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·Original Article·

Characterizing mouse male germ cell-specific actin capping protein α3 (CPα3): dynamic patterns of expression in testicular and epididymal sperm

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Abstract

Aim: To characterize mouse capping protein α3 (CPα3) during spermatogenesis and sperm maturation. **Methods:** We produced rat anti-CPα3 antiserum and examined the expression of CPα3 in various mouse tissues using Western blot analysis and the localization of CPα3 in testicular and epididymal sperm using immunohistochemical analyses. We also examined how the localization of CPα3 and β-actin (ACTB) in sperm changed after the acrosomal reaction by performing immunohistochemical analyses using anti-CPα3 antiserum and anti-actin antibody. **Results:** Western blot analysis using specific antiserum revealed that CPα3 was expressed specifically in testes. Interestingly, the molecular weight of CPα3 changed during sperm maturation in the epididymis. Furthermore, the subcellular localization of CPα3 in sperm changed dynamically from the flagellum to the post-acrosomal region of the head during epididymal maturation. The distribution of ACTB was in the post-acrosomal region of the head and the flagellum. After inducing the acrosomal reaction, the CPα3 and ACTB localization was virtually identical to the localization before the acrosomal reaction. **Conclusion:** CPα3 might play an important role in sperm morphogenesis and/or sperm function. (*Asian J Androl 2008 Sep; 10: 711–718*)

Keywords: acrosome; male germ cell-specific; spermatogenesis; testis

1 Introduction

Spermatogenesis is a highly specialized and complicated process. First, spermatogonial stem cells proliferate and differentiate into spermatocytes. Spermatocytes differentiate through meiosis to generate haploid round spermatids, and then the round spermatids undergo drastic morphological changes: the nucleus is shaped, the mitochondria are rearranged, the flagellum develops, and the acrosome is generated to differentiate spermatozoa [1]. Finally, during epididymal transit, spermatozoa acquire motility and the ability to recognize and to fuse

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with the oocyte [2]. Many proteins are associated with small membrane vesicles named "epididymosomes". Secreted epididymal proteins from epididymosomes are transferred to spermatozoa and play an important role in sperm maturation [3].

To elucidate the molecular mechanisms of spermatogenesis, we have cloned many genes that are specifically expressed in haploid germ cells from a complementary DNA (cDNA) library generated by subtracting messenger RNA (mRNA) derived from mutant (W/W^v) testis from wild-type testis cDNA [4]. A detailed analysis of the mRNA expression of various genes reveals that gene expression is controlled developmentally. We have analyzed these genes individually [5–9].

Previously, we cloned a novel capping protein α subunit gene from a subtracted cDNA library of mouse testis: germ cell-specific gene 3 (*Gsg3*) [4] (later referred to as capping protein α3 [CPα3]) [10]. Genomic analysis reveals that mouse *cpα3* is an intronless gene on chromosome 6 and the putative transcriptional promoter region contains cyclic adenosine monophosphate (AMP)-response element motifs [8, 11]. In rats, a testis-specific actin capping protein (TS-ACP) is expressed postmeiotically in round spermatids and its localization coincides with the position of the developing acrosome [12]. These results suggest that TS-ACP has an important role in the reorganization of the actin cytoskeleton during the shaping of the acrosome [12]. Human CPα3 is mainly localized in the neck region of ejaculated sperm, with moderate and faint signals also in the tail and postacrosomal region, respectively [13]. Furthermore, bovine CPα3 and two other actin-regulatory proteins exhibit dynamic distribution changed in both the head and tail of sperm during epididymal maturation and the acrosomal reaction [14].

In this investigation, we examined the distribution of mouse CPα3 during spermatogenesis and epididimal maturation. The CPα3 was specifically expressed in testes and the distribution was changed from the flagellum to the head during epididymal maturation. These results suggest that CPα3 might play an important role in sperm morphogenesis and/or sperm function.

2 Material and methods

2.1 Animals

All mice were bred and maintained in our laboratory animal facilities and used in accordance with guidelines for care and use of laboratory animals set by the Japanese Association for Laboratory Animal Science. Mice were kept under controlled temperatures and light conditions during experiments and were provided food and water *ad libitum*.

2.2 Preparation of antiserum

Production of the antiserum is described in our previous report [5]. Briefly, the full-length open reading frame of mouse *cpα3* (*mcpα3*) cDNA was subcloned into the pGEX-1 vector [15]. Glutathione S-transferase fusion protein was produced in *Escherichia coli* by isopropyl-β-D-thiogalactopyranoside induction and purified with glutathione-agarose beads. Polyclonal antiserum was raised by injection of the above antigens, followed by several booster injections into rats at 3 week intervals.

2.3 Preparation of protein extract

Various organs freshly removed from C57BL/6 mice and testes at different ages were homogenized on ice in lysis buffer (10 mmol/L Tris-HCl [pH 7.5], 160 mmol/L NaCl, 1% Triton X-100, 1% deoxycholic acid, 0.3% sodium dodecyl sulfate [SDS], and 2 mmol/L phenylmethylsulfonyl fluoride [Sigma, St. Louis, MO, USA]). After centrifugation at 17 800 × *g* for 10 min at 4ºC, the protein concentration of each supernatant was estimated using a Bradford Protein Assay kit (Bio-Rad, Richmond, CA, USA).

2.4 Western blot analysis

Protein from each extract (50 μg) was subjected to SDS-polyacrylamide gel electrophoresis (PAGE), followed by electroblotting to polyvinylidenedifluoride membrane filters (Millipore, Bedford, MA, USA). The filters were blocked with blocking solution in Tris-buffered saline (TBS; Nacalai, Kyoto, Japan). The filters were then reacted with diluted anti-CPα3 rat antiserum (1:1 000) in Can Get Signal (ToYoBo, Osaka, Japan) for 1 h at room temperature and washed in TBS (100 mmol/L Tris-HCl [pH 7.5], 150 mmol/L NaCl) three times for 10 min each. Finally, the filters were incubated with peroxidase-conjugated anti-rat immunoglobulin (1:1 000; Amersham Pharmacia Biotech, Tokyo, Japan) for 1 h at room temperature. After further washing, reactive bands were visualized by development with a POD staining kit (Wako, Osaka, Japan).

2.5 Construction of mCPα3 expression vector and trans-

fection into cultured cells

An expression vector carrying *mcpα3* was constructed by polymerase-chain-reaction cloning of amplified mouse *cpα3* cDNA into pEGFP-N1 (Clonetech, CA, USA). The resultant clone expressed the CPα3 protein fused with enhanced green fluorescent protein (CPα3-EGFP). Human embryonic kidney (HEK)-293 cells were transfected with the expression vector pEGFP-mCPα3 using Lipofect-Amine Plus reagent according to the manufacturer's protocol. Cells were observed with a fluorescent microscope 24 h after transfection and harvested for Western blot analysis. The filters for Western blotting were reacted with anti-CPα3 antibody (1:1 000 dilution) or anti-green fluorescent protein monoclonal antibody (1:500 dilution). Each filter was then incubated with peroxidase-conjugated anti-rat IgG (1:1 000 dilution; Dako Cytomation Norden A/S, Glostrup, Denmark).

2.6 Immunohistochemical analysis

Fresh testis samples were embedded in O.T.C. embedding medium (Sakura Finetek, Tokyo, Japan) and frozen at –20ºC. Eight-μm-thick sections were prepared using a cryomicrotome (HM 500 OM; Microm, Walldorf, Germany) and were fixed with 70% ethanol at 4ºC for 10 min. After blocking in 10% blocking solution in TBS (Nacalai) and normal rabbit serum in phosphate-buffered saline (PBS) at room temperature for 1 h, the sections were incubated overnight at 4ºC with diluted anti-CPα3 rat antiserum (1:1 000) or with pre-immune antiserum (1:1 000) as the control in Can Get Signal immunostain (ToYoBo). After three washes in PBS for 10 min each, sections were incubated with fluorescein isothiocyanate-conjugated anti-rat IgG antibody (Dako) diluted 1:10 000 in Can Get Signal immunostain (ToYoBo). Sections were counterstained with 4',6-diamino-2-phenylindole (DAPI; Nacalai). The slides were washed in PBS and examined under a fluorescent microscope. Each epididymis was minced with a razor brade in PBS and the supernatant that contained sperm and other cells were filtered through nylon mesh and centrifuged at 400 × *g* for 5 min. The pellet was washed in PBS, and a few drops were placed on glass slides and dried at 37ºC for 10 min. The slides were reacted using the same protocol as above. Diluted goat anti-actin antibody (1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and bovine anti-goat IgG antibody conjugated to rhodamine (1:300; Santa Cruz Biotechnology) were used. Sections were counterstained with DAPI (Nacalai) and/or 20 μg/mL tetramethylrhodamine isothiocyanate (TRITC)-conjugated peanut agglutinin (TRITC-PNA; Sigma-Aldrich, St. Louis, MO, USA) for 3 min, washed in PBS and examined under a fluorescent microscope.

2.7 Sperm induction of the acrosomal reaction

Sperm from the cauda epididymis were incubated in Toyoda Yokoyama Hoshi (TYH) medium (119 mmol/L NaCl, 4.8 mmol/L KCl, 1.7 mmol/L $CaCl_2$, 1.2 mmol/L KH_2PO_4, 1.0 mmol/L $MgSO_4$, 25 mmol/L $NaHCO_3$, 5.6 mmol/L glucose, 0.5 mmol/L sodium pyruvate, and 4 mg/mL bovine serum albumin) [16] at 37ºC in a humidified incubator with 5% CO_2 in air to allow capacitation. After 15 min, highly motile sperm were taken from the upper part of the medium and the calcium ionophore A23187 (Sigma) was added at a final concentration of 10 μmol/L to induce the acrosomal reaction. After an additional 15 min incubation, sperm were spotted onto a glass slide and examined using the same protocol as above. Acrosome status was evaluated by staining with TRITC-PNA, which binds to the outer acrosomal membrane and, therefore, does not stain acrosome-reacted sperm [17].

3 Results

3.1 Expression of CPα3 observed using Western blot analysis

To examine the specific reactivity of anti-CPα3 rat antiserum with mouse CPα3, a western blot analysis of HEK-293 cells transfected with recombinant CPα3-EGFP fusion protein was performed. HEK-293 cells transfected with the expression vector pEGFP-N1 alone were used as a negative control. The antiserum did not react with EGFP (Figure 1A). It specifically detected the CPα3-EGFP fusion protein (60 kDa; Figure 1A). Using this antiserum, we examined the expression of CPα3 in various mouse tissues. A single band with a molecular mass of approximately 50 kDa was detected exclusively in testis extracts (Figure 1B). A signal band with lower molecular mass (43 kDa) was detected in sperm extracts (Figure 1B). The expression of *mcpα3* mRNA occurs specifically in the testis, based on a northern blot analysis, so the band of approximately 40 kDa in the skeletal-muscle extracts was an extra band [10]. During germ-cell development, CPα3 was initially detected at 21 days of age (Figure 1C).

3.2 Immunohistochemistry of adult mouse testes

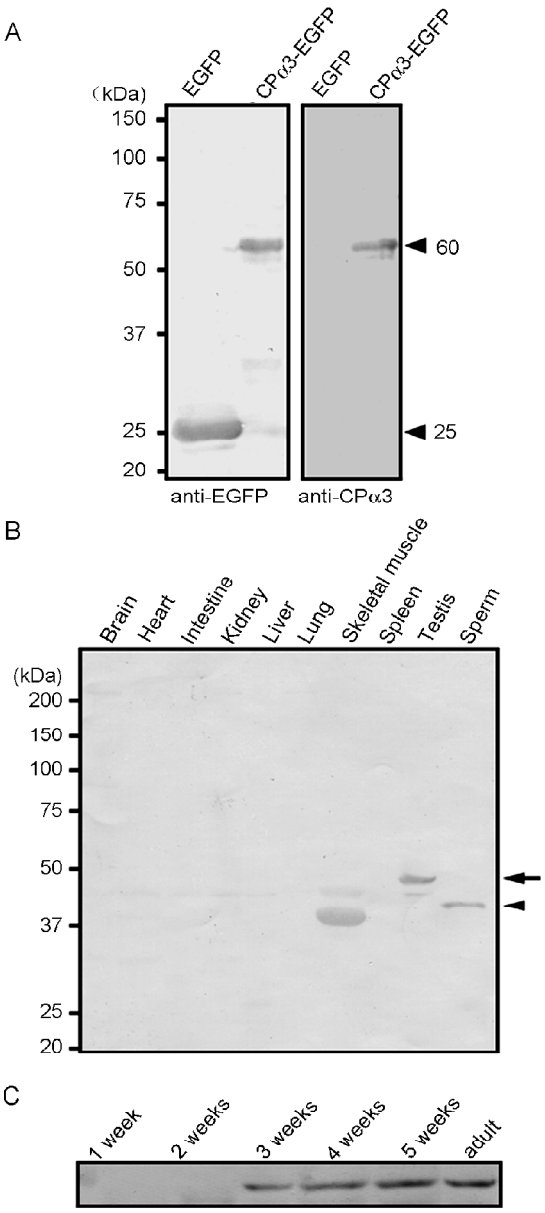

Figure 1. Western blot analyses using anti-capping protein α3 (CPα3) antiserum. (A): The lysates of HEK-293 cells transfected with pEGFP-N1 and pEGFP-mCPα3 were subjected to SDS-PAGE, followed by Western blotting, to identify reactivity with the recombinant mouse protein. EGFP (25 kDa) alone as control was not recognized by the anti-CPα3 antiserum but was recognized by the anti-EGFP antibody. CPα3-EGFP detected with both the anti-CPα3 antiserum and anti-EGFP antibody as a 60-kDa fusion protein. (B): Western blot analysis of various mouse tissues using anti-CPα3 antiserum. The arrow and arrowhead indicate a single band in the testis lane and cauda epididymal sperm lane, respectively. The approximately 40-kDa band in the skeletal muscle lane was assumed to be cross-reactive material. (C): The expression of CPα3 during male germ-cell development.

Immunohistochemical analysis of CPα3 in adult mouse testes showed that it was detected predominantly in elongated spermatids (Figure 2D–L). Without background signals in Leydig cells and basal membranes, no signal was detected using preimmune rat antiserum (Figure 2A–C) and we previously demonstrated that *cpα3*

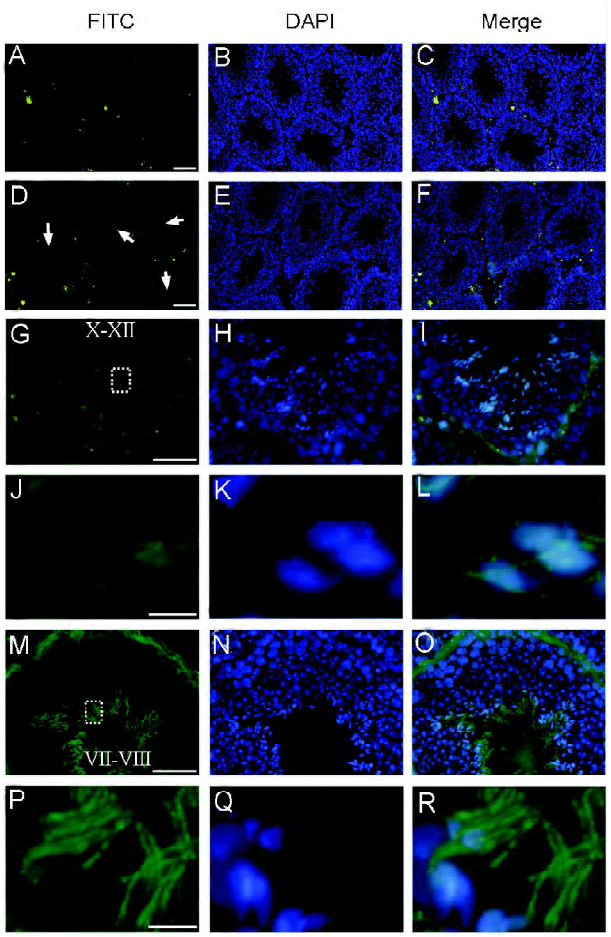

Figure 2. The localization of capping protein α3 (CPα3) in the testis. Frozen sections of adult mouse testis were stained with anti-CPα3 antiserum. (A–C): Sections stained with preimmune rat serum. No signal was detected without background signals in Leydig cells and basal membranes. (D–R): Sections stained with anti-CPα3 antiserum. Arrows indicate seminiferous tubules that were positive for CPα3. The signal was detected predominantly in elongated spermatids at the late steps (9–12) of haploid germ-cell development. (J–L) and (P–R) show higher-magnification views of the boxes in (G) and (M), respectively. Roman numerals indicate the stages of seminiferous tubule development (G and M). Later in development (steps 15–16), CPα3 localized to the midpiece of the flagellum and the signal at the head almost disappeared (M–R). The nuclei are shown in blue. Scale bar = 100 μm in (A), (D), (G) and (M), and 12.5 μm in (J) and (P). DAPI: 4',6-diamino-2-phenylindole.

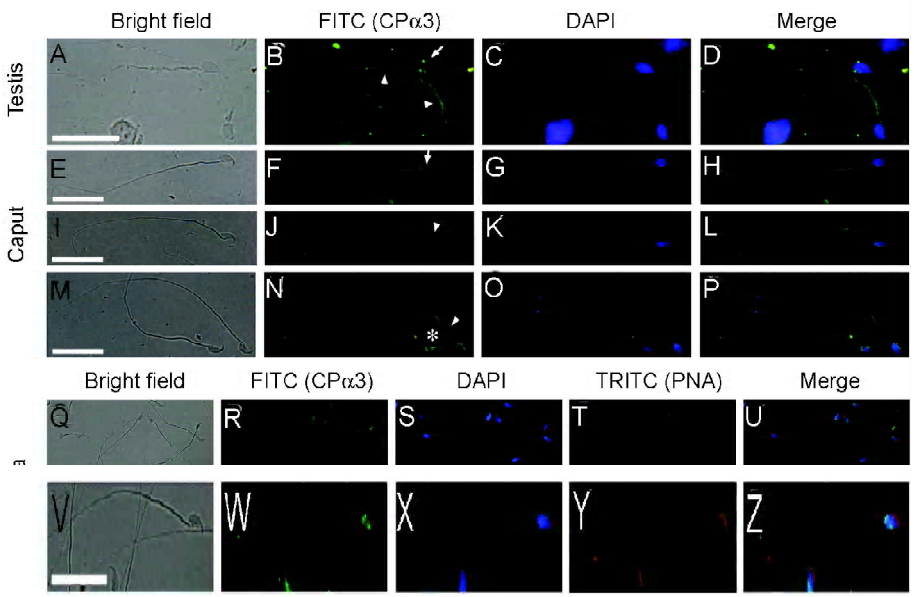

Figure 3. The subcellular localization of capping protein α3 (CPα3) in testicular and epididymal sperm. Testicular and epididymal sperm were stained with anti-CPα3 antiserum. A green signal indicates the localization of CPα3. The nuclei were stained with 4',6-diamino-2-phenylindole (DAPI, blue). (T and Y): Samples were stained using tetramethylrhodamine isothiocyanate (TRITC)-conjugated peanut agglutinin (TRITC-PNA, red) to visualize the acrosome. (A–D): In testicular sperm, CPα3 localized to the post-acrosomal region (arrow) and the midpiece of the flagellum (arrowheads). (E–P): In caput epididymal sperm, three patterns of localization were detected: localization only to the midpiece of the flagellum (arrow), to both the midpiece of the flagellum and the post-acrosomal region of the head (arrowhead), and to only the post-acrosomal region of the head (asterisk). (Q–Z) In cauda epididymal sperm, CPα3 was located in post-acrosomal regions only. Scale bar = 25 μm.

was not expressed in the supporting cells without germ cells [4]. At steps 9–12 of haploid germ-cell development, CPα3 localized to both the head and midpiece of the flagellum; at later steps (steps 15–16), there was almost no signal from the head (Figure 2M–R). These results are consistent with the age blot analysis (Figure 1C). The subcellular localization of CPα3 changed dynamically at each developmental stage.

3.3 Immunohistochemistry of testicular and epididymal sperm

To examine the localization of CPα3 in testicular and epididymal sperm, we performed immunohistochemical analyses. In testicular sperm, a signal was detected from the midpiece of the flagellum and a slight signal was detected in the post-acrosomal region of the head (Figure 3A–D). In sperm from the caput epididymis, three patterns of localization were detected: (i) both the midpiece of flagellum and a line on the curvature of head (Figure 3E–H); (ii) the midpiece of the flagellum, the post-acrosomal perinuclear theca and a thin line on the ventral curvature of nucleus (Figure 3I–L); and (iii) the post-acrosomal perinuclear theca and a line on the curvature of the head (Figure 3M–P). Furthermore, in sperm from the cauda epididymis, the CPα3 signal was detected only in the post-acrosomal perinuclear theca and a line on the curvature of the head. These results show that the localization of CPα3 changed drastically during sperm maturation in the epididymis (Figure 3Q–Z).

We examined how the localization of CPα3 and β-actin (ACTB) changed after the acrosomal reaction. Immunohistochemical analyses were performed using anti-CPα3 antiserum and anti-actin antibody. After inducing the acrosomal reaction, the CPα3 localization was virtually identical to the localization before the acrosomal reaction (Figure 4A–J). The subcellular distribution of CPα3 was slightly diffuse in the post-acrosomal region. The distribution of ACTB did not change but the signal was slightly more intense in the post-acrosomal region and the flagellum (Figure 4A–J).

4 Discussion

Actin plays various roles in dynamic cellular processes,

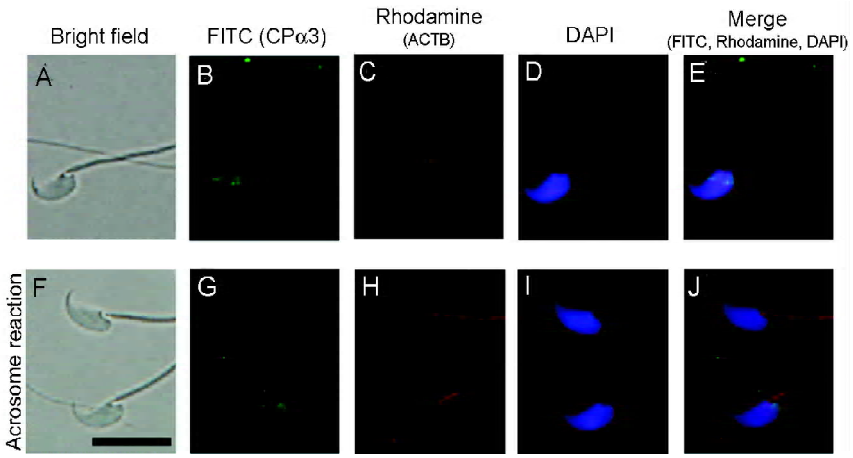

Figure 4. The localization of capping protein α3 (CPα3) and β-actin (ACTB) in sperm after the acrosomal reaction. Cauda epididymal sperm were stained with anti-CPα3 antiserum and anti-actin antibody after the acrosomal reaction (F–J) or before (A–E). The green and red signals indicate CPα3 and ACTB, respectively. The nuclei were stained with 4',6-diamino-2-phenylindole (DAPI, blue). Scale bar = 12.5 μm. ACTB colocalized with CPα3 in the post-acrosomal region after the acrosomal reaction.

including cell migration, cytokinesis, and membrane trafficking in somatic cells [18–20]. The roles of actin in male germ cells are less understood than its roles in somatic cells [21–23]. The subcellular localization of actin in the acrosomal region of several mammalian species has been reported [24–27]. These studies suggest that actin plays an important role in acrosome formation and that the acrosomal reaction is a crucial process that makes sperm able to penetrate the zona pellucida and fuse with the egg membrane. Furthermore, the localization of actin-binding proteins and actin-related proteins, such as calicin [28], destrin [29], thymosin β10 [29], testis-specific actin capping protein [29], CPβ3 [30], CPα3 [4], gelsolin [31], scinderin [32], Arp-T1 and T2 [33], and TACT1 and TACT2 [34] in the sperm head, suggest that actin polymerization and depolymerization might play important roles in sperm capacitation and the acrosomal reaction [35].

Actin capping protein (CapZ), an actin regulatory protein, is a heterodimer consisting of the capping protein α and β subunits [36, 37]. In the presemt study, we examined the distribution of CPα3 and its subcellular localization during spermatogenesis and maturation in epididymis. Using specific antiserum, we examined the expression of CPα3 in various mouse tissues using Western blot analysis. A single band with a molecular mass of 50 kDa was detected exclusively in testis extracts (Figure 1B), and in sperm extracts a signal band with lower molecular mass (43 kDa) was detected. The putative molecular mass of CPα3, calculated from its amino acid sequence, is 35 kDa. The size of the recombinant protein (fused to EGFP) expressed in somatic cells was similar to the predicted size (Figure 1A). A disulfide bond might have been broken because western blotting was performed with a reductant. The change in molecular mass might also reflect various modifications that are specific to germ cells and the band in testis lane between arrow and arrowhead in Figure 1B might be processing proteins. SPAM 1, also known as PH-20, is a glycosylphosphatidylinositol-linked sperm surface protein and the molecular mass of SPAM 1 is decreased as a result of progressive N-linked deglycosylation during epididymal transit [38]. The recombinant protein that expressed in mouse embryo fibroblast did not occur in some modifications, such as N-linked Glycosylation [39]. However, we confirmed the absence of N-glycosylation (data not shown). It is probable that CPα3 is tightly associated with other proteins for actin regulation or to protect it from degradation.

To identify the developmental expression pattern for CPα3, prepubertal mouse testes were examined. The transcript was not found in the testis until 3 weeks postpartum, when round spermatids differentiated into elongated spermatids (Figure 1C). Haploid spermatids undergo drastic morphlogical changes. Therefore, CPα3 may regulate actins and associated actin-like proteins to shape the sperm form.

Immunofluorescence analyses of mouse testes showed that CPα3 localized both in the head and flagellum at steps 9–12 (Figure 2G–L), and that the signal in the head slightly remained in later development (Figure 2M–R). Sperm morphogenesis requires drastic changes during head shaping and flagellum formation. Actin and CPα3 might support morphological changes during spermatogenesis. During epididymal maturation, the localization of CPα3 in sperm changed drastically in the post-acrosomal perinuclear theca and a line on the curvature of the head (Figure 3). In the cauda epididymis, CPα3 was only located in the post-acrosomal perinuclear theca and in a line on the curvature of head (Figure 3Q–Z). These results suggest that CPα3 might play an important role in controlling the actin cytoskeleton in the midpiece and post-acrosomal region during sperm maturation in the epididymis. Rat CPα3 protein was reported to be present mainly in the acrosomal region of rat testicular sperm and human CPα3 was detected in the acrosomal region, tail and neck region of ejaculated sperm [12, 13]. Human CPα3 was very similar to mouse CPα3 in the localization, but the molecular mass change was not detected in human sperm. Rat CPα3 was not localized in the flagellum of testicular sperm. After epididymal transit, the localization of rat CPα3 might change. Although the amino acid sequence of human and rat CPα3 is approximately 90%, CPα3 might have a slightly different role in each species.

To determine whether the localization of CPα3 and ACTB changed after the acrosomal reaction, immunohistochemical analyses were performed using anti-CPα3 antiserum and anti-actin antibody. After inducing the acrosomal reaction, ACTB still colocalized with CPα3 in the post-acrosomal region (Figure 4). CPα3 might regulate ACTB in the post-acrosomal region during the acrosomal reaction and other protein might regulate in the midpiece. *In vitro*, actin polymerization occurs during ram and bull sperm capacitation and F-actin breakdown occurs before the acrosome reaction [40]. Inhibition of actin depolymerization by phalloidin inhibits the acrosome reaction [41]. Therefore, actin might play an important role in fertilization and CPα3 might regulate remodeling of actin in the post-acrosomal region.

Spermatozoa acquire motility and the ability to recognize and fuse with the oocyte during epididymal transit. CPα3 might help sperm mature by controlling actin polymeration and depolymeration with other actin capping proteins. These results suggest that CPα3 might play an important role in spermatogenesis and/or acrosomal reactions by regulating remodeling of actin. Von Bulow *et al*. [28] showed that CPα3, a testis isoform of the CP subunit, is a component of the cytoskeletal calyx of the sperm head in the guinea pig. However, CPα3 has not been examined in detail. An analysis of CPα3 and CPα3 might provide a better understanding of the crucial role and regulation mechanisms of actin during spermatogenesis.

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