A hallmark of gamete interactions at fertilization is relative or absolute species specificity. A pig sperm protein that binds to the extracellular matrix of the egg in a species-specific manner was recently identified and named zonadhesin (Hardy, D. M., and Garbers, D. L. (1995) J. Biol. Chem. 270, 26025–26028). We have now cloned a cDNA for mouse zonadhesin (16.4 kb), and it demonstrates a large species variation in the numbers and arrangements of domains. Expression of mouse zonadhesin mRNA is evident only within the testis, and the protein is found exclusively on the apical region of the sperm head. There are 20 partial D-domains, found as tandem repeats, inserted between two of the four full D-domains and an additional partial D-domain. These domains are homologous to the D-domains of von Willebrand factor and a-tectorin. A region at the N terminus of the mouse cDNA contains three tandem repeats homologous to MAM domains. These are domains comprised of about 160 amino acids that are present in transmembrane proteins such as the meprins and receptor protein-tyrosine phosphatases, where they appear to function in cell/cell interactions. Additionally, mouse zonadhesin contains a mucin-like domain and a domain homologous to epidermal growth factor (EGF). A putative single transmembrane segment separates a short carboxyl tail from the extracellular region. The existence of MAM, mucin, D-, and EGF domains suggest that zonadhesin functions in multiple cell adhesion processes, where binding to the extracellular matrix of the egg is but one of the functions of this sperm-specific membrane protein.

Prior to fertilization, the sperm cell interacts with multiple cells including fellow sperm cells, Sertoli cells, epithelial cells within the male reproductive tract or female reproductive tract, cells associated with the egg, and the egg itself. In some cases, the interactions are attractive in nature and in others repulsive. In an attempt to identify sperm proteins that bind to the egg extracellular matrix, Hardy and Garbers (1) solubilized sperm membranes from the pig and determined which proteins bound to the pig zona pellucida. Subsequently, the cDNA encoding a protein that bound to the zona pellucida in a relatively species-specific manner was cloned and named zonadhesin (2). Pig zonadhesin is a transmembrane protein with a very short intracellular region. The putative extracellular region contains a mucin-like domain and five tandem repeats homologous to the D-domains of prepro-von Willebrand factor (ppvWF)3 (3, 4). The cloning of the pig sperm zonadhesin cDNA raised the questions of whether other species would contain homologs of zonadhesin and, if so, whether these would be variable within the above domain structure. Here, the mouse homolog of zonadhesin is cloned and shown to contain additional repetitive sequences within the D-domain region. Furthermore, three tandem repeats of a MAM domain were identified at the N terminus. The MAM domain is a module composed of about 160 amino acids including four conserved Cys; it is found in a number of functionally diverse transmembrane proteins such as the meprins and receptor-protein-tyrosine phosphatases (5). Thus, zonadhesin displays a large species variation between pig and mouse and also contains multiple domains previously suggested as involved in cell adhesion processes.

MATERIALS AND METHODS

RNA Isolation—Total RNA was isolated from 40 mouse testes or similar amounts of other mouse tissues (including brain, heart, kidney, liver, lung, small intestine, and spleen) in guanidinium thiocyanate and N-lauryl sarcosine, followed by extraction with acidic phenol/CHCl3 and precipitation with isopropyl alcohol (6). Poly(A)1 RNA was purified from total RNA by oligo(dT)2-cellulose chromatography (7).

Northern Blot—30 μg of total RNA from various mouse tissues was loaded on each lane of a formaldehyde-agarose gel (8) and blotted overnight using 10 × SSC as the transfer buffer. The blot was then fixed by UV light and hybridized with a random primer-labeled (Amersham Life Science, Inc.), 1.4-kb probe from the 3′-end of the mouse zonadhesin cDNA as described previously (1). After hybridization, the blot was washed with 0.2 × SSC and 0.2% SDS at 65 °C.

Screening of cDNA Libraries—The first mouse cDNA clone (#18) was obtained by a low stringency screening of a mouse testis cDNA library (Stratagene) using a cDNA fragment corresponding to the pig zonadhesin D4 domain as described previously (9). The same cDNA library was further screened with the mouse cDNA clone #18 as a probe under previously described conditions (1). The positive phage clones were converted to pBluescript plasmid by phage rescue and sequenced. The longest clone contained a 2.5-kb DNA fragment.

Construction of a Plasmid cDNA Library—To obtain additional 5′-end sequence, 2 μg of mouse testis mRNA was used to construct an

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3 The abbreviations used are: ppvWF, prepro-von Willebrand factor; kb, kilobase(s); PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis; DPF, diisopropyl fluorophosphate; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]1-propanesulfonic acid; PBS, phosphate-buffered saline; EGF, epidermal growth factor; ZP, zona pellucida; t-PA, tissue plasminogen activator; u-PA, urokinase-type plasminogen activator.
oligo-(dT) primed plasmid cDNA library using SUPERSCRIPT™ Plasmid System (Life Technologies, Inc.). The cDNA was ligated into a pSPORT1 vector and transformed into DH10B cells by electroporation according to the manufacturer instructions (Life Technologies, Inc.). The cDNA library was screened using a combination of inverse and anchor PCR.

**Inverse PCR and Anchor PCR**—Inverse PCR was carried out with two gene-specific primers, a 5′-end antisense primer and a 3′-end sense primer. The Expand™ Long Template PCR kit (Boehringer Mannheim) was used with an Erycop Twinblock System thermal cycler. The inverse PCR reaction was performed by denaturing at 95 °C for 2 min followed by 30 cycles of denaturation at 94 °C for 0 s, annealing at 55 °C for 1 min, and extension at 68 °C for 1 min. The PCR product was diluted 100-fold before use as a template for anchor PCR. Anchor PCR was performed using a sequence (5′-ATTACCCTGCTTAATAGCTACTATAGG-3′) on the vector pSPORT1 as the sense primer and a gene-specific primer, upstream of the 5′-end antisense primer used in the inverse PCR, as the antisense primer. The anchor PCR reaction was performed using an air thermal cycler (Idaho Technologies) by denaturing at 94 °C for 1 min, followed by 30 cycles of denaturation at 94 °C for 0 s, annealing at 55 °C for 0 s, and extension at 68 °C for 1 min. A mixture of Taq and Pfu (9:1) was used as polymerases for the anchor PCR (10) in a standard PCR reaction buffer (10 mM Tris-HCl, pH 9.0, 50 mM KCl, 2 mM MgCl₂, 0.1% Triton X-100, 0.2 mM dNTPs) and subcloned into a TA cloning vector (Invitrogen). The above process of combination of inverse and anchor PCR was repeatedly performed until no additional sequence could be obtained from the library. A total of an additional 6-kb composite sequence of cDNA was obtained.

**Gene-specific RT and 5′ RACE-PCR**—To obtain the full-length sequence of mouse zona pellucida, gene-specific reverse transcriptions (RT) and 5′ RACE (rapid amplification of cDNA ends) were performed using the Marathon™ cDNA amplification kit (CLONTECH). 2 μg of mRNA was used for synthesis of cDNA according to the manufacturer instructions. Gene-specific primers 5′-AGCCCGCTACGGCTAGGATGG-3′ and 5′-ATGGCATCTGACCTAACGTGTCG-3′ were used for the first and second general gene-specific reverse transcriptions, separately. RACE-PCR was carried out by nested PCR with two sets of primer pairs, each of them containing one gene specific primer and one adapter primer of the Marathon™ cDNA amplification kit. The RACE-PCR product was diluted 100-fold before use as a template for the nested PCR. The Advantage™ PCR kit (CLONTECH) was used for the RACE-PCR reactions. RACE-PCR reactions were performed by denaturing at 94 °C for 1 min, followed by 30 cycles of denaturation at 94 °C for 0 s, annealing at 55 °C for 0 s, and extension at 68 °C for 1 min. The PCR products were then purified on a 1% agarose gel and subcloned into a TA cloning vector (Invitrogen). An additional 7-kb composite sequence of cDNA was obtained by these methods.

**Preparation and Purification of GST Fusion Proteins**—The plasmid pGEX-KG (11) was used to express GST-D3c or GST-D3p17 fusion proteins, in which D3c represented the C terminus of the D3 domain which was derived from 17th D3 partial blotting (11). Primer pairs containing an EcoRI site at the 5′-end of the sense primer and a HindIII site at 3′-end of the antisense primer were used so that PCR products would be subcloned into the pGEX-KG vector in the correct reading frame. PCR reactions were performed using an air thermal cycler (Idaho Technologies) under the following conditions: 1 cycle of denaturation at 94 °C for 1 min; 1 cycle of denaturation at 94 °C for 0 s, annealing at 55 °C for 0 s, and extension at 68 °C for 1 min; 1 cycle at 72 °C for 2 min. PCR products were then purified on a 1% agarose gel and subcloned into a TA cloning vector (Invitrogen). A solution of 0.1M glycine/PBS solution overnight at 4 °C, samples were washed in PBS twice before being smeared on slides. When fixed in methanol, sperm cells were directly smeared on slides. For CDC, 4% paraformaldehyde/PBS was used for fixation, washed twice with 0.1M glycine/PBS solution overnight at 4 °C. The slides were washed in 0.01 M sodium phosphate buffer (pH 7.4) followed by washing twice with 0.1M glycine/PBS before being applied to nitrocellulose at 25 volts overnight at 4 °C. The blot was then washed three times in 10 ml of TBST for 15 min each and incubated with 1:20000 diluted horseradish peroxidase-conjugated goat anti-rabbit IgG (Biosource) for 1 h. The blot was then washed three times with 0.1M glycine/PBS before blotting. Peroxidase activity was detected with enhanced chemiluminescence (ECL, Amersham Life Science, Inc.).

**Cytoimmunoﬂuorescence**—Spermatozoa were collected in 200 μl of M199-ME medium (M199 with Earle’s salts, 50 μg/ml pyruvic acid, 4 mg/ml BSA, and 1 mM EDTA) (13) from the epididymis and washed twice with 15 ml of PBS by centrifugation at 1000 × g for 10 min. Sperm cells were then fixed either in a 4% paraformaldehyde/PBS solution or in 100% methanol. The fixation in 4% paraformaldehyde was performed on ice for 30 min followed by washing twice with 40 ml of PBS. After incubating in a 0.1% glycine/PBS solution overnight at 4 °C, sperm cells were washed twice with PBS. When fixed in methanol, sperm cells were directly smeared on slides and air dried. The slides were then placed in ice-cold methanol for 30 min followed by washing in 100% ethanol for 10 min before the immunofluorescence assay. 300 μl of blocking reagent (10% goat serum and 1 mM EDTA in PBS) was incubated with spermatozoa on a slide at room temperature for 30 min. Antisera P448 against GST-D3p17 fusion

**Preparation of Mouse Sperm Membrane Proteins**—The preparation of mouse sperm membrane protein was based on Hardy et al. (2) and Bleil et al. (13) with modifications. 40 male mice (ICR) were used for one preparation. Caudal epididymal and vas deferens were dissected from the mice, transferred to 2 ml of pre-warmed (37 °C) Earle’s modified medium 199 (Life Technologies, Inc.) containing 4 mg/ml BSA, 30 mM pyruvate, and 4 mM EGTA (M199-ME), covered with mineral oil, and then minced with a scissors and placed in a cell culture incubator for 10 min. The spermatozoa were then transferred to 30 ml of M199-ME and capacitated at 37 °C for 1 h. The spermatozoa were then centrifuged down at 1500 × g for 15 min and resuspended in 25 ml of ice-cold HE/diisopropyl fluorophosphate (DFP) buffer (50 mM NaHEPES, 1 mM NaEDTA, 1 mM DFP, pH 7.5) before being transferred to a Parr Bomb (30 ml) for N₂ cavitation at 0 °C and 650 p.s.i. for 30 min. Disrupted cells were centrifuged at 1500 × g for 10 min, and the supernatant centrifuged again at 51,000 rpm (Beckman Ti-70) for 45 min. The crude membrane pellet was resuspended in 5-ml of ice-cold HE/DFP, and washed twice by centrifugation at 100,000 rpm (TL-100.3 rotor) for 20 min. The membrane pellet was resuspended in 1 ml of ice-cold HE/DFP, and washed twice by centrifugation at 100,000 rpm (TL-100.3 rotor) for 15 min. The supernatant fluid was transferred to a new tube and stored at −20 °C.

**Solubilization of Sperm Membrane Proteins**—100 μl of 4 mg/ml sperm membrane protein was mixed with 100 μl of 2% CHAPS in 300 μl NaCl and 1 mM EDTA, 1 mM leupeptin, 1 μM pepstatin, and 4 mM Fabfloc (SC) and incubated at 4 °C for 1 h before centrifugation at 100,000 rpm (Ti-200.2 rotor) for 15 min. The supernatant fluid was transferred to a new tube and stored at −20 °C.
The finding of a pig sperm protein that bound to the egg extracellular region of the sperm head (Fig. 3A). This finding raised the important questions of whether homologs of this protein would exist in other species and, if so, if they would display significant species variation in structure. A mouse testis cDNA library was initially screened at low stringency with antibodies (S117 or P448) generated to zonadhesin fusion proteins. Expression was demonstrated as exclusively on the apical region of the sperm head (Fig. 3B), regardless of whether methanol- or paraformaldehyde-fixed sperm cells were used. The localization is consistent with zonadhesin interacting with the extracellular matrix of the egg or other cells and also supports the model of it as a transmembrane protein.

Zonadhesin was processed on mature and capacitated sperm cells as evidenced through detection with antisera S117 on Western blot (Fig. 3C). One band at 100 kDa and two major bands between 200 and 250 kDa are seen on non-reducing SDS-PAGE. The reason that S117 recognized three bands on the gel is very likely due to the high sequence similarities between the D3 partial domains. Processing of the pig zonadhesin also occurred, yielding 45 and 105 kDa mass proteins on reducing SDS-PAGE (2). The processing of zonadhesin and its relationship to function is not yet known, but presumably the unprocessed forms function prior to encountering the egg.

Analysis of the sequence of mouse zonadhesin revealed that it contained the domains present in the pig zonadhesin, including MAM domains (see below), a mucin-like domain, D-domains, an EGF-like domain, a transmembrane segment, and a short intracellular region. The similarity of the primary amino acid sequence within each domain (see below) together with Northern blot and cytoimmunofluorescence data strongly indicate that the composite cDNA sequence we obtained from mouse testis is a homolog of pig zonadhesin.

Mouse zonadhesin contains three tandem repeats of a domain known as the MAM domain, a new finding that strongly suggests a role for this region of zonadhesin in cell-cell interactions. Subsequently, we found that pig zonadhesin also contains one full and one partial MAM domain (Fig. 4). A MAM domain contains about 160 amino acids and four conserved cysteine residues, as well as conserved hydrophobic and aromatic amino acids (Fig. 5). The MAM domain is suggested to function in cell adhesion and is found in a diverse number of membrane proteins including meprins (16), A5 protein (17), and receptor protein-tyrosine phosphatases (18–20). The MAM-bearing meprins are zinc-metalloproteases that contain 39-amino acid intracellular C terminus with 30% basic residues. Cleavage of the putative signal peptide at Gly\textsuperscript{17} would produce a 5359-amino acid mature polypeptide chain with a calculated molecular mass of ~578,000 Da (Fig. 2). Mouse zonadhesin has 40 potential N-linked glycosylation sites (NXT/S) and a large number of potential O-linked glycosylation sites.

Like pig zonadhesin, mRNA of mouse zonadhesin is only detectable in testis, but the 16–17-kb mRNA is about twice as large (Fig. 3A).

The localization of zonadhesin is of particular importance since 1) the predicted structure to be discussed below suggests zonadhesin is a transmembrane protein, and 2) zonadhesin contains multiple extracellular domains, suggesting it potentially functions in multiple cell-cell interactions. By the use of antibodies (S117 or P448) generated to zonadhesin fusion proteins, expression was demonstrated as exclusively on the apical region of the sperm head (Fig. 3B), regardless of whether methanol- or paraformaldehyde-fixed sperm cells were used. The localization is consistent with zonadhesin interacting with the extracellular matrix of the egg or other cells and also supports the model of it as a transmembrane protein.

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The MAM domain has been suggested to mediate the dimerization or oligomerization of meprin subunits (5). A5 protein is a developmentally regulated cell surface molecule involved in neurite outgrowth or axonal guidance, and its MAM domain is critical for cell-cell interactions (5). Some of the receptor protein-tyrosine phosphatase family members, such as RPTPα, RPTPβ, RPTPγ, and PCP-2, contain MAM domains, where they have been suggested as

**FIG. 2.** Deduced amino acid sequence of mouse zonadhesin. A, N terminus including a 17-amino acid signal peptide (overlined) and three tandem repeats of the MAM domain; B, mucin-like domain showing 80 imperfect heptapeptide repeats; C, multiple sequence alignment of the tandem D-domains of zonadhesin; D, C terminus, including an EGF-like domain, transmembrane segment (underlined), and intracellular, highly basic domain.
essential for homophilic cell-cell interaction and the specificity of those interactions (5, 18). Another MAM-containing protein (gene B product) from *Xenopus* has been reported by Brown et al. (22). The function of this MAM-containing protein is unknown, but the protein is induced by thyroid hormone during *Xenopus laevis* metamorphosis (22). Interestingly, there is much higher sequence similarity within the MAM domains of a given protein group, suggesting slightly different functions within the different protein groups. The function of the MAM domains of zonadhesin is not known, but since this region can be eliminated by proteolysis with retention of zona pellucida binding properties (1, 2), it may function during sperm development, for example in interactions with Sertoli cells.

To the carboxyl side of the MAM domains is a mucin-like domain, The domain is very rich in Thr, Ser, Pro, Glu, and Val, containing 26, 8, 15, 17, and 14%, respectively. The mucin domain is composed of 7-amino acid imperfect repeats with a consensus sequence of PTE(E/V)(P/T)TV (Fig. 2). The similar heptapeptide repeats were also found in pig zonadhesin (2). It has been suggested that absolute length is not crucial to mucin function but rather that the core protein exists in an extended form as a scaffold for O-linked carbohydrate (23). The relatively short mucin-like domains present in some receptors have been suggested to lift the ligand-binding site above the glycocalyx (24). Additionally, membrane-associated mucins have been found in various instances to serve as ligands for cell surface receptor selectins (25). Therefore, the mucin-like domain in zonadhesin could serve to lift the MAM domain above the glycocalyx and thereby facilitate cell-cell interactions in the male reproductive tract and/or act as a repulsive barrier to

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**Fig. 3.** Expression of zonadhesin mRNA and protein. A, Northern blot showing that mouse zonadhesin is expressed only within the testis. Total RNA (30 µg) was loaded on each lane, and the blot was hybridized with the 1.4-kb 3’-end of the mouse zonadhesin cDNA. S, B, H, I, K, Li, Ln, and T represent spleen, brain, heart, intestine, kidney, liver, lung, and testis, respectively. The mRNA size is about 17 kb. B, cytoimmunofluorescence showing that zonadhesin exists only on the apical region of the sperm head. Slides were methanol fixed and incubated with antiserum P448. C, Western blot demonstrating that zonadhesin is processed.

**Fig. 4.** Schematic comparison of D-domain-containing proteins. *M. Zonadhesin* and *P. Zonadhesin* are mouse and pig zonadhesins; *M_.α-Teictorin, H_ppvWF, SW_Hemocytin*, and *H_MUC2* are mouse α-teictorin, human prepro-von Willebrand factor, silk worm hemocytin, and human intestinal mucin MUC2, respectively. MAM represents the MAM domain; Mucin represents the mucin or mucin-like domain; D0, D’, D”, D’, D1, D2, D3, D4, and D4’ represent the various D-domains; D3p1–20 represents the 20 tandem repeats of D3 partial domain; and ZP represents the zona pellucida domain conserved in various proteins including ZP-1, ZP-2, and ZP-3. Black boxes are regions having no sequence homologue to zonadhesin, orange boxes are the EGF-like domain, red boxes at the N terminus are the signal peptide and at C terminus are the transmembrane segment, and green boxes are the intracellular region.
Multiple Domains in Sperm-specific Membrane Protein

prevent nonspecific interactions between spermatozoa and other cells in the male or female reproductive tract (e.g., adhesion of spermatozoa within the oviductal isthmus).

Following the mucin-like domain, there is a partial D-domain (D0), 3 full D-domains (D1-D3), 20 tandem repeats of partial D3 domains (D3p1–20), and finally a D4 domain (Fig. 4). The D1, D2, D3, and D4 domains show relatively high similarity to the counterpart domains of pig zonadhesin (61, 68, 47, and 63, respectively). However, unlike pig zonadhesin which has only one partial D-domain (D0), mouse zonadhesin has at least 21 partial D-domains, a D0 domain, and 20 tandem repeats of a D3 partial domain. The newly identified 20 tandem repeats of D3p1–20 make up more than 40% of the protein mass of mouse zonadhesin. Like the D0 domain, D3p1–20 are very rich in cysteine, with 18 cysteines within each 120-amino acid repeat (15%). Each cysteine is conserved, suggesting that these residues are critical for the integrity of the protein structure (Fig. 2). We designated these 120-amino acid repeats as D3 partial domains instead of D0 domains since they have higher similarities to the C terminus of the D3 domain (56–67%) than to the D0 domain (26–37%) (Fig. 2). These partial D-domains are homologous to the D9 domain of vWF, in which D9 appears to support a specific binding site for procoagulant Factor VIII (3).

In addition to their presence in ppvWF, D-domains have recently been found in several functionally diverse extracellular proteins including some of the secreted mucins such as human, mouse, and intestinal mucin MUC2, mMuc2, and rMuc2 (23, 26, 27), Xenopus laevis integumentary mucin FIM-B, insect humoral lectin hemocytin (29), mouse inner ear matrix protein α-tectorin, and SCO-spondin, a glycoprotein secreted from the subcommissural organ (31). In Fig. 4, the domain structures of some of the above proteins are illustrated. The arrangement of D-domains can be divided into two groups with zonadhesin and α-tectorin in one group and vWF, MUC2, hemocytin, and SCO-spondin (not shown) in another. Of particular interest are two proteins, hemocytin and α-tectorin. Hemocytin is a humoral lectin cloned from Bombyx mori that plays an important role in a nonspecific self-defense mechanism; it possesses hemagglutinating activity. The carbohydrate-recognition domain in hemocytin overlaps with the D3 domain, and thus D3 domains could serve as lectins (29).

Mouse α-tectorin has been recently cloned from a mouse cochlea cDNA library (30), where it is one of the two major non-collagenous proteins of the mouse tectorial membrane of the inner ear. It interacts with another non-collagenous protein β-tectorin in the tectorial membrane. The α-tectorin contains a zona pellucida (ZP) domain which also exists in α-tectorin and thus contains components of the egg extracellular matrix as well as zonadhesin within the same molecule. The ZP domain may serve as a recognition site for filament formation. In the tectorial membrane, there are two distinct filament types, a light and dark staining filament, suggesting that the α- and β-tectorins may form homomeric filaments via their ZP domain while the two filament types interact with one another to form a striated sheet matrix via D-domain interactions. Thus, in the inner ear, the ZP domain and the D-domains can exist and potentially interact within the same extracellular matrix, whereas during fertilization, this interaction occurs between the different germ cells.

There are a total of 28 full-length D-domains in the proteins discussed above. The sequence alignment among these 28 D-domains indicates that a majority of Cys and two 4-amino acid motifs TFDG and GLCG are highly conserved to preserve a...
Like the D-domains in the pig, D1-D3 domains of mouse zonadhesin contain a CXXC motif. The CXXC motif is responsible for self-oligomerization of the ppWF (3) and MUC2 (27). Therefore, it is likely that the zonadhesin is also present as a multimer (1). The peptide of WREPFCACLS in bovine ppWF D2 domain is able to inhibit binding of ppWF to collagen (33). A similar sequence is also found in the mouse (WREPQFCPLV) and the pig (WRGPQFCPLA) zonadhesin D1 domains. Therefore, zonadhesin may also bind to collagen or collagen-like proteins.

During the cloning, we have also found variant fragments that could result from alternative splicing or gene rearrangement events. There was one PCR product missing the D3 partial domains from the 9th to 12th and another missing the 12th to 15th. One extra D3 partial domain was found on one occasion between the D3 domain and the second D3 partial domain. These products could be the result of alternative splicing. Additionally, rare PCR products were obtained that contained the C terminus of the D2 domain fused to the N terminus of the mucin-like domain and the C terminus of the D4 domain fused to the N terminus of the 16th D3 partial domain. These products suggest possible occasional gene rearrangement events. However, we cannot rule out possible cloning artifacts among these rare PCR products.

Zonadhesin has an EGF-like domain at the N terminus of the transmembrane segment (Fig. 4). The EGF-like repeat is one of the most shuffled domains in various animal extracellular proteins and has been identified in more than 70 different vertebrate and invertebrate proteins (34). It has been suggested that the EGF repeat may have a particular affinity for proteases because of the recurring role of proteolysis in the excision of growth factor from its precursor and the activation of many of the secreted soluble proteins such as factors VII, IX, and X, proteins C and S, and t-PA and u-PA (31). Therefore, the EGF domain in zonadhesin may also play a role in facilitating protease binding to zonadhesin since ZP affinity-purified pig zonadhesin contains two disulfide-bonded fragments, p45 and p105, where p45 is composed of a D0 and D1 domain while p105 contains the D2-D4 domains (1). Mouse zonadhesin was also processed based on Western blots (Fig. 3C). There are several dibasic residues in mouse zonadhesin that could serve as cleavage sites for endoproteinases (35). Extracellular proteinases have been suggested to play an important role in mammalian fertilization (36).

Based on the protein domain structure, zonadhesin is a multifunctional mosaic protein, typical of an adhesion molecule. The domains present in zonadhesin appear to be present within the extracellular regions of various proteins. Furthermore, the MAM, mucin, D-, and EGF-like domains are known to be involved in cell-cell, cell-matrix, and/or protein-protein interactions in the other proteins possessing these domains. Thus, zonadhesin likely serves in multiple cell-cell interactions as a sperm-specific membrane protein.

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