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Sperm Binding Glycoprotein (SBG) Produces Calcium and Bicarbonate Dependent Alteration of Acrosome Morphology and Protein Tyrosine Phosphorylation on Boar Sperm

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Abstract The oviduct is a dynamic organ which modulates gamete physiology. Two subpopulations of sperm have been described in the oviduct of sows, a majority with normal appearance in the deep furrows and a minority, centrally located, and showing damaged membranes. Sperm–oviduct interaction provides the formation of a sperm storage and allows the selection of sperm with certain qualities. Pig (*Sus scrofa*) oviductal sperm binding glycoprotein (SBG) binds to sperm and exposes Galβ1-3GalNAc. This disaccharide may be recognized by boar spermadhesin AQN1, which seems to be involved in sperm interaction with the oviduct. SBG is present at the apical surface of the epithelial cells that surround the lumen of the oviduct rather than at the bottom of the crypts. These characteristics imply it could be involved in sperm interaction with this organ. In this study, we evaluate the effect of SBG over boar sperm. We show that the presence of SBG produces alterations of the acrosome morphology of sperm only when they are incubated in capacitating conditions. SBG binds to the periacrosomal region of sperm undergoing capacitation. Its presence induces an increase on the tyrosine-phosphorylation of a polypeptide of apparent molecular mass 97 kDa, as occurs with a 95 kDa protein in other mammalian sperm upon acrosomic reaction. Altogether, these results suggest that SBG might be involved in sperm selection by alteration of the acrosome of sperm that have already begun the capacitation process when they arrive to the oviduct. J. Cell. Biochem. 103: 1413–1423, 2008.

Key words: sperm; boar; acrosome; SBG; glycoprotein; oviduct

During their journey in the female tract, sperm come into contact with the epithelial cells that line the tract and their secretions. The interaction between sperm and the oviduct results in a mechanism by which some sperm can be stored in the oviduct and allows the selection of sperm with certain qualities [Talevi and Gualtieri, 2004].

Sperm storage has been described in many mammalian species [Suarez, 1998], including sheep [Hunter and Nichol, 1983], mice [Suarez, 1987; Kan and Esperanzate, 2006], hamsters [Smith and Yanagimachi, 1991; Kan and Esperanzate, 2006], cows [Hunter et al., 1991; Kan and Esperanzate, 2006], and pigs [Hunter, 1981], and ensures the availability of a suitable number of viable spermatozoa for fertilization [Tienthai et al., 2004]. Sperm bind to oviductal epithelial cells via the plasma membrane overlying the acrosome [Gualtieri and Talevi, 2000] in a process that involves carbohydrate recognition [DeMott et al., 1995; Lefebvre et al., 1997; Suarez, 2001; Wagner et al., 2002]. Sperm interaction with the oviduct not only forms a sperm reservoir but also allows the selection of a

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higher quality sperm subpopulation [Talevi and Gualtieri, 2004]. Adhesion has been shown to occur only for sperm with intact acrosomes in cow [Gualtieri and Talevi, 2000], at an uncapacitated status for horse [Thomas et al., 1995], cow [Lefebvre and Suarez, 1996], and pig [Fazeli et al., 1999], with low internal free calcium content and reduced membrane proteins phosphorylation for pigs [Petrunkina et al., 2001a], of superior morphology for horse [Thomas et al., 1994] and with normal chromatin structure for human [Ellington et al., 1999]. It is likely that only a sperm subpopulation within the ejaculate exposes unknown surface proteins that confer the ability to adhere to oviductal epithelial cells and be stored in a fertile condition [Talevi and Gualtieri, 2004]. In fact, two sperm subpopulations have been described in the oviduct of sows, a majority present in the deep furrows or crypts which present normal appearance, and a minority centrally located, which present damaged plasma membranes and should be considered nonviable [Mburu et al., 1997; Tienthai et al., 2004].

The identity of the oviductal cells adhesion molecules involved in sperm binding and selection is still undefined [Talevi and Gualtieri, 2004]. In pig, binding inhibition experiments have led to the conclusion that galactose and mannose residues contained in glycoproteins are involved [Wagner et al., 2002] and spermadhesin AQN1 has been indicated as a probable sperm receptor for sperm adhesion [Ekhlasi-Hundrieser et al., 2005].

We have purified a sperm binding glycoprotein (SBG) from pig oviductal epithelial cells, which binds to components from the periacrosomal sperm membrane and to the heads of whole sperm [Marini and Cabada, 2003]. To our knowledge, this is the only oviductal epithelial cell membrane protein reported to interact with sperm. SBG exposes Galβ1-3GalNAc [Marini and Cabada, 2003] which may be recognized by spermadhesin AQN1 [Ekhlasi-Hundrieser et al., 2005]. This glycoprotein is present at the apical surface of epithelial cells, at the lumen rather than at the bottom of the folds and crypts of the oviduct, and at a larger amount in isthmus than in ampulla [Perez et al., 2006], thus being placed in zones where the presence of damaged sperm has been described. The biochemical properties of SBG, as well as its ability to bind to sperm indicate it may be involved in sperm interaction with oviductal epithelial cells. Its localization at the central part of the oviduct, where sperm with damaged membranes and probably nonviable are predominant [Mburu et al., 1997; Tienthai et al., 2004] suggests it may be involved in sperm selection. In order to establish the possible function of SBG in the oviduct we examined its effect on sperm physiological status, as well as the binding of SBG to sperm under capacitating conditions.

MATERIALS AND METHODS

Chemicals

Unless otherwise stated, chemicals were obtained from Sigma-Aldrich (Buenos Aires, Argentina).

Media

Sperm capacitating medium was Tyrode's medium, TALP [Parrish et al., 1988], which contains 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, 15 mM $NaHCO_3$, 5 mM glucose. When indicated variations were prepared lacking $CaCl_2$ or $NaHCO_3$ or both according to experimental requirements.

To avoid acrosome alterations by osmotic shock, the osmolarity of all of these solutions was maintained at 285–315 mOsm with NaCl.

Semen Collection and Treatments

Semen was collected from adult fertile boars by the glove-hand 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).

Quality of the samples was established by evaluating motility, viability, concentration, acrosomal, and morphological parameters [Althouse, 1997].

Spermatozoa were recovered by centrifugation at 700*g* for 5 min and washed twice in the medium used in each experiment, which is stated in each case. The pellet was resuspended to 10^7 sperm/ml in the same medium.

SBG Purification

SBG was purified as reported by Marini and Cabada [2003]. Briefly, oviducts of prepubertal gilts of approximately 120 days of age were used. The isthmic part was separated and

opened longitudinally. Epithelial cells were obtained by scrapping with the blunt side of a scalpel blade, disaggregated, washed and disrupted in Potter homogenizer. Fractions enriched in plasmatic membrane were prepared by differential centrifugation. Extracts of these fractions were obtained by incubation in 0.5 M NaCl—0.2% Triton X-100—10 mM Tris-HCl pH 7.5 during 1 h at 4°C. After centrifugation at 105,000*g* for 1 h at 4°C, the supernatants were dialyzed against TALP and used for affinity chromatography.

The affinity chromatography column used was prepared by coupling of a fraction of boar sperm enriched in components from the periacrosomal and acrosomal external membranes to Sepharose CL 4B [Marini and Cabada, 2003]. Oviductal cell membrane extracts prepared as indicated above were applied and, after washing, elution was achieved with 0.5 M NaCl—10 mM Tris-HCl pH 7.5. Only one protein peak was found on the bound fraction by measure of absorbance at 280 nm. Purification of the native form of SBG was confirmed by SDS–PAGE on 5–12% polyacrylamide gradient gels according to Laemmli [1970]; followed by silver staining (PlusOne™ Silver Staining Kit, GE Healthcare).

Acrosomal Status Analysis

Acrosomal status was examined by Wells and Awa [1970] staining. Dye solutions contained 2.9% sodium citrate, 95% ethanol, 1% Eosin B, and 1% Fast green FCF. Ten min before use, 1.7 parts ethanol, 1.4 parts fast green FCF, and 0.7 parts Eosin B were mixed.

Sperm suspensions were fixed for 15 min in 0.3% formol solution and centrifuged for 5 min at 8,700*g*. The supernatant was removed and sperm were resuspended in 1 ml 0.9% NaCl. Sperm suspension was centrifuged again at 8,700*g* for 5 min. Pellets were resuspended in 25 μl of sodium citrate and 50 μl aliquots of stain were added to each sample. After 10 min, a 10 μl aliquot of dye was smeared onto a slide and dried. Sperm were observed at magnification of 100× by bright field microscopy. Acrosome-intact sperm were considered to be those having a thickened blue-green region at the apex of the head, a blue-green cap covering the anterior two-thirds of the head and a pink color in the posterior one-third of the head (Fig. 1C, arrow head). A sperm was considered to have lost its acrosome when the apical thickened or cap was lost (Fig. 1D). Different patterns of acrosome alterations were observed as described in Results Section and shown in Figure 1. The number of sperm with altered acrosomes (AA) was estimated as the percentage of spermatozoa with any of this alterations or lacking the acrosome. At least 100 spermatozoa were scored in each experiment. Each value represents the mean of three replicates from different boars.

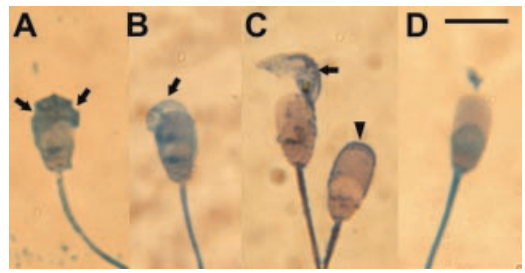

Fig. 1. Acrosome alterations produced by SBG. Micrographs of boar spermatozoa incubated in capacitating conditions in the presence of SBG and stained by Wells–Awa technique. (**A**): Irregularities at the edge of the periacrosomal region, (**B**) swelling of the acrosome, (**C**; arrow) periacrosomal membranes detached from the rest of the cell, (C; arrow head) sperm with intact acrosome. (**D**) Acrosome reacted sperm. Arrows show acrosomal alterations. Scale bars represent 10 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Evaluation of the Effect of SBG on Spermatozoa Under Capacitating and Noncapacitating Conditions

Sperm were suspended in TALP capacitating medium or variations of it lacking $CaCl_2$ or $NaHCO_3$ or both, which are considered non-capacitating media, according to experimental requirements. Media were supplemented with the indicated amounts of SBG (0, 100, 150 μg/ml) and sperm incubated at 37°C in a 5% CO_2 atmosphere. At the times stated in each case acrosomal status was examined by Wells–Awa staining as described, viability was analyzed by eosin–nigrosin staining method and motility was subjectively evaluated using a light microscope. Vitality was between 50% and 55% after 1 h of incubation and at least of 40% after 2 h of incubation.

Evaluation of the Effect of SBG on Previously Capacitated Spermatozoa

Sperm were suspended in TALP medium and incubated at 37°C in a 5% CO_2 atmosphere for 1 h. We have previously established that these conditions produce 15.5 ± 4.0% of pattern B

(capacitated) sperm as determined by chlortetracyclinestaining[Dapinoetal.,2006].Aliquotswere withdrawn and SBG (100 and 150 μg/ml), fetuin (150 μg/ml), or 1 μg/ml progesterone were added. Incubationwasfollowedandafter15and30minAA was determined by Wells–Awa staining as described before. An aliquot without any addition was also analyzed at each time as a control. Sperm viability was analyzed by eosin–nigrosin staining method, and was at least of 50% at the end of the experiment for every condition tested.

Detection of SBG Binding Sites

Sperm were incubated in TALP in the presence of 150 μg/ml of SBG for 1 h at 37°C. Suspensions were smeared on glass slides optimized for immunohistochemistry (Frosted HiFix^{NH}, TNT, Argentina). The slides were gently rinsed with PBS twice, blocked with 2% bovine serum albumin—0.2% tritón in PBS for 60 min and then treated with the primary antibodies overnight at 4°C. Antibodies were polyclonal anti-SBG (1:50) [Perez et al., 2006]. After being rinsed twice with PBS, the slides were treated with Cy3 conjugated anti-rabbit immunoglobulin (Chemicon International, Inc.) (1:750) for 60 min. After rinsing twice with PBS, the slides were covered with 0.22 M 1, 4-diazabicyclo [2,2,2] octane (Sigma-Aldrich) dissolved in glycerol/PBS (9:1) and cover slips. The preparations were examined under a microscope equipped with epifluorescence (BH 2, Olympus Optical Company Ltd, Tokyo, Japan).

Detection of Phosphotyrosine Containing Polypeptides

Sperm were incubated for 1 h at 37°C in a 5% CO_2 atmosphere in the following media: TALP lacking $CaCl_2$ and $NaHCO_3$, TALP capacitating medium, TALP containing 150 μg/ml SBG, TALP containing 150 μg/ml fetuin. Additionally, a sample was incubated in TALP capacitating media for 1 h at 37°C in a 5% CO_2 atmosphere and progesterone (1 μg/ml) was added, incubation was followed for further 15 min. Aliquots (1×10^6 sperm) from each treatment were used for protein separation on 12% SDS–PAGE and proteins transferred to nitrocellulose membranes (Amersham Biosciences, Arg.) using Miniprotean 3 System (Bio-Rad, Hercules, CA). Transferred proteins were visualized in the membrane by Pounceau S staining. Nonspecific binding sites were blocked by incubation with 5% dry nonfat milk in TBS (25 mM Tris-HCl, pH 7.4, 150 mM NaCl). Membranes were treated with anti-phosphotyrosine monoclonal antibodies (clone 4G10, Upstate, Lake Placid, NY) for 1 h at 1:2,000 in TTBS (TBS plus 0.5% Tween-20). After washing (three times for 10 min each), the blot was incubated with peroxidase-conjugated goat anti-mouse antibodies at 1:2,000 in TTBS for 60 min and washed again. Labeled proteins were revealed using enhanced chemiluminescence detection with ECL Kit (Amersham Biosciences, UK) according to manufacturer's instructions.

Statistical Analysis

Percentages of spermatozoa with AA were subjected to two-way analysis of variance (ANOVA). When *F*-test results were significant in ANOVA, individual means were further tested by Tukey's multiple range test [Motulsky, 1995].

RESULTS

Effect of SBG on Sperm Morphology

To examine the effect of SBG over sperm in capacitating conditions, spermatozoa were incubated in TALP medium containing 150 μg/ml of glycoprotein, and after 1 h of incubation sperm morphology was examined as described in Materials and Methods Section. Only few ($6 \pm 3\%$) sperm showed intact acrosomes (Fig. 1C, arrow head). Complete acrosome loss (Fig. 1D) was noted for $4 \pm 3\%$ of the sperm population. Morphological alterations were observed in $93 \pm 2\%$ of sperm. The number of sperm with AA was estimated as the percentage of spermatozoa with any of these alterations plus those lacking the acrosome. These alterations consisted mostly of irregularities at the edge of the periacrosomal region and apparent loss of portions of membrane (Fig. 1A). At a least extent sperm showed swelling of the acrosome (Fig. 1B). In some sperm periacrosomal membranes seemed to be detached from the rest of the cell (Fig. 1C, arrow). These patterns were rarely observed when sperm were incubated in the same conditions but in the absence of SBG (less than 1% of the population, data not shown) and differ from the complete lack of acrosome seen when physiological acrosomic reaction occurs (Fig. 1D). The rates of the alterations are stated in Table I. At the end of the incubation in

TABLE I. Rate of Different Acrosomal Alterations by SBG Over Sperm Incubated in Capacitating Condition

Irregularities and loss of portions of periacrosomal membrane	74.0 ± 1.73
Swelling of acrosome	16.6 ± 1.85
Periacrosomal membranes detached	1.0 ± 0.33
Lack of acrosome	4.6 ± 0.98
Intact acrosome	6.6 ± 0.33

Values are expressed as mean ± SEM.

the presence of SBG motility was completely suppressed.

Effect of SBG on Capacitating Sperm

The time and dose effect of SBG on acrosome morphology was analyzed. To this aim, sperm suspensions were incubated in TALP in the presence of different concentrations of SBG and samples were withdrawn at different time spans to test for acrosomal status examination by modified Wells–Awa staining as described in Materials and Methods Section. In the presence of SBG different patterns of acrosome alterations were observed as shown in Figure 1 and described in the previous section. The number of sperm with AA was estimated. Sperm lacking the acrosome were considered to have undergone spontaneous acrosomic reaction and represented 4 ± 3%.

The proportion of sperm that underwent spontaneous acrosomic reaction, and thus AA, increased with time in TALP capacitating medium in the absence of SBG (Fig. 2). In the presence of both concentrations of SBG tested

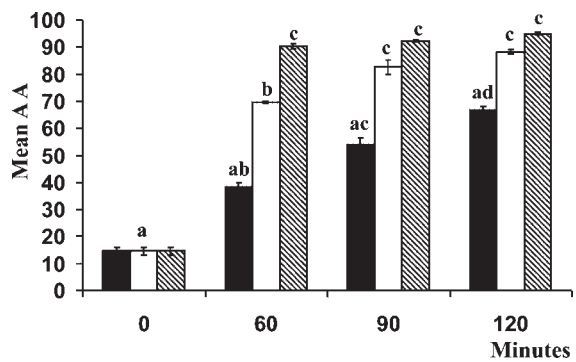

Fig. 2. Rate of acrosomal alteration produced by SBG over sperm incubated in capacitating conditions. Boar sperm were incubated in TALP for 1 h and the acrosomal state determined by Wells–Awa technique. AA includes sperm showing any of the acrosomal alterations shown in Figure 1 plus acrosome reacted sperm. AA is expressed as mean ± SEM, values with different letters are significantly different, n = 3. Incubation was in TALP media containing 0 (■), 100 μg/ml (□) or 150 μg/ml (▨) of SBG.

the fraction of sperm with AA was significantly greater than in the control at all the times tested (Fig. 2). At 1 h of incubation the proportion of AA sperm changed significantly ($P < 0.001$) with SBG concentration (Fig. 2). At longer incubation times the percentage of sperm with AA was greater for the higher SBG concentration, although not at a significant extent (Fig. 2).

Calcium and Bicarbonate Requirement for SBG Effect

In order to establish which components of TALP medium are required for SBG to affect sperm, experiments were done incubating spermatozoa in TALP medium lacking Ca^{2+} and HCO_3^- and containing different concentrations of SBG for up to 1 h. This time was chosen, as dose dependent significant results were observed in the previous section. As expected (Fig. 3A) no acrosome reaction was detected in this medium, as sperm are unable of capacitation and thus, of spontaneous acrosomic reaction [Jaiswal et al., 1998]. The presence of SBG did not produce any effect (Fig. 3A).

To examine if Ca^{2+} or HCO_3^- alone are needed in the media for SBG to alter the acrosome, sperm were incubated in TALP medium lacking each of them and containing different amounts of SBG for up to 1 h. Acrosome integrity was examined at different time spans. In the absence of Ca^{2+} no effect due to the presence of SBG was noted (Fig. 3B). In absence of HCO_3^- and presence of Ca^{2+}, a slight increase of spontaneous acrosome reaction was noted with time, regardless of the presence of SBG (Fig. 3C).

Effect of SBG on Previously Capacitated Sperm

As capacitating conditions, and not the presence of calcium or HCO_3^- alone, seem to be required for SBG to alter sperm acrosomes, the effect of the glycoprotein over previously capacitated sperm was examined. After capacitation of sperm in TALP medium for 1 h, SBG was added and the percentage of sperm with AA determined after further 15 and 30 min of incubation. When SBG was added after capacitation of sperm, an increase in the percentage of AA was observed for both concentrations used, at both times tested ($P < 0.001$; Fig. 4). As a control, sperm without SBG addition were further incubated for the same times. These showed only spontaneous

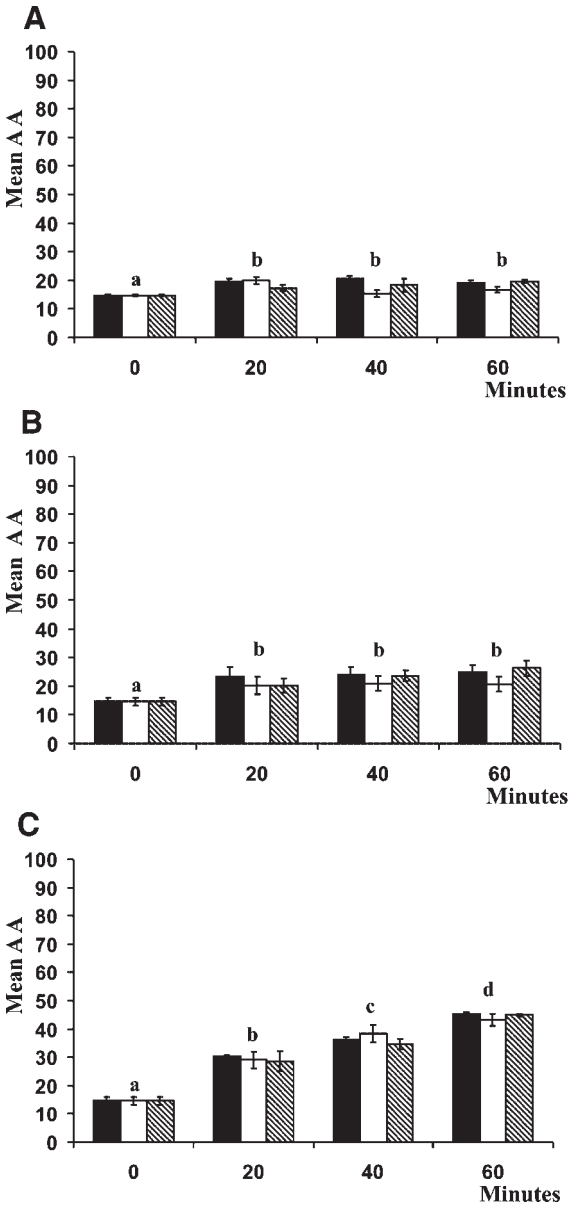

Fig. 3. Proportion of acrosomal alterations produced by SBG over boar sperm in noncapacitating conditions. AA includes sperm showing any of the acrosomal alterations shown in Figure 1 plus acrosome reacted sperm, as determined by Wells–Awa staining. AA is expressed as mean ± SEM, values with different letters are significantly different, n = 3. Media contain 0 (■), 100 μg/ml of SBG (□) or 150 μg/ml of SBG (▨). (**A**) TALP lacking Ca^{2+} and HCO_3^- ($P < 0.001$), (**B**) TALP lacking Ca^{2+} ($P < 0.0029$), (**C**) TALP lacking HCO_3^- ($P < 0.001$).

acrosome reaction (Fig. 4). Fetuin was added to an aliquot which was further incubated in the same conditions. This protein was used as a specificity control as the carbohydrates it exposes are different from Galβ1-3GalNAc, it has been reported not to inhibit sperm binding to oviductal epithelium explants [Wagner et al., 2002], and it does not bind to intact sperm [Marini and Cabada, 2003]. Fetuin did not produce any effect over acrosome integrity (Fig. 4).

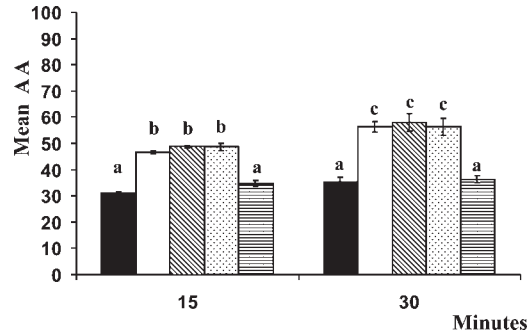

Fig. 4. Effect of SBG on previously capacitated boar sperm. Sperm were incubated in TALP capacitating medium for 1 h and 100 μg/ml of SBG (□), 150 μg/ml of SBG (▨), 1 μg/ml progesterone (⊞) or 150 μg/ml of fetuin (▤) were added. (■) Indicates TALP medium without further addition. Acrosomal status was examined by Wells–Awa technique. AA includes sperm showing any of the acrosomal alterations shown in Figure 1 plus acrosome reacted sperm AA is indicated as mean ± SEM. Values with different letters are significantly different, n = 3 ($P < 0.001$).

Another aliquot was treated with progesterone in the same conditions. This hormone is considered a physiological inducer of acrosome reaction, and triggered acrosome reaction at the same extent as did SBG for both times tested (Fig. 4).

Protein Phosphorylation in the Presence of SBG

Protein phosphorylation is one of the signals detected on mammalian sperm upon capacitation and acrosome reaction. Thus, the effect of SBG on protein tyrosine phosphorylation pattern was examined. Sperm were incubated in TALP capacitating medium and in TALP medium lacking Ca^{2+} and HCO_3^-, and the phosphotyrosin containing proteins from 10^6 sperm analyzed by western blotting followed by detection with anti-phosphotyrosine monoclonal antibodies (Fig. 5). When sperm were incubated for 1 h in TALP capacitating medium an increase in the phosphorylation of sp32 was observed (Fig. 5A, lane 3) as compared with nonincubated sperm (Fig. 5 A, lane 1) or sperm incubated in noncapacitating medium (Fig. 5 A, lane 2), as previously reported by Dube et al. [2005]. When sperm were incubated in TALP in the presence of SBG a remarkable increment in the phosphorylation of a protein of apparent molecular mass 97 kDa (p97) was detected (Fig. 5A, lane 5). This effect was not noted in

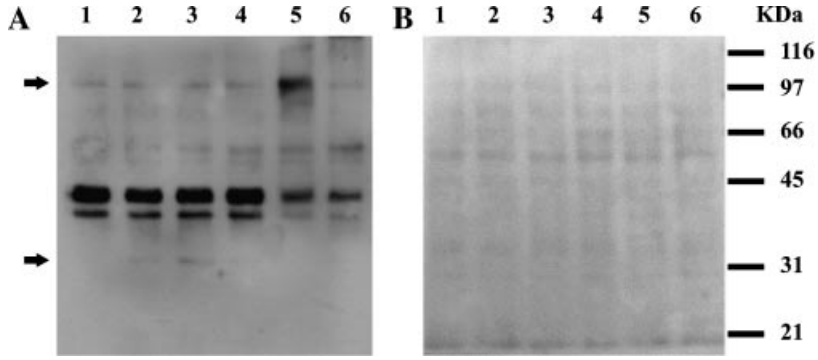

Fig. 5. Phosphotyrosine content of proteins from SBG treated sperm. Proteins from 1×10^6 sperm were used for SDS–PAGE and blotted on nitrocellulose membrane. Proteins were (**A**) assayed for immunoreactivity to anti-phosphotyrosin antibody and (**B**) observed by Pounceau Red staining. Sperm were incubated for 1 h in: **lane 2**—TALP without Ca^{2+} and HCO_3^-, **lane 3**—TALP, **lane 4**—TALP with 150 μg/ml fetuin, **lane 5**—TALP with 150 μg/m SBG. **Lane 6** corresponds to sperm incubated in TALP for 1 h and for 15 min more after the addition of 1 μg/ml progesterone. **Lane 1**—shows sperm without treatment. The arrows indicate polypeptides of apparent molecular mass 32 kDa (sp32) and 97 kDa (p97).

any other condition tested, and was not due to a difference in protein content, as confirmed by Pounceau Red staining of the membrane (Fig. 5B). Incubation in TALP containing fetuin was used as a specificity control and showed no effect (Fig. 5A, line 4). As SBG affects acrosome morphology, a sample in which acrosomic reaction was induced with progesterone was added. In this sample no increase on the phosphotyrosin level of p97 was noted (Fig. 5A, lane 6).

Localization of SBG Binding Sites on Sperm

We have previously reported the binding of SBG to the head of sperm [Marini and Cabada, 2003]. However, we had not established if SBG binding sites remain available when sperm change their physiological state. After incubation of sperm in TALP containing 150 μg/ml SBG for 1 h, SBG was localized by immunodetection with specific polyclonal antibodies [Perez et al., 2006].

Sperm with fluorescence covering the entire periacrosomal region (Fig. 6A) or presenting fluorescent stained patches on the periacrosomal region (Fig. 6B) were observed. The latter represent $86.1 \pm 2.02\%$ of the population. Controls were performed using only secondary antibody and with sperm incubated in TALP in absence of SBG. Under these conditions no fluorescence was detected (Fig. 6C).

DISCUSSION

The interaction between the sperm and the oviduct results in a mechanism by which some

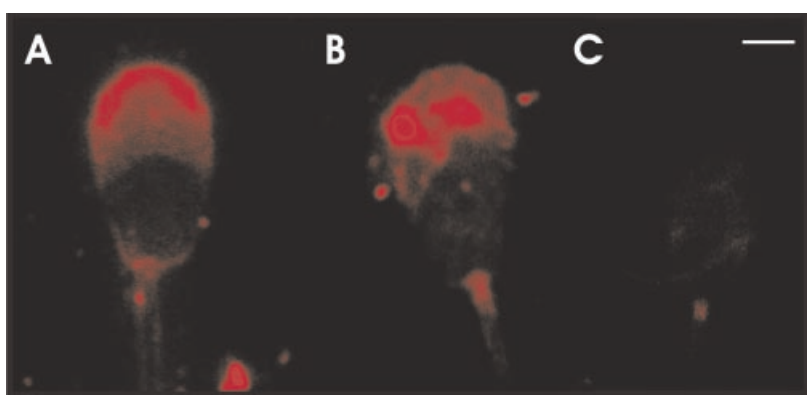

Fig. 6. Labeling of porcine sperm processed for the immunolocalization of bound SBG. (**A**), (**B**) Micrograph of the different patterns observed after spermatozoa were incubated for 1 h in TALP medium containing SBG (150 mg/ml) and revealed with anti-SBG polyclonal antibodies, followed by Cy3 conjugated anti-rabbit immunoglobulin. The pattern shown in (B) represents $86.1 \pm 2.02\%$ of the sperm population. (**C**) Incubation in TALP without SBG. Scale bar represents 5 mm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

sperm can be stored in the oviduct for several days and allows the selection of sperm with certain qualities. The role of oviductal secretions in sperm function has been extensively studied [Killian, 2004; Tardif et al., 2004], and the influence of oviductal cells on sperm viability and capacitation has been established for several species [Suarez, 1987; Pollard et al., 1991; Lefebvre and Suarez, 1996; Ellington et al., 1999; Fazeli et al., 1999; Gualtieri and Talevi, 2000; Scott, 2000]. However, the identity of the oviductal cell components responsible for sperm adhesion is still unknown. Recently, efforts have been made to define the oviductal cell surface proteome [Sostaric et al., 2006] in order to provide a basis to the understanding of the processes that take place at the oviduct. To our knowledge, the only protein from oviductal epithelial cells that has been shown to interact with sperm is SBG. This is a sperm binding glycoprotein isolated from plasma membrane enriched extracts of pig isthmus oviductal epithelial cells, based on its capacity of association to sperm periacrosomal membranes [Marini and Cabada, 2003]. It localizes to the apical surface of epithelial cells near the lumen of the oviduct [Perez et al., 2006] and exposes the O-linked disaccharide Galβ1-3GalNAc, which may bind to spermadhesins AWN [Dostalova et al., 1995], AQN-1 and AQN-3 [Calvete et al., 1996]. This facts make it tempting to propose that SBG's interaction with one or several of these spermadhesins is partly responsible for the interaction between oviductal cells and sperm in pig.

Adhesion to the oviduct occurs for a selected sperm subpopulation [Talevi and Gualtieri, 2004]. Kinetic studies suggest that sperm–oviduct interaction may develop in three main modes: selection of spermatozoa, maintaining a "low capacitation" state within a certain time window, and promotion of capacitation [Petrunkina et al., 2001b; Talevi and Gualtieri, 2004]. To examine the possible function of SBG in sperm–oviduct interaction, the morphology of spermatozoa incubated under capacitating conditions in the presence of the glycoprotein was analyzed. The presence of SBG produced time and dose dependent alteration of the acrosome (Fig. 2), visualized as swelling of the acrosome and detachment of the periacrosomal membrane (Fig. 1). This effect was not due to osmotic changes by the presence of SBG, as the osmolarity of the media was controlled and sperm incubated in noncapacitating media containing SBG showed no effect (Fig. 3). Incubation in the presence of SBG also produced suppression of motility, as has been reported for proteins derived from oviductal epithelial cells plasma membranes, as part of a sperm selection mechanism [Satake et al., 2006].

SBG binding to the head of intact acrosomes has already been reported [Marini and Cabada, 2003]. However, the distribution of SBG binding sites on sperm undergoing capacitation or acrosomic reaction had not been established. In these processes a redistribution of certain proteins and carbohydrates along the head of sperm has been reported [Kan and Esperanzate, 2006]. Immunodetection established that SBG is bound to sites located at the periacrosomal membrane of sperm even after acrosome alteration has occurred (Fig. 6). The proportion of sperm with SBG bound as patches is coincident with the rate of sperm with acrosomal alterations produced by SBG. It might be that SBG binds to specific sites in the periacrosomal membrane and remains bound even after acrosomal alteration occurs or, alternatively, that SBG binding sites located at the periacrosomal region stay at their locations and in a conformational state that allows SBG binding even after acrosome alteration takes place. Whichever alternative, SBG binding does not seem to be the limiting step for SBG to affect acrosome morphology. To examine the requirements for SBG to alter acrosomes, experiments where done in the absence of Ca^{2+} and HCO_3^-, which are noncapacitating conditions. In this medium no effect of SBG on acrosomes was observed (Fig. 3A). To analyze the possibility that SBG requires Ca^{2+} or HCO_3^- to affect the acrosomes the assays were repeated in the absence of each of these compounds. Again no effect of SBG over acrosome integrity was observed (Fig. 3B,C). This could lead to the conclusion that SBG requires both inorganic compounds for its effect or, more probable, that only sperm that had begun the capacitation process can be affected by SBG.

When SBG was added to previously capacitated boar sperm, it promoted AA at the same extent as the acrosomic reaction inducer progesterone (Fig. 4). The effect is specific for SBG as fetuin, used as a control, produced no AA. Progesterone hormone is currently used as a physiological inducer of acrosome reaction in vitro and is thought to be related to sperm

physiological state in the female tract in vivo [Jaiswal et al., 1999]. Progesterone, as well as zona pellucida, induce acrosomal exocytosis through pathways that involve protein tyrosine phosphorylation at least in mouse [Murase and Roldan, 1996], stallion [Rathi et al., 2003], and humans [O'Toole et al., 1996]. In pigs, an increment in protein tyrosine phosphorylation has been detected upon capacitation [Dube et al., 2005]. And, when acrosomic reaction is induced with calcium ionophore A23187 a significant decrease of tyrosine phosphorylation of proteins of apparent molecular mass 93, 175, and 220/230 is noted [Kalab et al., 1998].

When the effect of SBG on protein tyrosine phosphorylation was examined an increase on the phosphorylation of a protein of apparent molecular mass 97 kDa was noted (Fig. 5). This protein is not SBG itself, as the apparent molecular mass of this glycoprotein is greater than 220 kDa [Marini and Cabada, 2003]. Tyrosine phosphorylation of a 97 kDa protein from pig sperm has been detected in capacitating medium after prolonged (4.5 h) exposition to a cAMP–analogue combined with a nonspecific phosphodiesterase inhibitor [Tardif et al., 2004]. Enhancement of the phosphotyrosilation of the same protein was seen upon treatment with PK-A specific inhibitor H-89 [Tardif et al., 2004]. As the phosphotyrosin containing protein described by Tardif et al. [2004], and the one influenced by SBG show the same apparent molecular mass, it should be interesting to determine whether tyrosine phosphorylation enhancement of the 97 kDa polypeptide by SBG is related to cAMP and to the PKA pathway. Experiments are actually being done in our laboratory to examine this possibility.

As SBG produces modifications in the phosphorylation of pig sperm proteins, its effect seems to involve signaling processes. Phosphoryation of the 97 kDa protein is probably a step of the mechanism of action of SBG over sperm. Interestingly, an enhancement of the phosphorylation of a protein of similar apparent molecular mass (95 kDa) that binds to ZP has been detected upon human [Naz et al., 1991], and mouse [Leyton and Saling, 1989] acrosomal exocytosis. This protein was not identified in rat nor rabbit sperm [Naz et al., 1991] and, to our knowledge, has not been subject of molecular cloning.

Considering that porcine ZP exposes galactose [Jaiswal et al., 1999; Yonezawa et al., 2005] as does SBG, and human ZP binding to sperm promotes tyrosine phosphorylation of a 95 kDa protein and acrosome alteration, as does SBG, it is tempting to propose that in pig SBG interacts with sperm that undergo capacitation in a similar manner to that of ZP with human sperm.

SBG localizes to the lumen of the oviduct [Perez 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]. This glycoprotein produces alteration of pig sperm acrosome integrity and suppression of motility. It binds specifically to boar periacrosomal membranes of capacitating sperm. The AA effect is detected only for spermatozoa that have began the capacitation process and involves tyrosine-phosphorylation of a 97 kDa protein, as has been seen for a protein of similar molecular mass upon physiological acrosome exocytosis in rats and human. Altogether these results suggest that SBG may interact with sperm that have begun capacitation when they arrive to the oviduct, and produce acrosome rupture. These sperm would not be able to reach the ampulla or form the reservoir, as only uncapacitated sperm are attached [Fazeli et al., 1999]. Alternatively, SBG may be involved in sperm attachment to the oviduct, and produce alteration of acrosomes in a sperm-subpopulation specific manner that may involve calcium and bicarbonate microenvironment availability. These sperm would probably become nonviable due to membrane rupture. Thus, SBG is probably one of the oviductal components involved in the sperm selection function.

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