Role of zonadhesin during sperm–egg interaction: a species-specific acrosomal molecule with multiple functions

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ABSTRACT: Sperm–zona adhesion is an essential event in mammalian fertilization, failure of which causes sterility. However, the molecular mechanisms involved in this process are still poorly understood. It has been suggested by few laboratories studying gamete interaction that acrosomal molecules are implicated in sperm–zona pellucida adhesion prior to the acrosome reaction (AR). Zonadhesin, a sperm-specific protein located in the acrosome is critically involved in zona binding. Here we describe the cellular and molecular interaction of zonadhesin during fertilization and also discuss its role in species-specific gamete interaction—an intriguing question in biology. We propose a model in which sperm could transiently expose acrosomal molecules that adhere to the zona independently of the AR in a ‘kiss and run’ mechanism. This could be a valuable framework for further investigations and a detailed understanding of the molecular events during gamete adhesion is likely to provide new approaches for the design of more effective male contraceptives and better diagnostic methods for sperm dysfunction.

Key words: fertilization / spermatozoa / zona pellucida / acrosome reaction / cell–cell interactions

Introduction

Infertility is a global problem affecting ~1.7 (~80 million) couples worldwide (Irvine, 1998; Sharpe and Irvine, 2004; Boivin et al., 2007). The single commonest cause is sperm dysfunction (20%) and 28% of the cases have no identifiable cause and are classified as unexplained (Hull et al., 1985; Brandes et al., 2010, 2011). Surprisingly there is no non-assisted reproduction treatment (ART) for sperm dysfunction and a primary reason for this is our lack of understanding of the cellular and molecular workings of the mature cell (Barratt et al., 2011). This is particularly so in the understanding of the molecular events involved in sperm–zona pellucida (ZP) adhesion where there is a wealth of robust clinical data to show that sperm ZP interaction is a clear and defined cause of sperm dysfunction (Liu et al., 2007).

A detailed understanding of the molecular events during gamete recognition is likely to provide (i) improved diagnostic tests (ii) potential development of non-ART-based therapy possibly via drug development (iii) new approaches for the design of more effective male contraceptives. A key protein that has received particular attention in the last decade is the sperm-specific protein zonadhesin. Zonadhesin is involved in one of the essential steps of fertilization: sperm–ZP adhesion. Additionally, it is unique among putative sperm–ZP receptors elucidated to date, as it is the only ZP-binding molecule with species-specific gamete recognition in mammals. Therefore zonadhesin is functionally important for sperm–ZP adhesion and has a wider potential significance as key molecule regarding the fundamental question of biological diversity (species specificity). This review presents a synthesis of the more recent data relating to zonadhesin and discusses its relevance to both fertility and evolution.

Origin of zonadhesin

Zonadhesin was discovered following the pioneering work of Hardy and Garbers (1994). It was isolated by its ability to adhere to native ZP and described as a male germ cell protein associated with species-specific properties (Hardy and Garbers, 1994, 1995). Since its discovery 17 years ago, it remains the only mammalian molecule showing such species specificity. In marine invertebrates, species-specific interaction involve the sperm and the egg’s vitelline layer via a single acrosomal protein with its complementary molecule on the egg (Swanson and Vacquier, 2002). The primary
function of the acrosomal molecules bindin and lysin is to confer species specificity during fertilization of sea urchin and abalone, respectively (Vacquier and Moy, 1977; Vacquier et al., 1990). The involvement of zonadhesin in species specificity and its location in the acrosomal matrix, similar to marine invertebrates (bindin and lysin), suggest that this acrosomal function has been conserved between marine invertebrates and mammals. ZP3 glycoprotein has a role in the regulation of species-restricted gamete recognition in mammals (Han et al., 2011) and it has been suggested that structural features of gamete recognition proteins on the egg have been conserved over billions of years of evolution (Swanson et al., 2011). However, species-specific molecules found in the acrosome such as zonadhesin, bindin or lysin are evolutionarily unrelated (Swanson and Vacquier, 2002). Despite species-specific recognition between spermatozoa and ZP as one of the crucial steps in fertilization, little is known about the molecular mechanisms that underlie this process.

Zonadhesin is a mosaic protein organized in tandem

Zonadhesin is a multiple domain protein, first isolated from porcine sperm membranes (Hardy and Garbers, 1994). In the pig, its mRNA spans 7785 kb and encodes for Mr 270 234 protein (Fig. 1). Zonadhesin precursor encompasses three different domains related to cell adhesion: MAM (meprin/A5 antigen/mu receptor tyrosine phosphatase), mucin and von Willebrand D domains (VWD). Zonadhesin cDNA from pig (Hardy and Garbers, 1995), mouse (Gao and Garbers, 1998), human (Wilson et al., 2001), rabbit (Lea et al., 2001), donkey (HM070406.1 NCBI; partial) and stallion testis (Tardif et al., 2010a; HM064497.1 NCBI; partial) have been isolated and sequenced (Hardy and Garbers, 1995). Zonadhesin molecules share a remarkably common structure between species, but interestingly exhibit considerable inter-species variation in terms of amino acid identity (Tardif et al., unpublished), making this a potential mechanism for species specificity. Although zonadhesin’s structure seems highly conserved between non-rodent species (pig, human and rabbit), this molecule evolved dramatically in rodent species, for example, the size of zonadhesin mRNA doubled (16.481 kb) encoding for a protein of Mr 579 915. The domain structure found in other species is similar in mouse, but an additional region unique to mouse is localized between D3 and D4 VWD, named D3 partial domains (D3p; see Fig. 1; Gao and Garbers, 1998). We have demonstrated that this extra portion originated from a gene duplication event of the two last exons of D3 VWD (Tardif et al., unpublished). Moreover, this additional region in the mouse seems common in rodents. The complementary DNA encoding for D3 partial domains in hamster and rat has been isolated (Olson et al., 2004). Interestingly, Hickox and collaborators concluded that the last two exons of D3 VWD are possibly involved in porcine sperm–egg adhesion (Hickox et al., 2001). This relationship between adhesion and the origin of the duplicated region (D3 VWD) may suggest that this expansion between rodent species contributed to species specificity maintaining so many independent species in the rodentia order. This is particularly interesting because the mechanisms of the reproductive barrier in mouse (2277 independent rodent species) remain enigmatic.

![Figure 1](https://academic.oup.com/molehr/article-abstract/17/11/661/1028448)

**Figure 1** The domain structure of zonadhesin of three different mammals (human, pig and mouse). Note that the zonadhesin structure changed dramatically in mouse compared with human or pig. The consensus sequence of the testicular processing is shown for pig zonadhesin, the arrows denote the emplacement of these sites present in the D1, D2 and D4 VWD domains. Testicular precursor is hydrolysed at the position 807Asp→808Pro, 1191Asp→1192Pro, 1972Asp→1976Pro (aspartate–proline bond) producing zonadhesin polypeptides of Mr 300 000 (p300), Mr 105 000 (p105) and Mr 45 000 (p45) in the mature spermatozoa.
Localization of zonadhesin in male germ cells

Zonadhesin is detectable first in round spermatids

Zonadhesin is produced during spermatogenesis in a polarized fashion located at the nascent acrosome in the early spermatid (Fig. 2; Bi et al., 2003; Olson et al., 2004). It is modified by proteolytic enzymes very quickly after translation as no precursor form (full length) is detected, but predominant zonadhesin polypeptides found in pig spermatozoa are p300 (M, 300 000), p105 (M, 105 000) and p45 (M, 45 000) (see Fig. 1). Figure 2A summarizes zonadhesin formation during spermatogenesis. Testicular processing of zonadhesin occurs at the consensus site GDPHY at the aspartate and proline bond as shown in Fig. 2C, producing different polypeptide sizes in mature spermatozoa (Hickox et al., 2001; Bi et al., 2003). This site is conserved between porcine and equine species (Fig. 2C; GDPHYL) and probably also in other species (Tardif et al., 2010a).

Zonadhesin localize in the acrosomal matrix of spermatozoa

Zonadhesin is localized at the apical head region of spermatozoa released in the seminiferous lumen (Fig. 2D). Interestingly, co-localization of zonadhesin and *Pisum sativum*-FITC clearly showed zonadhesin within the acrosomal matrix. Zonadhesin is undetectable at the sperm surface of live uncapacitated cells, as it is sequestered inside of the acrosomal matrix before capacitation. Sperm membranes have to be permeabilized to allow access. Moreover, when spermatozoa undergo the acrosome reaction (AR), zonadhesin is undetectable. This has been observed in a number of mammalian species: mouse, hamster, rat, horse, donkey, zebra, human, pig, rabbit and dog (Tardif et al., unpublished).

Zonadhesin function during fertilization

Zonadhesin is exposed at the sperm surface during capacitation

In contrast to earlier expectations, detailed ultrastructural studies show that zonadhesin is located at the outer acrosomal membrane (OAM) and not the plasma membrane (PM) (Bi et al., 2003; Olson et al., 2004). Consequently, the function of zonadhesin was originally thought to be confined to after the AR rather than sperm–ZP recognition. However, zonadhesin is exposed or at the very least accessible at the sperm surface simultaneous with capacitation in mouse spermatozoa (Tardif et al., 2010b). Moreover, sperm–egg adhesion or sperm penetration was significantly reduced when sperm cells were incubated in the presence of zonadhesin antibody (to D3p18 domain) during mouse capacitation (Tardif et al., 2010b). This is a very important result because zonadhesin exposure during capacitation strongly supports the ‘acrosomal dynamic concept’ previously proposed by Gerton and colleagues (Buffone et al., 2008).
hypothesized direct interactions of intra-acrosomal proteins with the egg’s extracellular matrix prior to full vesiculation of the acrosome. These observations on zonadhesin exposure support a recent study showing that fertilizing spermatozoa begin the AR before physical contact with ZP in mouse, suggesting that the dogma of ZP induced AR needs revision (Jin et al., 2011; Tardif, 2011).

Zonadhesin is involved in gamete species specificity

Although data on the species-specific nature of zonadhesin was demonstrated using extract of zonadhesin and native zona almost 17 years ago, recent information at the cellular level provides details of its function (Tardif et al., 2010b). Using a sperm competition approach, spermatozoa from zonadhesin-zan-null mice had a higher level of sperm adhesion to heterologous ZP (bovine, pig and rabbit). Even though 3-fold the number of wild type spermatozoa was added simultaneously with zonadhesin-null cells, the sperm ratio bound to orthologue ZP (bovine, pig or rabbit) was preferential to zan-null cells. In contrast, the ability of mouse ZP to bind either wild type or zan-null mouse spermatozoa was identical for both genotypes, i.e. no selectivity. Zonadhesin removal by gene disruption did not affect the acrosomal structure and/or sperm physiology, but importantly the gamete specificity for other species was impaired. Furthermore, the profile of a number of different sperm proteins implied in zona adhesion was evaluated in zan-null spermatozoa and no alteration was observed, e.g. ZP3R/SPS6, SPAM1/PH-20, SED-1, β-1,4-galactosyltransferase (Tardif et al., 2010b). Taken together, these data suggest that zonadhesin is a potential checkpoint for species specificity—its absence allows inter-species adhesion.

Post-translational modifications of zonadhesin

Proteolytic activity and protein glycosylation cause an important size variation in zonadhesin polypeptides between species (Bi et al., 2003; Tardif et al., 2010a). In addition, Gasper and Swanson (2006) identified 19 non-synonymous polymorphisms (amino acid changes) in human zonadhesin over 48 individuals. This study established that five polymorphisms changed the charge and the degree of hydrophobicity implying a secondary structure change potentially affecting function. In the same context, five different mRNA variants of human zonadhesin have been isolated (AY046055). These variants were produced by alternative splicing, removing a portion of coding sequence between exon 42 and 45. Testicular mRNA collected from five different men was used to isolate zonadhesin splice variants. Therefore, it is unknown if all variants are present in one individual. We hypothesized that one or more particular variants could be associated with altered sperm–ZP adhesion, potentially making this sperm population less functional (Fig. 3). This putative model suggests that some zonadhesin sperm proteins combinations are sub-optimal compared with others and may be an explanation for idiopathic infertility, i.e. reduced compatibility between partners based on different isoforms (produced by post-translational modifications) of sperm–ZP receptors. This model is concordant with the adaptive hypothesis determining selective forces driving reproductive protein evolution (Findlay and Swanson, 2011) which involves the co-evolution of both male and female gamete proteins (Dorus et al., 2004; Clark et al., 2006; Calkins et al., 2007). Alternative splicing in human and interspecies variation may contribute to positive adaption, defined by a sperm–ZP adhesion advantage and promoting rapid evolution (Swanson and Vacquier, 2002). The evidence of positive selection in zonadhesin determined by different approaches suggests a species-specific advantage in gamete adhesion and post-translational modifications potentially changing zonadhesin adhesion activity (Herlyn and Zischler, 2005; Gasper and Swanson, 2006).

Zonadhesin in the mammalian kingdom

Zonadhesin changed dramatically in rodent species

As discussed, the gene structure of mammalian zonadhesin has changed dramatically within rodent compared with non-rodent species (Fig. 1). Mammals are segregated into 29 different orders, a taxonomic rank used to classify organisms, including ~5490 mammalian species (The International Union for Conservation of Nature). Almost half of mammals are included in Rodentia order (rodent). This order is constituted by two different sub-orders: hystricognathi and sciurognathi. The primary character used to classify mammals in one or the other sub-order is the shape of the lower jaw. Animals
forming the hystricognathi sub-order (guinea pig, capybara, chinchilla) are mostly found in South America (new world animals) with only a few species living in North America. The second sub-order (sciurog- 
nathi) encompassing other rodents originated from the 'supercountry' now known as North America, Europe and Asia. The 
sciurognathi sub-order includes common rodents, e.g. species of 
mouse, rat, hamster, beaver, squirrels and chipmunk. Among the 
2277 species of rodents some are very similar in size and live in the 
same ecological region, however, they remain independent. The implication of this is that molecular mechanisms make species in close proximity reproductively incompatible.

Zonadhesin was shown to be present in spermatozoa of all rodent species tested namely: mouse, rat, hamster, vole, chinchilla and guinea pig. However, the expansion of the partial D domain exclusive to rodent species was not detected in guinea pig zonadhesin at least using an affinity-purified antibody to WVD partial D domain. This observation suggested that partial D domain found in mouse (sciurognathi) occurred by gene duplication during evolution that did not happen in the hystricognathi sub-order. Although, zonadhesin mRNA has not been sequenced in the guinea pig, the genomic sequences are available. The zonadhesin gene is located on the guinea pig chromosome #30, in a syntenic region of mouse genome. Interestingly, the partial D domain region (exon #38–77 in mouse) seem to be absent in the guinea pig genomic sequence, only two exons were encoding downstream of the D3 domain, but all seven exons encoding for the D4 domain were present (Supplementary data, Fig. S1, S2 and Table S1). The coding regions down stream to the VWD3 domain look to be encoding for the VWD4 domain. These two independent pieces of information strongly suggest that the hystricognathi sub-order could have evolved differently than sciurognathi species. Previously, it was suggested that hystricomorph rodents should be part of a different taxonomical rank and treated as a separate mamma- 
lian order, distinct from Rodentia (Graur et al., 1991; D’Erchia et al., 
1996). Hystricomorph rodents from the new world often show molecular discrepancies in comparison with other murine species (Wolfe and 
Cebra, 1980; Sarkar et al., 1990; Shi et al., 2000). Moreover, the rate 
of evolution of a peptide and its receptor may reflect the phylogenetic 
relationship between different species. In agreement with this, 
sequences of several neuropeptides and receptors reflect taxonomy 
and phylogeny as morphological features (Larhammar and Salaneck, 
2004). For instance, the neuropeptide Y4 (Hoyle, 1999) as well zonad- 
hesin support the view that the guinea pig species belong to a distinct 
order. On the other hand, some molecular evidence support the mono- 
phyly hypothesis for the rodentia order, whether hystricognathi and 
sciurognathi species have a common ancestor and should be in the 
same taxonomic rank (Cao et al., 1994; Robinson-Rechavi et al., 
2000). This controversy remains unresolved. More gene analysis will 
be necessary to confirm the polyphyly or the monophyly hypothesis.

**Figure 4** Time line of mammalian fertilization and the importance of an acrosomal molecule such as zonadhesin in sperm adhesion, a species-specific process. Zonadhesin is exposed during capacitation (in red) using a putative model including the participation of a ‘fusion pore’ periodically in the open and close state. P4, progesterone; PM, plasma membrane; OAM, outer acrosomal membrane; IAM, inner acrosomal membrane; Zan, zonadhesin; ZP, zona pellucida.


**Putative mechanism of zonadhesin exposure**

The release of the acrosomal contents was previously suggested to take place during the AR, an exocytic phenomenon made possible by the fusion between the PM and OAM (Yanagimachi, 1994). However, instead of a one-off event this process may be a more controlled continuum although a clear model including molecular mechanisms has not been fully elaborated.

Exposure of zonadhesin during capacitation supports the latest model proposed to explain sperm–ZP interaction in which acrosomal proteins are directly involved in sperm–ZP adhesion. The diagram in Fig. 4 illustrates the sequence of zonadhesin exposure during fertilization. As discussed, zonadhesin exposure is directly related to capacitation (Tardif et al., 2010b) which is important as mammalian sperm gain fertilizing ability and the capability to penetrate an oocyte only after capacitation in the female tract (Austin, 1951, 1952; Chang, 1951). Various maturational events have been associated with sperm capacitation, such as calcium uptake (Publicover et al., 2007) and tyrosine phosphorylation in different species (Visconti et al., 1995; Leclerc et al., 1996; Tardif et al., 2001, 2003). The latter post-translational modifications could prime sperm cells to expose acrosomal proteins or make cells competent to interact with ZP and readily undergo exocytosis.

Mammalian exocytosis is associated with an increase of intracellular calcium (Florman et al., 1998), similarly observed with synaptic vesicles. The SNARE hypothesis suggests that a SNAP (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) present on the vesicle (OAM) named v-SNARE interacts with a complementary molecule on the target membrane (PM) named t-SNARE (see Fig. 4). This interaction could provoke localized fusion and docking the PM with OAMs allowing access of acrosomal proteins to the outside, but without an uncontrollable destabilization of the PM. SNARE complexes were shown to be associated with AR, involving different proteins as such syntaxin, synaptotagmin (Hutt et al., 2005), rab3 (Michaut et al., 2000), complexin (Roggero et al., 2007) and SNAP.25 (Tomes et al., 2002). Complexin I was suggested to be essential for the formation of SNARE complexes. Interestingly, complex I-deficient spermatozoa were unable to undergo induced AR suggesting that SNARE complexes are required in this process (Zhao et al., 2007). Whilst some of the details are known much remains to be explained regarding SNARE proteins during AR.

Exposure of zonadhesin and/or proteins from the acrosome at the sperm surface could be the result of the initial stage of sperm exocytosis (Abou-Haila and Tulsiani, 2003; Kim and Gerton, 2003). However, studies investigating SNARE proteins indicate that exocytosis has to be completed in a relative short period of time, calculated in seconds (Tollner et al., 2003), especially when Rab3 (small GTPase) (Michaut et al., 2000) is activated involving calcium channel opening (SOC: store operating channel) (Mayorga et al., 2007). Our results on zonadhesin exposure suggest that exposure is not the start of the AR as soluble components from the acrosomal matrix were not observed to leak out during this exposure (during capacitation).

We suggest that zonadhesin exposure during capacitation is a slower process than AR and potentially a reversible event. For instance, the mechanism of zonadhesin exposure during capacitation could be explained by a ‘kiss and run-like’ event (Fesce et al., 1994; Abou-Haila and Tulsiani, 2003). This model of fusion refers to the opening of a small pore termed ‘fusion pore’, followed by its rapid closure without collapsing the whole membrane (Staal et al., 2004). Moreover, synaptic cells showed that the kiss and run mechanism was preferred in a higher calcium concentration (Ales et al., 1999). As discussed earlier, calcium is essential for sperm capacitation (Visconti et al., 1995) and for zonadhesin exposure (Tardif et al., 2010b). Tardif et al. (2003) showed striking images of human spermatozoa at the swollen stage, at the instant when the PM was docked (tightly apposed to OAM) and showing fenestration of the OAM. In addition, human spermatozoa in transit through cumulus matrix were shown regularly exhibiting the swollen stage, but these spermatozoa were not demonstrated to have initiated exocytosis (White et al., 1990). However, spermatozoa at this stage could transiently expose acrosomal molecules and as such sperm could use such ‘kiss and run’ mechanisms (Florman and Ducibella, 2006). Taken together, it is possible that acrosomal protein exposure during capacitation is an independent event from AR. Further investigations are presently underway to determine more precisely the mechanism of zonadhesin exposure in human cells.

**Conclusions**

Notwithstanding a very active research period in the last two decades, the molecular basis controlling gamete adhesion and species specificity in mammals remains a conundrum. Sperm–ZP adhesion in mammals requires more than molecules located in the PM. Freshly ejaculated spermatozoa are functionally incompetent in the ability to fertilize an oocyte and post-translational modifications are needed. It is critical for the comprehension of mammalian fertilization to increase our knowledge on the molecular mechanisms leading to the fertilizing acquisition of spermatozoa. The importance of events related to sperm capacitation is frequently underestimated, because they are subtle and hard to follow by conventional techniques. However, this review presents information that may contribute to increase our comprehension in the critical events that prime spermatozoa to interact specifically with the extra cellular matrix of the egg. Moreover, experimental information gathered presents a new horizon of gamete interaction involving acrosomal molecules. Like all new models, several questions are still unanswered. However, it is important to express such hypothesis in a way to obtain more answers in a near future in the objective to clarify gamete interaction processes at the molecular level and sperm infertility.

**Supplementary data**

Supplementary data are available at http://molehr.oxfordjournals.org/.

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References


