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Multiple proteins present in purified porcine sperm apical plasma membranes interact with the zona pellucida of the oocyte

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An important step in fertilization is the recognition and primary binding of the sperm cell to the zona pellucida (ZP). Primary ZP binding proteins are located at the apical plasma membrane of the sperm head. In order to exclusively study primary zona binding proteins, plasma membranes of sperm heads were isolated, highly purified and subsequently solubilized with a mild or a strong solubilization procedure. Native, highly purified ZP ghosts were used as the binding substrate for solubilized sperm plasma membrane proteins, and a proteomic approach was employed to identify ZP binding proteins. Two-dimensional gel electrophoresis of ZP fragments with bound sperm proteins showed very reproducibly 24 sperm protein spots to be associated to the zona ghosts after mild plasma membrane solubilization whereas only three protein spots were detected after strong plasma membrane solubilization. This indicates the involvement of multiple sperm proteins in ZP binding. The three persistently bound proteins were identified by a tandem mass spectrometry as isoforms of AQN-3 and probably represent the main sperm protein involved in ZP binding. P47, fertilin β and peroxiredoxin 5 were also conclusively identified. None of the identified proteins has a known acrosomal origin, which further indicated that there was no sample contamination with secondary ZP binding proteins from the acrosomal matrix. In this study, we showed and identified multiple zona binding proteins involved in primary sperm–zona binding. Although we were not able to identify all of the proteins involved, this is a first step in understanding the event of primary sperm–zona interactions and the relevance of this for fertilization is discussed.

Key words: sperm zona binding; plasma membrane; spermadhesin; fertilin; lactadherin

Introduction

An important step in fertilization is the recognition and primary binding of the sperm cell to the zona pellucida (ZP), which is the extracellular coat of the oocyte. Directly after ejaculation, the sperm cell is not able to bind the ZP. The capability for zona binding is only achieved after activation in the female genital tract in a process called capacitation. The ZP receptors involved in the primary binding are exclusively located at the apical plasma membrane of the sperm head and zona binding is thought to be a species-specific event (Yanagimachi, 1994). This binding event serves to not only fix the sperm cell to the ZP but also initiates the signaling cascade that finally leads to the acrosome reaction. During the acrosome reaction, the apical plasma membrane fuses with the outer acrosomal membrane, thereby releasing the acrosomal contents and this enables the sperm cell to penetrate the ZP. During this penetration process, the sperm cell stays attached to the ZP by an interaction between proteins located in the acrosomal matrix and the ZP (termed secondary binding).

Our present understanding is that the porcine ZP consists of three glycoproteins, referred to as ZP2, ZPB and ZP3 [nomenclature according to Hughes and Barratt (1999) and Conner and Hughes, (2003)]. In pig, both ZPB and ZP3 are thought to be directly involved in sperm binding (Yurewicz *et al.*, 1998) and it is known that the oligosaccharide chains of the heterogeneously glycosylated ZP proteins play an important role in sperm binding (Nakona and Yonezawa, 2001). *In vivo*, the ZP proteins form a coherent network, which is probably necessary for sperm binding activity (Yurewicz *et al.*, 1998). However, less is known about the sperm candidates involved in this interaction, although numerous candidates such as fertilins and sperm adhesions have been proposed in previous studies (Topfer-Petersen *et al.*, 1998; Brewis and Wong, 1999; Primakoff and Myles, 2002). β1,4-Galactosyltranserase-I is one of the proposed candidates and has been studied most extensively. It has been shown to bind to the ZP, to be located at the apical plasma membrane and to evoke the acrosome reaction following ZP binding (Miller *et al.*, 1992). Hence, it seems to be the perfect candidate for a sperm receptor, but a confounding result is that β1,4-galactosyltranserase-I knock out mice are still fertile (Lu and Shur, 1997). This finding may indicate that β1,4-galactosyltranserase-I is not involved in ZP binding or that more than one protein is involved. Other postulated proteins, like

sp56 and sp38, are located in the acrosome and must therefore play a role in secondary instead of primary binding (Mori *et al.*, 1995; Kim *et al.*, 2001). Overall, the definitive identity of the sperm receptor has remained elusive. In particular, the question remains as to whether there is just one sperm receptor involved in the binding to the ZP or whether multiple (low affinity) proteins are involved in the binding to the multivalent ZP as Thaler and Cardullo (1996b) have suggested. In this study, we examined this possibility.

Here, we describe a direct primary ZP-binding assay in which unmodified sperm plasma membrane proteins and native ZP fragments were used to approach the biological conditions as much as possible. New to this direct approach was the isolation and purification of the sperm head (apical) plasma membranes (no detection of secondary ZP binding) and the use of highly purified zona ghosts (that retained their native quaternary zona protein matrix structure). Flesch *et al.* (2001) have used a similar approach to isolate ZP binding proteins on a column of ZP ghosts using high salt elution; in this study, we loaded the ZP ghosts with all bound sperm surface proteins directly on gel. Although others have used solubilized zona proteins (Ensslin *et al.*, 1995, 1998; Lea *et al.*, 2001), we have preferred to use native zona matrices as a binding substrate to identify primary ZP binding proteins. To support this approach, there is good evidence in studies using isolated plasma membrane vesicles from porcine sperm that these have affinity for the hetero-oligomerized form of ZPB and ZP3 (which is actually the physiological texture of the intact ZP). However, no such affinity was observed for the solubilized forms of ZPB or ZP3 (Yurewicz *et al.*, 1998).

The sperm plasma membrane proteins were solubilized (with mild or strong detergents), and after co-incubation, the ZP fragments with bound sperm head plasma membrane proteins were isolated. The sperm plasma membrane proteins, although present in low amounts, could be identified using highly sensitive techniques (capillary liquid chromatography combined with tandem mass spectrometry). This report presents data showing that multiple sperm proteins are involved in binding to the ZP after mild plasma membrane solubilization. In the presence of strong detergent, only three protein spots (isoforms of the same protein) were bound to the ZP. In contrast to secondary binding, the primary binding to the ZP in our direct binding assay was calcium independent. This is the first time that multiple zona binding proteins have been directly identified in the same study. Finally, the functional implications of this data are discussed.

Materials and Methods

Semen preparation and incubation

Semen was obtained from the Cooperative Centre for Artificial Insemination in Pigs 'Utrecht en de Hollanden' (Bunnik, The Netherlands). Semen was filtered through gauze to remove gelatinous material. Sperm cells were washed on a discontinuous Percoll® (Amersham Biosciences, Uppsala, Sweden) gradient as described (Flesch *et al.*, 1998). To induce capacitation, Percoll® washed sperm cells were incubated in a medium containing 15 mM bicarbonate equilibrated at 5% CO_2 as described previously (Flesch *et al.*, 1999). The sperm cell suspensions were incubated at 38.5°C in a cell incubator for 3–4 h.

Isolation of apical plasma membranes

Plasma membranes from the sperm head were isolated as described by Flesch *et al.* (1998). The plasma membranes were resuspended in Hepes buffered saline (HBS: 5 mM Hepes, 2.7 mM KCl, 146 mM NaCl, pH 7.4) containing a protease inhibitor cocktail (Complete, Roche, Mannheim, Germany), frozen in liquid nitrogen and stored at −20°C. The amount of protein was determined by the method of Lowry *et al.* (1951), using bovine serum albumin as a standard. For the determination of the purity of the isolated plasma membranes, the marker enzymes alkaline phosphatase and acrosin were used as described previously (Soucek and Vary, 1984; Flesch *et al.*, 2001). The purity of the apical plasma membranes used in the binding assay was checked with enzyme assays using well-established techniques in our group [see Flesch *et al.* (1998) for full validation of this method]. Alkaline phosphatase was used as a marker for the plasma membrane and proved to be 17.9 ± 2.2 (SEM) times enriched in plasma membrane preparations ($n = 18$). For the determination of acrosomal contamination, the marker enzyme acrosin was used. The apical plasma membranes were highly purified since only 2.2 ± 0.8 (SEM)% acrosin activity was detected ($n = 18$) compared with whole sperm samples.

Isolation of ZP fragments

ZP fragments were isolated as described previously (Flesch *et al.*, 2001) from ovaries collected in the slaughterhouse from slaughtered pigs (material kindly provided by 'Hendrix' vlees Druten BV', Druten, The Netherlands). The ZP fragments were frozen in liquid nitrogen and stored at −20°C.

The amount of protein was determined according to Bohlen *et al.* (1974). The purity was checked by two-dimensional (2D) gel-electrophoresis (as described later) and with lectins as described earlier (Flesch *et al.*, 2001). The lectins WGA-FITC (marker for the ZP) and LPA-FITC (marker for oocyte cytoplasm and cumulus cells) were used to check the purity of the ZP fragments. No oocyte contamination was detected with LPA-FITC whereas the ZP fragments were clearly stained with WGA-FITC [for staining patterns see Flesch *et al.* (1998)]. Furthermore, hardly any cellular protein contamination of ZP fragments was detected with 2D electrophoresis (Fig. 1A), whereas the typical components of the ZP appear as distinctive trains of protein spots. This is caused by their heavy and heterogeneous glycosylation, and previous work has clearly identified these proteins as zona proteins (Lefievre *et al.*, 2003).

Isolation of ZP binding proteins

For the mild solubilization procedure, the sperm plasma membrane proteins were solubilized with 0.05% (v/v) Tween-20 and diluted with HBS (final concentration 1 mg protein/ml). This condition was maintained during the zona-binding assay. The mild solubilization method was used to minimize the risk of denaturation of membrane proteins during solubilization and the binding assay. ZP fragments (100 μg protein) were added to this solubilized sperm plasma membrane proteins (1 mg protein) and this suspension was incubated at 4°C under constant rotation. After 3–4 h, the ZP fragments with bound sperm plasma membrane proteins were spun down (10 min, 2000 *g*, 4°C) and washed twice with HBS containing 0.05% (v/v) Tween-20 to remove unbound sperm proteins. Incubations at room temperature and 37°C gave similar results (data not shown); however, since protease activity is increased at elevated temperatures, we performed further experiments at 4°C.

For a strong solubilization, 60 μl of a 1% sodium dodecyl sulfate (SDS) solution (1% SDS in HBS containing protease inhibitors) was added to 1 mg of sperm plasma membrane proteins. After 30 min, 120 μl of 5% Nonidet-P40 in HBS with protease inhibitors was added and after another 30 min HBS containing protease inhibitors was added up to a total volume of 1 ml. Subsequently, the solubilized plasma membranes were centrifuged (30 min, 100 000 *g*, 4°C). After strong solubilization, the sperm membrane protein fraction was diluted in HBS containing protease inhibitors to final concentrations of 0.06% SDS and 0.6% for Nonidet-P40. ZP binding was performed with this detergent buffer as described above. The strong solubilization method was used to ensure complete membrane solubilization with the risk of protein denaturation.

For the experiments with ethylene-glycol tetra-acetic acid (EGTA) or Ca^{2+}, 10 mM EGTA or 2.5 mM $CaCl_2$, respectively, was added to the incubation and wash buffer.

The ZP fragments with bound proteins were solubilized in rehydration buffer [7 M urea, 2 M thiourea, 4% (w/v) 3-[(3-Cholamidopropyl)-dimethylammonio]-propane-sulfonate (CHAPS), 18 mM dithiothreitol (DTT), 2% (v/v) IPG buffer (pH 3–10) (Amersham Biosciences) and a trace of bromophenol blue]. Proteins of ZP fragments with bound sperm proteins were subjected to 1D SDS–PAGE analysis. Proteins were separated on 11% gel. Subsequently, the proteins were visualized using silver staining according to Blum *et al.* (1987).

For the 2D electrophoresis, ZP fragments with bound sperm proteins were solubilized in rehydration buffer. In the first dimension, proteins were subjected

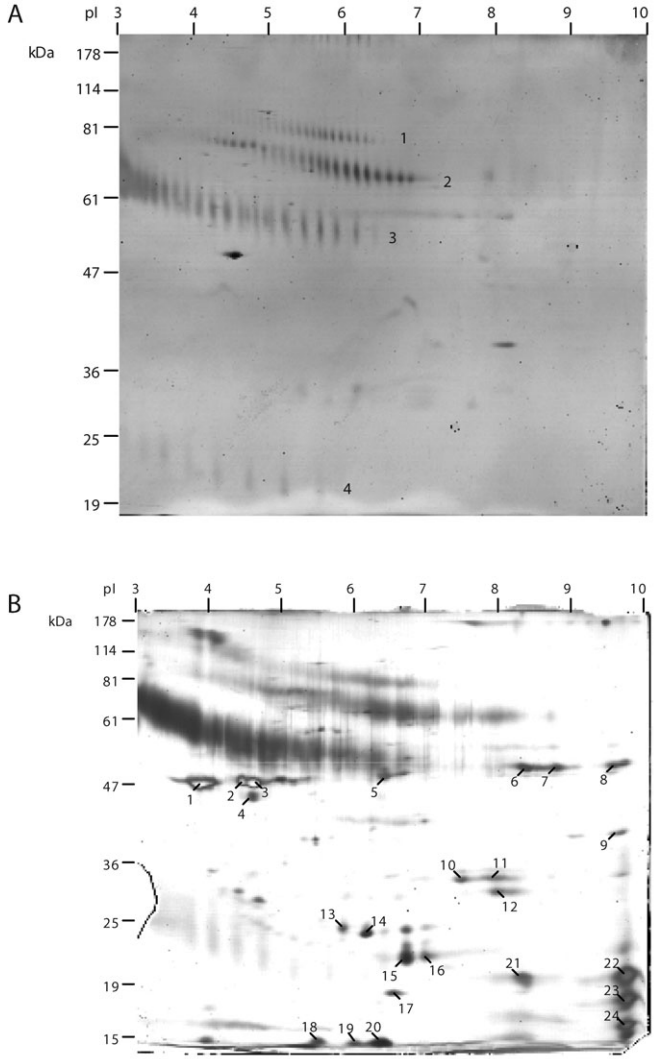

Figure 1: 2D gel of the proteins of the isolated porcine ZP (**A**) and the ZP fragments with bound sperm apical plasma membrane proteins after mild solubilization (**B**) (A) The ZP consists of at least three glycoproteins, provisionally identified as ZP2, ZP3 and ZPB: (1) ZP2 or ZP1, (2) cleavage product of ZP2, (3) ZP3 and ZPB and (4) cleavage product of ZP2 (Lefievre *et al.*, 2003). Hardly any contamination of cumulus cells or oocyte cytoplasm can be detected in the isolated ZP fragments. (B) After incubation of the ZP fragments with sperm plasma membrane proteins, the ZP fragments were washed to remove unbound proteins. The fragments with bound sperm proteins were solubilized and separated by 2D electrophoresis. Comparison of this gel with Fig. 1A (ZP fragments that were not incubated with sperm proteins) showed a number of additional spots which corresponded to the sperm proteins bound to the ZP. A set of 24 sperm protein spots was bound to the ZP (labeled with numbers)

to iso-electric focusing on linear IPG strips pH 3–10 18 cm (Amersham Biosciences) with a Multiphor II system [Amersham Biosciences; 150 V (30 min), 300 V (1 h), 1500 V (1 h) and 3000 V (8 h)].

The focused strip was rotated in 65 mM DTT in equilibration buffer [50 mM Tris/HCl (from stock 1.5 M Tris/HCl, pH 8.8), 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, 0.005% (w/v) bromophenol blue; 10 ml for 15 min] and subsequently in 135 mM iodoacetamide in equilibration buffer (10 ml for 15 min).

For the second dimension, 11% SDS–PAGE was performed and molecular weight markers (Benchmark prestained protein ladder, Invitrogen) were also run on each gel. The 2D gels were silver stained according to Blum *et al.* (1987) with small modifications. The gels were fixed in 40% (v/v) ethanol with 10% (v/v) acetic acid, washed twice with 30% (v/v) ethanol and once with distilled water. The gels were sensitized for 1 min in 0.02% (w/v) sodium thiosulfate and subsequently washed three times with water. The gels were incubated in a cold (4°C) 0.1% (w/v) silver nitrate solution and subsequently washed three times with water. The gels were developed in 1% (w/v) sodium carbonate, 0.017% (v/v) formaldehyde solution, and the staining was terminated with 5% (v/v) acetic acid.

Identification of ZP binding proteins

Spots that were consistently present (at least in 8 of 10 gels) were considered to be of interest and were excised and digested with trypsin. For the in-gel digestion, excised spots were washed with water and destained in a solution containing 30 mM potassium ferricyanide and 100 mM thiosulfate. The spots were washed with water and dehydrated with acetonitrile (ACN). Subsequently, they were reduced with DTT (6.5 mM DTT in 50 mM ammonium bicarbonate, pH 8.5), dehydrated with ACN and alkylated (54 mM iodo-acetamide in 50 mM ammonium bicarbonate, pH 8.5). After alkylation, the gel plugs were washed with 50 mM ammonium bicarbonate and dehydrated with ACN and this was repeated a total of three times. After the last ACN step, the gel plugs were dried in air and swollen with 12 μl 10 ng/μl trypsin (modified sequencing grade, Roche, Mannheim, Germany) in 50 mM ammonium bicarbonate on ice. After 30 min on ice, the gel pieces were digested overnight at 37°C. Digestion was stopped with 1% (v/v) trifluoroacetic acid (TFA) and the peptides were removed from the gel plug and stored in Eppendorf tubes at −20°C.

The peptides (in 50 mM ammonium bicarbonate with 0.1% (v/v) TFA; injection volume 5 μλ) were separated using capillary liquid chromatography (Waters Ltd, Elstree, Hertford, UK) and analyzed with a nano-electrospray ionization (ESI) quadruple time of flight (Q-Tof) tandem mass spectrometer (MS/MS) (Q-Tof Ultima GLOBAL, Micromass UK Ltd, Manchester, UK). The peptide mixture was trapped on the precolumn [PepMap C18 column 300 μm i.d. × 5 mm, Dionex (UK) Ltd] with 93% solvent A [0.1% (v/v) formic acid] and 7% solvent B [ACN with 0.1% (v/v) formic acid]. The peptides were gradient eluted (7–90% B) on a PepMap C18 column (75 μm i.d. × 15 cm; Cat. No. 160396, Dionex (UK) Ltd) over 45 min into the mass spectrometer. The raw data were processed using MassLynx 3.5 software (Micromass). MS/MS spectra were matched to sequences in two protein databases (SwissProt and the NCBI non-identical protein sequence database) using Mascot software (Matrix Science Ltd, London, UK). In these searches, a static modification of cysteine (carbamidomethylation) and a differential modification of methionine (oxidation) were selected. Matching of a peptide to a protein was done by the evaluation of the quality of the raw MS/MS data. Finally, each of the peptides was used to BLAST search to confirm that the protein identified by Mascot was the only relevant match in the non-redundant protein database for a particular peptide sequence (Kinter and Sherman, 2000).

Results

Zona binding of sperm proteins

The zona ghosts were incubated with mildly (0.05% Tween) or strongly [SDS and Nonidet-P40 (NP-40)] solubilized plasma membrane proteins from the sperm head and subsequently washed to remove unbound proteins. The ZP fragments with bound sperm proteins were first analyzed with 1D SDS–PAGE. Unfortunately, the ZP proteins showed severe smearing all over the 1D gel, which made detection of bound sperm proteins impossible. Therefore, the washed zona ghosts (containing the immobilized primary ZP receptors from the sperm head plasma membrane) were subjected to 2D electrophoresis (Figs 1B and 2B). The 2D gels depict ZP proteins (protein trains) and the bound sperm plasma membrane proteins. After the mild solubilization procedure, a set of 24 proteins showed affinity to the purified zona ghosts. These proteins are indicated and numbered in Fig. 1B. This experiment was repeated a number of times ($n = 10$) and 24 protein spots were consistently observed. When the strong solubilization procedure was applied, it was more difficult to detect the binding proteins. The amount and number of proteins bound to the ZP was reduced, probably by the presence of strong detergent. To be able to detect the binding proteins, it was necessary

to stain the 2D gels for a longer time. When the same staining procedure was applied to ZP fragments without bound proteins, more contaminated spots were detected (Fig. 2A). However, the amount of contamination is still very small compared with the total amount of ZP and it is unlikely that this small amount of contamination disturbs the binding assay. After strong solubilization in the presence of SDS and NP40, only three spots showed affinity to the ZP (Fig. 2B). These spots were indicated with A, B and C in Fig. 2B. These spots were also detected with the mild solubilization procedure (spot 22, 23 and 24). This experiment was repeated five times and the spots were detected in each experiment.

Identification of zona binding proteins

The resulting ZP binding sperm protein spots were cut out from the gel and digested with trypsin. Resulting peptides were subjected to Q-Tof tandem mass spectrometry (Q-Tof MS/MS) to enable amino acid microsequencing of peptides (for identification and sequences of matching peptides in spots 1–24 and spot A–C, Table 1). Not all proteins could be identified which can be attributed to the fact that the sequence database for the pig is incomplete, or the amount of protein in the spot is too low for adequate identification (note that we used silver staining for spot detection). Some of the identified proteins, such as fertilin β (Cho *et al.*, 1998), AQN-3 (Calvete *et al.*, 1996) and P47 (Ensslin *et al.*, 1998), are known sperm plasma membrane proteins and their possible involvement in ZP binding has been previously described. Other proteins like peroxiredoxin 5 have not been described in sperm cells before.

Four peptides present in spot number 9 were identified as a K^{+-} channel. However, the four peptides were identified as different K^+ channels. This is probably due to the fact that this porcine K^+ channel is not yet available in the pig proteome database but has similarity with other mammalian K^+ channels. Similarly, two different phosphatase hits were found for spot number 14 and two different carbonyl reductase hits for spot number 16.

Spot numbers 10, 11, 19 and 20 were all identified as SP32 precursor (pre SP32). None of the pre SP32 spots 10, 11, 19, 20 has the predicted correct molecular weight (61 kDa) and pI (4.8) of the complete

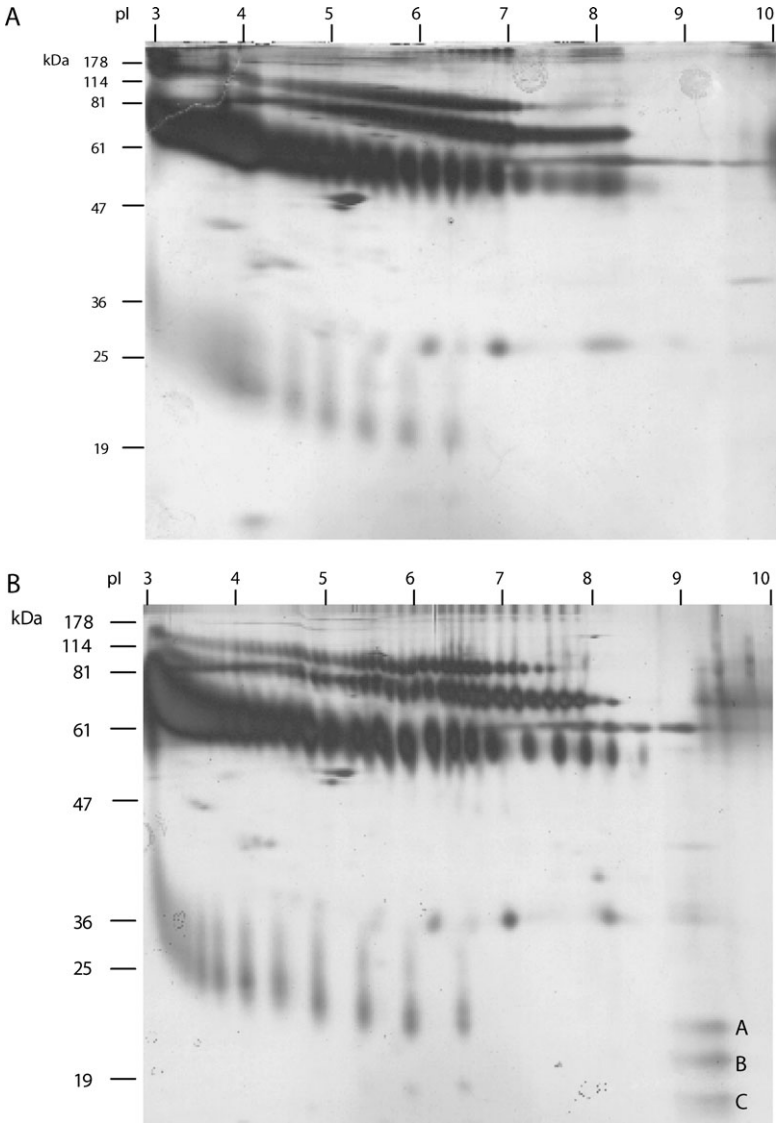

Figure 2: 2D gel of the proteins of the isolated porcine ZP (**A**) and 2D gel of ZP fragments with bound sperm apical plasma membrane proteins after strong solubilization (**B**) After incubation of the ZP fragments with sperm plasma membrane proteins, the ZP fragments were washed to remove unbound proteins. The fragments with bound sperm proteins were solubilized and separated with 2D electrophoresis. Comparison of gel 2B to gel 2A (ZP fragments that were not incubated with sperm proteins) showed three additional spots, which corresponded to the sperm proteins, bound to the ZP

Table 1: MS/MS identification of ZP binding sperm proteins

Spot number	Protein assigned from database search of sequenced peptides	Peptides identified and used for searching			Accession number	Mascot score	Comment
1	Fertilin beta	DGTVCGENKVCR ICVTVDYPYNDR	MWVKDGTVCGENK TDESGACGLTASGYIR	CIDSSYLNYDCTAEK ICVTVDYPYNDRNSEK	28564477	204	[c]
5	P47	WAPELAR LVPIICHR VTGIITQGAR QITASSFYR VTGVVTQGASR RVTGIITQGAR	GDVFTEYICK NPWIQVNLLR NMFETPFLTR IFMGNLDNSGLK IFPGNLDNNSHK KNMFETPFLTR	FQFIQGAEESGDK AGIVNAWTASNYDR KFQFIQGAEESGDK VNLFEVPLEVQYVR FELLGCELSGCAEPLGLK FNAWTAQSNSASEWLQIDLGSQR	7459686	725	[b]
8	P47	LVPIICHR VTGIITQGAR VTGVVTQGASR GDVFTEYICK NMFETPFLTR	IFMGNLDNSGLK DFGHIQYVAAYK FQFIQGAEESGDK KFQFIQGAEESGDK	VNLFEVPLEVQYVR QITASSFYR RVTGIITQGAR AGIVNAWTASNYDR	7459686	418	[c]
9	Potassium channel	IIINVGGTRHETY PSMLS——-KLLLG	NSHMVSAQIRCK	RLLIKYASNLDSGVPAR	5542588; 28373976; 17942553 14719795	[a]	[d]
10	Pre SP32	FYGLDLYGGLR	LEQCHSETNLQR		1082952	82	[c]
11	Pre SP32	SEDLVLR	FYGLDLYGGLR	LEQCHSETNLQR	1082952	104	[c]
14	Phosphatase	AVIPQGAAESDGRIHK	ETHGGEDVAVF		22218629, 13786807	[a]	[d]
16	Carbonyl reductase	VVAVTRTNSDLVSLAK	GIGFAITRDLCR		1827692, 27066006	[a]	[d]
17	Peroxiredoxin 5	VNLAELFK LLADPTGAFGK FSMVIEDGIVK	THLPGFVEQAEALK GVLFGVPGAFTPGCSK	VGDAIPSVVVFEGEPEKK ETDLLLDDSLVSLFGNR	10305336	305	[b]
19	Pre SP32	LEQCHSETNLQR	HLAACSLCDFCSLK		1082952	64	[c]
20	Pre SP32	SVCDSLGR LEQCHSETNLQR	AWQYLEDETLGFGK HLAACSLCDFCSLK	TPFISPLLASQSMSIGTQIGTLK	1082952	215	[c]
22 and A	AQN-3	GSDDCGGFLK	DSHHPASSFNVYFYGIPQGAK		543109	98	[b]
23 and B	AQN-3	EYLEVR	GSDDCGGFLK	DSHHPASSFNVYFYGIPQGAK	543109	129	[b]
24 and C	AQN-3	EYLEVR	IILQILPLNLTCGK		543109	54	[b]

The zona binding proteins from the mild solubilized sperm membranes that could be identified by MS/MS are listed. Note that the sperm protein spots 22–24 also bound to the zona ghosts after complete solubilization in SDS and NP40 (denoted by A, B and C).
Proteins were cut out from silver-stained 2D gels, digested with trypsin and microsequenced by tandem mass spectrometry (MS/MS). To assign an excised spot to a particular protein, at least two peptides with validated MS/MS data from a single spot matched the database sequence for the assigned protein. The amino acid sequences of identified peptides and the Mascot scores are indicated for each identified protein.
[a]The different forms of potassium channels, phosphatases and carbonyl reductases identified within one protein spot are detailed in the discussion section.
[b]Conclusive protein identification and protein mass and pI on gel were the same as previously reported.
[c]Conclusive identification by MS/MS data with lower protein mass than expected so possibly a fragment of this protein.
[d]Provisional identification by MS/MS data where the identified peptides are restricted to the same protein family.

pre SP32. Possibly, these spots are cleavage of the original protein as all of the spots are much smaller than predicted. In fact, they all showed homology to a putative signaling domain of this precursor part (Baba *et al.*, 1994) whereas no homologies with the mature part of the protein were detected.

Spots 22, 23 and 24 all were identified as AQN-3. The molecular weight and pI of spot 24 corresponds with the theoretical values for the secretory protein AQN-3 which has a molecular weight of 12.8 kDa and pI 8.9 (Evans, 2001). None of the pre SP32 spots 10, 11, 19 and 20 has the predicted correct molecular weight (61 kDa) and pI (4.8) of the complete pre SP32. Possibly, these spots are cleavage products of the original protein as all of the spots are much smaller than predicted.

Examination of binding specificity

To show that only a specific subset of sperm plasma membrane proteins is involved in the binding to the ZP, we separated a total plasma membrane protein extract using 2D electrophoresis (Fig. 3). This gel shows that ~200–300 proteins are detected in the plasma membrane of the sperm head with this method. Thus, only a relatively small number (24 or 3, dependent on the solubilization procedure) of protein spots from the sperm head plasma membrane had affinity for the ZP (indicating that the binding was selective). Moreover, these proteins remained bound during the washing procedure, in which Tween-20 or SDS/NP40 was used to prevent non-specific binding.

Since the binding of some secondary binding proteins is calcium-dependent (Mori *et al.*, 1993; Geng *et al.*, 1997), we determined whether the primary binding is calcium dependent as well. Therefore, the experiment was repeated in the presence and absence of calcium (for this experiment, the mild solubilization procedure was used). ZP fragments were incubated and washed with incubation medium either containing 2.5 mM Ca^{2+} or 10 mM EGTA. Figure 4 shows 2D gels of ZP fragments with bound plasma membrane proteins from the sperm head in the presence (Fig. 4A) and absence (Fig. 4B) of Ca^{2+}. The protein spots that emerged were similar (qualitatively as well as quantitatively) and thus primary zona binding

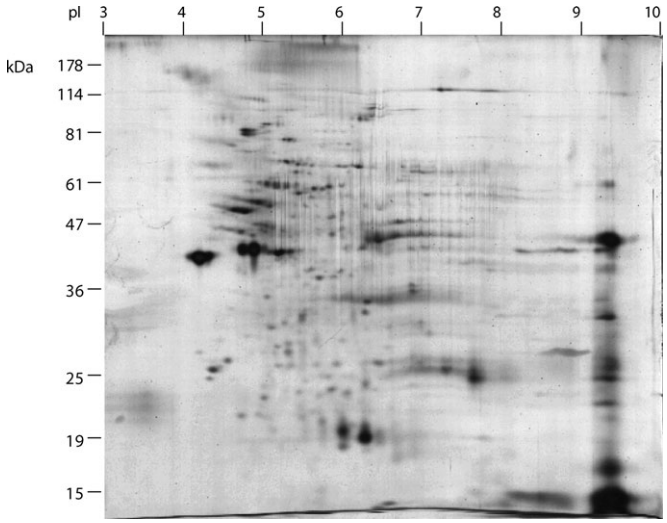

Figure 3: 2D gel of sperm apical plasma membrane proteins Apical plasma membrane proteins were solubilized and separated with 2D electrophoresis. This revealed ~300 spots. Note that only a minor number of the total amount of proteins present in the apical plasma membrane appears to have affinity to the ZP (Figs. 1B and 2B)

appears at least under the binding assay conditions to be Ca^{2+} independent.

Discussion

The primary binding of the sperm cell to the ZP of the oocyte is an essential step in the fertilization process. The sperm cell binds with the apical tip of the head to the ZP of the oocyte. This binding triggers the acrosome reaction, which in turn enables the sperm cell to penetrate the ZP and reach the oocyte. During this penetration process, the secondary binding between the intra-acrosomal proteins of the sperm and the ZP of the oocyte takes place (Yanagimachi, 1994). Over the past few decades, a lot of progress has been made on the side of the ZP but the complementary sperm proteins involved in this binding process remain the subject of much debate.

The confusion in the literature is due to a large extent to limitations in many of the approaches to identify ZP binding proteins. First, a number of proteins postulated to be involved in this binding turned out to be located in the acrosome (Mori *et al.*, 1995; Kim *et al.*, 2001) and must therefore play a role in secondary binding. The identification of acrosomal proteins is probably related to the fact that whole sperm lysates were used (i.e. secondary zona binding proteins were also allowed to bind to the ZP). Secondly, in many assays, ZP binding was only demonstrated indirectly by the addition of a competitive ligand [e.g. isolated putative ZP binding proteins, or antibodies raised against the putative zona binding protein or a competitive ligand that blocks ZP interaction (Talbot *et al.*, 2003)] steps that interfere in sperm–zona interaction. The problem with these indirect data is that they do not provide information as to how the ZP interaction is inhibited. It may either be directly by masking/competing with the ZP binding protein or indirectly by steric hindrance (e.g. by capping) of another protein not recognized by the antibody/ligand. Thirdly, many of the direct ZP-binding assays have made use of solubilized ZP material (Ensslin *et al.*, 1995, 1998; Lea *et al.*, 2001). Solubilization leads to loss of the quaternary structure of the polymerized ZP matrix with the potential risk of loss of bioactivity of the zona proteins (Yurewicz *et al.*, 1998), and the solubilization method may induce protein denaturation. Finally, the studies have all used focused approaches that concentrated on one particular protein rather than an approach that might identify more than one binding protein. Taken together, due to the limitations of these approaches definitive identification of sperm receptor(s) on the sperm plasma membrane remains to be elucidated.

We have designed a direct binding assay using biochemical purification approaches to detect and identify primary ZP binding proteins from porcine sperm. For this purpose, we simply isolated the two players involved in this primary ZP binding: (i) the apical plasma membrane of the porcine sperm head (where the primary ZP receptors are localized) and (ii) porcine zona fragments with the native quaternary structure of the ZP (the specific physiological substrate for primary ZP binding). Two solubilization methods were applied: a mild solubilization procedure in which 0.05% Tween-20 was added to the sperm plasma membranes and a strong solubilization method in which the plasma membranes were solubilized with SDS and NP40. The membrane lysates were brought together with the ZP fragments and following co-incubation unbound sperm proteins were removed. The ZP proteins together with bound sperm proteins were subjected to 2D electrophoresis. Finally, the immobilized sperm protein spots

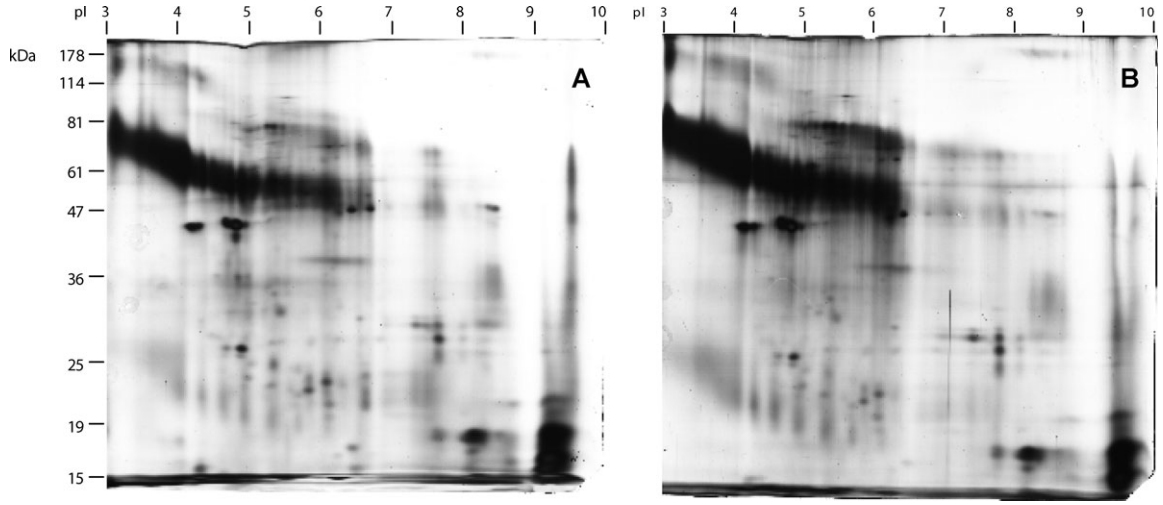

Figure 4: Primary binding of sperm proteins to the ZP appears to be Ca^{2+}-independent The sperm–ZP-binding assay was performed in the presence and absence of calcium. All ZP binding spots present in the 2D gel of ZP fragments incubated with sperm proteins in the presence of 2.5 mM $CaCl_2$ (**A**) could also be detected in the equivalent experiment, where the ZP fragments were incubated with sperm proteins in calcium-free medium containing 10 mM EGTA (**B**)

were analyzed by nano-LC-MS/MS peptide microsequencing. This fully direct approach clearly showed very reproducibly that not one but a set of 24 sperm protein spots were interacting with ZP when the mild solubilization was used, whereas three protein spots were interacting using the strong solubilization method (three isoforms of AQN3). We must note here that oviduct-specific glyocproteins (OGPs) will coat the ZP of the ovulated oocyte in the oviduct and that those peripheral zona OGPs facilitate sperm–zona adhesion in a ZP3 independent manner (Rodeheffer and Shur, 2004). With the assay used in the present study, sperm proteins involved in OGP mediated zona binding will not be detected, as the zona ghosts were prepared from ovarian material.

We also attempted to identify sperm membrane proteins with affinity for the ZP using a more traditional protein handling approach. Protein bands cut from 1D SDS–PAGE gels were trypsinized for MS identification, but we were only able to identify ZP derived peptides due to their relatively large abundance in such protein bands (data not shown). We anticipate that similar results would result with most proteomic approaches due to issues of dynamic range. The relatively large amount of ZP proteins present in the glycosylation trains over the 2D gels may well be hiding sperm–zona binding proteins. To overcome this, sperm surface proteins could be biotinylated before performing the ZP-binding assay. This allows visualization of eventual cryptive ZP binding proteins on a western blot. However, the biotinylation may well-introduce alterations in the ZP affinity of proteins as the addition of biotin groups could very well take place on the ZP binding domains. Furthermore, this approach does not allow protein identification as high amounts of antibodies and probably zona proteins still make the identification of a low amount of ZP binding proteins improbable. At any rate, it is possible that cryptic ZP binding proteins are not identified in this study. This is a limitation of our approach, but we believe that this method has been well suited to the direct identification of multiple zona binding proteins from purified apical sperm plasma membranes. Such hidden proteins could be visualized on a western blot.

Although *in vivo* capacitation is required for ZP binding to take place (Flesch *et al.*, 2001), in this assay not only the plasma membrane proteins from capacitated but also those from uncapacitated sperm cells were shown to bind to the ZP (data not shown). In contrast to the assay of Flesch *et al.* (2001), we have not eluted the ZP bound sperm proteins with high salt, but directly processed the ZP ghosts with all bound sperm proteins for gel electrophoresis. This different approach may explain the discrepancies with the observations made by Flesch *et al.* (2001). For instance, Savage *et al.* (1993) and Strom *et al.* (2000) reported that high salt concentrations might alter sperm binding properties of the ZP. The sperm plasma membrane proteins were solubilized before they contacted the ZP (otherwise all sperm plasma membrane proteins would have been immobilized to the ZP. In uncapacitated sperm cells, specific peripheral proteins inhibit sperm capacitation as well as zona binding (Florman and First, 1988). These so-called decapacitation factors are likely to be solubilized from the primary zona binding proteins and thus do not prevent the interaction anymore. The fact that proteins derived from uncapacitated apical sperm plasma membranes are able to bind to the ZP indicates that this binding is probably not dependent on post-translational modifications, like tyrosine phosphorylation, that occur during capacitation.

Before discussing the identity of isolated proteins, it is relevant to mention that we did not detect any secondary zona binding proteins. Furthermore, it is claimed that these secondary binding proteins (sp38 and proacrosin) bind in a calcium-dependent way to the ZP. In contrast, in our assay, primary zona binding appears to be calcium independent.

It was remarkable that most ZP binding proteins had a high pI value, especially when compared with the relatively acidic ZP glycoproteins. This suggests that electrostatic interactions may play an important role in this binding event. However, the finding that many basic proteins in the sperm head plasma membrane were not able to interact with the ZP ghosts indicates that electrostatic interactions are not exclusively responsible for primary sperm–zona binding. We should also again emphasize that in part this may be due to the fact that certain sperm proteins may run into the same position as the bulk ZP proteins in the 2D gels. Further to this, we would like to mention that this study provides preliminary work and identified multiple putative ZP binding protein candidates on the surface of porcine sperm. Detailed analysis of the type of ZP interaction remains to be elucidated.

Mild membrane solubilization

With the mild solubilization method, three AQN-3 were identified. This finding confirms and extends the work of Calvete *et al.* (1996) who have previously shown that AQN-3 was able to bind to the ZP. AQN-3 is particularly interesting as it was the only protein that was able to bind to the ZP after strong solubilization and in the presence of strong detergents. It also indicates that AQN-3 is a very potent sperm binding protein and probably the main sperm receptor in the porcine system. AQN-3 belongs to the spermadhesin family: a family of secretory proteins expressed in the epididymis [for a review see Topfer-Petersen *et al.* (1998)]. The spermadhesins are peripherally associated to the sperm cell and are located at the plasma membrane covering the acrosome (Topfer-Petersen *et al.*, 1998) which is indeed the plasma membrane area isolated for our primary ZP-binding assay (Flesch *et al.*, 1998). Ensslin *et al.* (1995) identified AQN-3 as one of the major ZP binding proteins using affinity chromatography, and in our assay, AQN-3 is one of the major spots as well, again indicating that AQN-3 is a main sperm receptor. Of the three spots (numbers 22, 23 and 24) that were identified as AQN-3, only the protein with the lowest molecular weight (number 24) had the correctly predicted molecular weight and pI. We suppose that the other two spots are differentially glycosylated forms of AQN-3, since this secretory protein has a conserved glycosylation sequence on an asparagine residue (Calvete *et al.*, 1993). We should note that a specific glycosylated isoform of AQN-3 has been shown to lose its ZP affinity properties by Calvete *et al.* (1993). Perhaps another residue for glycosylation has led to the isoforms identified in our study or other type of post-translational modification or protein truncation caused these three ZP binding AQN-3 isoforms. Future research should be devoted to reveal how AQN-3 becomes so tightly associated to the sperm surface.

Besides, AQN-3 several other spots were also detected. This may indicate that sperm–zona binding is orchestrated by multiple proteins. Theoretically, the formation of a multiple ZP binding protein complex functionally regulates enhanced sperm–zona binding. Such a potential protein complex of ZP binding proteins may also enable further ZP induced sperm signaling. In fact, the identity of some of the isolated proteins supports this view as they play a role in signal transduction whereas others have previously been shown to have ZP binding properties. Furthermore, a set of proteins involved in sperm–zona binding may easily act cooperatively to induce relatively firm binding to the ZP, and this will optimize the chance of fertilization by ensuring this important binding process. In line with our idea of cooperative binding mechanisms involved in sperm-ZP binding are the findings of Thaler and Cardullo (1996a). Thaler and Cardullo (1996a) described that galactosyltransferase and fucosyltransferase bound to the ZP with different affinities and proposed a model in which multiple low-affinity receptors attach to the ZP thereby enabling the sperm cell to attach firmly to the oocyte. We did not identify these transferases

using our ZP-binding assay. It may be possible that these proteins migrated on the 2D gel into the area with abundant ZP glycoproteins. A limitation of our assay is that if this situation takes place such proteins cannot be identified and may therefore be missing from the ZP binding protein list. Thus, the list of identified ZP proteins is probably incomplete. We cannot exclude this possibility, as we did not find the sequence for porcine galactosyl transferase in a database. However, human, mouse and bovine galactosyltransferases have a pI of ~9.3 and a molecular weight of ~44 kDa which is in the area where ZP glycoproteins are not present. Under the circumstances used, galactosyltransferases probably have a different pI and molecular weight due to glycosylation, which could lead to a gel migration into the ZP glycoprotein area diabling their identification. The only study on the involvement of porcine sperm surface galactosyltransferase in zona binding showed a 66 kDa protein which was shifted to 55 kDa band after deglycosylation (Rebeiz and Miller, 1999).

P47 was one of the 24 spots that were bound after mild solubilization, and it has also been previously shown to have ZP binding capacity (Ensslin *et al.*, 1998). P47 shares some properties with AQN-3 in that it is also secreted in the epididymis and becomes associated to the sperm plasma membrane with a similar lateral distribution over the anterior plasma membrane of the sperm head. This pig sperm protein is homologous to the lactadherins which are major components of the milk fat globule membrane of the mammary gland (Ensslin *et al.*, 1998). Its sequence contains two epidermal growth factor (EGF)-like domains (of which one contains an integrin-binding sequence, RGD) followed by two tandem repeats that are similar to the C1 and C2 domain of the blood clotting factors V and VIII. The C2-like domain contains a putative phospholipid-binding motif. Its function is largely unknown, but it has been described that P47 has affinity for the ZP (Ensslin *et al.*, 1998). Two spots (5 and 9) were identified as P47 of which spot 5 has the theoretically predicted mass of 47 kDa and pI of 6.6 (Ensslin *et al.*, 1998). The smaller protein with a pI value of 9.0 may well be a cleavage product of the mature P47. It is interesting to mention that Ensslin and colleagues (Ensslin *et al.*, 1995; Ensslin and Shur, 2003) have identified SED1, the mouse homologue of p47, as a protein required for mouse sperm binding to the ZP. SED1 is also a secretory protein and it contains Notch-like EGF repeats and discoidin/F5/8 type C domains. SED1 null males are subfertile and their sperm are unable to bind the ZP *in vitro*. Like AQN-3, it is not clear how P47 becomes attached to the sperm plasma membrane.

Another primary zona binding protein spot very specifically matched the identity of fertilin β (previously also named ADAM2 and PH-30) which is a transmembrane protein localized in the plasma membrane of the sperm head (Cho *et al.*, 1998). It is a member of the A Disintegrin and A Metalloprotease domain (ADAM) family and was originally thought to be involved in integrin-mediated sperm–oocyte plasma membrane adhesion and fusion (Cho *et al.*, 2000). Recently, it was shown that knock out mice lacking fertilin β were still fertile but were severely limited in binding to the ZP (Cho *et al.*, 1998). Since in the fertilin β knockout mice at least two other ADAM proteins (fertilin α and cyritestin) were also absent or severely reduced, it was not clear what the function of each protein was in the fertilization process. Here, we have a good indication that fertilin β also plays a role in the primary binding to the ZP. We did not detect fertilin α as a primary zona binding protein in porcine sperm.

We identified four spots as the pre SP32. The testis-specific 32-kDa protein SP32 is produced by post-translational modification of the 61-kDa pre SP32. SP32 is one of the major acrosomal proteins (Noland *et al.*, 1994); therefore, the presence of pre SP32 protein may be an indication for acrosomal contamination. In the acrosome, SP32 binds to (pro)acrosin (Baba *et al.*, 1994)—the most abundant secondary ZP binding (pro)protein. However, mature SP32 as well as (pro)acrosin were not present in any of the protein spots on the 2D gel with the primary zona binding protein. Furthermore, the precursor protein spots matched to amino acid sequences in the precursor part of SP32 only, and the four proteins containing pre SP32 sequences were much smaller (10–30 kDa) than the predicted full size of pre SP32 (61 kDa). Therefore, it is unlikely that any of these proteins is derived from mature SP32. More likely, the precursor part (or degradation products thereof) becomes associated with the plasma membrane after cleavage of pre SP32. Another possibility is that this precursor sequence is present in other proteins (e.g. by the result of gene amplification). The precursor protein seems to be cleaved into smaller parts sharing a consensus region that may enable primary zona binding. In fact, all contained a putative signaling domain of this precursor part (Baba *et al.*, 1994). The largest pre SP32 part may be a substrate for tyrosine kinases as a tyrosine phosphorylated protein spot emerged after capacitation (pI 8 and 30 kDa) as detected with western blotting using anti-phosphotyrosine antibodies (data not shown). This implies that spot number 10 represents the phosphorylated form of spot number 11. Indeed, in our lab (Flesch *et al.*, 1999) as well as in others (Urner and Sakkas, 2003), it has been demonstrated that capacitation induced tyrosine phosphorylation is involved in sperm binding to the ZP.

Another aspect of primary sperm–zona binding is the induction of the acrosome reaction. Interestingly, in this regard, one spot was identified as a K^+ channel (and thus must be a transmembrane protein). It has been proposed that binding to the ZP activates a K^+ channel in sperm and that this would lead to sperm membrane depolarization and thereby open voltage-sensitive T-type Ca^{2+} channels (Florman *et al.*, 1998). Not much is known about K^+ channels in sperm cells, although diverse types have been identified in mouse sperm by the group of Darszon (Munoz-Garay *et al.*, 2001; Felix *et al.*, 2002; Acevedo *et al.*, 2006) who also provided evidence that these channels have a role in sperm capacitation. The presence of K^+ channels in a putative ZP binding protein complex may therefore be functionally linking the sperm binding to the ZP with the ZP induced acrosome reaction (Acevedo *et al.*, 2006). The peptides detected in spot number 9 did not give a definite hit for a single K^+ channel, but the various peptides gave hits for different K^+ channels. Identified amino acid sequences (Table 1) were matching to (i) the tetramerization domain from the Akv3.1 (shaw-subfamiliy) voltage-gated K^+ channel (sequence IIINVGGTRHETY), (ii) the D chain from the Kcsa K^+ channel (sequence PSMLS-----KLLG), (iii) the chain A of the cytoplasmic domain of G-protein activated inward rectifier K^+ channel (sequence NSHMVSAQIRCK) and (iv) the chain B of the K^+ channel Kcsa-Fab complex (RLLIKYASNLDSGVPAR). This suggests that the peptides found are derived from a new protein that is not in the database but possesses a number of K^+ channel specific peptides or from diverse sperm K^+ channels with an identical migration behavior on the 2D gels.

The same is probably the case for the phosphatase (spot 14) which contained two peptide sequences (Table 1) that matched with the A chain (i) of the solution structure of the Pdz2 domain from human phosphatase HPTP-1e complexed with a peptide (sequence AVIPQGAAESDGRIHK) and (ii) of the crystal structure of a human phosphatase (sequence ETHGGEDVAVF). A similar scenario was found for the carbonyl reductase (spot 16) identifications (Table 1). One sequence was identified as the D chain for carbonyl reductase (sequence VVAVTRTNSDLVSLAK) and the other as the A chain of porcine testicular carbonyl reductase 20 beta hydroxysteroid dehydrogenase. Sullivan and Bleau (1985) have described a 26 kDa hamster sperm glycoprotein, called P26 h, that is 85% identical to

porcine lung carbonyl reductase. Like AQN-3 and P47, P26 h is acquired during epididymal transit, but in contrast to AQN-3 and P47, it is known that P26 h becomes attached to the sperm surface as a GPI anchored protein. P26 h is localized on the sperm plasma membrane covering the acrosome, and recently, Montfort *et al.* (2002) showed that inhibition of the carbonyl reductase activity decreased the sperm–ZP interaction without affecting total sperm progressive motility and acrosome integrity.

Another sperm surface protein that was isolated as a binding protein to the zona fragments was identified as peroxiredoxin 5, which belongs to a novel family of peroxidases. Their function is still not totally clear but some isoforms provide defense against oxidative damage, yet others appear to participate in signaling by controlling the H_2O_2 concentration (Rhee *et al.*, 2001; Wood *et al.*, 2003). So far, peroxiredoxins have only been detected intracellularly (Knoops *et al.*, 1999). The role of peroxideroxin 5 in ZP binding is unclear but one could hypothesize that peroxiredoxin 5 could indirectly be involved in this zona binding by associating to the ZP binding protein. If that is true, peroxiredoxin might be involved in (redox) signaling events induced by this ZP binding.

Strong membrane solubilization

With the strong solubilization method, only three protein spots, all identified as AQN-3, were detected. All other proteins showing ZP affinity under mild solubilization conditions lost this property under strong solubilization. One possible explanation is that mild solubilization incompletely solubilized sperm membrane proteins and that proteins complexed to AQN-3 proteins remain associated in the insoluble particles (detergent micelles). The other possible explanation is that only the three AQN-3 isoforms kept their native zona binding properties after strong solubilization, whereas the other ZP binding proteins faced protein denaturation and thus lost their ZP binding properties. We cannot discriminate between these two possibilities. However, we can conclude that AQN-3 is the only 5% NP40 and 1% SDS insensitive ZP binding protein that showed ZP affinity in completely solubilized form.

Although this study is based on a porcine model system, it implies the presence of multiple zona binding proteins and a knock out animal for one specific primary ZP binding protein could still be fertile (the remaining primary ZP binding proteins can still suffice to achieve fertilization). Indeed, it has been shown that knock out male mice for ZP binding proteins remain fertile (Lu and Shur, 1997; Nishimura *et al.*, 2001). The way to establish a role of the protein of interest would be to obtain sperm from the knock out mice, mix it 1 : 1 (on cellular basis) with wild-type sperm of the parental strain (since the latter contains all proteins involved in ZP interaction). In the case of a ZP binding protein, the percentage of wild-type offspring will be higher than 50%.

Finally, it is of considerable interest to note that many of the identified primary zona proteins (namely fertilin β, pre SP32, peroxiredoxin 5 and the three isoforms of AQN-3) have previously been identified as abundant components of sperm membrane rafts by ourselves (Van Gestel *et al.*, 2005). This finding may be relevant for future studies as under capacitation conditions the organization of membrane rafts changes, and this becomes specifically manifested in the apical plasma membrane region of the sperm head (Cross, 2004; Shadan *et al.*,2004; Sleight *et al.*, 2005; Van Gestel *et al.*, 2005) which is exactly matching the site where indeed sperm–zona binding is supposed to take place. Thus, reorganization in sperm membrane rafts may function in the assembly of the postulated putative cooperative ZP binding protein complex.

Overall, we have developed an assay to isolate in a direct way those porcine sperm proteins involved in the primary binding to the porcine ZP of the oocyte. Multiple proteins have been shown to be involved in the binding to the ZP. Three adhesion proteins originate from the epididymis and become firmly attached to the sperm surface. Two transmembrane proteins were identified: one with cellular adhesion properties and the other with K^+ membrane transport properties. Three cytosolic proteins were associated to the zona ghost under mild solubilization conditions: one of the identified proteins is thought to act as a protein phosphatase, another to regulate sperm redox signaling and one as a tyrosine kinase substrate. Further, the characterization of all these proteins and the mode of interactions will be required to establish their (different) roles in ZP binding and the subsequent induction of the signaling cascade leading to the acrosome reaction. This may lead to better understanding of sperm–zona recognition and the subsequent fertilization efficiency.

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