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The complete primary structure of the spermadhesin AWN, a zona pellucida-binding protein isolated from boar spermatozoa

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AWN is a boar protein which originates in secretions of the male accessory glands and which becomes sperm surface-associated upon ejaculation. It is one of the components thought to mediate sperm adhesion to the egg's zona pellucida through a carbohydrate-recognition mechanism. AWN may, thus, participate in the initial events of fertilization in the pig. In this report we describe its complete primary structure by combination of protein-chemical and mass spectrometric methods. AWN exists as two isoforms, AWN-1 and AWN-2, which differ in that AWN-2 is N-terminally acetylated. The amino acid sequence of AWN contains 133 amino acid residues and two disulphide bridges between nearest-neighbour cysteine residues. Analysis of the amino acid sequence of the AWN proteins showed significant similarity only to AQN-1 and AQN-3, two other boar spermadhesins.

Boar sperm protein; AWN; Spermadhesin; Primary structure

1. INTRODUCTION

Gamete recognition through specific complementary molecules located on the external surfaces of the spermatozoon and the oocyte is an essential event in mammalian species-specific fertilization. In the mouse, carbohydrate-binding proteins on the plasma membrane surrounding the sperm head recognize sulfated fucose- containing glycoconjugates attached to the protein core of the zona pellucida glycoprotein 3 (ZP3) [1–3]. This sperm receptor has been cloned and sequenced in a number of species [4–7], and its genomic organization, oocyte-specific gene expression, and developmental regulation have been studied [8–10].

Although a number of sperm surface components have been described which mediate initial gamete interaction in different mammalian species [1,11], until recently the only primary sperm receptor-binding protein for which structural data were available was the membrane-bound enzyme β1,4 galactosyltransferase [12,13].

Recently, we have isolated and structurally characterized several members of a low molecular mass, boar sperm surface-associated novel protein family that originate in secretions of the male accessory glands and which may mediate sperm binding to zona pellucida component(s) trough carbohydrate recognition [14–17]. We have coined the term 'spermadhesin' to collectively denominate this protein family involved in fertilization. The complete primary structure of two boar spermadhesins, AQN-1 and AQN-3, have been reported [15,17]. Here we describe (i) the topography of two isoforms of another spermadhesin, AWN, on the sperm surface investigated by indirect immunofluorescence microscopy, (ii) the ability of the isolated proteins to inhibit the capacitated sperm-egg interaction, and (iii) their complete primary structure elucidated by protein-chemical and mass spectrometric analyses.

The availability of the primary structure of AQN-1 AQN-3 and AWN-1/2 may aid in the identification of spermadhesins in other species, and thus may contribute to a better understanding of the sperm-egg recognition mechanism.

2. MATERIALS AND METHODS

Ejaculated boar spermatozoa were collected, washed and extracted as described [18]. Isolation of zona pellucida-binding proteins was done following the procedure used in [14]. Isolation of AWN-1 and AWN-2 was done following [16]. The last purification step was performed by reverse-phase HPLC on a Lichrospher RP-100 (Merck, Darmstad, Germany) C18 column (25×0.4 cm, 5 μm particle size) eluting at 1 ml/min with a linear stepwise gradient of 0.1% TFA in (A) water and (B) acetonitrile (isocratically (20% B) for 5 min, followed by 20–38% B in 15 min, 38–46% B in 32 min and 46–70% B in 24 min).

Polyclonal antibodies against AWN-1 were reased in chicken, the IgG fraction was furified from the egg's yolk following [19], dialyzed

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against 20 mM phosphate, 135 mM NaCl, pH 7.4 (PBS buffer), and stored at −20°C until used.

Capacitation of spermatozoa was done as described [17].

The topography of AWN on boar sperm was studied by indirect immunofluorescence. Briefly, 10 μl of the capacitated spermatozoa suspension (10^6/ml) were spread on slides, air-dried, fixed for 15 min in methanol, incubated with PBS buffer containing 5% (w/v) BSA for 2 h at 37°C followed by incubation with either PBS buffer containing 5% (w/v) pre-immunized hen's yolk total proteins [19], or with a 1:4,000 (v/v, in PBS/BSA buffer) dilution of the chicken anti-AWN-1 polyclonal antibody. After washing with PBS/BSA buffer, both samples were incubated with a 1:300 (v/v) dilution of a biotinylated rabbit anti-chicken IgG antibody for 2 h at 37°C, washed with PBS/BSA buffer, incubated for 2 h at 37°C with FITC-labeled streptavidin (Calbiochem) at the manufacturer's recommended dilution, and washed exhaustively with PBS/BSA buffer. Finally, 10 μl of PBS:glycerin (1:9, v/v) were added to each sample and observed under a fluorescence microscope (Zeiss, 100×10 magnification).

Zona-encased oocytes were recovered from frozen-thawed ovaries by passage through nylon screens of decreasing pore size (2,000–80 μm) as described [20].

The ability of the isolated AWN proteins to inhibit the binding of capacitated spermatozoa to zona-encased oocytes was tested as described [17].

The isolated proteins (5 mg/ml in 100 mM Tris/HCl, 2 mM $CaCl_2$, pH 7.8) were digested either with endoproteinase Asp-N (endo Asp-N), endoproteinase Lys-C (endo Lys-C) (Boehringer-Mannheim), or TPCK-trypsin (Sigma) at 37°C overnight, at an enzyme:substrate ratio of 1:100 (w/w). Peptides were isolated by reverse-phase HPLC [18] and characterized by N-terminal sequencing and fast atom bombardment mass spectrometry as described in [15,17].

Time-of-flight plasma desorption mass spectrometry was done using a Bioion (Uppsala, Sweden) spectrometer with a ^{252}Cf ionization source.

3. RESULTS AND DISCUSSION

In a previous paper [16] we have described the isolation and partial biochemical and functional characterization of two isoforms of AWN, a carbohydrate- and zona pellucida-binding boar sperm-associated protein. The aim of this work was to study its topography on boar sperm, to test the ability of the isolated protein to inhibit the sperm-egg interaction, and to complete its primary structure.

The topography of AWN on boar sperm was studied by indirect immunofluorescence using a monospecific chicken polyclonal antibody against AWN-1 which cross-reacts with AWN-2. For specificity control, the same experiment was done this time using pre-immune hen's egg yolk protein extract. Fig. 1 shows that the AWN proteins are predominantly localized on the membrane overlying the acrosomal cap region of the sperm head.

In vitro, both AWN-1 and AWN-2 blocked the sperm–oocyte interaction in a dose-dependent manner when freshly isolated zona-encased oocytes were pre-incubated with different concentrations of the proteins: a solution of 0.2 mg/ml of AWN-1 almost completely abolished the subsequent binding of spermatozoa to oocytes (Fig. 2).

The above results show that AWN fulfills the criteria expected for a sperm zona pellucida-recognition molecule: it is located on the acrosomal cap, where sperm initiate binding to the zona pellucida, and the isolated protein competes with capacitated spermatozoa for zona pellucida binding. These data reinforce our conclusion that AWN may be one of the sperm components implicated in sperm–zona pellucida binding.

AWN-1 and AWN-2 were isolated from acid extracts of fresh ejaculated boar sperm as described [16]. Sequence analyses of HPLC-isolated peptides obtained by proteolytic digestion of native AWN-1 with endoproteinases Asp-N and Lys-C provided the neccesary overlapping peptides to derive the complete primary structure of the protein (Fig. 3). The final structure, including the disulphide bonds between Cys^9–Cys^{30} and Cys^{53}–Cys^{74} [16], was confirmed by fast atom bombardment mass spectrometric analyses of the proteolytic peptides (Fig. 3). The average molecular mass calculated for AWN-1 (Fig. 3) is 14471.2, which is close to the experimental value measured by plasma desorption mass spectrometry (14452 ± 0.1%), indicating that the amino acid sequence of AWN-1 shown in Fig. 3 corresponds to its complete primary structure.

Previous structural work showed that AWN-2 might be an N-terminal acetylated form of AWN-1. To confirm this point AWN-2 was cleaved with trypsin and the tryptic peptides analyzed by fast atom bombardment mass spectrometry. All the quasimolecular ions (M+H+) found matched to the primary structure of AWN-1 (Fig. 3), except the peptide containing the N-terminus, whose molecular mass was 42 mass units greater than the corresponding peptide of AWN-1, as expected for the presence of an N-acetyl blocking group.

A computer search for similar amino acid sequences in the MIPS data bank failed to identify any entry. However, the amino acid sequence of AWN-1 is 52.5% and 38.6% identical to the recently reported primary structures of AQN-1 [17] and AQN-3 [15] (Fig. 4), and thus identifies AWN-1/2 as a member of the boar spermadhesin protein family.

Although the three spermadhesins (AQN-1, AQN-3 and AWN) bind solubilized zona pellucida [14–17], their ligand specificity as well as the protein ligand-binding domain remain to be characterized. As with AQN-1 and AQN-3, the polypeptide region between residues 69 and 109 fulfills the general criteria of known saccharide-binding protein domains [21] since it is rich in polar and hydroxyl-containing residues usually involved in the network of hydrogen bonds formed in the protein–sugar complex. Its participation in the carbohydrate recognition of spermadhesins is currently under investigation.

It is conceivable that species-specific recognition during fertilization may have evolved either by (i) co-evolution of complementary molecules on both sperm and egg surfaces, including subtle changes in the sequence

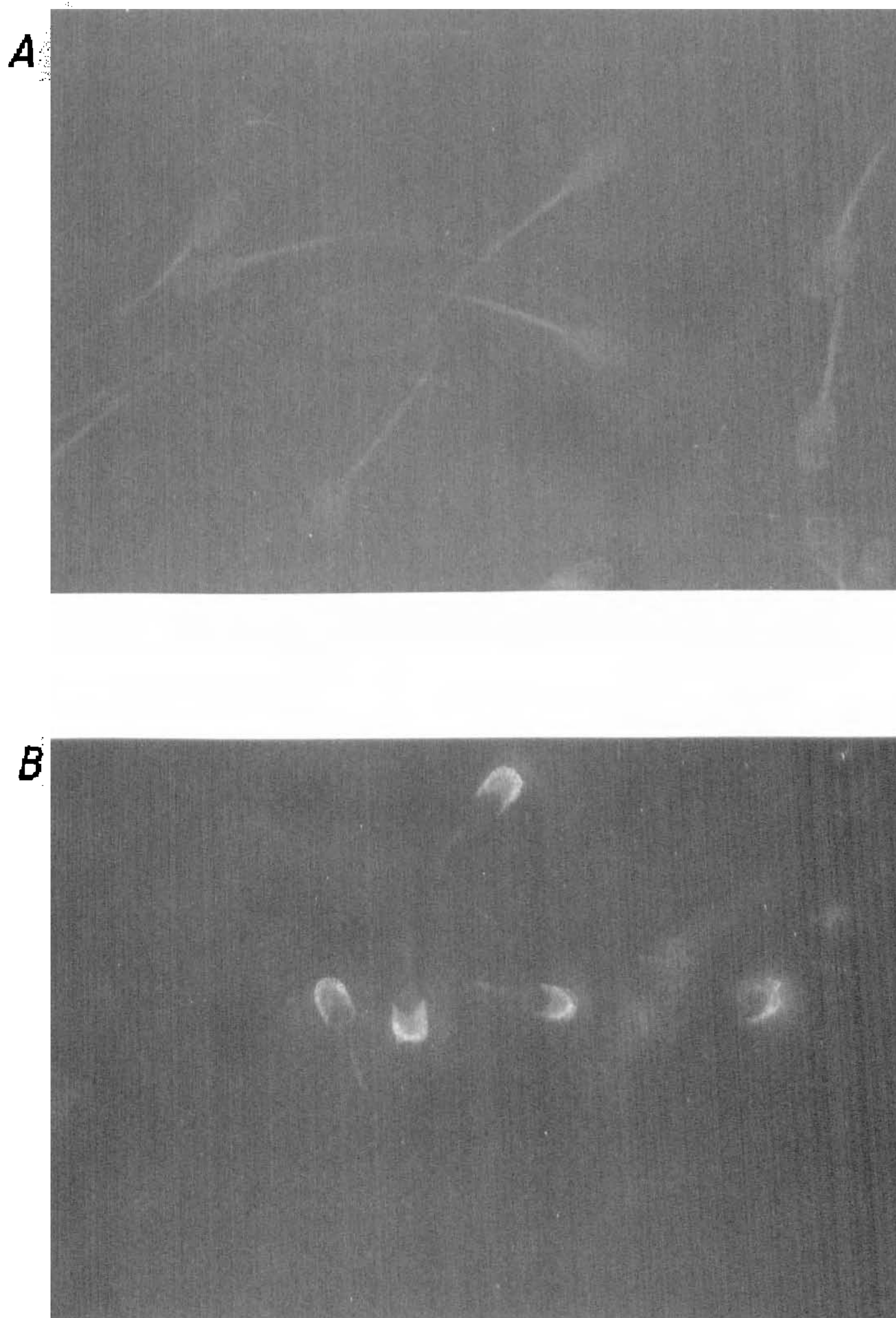
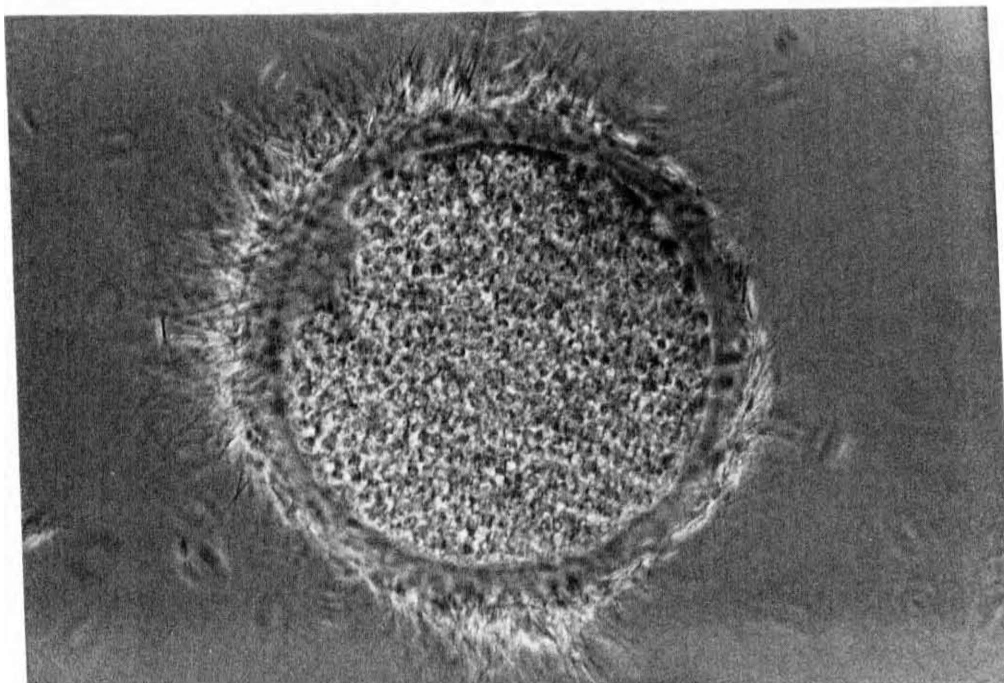


Fig. 1. Topographical localization of AWN-1 on the boar sperm surface by indirect immunofluorescence. The fixed spermatozoa were incubated with (A) pre-immunized hen's yolk total proteins, and (B) with chicken anti-AWN-1 polyclonal antibodies.

and/or conformation of the carbohydrate component on the oocyte and in its recognition site on the sperm receptor, (ii) as a consequence of utilization of different counter-receptors, or (iii) both.

A number of mammalian sperm surface components have been suggested to mediate gamete recognition [1,11,22]. Among them, the enzymes galactosyltransferase (GalTase) [12,13, 23] and α-D-mannosidase [24]

A



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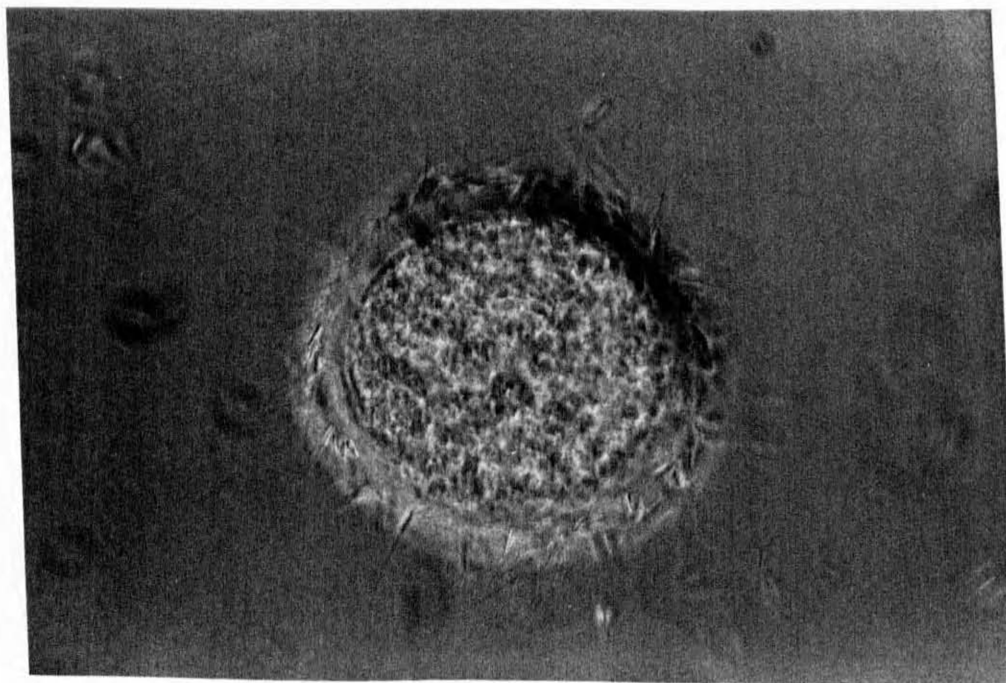

Fig. 2. Inhibition of the sperm-oocyte binding by isolated AWN-1. Washed zona pellucida-encased oocytes were incubated with (A) buffer or (B) 0.2 mg/ml isolated AWN-1, washed and then mixed with capacitated spermatozoa. The same result was obtained with AWN-2 (data not shown). Pictures were taken weith a Zeiss fluorescence microscope (magnification: 100xobjective).

have been reported to play a role in sperm–zona interaction. GalTase is thought to bind to terminal galactose residues on the zona pellucida glycoprotein 3 [25]. Since substrate specificity is essentially conserved between ho-

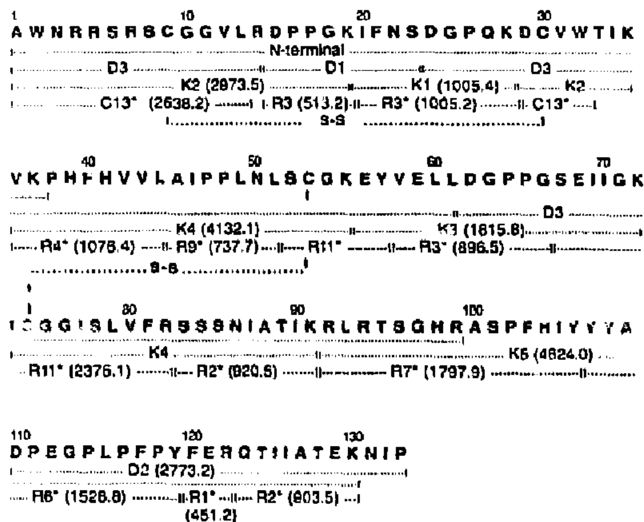

Fig. 3. The complete primary structure of AWN-1. D and K were peptides obtained by digestion of native AWN-1 with endoproteinases Asp-N and Lys-C, respectively. C* and R* denote chymotryptic and tryptic peptides from AWN-2, respectively. The numbers in brackets are the molecular mass of the peptides determined by fast atom bombardment mass spectrometry.

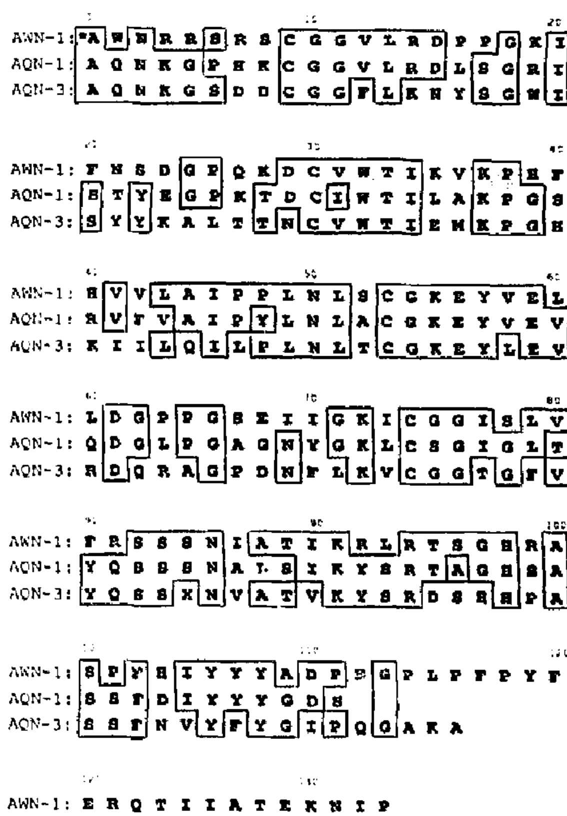

Fig. 4. Alignment of the primary structure of AWN with the known amino acid sequences of the boar spermadhesins AQN-1 [17] and AQN-3 [15]. *The primary structure of AWN-2 is identical with that of AWN-1 except that the former contains an N-terminal acetyl group [16].

mologous proteins from different species, enzymes may contribute to gamete interaction is a non-species-specific manner, as accessory adhesion molecules.

In addition to enzymes, other carbohydrate-binding proteins have been implicated in gamete recognition. Thus, Leyton and Saling [26] have shown that a 95 kDA protein localized on the mouse sperm surface binds zona pellucida glycoprotein 3, and subsequent patching of the 95 kDa protein leads to the acrosome reaction. Also in the mouse, Bleil and Wassarman [27] have identified, by cross-linking experiments, a 56 kDa sperm surface protein which has many of the properties expected for a sperm's zona pellucida recognition protein [11].

A group of low molecular weight (15–18 kDa) proteins, which possess zona pellucida-binding activity, have been found in each species so far tested [1,14,28,29] and are further candidates for species-specific gamete adhesion molecules. The paradigm molecules in this group are the rabbit sperm autoantigens (RSA) [22,30–32]. RSA-like molecules were also shown to be present in human and mouse spermatozoa [32]. In the boar, Hanqing and co- workers [28] have described similar zona pellucida- binding proteins, which show identical functional properties, cell compartimentalization and biological origin as AWN-1. Although no structural data of the rabbit or the boar proteins were presented we believe that these proteins may be related to the spermadhesin protein family.

It is tempting to speculate that spermadhesins are responsible for species-specific gamete recognition. The relative contribution of the different sperm adhesion molecules to the processes of sperm–egg recognition and binding remains to be investigated. The availability of the primary structures of the members of the spermadhesin family may enable us or others to identify homologous proteins in other species and, thus, may lead to a better understanding of the molecular basis of species-specific gamete interaction.

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