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The Proacrosin Binding Protein, sp32, Is Tyrosine Phosphorylated During Capacitation of Pig Sperm

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ABSTRACT: Mammalian sperm must undergo capacitation, a preparation period in the female reproductive tract or in vitro, in order to fertilize. We have previously described a Mr 32 000 tyrosine phosphorylated protein, ''p32,'' that appears in pig sperm during capacitation. The identity of p32 remains unknown; if and how it is involved during capacitation is not understood. The objective of the present study was to identify p32 by proteomic techniques. Western blotting of proteins separated successively under nonreducing and then reducing conditions showed the appearance of the tyrosine phosphorylated p32 only when sperm were incubated in capacitating conditions. The spot was sequenced by mass spectrometry/mass spectrometry and identified as ''sp32,'' a protein implicated in proacrosin maturation. The same membranes probed with anti-sp32 antibody demonstrated that sp32 is present in both noncapacitating and capacitating conditions and revealed exactly the same spot as p32. Immunoprecipitation with either anti-phosphotyrosine or anti-sp32 antibody corroborated these results. Indirect immunofluorescence with anti-phosphotyrosine antibody or anti-sp32 antibody show similar labeling of capacitated sperm, supporting the hypothesis that p32 is a tyrosine phosphorylated form of sp32. After ionophore treatment to induce the acrosome reaction, anti-sp32 and anti-phosphotyrosine labeling on the acrosome disappeared. These results demonstrate that sp32, a (pro)acrosin binding protein, is the p32, a tyrosine phosphorylated protein related to capacitation. We will now focus on the significance of tyrosine phosphorylation on sp32 function during fertilization-related events.

Key words: Fertilization, p32, acrosin, acrosome reaction.
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To fertilize the oocyte, sperm must undergo ''capacitation,'' defined as the biochemical molecular modifications experienced by the sperm during their transit through the female reproductive tract (Austin, 1951; Chang, 1951) or during incubation in appropriate media in vitro (Yanagimachi, 1994). Capacitated sperm penetrate the cumulus oophorus and bind to and finally penetrate the zona pellucida of the egg. To penetrate the zona, sperm must undergo exocytosis of the acrosome, or the acrosome reaction (Florman and Storey, 1982).

Capacitation is regulated by several specific signal transduction pathways (Visconti et al, 1995a; Baldi et al, 2002) involving sperm protein phosphorylation (Visconti and Kopf, 1998; Tardif et al, 1999). In various species, such as the mouse (Visconti et al, 1995b), human (Leclerc et al, 1996), bovine (Galantino-Homer et al, 1997), stallion (Pommer et al, 2003), and pig (Kalab et al, 1998; Flesch et al, 1999; Tardif et al, 2001), capacitation is associated with the tyrosine phosphorylation of sperm proteins. The identification of these tyrosine phosphorylated substrates, such as the calcium-binding tyrosine phosphorylation–regulated fibrous sheath protein CABYR (Naaby-Hansen et al, 2002) and the A-kinase anchoring protein AKAP3 (Ficarro et al, 2003) in human sperm; the pro-A-kinase anchoring protein pro-AKAP83 and the A-kinase anchoring protein AKAP83 (Jha and Shivaji, 2002) in hamster sperm; and the A-kinase anchoring protein AKAP82 (Carrera et al, 1994) in the mouse sperm, allows a better understanding of capacitation. However, the presence of these proteins in the flagellum indicates that they are more involved in motility changes during capacitation than sperm-zona binding or the acrosome reaction.

Recently, we reported that a tyrosine phosphorylated protein in porcine sperm, named ''p32,'' appears only during incubation under capacitating conditions (Tardif et al, 2001, 2003). We also described a tyrosine kinase, termed TK-32, in pig sperm (Tardif et al, 2001), which

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was then found to more likely be a dual-specificity kinase such as a MAPK (Tardif et al, 2003). The activity of this enzyme increased during sperm capacitation, and immunoprecipitation experiments clearly showed that ''p32'' and ''TK-32'' are likely to be two different proteins (Tardif et al, 2003). Therefore, the identity of the p32 tyrosine phosphorylated protein remains unknown. Moreover, if and how p32 is involved during the capacitation process at the molecular level is not understood. Our general hypothesis is that the appearance of the p32 tyrosine phosphorylated protein is implicated in boar sperm capacitation. The objective of the present study was to identify p32 by proteomic techniques. In this report, we establish that ''p32'' is, in fact, ''sp32,'' a binding protein implicated in (pro)acrosin maturation (Baba et al, 1989).

Materials and Methods

Chemicals

The research was conducted with the approval of the university committee for chemical safety. Monoclonal mouse anti-phosphotyrosine antibody (clone 4G10) and peroxidase-conjugated goat anti-mouse antibody and peroxidase-conjugated goat anti-rabbit antibody were obtained from Upstate Biotechnology (UBI, Lake Placid, NY). Affinity-purified anti-sp32 antibody was prepared as described previously (Baba et al, 1994). FITC-conjugated goat anti-rabbit antibody was from Biosource International (Camarillo, Calif), and CY™3-conjugated goat anti-mouse antibody was from Zymed Laboratories Inc (San Francisco, Calif). Protein G Sepharose™ 4 Fast Flow was obtained from Amersham Biosciences AB (Uppsala, Sweden). The protease inhibitor cocktail Complete™ was from Roche Diagnostics GmbH (Mannheim, Germany). Molecular weight markers were obtained from Amersham International (Oakville, Canada) and acrylamide N, N′-methylene bisacrylamide, ammonium persulfate, and tris (hydroxymethyl) aminoethane (Tris) were from Bio-Rad. Other chemical products were from the Sigma Chemical Company (St Louis, Mo).

Culture Media

The principal culture media used were based on Krebs Ringer Bicarbonate (Toyoda and Chang, 1974). Capacitating medium (CM) was composed of 4.8 mM KCl, 1.2 mM KH_2PO_4, 95 mM NaCl, 5.56 mM glucose, 25 mM $NaHCO_3$, 2 mM $CaCl_2$, 0.4% bovine serum albumin (BSA; type V, fatty acid-free), and 2 mM pyruvate, pH 7.4. The noncapacitating medium (NCM) was similar to CM but was without calcium, bicarbonate, and BSA (4.8 mM KCL, 1.2 mM KH_2PO_4, 95 mM NaCl, and 5.56 mM glucose, pH 7.4). The CM has been previously shown to support capacitation, whereas NCM acts as a negative control (Tardif et al, 2001).

Sperm Preparation

The research was conducted with the approval of the university committee for animal care. The sperm-rich semen fraction was collected from fertile boars at the Centre d'Insémination Porcine du Québec (St Lambert, Canada) and was transported to the laboratory at 16°C–18°C within 30 minutes, as previously reported (Tardif et al, 2001). The sperm-rich semen was diluted to 40×10^6 sperm/mL directly in either NCM, as a noncapacitating negative control, or in CM to induce capacitation. Sperm were then incubated at 38.5°C, the internal body temperature of the pig, in a humidified 5% CO_2 atmosphere.

Isolation of Pig Sperm Proteins

Sperm proteins from pig sperm were isolated essentially as described previously (Dubé et al, 2003). Briefly, aliquots (5×10^6 sperm) in either NCM or CM were taken before and during incubation at different times. Sodium orthovanadate (0.2 mM final concentration) was added, and the samples were centrifuged to obtain a sperm pellet (4 minutes, $16\,060 \times g$, room temperature), which was resuspended in sample buffer without 2-mercaptoethanol and heated for 1 minutes at 95°C. The sperm solution was recentrifuged (4 minutes, $16\,060 \times g$) and 2-mercaptoethanol (5%, final concentration) was added to the resulting supernatant. For nonreduced samples, the addition of 2-mercaptoethanol was omitted. At this point, samples were stored immediately at −80°C until the day of electrophoresis. The sperm protein samples were heated for 1 minute at 95°C. Sperm proteins were then ready for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE).

Immunoprecipitation

Sperm were solubilized by vortexing every 15 minutes (1 hour, 4°C) in lysis buffer (4% CHAPS; 50 mM Tris-HCl, pH 7.4; 0.2 mM Na_3VO_4; and protease inhibitor cocktail). Lysates were centrifuged, and 1–5 μg of either anti-sp32 antibody or anti-phosphotyrosine antibody were added to the supernatant. The sperm protein solution was incubated at 4°C overnight with gentle agitation. The immunocomplexes were sequestered by adding 100 μL protein G-coupled sepharose (Amersham Biosciences AB, Uppsala, Sweden) for 2 hours at 4°C. The immune complexes linked to the beads were collected by a quick spin (8 seconds, $16\,060 \times g$) and the supernatant was kept for acetone precipitation. The beads were washed 3 times with cold phosphate-buffered saline, resuspended in 2× sample buffer, heated for 2 minutes at 90°C, and centrifuged (5 minutes, $16\,060 \times g$). The supernatant, with or without 2-mercaptoethanol, was submitted to SDS-PAGE and immunoblotting.

SDS-PAGE and Western Blotting

As described in Dubé et al (2003), sperm proteins were subjected to SDS-PAGE, and separated proteins were electrophoretically transferred (overnight, 200 mA) to polyvinyldienne fluoride (PVDF) membranes (PerkinElmer Life Sciences, Boston, Mass). Nonspecific binding sites on the membrane were blocked with 5% dry nonfat milk in Tris-buffered saline (TBS: 25 mM Tris-HCl, 150 mM NaCl). Blots were incubated with anti-phosphotyrosine (1:20 000) or anti-sp32 antibodies (1:10 000) for 1 hour in Tween TBS (TTBS; 0.1% Tween 20, 25 mM Tris-HCl, 150 mM NaCl) at room temperature, washed 3 times for 10 minutes each time with fresh TTBS, and then incubated with peroxidase-conjugated goat anti-mouse antibody (1:3000) or peroxidase-

conjugated goat anti-rabbit antibody (1:3000) in TTBS for 45 minutes at room temperature. Membranes were washed again, 3 times for 10 minutes each time, with fresh TTBS. Labeled proteins were visualized using a chemiluminescence detection kit (ECL, Amersham) according to the manufacturer's instructions.

For some experiments, membranes were reprobed with another antibody. Therefore, the membranes were first stripped at 50°C for 20 minutes in a solution of 62.5 mM Tris-HCl (pH 6.8), 2% SDS, and 100 mM 2-mercaptoethanol to remove the original antibodies. The membranes were then washed in TBS 1×, blocked with 5% milk, and reprobed as described above.

Consecutive Nonreducing/Reducing SDS-PAGE

Sperm proteins were extracted in nonreducing conditions (without 2-mercaptoethanol) and separated by 1-dimensional 12% SDS-PAGE. At the end of the migration, the lane of interest on the gel was cut out using a scalpel and straight edge to slice from the well comb to the bottom (the adjacent wells were left empty to prevent contamination from neighboring proteins), then incubated under reducing conditions (sample buffer with 5% 2-mercaptoethanol) for 2 hours at room temperature with gentle agitation. The gel strip was layered in the application well made in the stacker of another 12% SDS gel for a second consecutive round of 1-dimensional SDS-PAGE under reducing conditions. Before and after placing the strip, 200 μL of reducing sample buffer were poured into the well. Electrophoresis was carried out at 200 V for approximately 45 minutes. Proteins were then transferred on PVDF membranes and probed with antibodies as described previously.

Identification by MS/MS

Proteins were visualized by Coomassie Blue staining of the gels after protein electrophoresis under nonreducing followed by reducing conditions. Selected spots of interest were excised from the gel and trypsin-digested for peptide mass spectrometric analysis using the mass spectrometry/mass spectrometry (MS/MS) technique (Zhu et al, 2003) at the Institute for Biomolecular Design, University of Alberta (Edmonton, Canada). Identification was performed by ''mass fingerprinting'' in a peptide mass computer data bank (http://www.matrixscience.com).

Indirect Immunofluorescence

Pig sperm were incubated in appropriate media at 40×10^6 sperm/mL, centrifuged (10 minutes, $270 \times g$), fixed with 2% formaldehyde for 1 hour, centrifuged again (10 minutes, $270 \times g$), and then blocked with 2% BSA overnight to eliminate nonspecific binding. Approximately 8×10^6 sperm were air dried onto Polysine™ microscope slides (Esco, Portsmouth, NH) and then permeabilized in anhydrous ethanol or not. Acrosome-reacted sperm were obtained by incubation with 10 μM of the nonfluorescent calcium ionophore Br-A23187 (Molecular Probes, Eugene, Oreg) for 60 minutes (Moos et al, 1993a). For double labeling, samples on slides were incubated for 1 hour with a mixture of anti-sp32 antibody and anti-phosphotyrosine antibody with 5% goat serum, washed in TBS, then coincubated for 1 hour with a mixture of the second antibody conjugated to FITC (green) and CY™3 (orange) diluted in TBS with 5% goat serum. One drop of 90% glycerol was added on the slide to limit photobleaching, and slides were mounted with coverslips. The sperm were examined by epifluorescence using a Nikon Eclipse E600 microscope equipped with fluorescence (FITC: maximal excitation: 495 nm, DM 510 filter; CY™3: maximal excitation: 550 nm, DM 400 filter; 40 × 0.75).

Results

Consecutive Nonreducing/Reducing SDS-PAGE Confirms p32 in Capacitating Conditions

The following results were obtained by pig sperm protein extraction under nonreducing conditions, separated by SDS-PAGE, submitted to reducing conditions, and then separated a second time by SDS-PAGE. This technique provides a direct means to identify proteins containing disulfide bonds (Yano et al, 2002) and to separate them. Figure 1 shows pig sperm proteins submitted to nonreducing/reducing SDS-PAGE, transferred to PVDF membranes, and hybridized with anti-phosphotyrosine antibody. Two bands of tyrosine phosphoproteins were present between Mr 44 000 and Mr 46 000 from sperm in either noncapacitating (Figure 1A) or capacitating conditions (Figure 1B). However, the incubation of pig sperm in capacitating conditions (Figure 1B) reveals the appearance on an additional band of approximately Mr 32 000, presumably p32 (Tardif et al, 2001, 2003; Dubé et al, 2003), that is not present in noncapacitating conditions (Figure 1A).

MS/MS Reveals that p32 Is sp32

To identify the spot appearing at Mr 32 000 on the nonreducing/reducing gel, it was excised and subjected to MS/MS. The matching of peptides by homology identified the protein as sp32, an acrosomal protein (Baba et al, 1989). Figure 2 shows the sequence of the sp32 precursor. The length of the sp32 precursor is 537 amino acids (www.expasy.ch/tools), with an estimated Mr of 60 539. The active protein sequence begins at amino acid 268 and ends at 537 and includes 7 phosphorylation sites on serine residues, 4 on threonine residues, and 3 on tyrosine residues (www.expasy.ch/tools). By MS/MS analysis, 8 peptides were matched with the entire sequence of sp32, all of which were found in the active part of the precursor (AA 268–537).

Immunoblotting the Same Membranes Indicates That p32 Is sp32

To confirm the relationship between ''p32'' and ''sp32,'' the same membranes used for the detection of tyrosine phosphorylated proteins (Figure 1) were stripped, blocked, and incubated with anti-sp32 antibody. The results (Figure 3) show the presence of ''sp32'' in either noncapacitating or capacitating conditions. As well, sp32

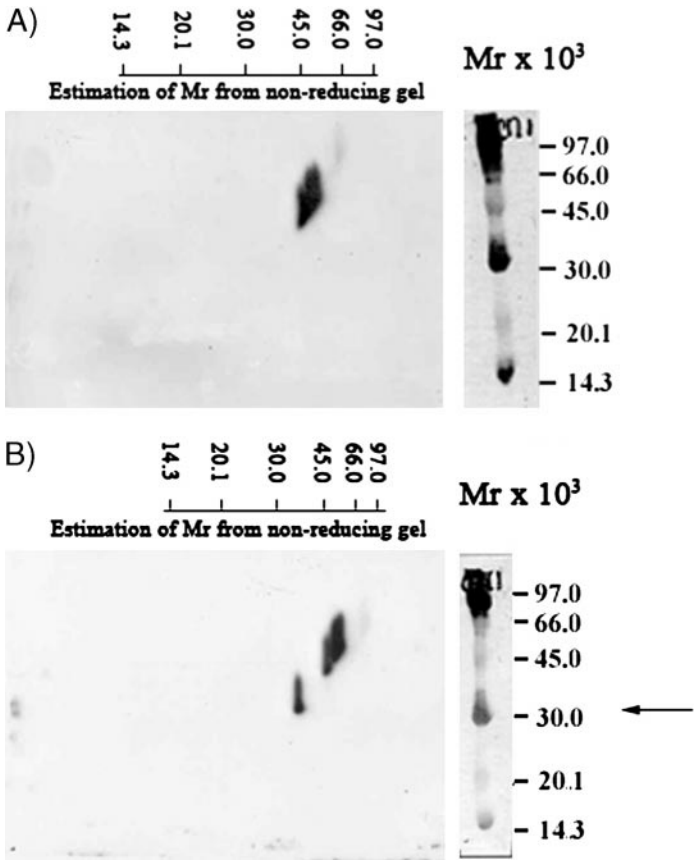

Figure 1. Western blot of boar sperm proteins labeled with anti-phosphotyrosine antibody. Fresh boar sperm were incubated in noncapacitating **(A)** and capacitating **(B)** conditions. Sperm proteins were subjected to 2 consecutive rounds of sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), with the first being under nonreducing conditions and the second under reducing conditions. Transfer to a polyvinyldienne fluoride (PVDF) membrane and immunoblotting were then performed. The molecular weight marker on the gel was Rainbow (Amersham). Since Rainbow is not visible on Western blots, the molecular weight marker shown is stained with Ponceau S directly on the membrane used for the Western blot. The tyrosine phosphoprotein p32 appears only in capacitating conditions **(B; arrow)**. This experiment was repeated 4 times, and representative blots are shown.

1 QLAAGSLLSL LKVLLLPLAP APAQDANSAS TPGSPLSPTE YERFFALLTP TWKAETTCRL

61 RATHGCRNPT LVQLDQYENH GLVPDGAVCS DLPYASWFES FCQFTQYRCS NHVYYAKRVR

121 CSQPVSILSP NSLKEVDTSS EVPITTMTSP VSSHITATGR QVFQPWPERL NNNVEELLQS

181 SLSLGGQEQG QEHKQEHKQE QGQEHKQDEG QEQEEQEEEQ EEEGKQEEGQ GTEESLEAMS

241 GLQADSEPKF QSEFVSSNPF SFTPRVR↓EVE STPMMMENIQ ELIRSAQEMD EMGDVYEEEN

301 IWRAQSPGSL LQLPHVDALL VLCYSIVENT **CVITPTAKAW QYLEDETLGF GKSVCDSLGR**

361 RHLAACSLCD FCSLK**LEQCH SETNLQR**QQC DNSHK**TPFIS PLLASQSMSI GTQIGTLK**SG

421 **RFYGLDLYGG LRMDFWCAR**L ATKGCEDNRV ASWLQTEFLS FQDGDFPTK**I CDTEYVQYPN**

481 **YCAFK**SQQCM MRNRDRKVSR MRCLQNETYT VLTQAK**SEDL VLR**WSQEFST LTLGQAG

Figure 2. Amino acid sequence of the sp32 precursor (http://www.matrixscience.com/). Amino acids are represented by standard single-letter codes. The split site of pro-sp32 into sp32 is indicated by an arrow between Arg267 and Glu268. Amino acids in bold represent the matched peptides sequenced by mass spectrometry/mass spectrometry (MS/MS).

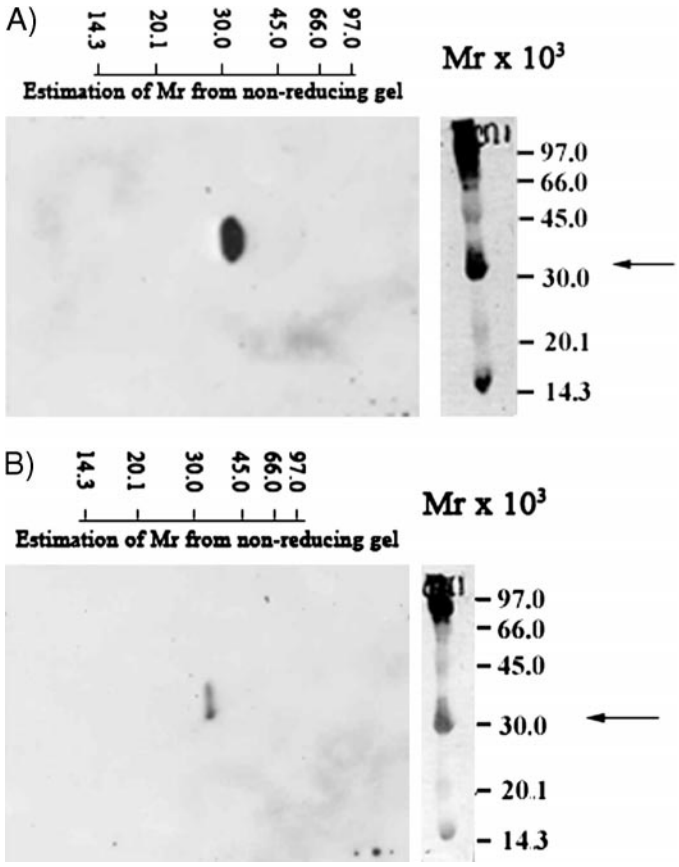

Figure 3. Western blot of boar sperm proteins labeled with anti-sp32 antibody. The identical polyvinyldienne fluoride (PVDF) membranes shown in Figure 2A and B were stripped, blocked, and rehybridized with anti-sp32 antibody. The molecular weight marker used on the gel was Rainbow (Amersham). Since Rainbow is not visible on Western blots, the molecular weight marker shown is stained with Ponceau S directly on the membrane used for the Western blot. The acrosomal protein sp32 appears in noncapacitating **(A)** and capacitating **(B)** conditions (arrows). This experiment was repeated 4 times, and representative blots are shown.

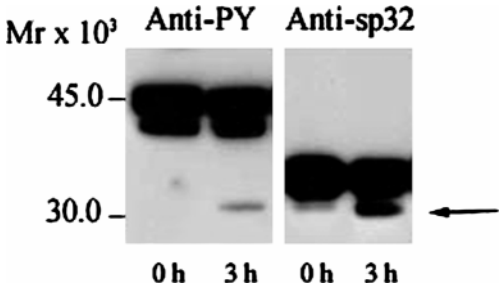

Figure 4. Western blots of boar sperm proteins labeled with anti-phosphotyrosine antibody and anti-sp32 antibody. Fresh boar sperm were incubated in capacitating conditions for 0 hours (precapacitation negative control), and 3-hour sperm proteins were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. Transfer to a polyvinyldienne fluoride (PVDF) membrane and immunoblotting were then performed. The tyrosine phosphoprotein p32 appears only in capacitating conditions (3 hours; arrow) and is detected on the same blot at both 0 hours and 3 hours by anti-sp32 antibody. This experiment was repeated 4 times, and a representative blot (probed first with anti-phosphotyrosine antibody then reprobed with anti-sp32 antibody) is shown.

appears to be more abundant in noncapacitating conditions (in all repetitions). Only small amounts of proteins on the membranes were evident by Ponceau S staining (not shown); however, similar levels were observed for both treatments, and this finding does not account for the relatively greater present of sp32 in noncapacitated vs capacitated sperm. Superposition of autoradiograph films obtained in Figures 1 and 3 indicates that ''p32'' is ''sp32,'' although the protein is tyrosine phosphorylated only when sperm are incubated under conditions supporting capacitation.

Figure 4 is a Western blot of total sperm extracts separated by SDS-PAGE. The sperm were incubated in CM for 0 or 3 hours to induce capacitation, and as reported earlier, p32 appears coincident with capacitation (Tardif et al, 2001, 2003). A Mr 44 000–46 000 doublet of tyrosine phosphorylated proteins is also present independent of the treatments in all samples, as previously observed (Tardif et al, 2001, 2003; Dubé et al, 2003). The same membrane used with anti-phosphotyrosine antibody was stripped, blocked, and incubated with anti-sp32 antibody

(Figure 4). The results showed that a Mr 32 000 protein is present in either noncapacitating or capacitating conditions. We observed that the intensity of the protein band present at Mr 32 000 in noncapacitating conditions seems to depend on the boar. An additional band of proteins was also detected by anti-sp32 antibody at approximately Mr 35 000.

Immunoprecipitation Corroborates That sp32 Is Tyrosine Phosphorylated in Capacitated Sperm

To confirm the results obtained in Figures 1 through 4, proteins from capacitated sperm were immunoprecipitated with anti-phosphotyrosine or with anti-sp32 antibodies. Figure 5A shows the Western blot of anti-phosphotyrosine and anti-sp32 immunoprecipitated proteins labeled with anti-phosphotyrosine antibody. The total extract in Figure 5A (lane 1) demonstrates again that p32 is tyrosine phosphorylated. Tyrosine phosphorylated proteins were immunoprecipitated as well by anti-phosphotyrosine antibody in the pellet (Figure 5A, lane 2), and some remained in the supernatant (Figure 5A, lane 3). When proteins are subjected to immunoprecipitation with anti-sp32 antibody, tyrosine phosphorylated proteins are detected in the pellet (Figure 5A, lane 4), but mostly in the supernatant (Figure 5A, lane 5), indicating that sp32 was immunoprecipitated as a tyrosine phosphorylated protein.

After stripping and rehybridizing the same membrane with anti-sp32 antibody (Figure 5B), the total extract (lane 1) revealed the presence of sp32 at the same molecular weight with relation to where the p32 appeared in Figure 5A. In addition, immunoprecipitation with anti-phosphotyrosine antibody detected with anti-sp32 antibody (Figure 5B, lanes 2 and 3) shows the presence of sp32 in the pellet (lane 2) and the supernatant (lane 3). As expected, immunoprecipitated proteins with anti-sp32 antibody are mostly found in the immunoprecipitate (lane 4). Consequently, those results confirm that sp32 is tyrosine phosphorylated and that sp32 and p32 are one and the same protein.

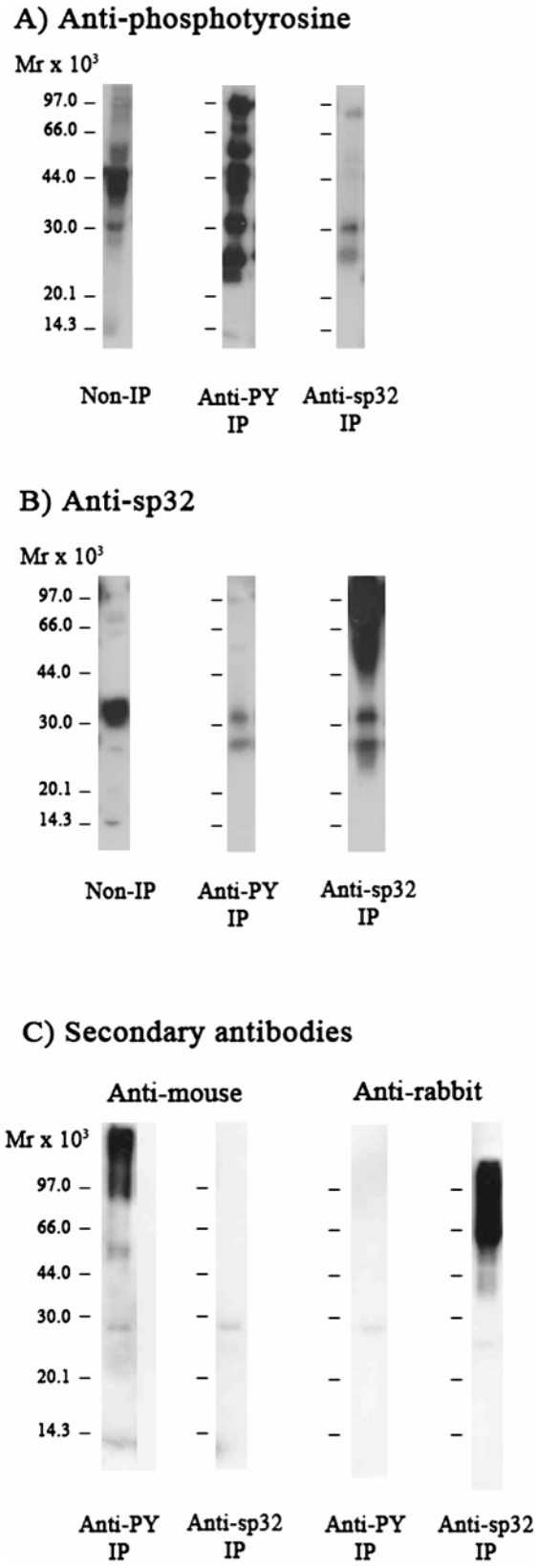

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Figure 5. Western blot of immunoprecipitated boar sperm proteins labeled with anti-phosphotyrosine antibody **(A)** and anti-sp32 antibody **(B)**. **Panel C** shows the controls with the secondary antibodies only. Fresh boar sperm were incubated in capacitating conditions. Sperm proteins were extracted (''Non-IP'') and immunoprecipitated with either anti-phosphotyrosine antibody (''Anti-PY IP'') or anti-sp32 antibody (''Anti-sp32 IP''). The same polyvinyldienne fluoride (PVDF) membrane in **(A)** was stripped, blocked, and rehybridized with anti-sp32 antibody for **B**. Note that sp32 is tyrosine phosphorylated, as **Panel A** (''Anti-sp32 IP'') shows that the anti-sp32 immunoprecipitate is tyrosine phosphorylated at approximately Mr 32 000, while **Panel B** (''Anti-PY IP'') shows that anti-sp32 recognizes an approximately Mr 32 000 protein in the antiphosphotyrosine immunoprecipitate.

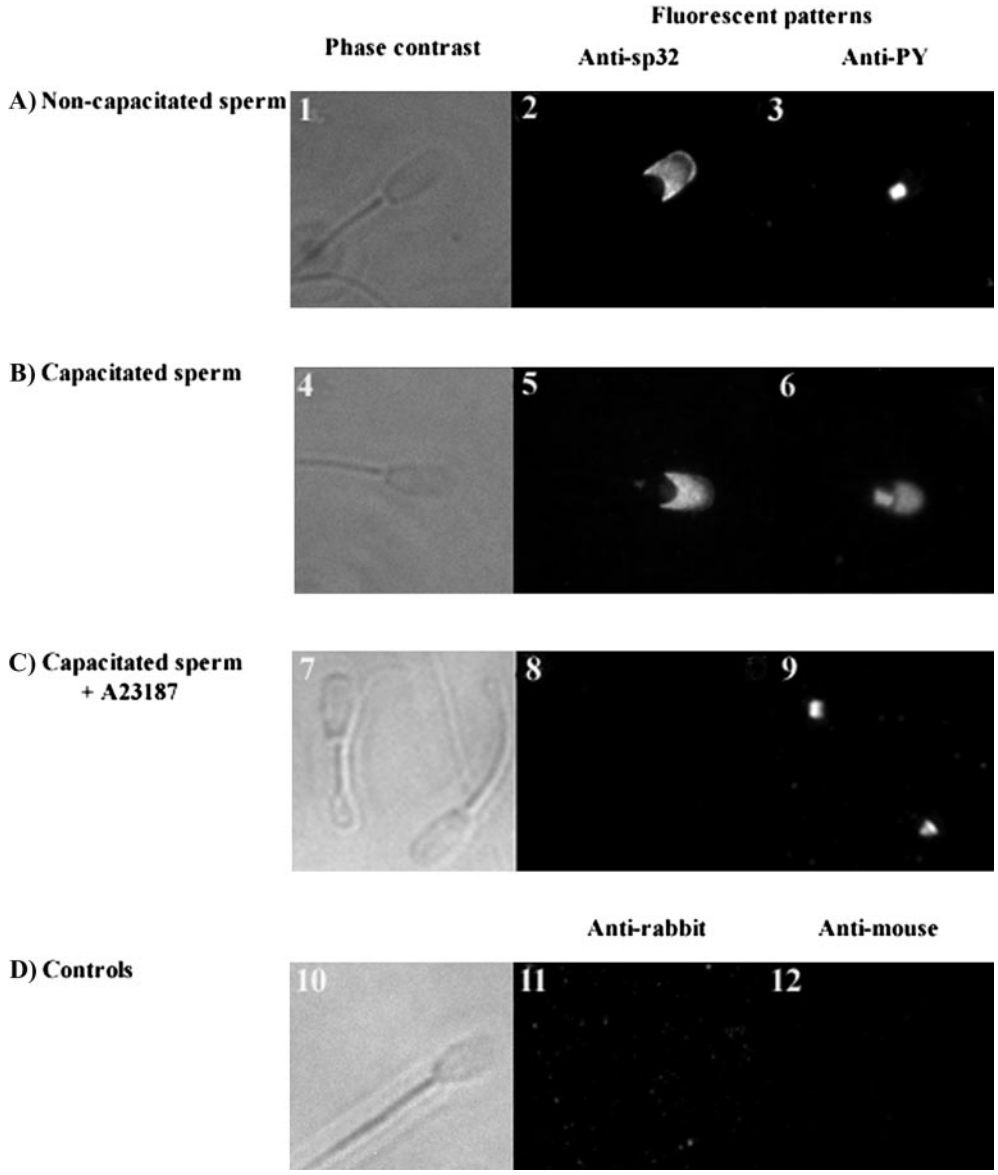

Figure 6. Indirect immunofluorescence of anti-sp32– and antiphosphotyrosine–double-labeled proteins in fixed porcine sperm. Sperm were incubated in noncapacitating conditions **(A)** and in capacitating conditions without **(B)** and with calcium ionophore A23187 **(C)** to provoke the acrosome reaction. Panels 1, 4, 7, and 10 are phase contrast, and fluorescent microscopy photographs of sperm are shown in Panels 2, 3, 5, 6, 8, 9, 11, and 12. The results showed labeling of both antibodies over the acrosomal region of capacitated sperm, which is consistent with the hypothesis that p32 is a tyrosine-phosphorylated form of sp32. Panels 8 and 9 show acrosome-reacted sperm. Secondary antibody only controls are presented in Panels 11 and 12. This experiment was repeated 4 times, and representative photographs are shown.

Localization of sp32 to the Acrosome

Figure 6 shows indirect immunofluorescence double labeling with anti-sp32 antibody and anti-phosphotyrosine antibody. As shown in Figure 6, sp32 is present on the acrosomal cap of spermatozoa, and the fluorescence is equally distributed. No signal is detected on the equatorial segment, the postacrosomal region, or on the flagellum. A similar pattern of anti-sp32 antibody fluorescence labeling was obtained when sperm were incubated in either noncapacitating or capacitating conditions (Panels 2 and 5) or when they were permeabilized (not shown). The anti-sp32 antibody labeling on the acrosome disappeared when the sperm were incubated in the presence of calcium ionophore A23187 to induce the acrosome reaction (Panel 8), confirming the localization of sp32 to the acrosome.

Sperm labeled with anti-phosphotyrosine antibody exhibited an intense spot of fluorescence on the equatorial segment in noncapacitating conditions (Panel 3); however, the fluorescence was displaced to the acrosomal cap after incubation in capacitating conditions (Panel 6), coincident with the sp32 labeling. When sperm were acro-

some reacted (Panel 9), only the spot of anti-phosphotyrosine antibody fluorescence on the equatorial segment remained, confirming the anti-sp32 antibody results. Controls incubated only with secondary antibodies validate our treatments (Panels 11 and 12).

Discussion

The discovery that sperm capacitation in all mammalian species studied to date is associated with the appearance of tyrosine phosphorylated proteins has been paramount in proving that capacitation is a regulated, signal transduction–mediated event. However, identification of these tyrosine phosphorylated proteins is incomplete, and their specific roles during capacitation are only poorly understood. We have previously shown that capacitation of pig sperm is associated with the tyrosine phosphorylation of p32, a protein of Mr 32 000 (Tardif et al, 2001, 2003; Dubé et al, 2003). Also, p32 tyrosine phosphorylation has also been reported in boar sperm by Flesch et al (1999) as a Mr 33 000 non–plasma membrane protein; this has also been reported by Green and Watson (2001) and more recently by Pyoung and Yi (2004) and Harayama et al (2004). However, the identity of p32 has remained unknown until the present report that p32 is the same protein as sp32, an acrosomal binding protein implicated in acrosin maturation. This study is the first to identify p32 as sp32.

Sequencing Reveals p32 to Be sp32

The p32 tyrosine phosphoprotein was isolated as described by Kalab et al (1994) using two consecutive series of SDS-PAGE, under nonreducing and reducing conditions, respectively (Figure 1). Amino acid sequencing of the peptides from p32 by MS/MS revealed 100% identity with the sp32 precursor (Figure 2), an acrosomal binding protein implicated in acrosin maturation (Baba et al, 1989). The identity of the putative sp32 was confirmed by rehybridizing the original membranes (Figure 1) with an anti-sp32 antibody (Figure 3). The presence of 3 tyrosine phosphorylation sites on the active form of sp32 is consistent with the results obtained with the anti-phosphotyrosine antibody. However, the molecular mass of the protein on the gel (Mr 32 000) indicates that the active sp32 is observed, not the precursor (Mr approximately 61 000). Moreover, the sp32 precursor is processed into the mature form sp32 in the testis during spermatogenesis (Baba et al, 1994). A protein of approximately Mr 35 000, detected by anti-sp32 antibody on Figure 4, is not present on Figure 3A and B. Presently we have no firm explanation about this protein detected with anti-sp32 antibody, but the difference between Figures 3 and 4 could be explained by different epitope conformations detected by anti-sp32 antibody due to the techniques. We are currently conducting 2-dimensional gel electrophoresis and Western blotting followed by sequencing to identify all proteins recognized by the anti-sp32 antibody.

The first report of a proacrosin binding protein was in 1977, when Polakoski and Parrish revealed that the proacrosin samples in their study were contaminated with a Mr 29 000 protein in boar sperm. Shortly thereafter, in 1978, Parrish and Polakoski (1978) demonstrated that this protein forms a complex with proacrosin. In 1989, Baba et al (1989) were the first to describe the function of the 32-kd protein as a facilitator of proacrosin conversion. In 1992, Yi et al (1992) detected a proacrosin binding protein between Mr 28 000 and Mr 32 000 in the boar sperm using a proacrosin binding protein antibody. A similar protein of Mr 28 000–29 000 was reported by Moos et al (1993b). As well, a protein of Mr 28 000–29 000 was detected in guinea pig sperm by Hardy et al (1991) and Noland et al (1994). Finally, in 1994, Baba et al reported the amino acid sequence of the sp32 (and its precursor), and a few years later, Huh and Yi (1999, 2001) determined the partial sequence of a Mr 28 000–29 000 protein similar to sp32. The importance of this sp32 protein was of greater interest when Howes et al (2001) and Howes and Jones (2002) presented evidence that proacrosin/acrosin are complementary binding proteins on sperm for ZP2, the secondary receptor that retains acrosome reacted sperm on the egg's zona pellucida. The importance of sp32 in reproduction is also underscored by the discovery that the sp32 precursor is a potential human cancer/testis antigen (Ono et al, 2001). Whether tyrosine phosphorylation of sp32 is involved in these putative roles has never been discussed.

A Tyrosine Phosphorylated Form of sp32 Is p32

Immunoprecipitation experiments confirm that the tyrosine phosphorylated sp32 is p32. Figure 5A shows that most of the tyrosine phosphorylated proteins are immunoprecipitated (pellet, lane 2), but some remain in the supernatant (lane 3). The anti-sp32 pellet (the immunoprecipitate) confirms that sp32 is tyrosine phosphorylated in capacitated sperm (Figure 5A, lane 4). The strong band appearing in the anti-sp32 supernatant (Figure 5A, lane 5) indicates that other proteins are tyrosine phosphorylated, and some of these are unlikely to be sp32. Moreover, the presence of a sp32 band in the anti-phosphotyrosine immunoprecipitate (Figure 5B, lane 2) indicates that some of the sp32 content is tyrosine phosphorylated, while the strong sp32 band remaining in the anti-phosphotyrosine supernatant (Figure 5B, lane 3) shows a major portion of the sp32 to be non–tyrosine phosphorylated. Taken together with our previous research (Tardif et al, 2001), we conclude that in pig sperm, some of the sp32 molecules are tyrosine phosphorylated during capacita-

tion. Ficarro et al (2003) showed that sp32 is also tyrosine phosphorylated in capacitated human sperm, lending support to our findings. To the best of our knowledge, it is not known if sp32 is one of the tyrosine phosphorylated substrates in the sperm of other species (such as the guinea pig, which contains sp32; Noland et al, 1994).

Tardif et al (2001) observed, in nonpermeabilized boar sperm, that tyrosine phosphoproteins relocalized from the equatorial segment of the sperm to the acrosome during capacitation, whereas permeabilized sperm always displayed labeling over the acrosome. By double labeling, we compared those patterns with those obtained using anti-sp32 antibody (Figure 6). The anti-sp32 antibody labeling was uniformly observed over the acrosomal cap in capacitated and noncapacitated sperm, regardless of permeabilization. We suggest that sp32, a (pro)acrosin binding protein, is constitutively present in the acrosome. Inducing the acrosome reaction confirmed that sp32 is present, because loss of the acrosome leads to the loss of the fluorescent anti-sp32 signal. We previously used Triton X-114 to extract sperm proteins, which revealed that p32 is present in the cytosolic fraction in uncapacitated pig sperm and in both the cytosolic and membrane (micellar) fractions after capacitation (Tardif et al, 2001). Given our current hypothesis that p32 is a tyrosine phosphorylated form of sp32, it is tempting to speculate that the partitioning of sp32 in the acrosomal compartments (soluble, the matrix, acrosomal membrane) is regulated by the phosphorylation status of the sp32. We will test this hypothesis in future experiments.

Hypothetical Roles of Tyrosine Phosphorylated sp32

The role of sp32 in acrosin maturation/capacitation in porcine sperm leads to different theories. First, Baba et al (1989) speculated that the proacrosin binding protein accelerates proacrosin activation. Hardy et al (1991) then co-localized proacrosin and its binding protein in the acrosomal matrix. Finally, Yi et al (1992) hypothesized that proacrosin binding protein might be involved in proacrosin processing. In 2001, Kim et al demonstrated that AM67, a proacrosin binding protein, was localized in the acrosomal matrix, a finding that was in agreement with those of other reports (Hardy et al, 1991; Yi et al, 1992; Baba et al, 1994). Those studies suggest that proacrosin binding proteins immobilize and stabilize proacrosin until the acrosome reaction. In addition, proacrosin might play a role in the secondary sperm binding to the zona pellucida via ZP2 (Howes et al, 2001, 2002), and sp32 could anchor proacrosin in the acrosomal matrix until a later phase of capacitation or until early in the acrosome reaction. Here our indirect immunofluorescence results on acrosome-reacted sperm showed no sp32 signal after the acrosome reaction; the sp32 could be liberated or proteolytically degraded (Hardy et al, 1991; Moos et al, 1993b) during this process and thereby might no longer be detected by the anti-sp32. The tyrosine phosphorylated portion of the degraded sp32 could remain in the sperm, as reflected by the persistent anti-phosphotyrosine labeling following the acrosome reaction (Figure 6).

During capacitation, maturation of some proacrosin to acrosin could be facilitated by tyrosine phosphorylation of sp32. Since sp32 does not bind acrosin (Baba et al, 1989), the tyrosine phosphorylated sp32 would be released to the acrosomal cap as acrosin is transferred to the soluble compartment of the acrosome. Binding to the ZP leads to the acrosome reaction and release of the acrosomal contents, including acrosin. The secondary binding of the sperm to the ZP could thereby be accomplished through the proacrosin still anchored in the acrosomal matrix.

In conclusion, this study demonstrates that p32, a tyrosine phosphorylated protein related to capacitation of porcine sperm, is a tyrosine phosphorylated form of sp32, a proacrosin binding protein. Our interest will now focus on the actual implication of the tyrosine phosphorylation of sp32 during capacitation and acrosin maturation.

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