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Short communication

Molecular identification of the sperm selection involved porcine sperm binding glycoprotein (SBG) as deleted in malignant brain tumors 1 (DMBT1)

Juan Manuel Teijeiro [1], María Lorena Roldán [1], Patricia Estela Marini [*,2]

Instituto de Biología Molecular y Celular de Rosario (IBR-CONICET), Área Biología, Departamento de Ciencias Biológicas, Facultad de Ciencias Bioquímicas y Farmacéuticas (UNR) Suipacha 590–Rosario (S2002LRK), Santa fe, Argentina

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ABSTRACT

Porcine sperm binding glycoprotein (SBG) is involved in sperm–oviduct interaction. Here we use mass spectrometry to identify SBG, finding peptides corresponding to deleted in malignant brain tumors 1 (DMBT1), at scavenger receptor cysteine-rich (SRCR) and CUB domains. RT-PCR allowed the cloning of unique sequences, belonging to porcine DMBT1. Western blot and immunofluorescence of oviductal tissues using anti-SBG and anti-hDMBT1 antibodies showed identical results. The biochemical characteristics of both proteins are coincident. We conclude that porcine SBG is an oviductal form of DMBT1, and thus assign this protein a novel location and function.

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1. Introduction

The oviduct is a dynamic organ in which final gamete maturation takes place. The interaction between sperm and the oviductal cells and secretions results in storage of a selected subpopulation of sperm in most analyzed mammals, ensuring the availability of a suitable number of viable spermatozoa for fertilization (for review see [1]). Detachment from the oviduct has been linked to protein loose upon capacitation [2], to hyperactivated motility [3,4] and to protein disulfide reduction [5]. A less studied but already accepted event is sperm negative selection in the oviduct (for review see [1,6]. Sperm subpopulations with altered plasma membranes have been detected in the lumen of the porcine oviduct in vivo [7,8] and a porcine sperm binding glycoprotein (SBG), which produces an acrosomal alteration effect on sperm incubated under capacitating conditions, has been described [9,10]. SBG localizes to the apical surface of cells at the lumen of the oviduct, as determined by immunohistochemistry [11], in coincidence with the region where altered sperm are seen [7,12]. This glycoprotein was first isolated due to its capacity of association to sperm periacrosomal membranes [13] and produces, in vitro, alteration of pig sperm acrosome integrity, suppression of motility and tyrosine phosphorylation of a 97 kDa protein on capacitating spermatozoa [9]. These data support a sperm negative selection function for SBG, and lead us to proceed to the molecular characterization of the glycoprotein.

2. Materials and methods

2.1. Chemicals and samples

Chemicals were obtained from Sigma–Aldrich (Argentina), unless otherwise stated. Oviducts were obtained at a local abattoir and transported to the laboratory in ice-cold PBS.

2.2. Protein Assays and electrophoresis

Oviductal cell fractions enriched in plasmatic membranes were obtained as described in [13]. Membranes were incubated with 7 M urea-2 M thiourea–4% CHAPS-50 mM DTT, centrifuged at 21,000 g for 20 min and supernatants recovered. Protein concentrations were determined using a Qubit 2.0 device (Invitrogen, Argentina).

Samples for 2D were suspended 125 μl of buffer to rehydrate 7-cm, pH 3-10 strips (ReadyStrip™ IPG Strip, Bio-Rad). Proteins were resolved in the first dimension by IEF for a total of 10,000 V-h using Protean® IEF Cell (Bio-Rad). After focusing, strips were equilibrated in an appropriated buffer and affixed onto 8% SDS-

Abbreviations: SBG, sperm binding glycoprotein; SRCR, scavenger receptor cysteine-rich; DMBT1, deleted in malignant brain tumors 1.

* Corresponding author. Tel.: +54 341 4350661x122; fax: +54 341 4804601.
E-mail addresses: pmarini@fbioyf.unr.edu.ar, marini@ibr.gov.ar (P.E. Marini).

[1] Contributed equally to this work.

[2] Member of the Research Career CIC-UNR.

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Fig. 1. Alignment of SBG/DMBT1 sequences. The aligned cDNA and amino acid derived sequences of porcine DMBT1 from NM-001048188.1 and from SBG obtained in this work are shown. Mismatches are in capital letters. Boxes indicate the peptides sequenced from oviductal SBG. The domains are underlined: SRCR (−), CUB (….), ZP (-·-) and numbered at the right. The transmembrane region is underlined and in italics. The oligonucleotides used in RT-PCR experiments are indicated in bold and the first pair used doble underlined.

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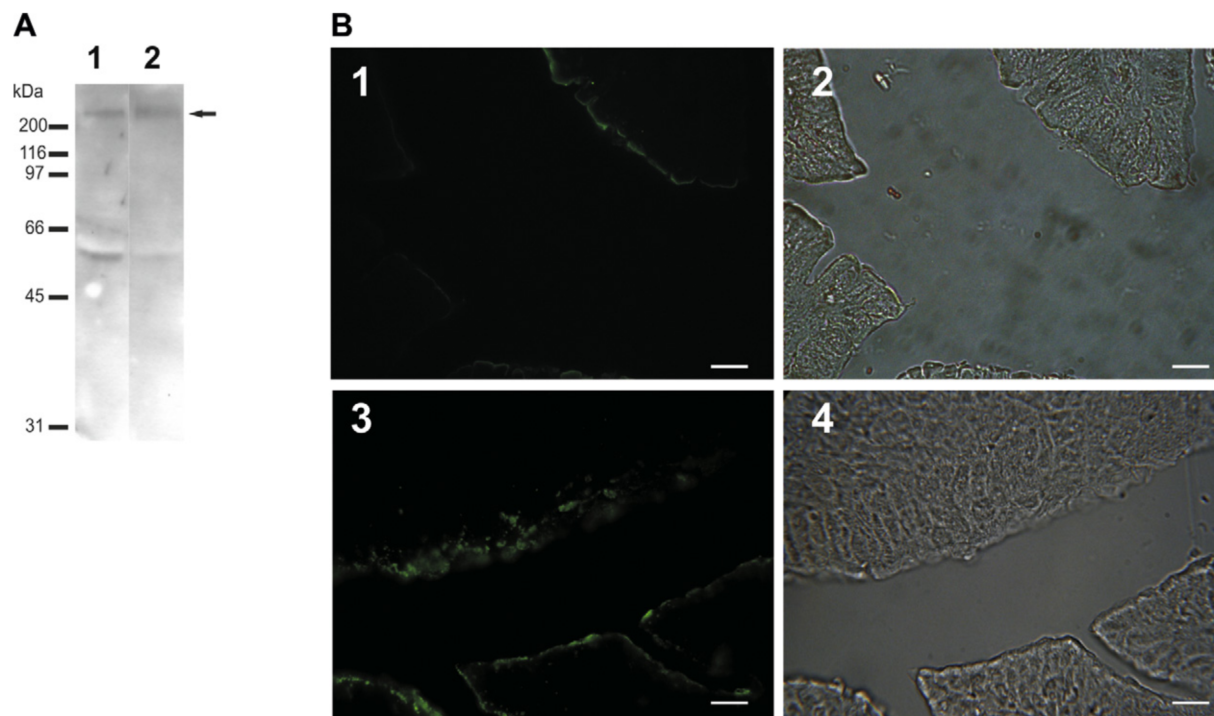

Fig. 2. SBG/DMBT1 protein in the porcine oviduct. A- SBG and DMBT1 content in oviductal cell extracts (20 μg protein) was analyzed by Western blotting with: (1) anti-SBG and (2) anti-hDMBT1 antibodies. Molecular mass markers are displayed on the left of the gel (numbers indicate the molecular mass in kDa). B- Immunofluorescence localization of SBG/DMBT1 in porcine oviductal tissue. Longitudinal sections from oviductal isthmus were treated with: (1) anti-SBG and (3) anti-hDMBT1 antibodies as described in materials and methods. (2–4) Bright fields. Bars represent 10 μm.

polyacrylamide gel. The second dimension and regular SDS-PAGE were performed in Mini-PROTEAN® 3 Cell (Bio-Rad).

2.3. Western blot

For western blots proteins were transferred to nitrocellulose membranes. Membranes were blocked with 5% dry milk in TBS-T (TBS plus Tween® 0.05%) for 1 h, incubated with the corresponding anti-SBG [11] (1:1000) or anti-hDMBT1 (1:400) antibodies, which were gently provided by Dr. Jan Mollenhauer (University of Southern Denmark). After washing with TBS, incubating with anti-rabbit IgG-HRP (GE Healthcare, Argentina) 1:5000 (v/v) in TBS, during 1 h at room temperature, and washing again with TBS and TTBS, peroxidase activity was revealed using ECL (GE Healthcare, Argentina).

2.4. Mass spectrometry

SBG was excised after 2D electrophoresis and submitted to ProtTech, Inc. (Norristown, USA) for identification. NanoLC–MS/MS peptide sequencing technology was used. The data acquired by MS/MS were used to search protein databases from NCBI with ProtTech's software. Further details can be found at www.prottech.com. Protein identification was limited to peptide hits with 95% statistical significance.

2.5. RT-PCR and determination of cDNA sequences

Epithelial cells from the isthmic region of the oviduct were obtained by scrapping with a scalpel blade in TRIzol™reagent (Invitrogen, Argentina). Total RNA was isolated and 5 μg aliquots reverse transcribed by oligodT using SuperScript™II reverse transcriptase (Invitrogen, Argentina). The cDNA (1 μl) was used as template in PCR in a Mastercycler thermocycler (Eppendorf). The oligonucleotides were designed with a BamHI (sense) or EcoRI (antisense) restriction sites to facilitate posterior cloning. Amplified products were cloned and sequenced.

2.6. Immunofluorescence detection in oviductal tissues

The isthmic portions of oviducts were separated by dissection into 1-cm segments and fixed in 4% formaldehyde. Tissue was dehydrated, embedded in paraffin, cut into 5-μm sections, and mounted on slides optimized for immunohistochemistry (Frosted HiFix^{NH}, TNT, Argentina). Tissue sections were deparaffinized and treated with 2% BSA in buffer to minimize nonspecific binding before incubating overnight at 4 °C with anti-SBG (1:100) or anti-hDMBT1 (1:50) antibodies. Slides were rinsed with TBS and incubated 1 h with goat anti-mouse Alexa Fluor® 488-conjugated IgG (Invitrogen, Argentina) at a dilution of 1:100. After rinsing with TBS, slides were covered with 0.22 M 1,4-diazabicyclo (14) octane (Sigma–Aldrich, Argentina) dissolved in glycerol:PBS (9:1) and cover slips added. Preparations were examined with a microscope equipped with epifluorescence (BH 2, Olympus Optical Company Ltd., Japan). Controls were run by omission of the primary antibody (data not shown).

3. Results

3.1. Identification of SRCR domains in SBG

SBG was identified in 2D separated porcine oviductal cell membrane extracts by western blot using specific anti-SBG antibodies. The corresponding spot was cut from the 2D gel and used for peptide sequence determination by LC-MS/MS. The results were three peptide sequences (two of them identical) corresponding to

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scavenger receptor cysteine-rich domains (SRCR) of porcine DMBT1 (NP_001041653.1). Blast search for peptides in databanks showed homology only to DMBT1. The peptide found repeatedly by SBG mass spectrometry is present in SRCR 1, SRCR 3 and SRCR 4 of DMBT1, while the other is included in the SRCR 5 domain (Fig. 1).

3.2. Oviductal SBG/DMBT1 cDNA sequence determination

As SRCR domain is a module contained in a family of proteins [14], oligonucleotides were designed based on the sequences of the peptides obtained by mass spectrometry (Fig. 1, doble underlined) and used for RT-PCR with porcine oviductal cell cDNA as template. This experiment was performed in order to clone the cDNAs of SBG and all other possible SRCR coding sequences expressed in oviductal cells. A unique DNA fragment was obtained, which was cloned and sequenced, and showed 99% identity to the reported DMBT1 sequence (nt 1837–2513, Fig. 1). This region contains part of SRCR 4 and SRCR 5, the CUB domain contained between them, and the SRCR interspersed domain 4 (SID4). As there is high identity not only in SRCR domain's sequences but also in CUB and even SID regions, we conclude that SBG is most probably DMBT1, and not another SRCR domain containing protein. Oligonucleotides contained in sequences previously obtained as cDNA in this work combined with oligonucleotides designed based on DMBT1 reported sequence were used for further cloning and sequencing. The total cDNA sequence obtained covers from SRCR2 to the end of DMBT1, including the transmembrane region, which is exclusive of some of the DMBT1 variants [15]. The 102 nt from 3724 to 3826 were not sequenced in this work (Fig. 1), however a fragment of the corresponding length is present in the cDNA. Only 39 mismatches and 1 gap, 6 nucleotides long, were found comparing with the previously reported DMBT1 EST sequence [15] (Fig. 1).

3.3. Detection and inmuno-colocalization of SBG and DMBT1 in the oviduct

Anti-SBG antibodies [11] and anti-hDMBT1 antibodies, were used for protein detection on porcine oviductal cell extracts. The two antibodies recognize only one, coincident protein band, of apparent molecular mass 220 kDa (Fig. 2A). This further supports the identification of SBG as DMBT1.

We have previously reported the localization of SBG preferentially to the apical surface of epithelial cells, and at the lumen rather than at the bottom of the folds and crypts of the porcine oviduct [10,11]. Here, we compare the localization of SBG in the porcine oviduct with that of DMBT1 detected by anti-hDMBT1 antibodies. As seen, both antibodies detect proteins at the same localization, the apical surface of the epithelial cells at the luminal region of the oviduct (Fig. 2B).

4. Discussion

SBG is a porcine oviductal cell glycoprotein first identified and purified due to its ability to bind to homologous sperm [13]. This protein produces acrosome alteration on sperm incubated under capacitating conditions and is considered to function on sperm negative selection in the oviduct [9,10]. In this work, we identify SBG by mass spectrometry, sequencing of the corresponding cDNA and western blot as DMBT1. DMBT1 has been independently discovered in various animal species from human to fish. DMBT1 belongs to group B of the SRCR superfamily, composed mainly of transmembrane proteins [16]. In pig, two independent ESTs for DMBT1 have been reported, one of them lacking the transmembrane corresponding nucleotides. In mice, alternative splicing may produce membrane-anchored and secreted variants of DMBT1 [17]. The cDNA sequencing done in this work (Fig. 1) failed to find the shorter, transmembrane region lacking, transcriptional variant.

Comparing the biochemical characteristics previously reported for both proteins: molecular mass of the glycosylated form is approximately 220 kDa [13,18], intracatenary disulfide bonds are main components of SRCR domains [19] and are present in SBG [13], SBG exposes GaLβ1-3GalNAc attached by O-links [13] and DMBT1 may contain N- and O- glycosylations, depending on the species and tissue [16].

In epithelia where differentiation is continuous, the distribution of DMBT1 is suggestive that it might be involved in terminal differentiation. For instance, in the intestine, the crypt is the site of stem cells and the villus cells are terminally differentiated. DMBT1 is localized in the villus but not in the crypt [20]. Similarly, the localization of SBG in the oviduct is at the apical surface of isthmic and ampullary epithelial cells, preferentially at the ciliary cells and predominantly in cells near the lumen of the tube, covering two-thirds of the crypts in isthmus and the luminal projections of the folds in ampulla [11]. At the moment of its first localization [11] we considered SBG's location as appropriated for interaction with the arriving sperm cells. In this work, we perform immunohistochemistry of porcine oviductal tissue with anti-SBG and with anti-hDMBT1 antibodies showing that the exact same results are obtained for both (Fig. 2B, 1–3). This result is a further evidence of the identification of SBG as DMBT1, and also shows a new localization for DMBT1, at the oviductal epithelium. It also opens a new hypothesis of SBG/DMBT1 function in the oviduct, as the localization is compatible with the one previously accepted as related to cell differentiation in other epithelia.

DMBT1 is a multifunctional protein involved not only in cell differentiation/cancer, but also in the host innate immune defense system at mucosas. Although DMBT1 has not been previously reported in the oviduct, in the genital tract its presence has been detected in rodent and primate endometrial epithelium [21,22], and its expression by cervical and vaginal epithelial cells has been studied in relation to infections [23]. This role could be extended to the oviduct. The identification of SBG as DMBT1 also implies that it has another function, sperm selection in the oviduct. The interaction of SBG/DMBT1 with bacteria and virus might be similar to the one with the antigenically different sperm cells. Interestingly, DMBT1 deficient knock-out mice are fertile [24], indicating that the role of SBG/DMBT1 in reproduction is not crucial or may be substituted by other mechanisms, as would be the case for sperm selection.

In whole, we identify porcine oviductal SBG as DMBT1, providing a new localization for the glycoprotein at the ciliated epithelial cells of the oviduct, and adding a new function for this SRCR protein in sperm selection.

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Appendix. Supplementary material

Supplementary data related to this article can be found online at doi:10.1016/j.biochi.2011.10.008.

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