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

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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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 acquire 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 100 μl PBS for caput and cauda regions and 50 μl PBS for vas deferens, and centrifuged at 800 × *g* for 10 min after incubation at room temperature for over 30 min. The supernatant was used as a luminal fluid following further centrifugation at 9100 × *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 ($1–5 \times 10^5$ 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 labeled 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