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Epididymal C4b-binding protein is processed and degraded during transit through the duct and is not essential for fertility

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Abstract

C4b-binding protein (C4BP) is known as one of the circulating complement regulators that prevents excessive activation of the host-defense complement system. We have reported previously that C4BP is expressed abundantly in the rodent epididymis, one of the male reproductive organs connecting the testis and vas deferens, where immature spermatozoa acquire their motility and fertilizing ability during their transit through the duct. Epididymal C4BP (EpC4BP) is synthesized androgen-dependently by the epithelial cells, secreted into the lumen, and bound to the outer membrane of the passing spermatozoa. In this study, we found that EpC4BP is secreted as a large oligomer, similar to the serum C4BP, but is digested during the epididymal transit and is almost lost from both the luminal fluid and the sperm surface in the vas deferens. Such a processing pattern is not known in serum C4BP, suggesting that EpC4BP and serum C4BP might have different functional mechanisms, and that there is a novel function of EpC4BP in reproduction. In addition, the disappearance of EpC4BP from the sperm surface prior to ejaculation suggests that EpC4BP works only in the epididymis and would not work in the female reproductive tract to protect spermatozoa from complement attack. Next, we generated C4BP-deficient (C4BP−/−) mice to examine the possible role of EpC4BP in reproduction. However, the C4BP−/− mice were fertile and no significant differences were observed between the C4BP−/− and wild-type mouse spermatozoa in terms of morphology, motility, and rate of the

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spontaneous acrosome reaction. These results suggest that EpC4BP is involved in male reproduction, but not essential for sperm maturation.

Keywords

C4BP; complement; epididymis; gene targeting; processing; reproduction; sperm maturation

Introduction

The complement system plays a pivotal role in both innate and adaptive immunity by defending against bacterial infection. The main component C3 is converted to C3b by the C3 convertases produced by three initiation pathways, and the covalent binding of C3b to the invader surfaces induces various antimicrobial activities such as phagocytosis, cell lysis, or activation of the adaptive immune system. To prevent the loss of C3 by excessive activation or C3b binding to autologous cells, several regulators exist in the plasma and on cell surfaces. C4b-binding protein (C4BP) was initially identified as one of the fluid-phase complement regulators (Ferreira et al. 1977). C4BP binds to the activated fourth component C4b, which is a constituent of the C3 convertase in the classical pathway, preventing the production of the C3 convertase or working as a cofactor during degradation of C4b by factor I (Gigli et al. 1979). C4BP also binds to C3b, although only weakly (Fujita and Nussenzweig 1979). C4BP is a large heterogeneous protein with M_r 540,000–590,000 and has been identified in several animals (Chung et al. 1985; Hillarp et al. 1997; Kristensen et al. 1987; Nonaka et al. 2001). The major form of human C4BP is composed of seven α chains of M_r 70,000 and one β chain of M_r 30,000, which are linked covalently via the cysteine residues in the C-terminal regions. The α and β chains are encoded by different genes and consist of eight and three short consensus repeat (SCR) —also called sushi or complement control protein (CCP) —domains, respectively, with a C-terminal region containing two cysteines. In mice, the α chain of C4BP is composed of six SCR domains and a C-terminal region, while the β chain is not expressed because its gene has become a pseudogene. The C-terminal region of the α chain of mouse C4BP contains no cysteine residues, but mouse C4BP also exists as a large protein composed of several non-covalently bound α chains (Kaidoh et al. 1981).

It is known that some non-complement proteins, such as heparin (Hessing et al. 1990), serum amyloid protein (SAP) (Schwalbe et al. 1990), and low density lipoprotein receptor-related protein (LRP) (Westein et al. 2002) bind to C4BP, although these proteins possess many other ligands. In humans, a portion of serum C4BP exists as a complex with anti-coagulation protein S via the β chain, suggesting the involvement of C4BP in the regulation of anti-coagulation (Dahlback 1991). C4BP also directly binds to the cell surface of some ovarian adenocarcinoma cell lines (Holmberg et al. 2001) and to B cells via CD40 (Brodeur et al. 2003). On the other hand, a number of bacteria such as *Streptococcus* and *Neisseria* bind to C4BP and use it as a protector from complement attack during invasion (reviewed in (Blom and Ram 2008)). In addition, many endogenous ligands for C1q such as C-reactive protein (CRP), DNA, prions, late apoptic and necrotic cells, and the extracellular matrix

proteins also intereact with C4BP as well as factor H (FH) (reviewed in (Sjoberg et al. 2009)).

Furthermore, we have shown that C4BP is expressed abundantly in the epididymis in guinea pigs (Nonaka et al. 2001) and mice (Nonaka et al. 2003). In both species, epididymal C4BP (EpC4BP) is expressed androgen-dependently, whereas serum C4BP is constitutively expressed in the liver, and different promoter regions of a single-copy gene were used in the epididymis and liver. The epididymis is a long sinuous duct that provides a path for the spermatozoa from the testis to the vas deferens. Immature spermatozoa released from the testis pass slowly along it for days and their motility and fertilizing ability during transit, interacting with the many proteins secreted from the epithelium (reviewed in (Robaire et al. 2006)). We have shown that EpC4BP synthesized in the epithelial cells is secreted into the lumen and binds to the outer membrane of the passing spermatozoa (Nonaka et al. 2003). However, the synthesis of C3 and C4 mRNA in the epididymis is low (Nonaka et al. 2003), and infiltration of the plasma proteins into the lumen is regulated by the blood-epididymis barrier constructed between the adjacent epithelial cells (reviewed in (Mital et al. 2011)). The C3 level in human semen is 1/40th of that in plasma (Bozas et al. 1993). Therefore, it was speculated that EpC4BP might work to protect the spermatozoa from complement attack, not in the male, but in the female reproductive tract where C3 has been detected to be abundant (Li et al. 2002). Otherwise, EpC4BP might be involved in the sperm maturation system. In this report, we followed the EpC4BP along the epididymal duct and investigated its possible role in developing the sperm motility and fertility by studying the C4BP-deficient mice created by gene targeting.

Materials and methods

Mice

The C57BL/6J strain was used for most experiments. The BALB/c strain was used only for mating analysis. Both mice were purchased from CLEA Japan, Inc (Tokyo, Japan). All animal protocols were approved by the Animal Care and Use Committee of the University of Tokyo and conducted in accordance with their guidelines for animal experiments.

RT-PCR

Total RNA extracted from various regions of the epididymal tract and vas deferens of the 3-month-old mice using Isogen (Nippongene, Tokyo, Japan) was reverse-transcribed, and the cDNA fragments were amplified by polymerase chain reaction (PCR), with denaturing at 95 °C for 3 min, followed by 20 cycles of 95 °C for 0.5 min, 50 °C for 1 min, and 72 °C for 1 min, and a final extension at 72 °C for 5 min. The primers used for amplification were designed at the SCR1 and SCR6 regions as follows: forward, 5′-ACCTGCTATACCCAATG and reverse, 5′-CCAGAGATCACATTGGAT. Those for actin were: forward, 5′-ATGGAGAAGATCTGGCA and reverse, 5′-CATCTCCTGCTCGAAGT.

Western blotting analysis

The caput and cauda regions of epididymal tract and vas deferens were minced and suspended in μl PBS for caput and cauda regions and 50 μl PBS for vas deferens, and

centrifuged at 800 ×*g* for min after incubation at room temperature for over 30 min. The supernatant was used as a luminal following further centrifugation at 9,100 ×*g* for 5 min to remove the debris. The sperm pellets were washed twice with PBS and suspended in 10–120 μl of the lysis solution containing 1% NP-40, 10 mM Tris-HCl buffer (pH 7.2), 0.15 M NaCl, 0.5% SDS, and 2 mM PMSF (1–5 × 10^5 sperm/ml). After placed for overnight at 4°C, the sperm suspensions were centrifuged at 13,000 ×*g* for 15 min, and 2 μl aliquots of the supernatant were subjected to Western blotting analysis as previously described (Nonaka et al. 2003). Briefly, the samples were separated by 8% SDS-PAGE or 7.5% native PAGE under non-reducing conditions, then transferred onto a Hybond-P membrane (GE Healthcare Japan, Tokyo, Japan), and treated with 1/2000 diluted rabbit anti-mouse C4BP antiserum followed by treatment with 1/10,000 diluted HRP-anti-rabbit IgG (GE Healthcare Japan). Bands were visualized using ECL detection reagents (GE Healthcare Japan). The antiserum supplied from Dr. R.T. Ogata was generated using the fusion protein of mouse C4BP and the membrane and cytoplasmic domains of human CR2 (Ogata et al. 1993). One-tenth diluted mouse serum was used as a reference. For neuraminidase treatment, 5 μl of luminal fluids or sperm lysates were incubated with the 25 and 50 units of neuraminidase (New England Biolabs Japan Inc., Tokyo, Japan) in a 10 μl reaction for 1 h at 37°C.

Electron microscopic immunohistochemistry

Electron microscopic immunohistochemistry was performed as previously described (Nonaka et al. 2003). Briefly, frozen sections of the epididymal tissue specimens were incubated with the HRP labelled anti-rabbit IgG after the incubation with the anti-mouse C4BP antiserum, and then visualized by 3,3′-diaminobenzidine tetrahydrochloride and H_2O_2. The sections were postfixed with 1% OsO_4, and then embedded in Epon 812 (Okenshoji Co., Ltd., Tokyo, Japan). The thin sections of 80 nm thicknesses were prepared, stained with lead citrate, and observed with an electron microscope (JEM-1010, JEOL, Tokyo, Japan).

Construction of C4BP-deficient (C4BP−/−) mice

A 1.0 kb region containing the exons encoding SCR2b and SCR3 of *C4bpa* was replaced by a neomycin-resistant gene cassette in the reverse transcriptional orientation. EZ-1 mouse 129/Svj embryonic stem cells were transfected with the linearized targeting vector. Four correctly targeted embryonic stem cell clones were identified by Southern blot analysis using a probe upstream of the targeting vector. Three targeted clones were microinjected into C57BL/6 blastocytes. Eight highly chimeric male offspring were generated, one of which when crossed with C57BL/6 females produced germline transmission of the mutant *C4bpa* allele. C4BP+/− mice were backcrossed to produce C4BP−/− mice. The C4BP−/− mice were backcrossed with C57BL/6 mice over ten times to establish the C4BP−/− mice on a C57BL/6 genetic background.

Northern blotting analysis

Total RNA was isolated from the tissues of the 3-month-old mice using the guanidine thiocyanate/CsCl method. Northern blotting analysis was performed using the glyoxal-denaturing method as previously described (Thomas 1983). The mouse C4BPα cDNA

fragments covering the regions from the signal peptide to SCR2a and from SCR2b to SCR5 were used as probes. The 1.4 kb fragment containing a thioester region and the 1.0 kb fragment containing the SCR18–20 and 3′ untranslated regions were used for C4 and FH, respectively. Probes were labeled with [α-^{32}P]dCTP using the Rediprime II DNA Labeling System (GE Healthcare Japan).

Analysis of sperm motility

To analyze the sperm motility, sperm were obtained from the cauda epididymidis of the 5-month-old mice by suspending in 100-μl TYH medium (LSI Medience Corp., Tokyo, Japan) with the addition of0.4 mg bovine serum albumin. After incubation for 1 h at 37°C under 5% CO_2 in air, the released sperm were collected and analyzed. Parameters of sperm motility were quantified by computer-assisted semen analysis (CASA) using integrated visual optical system (IVOS) software (Hamilton-Thorne Biosciences, Beverly, MA), as previously described (Bray et al. 2005). More than 200 sperm per sample were analyzed using standard settings.

Spontaneous acrosome reaction assay

The cauda epididymidis obtained from the 3–5-month-old mice was suspended in 0.2 ml of TYH medium, kept at room temperature for 10 min, and the released sperm were incubated at 37°C under 5% CO_2 in air. At 30, 60, 120, and 180 min after the starting of incubation, 15 μl of the sperm suspensions were transferred into a 1.5-ml tube containing 90 μl of PBS and some aliquots of the solution were spread onto a glass slide. Then the sperm were fixed with methanol for 10 min, washed with PBS, and stained with 50 ng/μl of Alexa Fluor 488 conjugates of peanut agglutinin (PNA) (Molecular probes, Eugene, OR) by reaction for 45 min at room temperature. After washing with PBS, the slides were mounted with DAPI-Fluoromount-G (SouthernBiotech, Birmingham, AL). The acrosome-reacted sperm were counted under a fluorescence microscope (Axioplan 2, Carl Zeiss Japan, Tokyo, Japan).

Results

Region-specific expression of the C4bpa gene in the murine epididymis

Fig. 1A shows male reproductive organs of the mouse. Testicular spermatozoa are sent to the epididymis via the efferent ductules and ejaculated via the vas deferens after the long journey through a long sinuous duct of the epididymis. The epididymal duct can be divided into four regions termed initial segment, caput, corpus and cauda, based on the morphology of the epithelium and its function (Abe et al. 1983). Briefly, the initial segment is involved in absorption of testicular fluid and concentration of spermatozoa. Sperm maturation occurs mainly in the caput and corpus regions, and the spermatozoa are stored in the cauda region. In a previous paper (Nonaka et al. 2003), immunohistochemical analysis detected C4BP on the luminal wall of the epithelium and on the surfaces of the spermatozoa in the caput and cauda regions, and we speculated that C4BP is preferentially synthesized in the caput region because the Golgi apparatus in the caput epithelium was strongly stained. To confirm the precise region of mRNA synthesis, RT-PCR using RNA obtained from each region was performed. As shown in Fig. 1B, EpC4BP mRNA was synthesized mainly in the caput region, decreased in the corpus region, and was at very low levels in the cauda region and

vas deferens. This indicates that the C4BP detected by immunostaining in the cauda was derived mostly from the caput region.

Processing of the EpC4BP

To further characterize the region specificity of the murine EpC4BP, Western blotting analysis was performed. The luminal fluids and sperm lysates were subjected to blotting after SDS-PAGE (Fig. 2A) or native-PAGE (Fig. 2B) under non-reducing conditions. In SDS-PAGE, a monomer of C4BP with a molecular mass of approximately 61–65 kDa was detected. The caput C4BP showed a molecular mass of about 63 kDa slightly lower than that of serum C4BP of about 65 kDa, and the cauda C4BP showed a molecular mass of about 61 kDa even lower than that of caput C4BP in both the luminal fluid and sperm lysate. Because most of the cauda C4BP were derived from caput C4BP (Fig. 1B), it was suggested that C4BP secreted in the caput region was processed during the transit through the epididymal duct, both in the luminal fluid and on the sperm surfaces. In the vas deferens, only a faint band of C4BP was detected with the same molecular mass as the cauda C4BP in both the luminal fluid and sperm lysate, suggesting that most of EpC4BP on the sperm surfaces were dissociated and absorbed by the epithelial cells of the vas deferens together with the C4BP in the luminal fluid, or degraded to smaller fragments at a level undetectable by the polyclonal antibodies used. In native-PAGE, both the caput and cauda C4BP were detected as a large molecule near the start point in both luminal fluids and sperm lysates, similar to serum C4BP, suggesting that EpC4BP also exists as a large protein composed of several non-covalently bound α chains. This also suggests that processed cauda C4BP still exists as a large complex, but the size differences between caput and cauda C4BP were not clearly shown by native-PAGE.

Electron microscopic immunohistochemistry

A very low level of C4BP in the vas deferens was confirmed by immunoelectron microscopic analysis. Immunostaining using a polyclonal antibody detected C4BP on the apical surfaces of the epithelial cells including the microvilli (Fig. 3, a and b, shown by arrowheads), and sperm surfaces (Fig. 3, d and e, shown by arrows), in both the corpus and cauda region. By contrast, C4BP was rarely detected on the surfaces of both the epithelium and sperm in the vas deferens (Fig. 3, c and f), in good agreement with the results of the Western blotting analysis (Fig. 2A). The loss of EpC4BP from the surface of the spermatozoa before ejaculation suggests that EpC4BP does not play a role in the female reproductive organs to protect sperm from complement attack.

C4BP was detected on the entire surfaces of the spermatozoa in the corpus region (Fig 3, d), but was detected preferentially on the sperm head in the cauda region (Fig 3. e), suggesting that C4BP on the sperm surface were gradually lost from the tail during the transit through the epididymal duct.

Neuraminidase treatment

To analyze whether the size differences of C4BP in the epididymal duct regions were due to the amount of carbohydrates or proteolytic degradation, the luminal fluids and sperm lysates were treated with neuraminidase (Fig. 4). Twofold dilutions (25 and 50 units in a 10 μl

reaction mixture) of the neuraminidase were tested and the same results were obtained in both concentrations, showing that the reaction was complete. The size difference between serum and caput C4BP seems to be due largely to the carbohydrates because their sizes were similar in both luminal fluids and sperm lysates after the neuraminidase treatment. The sizes of the neuraminidase-treated serum and caput C4BP were also similar to the untreated cauda C4BP. However, the band size of the cauda C4BP decreased further after neuraminidase treatment, suggesting that the size difference between caput and cauda C4BP is not due to deglycosylation, but is due to digestion of the peptide in the cauda region. From these results, it is likely that EpC4BP molecules synthesized in the epithelium of the caput region are processed proteolytically during transit through the epididymal duct.

Targeting of the C4bpa gene

To analyze the possible role of C4BP in reproduction, C4BP-deficient mice were generated. To target the *C4bpa* gene, the region including the exons encoding SCR2b and SCR3 of the *C4bpa* gene was replaced by a neomycin-resistant gene cassette (Fig. 5A). The SCR1–3 region of C4BP has been reported to be essential for binding to C4b in both mice (Ogata et al. 1993) and humans (Blom et al. 2001). In addition, because the intron between the exons encoding SCR2a and SCR2b is inserted in phase 2 whereas other introns are inserted in phase 1, the disruption of the exon encoding SCR2b would cause a frame shift in the next exon, causing the stop codon to occur sooner. Fig. 5B shows genomic Southern blotting analysis using mouse-tail DNA demonstrating germ-line transmission of the *C4bpa* null allele. The 7.9-kb band corresponds to the mutated *C4bpa* in heterozygous (+/−) and homozygous (−/−) mice when hybridized with probe 1. In the Western blotting analysis, the serum samples obtained from the +/+ and −/− mice were separated on an SDS-PAGE gel (Fig. 5C, left panel) or a native gel (Fig. 5C, right panel) under non-reducing conditions. C4BP was not detected in the −/− mice in both experiments. Fig. 5D shows the results of Northern blotting analysis of C4BP using total RNA obtained from the +/+, +/−, and −/− mice. The probes used were cDNA fragments encoding the region from the signal peptide to SCR2a (left panel), and the region from SCR2b to SCR5 (right panel). Both probes detected no C4BP mRNA in the liver and epididymis of the −/− mice. Although the probe containing the SCR1 and SCR2a regions detected a faint smaller band (shown by open arrowhead in the left panel) in the +/− and −/− mice, this transcript is likely to be untranslated because the protein band corresponding to this transcript was not observed in the Western blotting analysis (Fig. 5C). Fig. 5E shows the results of Northern blotting analysis of complement C4 and factor H (FH). The transcripts of C4 and FH were detected at a similar level in the +/+ and −/− mice in both liver and epididymis, suggesting that the C4BP deficiency did not affect the mRNA expression level of these complement components.

Analysis of the C4BP−/− mice

The C4BP −/− mice showed normal development and fertility in both the males and females. No significant difference was observed in the numbers of pups between the wild type (WT) and C4BP −/− mice (Table 1). The morphology of the spermatozoa of the C4BP −/− mice seemed to be normal in both the caput and cauda regions under light microscopy (Fig. 6A) and electron microscopy (data not shown). The motility of the spermatozoa was analyzed by a CASA system using the sperm obtained from the cauda epididymidis. As

shown in Table 2, no significant differences were observed between the motility of the WT and C4BP−/− mouse sperm. In addition, the rate of the spontaneous acrosome reaction was examined using spermatozoa obtained from cauda epididymidis, and no significant differences were observed between sperm of the WT and C4BP−/− mice (Fig. 6B). These results suggest that EpC4BP is not essential in acquiring sperm motility and fertility.

Discussion

C4BP, one of the circulating regulators in the host-defense complement system, has been identified as one of the epididymal proteins in rodents (Nonaka et al. 2003; Nonaka et al. 2001). In this study, we showed that murine EpC4BP synthesized in the caput region is secreted into the lumen as a large homo-oligomer composed of several α chains, similar to serum C4BP, although each monomer is less glycosylated compared with serum C4BP. We then showed that each monomer, in both the luminal fluid and on the surface of spermatozoa, was processed to a lower molecular weight form during the transit from the caput to the cauda epididymidis, and was detected at a remarkably low level in the vas deferens. It is likely that most of the processed EpC4BP was absorbed by the epithelial cells in the vas deferens, or further degraded to smaller fragments at a level undetectable by the polyclonal antibodies used, or both. The C4BP on the sperm surface may be dissociated or degraded prior to ejaculation, suggesting that the EpC4BP would not work in the female reproductive organs as a complement regulator to protect the spermatozoa from complement attack, and that EpC4BP could only function in the male reproductive organs. However, complement components has been detected at only a low level in the epididymal fluid (Bozas et al. 1993; Nonaka et al. 2003), this strongly suggest that EpC4BP is involved in reproduction, but not in complement regulation.

To our knowledge, this is the first report of C4BP processing. This study suggests that EpC4BP and serum C4BP might have different functional mechanisms. By contrast, there have been many reports of epididymal proteins undergoing processing such as proteolysis, deglycosylation, and/or relocation on spermatozoa during passage through the epididymis (reviewed in (Cuasnicú et al. 2002)). For example, the rat and mouse sperm/egg fusion protein Crisp-1 (or proteins D/E) —synthesized by the epithelial cells in the caput region, secreted into the lumen, and bound to the passing spermatozoa in a similar fashion to EpC4BP —is degraded into smaller fragments by proteolysis during epididymal transit in both the fluid and sperm-bound forms, although the functional domain remains on the sperm surface after proteolytic processing (Roberts et al. 2002).

Furthermore, various testicular sperm proteins also undergo processing or repositioning for sperm maturation during epididymal transit (reviewed in Cuasnicu et al. 2002). For example, sperm adhesion molecule 1 (SPAM-1, also known as PH-20), which is expressed on the sperm surface playing a role in sperm-egg binding, is located in the entire head region of testicular spermatozoa, but in the cauda and ejaculated sperm it is found only in the posterior head region and its molecular weight is slightly decreased (Deng et al. 1999; Rutllant and Meyers 2001).

It is worth noting that the mouse testicular sperm protein, zona pellucida 3 receptor (ZP3R/sp56) that is known as a sperm-zona pellucida binding protein, is processed during the course of the acrosomal reaction (Buffone et al. 2009). ZP3R/sp56 is an SCR-containing protein composed of seven SCR domains with a C-terminal region, and is related to the α chain of C4BP. Its gene is found in tandem next to the *C4bpa* gene in various mammalian species, suggesting that ZP3R/sp56 and C4BP were generated by recent gene duplication, although the human counterpart of the *ZP3R/sp56* gene has become a pseudogene (termed *C4BPAP1*). ZP3R/sp56 is synthesized as a 67-kDa molecule and exists as a large homo-oligomer similar to murine C4BP, although each monomer is bound covalently. Interestingly, each monomer of ZP3R/sp56 is processed proteolytically into 43-kDa and 27-kDa peptides coincident with release from the acrosome, decreasing their ability to maintain the oligomeric structure. The putative cleavage site is estimated to be located in the unknown peptide region inserted between the sixth and seventh SCRs. Although the cleavage pattern is different, it is intriguing that processing occurs in both EpC4BP and ZP3R/sp56, which have similar structure and are presumably both involved in reproduction.

Endocytosis is an important component of the epithelial cell function in the epididymis and vas deferens, as well as secretion. Many proteins secreted in the caput region are absorbed by epithelial cells (reviewed in (Dacheux and Dacheux 2002)). For rat clusterin, endocytosis following processing and degradation has been suggested. Clusterin, which shows many biological activities including the regulation of the construction of the membrane attack complex in the complement system, is a major component of the epididymal fluid in many mammalian species. Rat clusterin is synthesized in the testis and epididymis, and testicular clusterin is absorbed once it is in the proximal initial segment. Clusterin is secreted into the lumen again in the caput region and binds to the passing spermatozoa, and decreases during transit and is almost lost from both the luminal fluid and sperm surfaces in the cauda region. It is presumed that proteolytic processing and endocytosis by the epididymal epithelium occur in the cauda region (Hermo et al. 1991; Mattmueller and Hinton 1992). The low-density lipoprotein receptor-related protein-2 (LRP2) may be involved in endocytosis of clusterin in the cauda region (Morales et al. 1996), and apolipoprotein E receptor-2 (also called LRP8) might be involved in absorption of testicular clusterin in the initial segment (Andersen et al. 2003). LRP2 and LRP8 are members of the low-density lipoprotein receptor (LDLR) family, which plays a role in endocytosis and degradation of various extracellular ligands. Because C4BP binds to LRP, another member of the LDLR family (Westein et al. 2002), it is possible to speculate that endocytosis of processed EpC4BP might occur through a member of the LDLR family in the vas deferens.

We created C4BP-deficient mice by gene targeting and examined the possible participation of C4BP in sperm maturation and acquisition of fertility and motility. However, the C4BP−/− mice developed normally and both the males and females showed normal fertility. The spermatozoa showed no differences in morphology or motility compared with those of the WT mice. In addition, the spontaneous acrosome reaction of the C4BP−/− mouse sperm was not significantly different from that of the WT mice. These findings suggest that EpC4BP is not essential for sperm maturation. At this point, FH, another circulating complement inhibitor, could not be the substitute for C4BP because FH mRNA is expressed at a very low

level in the male reproductive organs, and no increase was observed in the C4BP−/− mice (Fig. 5E). Among the proteins involved in the reproductive system, some showed no significant differences after the targeting disruption. For example, the knockout mice of PH-20 (Baba et al. 2002) and clusterin (Bailey et al. 2002) discussed in the previous paragraphs showed normal fertility. Moreover, unexpectedly, gene-targeting of the C4BP-related protein, ZP3R/sp56, had no effect on mouse fertilization, and it has been suggested that the sperm-zona pellucida binding system might be functionally redundant (Muro et al. 2012). The sperm maturation system is likely to involve multiple redundant proteins, and C4BP might be one of them.

Besides C4BP and clusterin, the complement inhibitors of membrane-bound types, such as membrane cofactor protein (MCP, CD46), decay accelerating factor (DAF, CD55), and CD59, are expressed on spermatozoa in both humans and mice, and have been speculated to play a non-complement role in reproduction as well as complement regulation in the female reproductive organs (reviewed in (Harris et al. 2006)). MCP is found on the inner acrosomal membrane, and DAF is detected primarily on the acrosomal outer membrane and weakly on the surface of the tail. CD59 is expressed on the entire outer surface of spermatozoa. Especially in mouse CD59, two isoforms are known: CD59a is widely expressed and including on the sperm surface playing a regulatory role in the membrane attack complex assembly (Baalasubramanian et al. 2004), whereas CD59b is expressed only on the spermatids and spermatozoa (Harris et al. 2003). Functional analysis using the knockout mice for these proteins was conducted, but any abnormality in fertility caused by the disruption of MCP and DAF has not been reported. Only a small increase in the number of pups and an accelerated spontaneous reaction was observed in the case of MCP (Inoue et al. 2003). However, the targeting of CD59b causes abnormal sperm morphologies in the testis, resulting in sperm immobility and progressive loss of fertility, in addition to the spontaneous hemolytic anemia caused by down-regulation of CD59a (Qin et al. 2005). This damage to sperm is complement-independent, while hemolytic anemia is complement-dependent, suggesting that CD59b is involved in spermatogenesis in the male reproductive system. Approaches other than gene targeting are needed to clarify the function of EpC4BP as another bridge between the complement system and reproduction.

In conclusion, this study demonstrates that EpC4BP is a large homo-oligomer composed of several α chains, similar to serum C4BP. However, EpC4BP is different from serum C4BP in that each α chain is less glycosylated and is processed proteolytically during passage through the epididymal duct, suggesting that EpC4BP and serum C4BP might play different functional roles. EpC4BP on the sperm surface is lost prior to ejaculation, suggesting that EpC4BP would not play a role in complement regulation in the female reproductive tract, but is involved in male reproduction. However, the analysis of the C4BP knockout mouse showed that EpC4BP is not essential for sperm motility and fertility.

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Abbreviations

C4BP	C4b-binding protein
EpC4BP	epididymal C4BP
DAF	decay accelerating factor
MCP	membrane cofactor protein
LDLR	low-density lipoprotein receptor
LRP	low-density lipoprotein receptor-related protein
SCR	short consensus repeat
WT	wild type
ZP3R	zona pellucida 3 receptor

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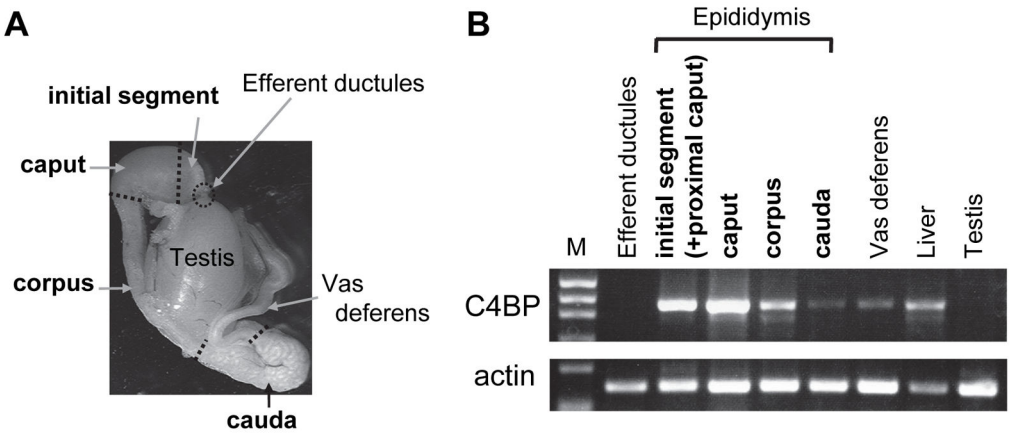

Fig. 1.

Region-specific expression of epididymal C4BP mRNA. (A) Sectioning of the epididymis. The epididymal duct is divided into four main regions: initial segment, caput, corpus, and cauda (shown in bold). The approximate boundaries are shown with dotted lines. The sectioned tissues of each region may contain a small part of the neighboring regions because the duct is sinuous and the boundary of each region is blurred. (B) The results of the RT-PCR. The C4BP mRNA was observed at a significantly high level in the caput region, at a lower level in the corpus region, and at very low levels in the cauda region and vas deferens. It was undetectable in the efferent ductules and testis. Actin was used as a reference. M, size marker.

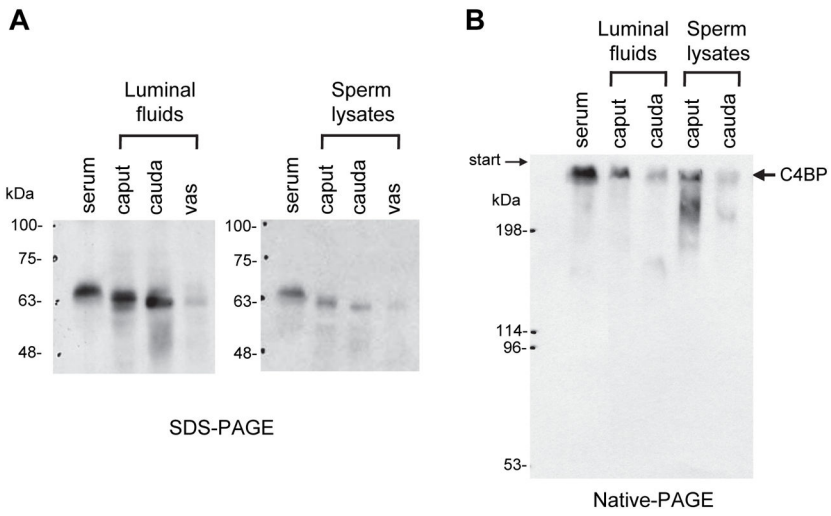

Fig. 2.

Characterization of epididymal C4BP in the luminal fluids and sperm lysates. Samples obtained from the caput and cauda epididymidis and vas deferens were hybridized with the polyclonal antibody to mouse C4BP after 8% SDS-PAGE (A) or 7.5% native PAGE (B) under non-reducing conditions using one-tenth diluted serum as a reference. In SDS-PAGE, the caput C4BP showed a molecular weight slightly lower than that of serum C4BP, and the cauda C4BP showed a molecular weight slightly even lower than that of caput C4BP in both the luminal fluids and sperm lysates. In the vas deferens, only a faint band of C4BP with the same size as the cauda C4BP was detected. In native PAGE, both serum and epididymal C4BP were detected as a large molecule near the start point in both the luminal fluids and sperm lysates, showing that epididymal C4BP exists as a homo-oligomer similar to serum C4BP. However, the size differences between serum, caput, and cauda C4BP were not clear in the native PAGE.

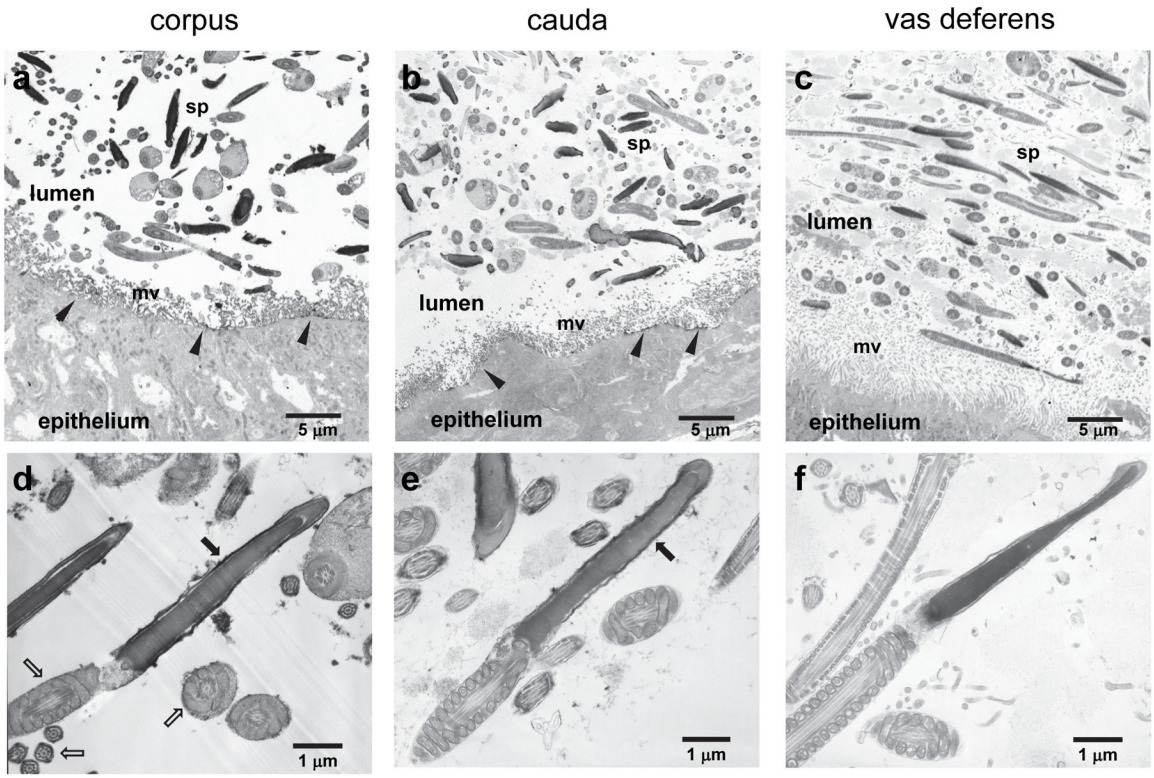

Fig. 3.

Electron microscopic immunohistochemistry of the epididymis and vas deferens. The corpus and cauda epididymidis and vas deferens were immunostained with the polyclonal antibody to mouse C4BP. a, b, and c show the epithelium and lumen, and d, e, and f show the magnified spermatozoa (sp) of each region. C4BP was detected on the apical surfaces of the epithelial cells including the microvilli (mv) in the corpus (a) and cauda (b) epididymidis, as shown by *arrowheads*. C4BP was also detected on the sperm surfaces at the sperm head (solid arrow) and tail (open arrow) in the corpus region (d) and at the sperm head in the cauda region (e). In contrast, C4BP was rarely detected on the surface of both the epithelium (c) and spermatozoa (f) in the vas deferens.

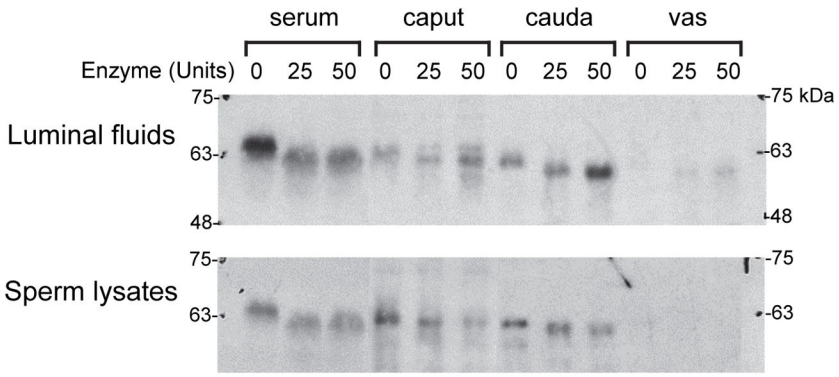

Fig. 4.

Neuraminidase treatment of serum and epididymal C4BP. Five μl of the luminal fluids and sperm lysates obtained from the caput and cauda epididymidis and vas deferens were treated with 25 and 50 U of neuraminidase in a 10-μl reaction mixture. One-tenth diluted serum was used as the reference. C4BP was detected by Western blotting analysis after 8% SDS-PAGE under non-reducing conditions. The lanes for serum were exposed for a shorter time than the other lanes. Twofold dilutions of the neuraminidase showed the same results, indicating that the reaction was complete. In both the luminal fluids (upper panel) and sperm lysates (lower panel), the neuraminidase-treated serum and caput C4BP showed similar sizes, suggesting that the size difference between serum and caput C4BP is due largely to the carbohydrates. However, caput and cauda C4BP decreased their molecular weights to an almost equal degree after the neuraminidase treatment, suggesting that the size difference between caput and cauda C4BP is due to the digestion of the peptides during transit through the epididymal duct. It appears that EpC4BP in the sperm lysates (lower panel) decreased the molecular weight after the neuraminidase treatment less than the EpC4BP in the luminal fluids (upper panel). This suggests that EpC4BP may be somewhat deglycosylated when it binds to the spermatozoa, although further analysis was not done.

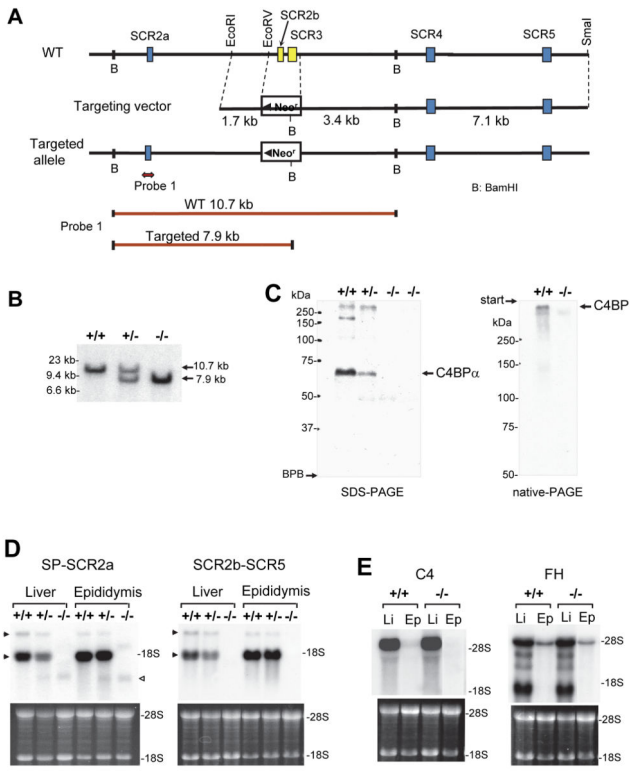

Fig. 5.

Targeted disruption of mouse *C4bpa* gene. (A) Targeting strategy. Exons encoding SCR2b and SCR3 of the *C4bpa* gene of the wild type (WT) were replaced by a neomycin-resistant gene in the reverse transcriptional orientation. Probe 1 shows the position of the probe used for screening by Southern blot analysis, and the expected lengths of the *Bam*HI-digested genomic DNA for the WT and targeted allele are shown below. (B) Representative results of Southern blot analysis obtained by using mouse-tail genomic DNA. Three genotypes, WT (+/+), heterozygous (+/−), and homozygous (−/−) are shown. (C) Western blotting analysis of the serum obtained from the +/+, +/− and −/− mice. One-tenth diluted serum samples were electrophoresed under non-reducing conditions in 8% SDS-PAGE (*left panel*) and 7.5% native-PAGE *(right panel*). (D) Northern blotting analysis using total RNA obtained from the +/+, +/−, and −/− mice. The probes used were cDNA fragments encoding the region from the signal peptide to SCR2a (*left panel*), and the region from SCR2b to SCR5 (*right panel*). Solid arrowheads show the 1.9-kb transcript and the 3.0-kb transcript with the longer 3′ untranslated region of C4BP. Open arrowhead shows the untranslated transcript. (E) Northern blotting analysis of C4 and factor H (FH) using the liver (Li) and epididymis (Ep) of the +/+ and −/− mice. An approximately 5-kb transcript of C4 (*left panel*) and four transcripts using the liver and one transcript using the epididymis of FH (*right panel*) were expressed at a similar level in the +/+ and −/− mice.

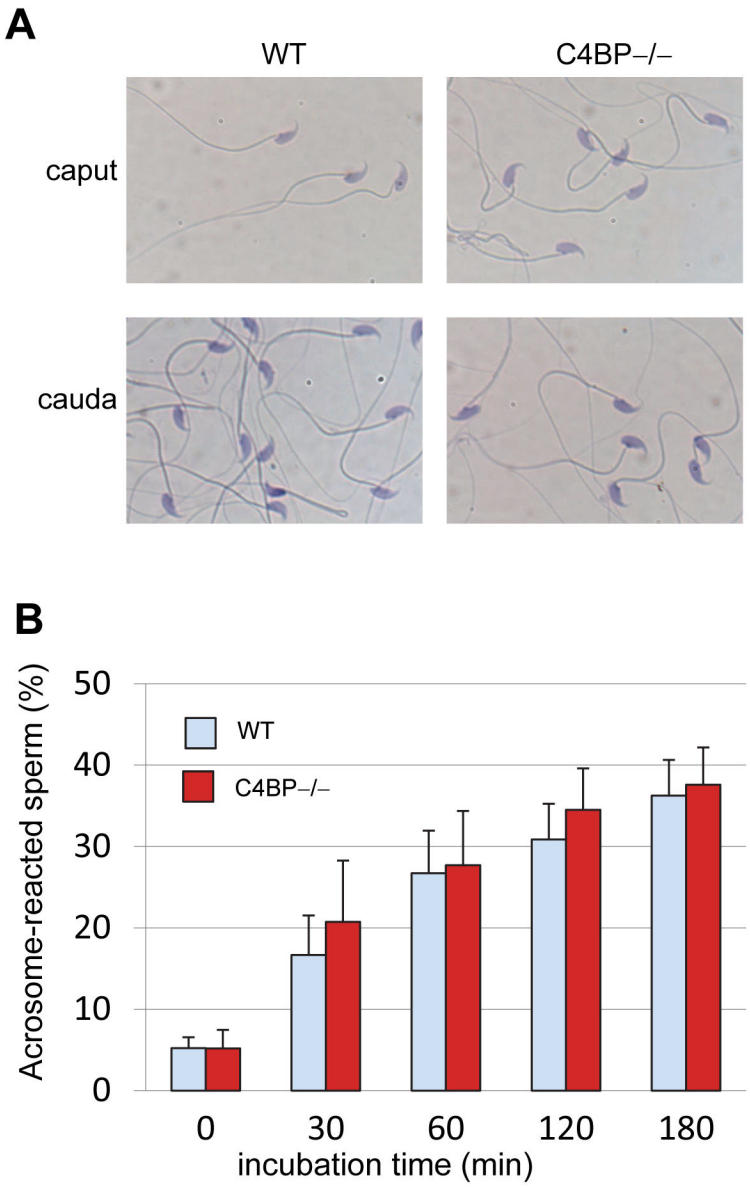

Fig. 6.

Analysis of spermatozoa. (A) Hematoxylin-stained spermatozoa, ×40. The sperm from the WT and C4BP−/− mice were not morphologically different in either the caput or cauda region. (B) Spontaneous acrosome reaction. The sperm obtained from the cauda region were incubated in TYH medium to induce the spontaneous acrosomal reaction. The acrosomal statuses were analyzed by staining the acrosomal membrane using fluorescent dye-conjugated PNA at the indicated time points after the incubation. Five independent experiments were performed and the percentages of the acrosome-reacted sperms are shown as means ± standard deviations for each time. No significant differences were observed between the sperm of the WT and C4BP−/− mice.

Table 1

Comparison of the numbers of pups[a]

male	WT	C4BP−/−	WT	C4BP−/−	WT	C4BP−/−
female	WT		C4BP−/−		BALB/c	
No. of pups	7.2 ± 1.2 (30)	8.1 ± 0.9 (10)	8.2 ± 1.9 (6)	7.9 ± 1.7 (30)	9.0 ± 1.6 (4)	10.2 ± 2.7 (11)

[a]WT (C57BL/6) and C4BP−/− male mice were mated with WT, C4BP−/−, and BALB/c female mice. BALB/c is known as a strain with a large litter size. The results are shown as means ± standard deviations. The number of deliveries is shown in parentheses. Only normal deliveries were analyzed. No significant differences were observed between the WT and C4BP−/− mice, regardless of the female genotype or strain used.

Table 2

Comparison of motility and hyperactivation of the capacitated epididymal sperm[a]

parameters	WT	C4BP−/−
Total motility (%)	76.7 ± 5.6	74.0 ± 2.4
Progressive motility (%)	63.3 ± 5.9	59.7 ± 2.6
Average path velocity (μm/s)	105.3 ± 2.9	107.0 ± 9.7
Progressive velocity (μm/s)	73.7 ± 3.8	76.3 ± 8.9
Amplitude of lateral head displacement(μm)	8.7 ± 0.5	8.2 ± 0.8
Beat frequency (Hz)	16.2 ± 1.4	16.9 ± 1.6
Straightness (%)	70.3 ± 1.7	71.0 ± 0.0
Linearity (%)	44.3 ± 1.7	47.0 ± 0.8
Hyperactivation (%)	21.0 ± 4.8	21.8 ± 4.3

[a]Data show the means ± standard deviations of three independent experiments. At least 200 sperm per sample were analyzed in each experiment. No significant differences were found between WT and C4BP−/− mice for any of the parameters.