J. Mamm. Ova Res. Vol. 14, 1997

—Review—

Molecular Mechanism in Mammalian Fertilization

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In mammalian fertilization, the sperm at first binds to a carbohydrate moiety of the extracellular glycoprotein matrix of the egg called zona pellucida (ZP) [1–4] and then penetrates through it to adhere to the egg plasma membrane with its postacrosomal membrane, followed by the fusion of two gametes [5, 6].

In mice, ZP is reported to be composed of three glycoproteins, ZP1, ZP2, and ZP3, with apparent average molecular sizes of 200, 120 and 83 kDa, which are associated with each other to construct a specific structure [7]. So far, it has been shown in mice that the mucin type sugar chains of ZP [8, 9] and/or the galactosyl transferase on sperm plasma membrane mediates the binding of sperm and egg [2, 10, 11]. In spite of these reports suggesting the involvement of sugar moieties of ZP in the ligand-receptor relationship for sperm, no direct structural analysis of the sugar moieties of ZP glycoproteins has been reported. We have recently performed the structural analysis on all of sugar chains of porcine ZP obtained from relatively large amounts of porcine ZP. Porcine ZP is also composed of three glycoproteins, one with a molecular size of 90 kDa and the other two with a molecular size of 55 kDa α, β [12, 13]. Although a difference between the size of porcine and murine ZP glycoproteins is found, their structural similarity has been shown by cDNA cloning of each ZP core protein [14, 15]. In our recent studies, all structures of the N-linked, O-linked and neutral/acidic sugar chains were analysed in detail to elucidate the roles of the sugar moiety in mammalian fertilization. The molecules reported as ZP-receptors on the plasma membrane of sperm include galactosyl transferase [3, 10, 11], sp56 [16] and a substrate molecule for tyrosine kinase with a molecular size of 95 kDa [17]. All of these proteins have been reported to bind to a specific component of murine ZP3, suggesting the induction of the acrosome reaction by aggregating its receptors on sperm plasma membrane to introduce signals for acrosomal activation [18]. The acrosome reaction of sperm cells is induced while passing through the cumulus cells surrounding the ZP, or on the surface of the ZP matrix [19, 20]. The morphological consequence of the acrosome reaction involves the vesiculation of the outer acrosomal membrane, loss of the acrosomal components, and removal of the vesiculated membranous ghost from the sperm head [21]. The biochemical incidents, including the increase in intracellular pH, massive influx of Ca^{2+}, increase in intracellular cAMP and activation of protein kinase systems are closely related to the morphologically observed consequences [22–25]. Moreover, the sperm's ZP-binding molecules are located not only on the plasma membrane but also on the inner surfaces exposed during the acrosome reaction, possibly involved in the various phases of the interaction between sperm and ZP. ZP-binding molecules exposed during the acrosome reaction include PH-20 [26], proacrosin, a zymogen of a serine protease acrosin [27,28], and "the second ZP receptor" that binds to ZP2 [29].

In a previous study [30] we found a new boar sperm protein, sp38, with an apparent molecular size of 38 kDa that specifically binds to the 90-kDa family glycoprotein of ZP (ZP2) in a calcium-dependent manner. The ZP-binding properties of sp38 were similar to those of proacrosin. The binding of sp38 to ZP was inhibited by the addition of proacrosin. We now report the cDNA sequence encoding porcine sp38 and the protein localization in sperm. Potential sequences required for the ZP-binding in sp38 and proacrosin are also described.

Furthermore, besides the recognition molecules that modify the binding of sperm to ZP, it has been considered that another set of recognition molecules exist in the postacrosomal region of the sperm and egg plasma membrane. Although the expression of the MHC molecule in mammalian reproductive tissues has been controversial [31–33], the expression of MHC class II

Received: December 18, 1996
Accepted: January 7, 1997
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structure on the surface of human and murine sperm head was recently demonstrated at serological, cellular and genetic levels by Bishara *et al.* [34] and by us [35–37]. We have shown in a previous paper that the monomorphic related region of MHC class II molecules in the posterior region of sperm head plays an adhesive role in the recognition between sperm and egg during fertilization [35]. Corresponding to the MHC class II molecule on murine sperm, CD4 proteins have been demonstrated on the plasma membrane of murine egg [36]. We have also shown that the adhesion between the postacrosomal membrane of the sperm and the plasma membrane of the egg was mediated by MHC class II on the sperm and CD4 on the egg. To examine the direct interaction of the CD4 molecule from the egg with murine sperm cells bearing MHC class II molecules, we employed a baculovirus expression system to generate CD4 proteins on the surface of *Spodoptera frugiperda* (Sf9) cells [38]. We also demonstrated that the expression of $p56^{lck}$, a member of the src protein tyrosine kinase family, is associated with CD4 molecules beneath the plasma membrane of the egg in the same manner as that of T cells [39] and suggested the possibility that these molecules may play the signal transducing role in the egg at the fusion step in fertilization [40].

Experimental Results

I. Analysis of sugar structure of porcine ZP

N-linked sugar chains from ovarian follicular oocytes were analyzed [3]. The structural analysis was performed by paper electrophoresis, serial lectin column chromatography, Bio-Gel P-4 column chromatography, sequential glycosidase digestion and by methylation analysis. The results revealed that the sugar chains are of bi-, tri-, and tetra antennary complex type with a fucosylated trimannosyl core. A characteristic feature is that 39% of the sugar chains contain N-acetylglucosamine residues at their nonreducing termini. The acidic sugar chains contained sialic acid and/or sulfate residue. Most of the sulfate residues were located at C-6 position of N-acetyl glucosamine residues included in polylactosamin repeating units and in nonrepeating antennae. The results are summarized in Fig. 1.

O-linked sugar chains were analyzed by alkaline-borohydride treatment of porcine ZP glycoprotein [41]. The major oligosaccharides were shown to have the following structures with type-1 cores: (Glaβ1-4 GlcNAcβ1-3)$_{1\sim3}$-Galβ1-3N-acetylgalactosaminitol (54%) and GlcNAcβ1-3(Glaβ1-4GlcNAcβ1-3)$_{0\sim1}$-Galβ1-3N-acetylgalactosaminitol (16%). Approximately 6% of the oligosaccharides had a structure with a type-3 core, Galβ1-4GlcNAcβ1-3N-acetylgalactosaminitol. Oligosaccharides with α-galactose (2%) and β-GalNAc (4%) at their non-reducing termini were also found as minor components.

Sulfation and sialylation occurred also in O-linked sugar chains in the same manner as in N-linked sugar chains.

II. Purification and structural analysis of a ZP2-binding protein, sp38

By means of reverse phase HPLC, a 38 kDa protein, sp38, was purified from the detergent extract of porcine epididymal sperm. Sp38 showed ZP2-binding properties similar to those of proacrosin. These two proteins specifically bound to the 90 kDa glycoprotein of ZP com-

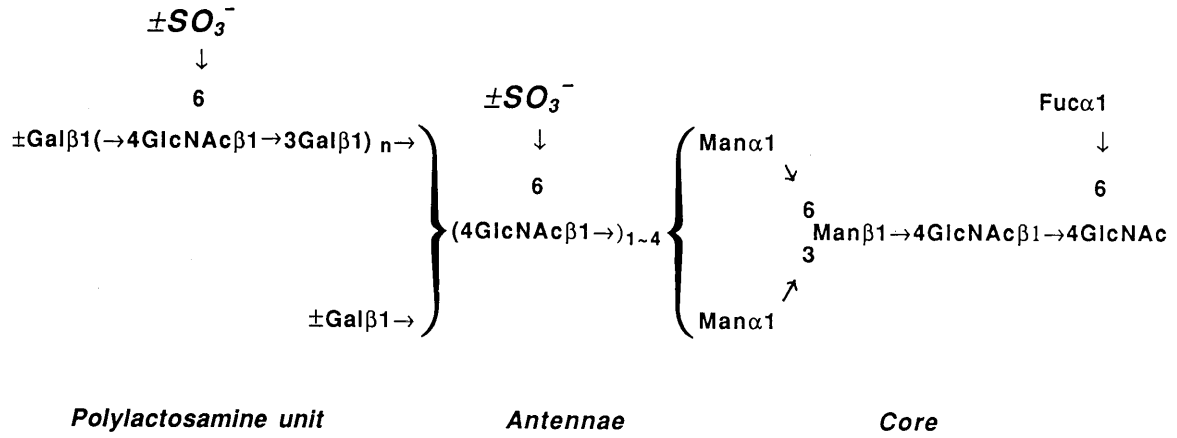

Fig. 1. Structure of the N-linked sugar chains of porcine Zona pellucida glycoproteins.

ponents in a calcium-dependent manner. The binding of sp38 to ZP glycoprotein was inhibited by proacrosin. These findings suggest that the two proteins competitively interact with ZP during the early stage of fertilization. The purified sp38 protein was therefore used for the aminoacid sequence. The preparation of sp38 by HPLC and its electrophoresed profile are shown in Fig. 2.

Isolation and characterization of cDNA clones of sp38

To isolate cDNA clones coding for porcine sp38, an oligonucleotide was synthesized according to the 9-residue sequence, KVYVMLHQK, of AP-2, which was obtained by *in situ* digestion of sp38 with Achromobacter protease I [30], and used to screen approximately 7×10^5 recombinant phage from a boar testis cDNA library in lgt11. We then, selected four clones from among twenty-four positive clones for nucleotide sequence analysis.

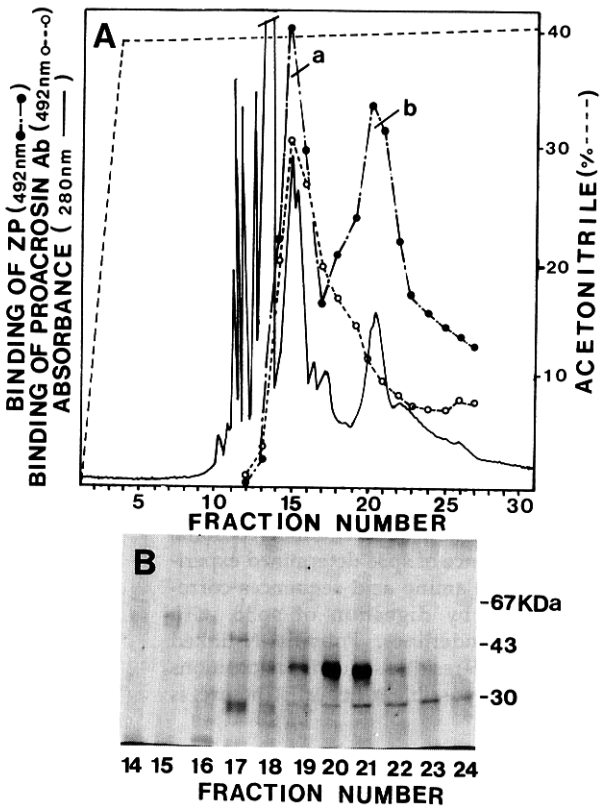

Fig. 2. Reverse phase HPLC of ZP-binding proteins (A) and SDS-PAGE of the effluents (B). (A): The ZP-binding proteins were subjected to a Bakerbond Wide Pore Butyl column and eluted with acetonitrile increased from 0 to 38% during the first 5 min then to 43% during the next 120 min. The effluents were examined for ZP-binding and antiproacrosin antibody-binding by a solid-phase binding assay. (B): Fractions from 14 to 24 were subjected to SDS-PAGE.

The composite nucleotide sequence of the overlapping cDNA inserts encodes a 1,053-open reading frame that is flanked by 5'- and 3'-untranslated regions of 14 and 83 nucleotides, respectively (Fig. 3). The open reading frame encodes a polypeptide of 350 amino acids with a calculated molecular mass of 39,609 Da. The deduced amino acid sequence at residues 52–71 matches the amino-terminal sequence of sp38 determined by protein analysis [30]. In addition, the sequences of six peptides derived from the Achromobacter protease I-digested sp38 were all found in the deduced amino acid sequence of sp38. These data clearly confirm that the isolated cDNA clones code for porcine sp38, and demonstrate that the mature protein contains 299 amino acids with a molecular mass of 34,103 Da. This value is consistent with the molecular size (33 kDa) of sp32 with N-glycanase treatment. Potential N-linked glycosylation sites are located at residues 113, 186 and 339 (Fig. 3). The sequence of sp38 shows no significant similarity with those of known proteins listed in the National Biomedical Research Foundation protein library.

The 51-residue sequence preceding the amino terminus of sp38 is different from that of a typical signal peptide in length. Computer-aided analysis of the 51-residue sequence demonstrates that the 16-residue sequence at positions 22–37 is strongly hydrophobic and forms an α-helical structure. This 16-residue sequence therefore appears to possess the membrane-associated properties. Although the precise function of the 51-residue sequence is not yet certain, the mature sp32 is produced from its precursor protein by post-translational modification, probably cleavage of the peptide bond between R51 and S52 by a serine protease.

Nothern blot analysis of sp38 in various porcine tissues

Total cellular RNA was isolated from porcine testis, liver, kidney and spleen, and subjected to Northern blot analysis. A 1.4-kb transcript for the sp38 gene is found only in the testis. The size of the overlapping cDNA sequence is therefore less than the full length of the mRNA, but the cDNA sequence appears to cover the entire region of the open reading frame, as mentioned above.

Preparation of antibody to sp38 fusion protein and Western blotting analysis

A protein-coding region of the sp38 cDNA, corresponding to the 287-residue sequence at positions 64–350 (Fig. 3), was introduced into a pGEMEX-1 vec-

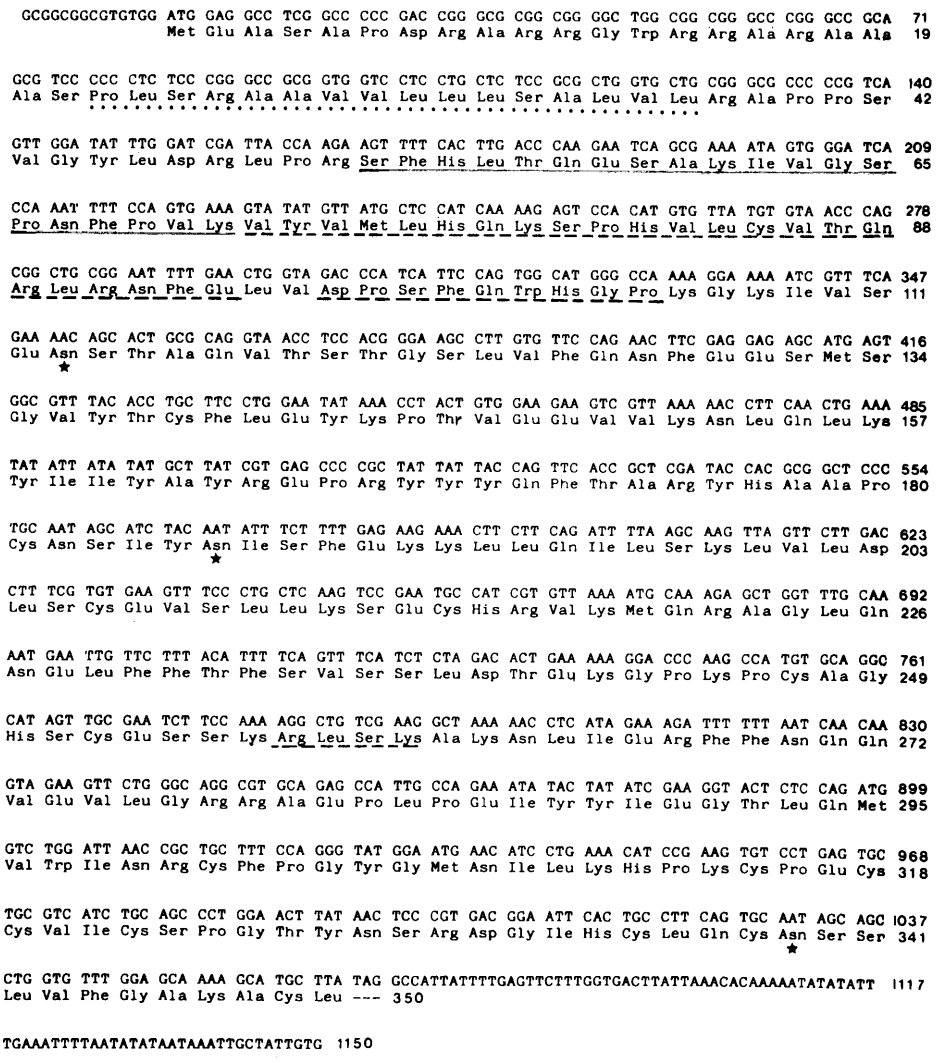

Fig. 3. Nucleotide sequence of boar sp38 cDNA and its deduced amino acid sequence. The amino acid sequence is shown below the nucleotide sequence numbered in the 5′- to 3′-direction. The N-terminal amino acid sequence of sp38 determined experimentally (30) is shown by a solid underline. The amino acid sequences corresponding to the peptide fragments obtained by digestion of sp38 with Achromobacter protease I are shown by broken underlines. Potential N-linked glycosylation sites are indicated by asterisks. The 16-residue sequence at positions 22–37, which is strongly hydrophobic and forms a possible α-helical structure, is represented by a wavy underline.

tor, and expressed in *E. coli*. A fusion protein of sp38 with a T7 gene10 protein expressed bound to biotin-labeled ZP. The antibody to the sp38 fusion protein was prepared by immunizing a polyacrylamide gel slurry containing the fusion protein. Western blot analysis demonstrated the immunoreactivity of the affinity-purified antibody with the purified sp38. Extracts of epididymal sperm cells gave a major immunoreactive band of the 38-kDa protein. A minor 43-kDa band was also detectable. Thus these data suggest that most of the sp38 precursor is already processed into the mature form in the epididymis.

Localization of sp38 in porcine sperm by immunohistochemical method

Sperm cells with or without induction of the acrosome reaction by calcium ionophore A23187 were stained with affinity-purified anti-sp38 antibody or preimmune IgG.

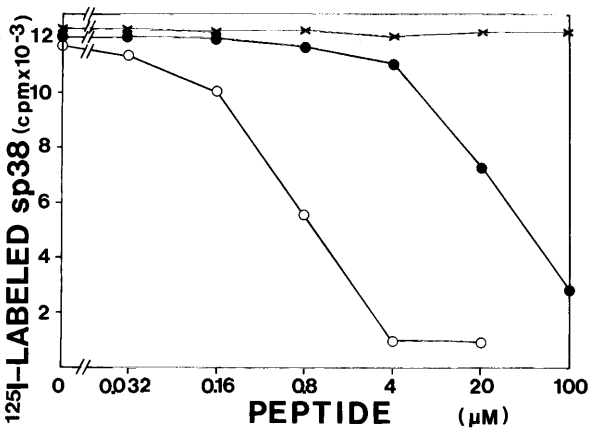

Fig. 4. Inhibitory effects of synthetic peptides on the binding of ^{125}I-labeled sp38 to ZP glycoprotein. ^{125}I-Labeled sp38 was incubated with ZP glycoprotein coated on the wells of ELISA plates in the presence of various concentrations of synthetic peptides corresponding to the sequences of sp38 (●) and proacrosin (○). Free ^{125}I-labeled sp38 was removed by washing with 10% SDS, and the remaining radioactivity was measured with an auto-counter.

No staining was observed in sperm cells before induction of the acrosome reaction. After 30-min incubation with A23187, approximately 90% the of sperm cells stained positively in the anterior portion. When sperm cells without the induction of the acrosome reaction were permeabilized with methanol, the anterior portion underneath the plasma membrane was stained positively. These results indicate that sp38 is initially localized in an intraacrosomal region, exposed outside during the acrosome reaction, and then released after the completion of the reaction.

Determination of the amino acid sequence of sp38 and acrosin involved in the ZP-binding

To identify the binding region of proacrosin to ZP, proacrosin (a mixture of 55- and 53-kDa forms) was autoactivated *in vitro* at pH 8.5, and the ZP-binding properties during the activation process were examined. SDS-PAGE analysis implied that conversion of proacrosin into a 43-kDa intermediate form of acrosin was critical for the decrease in the ZP-binding activity. In porcine, the 55-kDa proacrosin is converted into the 43-kDa intermediate via the 53-kDa proacrosin and a 49-kDa acrosin intermediate by two processes: removal of proenzyme segments from the C-terminus and cleavage of the peptide bond between R23 and V24 [42, 43]. The above results therefore suggested that the N- and/or C-terminal regions of proacrosin are implicated in the ZP-binding.

Sequence comparison of the C-terminal region of proacrosin with sp38 revealed a regional similarity between these two proteins: KRLQQLIE at residues 365–372 [40] and KRLSKAKNLIE at residues 256–266 in proacrosin and sp38, respectively. These two peptides were practically synthesized, and their inhibitory effects on the binding of sp38 to ZP were examined by a solid-phase binding assay. As shown in Fig. 4, both of the synthetic peptides inhibited the binding of ^{125}I-labeled sp38 to the ZP glycoproteins coated on the wells of ELISA plates. The concentrations of the peptides for sp38 and proacrosin, which gave 50% inhibition for the binding, were 20 and 0.8 μM, respectively. The profile of sp38 was described in detail in our previous studies [30, 45].

III. Participation of Ig superfamily and its related molecules in mammalian fertilization

The notion that the major histocompatibility complex (MHC) molecule and related members of the immunoglobulin (Ig) superfamily may participate directly in fertilization has been controversial not only with regard to marine species of immediate ancestral forms of vertebrates [44] but also with regard to mammalian species [31–33]. These observations support the hypothesis that vertebrates' histocompatibility genes might have evolved from gametic recognition systems [46, 47].

Presence of MHC class II antigen on murine sperm and its roles in fertilization

The expression of MHC class II antigen on the membrane of mouse sperm head was clearly demonstrated by a indirect immunofluorescence (IIF) test as shown in Fig. 5A, enzyme immunoassay, mixed lymphocyte sperm reaction and immunoblotting as well as the examination of its expression at transcriptional levels by northern dot blotting. Furthermore, the roles of the MHC class II molecule of sperm in fertilization were investigated by means of an *in vitro* fertilization (IVF) system. The rate of successful IVF was significantly decreased by treatment of sperm with anti monomorphic region of class II monoclonal antibody (mAb) but not with anti polymorphic region mAb, which strongly suggested that the monomorphic or its related region of MHC class II molecule on sperm plays an important role in recognition between sperm and egg in fertilization [35–37].

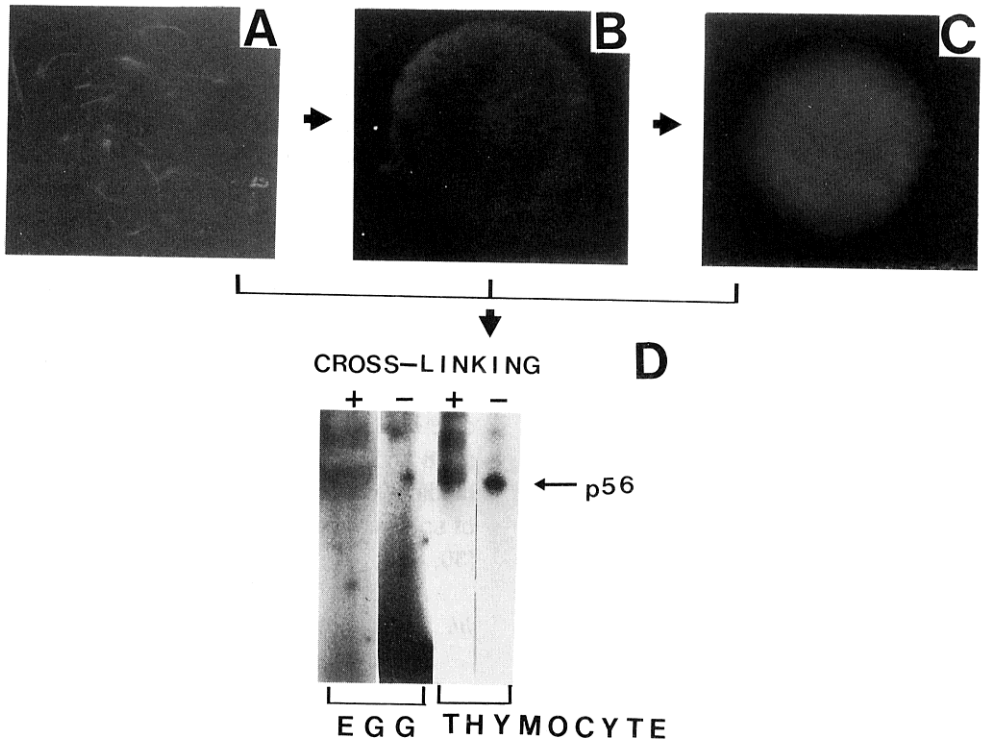

Fig. 5. IIF staining of murine sperm/egg and immunocomplex kinase assay of $p56^{lck}$. The motile sperm cells were stained with anti-MHC class II mAb (A). An unfertilized murine egg freed of zona pellucida was stained with anti-CD4 (B) or anti-mouse $p56^{lck}$ mAb (C) followed by incubation with FITC-labeled second antibody (× 200). Eggs or thymocytes were immunoprecipitated by anti $p56^{lck}$ serum and autophosphorylated after crosslinking with anti CD4 mAb (D, +lanes) and without cross-linking (D, −lanes).

Transcriptional expression of CD4 gene and the signal transducing region of lck gene in murine egg by RT-PCR followed by sequencing

One μg of each total RNA preparation from various murine cells and tissues was subjected to reverse transcriptase-polymerase chain reaction (RT-PCR) with the primers which should produce the full-length CD4 cDNA. An aliquot of each PCR product was then subjected to Southern blot hybridization analysis with an end-labeled internal oligomer probe. As shown in Fig.6, the PCR products from thymus (lane 2), ovary (lane 3), oocytes (lane 4) and eggs (lane 5) showed a positive signal at the position corresponding to the size of the full-length CD4 cDNA. On the other hand, the reaction mixture without cDNA (lane 1) and product from granulosa cells (lane 6) showed a negative reaction. The CD4 band obtained from eggs was cloned into a plasmid vector, pCRTM II with the TA cloning TM kit as shown in Fig. 9. The sequencing results show that the sequence of CD4 cDNA obtained from eggs was completely identical to that of T lymphocytes. Furthermore, to examine the expression of the *lck* gene in eggs at the transcriptional level, RT-PCR amplification was performed along with several control tissues. Total RNA preparations from freshly isolated sperm, brain, thymus, ovary and eggs were reverse transcribed, and their cDNAs were amplified by PCR with the primers which should amplify the sequence of the unique N-terminal region of lck cDNA [48] as shown in Fig. 7C. Thymus, ovary and eggs gave PCR products of the predicted size of 201 base pairs long (Fig. 7A), whereas sperm and brain did not give any PCR products. Restriction enzyme analysis of the PCR products was performed (Fig. 7B). The PCR products from thymus, ovary and eggs cleaved with the restriction enzyme Bgl II gave DNA fragments consistent with the predicted sizes of 97 and 104 base pairs. The DNA fragments detected corresponded in size to the N-terminal region of *lck* cDNA of T lymphocytes [48]. Following the subcloning of PCR products, the sequence of PCR products from eggs was also examined by the dideoxy chain termination method. As shown in Fig. 7C, analysis of the nucleotide seqences of two

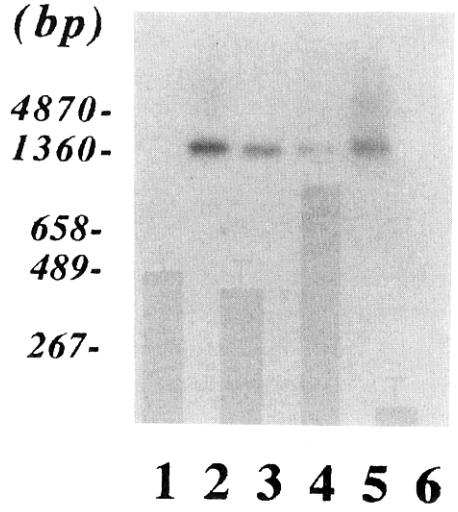

Fig. 6. Southern blot hybridization analysis of RT-PCR product with an internal oligomer probe for CD4 cDNA. One μg of each total RNA preparation isolated from various cells and tissues was reverse transcribed to cDNA, PCR amplified with forward and reverse primers, separated with 1% agarose gel, transferred onto nylon membrane, and hybridized with an end-labeled internal oligomer probe for CD4 cDNA. Each lane shows RT-PCR products from the reaction mixture without cDNA (lane 1), thymus (lane 2), ovary (lane 3), oocytes (lane 4), eggs (lane 5) and granulosa cells (lane 6).

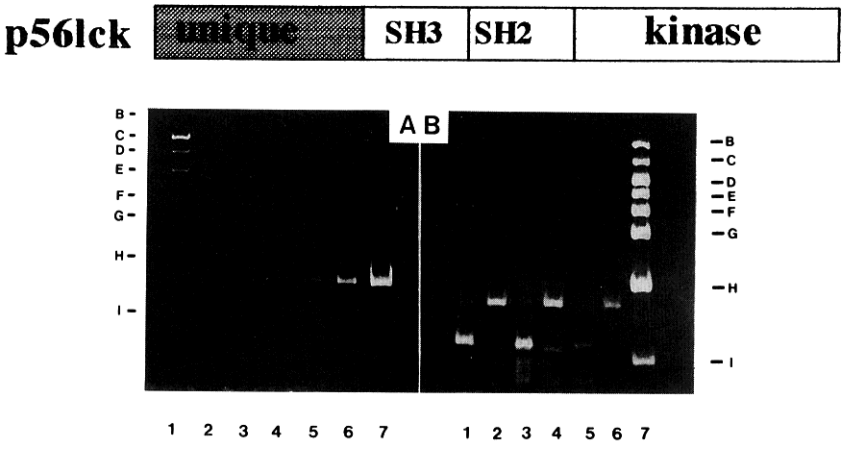

Fig C

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Met Gly Cys Val Cys Ser Ser Asn Pro Glu Asp Asp Trp Met Glu Asn
ATG GGC TGT GTC TGC AGC TCA AAC CCT GAA GAT GAC TGG ATG GAG AAC
             9          18         27          36          45

Ile Asp Val Cys Glu Asn Cys His Tyr Pro Ile Val Pro Leu Asp Ser Lys Ile
ATT GAC GTG TGT GAA AAC TGC CAC TAT CCC ATA GTC CCA CTG GAC AGC AAG ATC
          57          66          75          84          93         102

                                                                Bgl II
Ser Leu Pro Ile Arg Asn Gly Ser Glu Val Arg Asp Pro Leu Val Thr Tyr Glu
TCG CTG CCC ATC CGG AAT GGC TCT GAA GTG CGG GAC CCA CTG GTC ACC TAT GAG
        111         120         129         138         147         156

Gly Ser Leu Pro Pro Ala Ser Pro Leu Gln Asp Asn Leu Val Ile
GGA TCT CTC CCA CCA GCA TCC CCG CTG CAA GAC AAC CTG GTT ATC
        165         174         183         192         201
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Fig. 7. Detection of PCR products amplified from *lck* cDNA of eggs and other tissues (A), restriction enzyme analysis (B) and nucleotide/deduced amino acid sequence of the unique N-terminal region of *lck* cDNA (C). A: Each lane shows PCR products from reaction mixture without cDNA (lane 2), thymus (lane 7), ovary (lane 6), eggs (lane 5), brain (lane 4) and sperm cells (lane 3). Lane 1, DNA markers. B: Lanes 2, 4 and 6, PCR products amplified from thymus, ovary and egg cDNA respectively. Lanes 1, 3 and 5, results of Bgl II cleavage of PCR products shown in lanes 2, 4 and 6, respectively. Lane 7, DNA markers. C: Nucleotide/deduced amino acid sequences of the unique N-terminal region of *lck* cDNA, PCR primers are shown underlined.

independent clones demonstrated that each clone contained an open reading frame corresponding to an amino acid sequence identical to the N-terminal domain of the p56^{lck} molecule of murine thymocytes. This is in agreement with the finding obtained by Northern blotting of its expression in human ovary [49].

Presence of CD4 structure on murine egg plasma membrane and its roles in fertilization

The presence of the CD4 molecule on murine egg plasma membrane was demonstrated by IIF test as shown in Fig. 5B and an immunoprecipitation method. This molecule was of almost the same size as that of the authentic CD4 molecule from T cells on SDS-PAGE. This molecule also appeared to bind to MHC class II structure on sperm during fertilization because anti CD4 mAb blocked IVF [36]. Furthermore, the adherence between sperms labeled with anti MHC class II mAb and eggs labeled with anti CD4 mAb was strongly disturbed as shown in Fig. 8.

Construction of baculovirus (AcNPV) expression vectors for CD4 cDNA of murine egg in Sf9 cells

The generation of CD4 molecules in Sf9 cells was performed with the baculovirus (AcNPV) expression vector system according to the standard method [50]. The strategy used for the construction of the recombinant AcNPV expression vectors and the full-length murine egg CD4 cDNA is outlined in Fig. 9. A cDNA clone encoding the entire region of murine egg CD4 was excised from pCRTM II and then ligated into the EcoRI excised PVL1393 that had been treated with bacterial alkaline phosphatase. The resultant recombinant plasmids PVL1393-CD4 containing a single copy of full length CD4 cDNA in the correct orientation were identified by restriction mapping.

The Sf9 cells were co-transfected with the stocks of the AcNPV combined with the plasmid PVL1393 encoding CD4 cDNA (AcNPV-CD4) or the plasmid PVL1393 (AcNPV-1393). The expression of CD4 in Sf9 cells infected with the recombinant viruses was then examined by using IIF test with anti-CD4 mAb. As shown in Fig. 10A, upper panel, approximately forty percent of AcNPV-CD4-infected Sf9 cells showed intense fluorescence on their cell surface membrane, whereas specific staining was not obtained in AcNPV-1393-infected Sf9 cells (Fig. 10B, upper panel).

Sperm adhesion to Sf9 cell expressed with CD4 protein

The adhesion of AcNPV-CD4-infected Sf9 cells to sperm cells was examined. Sperm cells adhered to

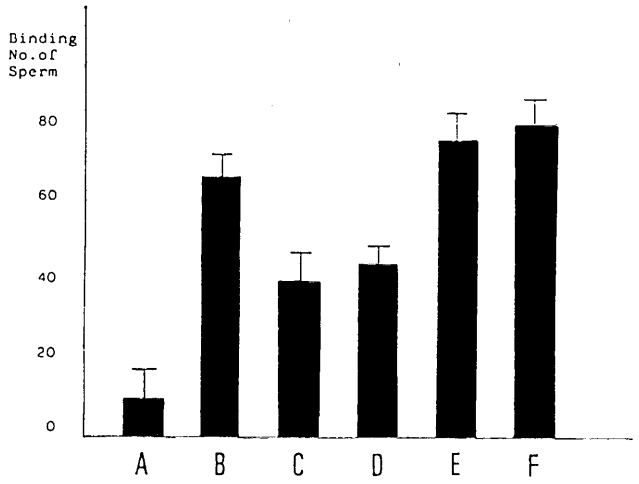

Fig. 8. Blocking effects of anti-MHC class II and/or anti CD4 mAb on the adhesion of two gametes. The pretreatment of eggs or sperm with anti-CD4 or anti-MHC class II mAb. A: egg, labeled with anti-CD4 mAb. sperm, labeled with anti-MHC class II mAb. B: sperm, labeled with anti-CD4 mAb. egg, labeled with anti-MHC class II mAb. C: untreated egg, sperm, labeled with anti-MHC class II mAb, D: egg, labeled with anti-CD4 mAb, untreated sperm, E: untreated egg, untreated sperm, F: egg and sperm, treated with IgG. Each column and vertical bar represents the mean ± SD of triplicate experiments.

AcNPV-CD4-infected Sf9 cells (Fig. 10A, lower panel) but not to AcNPV-1393-infected Sf9 cells (Fig. 10B, lower panel). Treatment of sperm cells with calcium ionophore A23187 to induce the acrosome reaction slightly decreased the adherent activity ($P<0.05$). The adherence of sperm cells to AcNPV-CD4-infected Sf9 cells was inhibited specifically by treating either sperm cells with anti monomorphic related region of MHC class II mAb or AcNPV-CD4-infected Sf9 cells with anti-CD4 mAb, or synergistically by both treatments as summarized in Fig. 11. In the reverse experiment, the adherence between the two cells was not influenced by treating either AcNPV-CD4-infected Sf9 cells with anti MHC class II mAb or sperm cells with anti-CD4 mAb. The construction procedure for AcNPV-CD4-infected Sf9 cells and their interactions with sperm cells were presented in our previous report [38].

Binding of MHC class II to sperm and CD4 to egg triggers the signals of fertilization through p56^{lck} underneath egg plasma membrane

When anti MHC class II and CD4 mAb were applied to murine sperm and egg freed of zona pellucida, the

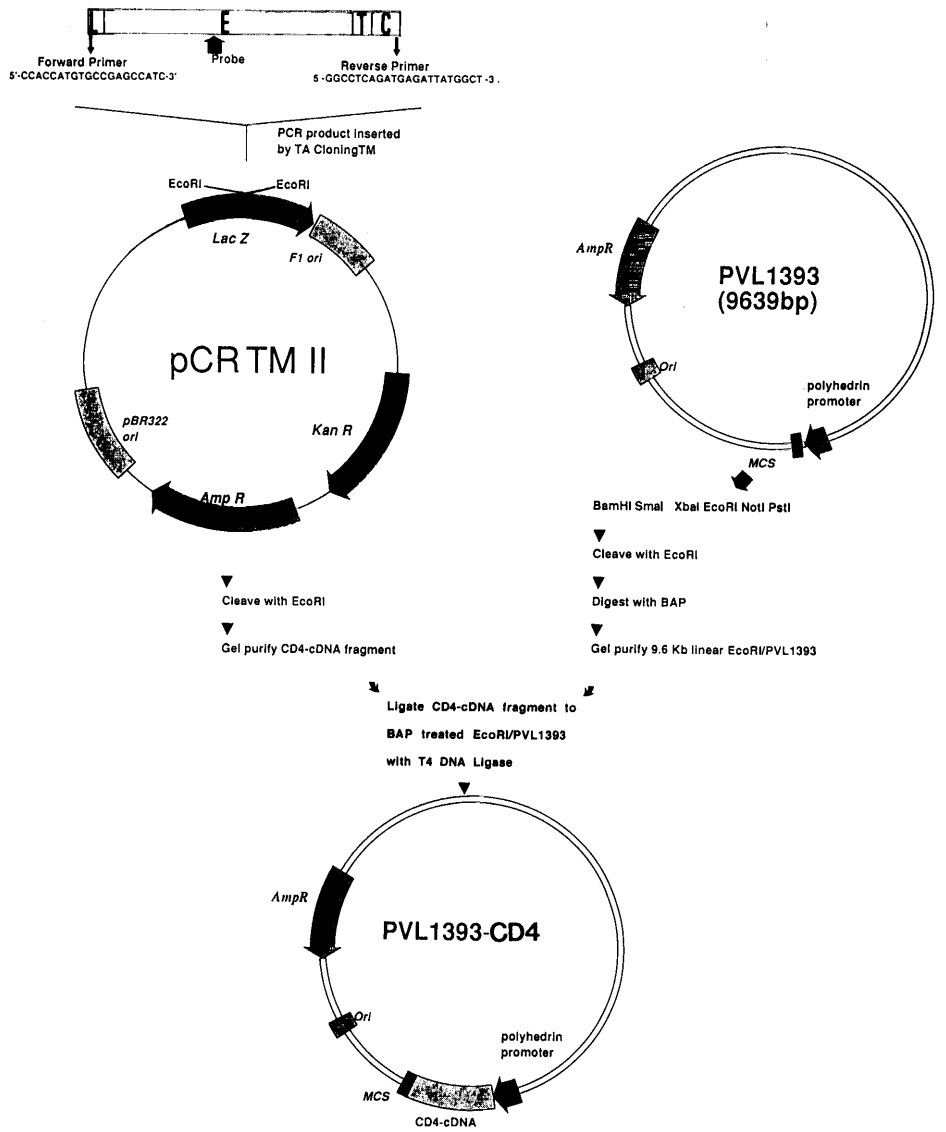

Fig. 9. The process of PVL1393-CD4 construction. The PCR products were gel-purified and subcloned into a plasmid pCR TM kit followed by DNA sequencing. The 1.4 kilobase EcoRI-EcoRI restriction fragment of CD4 cDNA was then recloned into PVL1393. The recombinant plasmid PVL1393-CD4 encodes the entire CD4 coding region beginning at the translation initiator of the polyhedrin promotor. L, leader sequence; E, extracellular region; T, transmembrane region; C, cytoplasmic region; MCS, multicloning site; BAP, bacterial alkaline phosphatase.

IIF pattern of sperm cells appeared to be localized in the posterior region of the sperm head, as shown in Fig. 5A and in Fig. 5B. The IIF pattern of the egg appeared as patchy profiles and resulted in the localization of CD4 molecules, presumably due to their aggregation by crosslinking. As shown in Fig. 5C, IIF test of eggs fixed with ethanol was performed with anti N-terminal region of $p56^{lck}$ mAb, and the inner plasma membrane of eggs fluoresced clearly. When the signal transducing role of $p56^{lck}$ was examined by immunocomplex kinase assay, similar results to those obtained with the CD4 positive murine thymocytes were obtained in eggs. As shown in Fig. 5D, CD4 of eggs and thymocytes crosslinking with anti-CD4 mAb resulted in an increase in the tyrosine-specific protein kinase activity of $p56^{lck}$ and $p60^{lck}$ appeared with an autophosphorylated form. The kinase

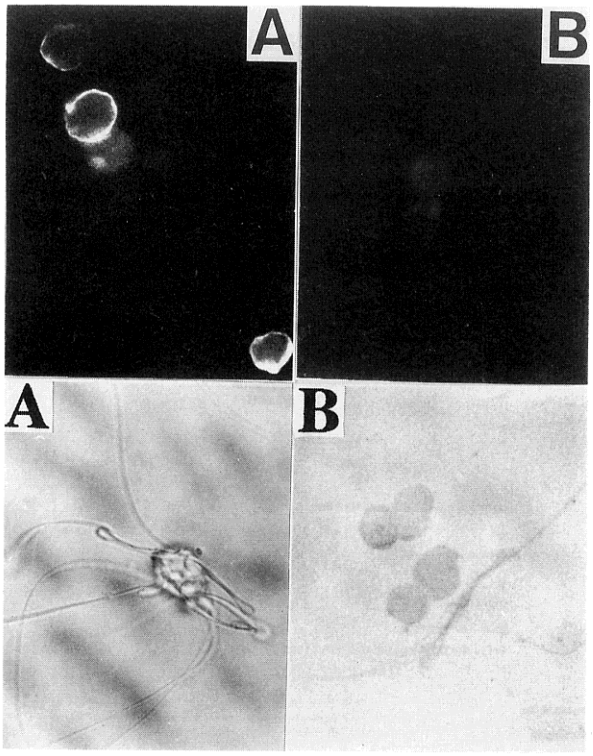

Fig. 10. The expression of CD4 in Sf9 cell infected with AcNPV-CD4 and sperm adhesion to Sf9 cell infected with AcNPV-CD4. The membrane immunofluorescene of AcNPV-CD4-infected Sf9 cells (A, upper panel) and AcNPV-1393-infected Sf9 cells (B, upper panel) obtained by IIF test with anti-CD4 mAb. A, lower panel: AcNPV-CD4-infected Sf9 cells adhered to sperm cells. B, lower panel: No AcNPV-1393-infected Sf9 cells adhered to sperm cells. Magnification in each panel, × 400.

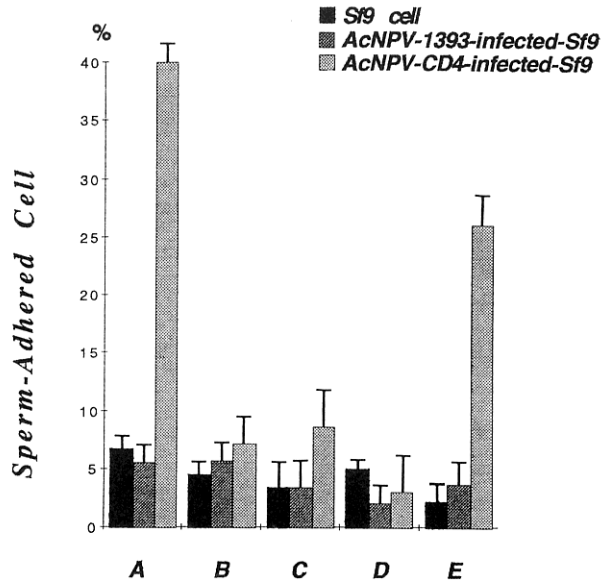

Fig. 11. Percentage of different Sf9 cell with adherent sperm. The three cell types are grouped according to the pretreatment of the Sf9 cell or sperm with anti-CD4 or anti-MHC class II mAb. A: untreated Sf9 cell, untreated sperm, B: Sf9 cell, labeled with anti-CD4 mAb, untreated sperm, C: untreated Sf9 cell, sperm, labeled with anti-MHC class II mAb, D: Sf9 cell, labeled with anti-CD4 mAb, sperm, labeled with anti-MHC class II mAb. E: untreated Sf9 cell, sperm, treated with A23187. Each column and vertical bar represents the mean ± SD of triplicate experiments.

activity of the immunocomplex with rabbit anti-p56^{lck} serum alone was seen in thymocyte lysate but scarcely seen in egg lysate. In the conversion of p56^{lck} to p60^{lck} in eggs it was therefore definitely demonstrated that the CD4 molecule and p56^{lck} are present in eggs in a physically associated form, and *in vitro* protein kinase activity of p56^{lck} was activated by cross-linking of CD4 molecules in the same manner as described in thymocytes [51]. The results obtained suggested that the adherence and binding of MHC class II molecules on sperm to CD4 of egg triggers the activation of p56^{lck} associated with the cytoplasmic domain of CD4 underneath the egg plasma membrane. This signal transducing pathway is considered to lead to the ionic activation of the egg generating G protein-phosphoinositide cascade as well as that of the somatic cells [52]. Further studies are continuing to clarify the signaling pathway into the cortical reaction and the cleavage of the fertilized egg.

Discussion

The sugar moieties of ZP glycoproteins play an adhesive and binding role in the interaction of mammalian sperm with eggs at the initial stage of fertilization. One of the well-studied examples is that O-linked oligosaccharides with α-linked galactose residues of mouse egg ZP3 have a sperm receptor activity [9, 53]. Another example also suggested was that galactosyl transferase located on the plasma membrane of the mouse sperm head mediates the binding of sperm to eggs [10, 11, 54]. In this context, certain oligosaccharides with exposed N-acetylglucosamine residues might be recognized as acceptors for the enzyme, although their structures have not been clarified in the mouse system. The present study showed that considerable quantities of N-linked, neutral oligosaccharides in porcine ZP glycoproteins are terminated with N-acetylglucosamine residues. It was preliminarily observed that α-lactal-

bumin, an inhibitor of galactosylation of N-acetylglucosamine by galactosyltransferase, inhibits the binding of boar sperm to its eggs. This fact may indicate that exposed N-acetylglucosamine residues in the oligosaccharides are at least one of the sperm binding determinants in the porcine system. Of interest also is the occurrence of lectin-like proteins on boar sperm which bind to fucose [55]. Whether fucosyl residues found in almost all of the neutral oligosaccharides are recognized by the sperm lectin is a subject to be investigated in the future.

It is known that sperm are loosely attached to the ZP immediately after mixing with eggs and then the attached sperm bind more tightly to ZP. The bound sperm undergo the acrosome reaction, by which the inner acrosome membrane of the sperm is exposed, penetrates the ZP, and finally fuses with egg plasma membrane [56, 57]. It is therefore quite possible that multiple recognition mechanisms are involved in these processes. As mentioned above, the present study on the N-linked, neutral oligosaccharides revealed interesting structural aspects in the elucidation of proposed roles of the sugar moieties of ZP glycoproteins in the sperm-egg interaction. Fifty percent of N-linked and O-linked acidic oligosaccharides are sulfated. This characteristic is also notable in view of the evidence that sulfated glycans such as dextran sulfate and fucoidin inhibit the binding of the sperm to the egg in several mammalian species including pig [58, 59]. Detailed analysis of the sugar moieties of ZP glycoproteins will therefore help us to find a clue to the understanding of the molecular mechanism of fertilization.

We have isolated cDNA clones encoding porcine sp38 with ZP-binding activity. The cDNA sequence of sp38 indicates that this protein is originally synthesized as a precursor protein of 350 amino acids, including a 51-residue presequence at the N-terminus. Sp38 contains three potential N-glycosylation sites. Two of them are probably glycosylated, since the molecular size is decreased 5 kDa by N-glycanase treatment. Immunostaining of sperm cells with affinity-purified anti-sp38 antibody reveals that sp38 is an intraacrosomal protein and exposed to the outer surface during the acrosome reaction. The sequence of sp38 shares no significant similarity with those of PH-20 [26] and sp10 [60] that possess similar properties to sp38.

We previously [31] reported that sp38 has similar ZP-binding properties to proacrosin, and these two proteins specifically bind to the 90-kDa family of porcine ZP glycoproteins in a calcium-dependent manner [30]. The binding is effectively inhibited by dextran sulfate, but poorly by keratan sulfate. Moreover, the binding of ^{125}I-labeled sp38 to the ZP glycoprotein is competed by proacrosin. In this study, six residues of the 11-residue sequence KRLSKAKNLIE in sp38 are conserved in the 8-residue sequence, KRLQQLIE in proacrosin. In fact, the synthetic peptides for these two sequences inhibit the binding of ^{125}I-labeled sp38 to ZP glycoprotein. The binding affinity of the peptide for proacrosin to the ZP glycoprotein is almost 25-fold higher than that of the peptide for sp38. These results are supported by the fact that the heavy chain sequence of proacrosin is responsible for the ZP-binding property [61]. At any rate, the amino acid sequence KRLXX(XXX)LIE most likely plays a key part in binding the ZP glycoprotein. The ZP-binding activity of proacrosin largely decreases during its maturation. This is explained by the conversion of the 53-kDa proacrosin into the 43-kDa acrosin intermediate that is lacking in the above 8-residue sequence [43, 44].

It has been reported that the ZP glycoprotein binds to the acrosome-reacted or membrane-permeabilized sperm, but not to the membrane-intact sperm, in contrast to the case reported in mice [30]. The apparent difference in the binding mechanism among species is considered to be due to the difference in the amount of expression of each molecule involved in the binding with ZP, as well as the difference in the affinity to the ZP, among species. In porcine, the molecules exposed during the acrosome reaction seem to play the major role in the binding to ZP. A possible function of sp38 may therefore be the regulation of the ZP-binding and/or the proteolytic activity of proacrosin /acrosin, which enables sperm to bind to and penetrate the ZP properly. The testis-specific, abundant expression of sp38 mRNA shown by Northern blot analysis suggests the importance of sp38 not only in fertilization but also in spermatogenesis.

In the late stage of fertilization (fusion stage), we found the participation of the Ig superfamily and its related molecules in fertilization. We have reported the haploid expression of the MHC class II molecule on the sperm head as shown in our previous studies [35–37], and we also demonstrated that this molecule in the posterior region of the sperm plays an adhesive role in the processes of adhesion and fusion between the sperm and egg because the anti-monomorphic related region of MHC class II mAb decreased the successful ratio of fertilization in the IVF system [36]. We also observed that a foreign DNA bound to sperm mediated by its MHC class II structure is diffused on the egg plasma membrane in the fusion steps as revealed by auto-

radiography [37].

Corresponding to these lines of evidence for the presence of MHC class II molecules on the sperm head, we found expression of the CD4 molecule on the plasma membrane but not on the zona pellucida of murine eggs. Subsequent immunoprecipitation indicated that the CD4 molecules in eggs had almost the same molecular size as those in thymocytes. We further demonstrated that these molecules worked at the stage of adhesion and fusion between two gametes by finding that covering of the CD4 molecules on egg plasma membrane with anti-CD4 mAb resulted in the blocking of fertilization [36, 38].

We further confirmed the expression of the CD4 molecule on egg plasma membrane by the IIF method by using two different clones of anti-CD4 mAb as used previously. In addition, the full-length of CD4 cDNA was synthesized by RT-PCR from an RNA preparation of ovulated eggs or ovarian oocytes but not from that of granulosa cells. Subsequent DNA sequencing of the PCR product indicated that the full length of CD4 cDNA from eggs was completely identical to that from immune T cells [49].

We further showed the direct interaction between Sf9 cells expressed with the CD4 molecule from the murine egg and the sperm bearing MHC class II molecule. The Sf9-baculovirus expression system was employed because of the low background adhesion of sperm to Sf9 cells compared to other cells. The adhesion between CD4-expressing Sf9 cells and MHC class II molecule bearing sperm cells was specifically blocked by anti-CD4 mAb or the anti-monomorphic related region of MHC class II mAb.

On the other hand, the involvement of a sperm protein (PH-30) with a structure similar to viral fusion proteins [62] or a protein with RGD amino acid sequence [63, 64, 66] in the fusion of two gametes has been reported. Also mutant class II -/- knockout mice did not bleed well [65]. Taken together, the results strongly suggested that the adhesion and fusion step in fertilization would be mediated not only by a single set of recognition molecules but also by multiple mechanisms including the binding of MHC class II molecules on the sperm head to the CD4 molecules on the egg plasma membrane.

It has also been revealed that there is some restriction on the ability of the gametes from two different species to interact, especially in the fusion step in fertilization. Even though hamster eggs are known to fuse with a wide variety of mammalian sperm, they show a preference for fusing with hamster sperm [6, 66]. Conversely guinea pig sperm will fuse more readily with guinea pig eggs than they will with hamster eggs [67]. The basis for these cross-species differences is considered to reflect a reduced and variable affinity of eggs and sperm of different species in a bind step that leads to fusion.

The molecular basis of the allogeneic recognition in mammalian fertilization involves the species-specific domain-domain interaction of MHC class II molecules on sperm and CD4 molecules on egg plasma membrane. Actually the part of the amino acid sequence of the CD4 molecule involved in the interaction with MHC class II showed a reasonable heterogeneity between human and mouse [68, 69]. Previous homology-scanning mutations indicated that the interaction of CD4 with MHC class II molecules is complex, involving several parts of CD4 domains 1 and 2 [70–72]. On the other hand, the HIV gp120 binding site is localized to residues near the C'C" ridge of domain 1 [73, 74]. Moebius *et al.* have recently characterized the CD4 residues involved in MHC class II binding by using genetically typed B-cell lines expressing defined HLA-DR, -DP, and -DQ gene products. It was further reported that residues in the C'C" region were critical for interaction with class II molecules, independent of allelic polymorphisms [68], and β_2 domain in the HLA-DR molecules was identified as a CD4 binding site [69]. These findings in the molecular interaction site between the MHC class II molecule and CD4 molecule are summarized in Fig. 12. Our experimental results demonstrated here clearly explain the species-specific adhesive mechanism through the molecular interaction between MHC class II on the sperm and CD4 on the egg in the mammalian fertilization.

Although a number of studies [75–77] have demonstrated that the MHC molecules and related members of the Ig superfamily are well developed in vertebrates and play central roles in their immune response, a series of our studies proposed for the first time the molecular basis that supports the hypothesis that vertebrate histocompatibility and related members of the Ig superfamily gene might be evolved from the gametic allorecognition system [46] in accordance with recent studies on the gamete interactions in marine species [78]. We are now promoting further studies on the signal transducing pathway into the egg through MHC class II on sperm to the CD4/p56^{lck} complex of the egg.

Comparison of N-terminal amino acid sequence of CD4 domain 1 in mouse L3T4 and human CD4 *; amino acids involved in the interaction with MHC class II.

```
                                  *                                  * * * * * * * * * * *
L3T4D1: VTQGKTLVLGKEGESAELPCESSQKKITVFTWKFSDQRKILGQHGKGVL
CD4D1:  ATQGNKVVLGKKGDTVELTCTASQKKSIQFHWKNSNQIKILGNQG-SFL
          A-strand        B-strand          C-strand      C'-strand

          * * * * * *       * * * *                       *
L3T4D1: IRGGSPSQFDRFDSKKGAWEKGSFPLIINKLKMEDSQTYICELENRKEEVEL
CD4D1:  TKGPSKLN-DRADSRRSLWDQGNFPLIIKNLKIEDSDTYICEVEDQKEEVQL
        C"-strand  D-strand          E-strand          F-strand  G-strand
```

Comparison of amino acid sequence of MHC class II β_2 domain in mouse I-E and human HLA-DR ▲; amino acids involved in the interaction with CD4

```
                                                    ▲ ▲ ▲ ▲ ▲ ▲ ▲ ▲
I-E β2:       VEPTVTVYPTKTQPLEHHNLLVCSVSDFYPGNIEVRWFRNGKEEKTG
HLA-DR β2:    VEPKVTVYPSKTQPLQHHNLLVCSVSGFYPGSIEVRWFRNGQEEKAG

              ▲ ▲ ▲ ▲ ▲ ▲ ▲
I-E β2:       IVSTGLVRNGDWTFQTLVMLETVPQSGEVYTCQVEHPSLTDPVTVEW
HLA-DR β2:    VVSTGLIQNGDWTFQTLVMLETVPPSGEVYTCQVEHPSVTSPLTVEW
```

Fig. 12. Comparison of N-ternminal amino acid sequence of CD4 domain 1 and MHC class II β2 domain in murine L3T4/I-E and human CD4/HLA-DR. *: amino acids involved in the interaction with MHC class II. ▲: amino acids involved in the interaction with CD4.

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