Molecular Human Reproduction, Vol.18, No.6 pp. 298–307, 2012
Advanced Access publication on February 21, 2012 doi:10.1093/molehr/gas009

MHR Basic science of reproductive medicine

ORIGINAL RESEARCH

Alteration in the processing of the ACRBP/sp32 protein and sperm head/acrosome malformations in proprotein convertase 4 (PCSK4) null mice

Steve Tardif[1,2], Benoit Guyonnet[1], Nathaly Cormier[1], and Gail A. Cornwall[1,*]

[1]Department of Cell Biology and Biochemistry, Texas Tech University Health Sciences Center, 3601 4th Street, Lubbock, TX 79430, USA

*Correspondence address. Tel: +1-806-743-2700; Fax: +1-806-743-2990; E-mail: gail.cornwall@ttuhsc.edu

Submitted on November 17, 2011; resubmitted on February 10, 2012; accepted on February 13, 2012

ABSTRACT: Proprotein convertase 4 (PCSK4) is a member of a family of proprotein convertases that convert inactive precursor proteins into their mature and active forms. PCSK4 is expressed by testicular germ cells and localizes to the sperm acrosome, suggesting roles in fertilization. Mice lacking PCSK4 exhibit a profound fertility defect; yet, to date, few substrates for PCSK4 are known. In this study, two-dimensional differential in-gel electrophoresis analysis was carried out in order to identify proteins that are altered in spermatozoa from PCSK4 null mice. Herein, we report that the sperm fertilization molecule acrosin-binding protein (ACRBP)/sp32, which normally undergoes processing from a 58.5 kDa precursor to a 27.5 kDa mature form, is not proteolytically processed in PCSK4 null mice and thus may be a substrate for PCSK4. However, analysis of the ACRBP sequence did not show a strong consensus site for convertase cleavage, suggesting that ACRBP processing may require the activity of a yet unknown enzyme that itself may be a PCSK4 substrate. Further analysis of spermatozoa from the PCSK4 null mice showed that proacrosin did not undergo autoactivation, supporting a role for the mature form of ACRBP in the regulation of proacrosin conversion into different acrosin isoforms. Finally, examination of ACRBP localization revealed a previously undetected morphological defect in the head/acrosomes of spermatozoa from PCSK4 null mice. Taken together, these results demonstrate that the fertility defect in the PCSK4 null mice may in part be due to altered ACRBP protein processing as well as abnormalities in the sperm head/acrosome.

Key words: ACRBP / acrosome / PCSK4 / proprotein convertase / spermatozoa

Introduction

Proprotein convertase 4 (PCSK4) belongs to a family of calcium-dependent subtilisin/kexin-like serine proteases that cleave precursor proteins after dibasic residues converting inactive into active proteins (Seidah, 2011). Of the nine convertase family members known, most exhibit fairly broad patterns of expression with PCSK1 and PCSK2 primarily found in the endocrine and neuroendocrine systems. PCSK4, however, is restricted to the reproductive tract and expressed primarily in testicular germ cells, suggesting specific roles in reproduction (Nakayama *et al.*, 1992). Low levels of PCSK4 mRNA have also been detected in ovaries and the placenta (Tadros *et al.*, 2001; Qiu *et al.*, 2005). Immunolocalization studies show that PCSK4 localizes to the mouse sperm plasma membrane overlying the sperm acrosome and is thought also to be intraacrosomal and thus may play a role in fertilization (Gyamera-Acheampong *et al.*, 2006). Indeed, mice lacking PCSK4 exhibit reduced fertility in the absence of any apparent spermatogenic defect (Mbikay *et al.*, 1997). Further studies of the PCSK4 null mice revealed that spermatozoa were more sensitive than wildtype (WT) spermatozoa to capacitating conditions, resulting in enhanced protein tyrosine phosphorylation during capacitation and lower levels of solubilized zona pellucida proteins required to induce an acrosome reaction (Gyamera-Acheampong *et al.*, 2006, 2010). Spermatozoa from PCSK4 null mice also showed reduced zona pellucida binding *in vitro* (Gyamera-Acheampong *et al.*, 2006). These studies suggest that PCSK4 enzyme activity is critical for normal fertilization events perhaps by its processing of other acrosomal proteins with roles in fertilization. A high degree of conservation (91%) between

[2] Present address: Reproductive and Developmental Biology Group, Level 4 MACHS Labs, Centre for Oncology and Molecular Medicine, Division of Medical Sciences, Ninewells Hospital, University of Dundee, Dundee DD1 9SY, UK.

the mouse and human sequences implies that PCSK4 may carry out similar biological functions during human fertilization.

Thus far, few endogenous substrates of PCSK4 within the male germ cells are known. Within the testicular germ cells, proPACAP (pituitary adenylate cyclase activating peptide) with putative roles in cell signaling has been shown to be a substrate and its active products PACAP38 and PACAP27 are not detected in the PCSK4 null mice (Li *et al.*, 2000). However, PACAP null male mice are fertile, suggesting that the loss of these peptides in the PCSK4 null mice is not the primary cause of their infertility (Gray *et al.*, 2001; Sherwood *et al.*, 2007). More recently, it was shown that sperm protein ADAM2 exhibited enhanced processing in the PCSK4 null mice compared with that in WT mice (Gyamera-Acheampong *et al.*, 2010). However, this may reflect that overall proprotein convertase activity is increased in spermatozoa from PCSK4 null mice (Iamsaard *et al.*, 2011) (and our unpublished results). This activity is believed to represent a compensatory up-regulation of proprotein convertase 7, which is also expressed in male germ cells (Bergeron *et al.*, 2000) and which may carry out some, but not all, of the functions of PCSK4 (Scamuffa *et al.*, 2006). More direct evidence for a role of PCSK4 in ADAM2 processing are studies showing that a PCSK4 peptide, representing the prodomain of PCSK4 which inhibits activity of the mature enzyme, inhibited ADAM2 processing during capacitation as well as slowed the capacitation process itself based on chlortetracycline staining of spermatozoa (Iamsaard *et al.*, 2011). Taken together, these studies support a critical role for PCSK4 during fertilization.

The acrosome is an exocytotic vesicle located on the surface of the sperm head and, following the acrosome reaction, its contents are released and interact with the oocyte zona pellucida during fertilization. Acrosin, a serine protease involved in sperm–oocyte interactions, is present in the acrosome in its zymogen form proacrosin, which undergoes autoactivation at basic pH and is converted into the mature isoforms (Polakoski and Parrish, 1977; Brown, 1983; Baba *et al.*, 1989). Proacrosin-binding protein (ACRBP) or sp32 is synthesized as a 58–60 kDa precursor protein that is processed to a mature 28–32 kDa form corresponding to the C-terminal half of the precursor (Baba *et al.*, 1994). In guinea pigs, ACRBP colocalizes with proacrosin in the sperm acrosomal matrix and, in boars ACRBP has been shown to bind to the intermediate forms of proacrosin and mediate its activation *in vitro* (Hardy *et al.*, 1991; Baba *et al.*, 1994). Although the biological function of ACRBP in spermatozoa and fertilization is unknown, based on *in vitro* studies, it may regulate acrosin release from the matrix during the sperm acrosome reaction (Baba *et al.*, 1994). In humans, the roles of acrosin and ACRBP in fertilization are less understood. However, both human proteins show high conservation (76% acrosin and 86% ACRBP, respectively) with the mouse proteins, suggesting conserved biological functions. Furthermore, *in vitro* studies support a role for acrosin in the human sperm–zona interaction (Veaute *et al.*, 2010).

Because of the paucity of information regarding putative PCSK4 substrates in male germ cells, the main objective of this study was to identify changes in sperm proteins from PCSK4 WT and null mice that may indicate altered protein processing. Herein, we describe the identification of several known fertilization molecules as potential substrates of PCSK4 activity, and in particular, focus on one such protein, ACRBP.

Materials and Methods

Animals

New Zealand White rabbits were purchased from Charles River Laboratories (Wilmington, MA, USA). B6 mice WT (+/+) or null congenic (−/−) for the PCSK4 allele were generously provided by M. Mbikay, Ph.D. (Ottawa Hospital Research Institute, ON, Canada) and were used to establish a breeding colony in house. Mice were maintained under a constant 12 h light/12 h dark cycle with food and water *ad libitum*. All animal studies were conducted in accordance with the NIH Guidelines for the Care and Use of Experimental Animals and with approval by the Texas Tech University Health Sciences Center Institutional Animal Care and Use Committee.

Two-dimensional differential in-gel electrophoresis analysis

Caudal epididymal spermatozoa from age-matched $PCSK4^{+/+}$ (WT) and $^{-/-}$ [null or knockout (KO)] mice were dispersed separately into phosphate-buffered saline (PBS; 2 ml; pH 7.4) for a period of 15 min at 37°C (100% air), motility was observed and sperm concentration calculated using a hemocytometer. Spermatozoa were centrifuged at 10 000*g*. The pellets were solubilized in 2× Laemmli buffer under reducing conditions at 1×10^6 spermatozoa/μl and incubated at 75°C for 5 min. The sperm solution was sonicated to shear DNA, and the insoluble material was discarded after 5 min of centrifugation at 13 000*g*. Extracted proteins representative of $20–25 \times 10^6$ spermatozoa/mouse from both PCSK4 genotypes ($n = 3$ pairs) were sent on dry ice to the Vanderbilt University School of Medicine, Proteomics Laboratory, Mass Spectrometry Research Center, Nashville, TN under the direction of David Friedman, Ph.D. for two-dimensional (2D) differential in-gel electrophoresis (DIGE) analysis.

Briefly, for 2D-DIGE, the proteins in sperm samples from WT and null mice were first labeled with Cy3 and Cy5 fluorescent dyes, respectively, while a mixed sample was labeled with Cy2 and served as an internal control. All three samples were mixed together prior to separation by 2D gel electrophoresis (pH 4-7) to resolve protein spots. The gels were then scanned with the excitation wavelength of each dye and normalized to that of the internal standard. DIGE data were then analyzed using the deCyder software version 6.5. By comparing the Cy3 and Cy5 fluorescence values for each spot, differences in the abundance of a particular sperm protein from PCSK4 WT and KO (null) mice were determined. Gels were post-stained with SyproRuby to allow accurate excision of proteins of interest, which were then identified by matrix-assisted laser desorption/ionization-time of flight mass spectrometry.

ACRBP recombinant protein expression and antibody production

An antigenic region of mouse ACRBP determined using Jameson–Wolf algorithm (DNASTAR software) encoded by the regions 409–512 (data base accession number: sp Q3V140) was selected to produce mouse ACRBP antigen. Recombinant DNA was produced by RT–PCR using mouse testis cDNA (sense primer: 5′-GAGCATATCCACGGGCA ACCA-3′; antisense primer: 5′-GTACCTCTCATTCAGCATACACC-3′). Briefly, total RNA (2 μg) was isolated using TRIzol procedure (Invitrogen, Grand Island, NY) from mouse testes flash-frozen in liquid nitrogen and reverse-transcribed according to the manufacturer's instructions with 200 units of superscript III reverse transcriptase (Invitrogen) and 25 ng/ml oligo dT_{15} (Promega, Madison, WI, USA). ACRBP cDNA was cloned into the pQE-9 vector (Qiagen, Valencia, CA, USA), and His-tag fusion proteins were expressed in *Escherichia Coli* (BL21 strain) induced by 1 mM isopropyl β-*d*-thiogalactoside for 3 h at 37°C. Expressed proteins

were extracted by incubating the bacterial cell pellets in 7 M urea solution (7 M urea, 10 mM Tris–HCl pH 8.0 and 100 mM NaH_2PO_4), and purified by affinity chromatography on nickel–sepharose column (Ni-NTA, Qiagen) using a GradiFrac system (GE Healthcare Life Sciences, Piscataway, NJ, USA). The column loaded with ACRBP His-tag protein was washed with 7 M urea solution (7 M urea, 10 mM Tris–HCl pH 6.0 and 100 mM NaH_2PO_4) and recombinant proteins were eluted with 7 M urea (7 M urea, 10 mM Tris–HCl pH 4.5 and 100 mM NaH_2PO_4). Antibodies to mouse ACRBP were raised in two female New Zealand White rabbits using complete and incomplete Freund adjuvant (F5506, F5881, Sigma-Aldrich, St. Louis, MO, USA) as described by Tardif *et al.* (2010).

Evaluation of ACRBP in sperm protein extractions

Spermatozoa were isolated from the cauda epididymis as described above and the sperm pellet was solubilized in a 1% SDS-TN buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl) for 5 min at room temperature. Three volumes of 0.5% Triton X-100 were added and after incubation for 15 min at room temperature, the insoluble material was discarded after 5 min centrifugation at 13 000*g*. The supernatant was recovered and proteins were precipitated using a 2D Clean-Up Kit™ (GE Healthcare Life Sciences). The number of sperm cells recovered from the cauda epididymis per mouse ($16.1 \times 10^6 \pm 0.81$ versus $16.2 \times 10^6 \pm 1.6$, respectively; $n = 3$) and also the amount of total protein extracted (23.2 ± 6.9 versus 27.3 ± 7.3 μg/million of cells, respectively) were comparable between PCSK4 WT and PCSK4 null mice. The resulting pellet was separated by immobilized pH gradient (IPG) after resolubilization with 2D sample buffer pH 4-7 (9.8 M urea, 2% Triton X-100, 18 mM dithiothreitol (DTT), 2 M thiourea) added to 125 μl IPG buffer (GE Healthcare Life Sciences). After the IPG strips (GE Healthcare Life Sciences) were rehydrated with the protein solution in a swelling tray (25°C, overnight), the strips were applied on the Ettan™ IPGphor™ three isoelectric focusing (IEF) unit (GE Healthcare Life Sciences) and the focusing was stopped after reaching ~5000 V h^{-1} (200 mA/strip; 500 V 30 min; 1000 V 30 min; 5000 V 1.5 h). Proteins extracted from 15×10^6 spermatozoa were loaded/strip.

Sperm proteins were next resolved in a second dimension IEF using 4–15% SDS–PAGE gradient gels. IEF strips were first incubated in equilibration buffer containing 6 M urea, 75 mM Tris–HCl pH 8.8, 29.3% glycerol, 2% SDS, 0.002% bromophenol blue and 100 mg DTT/10 ml for 15 min at room temperature to reduce protein disulfides followed by incubation in equilibration buffer containing 250 mg iodoacetamide/10 ml for 15 min at room temperature to alkylate sulfhydryl groups. After the second dimension was completed, the proteins were transferred on to a PVDF membrane (polyvinylidene fluoride, Immobilon-P, 0.45 μm pore size, Millipore, Billerica, MA, USA) with an electro-blotting system and incubated overnight at 4°C in the presence of rabbit anti-mouse ACRBP sera (dilution 1:25000). The detection of ACRBP was performed as described in Tardif *et al.* (2003). Briefly, the membrane was washed three times (10 min each at room temperature) with tris-buffered saline with Tween-20 (TBST) (25 mM Tris–HCl pH 7.4, 150 mM NaCl, 0.1% Tween-20) and incubated for an additional 45 min with horse-radish peroxidase (HRP)-conjugated secondary antibody [goat anti-rabbit immunoglobulin G (IgG); BIOSOURCE International, Camarillo, CA] diluted 1:50 000 in TBST. The membrane was then washed as described above followed by incubation with chemiluminescence reagent (SuperSignal, Thermo Scientific, Rockford, IL, USA).

Testicular spermatozoa and spermatozoa from different epididymal regions (caput, regions 1–3; corpus, region 4; cauda, region 5) were isolated from WT and PCSK4 null mice. A crude preparation of testicular spermatozoa was prepared by removing the tunica albuginea and mincing the testis in PBS and centrifuging the sample (20 000*g* 20 min) through a 3-ml 52% Percoll (Sigma Chemical Co., St. Louis, MO, USA) density column to separate spermatozoa from germ cells. The bottom fraction contained testicular spermatozoa as well as likely late-stage spermatids and sperm cells that had not yet undergone spermiation but were released as a result of the mincing process. Testicular spermatozoa were then washed free of Percoll and resuspended in 2× Laemmli buffer containing 10% β-mercaptoethanol. Epididymal spermatozoa were purified from contaminating epithelial cells by centrifuging cell suspensions isolated in PBS through 20–40% Percoll density gradients as described by Syntin and Cornwall (1999). After spermatozoa were washed free of Percoll and resuspended in 2× Laemmmli buffer in the presence of 10% β-mercaptoethanol. Proteins from approximately 1 to 2.5×10^5 sperm cells were separated by SDS–PAGE using Bio-Rad 15% Criterion gels (Bio-Rad, Hercules, CA, USA), and western blotting was performed using the rabbit anti-mouse ACRBP antibody following a protocol described previously (Chau and Cornwall, 2011). Briefly, separated proteins were transferred to Immobilon-P for 1.5 h at 100 V. Membranes were blocked with 3% non-fat dry milk in TBS with 0.2% Tween-20 (TBST) for 1 h at room temperature followed by incubation with rabbit anti-mouse ACRBP antibody at 1:10 000 dilution in 3% milk/TBST (0.2% Tween-20) overnight at 4°C. Blots were then washed with TBST three times for 10 min each and incubated for 2 h at room temperature with a goat anti-rabbit HRP secondary antibody (1:30 000)(Thermo Scientific, Rockford, IL, USA) in 3% milk/TBST. Blots were washed with TBST three more times for 10 min each and once with TBS before developing with SuperSignal West Pico chemiluminescent substrate (Thermo Scientific).

Evaluation of proacrosin activation

Sperm proteins were extracted using 0.5% SDS-TN buffer as described, above and this solution was diluted 10-fold with 0.5% Triton X-100, 50 mM Tris–HCl pH 3.5, 50 mM $CaCl_2$, to prevent autocatalytic activation of proacrosin. An aliquot was saved for western blot analysis, and the remaining sperm protein extract was adjusted to basic pH (pH 8.8) using 10 N NaOH to initiate proacrosin conversion to acrosin. The sperm protein extract was incubated at room temperature with agitation, and aliquots were removed at various times for western blot analysis. Upon removal, the samples were immediately mixed with 2× Laemmli buffer and flash frozen for western blot analysis. The next day, 25 mM DTT was added to the sperm extracts to reduce protein disulfides, and samples were loaded on a 4–15% SDS–PAGE gradient gel (2.5×10^6 sperm cells/lane). The separated proteins were transferred to a PVDF membrane as previously described. The blots were blocked in 2.5% non-fat dry milk diluted in TBS (25 mM Tris–HCl pH 7.4, 150 mM NaCl) for 1 h at room temperature and incubated overnight with a rabbit anti-guinea pig proacrosin/acrosin antibody 1:10000 (generously provided by Daniel Hardy, Ph.D. from Texas Tech University Health Sciences Center) diluted in TBST (0.1% Tween-20). The next day, the membranes were washed 3 × 10 min each in TBST and incubated with a goat HRP-labeled secondary antibody (goat anti-rabbit IgG; BIOSOURCE International) diluted 1:50000 in TBST for 45 min at room temperature. After washing 3 × 10 min each in TBST, the membrane was incubated with chemiluminescence reagent (SuperSignal, ThermoScientific).

Immunofluorescence analysis

Testicular spermatozoa were released from the testis by removing the tunica albuginea and dispersing the tubules using a scissors. Spermatozoa

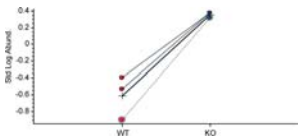
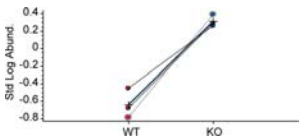
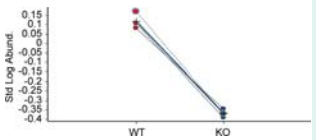
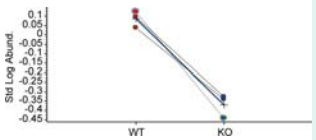
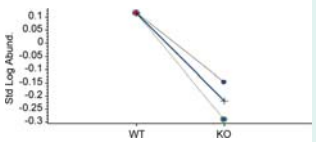
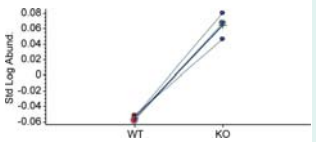
were allowed to disperse at 37°C for 10–15 min. Caput and cauda epididymal tubules were punctured with a needle in PBS containing a protease inhibitor cocktail (PI) (Roche, San Francisco, CA, USA) and spermatozoa allowed to disperse for 10 min at 37°C. The sperm suspension was then filtered through a 10-μm nylon mesh and the collected spermatozoa were washed two times in PBS/PI by centrifugation at 500*g* for 5 min. The final pellet was resuspended in PBS/PI and 5 μl of each sample (1.2×10^4 cells/μl in PBS/PI) was spread by slide and allowed to dry overnight at room temperature. The sperm samples were fixed/permeabilized for 10 min in 100% methanol bath at room temperature and then washed at room temperature in Dulbecco's PBS (DPBS) containing 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$ (Cellgro, Manassas, VA, USA). The slides were incubated for 1 h with 100% heat-inactivated goat serum (HIGS) (Vector Laboratories, Burlingame, CA, USA) at 37°C followed by rabbit anti-mouse ACRBP (1:2000) in DPBS containing 5% HIGS for 1 h at 37°C. Control slides were incubated with normal rabbit serum (1:2000) in place of the ACRBP antiserum. Slides were washed with DPBS 3 × 7 min each followed by incubation with a goat anti-rabbit Alexa594 conjugated secondary antibody (2 μg/ml) (Invitrogen) in DPBS containing 5% HIGS for 1 h at 37°C in the dark. Slides were rinsed with DPBS 2 × 7 min each and then incubated with peanut agglutinin (PNA; *Arachis hypogaea*) conjugated to FITC (10 μg/ml in DPBS) for 20 min at room temperature in the dark. The samples were washed with DPBS 2 × 7 min each, followed by Tris buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl) for 7 min at room temperature in the dark and then rinsed 1× with MilliQ water and coverslips mounted with Fluoromount G (Southern Biotech, Birmingham, AL). Samples were examined using an Olympus BX-60 microscope equipped with epifluorescence.

Results

2D-DIGE analysis

To identify proteins that may be targets of PCSK4 processing and contribute to the sperm fertility defect observed in the PCSK4 null mice, 2D-DIGE analysis was performed on cauda epididymal spermatozoa isolated from WT and PCSK4 null mice. 2D-DIGE is a commonly used approach that is used to detect global changes in protein expression in response to different experimental conditions. As shown in Table I, three proteins were identified with high confidence by mass spectrometry, including acrosin-binding protein (ACRBP), also known as sp32, zona pellucida 3 receptor or sp56 and glutathione

Table I 2D-DIGE analysis of proteins extracted from PCSK4 WT and null (KO) mouse spermatozoa.

Protein name	UniProtKB ID	MW, pI	Av. Rat[a]	T-test[b]	DIGE profile[c]
Acrosin-binding protein	ACRBP MOUSE	58.5 kDa, 4.97 pI	8.29	0.0032	
Acrosin-binding protein	ACRBP MOUSE	58.5 kDa, 4.97 pI	8.58	0.00088	
Acrosin-binding protein	ACRBP MOUSE	27.5 kDa, 5.5 pI	−3.09	0.000078	
Acrosin-binding protein	ACRBP MOUSE	27.5 kDa, 5.5 pI	−2.85	0.00049	
Zona pellucida 3R/sp56	ZP3R MOUSE	61 kDa, 6.9 pI	−2.14	0.043	
Glutathione *S*-transferase mu5	GSTM5 MOUSE	27 kDa, 6.8 pI	1.32	0.00029	

[a,b]Av. Rat, average volume ratios and Student's *t*-test *P*-values calculated using DeCyder software version 6.5 utilizing the mixed sample internal standard methodology.
[c]DIGE profiles created using DeCyder software version 6.5. Graphs depict normalized log abundance ratios relative to the cognate signals present in the internal standard specific for each protein shown. Dashed lines represent changes in protein abundance between WT and KO mice for $n = 3$ replicates while the solid line represents the averaged values.

S-transferase mu5, all of which exhibited significant changes in protein levels between WT and PCSK4 null mice. Four protein spots for zona pellucida 3R were identified (only one representative spot is shown), all showing significant decreases in the level of the 61 kDa protein in spermatozoa from the PCSK4 null mice. In contrast, of the four protein spots that were identified as glutathione *S*-transferase mu5 (one representative spot is shown), all showed significant increases in spermatozoa from PCSK4 null mice compared with WT. Several protein spots for ACRBP were identified by mass spectrometry to represent the precursor (58.5 kDa) and processed (27.5 kDa) forms of the protein with an increase in precursor and decrease in processed form observed in the PCSK4 null mice. In addition to ACRBP, zona pellucida 3R and glutathione *S*-transferase mu5, several other protein spots showed differences between the PCSK4 WT and null mice following 2D-DIGE analysis. However, insufficient protein was extracted from the gel to allow a confident identification by mass spectrometry (data not shown). Since the observed changes in protein levels for zona pellucida 3R and glutathione *S*-transferase mu5 may represent effects of the loss of PCSK4 independent of protein processing, we decided to focus on ACRBP in the remainder of the studies to examine its processing and the putative biological consequences of altered ACRBP processing in sperm function in the PCSK4 null mouse model.

To confirm the 2D-DIGE analysis, Percoll-purified spermatozoa isolated from the testis and epididymis from PCSK4 WT and null mice were examined by one-dimensional and two-dimensional electrophoresis and western blot analysis using a rabbit anti-mouse ACRBP antibody generated in our laboratory. As shown in Fig. 1A, although some of the mature form (27.5 kDa) of ACRBP was detected, the precursor (58.5 kDa) was the predominant form of the protein present in testicular spermatozoa from WT mice. The examination of a mixed population of testicular germ cells also showed both precursor and mature forms of ACRBP (data not shown). However, the analysis of spermatozoa isolated from the caput-corpus (regions 1–4) (Fig. 1A) and cauda epididymis (Fig. 1A and B) showed that the majority of the ACRBP in WT mice was present as the 27.5 kDa mature form, suggesting that ACRBP processing primarily occurs within the testis and possibly during the migration of spermatozoa through the efferent ducts into epididymis. In both the testis and epididymal spermatozoa from WT mice, two mature forms of ACRBP were detected, which may represent differential phosphorylation (Dube *et al.*, 2005). In contrast to that observed in the WT mice, ACRBP remained unprocessed in both testicular and epididymal spermatozoa from PCSK4 null mice, suggesting that the PC4 convertase plays a role in the processing of ACRBP to its mature form.

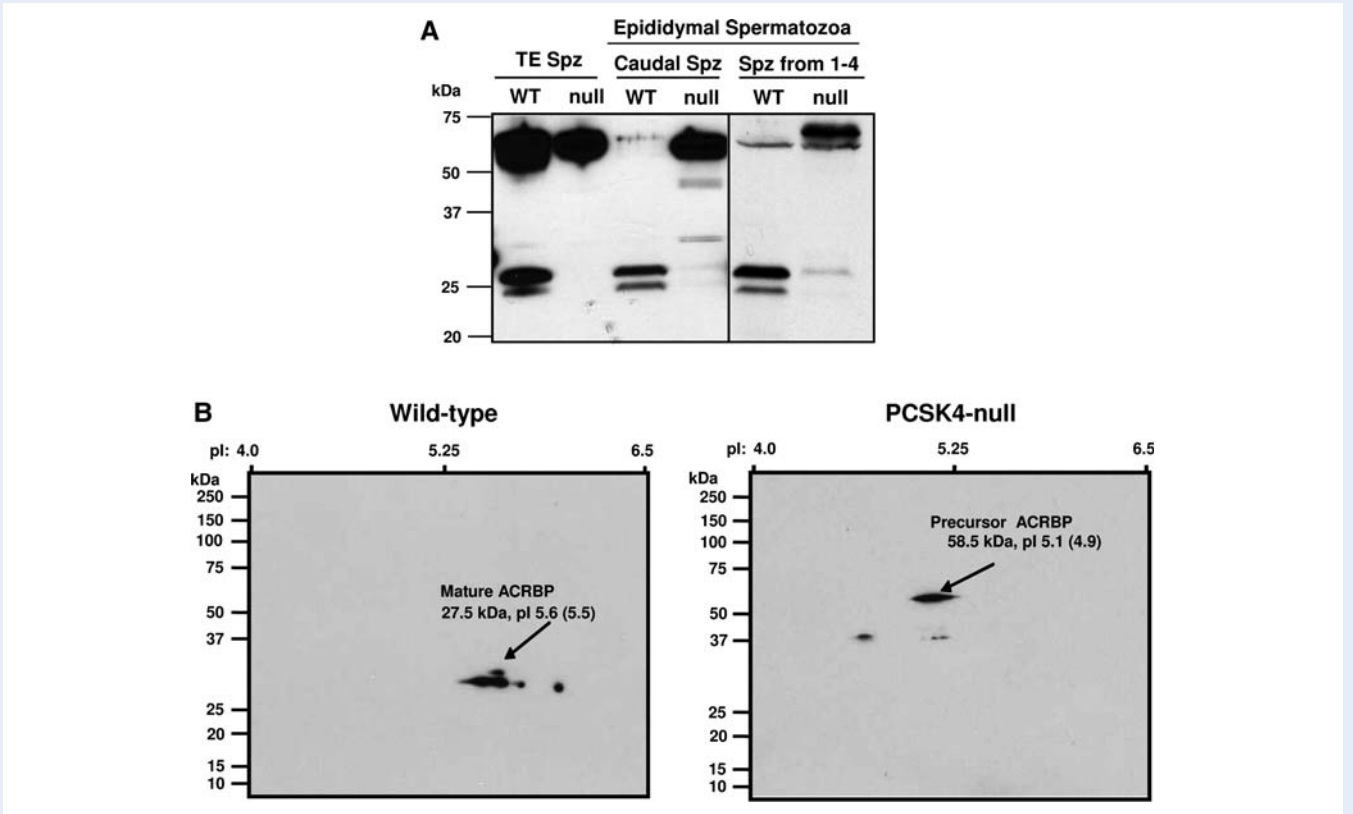


Figure 1 The absence of ACRBP protein processing in spermatozoa from PCSK4 null mice. (**A**) Western blot analysis of ACRBP in 2×10^5 Percoll purified testicular and epididymal spermatozoa from PCSK4 WT and null (KO) mice. TE spz, testicular spermatozoa: 1–4 spz, spermatozoa isolated from epididymal regions 1–4 representing the caput and corpus epididymidis; cauda spz, spermatozoa isolated from epididymal region 5 representing the cauda epididymidis. (**B**) Proteins extracted from 15×10^6 cauda epididymal spermatozoa from PCSK4 WT and null mice were separated by 2D gel electrophoresis followed by western blot analysis using the ACRBP antibody. The calculated pI and known pI (in parentheses) are noted.

ACRBP has previously been shown to localize to the sperm acrosome, specifically the acrosomal matrix in guinea pig and boar, and to bind to proacrosin. Similarly, we have determined that in mice, ACRBP also localizes to the acrosomal matrix (Guyonnet and Cornwall, unpublished observations). Furthermore, studies *in vitro* demonstrated that ACRBP accelerated proacrosin autoactivation and may function in the release of acrosin from the matrix during the sperm acrosome reaction (Baba *et al.*, 1994). Therefore, studies were next carried out to first examine whether ACRBP localization was altered in spermatozoa from PCSK4 null mice and second to determine whether the absence of a mature form of ACRBP in the null mice affected proacrosin activation.

Immunolocalization of ACRBP

The localization of ACRBP in testicular and epididymal spermatozoa isolated from WT and PCSK4 null mice was examined by the indirect immunofluorescence analysis using the ACRBP antibody. As expected and as previously shown, ACRBP localized to the acrosome in spermatozoa isolated from both the testis and epididymis from WT mice (Fig. 2A and B). Although acrosomal staining was also detected in spermatozoa from PCSK4 null mice, the pattern was different from that in WT mice. In particular, PCSK4 null mice showed an additional punctate localization of ACRBP that varied between individual spermatozoa and included localization near the apex of the sperm acrosome (Fig. 2B, 2), near the top of the acrosomal cap (Fig. 2B, 3), or overlaying the acrosomal cap and equatorial segment (Fig. 2B, 4). Staining of the sperm samples with PNA, a marker for the sperm acrosome, indicated that the punctate structure was PNA-positive, suggesting that it represented acrosomal material (Fig. 2A and B). These abnormalities were detected in all the sperm populations examined, including testicular, caput and cauda epididymal spermatozoa (Fig. 2A). Furthermore, the majority of spermatozoa from PCSK4 null mice possessed these deformities, with approximately $<5\%$ of the spermatozoa showing what appeared to be normal morphology.

Examination of the spermatozoa under phase microscopy and at higher magnification showed that the areas of punctate immunofluorescence corresponded to PNA-positive vesicular material around the acrosome (Fig. 2B, 2–4, arrowhead), suggesting abnormal acrosome formation during spermatogenesis. Other material was also observed associated with the concave surface of the sperm head (Fig. 2B, 2–5, arrow), which may arise from a different source since this material was not ACRBP/PNA-positive. Spermatozoa with apparently profoundly abnormal acrosomes were also present as evidenced by spermatozoa that possessed only a diffuse staining for ACRBP and PNA (Fig. 2B, 5). These spermatozoa had a sickle-shaped head but lacked the pointed apex. Spermatozoa incubated with the control normal rabbit serum did not show any specific punctate and/or acrosomal staining on spermatozoa for either WT or null mice, indicating the specificity of the ACRBP antibody (Fig. 2A).

Proacrosin autoactivation

To determine whether proacrosin autoactivation was affected in the PCSK4 null mice compared with that in WT mice, sperm extracts were isolated under acidic conditions (pH 3.5) and the pH of the extracts then adjusted to 8.8 to allow autoactivation of proacrosin. In WT spermatozoa, mature isoforms of acrosin were detected, suggesting that autoactivation of proacrosin had occurred. In contrast, little to no acrosin was detected in the sperm extracts from PCSK4 null mice, suggesting that the autoactivation of proacrosin was less efficient (Fig. 3).

Potential PCSK4 cleavage sites in the ACRBP sequence

The absence of the mature form of ACRBP in spermatozoa from PCSK4 null mice suggests that it may be a substrate for PCSK4 processing. Like other prohormone convertases, PCSK4 has been shown *in vitro* to cleave synthetic peptide substrates after an Arg (R) in a basic sequence context, most often after paired basic residues KR, RR, (Remacle *et al.*, 2008) but is thought to cleave better after a single Arg preceded by a Lys at P4 (KXXR) Basak *et al.* (1999, 2004). Examination of the mouse ACRBP sequence showed two possible sites for PCSK4 activity (KRVR$\downarrow_{120-123}$ and KMSR$\downarrow_{500-503}$) (Fig. 4), both of which are conserved in the porcine and guinea pig sequences and only one of which is conserved in the human (KVSR$\downarrow_{500-503}$) sequence. However, based on the presence of cysteine residues adjacent to or near these cleavage sites, neither site is predicted to be a strong candidate for PCSK4 cleavage due to the possibility of disulfide bond formation, which would prevent enzyme access. Furthermore, if cleavage did occur at the KRVR site, the predicted processed C-terminal form would be approximately 42 kDa, which is not what is observed *in vivo*. The same holds true for the KMSR (KVSR$\downarrow$ in human) site as the predicted processed form would be 3.7 kDa. The site predicted by Baba *et al.* (1994) (FTPRVR$\downarrow$EVES) is more likely the cleavage site for ACRBP processing, as this site is also conserved in porcine, guinea pigs and humans and predicts a mature ACRBP form of $\sim$26.8 kDa which is very close to that of the mature ACRBP (27.5 kDa) observed in spermatozoa. Taken together, these data suggest that ACRBP is not a direct substrate of PCSK4 but rather is a substrate of another enzyme that itself may require activation by PCSK4.

Discussion

The processing of inactive precursor proteins to their mature and active forms is a critical site of regulation at the posttranslational level. The proprotein convertases are a family of proteins that cleave at the carboxy terminus of the consensus sequence RXXR. A wide variety of proteins, including hormones, growth factors, receptors, proteases and neuropeptides, are activated by proprotein processing and thus proprotein convertases play critical roles throughout many organ systems. The proprotein convertase PCSK4 (PC4) is especially unique, since it is primarily expressed in the testicular germ cells and mice lacking PCSK4 are infertile. However, to date, few substrates for PCSK4 are known.

In this report, we demonstrated that spermatozoa from mice lacking PCSK4 exhibit changes in protein levels of several key fertilization molecules, including ACRBP and zona pellucida 3R, suggesting that they are possible substrates for PCSK4 activity and that alteration in these proteins may contribute to the PCSK4 fertility defect. In particular, our studies show that ACRBP does not undergo proteolytic processing from its precursor (58.5 kDa) form to its mature form (27.5 kDa) in mice lacking PCSK4. However, analysis of the ACRBP

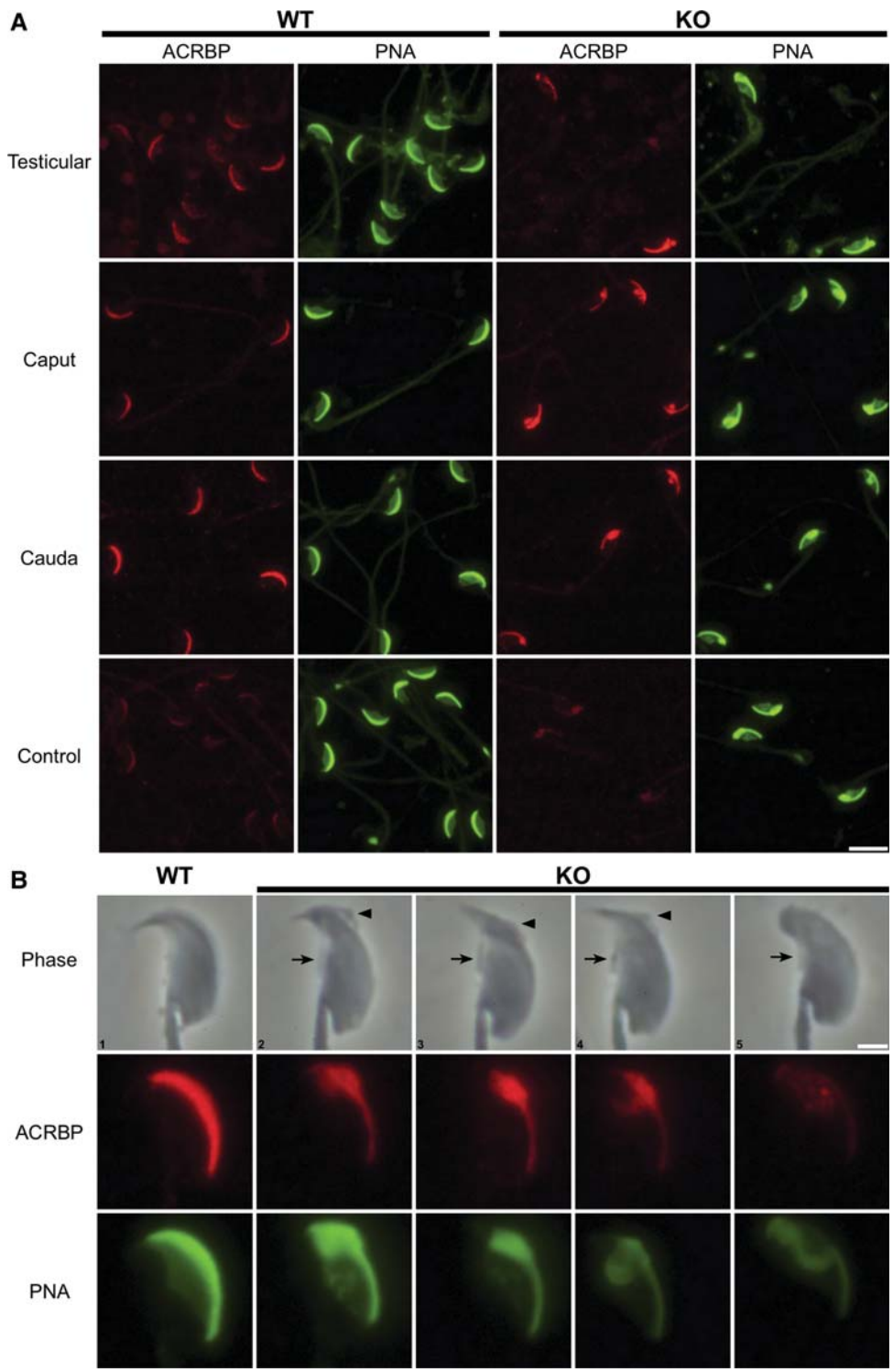


Figure 2 Immunofluorescence analysis of ACRBP in spermatozoa isolated from PCSK4 WT and null (KO) mice. (**A**) Testicular, caput (regions 1–3) and cauda (region 5) epididymal spermatozoa were incubated with ACRBP antibody followed by PNA as a marker for the sperm acrosome. Control, cauda epididymal spermatozoa incubated with normal rabbit serum followed by PNA. Scale bar = 10 μm. (**B**) Higher magnification of WT and KO epididymal spermatozoa showing the different types of defects observed in the sperm head from PCSK4 KO mice. Arrow head, ACRBP/PNA positive material; Arrow, ACRBP/PNA negative material. Scale bar = 2 μm.

sequence showed a lack of consensus proprotein convertase cleavage sites in good context, suggesting that ACRBP is not a direct target of PCSK4 activity but rather more likely is a substrate for an as yet unknown enzyme that itself requires activation by PCSK4. Examination of the zona pellucida 3R and glutathione *S*-transferase mu5 sequences also did not show good consensus proprotein cleavage sites suggesting that they also may be indirect targets of PCSK4. Together, these observations suggest that PCSK4 activity may be required early in protein-processing pathways resulting in the activation of one or more sperm proteases that may in turn affect a multitude of downstream targets, including ACRBP, zona pellucida 3R, glutathione *S*-transferase mu5 as well as likely other proteins. This would be consistent with the variety of fertilization defects observed in the PCSK4 null mice including altered capacitation, acrosome reaction and sperm-zona binding. In several human cell systems, a number of proprotein convertases are known to activate other enzymes including that of ADAMTS4 and 5, which function as aggrecanases that cleave the cartilage aggrecan (Malfait *et al*., 2008) and proprotein cleavage of BACE, the β-secretase that cleaves amyloid precursor protein to initiate β-amyloid formation (Bennett *et al*., 2000).

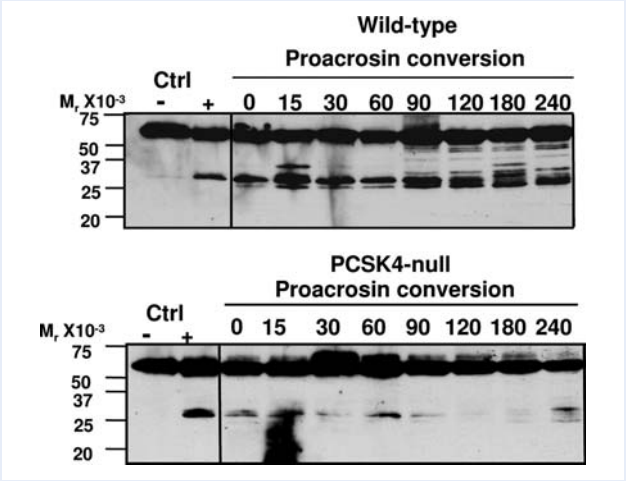


Figure 3 Altered proacrosin autoactivation in spermatozoa from PCSK4 null mice. Protein extracts were prepared from spermatozoa from PCSK4 WT and null mice under acidic conditions (pH 3.5) and adjusted to pH 8.8 to initiate autoactivation of proacrosin and aliquots removed at the indicated times (minutes). Proteins representative of 2.5×10^6 spermatozoa were loaded in each lane, separated by SDS–PAGE under reducing conditions and incubated with the proacrosin antibody in western blot analysis. Control lanes in both blots were protein extracts of cauda epididymal spermatozoa from PCSK4 WT mice that were removed prior to initiation of autoactivation (−) or after autoactivation (+) to show expected isoforms of proacrosin and acrosin.

ACRBP has been shown to mediate proacrosin autoactivation *in vitro* (Baba *et al*., 1994), suggesting a role for ACRBP in the regulation of proacrosin release from the acrosomal matrix during the acrosome reaction. Our studies support a role for ACRBP in proacrosin autoactivation *in vivo* since sperm extracts prepared from PCSK4 null mice, and which contained only the precursor form of ACRBP, were unable to carry out appropriate proacrosin autoactivation compared with WT mice. Mice lacking acrosin show a delay in fertilization due to a delay in the release of several acrosomal proteins, suggesting that acrosin is needed for the dispersal of acrosomal contents (Yamagata *et al*., 1998). Together, these studies support that ACRBP function during fertilization is to regulate proacrosin autoactivation to the mature isoforms of acrosin, which in turn accelerates the dispersal of the acrosomal contents during the acrosome reaction.

Our studies of ACRBP localization also revealed a previously undetected defect in the morphology of the sperm acrosome and head from PCSK4 null mice. In contrast to WT spermatozoa that showed the expected ACRBP localization to the sperm acrosome, spermatozoa from PCSK4 null mice showed an additional ACRBP and PNA-positive structure associated with various aspects of the sperm acrosome and sperm head. While conclusive evidence for what the defect is requires a full ultrastructural analysis, examination of the spermatozoa from PCSK4 null mice by phase contrast suggested that this material could represent a defect during acrosomal biogenesis. The presence of a vesicle-like structure, both ACRBP and PNA positive, associated with the sperm acrosomes suggests that some proacrosomal vesicles may have not fused completely to form the single acrosomal vesicle that occurs during the Golgi phase. Additional abnormal material

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1   MMNLAAGFLL MLLEVLLLPG TPLSAEESPA STPGSPLSST EYERFFALLT 50
52  PTWKAETTCR LRATHGCRNP TLVQLDQYEN HGLVPDGAVC SDLPYASWFE 101
102 SFCQFAQYRC SNHVYYAKRV RCSQPVSILS PNTLKEVESS AEVPPTSMTT 150
151 PIVSHATATE HQAFQPWPER LNNNVEELLQ SSLSLGGKDQ QSSRRPGQEQ 200
201 RKQEQIQEHK LEEAQEQEEQ EEEEEEEEAK QEEGQGTEAG LESVSRLQSD 250
251 SEPKFQSQSL SSNPSFFTPR VREVESAPLM MKNIQELIRS AQEMDEMNEL 300
301 YDDSWRSQST GSLQQLPHME TLMVLCYSIM ENTCTMTPTA KAWSYMEEEI 350
351 LGFGDSVCDN LGRRHTAACP LCAFCSLKLE QCHSEASVLR QKCDASHKIP 400
401 FISPLLSAQS ISTGNQARIP DKGRFAGLEM YGGLSSEFWC NRLAMKGCED 450
451 DRVSNWLKAE FLSFQEGDFP TKICDTNYIQ YPNYCSFKSQ QCLLRNQNRK 500
501 MSRMRCMLNE RYNVLSLAKS EEVILRWSQE FSTLAIGQFG 540
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Figure 4 Amino acid sequence of the mouse ACRBP sequence. The amino acids indicated in bold show the peptides identified by mass spectrometry in the 2D-DIGE analysis. The putative cleavage sites by PCSK4 are indicated by an underline while the site predicted by Baba *et al*. (1994) is indicated by the closed arrow.

was also associated with the sperm head that was not ACRBP/PNA positive, suggesting that this material could represent residual, possibly cytoplasmic, material that was not removed during spermiation. Thus, PCSK4 protein processing may not only directly or indirectly affect specific molecules with known roles in fertilization but may also target other proteins with roles in acrosome formation and spermiation. Although the lack of other proteins from spermatozoa including α-*L*-fucosidase (Veeramachaneni *et al*., 1998), Hrb (Kang-Decker *et al*., 2001), GOPC (Yao *et al*., 2002) and PICK1 (Xiao *et al*., 2009) have resulted in abnormalities in the formation of the acrosome, most of these defects were characterized by a complete lack of the sperm acrosome and the presence of round headed spermatozoa (globozoospermia), a more severe defect than observed with the PCSK4 mice. Therefore, the sperm acrosome/head morphology defect that was observed appears unique to the PCSK4 null mice.

Taken together, our studies show that ACRBP is an indirect target of PCSK4 protein processing. Although PCSK4 has been localized to the plasma membrane overlying the sperm acrosome, it has not been determined whether the enzyme is also associated with the acrosomal matrix. It is of interest, however, that two of the proteins identified by the 2D-DIGE analysis to be potential targets of PCSK4 activity, ACBRP and zona pellucida 3R, are known to be associated with the acrosomal matrix and thus interactions between these molecules could occur in this compartment. Alternatively, the enzyme that cleaves ACRBP and that itself may be a target of PCSK4 may localize to several compartments, thus allowing interactions with both PCSK4 and ACRBP. Our studies also support a role for ACRBP in the autoactivation of proacrosin since spermatozoa from PCSK4 null mice, which contained only the precursor form of ACRBP, were unable to efficiently activate proacrosin conversion, which, in turn, could contribute to the fertility defect in these mice. However, the PCSK4 fertility defect likely arises from a multitude of effects, including altered PCSK4 substrate processing, inappropriate processing of substrates by up-regulation of the proprotein convertase PCSK7 and based on the studies presented herein, a defect in sperm head/acrosome morphology. Inappropriately formed acrosomes could contribute to the precocious capacitation and acrosome reaction observed in spermatozoa from PCSK4 null mice since important fertilization molecules may not be in the correct context. The profound fertility defect observed in the PCSK4 null mice and the fact that several key fertilization molecules are affected by the loss of PCSK4 supports that PCSK4 may be an appropriate target for the development of male-based contraceptives. Indeed, our studies showing the PCSK4 may function early in protein processing pathways suggest that multiple downstream processes would be affected if PCSK4 were inhibited. Furthermore, the PCSK4 prodomain is known to be inhibitory to the active enzyme and has been shown to inhibit fertilization *in vitro* (Iamsaard *et al*., 2011). Human PCSK4, like mouse, has been detected on the human sperm plasma membrane overlying the acrosome suggesting comparable roles in human sperm function to that in the mouse (Gyamera-Acheampong and Mbikay, 2009). Clearly, further studies on the PCSK 4 null mouse model would provide valuable insight regarding the key proteolytic processing events that are critical for spermatogenesis and fertilization. These studies would include identifying the upstream protease(s) that are the direct targets of PCSK4 processing and which in turn may control multiple downstream regulatory pathways in spermatozoa.

Acknowledgements

The authors acknowledge Daniel Hardy, Ph.D. for his assistance with the fluorescence microscopy studies and his partial financial support of Steve Tardif, Ph.D.

Authors' roles

S.T. generated the ACRBP antibody and carried out the 2D gel electrophoresis analyses and proacrosin conversion experiments, B.G. performed the IIF experiments, N.C. prepared the samples for 2D-DIGE analysis and G.A.C. designed the overall study and prepared the manuscript.

Funding

This work was supported by the National Institutes of Health (HD56182 to G.A.C.).

Conflict of interest

None declared.

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