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ORIGINAL ARTICLE

S100A7 is present in human sperm and a homologous pig sperm protein interacts with sperm-binding glycoprotein (SBG)

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Summary

In sows, the oviductal sperm-binding glycoprotein (SBG), which binds to the periacrosomal region of boar sperm, has been shown to be involved in sperm selection. In this work, we isolated porcine sperm proteins that interact with SBG. One of them is identified as a homologue of human S100A7 (psoriasin). Anti-human S100A7 antibodies show that this homologous protein localises to the head of sperm. The isolation of a homologue of S100A7 based on affinity to SBG and its localisation at the head of sperm leads us to suggest that S100A7's homologous protein may be involved in the negative selection of sperm by SBG in pigs. Human S100A7 shows antibacterial properties, particularly over *Escherichia coli*, a species that has demonstrated deleterious effects on human sperm. We searched for S100A7 in human sperm and found that it is present and localises at the acrosomal region. Thus, we report the presence of S100A7 in human sperm and of a homologous protein in pig, with similar localisations. In humans, an antimicrobial role seems likely for psoriasin; in porcine sperm the studied protein binds to SBG suggesting a function in sperm selection, but an antimicrobial function cannot be ruled out.

Introduction

In most analysed mammals, sperm comes into contact with the epithelial cells that line the female tract and their secretions, resulting in the storage of some spermatozoa and allowing the selection of sperm with certain qualities (Talevi & Gualtieri, 2010). Sperm binds to oviductal epithelial cells via the plasma membrane overlying the acrosome (Gualtieri & Talevi, 2000). In bovine, the protein PDC-109, which is found adsorbed to sperm, plays a role in forming the oviductal sperm reservoir (Gwathmey *et al.*, 2003) and oviductal annexins interact with bull sperm, in particular with PDC-109, in relation to sperm adhesion (Gwathmey *et al.*, 2003). In porcine, spermadhesin AQN1 is a candidate receptor molecule for the formation of the oviductal sperm reservoir (Ekhlasi-Hundrieser *et al.*, 2005), and oviductal Annexin A2 is probably involved in this process, as it is in bovine (Teijeiro *et al.*, 2009). The proteins involved in sperm selection are still under study. In pig, we propose that oviductal sperm-binding glycoprotein (SBG) is involved in sperm selection, producing acrosome alteration in sperm that have begun the capacitation process (Teijeiro *et al.*, 2008). In this work, we search among pig sperm proteins, for those that bind to SBG, and identify a pig homologue of human S100A7 as one of the proteins that interact with the glycoprotein. Due to the vital importance demonstrated for S100A7 in innate immunity through its *Escherichia coli*-cidal effect (Glaser *et al.*, 2005; Lee & Eckert, 2007; Abtin *et al.*, 2008; Meyer *et al.*, 2008; Mildner *et al.*, 2010) and the well documented deleterious effect of *E. coli* on human sperm (Diemer *et al.*, 2000, 2003; Villegas *et al.*, 2005; Fraczek *et al.*, 2007; Prabha *et al.*, 2010; Schulz *et al.*, 2010), we also investigate the possible presence of S100A7 on the human sperm.

Materials and methods

Chemicals

Chemicals were obtained from Sigma-Aldrich (Buenos Aires, Argentina), unless otherwise stated. The following antibodies were used: horseradish peroxidase-conjugated anti-rabbit IgG (GE Healthcare, Buenos Aires, Argentina);

Cy3 conjugated anti-rabbit immunoglobulin (Chemicon International Inc., Temecula, CA, USA); anti-S100A7 polyclonal antibody that specifically recognises a 14 amino acid peptide corresponding to the carboxi-terminal end of human S100A7 (KQSHGAAPCSGGSQ), gently gifted by Dr. Peter Watson (Deeley Research Center, BC Cancer Agency, Victoria, Canada), from now on anti-C-S100A7; anti-S100A7 polyclonal antibodies generated against human recombinant GST-S100A7 developed in this work; and anti-S100A9 polyclonal antibodies which recognise amino acids 25–114 of human Calgranulin (Santa Cruz Biotechnology®, Inc, Santa Cruz, CA, USA). The three last antibodies were developed in rabbit.

Semen collection

Boar semen was collected from adult fertile boars from different breeds by the glove-handed method. Sperm rich fraction was diluted in Cronos (Laboratorio Medi Chimica, Reggio Emilia, Italy) and conserved at 16 °C, until use (no more than 24 h from collection). The quality of the samples was established by evaluating motility, viability, concentration, acrosomal and morphological parameters (Althouse, 1997). Human semen samples ($n = 6$) from healthy donors with normal semen parameters according to WHO criteria (WHO, 2010) were gently gifted by Dr. S. Ghersevich from the Clinical Biochemistry Area, School of Biochemistry, University of Rosario.

Preparation of sperm surface proteins

Porcine sperm were recovered from seminal doses (40 ml of 3×10^7 sperm ml^{-1}) by centrifugation at 700 ***g*** for 5 min at 16 °C and washed twice with TALP (96 mM NaCl, 3.1 mM KCl, 2.0 mM $CaCl_2$, 0.4 mM $MgSO_4$, 0.3 mM NaH_2PO_4, 20 mM HEPES, 21.6 mM sodium lactate, 1 mM sodium pyruvate and 15 mM $NaHCO_3$) (Parrish *et al.*, 1988). Washed sperm were incubated with 20 ml of 10 mM Tris-HCl, 0.145 M NaCl, pH 7.5 for 1 h at 4 °C. Suspensions were centrifuged at 3500 ***g*** for 5 min at 4 °C and the supernatants dialysed against water and lyophilised. The sperm surface proteins were resuspended in TALP. After treatment, morphology of spermatozoa was evaluated by Wells-Awa staining according to Teijeiro *et al.* (2008) showing no alterations.

SBG purification

SBG was purified by affinity chromatography as usually performed in our laboratory, according to Marini & Cabada (2003). Purity was assessed by SDS-PAGE, followed by silver staining.

Purification of proteins that bind to SBG

SBG was coupled to cyanogen bromide-activated Sepharose CL 4B as described elsewhere (Martínez *et al.*, 2000). One-hundred micrograms of sperm surface proteins from three different boars were pooled and applied to the affinity column (1 ml bed volume and 200 μg of protein) equilibrated with TALP, followed by washing with 20 volumes of the same solution. Bound proteins were eluted with 20 ml of 200 mM galactose in TALP and the whole eluted volume was collected. The eluted proteins from three chromatographic runs were dialysed against water and lyophilised.

Protein assays and two-dimensional electrophoresis

Protein concentrations were determined by Qubit® device (Invitrogen, Buenos Aires, Argentina) according to manufacturer's instructions. Lyophilised samples were resuspended in buffer containing 9.8 M urea, 4% (w/v) CHAPS, 0.2% 3–10 Ampholyte, DTT 100 mM. To rehydrate 7-cm, pH 3–10 strips (ReadyStrip™ IPG Strip; Bio-Rad, Hercules, CA, USA) 125 μl of the mentioned solution were used. Proteins were resolved in the first dimension by isoelectrofocusing (IEF) at 10 000 V-h using Protean® IEF Cell (Bio-Rad). After focusing, IPG strips were equilibrated 15 min in buffer 6 M urea, 0.375 M Tris-HCl, pH 8.8, 2% SDS, 20% glycerol, 2% (w/v) DTT, and then 15 min in buffer 6 M urea, 0.375 M Tris-HCl, pH 8.8, 2% SDS, 20% glycerol, 2.5% (w/v) iodoacetamide. These strips were then affixed onto 15% SDS-polyacrylamide gel. The second dimension was performed in Mini-PROTEAN® 3 (Bio-Rad). The gels were silver stained.

Mass spectrometry analysis

For protein identification two-dimensional electrophoresis separated spots were sent to ProtTech, Inc (Norristown, PA, USA). Samples were analysed using NanoLC-MS/MS peptide sequencing technology. More experimental details can be found at www.ProtTech.com.

Development of anti-human S100A7 antibodies

The coding sequence for human S100A7, cloned into pBR322 was a gentle gift from Dr. Peder Madsen (Aarhus University, Aarhus, Denmark). This sequence was subcloned into pGex-2T (GE Healthcare, UK) and the recombinant protein GST-S100A7 was expressed in *E. coli* DH5α. Protein expression was induced with isopropyl-β-D- 1-thiogalactopyranoside for 20 h and purification was performed with Gluthatione Sepharose™ 4 Fast Flow (GE Healthcare, Amersham, UK). Recombinant protein was used for polyclonal antibody development in rabbit

as described (Pérez *et al.*, 2006). The later clone was used to subclone S100A7 cDNA into pET-tev as histidine protein fusion. Purified His-6-S100A7 was used to purify anti-GST-S100A7 antibodies for immunocytochemistry (Plaxton, 1989).

Western blots

Sperm protein extracts were obtained suspending human or porcine sperm on RIPA buffer (25 mM TrisHCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% deoxicolato de sodio, 0.1% SDS), boiling for 3 min, and centrifuging 15 min at 16 000 ***g***. The supernatants were used for 15% SDS-PAGE and proteins transferred to nitrocellulose membranes (GE Healthcare, Buenos Aires, Argentina). Nonspecific binding sites were blocked by incubation with 5% dry non-fat milk in TBS (25 mM Tris-HCl, pH 7.4, 150 mM NaCl). Membranes were treated for 1 h with the corresponding antibodies. After washing (three times for 10 min each), the blot was incubated with peroxidase-conjugated anti-rabbit IgG for 1 h and washed again. Labelled proteins were revealed using enhanced chemiluminescence detection with ECL Kit (GE Healthcare, Buenos Aires, Argentina).

Immunocytochemistry

Sperm suspensions were smeared on glass slides optimised for immunohistochemistry (Frosted HiFix^{NH}, TNT, Buenos Aires, Argentina) and fixed with 0.2% glutaraldehyde. Slides were gently rinsed with PBS twice, blocked with 2% BSA, 0.2% TritonX-100 in PBS for 1 h and treated with the primary antibodies overnight at 4 °C. Antibodies were polyclonal anti-S100A7 purified by affinity to His-6-S100A7 immobilised on nitrocellulose membranes (Plaxton, 1989). After rinsing twice with PBS, the slides were treated with Cy3 conjugated anti-rabbit immunoglobulin (1 : 2000) for 1 h. After rinsing twice with PBS, slides were covered with 0.22 M 1,4-diazabicyclo [2,2,2] octane dissolved in glycerol : PBS (9 : 1) and cover slips were applied. The preparations were examined under a microscope equipped with epifluorescence (BH 2; Olympus Optical Company Ltd., Tokyo, Japan).

Results

Purification of proteins with affinity for SBG

An affinity chromatography column containing pig oviductal glycoprotein SBG was built to search for pig sperm proteins with SBG-binding capacity. Proteins loosely adsorbed to the porcine sperm surface were obtained by low ionic strength treatment with an isoosmotic solution. This treatment did not affect sperm morphology, as stated by Wells-Awa staining. The sperm surface protein containing samples (Fig. 1a) were applied to the affinity column and extensive washing was performed with TALP. Sperm-oviduct interaction is thought to be based on carbohydrate-recognition systems (Töpfer-Petersen *et al.*, 2008) and SBG exposes Galβ1-3GalNAc (Marini & Cabada, 2003), thus galactose was chosen for the elution of SBG-bound proteins. The eluted proteins were resolved in two-dimensional gel electrophoresis resulting in the pattern shown in Fig. 1b.

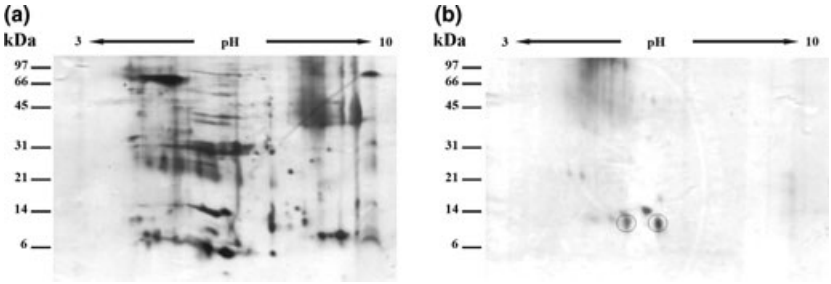

Fig. 1 Purification of sperm surface proteins that interact with sperm-binding glycoprotein (SBG). Two dimensional gel electrophoresis of (a) surface proteins (250 μg) extracted from sperm, (b) proteins eluted from affinity chromatography to SBG, using 200 mM galactose. Spots used for protein sequencing by LC-MS/MS are shown in circles. The first dimension was run in 3–10 pH range. Proteins were silver stained.

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Human S100A7 MSNTQAERSIIGMIDMFHKYTRRDDKIDKPSLLTMMKENFPNFLSACDKKGTNYLADVFEKKDKNEDKKIDFSEFLSLLGDIATDYHKQSHGAAPCSGGSQ
Porcine S100A7 MSTTPAEKSMMDTIDLFHKFTDDSDTMDKEGLLKLLQENFPNFLSACDKNGVDYLANIFEQKDKNKDQRIDFSEFLSLLGDIATDYHKHSHQEELCPPEHQ
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Fig. 2 Alignment of human S100A7 and porcine similar to S100A7 reported sequence. Sequences of human S100A7 and the porcine sequence EW648856.2 (GenBank, NCBI) are aligned, the peptides obtained by LC-MS/MS from sperm-binding glycoprotein interacting porcine surface sperm proteins are shown in boxes. The peptide recognised by anti-C-S100A7 antibodies is in bold.

Identification of a pig homologue of S100A7

From the porcine sperm proteins that interact with SBG, separated by two-dimensional electrophoresis, two main spots of pI 6.2 and 6.4, with apparent molecular mass 11 kDa (Fig. 1b, in circles) were excised and analysed by LC-MS/MS. The obtained peptide sequences, coincident for both spots, (Fig. 2) are included in human S100A7 and cover, from its 101 amino acids, sequences 38–50 and 70–88. Databank search allowed the identification of a porcine tongue EST (GenBank entry: EW648856.2. Library: dbEST21727.ton, NCBI GenBank) that upon *in silico* transduction shows a polypeptide highly homologous to human S100A7 (Fig. 2). The sequenced peptides correspond to conserved domains involved in dimerization.

Western blot detection of a pig protein homologous to S100A7 and human S100A7 in sperm protein extracts

Porcine sperm surface proteins and human whole sperm extracts were analysed by western blotting. Anti-GST-S100A7 antibodies recognise a protein of apparent molecular mass 11 kDa in both samples (Fig. 3a). Controls were performed with preimmune serum showing no bands (data not shown). When anti-C-S100A7 antibodies, which recognise an S100A7 peptide indicated in Fig. 2, were used (Fig. 3b) human sperm showed the expected band, but no signal was observed for pig sperm surface proteins (Fig. 3b, line 2). As calgranulin (S100A9) is frequently expressed together with S100A7 in other cell types (Carlsson *et al.*, 2005) and has already been detected by proteomic methods in human sperm, we used anti-calgranulin antibodies to search for the presence of this protein in boar and human whole sperm extracts (Fig. 3c). S100A9 was not detected in pig samples, but it was in human sperm extracts, in coincidence with previous reports (Martinez-Heredia *et al.*, 2006). Positive control to anti-S100A9 reaction was performed with leucocyte protein extracts (Fig. 3c, line 4). Even when two spots of a protein homologous to S100A7 appeared in two dimensional gel analysis for pig sperm, only one spot of pI 6.2 was detected in human sperm protein extracts.

Localisation of S100A7 and a porcine homologue of S100A7 in human and pig sperm respectively

For the immunolocalisation of S100A7, anti-GST-S100A7 antibodies were purified by affinity to His-6-S100A7. Upon exposure of pig sperm to these antibodies (Fig. 4a), fluorescence was localised to the whole head of sperm. However, the distribution of S100A7 on human sperm

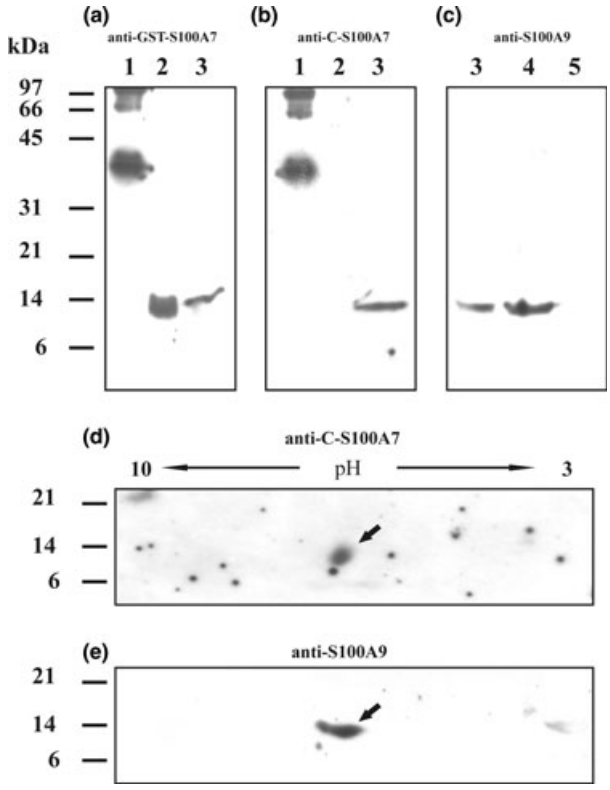

Fig. 3 Identification of pig S100A7 homologue and human S100A7 using specific antibodies. Western blots using (a) anti-GST-S100A7 antibodies (1 : 1000); (b, d) anti-C-S100A7 (1 : 1000); (c, e) anti-calgranulin antibodies (1 : 1000). Samples are 1: 1 μg of human recombinant GST-S100A7, 2: 40 μg of porcine sperm surface proteins, 3: 40 μg of human sperm protein extract, 4: 40 μg of leucocyte extract, 5: 40 μg of porcine sperm protein extract. (e, f) Western blot of human sperm proteins resolved in two dimensional gel electrophoresis. Arrows point to spots of pI 6.2 and 6 correspond to S100A7 and S100A9 respectively.

was limited to the acrosomal region (Fig. 4e). Controls performed with preimmune serum gave no signal for pig nor human sperm (Fig. 4c, G).

Discussion

In pig, sperm are selected in the oviduct for morphological intactness (Waberski *et al.*, 2006), maturity (Petrunkina *et al.*, 2001) and uncapacitated status (Fazeli *et al.*, 1999). In this mammal, a model has been proposed in which uncapacitated sperm are trapped in the oviduct by the formation of a sperm reservoir due to the binding of the sperm surface associated spermadhesin AQN-1 to glycans exposed by oviductal glycoproteins (Töpfer-Petersen *et al.*, 2008). So far, only annexins have been identified as probable mediators of sperm reservoir formation in pig

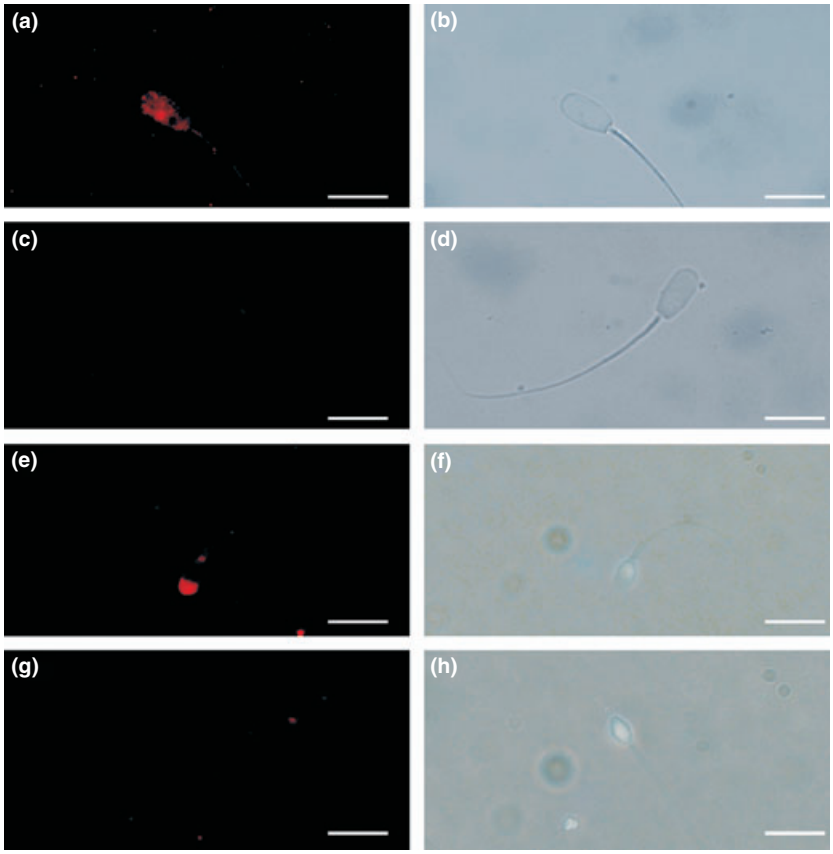

Fig. 4 Immunolocalisation of S100A7 and its porcine homologue in sperm. Micrograph of sperm treated with anti-S100A7 affinity purified antibodies, followed by Cy3 conjugated anti-rabbit immunoglobulin. (a–d) Boar sperm; (e–h) human sperm. (c, d, g, h) Incubation with preimmune serum as control. (a, c, e, g) Fluorescence detection; (b, d, f, h) bright field.

(Teijeiro *et al.*, 2009). This function has also been proposed for annexins in cow (Ignotz *et al.*, 2007).

In relation to the role of the oviduct in sperm selection, we have previously isolated SBG from pig oviductal epithelial cells, based on its capacity of binding to periacrosomal membranes of sperm (Marini & Cabada, 2003). SBG localises to the lumen of the oviduct (Pérez *et al.*, 2006), where sperm subpopulations with altered plasma membranes have been detected (Mburu *et al.*, 1997; Jaiswal *et al.*, 1999; Tienthai *et al.*, 2004), and it damages capacitating sperm, at least *in vitro* (Teijeiro *et al.*, 2008). These lead us to propose that SBG is involved in sperm negative selection, by damaging those sperm that have begun capacitation when they arrive to the oviduct (Teijeiro *et al.*, 2008).

In this work, we search for sperm-associated proteins that interact with SBG, and may thus mediate the physiological effect produced by this glycoprotein. It is actually accepted that complex interactions as that of sperm and oviductal cells are due to the relation between several proteins, which form part of the cell interactome (Ramirez *et al.*, 2007). In accordance with this, several porcine sperm surface proteins were retained by affinity to SBG, upon chromatography and elution with galactose (Fig. 1b). The fact that these proteins are eluted by competition with galactose may be an evidence of carbohydrate-mediated interaction between the retained sperm proteins and the Galβ1-3GalNAc disaccharide exposed by SBG.

From the SBG-interacting proteins two spots, probably two isoforms, showed homology to human S100A7 (Fig. 1b). Western blot analysis using anti-GST-S100A7 showed the presence of a homologue of S100A7 in boar sperm (Fig. 3a). However, when antibodies which specifically recognise a carboxyterminal peptide contained in human S100A7 were used no signal was observed (Fig. 3b). This is in accordance with the lack of detection of peptides that belong to this region by LC/MS-MS of the S100A7-like isolated pig sperm proteins. The apparent molecular mass of the isolated pig proteins is similar to that of human S100A7 (Fig. 3a), indicating that the carboxyterminal region of both proteins probably present low homology. The mutation of the carboxyterminal region of human S100A7 slightly reduces psoriasin's antibacterial

activity (Lee & Eckert, 2007). In this regard, the lack of homology of porcine proteins at the carboxyterminal end might indicate that innate immunity is not their main function in porcine. S100A7 and S100A9 are co-expressed in relation to epithelial differentiation (Martinsson *et al.*, 2005). In this sense, we searched for S100A9's presence in pig sperm; however, we failed to find this protein by immunological methods (Fig. 3c).

S100A7 has not been previously reported in human sperm, instead S100A8 and S100A9, other members of the S100 family, have been reported (Martinez-Heredia *et al.*, 2006). Psoriasin is a component of the innate immune system, which is eventually overexpressed, together with other immunological proteins as β-defensin-2 (Schlapbach *et al.*, 2009). Interestingly, β- defensins have been found in sperm: β-defensin 126 covers the whole macaque sperm (Yudin *et al.*, 2003) and β-defensin 22 is found principally at the acrosomal region of mouse sperm (Yudin *et al.*, 2008). Thus, the presence of S100A7, another protein with immune activity, reported here in this location seems natural and an immunological function for it plausible. β-defensin 2D6 is an epididymally secreted glycoprotein and a pore-forming glycopeptide that has been found attached to the plasma membrane of rat sperm (Zanich *et al.*, 2003). The epithelial origin of S100A7 and its pore-forming activity (Michalek *et al.*, 2009) suggest a possible epididymal origin for it and a similar function for both proteins in sperm. Some sperm-associated immunological proteins have been related to reproductive functions. β-defensin 126, which is found in macaque sperm, is involved in its attachment to oviductal epithelia (Tollner *et al.*, 2008), and CAP18 has been found attached to human sperm (Malm *et al.*, 2000) not only in relation to its antimicrobial properties but also to its involvement in zona pellucida binding (Doussau *et al.*, 2008). All these reports permit to infer a possible role for sperm psoriasin in immunity, without ruling out a possible reproductive-specific function. The fact that *E. coli*-induced alterations of human spermatozoa affect the inner and outer acrosomal membranes (Diemer *et al.*, 2000), in coincidence with the localisation of antimicrobial psoriasin at the human sperm acrosome (Fig. 4) strongly supports its immunological function in this cells.

In this work, we isolate a porcine sperm protein similar to S100A7, with SBG-binding ability, and probably involved in sperm selection. We also report the presence of S100A7/psoriasin in human sperm. Even though both proteins localise to the sperm head, the different distribution and characteristics seem to entail a mainly immunological function for human S100A7 and a mainly physiological function for the porcine version. However, a reproductive function for psoriasin may not be ruled out. It should be interesting to explore the possibility of the existence of a human glycoprotein similar to SBG, and to analyse the possible immunological activity of porcine S100A7 homologue.

Acknowledgements

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