Focus on Fertilization

The mechanism of sperm–oocyte fusion in mammals

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Abstract

Sperm–oocyte fusion is one of the most impressive events in sexual reproduction, and the elucidation of its molecular mechanism has fascinated researchers for a long time. Because of the limitation of materials and difficulties in analyzing membrane protein–protein interactions, many attempts have failed to reach this goal. Recent studies involving gene targeting have clearly demonstrated the various molecules that are involved in sperm–oocyte binding and fusion. Sperm ADAMs (family of proteins with a disintegrin and metalloprotease domain), including fertilinα, fertilinβ and cyritestin, have been investigated and found to be important for binding rather than for fusion and painstaking studies have raised suspicions that their putative receptors, oocyte integrins, are necessary for the sperm–oocyte interaction. Recently, several studies have focused the spotlight on CD9 and glycosylphosphatidylinositol (GPI)-anchored proteins on oocytes, and epididymal protein DE on sperm, as candidate molecules involved in sperm–oocyte fusion. Lack of, or interference with the function of, these proteins can disrupt the sperm–oocyte fusion without changing the binding. In this review we highlight the candidate molecules involved in the sperm–oocyte interaction suggested from the recent progress in this research field.

Introduction

Fertilization is a complicated process involving many molecules. In mammals, ovulated oocytes are surrounded by two substantial layers; the cumulus cell barrier and the zona pellucida (Fig. 1). Normally, only one sperm penetrates these two layers, and reaches and interacts with the oocyte plasma membrane, resulting in fusion and subsequent formation of a zygote. This process of penetrating these layers is important for sperm not only to reach the oolemma, but also to acquire fusibility. During this process, sperm undergo cellular exocytosis, called the acrosome reaction (Fig. 1). The acrosome is a large, Golgi-derived lysosome-like organelle that overlies the nucleus in the apical region of the sperm head. This organelle secretes enzymes such as the serine protease, acrosin, which helps sperm penetration into the zona pellucida (Abou-Haila & Tulsiani 2000). In addition to the secretion of enzymes, the acrosome reaction brings exposure of the inner acrosomal membrane to the outside by breaking the organelle. This process is necessary for the sperm to acquire fusibility with the oocyte plasma membrane. Sperm–oocyte fusion begins from the equatorial segment located between the inner acrosomal membrane and the plasma membrane overlying the nucleus in the posterior region of the sperm head (Yanagimachi & Noda 1970, Bedford et al. 1979).

Oocytes have a unique structure on their surface (Longo & Chen 1984). The surface of oocytes is covered with microvilli with the exception of the region overlying the meiotic spindle. Oocyte microvilli surround the sperm head preceding the sperm–oocyte fusion. It has been observed that sperm rarely fuse with oocytes at the region lacking microvilli. Therefore, oocyte microvilli, together with the sperm equatorial segment, are thought to be rich in molecules involved in sperm–oocyte fusion.

For a long time, many efforts have been made to find molecules involved in the process of sperm–oocyte fusion. Pioneering studies using anti-sperm mAbs that inhibited fertilization suggested candidate molecules involved in the sperm–oocyte fusion process. Following such antibody studies came gene targeting, which has proved to be a powerful tool to reveal the function of candidate molecules involved in sperm–oocyte binding and fusion (Table 1). Although the detailed molecular mechanisms of this process have not yet been clarified, recent studies have elucidated certain molecules involved in the fusion or binding reaction (Cuasnicu et al. 2001, Evans 2002). In this review we highlight molecules participating...
Candidate molecules involved in the sperm–oocyte interaction

CD9 on oocytes

CD9 is a member of the ubiquitous tetraspanin family of proteins (also known as tetraspans or transmembrane four superfamily proteins), whose members are characterized by four transmembrane regions and two extracellular loops (Boucheix et al. 1991). The second loop is larger than the first and has highly conserved amino acid residues (Seigneuret et al. 2001). Although the tetraspanins have no known functional domains common to other protein families, as well as interacting with other tetraspanins they reportedly interact with other proteins including integrins, immunoglobulins, proteoglycans, complement regulatory proteins and growth factor receptors. For this reason, tetraspanins are thought to form multi-molecular complexes with these associated molecules on the plasma membrane, and thus have been implicated in cell adhesion, motility, proliferation, and differentiation (Boucheix & Rubinstein 2001).

CD9 has been reported to be composed of a variety of multi-protein complexes involved in various cellular and physiological processes, including viral infection in canine and feline cells, muscle cell fusion, cell adhesion and motility (Loffler et al. 1997, de Parseval et al. 1997, Tachibana & Hemler 1999).

The first indication that CD9 is functional in fertilization was obtained from experiments using an antibody against CD9 (Chen et al. 1999). In this study, Chen and colleagues found that the anti-CD9 antibody inhibited the sperm–oocyte binding and fusion dose dependently in vivo. Consistent with this finding, CD9 is distributed over the entire oocyte surface except the region overlying the mitotic spindle, which is the only area lacking microvilli on the oocyte surface and where sperm inefficiently fuse with oocytes (Kaji et al. 2000). This observed distribution pattern of CD9 corresponds with that found for a6 integrin. This integrin can be a receptor for sperm fertilin, an ADAM (a disintegrin and a metalloprotease domain) family member, and also interacts with CD9 on the egg surface (Miyado et al. 2000, discussed below).

We and two other groups reported that CD9-deficient female mice were sterile due to a lack of fusibility of their CD9-deficient oocytes (Kaji et al. 2000, Le Naour et al. 2000, Miyado et al. 2000). Sperm could bind to oocytes from CD9-deficient mice in a similar manner as they could to those from wild-type mice. However, these CD9-deficient oocytes rarely fused with sperm either
in vitro or in vivo. When sperm were injected into oocytes from CD9-deficient mice by an intracellular sperm injection (ICSI) technique, the fertilized eggs developed to term, revealing that CD9-deficient oocytes could apparently produce zygotes that developed normally (Miyado et al. 2000). Although rare, CD9-deficient females bore normal offspring. In the case of in vitro fertilization, the longer incubation time of CD9-deficient oocytes with sperm seemed to increase the number of successful fusions. These observations, as well as the fact that tetraspanins can form multi-protein complexes in the plasma membrane to modulate the function of the partners, suggest that CD9 may cooperate with some other molecules to facilitate the sperm–oocyte fusion.

In connection with the idea of a multi-protein complex, it is also possible that CD9 acts as a linker between the molecules that are functional in the sperm binding and the molecules facilitating fusion. The effects of anti-CD9 antibodies on the sperm–oocyte binding are not entirely clear. Some reports demonstrated that the antibody inhibited only the sperm–oocyte fusion (Miller et al. 2000, Miyado et al. 2000, Kaji et al. 2002). In contrast, CD9 was reported to play a role in sperm–oocyte binding. Zona pellucida-free oocytes treated with anti-CD9 antibodies showed reduced levels of binding to the ligands; fertilin α, fertilin β and cyritestin on sperm when the antibodies were immobilized on small beads (Chen et al. 1999, Takahashi et al. 2001, Wong et al. 2001, Zhu & Evans 2002). Although the functional difference between the lack of CD9 and treatment with anti-CD9 antibodies is not clear, the antibodies may disturb the strength of adhesions mediated by a molecule such as α6 integrin involved in the multi-protein complex together with CD9. In addition to treatment with antibodies, treatment of oocytes with a recombinant form of the large extracellular loop (LEL) of CD9 led to a reduction in the sperm–oocyte fusion when mouse oocytes were inseminated, though treatment of sperm with this had no effect (Zhu et al. 2002). This finding suggests that interaction between the LEL portion of intact CD9 and some molecule(s) on the oocyte surface was essential for the sperm–oocyte fusion and that this interaction became unstable due to competition between the recombinant LEL and the endogenous CD9 for interaction with this/these molecule(s). Interestingly, in mice, LEL from mouse CD9 inhibited only fusion, but LEL from human CD9 inhibited sperm–oocyte binding as well as fusion (Higginbottom et al. 2003). Although it is difficult to explain this difference, it may be possible to identify the regions responsible for the different effects by generating chimeric proteins composed of human LEL and mouse LEL.

Oocytes from CD9-deficient mice could be rescued by injection with CD9 mRNA (Kaji et al. 2002, Zhu et al. 2002). Using this method, Zhu et al. (2002) determined the functional domains of mouse CD9 acting in the process of sperm–oocyte fusion. One point mutation of the amino acid residue 174 (F to A) and triple mutations of the amino acid residues 173–175 (SFQ to AAA), in the second loop of CD9, severely reduced the ability of the mutated mRNA to rescue the fusibility of CD9-deficient oocytes. The experimental idea that these particular amino acids are important for CD9 function originated from the results of a study showing that the equivalent region of human CD81, also a tetraspanin, was crucial for CD81 binding to hepatitis C virus (HCV, Pileri et al. 1998). When human CD81 functions as an HCV receptor, a phenylalanine at the position of 186 is essential for HCV binding. CD81, and other tetraspanins, of other species lacking phenylalanine at this position also lose this function (Higginbottom et al. 2000). For fertilization,
human CD9 could restore the ability of CD9-deficient mouse oocytes to fuse with sperm and mouse CD81 was able to partially rescue the fusibility, even though the corresponding amino acids of SFQ in mouse CD9 are TFF for human CD9, and TPL for mouse CD81 (Kaji et al. 2002). Because this region in the second loop of the tetraspanins is thought to face to the outside structurally, it is possible that CD9 interacts with other molecules at this region on the oocyte plasma membrane and that this interaction may be essential for efficient fusion (Seigneuret et al. 2001).

CD9 has been implicated in other fusion events in addition to fertilization. The addition of anti-CD9 and anti-CD81 mAbs inhibited the conversion of cells of the mouse myoblast cell line (C2C12 cells) to elongated myotubes despite normal expression of muscle-specific proteins. In contrast, ectopic expression of CD9 increased myotube formation (Tachibana & Hamler 1999). CD9/CD81 double-null mice spontaneously developed multinucleate giant cells in their lungs, and showed enhanced osteoclastogenesis in their bones (Takeda et al. 2003). Anti-CD9 antibodies selectively blocked canine distemper virus-induced cell–cell fusion (syncytium formation; Schmid et al. 2000). The mechanism(s) of these cell–cell fusion events and function of CD9 in such fusion are not known. The role of CD9 in these fusion events is probably not the same as in the sperm–oocyte fusion, because the fusion efficiency of osteoclasts or multinucleate giant cells in the lungs is increased in CD9-deficient mice. These contrasting effects on cell–cell fusion may also be explained by the character of tetraspanins, which form unique multi-molecular complexes on individual types of cells.

**Glycosylphosphatidylinositol (GPI)-anchored proteins on oocytes**

In addition to CD9-deficient oocytes, oocyte-specific phosphatidylinositol glycan class-A (PIG-A)-deficient mice also showed severely reduced fusibility of oocytes with sperm (Alfieri et al. 2003). The PIG-A gene encodes a subunit of N-acetyl glucosaminyl transferase, which is the enzyme involved in the first step of the biosynthesis of the GPI-anchor (Tiede et al. 2000). Participation of oocytespecific GPI-anchored proteins in the sperm–oocyte interaction was suggested earlier in a study in which oocytes were treated with phosphatidylinositol-specific phospholipase C (PI-PLC; Coonrod et al. 1999). This caused release of two GPI-anchored proteins (~35–45 kDa and 70 kDa), and a reduction in the sperm–oocyte binding and strong blockage of the sperm–oocyte fusion. Complete deficiency of PIG-A resulted in embryonic lethality (Kawagoe et al. 1996), so oocyte-specific PIG-A-deficient mice were generated by use of the Cre-loxp system, in which Cre-recombinase was expressed under control of the oocyte-specific promoter ZP3. After natural mating, only 1.3% of the recovered healthy cells were successfully fertilized and developed normally into two-cell stage embryos, whereas 83% of those from wild-type females were fertilized, and developed normally. Multiple sperm were detected in the perivitelline space of several PIG-A(−/−) oocytes (Alfieri et al. 2003), as seen in the mating of CD9-deficient females (Miyado et al. 2000). In an in vitro fertilization assay in which zona-free oocytes were used, PIG-A(−/−) oocytes showed a low fertilization rate (~10%) and lower sperm binding (~60%) compared with wild-type oocytes. This result suggests that GPI-anchored proteins are more important for the sperm–oocyte fusion than for the binding.

One of the GPI-anchored proteins on oocytes, released by PI-PLC treatment, is CD55 (Alfieri et al. 2003), probably detected as a ~70 kDa protein by Coonrod et al. (1999). CD55-deficient mice showed normal fertility (Sun et al. 1999). Thus, identification and characterization of the ~35–45 kDa protein, also detected by Coonrod et al. (1999), or still undetected GPI-anchored proteins in lower abundance are needed to elucidate the phenotype of PIG-A-deficient oocytes, along with further examination of the interaction between this/these protein(s) and CD9.

**DE on sperm**

One candidate molecule expressed on sperm, which participates in fusion, is epididymal protein DE/cysteine-rich secretory protein 1 (CRISP1). DE was originally identified as a molecule synthesized in rats in an androgen-dependent manner by the proximal segment of the epididymis, and was reported to become localized on the surface of sperm during epididymal transit (Cameo & Blaquier 1976, Kohane et al. 1980a,b). Subsequently, many results indicating its participation in sperm–oocyte fusion in rats have been reported. Before the acrosome reaction occurs, DE is localized on the dorsal region of the rat sperm acrosome and subsequently migrates to the equatorial segments, where sperm begins to fuse with an oocyte (Rochwerger & Cuasnicu 1992). Polyclonal anti-DE antibodies significantly prevented sperm from fusing with zona-free oocytes without changing either the mobility or binding ability of the sperm (Cuasnicu et al. 1984). A purified DE protein could bind to the entire surface of oocytes except the region over the meiotic spindle, where sperm rarely fuse (Rochwerger et al. 1992), suggesting that there is a receptor for DE on the surface of oocytes for promoting the sperm–oocyte fusion.

The mouse epididymal protein, AEG-1/CRISP-1, possesses 70% homology to rat DE. Proteins recognized by anti-rat DE antibodies are localized on the dorsal region of the sperm acrosome, although it is not clear whether they are AEG-1 or not and mouse oocytes also have rat DE-binding sites similar to those of rat oocytes (Mizuki & Kasahara 1992, Cohen et al. 2000). Furthermore, anti-rat DE antibodies reduced the penetrability of mouse sperm without reducing their ability to bind to mouse oocytes (Cohen et al. 2000). The human epididymal protein
Integrins on oocytes and ADAMs on sperm

Before the discovery of a functional role for CD9 in the sperm–oocyte fusion, most attention had been given to integrins on oocytes and their ligands, fertilin α, fertilin β and cyritestin, on sperm. Fertilin α, fertilin β and cyritestin are members of the ADAM family, being ADAM1, ADAM2, and ADAM3, respectively, each of which has a disintegrin and a metallopeptase domain. Originally fertilin β was identified as the antigen recognized by the mAb, PH-30, an anti-sperm antibody that inhibits fertilization of guinea pig oocytes, and was found to form a heterodimer with fertilin α (Blobel et al. 1992), whereas cyritestin plays a role as a single polypeptide (Nishimura et al. 2001). Fertilin α has an amino acid sequence called the fusion peptide in addition to a disintegrin domain that can bind to integrins on oocytes. The fusion peptide fulfills the following criteria for such a peptide: (a) location in a membrane-anchored subunit, (b) relatively strong hydrophobicity, and (c) ability to be modeled as a ‘sided’ α-helix with most of the bulky hydrophobic residues on one face and charged amino acids on the other face. These are the same characteristics of viral fusion peptides involved in the penetration of the target cell membrane preceding fusion events between cells and virus particles (de Parseval et al. 1997). Therefore, it is likely that sperm ADAMs play a key role in the sperm–oocyte binding and fusion.

ADAM functions have been examined by use of in vitro fertilization in combination with antibodies, peptides and isolated proteins. Recombinant forms of fertilin α, fertilin β and cyritestin were shown to bind to the mouse oocyte plasma membrane and inhibit the sperm–oocyte binding, resulting in a reduced incidence of fertilization (Evans et al. 1997b, Takahashi et al. 2001). In addition to the data obtained in vitro, knockout mice specific for these genes have provided evidence for the participation of the sperm ADAMs in the sperm–oocyte interaction. For example, sperm from fertilin β-deficient mice were severely inhibited (~80%) in their oolemma-binding ability, though some of the few sperm that could bind were able to fuse with the oocyte (Cho et al. 1998). Cyritestin-null sperm also demonstrated a great reduction in their ability to bind to oocytes in spite of a having normal sperm–oocyte fusion (Nishimura et al. 2001). Interestingly, fertilin β-deficient sperm lacked fertilin α, and showed a greatly reduced amount of cyritestin, whereas cyritestin-deficient ones lacked fertilin α and showed reduced fertilin β expression, ~50% compared with the wild-type sperm (Nishimura et al. 2001). Although fertilin α-deficient mice, and their associated levels of fertilin β and cyritestin expression, have not been reported yet, these 3 ADAMs appear to play important roles in the sperm–oocyte binding but not in the fusion.

Besides sperm ADAMs, their putative receptors on oocytes, i.e. integrins, have also been well studied as candidate genes being responsible for the sperm–oocyte interaction. The first member of the integrin family shown to be active in this regard was α6β1. GoH3, a function-blocking mAb against the α6 integrin subunit, inhibited both sperm–oocyte binding and fusion, and peptides derived from a sequence of a part or all of the fertilin β disintegrin domain bound to α6β1 integrin (Almeida et al. 1995, Chen & Sampson 1999, Takahashi et al. 2000). However, the contribution of α6β1 integrin to the sperm–oocyte interaction remains questionable because some inhibition studies using GoH3 mAb under different conditions did not show blocking of the interaction (Evans et al. 1997a, Miller et al. 2000). Moreover, oocytes from α6 integrin-deficient mice showed normal binding and fusibility with sperm, demonstrating that the α6 integrin is not essential for the gamete binding and fusion (Miller et al. 2000). This result evoked the possibility that the function of α6 integrin is redundant, because some other members of the integrin family were also expressed on oocytes. Integrins expressed on the surface of mouse oocytes can be divided into 2 groups: β1 integrins (α2β1, α3β1, α5β1, α6β1 and α9β1) and αv integrins (αvβ1, αvβ3, αvβ5; He et al. 2003). In addition to α6 integrin-deficient mice, α3-, β3- and β5-deficient mice were also reported to show normal fertility (Hodivala-Dilke et al. 1999, Huang et al. 2000, He et al. 2003). Because these results could not exclude the possibility of the occurrence of functional redundancy among various integrins, He et al. (2003) performed some elaborate experiments. As β1 integrin-deficient mice were preimplantation lethal, they established oocyte-specific β1 integrin-deficient mice by use of the Cre-loxP system. The β1 conditionally deficient females showed the normal pregnancy rate, and their oocytes demonstrated normal binding and fusion with sperm in an in vitro fertilization assay. Moreover, in the presence of anti-β3 or αv integrin antibodies for functional blocking, oocytes showed normal sperm binding and fusibility. Thus, integrins on mouse oocytes are unlikely to be redundant with each other in the sperm–oocyte binding and fusion.

Conclusions and perspectives

Because of the limitation of materials and difficulties in analyzing membrane protein–protein interactions, the molecular mechanism of sperm–oocyte fusion remains to be elucidated, even though it is one of the most important events as it triggers the beginning of development. Advanced studies on virus infection and exocytosis
demonstrate that molecules having α helix-hemagglutinin (HA) in the viral membrane and SNAREs in intracellular vesicles, etc. play a key role to bring the two plasma membranes into extremely close proximity preceding fusion (Blumenthal et al. 2003). However, the specific ‘docking molecules’ used to achieve the stable association between the two membranes have not been identified in any cell–cell fusion events, such as myotube formation, osteoclastogenesis, giant cell formation, as well as fertilization.

The phenotype of CD9-deficient oocytes shows a quite severe abnormality in the fusion process with sperm. However, it is not likely that CD9 is the docking molecule by itself, because all cells expressing CD9 in various tissues, except oocytes, cannot fuse with sperm. Moreover, even if oocytes lack CD9, in rare cases some of them can still fuse with sperm. Thus, identification of oocyte-specific CD9-associating molecules might help to clarify the mechanism of sperm–oocyte fusion.

Furthermore, prior to defining such associating molecules, identification and characterization of the GPI-anchored ~35–45 kDa protein, a receptor for epididymal protein DE on oocytes, is needed. Further studies including the generation of knockout mice specific for these genes and analysis of the interaction between these molecules should help us in clarifying the molecular mechanisms of both binding and fusion aspects of the sperm–oocyte interaction.

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