SGG desulfation ((43) and our unpublished results on SGG docking to AS-A), it is unlikely that this binding involves the active site pocket of AS-A. With the availability of the active site pocket, it is tempting to speculate that AS-A may exert its desulfation activity on sulfated sugar residues present on the ZP glycans (50), as part of the mechanism of sperm penetration through the ZP layer. This type of mechanism might also be utilized by glycoenzymes such as alpha-D-mannosidase and PH-20 during the same event.

A few ZP binding proteins are not listed in Table 2 due to a lack of information on their peptide sequence. These include a 55 kDa protein in pig sperm (51,52) and a low molecular weight (~15 kDa) trypsin inhibitor binding component on the mouse sperm surface (53,54). Zona receptor kinase (ZRK), a 95 kDa ZP binding protein in human sperm with a homolog in mice (55-57), was also not listed in Table 2. In this case, its peptide sequence was published and claimed to be a novel protein tyrosine kinase (55). However, the validity of its sequence is questionable as it is identical to a truncated form of c-*mer*; this may be due to errors in molecular cloning and sequencing (58,59).

4. ORIGIN OF SPERM MOLECULES WITH ZP AFFINITY AND THEIR TARGETING TO THE ZONA PELLUCIDA BINDING SITES ON THE SPERM HEAD

Sperm molecules that are involved in the primary ZP binding need to be localized to the head anterior plasma membrane of capacitated acrosome-intact sperm, whereas those engaged in the secondary ZP binding can exist at the outer acrosomal membrane, as part of the acrosomal matrix and/or the inner acrosomal membrane. A number of primary ZP binding molecules are synthesized in spermatogenic cells and targeted to their plasma membrane (such as GALT, mannosidase, P26h, basigin, and SGG) (Table 2 and references therein). In most cases, the ZP binding molecules are compartmentalized to the sperm head anterior during spermiogenesis. However, basigin is first targeted to the sperm tail but is then relocalized to the sperm head anterior plasma membrane during sperm capacitation (60). An increase in sperm plasma membrane fluidity during capacitation (61), due to cholesterol efflux (17), may account for this significant movement of basigin.

Epithelial cells of the epididymis and oviduct as well as Sertoli cells synthesize ZP binding molecules, which are then secreted into luminal fluid, ready to be adsorbed onto the plasma membrane of male germ cells that come into contact with the fluid. Besides being synthesized in spermatogenic cells, SED-1 is additionally acquired onto the sperm plasma membrane from the epididymal luminal fluid during sperm transit/storage (31). In contrast, AS-A, P31m, P34H, spermadhesins and GST on the sperm surface are solely derived from the luminal and/or seminal fluid. AS-A originates from the epididymal fluid, spermadhesins from the epididymal and oviductal fluid and seminal plasma (secreted from the male accessory glands), and GST from seminiferous tubal fluid (secreted from Sertoli cells). Deposition of these extracellular proteins onto the sperm surface appears to be through two main mechanisms. First, P31m and P34H, containing a glycosyl phosphatidylinositol (GPI) link (see references in Table 2), integrate into the sperm plasma membrane via their lipid anchor. Second, proteins such as AS-A and SED-1, possessing inherent affinity for specific lipids on the sperm surface (SGG in the case of AS-A (43,46) and anionic phospholipids for SED-1 (62,63)), are peripherally deposited to the sperm plasma membrane via binding to these sperm membrane lipids.

In contrast to sperm molecules involved in primary ZP binding, secondary ZP binding molecules (proacrosin, Sp38, zonadhesin, Sp17 and sp56) are synthesized in spermatogenic cells and, except for PH-20, they are directly targeted to the acrosome. The targeting process of PH-20 in guinea pig sperm appears to be unique. PH-20 is synthesized in round spermatids with one pool targeted to the acrosomal membrane and the other to the plasma membrane. In testicular sperm, PH-20 exists uniformly on the whole head plasma membrane as well as the outer and inner acrosomal membranes. The localization of both PH-20 populations changes dramatically in mature epididymal acrosome-intact sperm. The plasma membrane population is localized to the postacrosomal region and the acrosome population to only the inner acrosomal membrane. Following acrosomal exocytosis, the PH-20 population that used to be on the postacrosomal plasma membrane moves to the inner acrosomal membrane with the population that has been there (37,64,65). In acrosome-intact sperm, there may exist a barrier between the postacrosomal plasma membrane region and the inner acrosomal membrane. This barrier may interact with PH-20, thus slowing down its diffusion rate, as observed in fluorescence recovery after photobleaching (FRAP) studies. Once this barrier breaks down during the acrosomal exocytosis, the diffusion rate of PH-20 increases. PH-20 gains free movement towards the inner acrosomal membrane where its density is the highest for ZP interaction (66). Recent single particle fluorescence imaging (SPFI) studies, using a fluorescent lipid reporter, 1,1'-dihexadecyl-3,3,3'3'-tetramethyindocarbocyanine (DiIC16), also indicate the presence of a barrier at the border of the postacrosome. In these studies, particles with a ~200 nm diameter were shown to be incapable of moving freely between the postacrosomal region and the equatorial segment/the anterior acrosomal area, whereas the free DiIC16 could (67). These results suggest that sperm surface molecules existing in microdomains with a diameter of 200 nm or larger are restricted from crossing this barrier. While the GPI anchor of PH-20 may allow its lateral diffusion in the sperm plasma membrane, it may also sequester the protein into microdomains such as lipid rafts (see the next section), thus preventing PH-20 in acrosome-intact sperm to move through this barrier.

In mice, PH-20 is synthesized by both spermatogenic cells and epididymal epithelial cells (68-70). PH-20 is present in the anterior head plasma membrane in mature mouse sperm. It plays an important role in sperm

penetration through the cumulus cell layer as it possesses a hyaluronidase domain (71), which is separate from the ZP binding domain utilized by PH-20 at the time of sperm-egg union (72). It is unclear at the present time how much PH-20 derived from spermatogenic cells versus the PH-20 population secreted from the epididymal epithelial cells is distributed on the mature mouse sperm head plasma membrane. Furthermore, Zhang and Martin-DeLeon (73) have described that PH-20 is present in the uterine/oviductal luminal fluid and it is deposited onto the transit sperm plasma membrane. Zhang and Martin-DeLeon argue that this additional deposition of PH-20 may ensure that the protein exists in a sufficient amount for their functions during sperm penetration through the cumulus layers of cumulus oocyte complexes (as hyaluronidase) and during sperm-ZP interaction (as a ZP adhesion molecule) (see Table 2 for more details). Nonetheless, future studies need to be performed to discern the mechanisms of how PH-20 is targeted to the inner acrosomal membrane following acrosomal exocytosis in the mouse system.

Targeting ZP binding molecules to sperm head anterior is the first essential step for their functionality during sperm-ZP interaction. However, it is generally believed that these ZP binding molecules, especially those involved in the primary binding, are not yet optimally exposed for ZP interaction until capacitation (2,17). They are masked by decapacitation factors present in the seminal plasma and male reproductive tract. These decapacitation factors include cholesterol containing membrane vesicles in the seminal plasma (74), and phosphoethanolamine binding protein 1 (75-77) and glycosides (27,78,79) in the epididymis. Through still unclear mechanisms, these decapacitation factors are removed during capacitation; thus, the ZP binding molecules are exposed on the sperm surface. Recent results of Aitken and colleagues (80) suggest that sperm inherent factors may also play a role during capacitation in exposing the ZP binding molecules on the sperm surface. They have described the tight correlation between tyrosine phosphorylation of proteins on the plasma membrane overlying the mouse sperm acrosome (ZP binding site) and the ZP binding ability of sperm. There appear to be three major proteins from this sperm entity that are tyrosine phosphorylated and two of them are identified to be molecular chaperones, heat shock protein 60 (hsp60) and endoplasmin 99 (erp99). Since pretreatment of capacitated sperm with anti-phosphotyrosine antibody does not result in inhibition of sperm-ZP binding, the authors have suggested that tyrosine phosphorylation of the two molecular chaperones may be important for ZP binding molecules becoming functional ZP receptor complexes; this may be through conformational changes of these molecules that lead to exposure of their ZP binding domains (80). It is also tempting to propose that these molecular chaperones may bring various ZP binding molecules into the same microdomains on the sperm head plasma membrane. This may facilitate co-operative binding activities of these ZP binding molecules, thus allowing sperm interaction with the ZP to be stable enough to withstand the pulling force generated by the ongoing sperm movement. We have recent evidence that these microdomains are likely sperm lipid rafts, and this is discussed in detail in the next section.

5. ROLES OF SPERM LIPID RAFTS IN SPERM-ZONA PELLUCIDA INTERACTION

Freeze fracture electron microscopy reveals the presence of elevated hexagonal particles (each with a diameter of ~20-30 nm) on the plasma membrane overlying the acrosome in both guinea pig and rat sperm (81). This finding corroborates the more recent concept that there exist liquid ordered microdomains termed lipid rafts on the plasma membrane (82,83). The "liquid ordered" property of lipid rafts refers to their intermediate state between the gel phase and the "fluid" liquid crystalline phase; the hydrocarbon chains of raft-resident lipids pack together in an orderly fashion, yet they can still move laterally in the bilayers (84-86). In most lipid rafts, cholesterol is an integral component. Glycolipids, sphingolipids and saturated phospholipids, as well as GPI-linked and acylated proteins, are also present selectively in lipid rafts (82,83). The saturated nature of the hydrocarbon chains of these lipids facilitates hydrophobic interaction between themselves and the planar rings and side chain of cholesterol. Hydrogen bonding between cholesterol and glycolipids further stabilizes the lipid raft microdomains. This results in the resistance of lipid rafts to solubilization by a number of non-ionic detergents (85,87,88) and they are often referred to as detergent resistant membranes (DRMs). Triton X-100 (0.5 to 1%) has been widely used at cold temperature to isolate lipid rafts from cells (85,89). Due to their low buoyant density (attributed to low proteins/lipids weight ratio), lipid rafts float up in a density gradient. In contrast, detergent solubilized proteins/lipids remain at the bottom (89). Lipid rafts have also been prepared using milder non-ionic detergents and even no detergents (90-94). Notably, similar types of lipid and protein components are present in rafts isolated either by detergent or non-detergent methods (92,94). Furthermore, lipid rafts have been isolated from a mixture of two HeLa cell populations: one metabolically labeled with tri-deuterated Leu (LeuD3) and the other cultured in medium with normal Leu but treated with a cholesterol binding molecule to disrupt lipid raft microdomains. The isolated lipid rafts only contained LeuD3-labeled proteins (92). These results argue against the controversy that lipid rafts are detergent induced aggregation artifacts. Nonetheless, high resolution imaging methods that can account for the dynamic nature of lipid rafts are desirable for validating the existence of lipid rafts *in situ*. Results from imaging studies reveal that lipid rafts of somatic cells are between 10 and 700 nm in size and are dynamic in their existence (95-99).

Numerous cell adhesion, signaling and trafficking molecules have been found in isolated lipid rafts (92,94,100-104). Therefore, lipid rafts have been considered as platforms on the plasma membrane for cell adhesion and signaling as well as final targets of protein/lipid sorting and trafficking (82,83). In addition, the current concept states that ligand-induced aggregation of lipid rafts leads to stabilization of the raft microdomains as well as activation of cell signaling (82,83,96). These results and new concept have made research in lipid rafts a hot topic. To date, approximately 2000 articles on lipid

rafts are reported in PUBMED. While sperm-egg interaction is an ideal physiological process for validating the implication of lipid rafts in cell adhesion and signaling, research on gamete lipid rafts is still limited. Only 17 research articles on sperm lipid rafts and 6 articles on egg lipid rafts have appeared in PUBMED. All egg lipid raft studies involved isolation of the raft vesicles and are thus restricted to the Xenopus and sea urchin systems (105-110), since eggs can be obtained in quantity from these animals. Most of these studies reveal the presence of signaling proteins in egg lipid rafts (e.g., xSrc and uroplakin in Xenopus egg rafts and Src and PLCγ in sea urchin egg rafts). The presence of GM1, a ganglioside found in somatic lipid rafts, is also reported in isolated Xenopus egg lipid rafts (109). For sperm lipid rafts, reports are available from both mammalian (47,111-123) and sea urchin (124-126) systems. To date, three lines of questions have been addressed in sperm lipid raft research. First, do sperm or isolated sperm lipid rafts (DRMs) contain markers known to exist in somatic cell lipid rafts? Second, is the formation of lipid rafts compromised in capacitated sperm due to the efflux of cholesterol, a raft integral component? Third, are sperm lipid rafts involved in egg binding? Positive answers have been given for the first question. Mammalian sperm and/or their isolated lipid rafts contain GM1 (114,117-120), caveolin (111,115,120,121,123) and flotillin (47,114,123,127). A number of GPI-anchored proteins are also present in isolated sperm lipid rafts, including CD59, CD52, TESP5 and PH-20. (112,114,117,122), and these findings are similar to what has been observed in somatic cells (82). In place of GM1, isolated sea urchin sperm lipid rafts contain unique acidic glycolipids, i.e., sulfated and non-sulfated poly-sialic acid containing gangliosides (124-126).

Two opposing answers have been obtained to the second question. Our studies, as well as those from Roy Jones' group, indicate an increase in the levels of lipid rafts in capacitated pig sperm (47,113). In contrast, Pablo Visconti et al. show a disappearance of lipid rafts in mouse sperm following capacitation (115), a finding that concurs with the known fact that cholesterol, an integral lipid raft component, is released from capacitated sperm. However, it should be remembered that there is still a substantial level of cholesterol remaining in capacitated sperm (~50% of non-capacitated sperm levels), and it is unlikely that all lipid rafts would have disappeared as described by Visconti et al. (115). We argue that this disappearance is due to the use of a high weight ratio of Triton X-100 to sperm proteins (47) in their lipid raft isolation. On the other hand, an increase in lipid raft levels following capacitation can be explained by the following events. First, cholesterol is released from the non-raft (fluid) domains of the sperm plasma membrane during capacitation. This release leads to activation of scramblase (128), which then allows the remaining cholesterol to regroup with its preferential lipid partners (i.e., SGG and saturated phospholipids), thus more patches of lipid rafts can be formed (47). This model is shown in Figure 2A.

Direct interaction of sea urchin sperm lipid rafts with SBP, a 350 kDa sperm binding protein, existing in the egg vitelline layer, has been documented (126). These investigators further show that the highly acidic sulfated and non-sulfated poly-sialic acid containing glycolipids are important for this binding. SGC (a structural analog of SGG), co-present with these highly acidic glycolipids in sea urchin sperm lipid rafts, is also involved in this interaction (126). For mammalian sperm lipid rafts, a number of reports indicate the presence of molecules with affinity for the ZP and egg plasma membrane. These include PH-20 (112), spermadhesins, proacrosin (123), basigin, IZUMO, CRISP1, TESP1 (115), SGG and AS-A (47). Finally, our recent work indicates that isolated pig sperm lipid rafts are able to bind directly to solubilized pig ZP with mechanisms similar to those employed by intact sperm and isolated sperm anterior head plasma membranes (47). In particular, the sperm lipid rafts-ZP interaction is greatly dependent on pig ZPB glycoprotein as well as the ZP carbohydrate moieties. Most significantly, our results reveal a high affinity and capacity of lipid rafts from capacitated sperm to bind to the ZP (which may reflect larger areas of lipid raft microdomains in these sperm). Of further interest is the finding that the majority of SGG exists in lipid rafts of capacitated sperm. Furthermore, SGG contributes to lipid raft formation via its interaction with cholesterol and saturated phospholipids, as well as the ZP binding ability of the sperm lipid rafts (47). However, since the galactosyl sulfate groups of SGG molecules rise only a short distance above the sperm plasma membrane bilayer, it is likely that another protein, well exposed on the sperm head surface, may be responsible in the initial capture of the ZP glycans to interact with these galactosyl sulfate groups of SGG. Our recent result indicates the presence of AS-A, a ZP binding protein, in isolated sperm lipid rafts. As an SGG binding partner and a peripheral plasma membrane protein, AS-A is likely engaged in capturing the ZP glycan for the subsequent carbohydrate-carbohydrate interaction with SGG head groups. This postulation is illustrated in Figure 2B. Our observations, as well as the proteomic analysis results of sperm lipid rafts described above, support the concept that sperm lipid rafts are platforms of ZP binding molecules on the sperm head plasma membrane for ZP interaction. Nonetheless, changes of lipid raft microdomains prior to or after the initial sperm-ZP binding may also be important for the fertilization process. Prior to the sperm-ZP binding (perhaps as part of sperm capacitation), angiotensin converting enzyme (ACE), possessing a GPI-anchored protein releasing activity, can release TESP5 and PH-20 from sperm lipid rafts, and this release appears to be essential for sperm binding to the ZP (129). Sperm retrieved from the infertile Ace-null mice cannot bind to the ZP (130). However, this ZP binding activity can be restored by pretreatment of sperm with ACE. It is likely that components of sperm lipid rafts involved in the initial sperm-ZP binding would be released from the sperm surface following the acrosome reaction. The movement of PH-20 out of these raft microdomains prior to the initial sperm-ZP binding would protect it from this release, so that it can serve as a ZP adhesion molecule during the secondary binding. In contrast, the interaction between multivalent oligosaccharides of ZP3 and their receptors on the sperm surface leads to

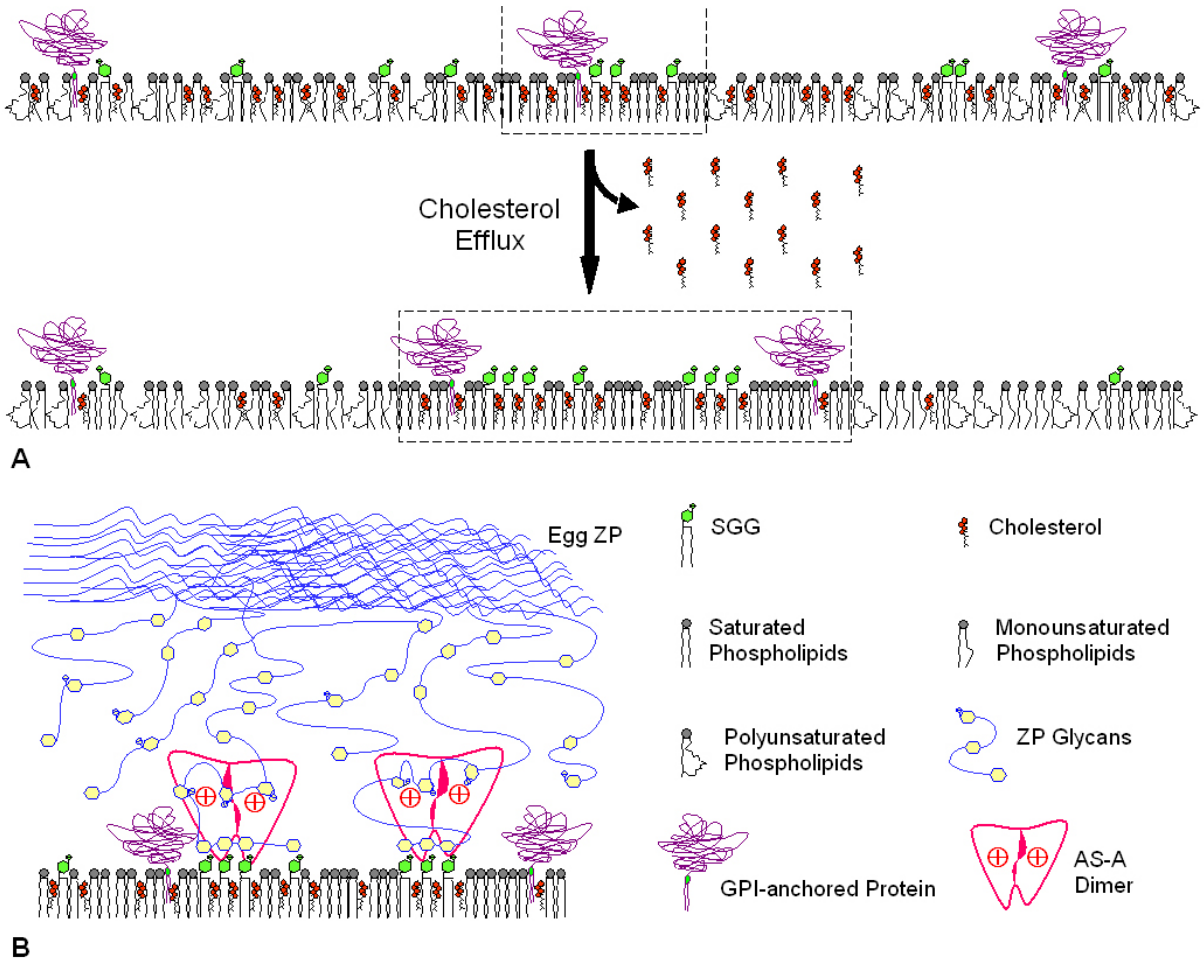

Figure 2. A. Hypothetical models for an increase in lipid rafts microdomains in the sperm plasma membrane following capacitation. For simplicity, only the outer plasma membrane leaflet is shown in this model as well as in the model in B. In control sperm, ~30% of SGG was in the lipid rafts (cropped in a dashed-line square). These SGG molecules interact with cholesterol and saturated phospholipids. Some GPI-linked proteins may also exist in control sperm lipid rafts via the interaction of their acyl chains with the saturated fatty acyl chains of phospholipids. The remaining SGG molecules are in the non-raft areas, which contain a high percentage of phospholipids with one of their acyl chains being mono- or polyunsaturated. However, the other acyl chain of these phospholipids is mainly saturated, thus interacting with the hydrocarbon chains of SGG in the non-raft areas. Cholesterol also coexists with SGG and phospholipids in the fluid non-raft areas, interacting with their saturated hydrocarbon chains or monounsaturated acyl chains with the double bond deep below the membrane interface. During capacitation, cholesterol is likely to be released from the non-raft areas, resulting in a further increase in fluidity. Scramblase is also activated allowing lipids to reorganize themselves (Flesch et al., 2001). SGG, cholesterol and saturated phospholipids would regroup together, thus forming new patches of lipid rafts, which may coalesce with preexisting lipid raft microdomains in the sperm plasma membrane. B. Hypothetical model of how SGG molecules in sperm lipid rafts interact with the ZP glycans. The ZP glycans likely interact with the galactosyl sulfate moiety of SGG. However, since this monosaccharide head group would rise only a short distance above the sperm membrane layer, it would be difficult for the ZP glycans to reach this head group without additional anchoring force. AS-A, a peripheral plasma membrane protein which exists in a dimeric form at physiological pH, has affinity for both SGG and the ZP (Carmona et al., 2002a,b). AS-A may first bind to the ZP glycans (possibly via interaction between positively charged amino acids of AS-A and sulfated sugar residues of the glycans) and attract them to the sperm plasma membrane for the interaction with the galactosyl sulfate lawn of SGG molecules. Although the carbohydrate-carbohydrate interactions between the galactosyl sulfate groups of SGG and ZP glycans are not strong, they may be stabilized by the multiplicity of SGG molecules in the lipid raft microdomains. Reproduced with permission from 47.

aggregation of these receptors and then activation of sperm signaling events (25). Since sperm lipid rafts contain a number of ZP binding molecules, it is likely that the raft microdomains are the sperm surface entities that become aggregated. This hypothesis corroborates the concept in lipid raft research that cell signaling

events are induced following aggregation of lipid raft microdomains (82,83,96).

6. PERSPECTIVES

Several explanations have been given to the existence of a large number of ZP binding molecules. The arguments that they can act as backups for one another and/or they act co-operatively via different mechanisms are supported by the ability of *Galt*-null mouse sperm to bind to intact ZP and to fertilize the egg. The concept that a group of molecules are involved in the initial primary ZP binding, whereas the other in the secondary binding is attested by the different localization of these two types of molecules on the sperm head. The former is on/in the plasma membrane overlying the acrosome and the latter in the acrosome. In addition, molecules involved in the initial interaction between sperm and the ZP have selective binding to ZP3, and those engaged in the secondary binding likely interact more with ZP2. However, the explanation that a certain set of ZP binding molecules serves for species/order specificity has not been well supported by data obtained so far. Only sp56 appears to be rodent-specific. The search for species/order specific ZP binding molecules will require extensive sperm proteomic analyses among different species/orders. Furthermore, information on the identities of ZP carbohydrate moieties, as well as the understanding of how ZP glycoproteins interact with sperm surface molecules, will be required in this search, as this species- or order-specific binding may also be attributed to the ZP glycoproteins (131).

If ZP binding molecules on the sperm surface can act co-operatively as well as act as backups for one another, it would be logical to speculate that they are in close proximity to one another. The observations that a number of ZP binding molecules exist in isolated sperm lipid rafts support this hypothesis. However, in order to prove this, co-localization of these molecules in the lipid raft microdomains on the sperm head surface needs to be shown *in situ*. A number of imaging techniques have been applied to detect lipid raft components, including fluorescence resonance energy transfer (FRET), fluorescence recovery after photobleaching (FRAP), single particle tracking, photonic force microscopy and spatial analysis by electron microscopy (95,98). Among these techniques only the electron microscopy approach gives the direct information on molecular localization, and can be easily used to localize more than one molecule. This is in contrast to other detection methods, which are based on the deduction of the diffusion rates of the molecules (98). As illustrated by freeze fracture electron microscopy done in 1974 that there exist elevated microdomains on the sperm head plasma membrane (81), it remains to be seen whether these microdomains house lipid raft components including those that are ZP binding molecules.

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