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Source: Biology of Reproduction, 80(6) : 1293-1304

Published By: Society for the Study of Reproduction

URL: https://doi.org/10.1095/biolreprod.108.073866

BIOLOGY OF REPRODUCTION **80,** 1293–1304 (2009)
Published online before print 4 February 2009.
DOI 10.1095/biolreprod.108.073866

CABS1 Is a Novel Calcium-Binding Protein Specifically Expressed in Elongate Spermatids of Mice[1]

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ABSTRACT

Single intraperitoneal injection of busulfan at 20 mg/kg body weight to mature male mice induced the deletion of the spermatogenic cells, followed by the restoration of the spermatogenesis by the surviving undifferentiated spermatogonia. The changes of the protein contents in testis during these processes were analyzed by two-dimensional gel electrophoresis in order to identify the proteins expressed at the specific stages of spermatogenesis. An acidic protein that disappeared and recovered in the same time course as spermatids after the busulfan treatment was identified as CABS1 by mass spectrometry. It was found that CABS1 was specifically expressed in the elongate spermatids at steps 13 to 16 in stages I to VIII of the seminiferous epithelium cycle of the mouse, and then it localized to the principal piece of flagellum of the mature sperm in the cauda epididymis. We have found for the first time that CABS1 is a calcium-binding protein that binds calcium during the maturation in the epididymis.

busulfan, calcium, signal transduction, spermatogenesis, sperm maturation

INTRODUCTION

Mammalian spermatogenesis is accomplished by the orderly series of differentiation steps. First, spermatogonia undergo multiplication by mitosis, leading to differentiation into spermatocytes. The spermatocytes then reduce the chromosomal number by meiosis, changing into haploid spermatids. The round spermatids finally differentiate into spermatozoa through the processes known as spermiogenesis. These steps are known to be regulated by the microenvironment surrounding the germ cells as well as by the stage-specific gene expression by the germ cells themselves. Although a number of genes and proteins have been identified in relationship to the specific stages of spermatogenesis, the molecular mechanisms involved in spermatogenesis remain largely unknown [1–8]. This is partly because of the lack of appropriate in vivo or in vitro model systems to analyze selectively the transcripts and/or proteins with specific localization in testis and the specific timing of expression during spermatogenesis.

In order to analyze the spermatogonial stem cell function, transplantation of germ cells into a host testis depleted of spermatogenic germ cells by treatment with busulfan has recently been employed [9–13]. Busulfan has been used as an antineoplastic through its alkylating property to inhibit DNA replication [14]. So, at adequate concentrations busulfan selectively attacks the dividing spermatogonia and spermatocytes, resulting in the depletion of the spermatogenic germ cells from testis without influencing the abilities of the testicular somatic cells to support spermatogenesis. Under such conditions, the busulfan-induced depletion of spermatogenic germ cells can be restored by surviving spermatogonial stem cells [12, 15–17].

In the present paper, spermatogenesis was temporarily ceased by a single intraperitoneal injection of busulfan to the mature male mice, and then we investigated the first cycle of recovered spermatogenesis in order to identify the proteins expressed at the specific stages of spermatogenesis. Among those proteins, here we focused on an acidic protein, NYD-SP26, which had been reported as a gene highly expressed in human testis with unknown function [18]. We have found that NYD-SP26 is a novel member of the calcium-binding proteins in spermatozoa that is specifically expressed in the elongate spermatids and then localized into the principal piece of flagella of matured spermatozoa. Therefore, we have named this protein calcium-binding protein, sperm-specific 1—CABS1, which has been approved by the Mouse Genomic Nomenclature Committee and the HUGO Gene Nomenclature Committee.

MATERIALS AND METHODS

Animals and Histology

Animal experiments were carried out in a humane manner after approval was received from the Institutional Animal Experiment Committee of the University of Tsukuba. IV-CS male mice (6 wk old) purchased from the Institute for Animal Reproduction (Kasumigaura-shi, Japan) received a single intraperitoneal injection of busulfan (20 mg/kg body weight; Sigma) dissolved in 50% dimethyl sulfoxide. Freshly dissected testes were fixed in Bouin fixing fluid, processed in paraffin, sectioned at 4 μm, and stained by hematoxylin and eosin.

Protein Extraction and Two-Dimensional Gel Electrophoresis

Testes were homogenized by Polytron (Kinematica AG) in 20 mM Tris-HCl, pH 7.4, containing 4% CHAPS and 20 μl/ml protease inhibitor cocktail (Sigma) and were incubated on ice for 60 min with periodic vortexing. The homogenate was centrifuged at 105 000 × *g* for 1 h at 4°C. The resulting supernatant was stored at −20°C until use.

A total of 450 μg of extracted proteins were separated on the pH 3–10, 7-cm immobilized pH gradient (IPG) strip (Bio-Rad) using a Protean IEF Cell

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Received: 30 September 2008.
First decision: 29 October 2008.
Accepted: 14 January 2009.

eISSN: 1259-7268 http://www.biolreprod.org
ISSN: 0006-3363

according to the manufacturer's instructions (Bio-Rad). After isoelectric focusing, the IPG strip was successively equilibrated in the equilibration buffer (6 M urea, 2% SDS, 20% glycerol, and 375 mM Tris-HCl, pH 8.8) containing 130 mM dithiothreitol or 135 mM iodoacetamide each for 10 min at room temperature. The IPG strip was laid on the SDS-polyacrylamide 10% or 15% gel and electrophoresed with a MiniProtean II (ATTO Technology) at 200 V. Gels were stained overnight with Rapid Stain CBB kit (Nacalai Tesque, Kyoto, Japan) and then destained with 30% methanol and 10% acetic acid until a clear background was achieved. For image analysis, the stained two-dimensional gel electrophoresis (2-DE) gels were scanned with a GS-710 densitometer (Bio-Rad) and analyzed with the software PDQuest Version 6.2 (Bio-Rad).

Protein Identification by Mass Spectrometry

The spots of interest were excised from 2-DE gel, destained with 50% (v/v) acetonitrile and 2.5 mM Tris-HCl, pH 9.0, and dried in a vacuum centrifuge. The gel pieces were covered with 50 μl of 2.5 mM Tris-HCl, pH 9.0, containing 0.04 mg/ml lysyl endopeptidase (Wako, Osaka, Japan) and then rehydrated at 4°C for 40 min. The supernatant was then replaced by 100 ml of 2.5 mM Tris-HCl, pH 9.0, and the gel pieces were incubated overnight at 37°C.

The digested peptides were loaded onto C18 Zip Tips (Millipore, Framingham, MA) for desalting. Matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectra were acquired using a PerSeptive Biosystems Voyager Elite-DE (Framingham, MA).

For protein identification, monoisotopic masses of digested peptides observed in the MALDI-mass spectrometry spectra were used to query NCBInr and SWISS-PROT sequence databases using Mascot (www.matrixscience.-com) search programs.

RT-PCR Analysis

Total RNA was isolated from testes with ISOGEN (Nippon Gene, Tokyo, Japan) and subjected to RT-PCR. First-strand cDNA reverse transcribed using an oligo(dT) primer was further amplified by PCR. Oligonucleotide primers used in this study were 5′-CTCTGGCACACTAGAGGACA-3′ and 5′-GAGGTCACTGAAGGAAGCTC-3′ (nucleotides 530–549 and 1129–1110 in Supplemental Fig. S1 [available online at www.biolreprod.org], respectively). Denaturation was for 30 sec at 94°C, annealing for 30 sec at 60°C, and synthesis for 2 min at 72°C.

Sequence Analysis of Cabs1 mRNA by RACE Methods

The nucleotide sequence of *Cabs1* mRNA was determined by RACE methods using a 5′-Full RACE core set and 3′-Full RACE core set (Takara, Shiga, Japan) according to the manufacturer's instructions. Briefly, first-strand cDNA of *Cabs1* was synthesized by reverse transcription from the mouse testis total RNA using 5′-end phosphorylated RT primer (nucleotides 549–536 in Supplemental Fig. S1). After digestion of RNA by RNase H, concatemers of single-stranded cDNA were formed by T4 RNA ligase. An unknown 5′-end region was amplified using inverse PCR (5′-RACE), and then its product was cloned into pGEM-T vector (Promega Corp.) for sequencing. For 3′-RACE, first-strand cDNAs were synthesized by RT of the mouse testis total RNA using Oligo dT-3sites Adaptor Primer contained in the 3′-Full RACE core set. Polymerase chain reaction was performed with a *Cabs1*-specific primer (nucleotides 1018–1057 in Supplemental Fig. S1) and the 3site Adaptor Primer, whose product was then cloned into the pGEM-T vector and sequenced.

Northern Blot Analysis

Total RNA prepared from various mouse tissues (10 μg each) was electrophoresed and transferred to the nylon membranes (Hybond-N^+; Amersham). The RNA blots were hybridized with 600 bases of cRNA labeled with digoxygenin (Boehringer-Mannheim Biochemica). The RT-PCR product obtained as mentioned above was ligated into pCR II Vector (TA Cloning Kit Dual Promoter; Invitrogen). After linearization with *Bam*HI or *Eco*RV, sense and antisense cRNA probes were synthesized by in vitro transcription with T7 or SP6 RNA polymerase (DIG RNA Labeling Kit; Roche Applied Science). The location of the cRNA probe in the *Cabs1* mRNA is shown in Supplemental Fig. S1.

In Situ Hybridization

Fresh mouse testis was frozen quickly in liquid nitrogen, embedded in CMC compound (FINETEC), and then sectioned at 4 μm by Cryostat (Leica). The sections were fixed with 4% paraformaldehyde and then treated with 1 μg/ml proteinase K. After postfixation with 4% paraformaldehyde, the sections were washed with PBS containing 0.2% Tween-20 and hybridized with 1 μg/ml DIG-labeled cRNA probe dissolved in 50% formamide, 5× saline-sodium citrate (SSC), 1% SDS, 50 μg/ml heparin, and 50 μg/ml yeast RNA at 65°C overnight in a moist chamber equilibrated with 50% formamide and 2× SSC, pH 4.5. The sections were successively washed with 50% formamide; 5× SSC, pH 4.5; and 1% SDS at 65°C for 30 min and three times with 50% formamide and 2× SSC, pH 4.5, at 65°C for 30 min each. The sections then were washed three times with 25 mM Tris-HCl, pH 7.4, containing 0.8% NaCl, 0.002% KCl, and 0.1% Tween-20 (TBST) at room temperature for 5 min each. After treatment with 0.5% blocking reagent (Roche Applied Science) in TBST at room temperature for 1 h, the sections were incubated with alkaline phosphatase-conjugated anti-DIG antibody (Roche Applied Science) at 4°C overnight. The colorization was performed using BM purple AP substrate according to the manufacturer's instructions (Roche Applied Science).

Expression and Purification of the Recombinant Proteins for Antigen Production and for Analysis of Calcium-Binding Activity

Two recombinant proteins were prepared for antigen production and for analysis of calcium-binding activity. The *Cabs1* cDNA fragments were synthesized by RT-PCR using testis total RNAs as template, and each was ligated into *Bam*HI and *Eco*RI sites of the bacterial expression vector, either pGEX-2T (for antigen production) or pGEX-6P-2 (for the calcium-binding assay), which was transformed into *Escherichia coli* DH5α. After the purification of the recombinant GST-CABS1 fusion proteins, CABS1 protein fragments were cut out by thrombin or PreScisson Protease (Amersham Pharmacia Biotech) and purified using Glutathion Sepharose 4B (GE Healthcare) according to the manufacturer's instruction. The location of both recombinant proteins in CABS1 protein is indicated in Supplemental Fig. S1.

Production of Rabbit Antisera Against CABS1 and Western Blot Analysis

Rabbit antisera against the CABS1 protein fragment were produced by subcutaneous injection of 250 μg of purified antigen with Freund complete adjuvant (Sigma). Three additional booster injections of 125 μg of purified antigen with Freund incomplete adjuvant (Sigma) were followed at intervals of 2 wk.

The mouse testis proteins were separated by 2-DE and electroblotted to polyvinylidene fluoride (PVDF) membranes under semidry conditions. Membranes were treated with the antisera against CABS1, followed by peroxidase-conjugated anti-rabbit IgG secondary antibody (Sigma). Signals were detected by incubating the membranes with 0.06% 4-chloro-1α-naphtol and 0.01% H_2O_2 in 50 mM Tris-HCl, pH 7.4.

Immunohistochemical Analysis of Mouse Testis

Mouse testes were fixed by soaking in Bouin fixing fluid at room temperature for 24 h, and they then were dehydrated and embedded in paraffin. The block was sectioned at a thickness of 4 μm and mounted on slides. After deparaffinization, the sections were soaked in 0.1 M sodium citrate buffer at 37°C and washed with distilled water.

Immunohistochemical analysis was carried out by an avidin-biotin complex method using a Vectastain ABC Kit (Vector Laboratories Inc.) according to Noguchi et al. [19]. Specificity control for the immunohistochemical reaction was carried out on adjacent sections, which were incubated with the neutralized antisera instead of the anti-CABS1 antisera. Anti-CABS1 antiserum was neutralized by incubation with the purified antigen (1 mg/ml) at room temperature for 30 min. Counterstaining of the sections was done by hematoxylin and eosin.

Immunocytochemical Analysis of Mouse Sperm

The mature sperm squeezed out from the mouse cauda epididymis were washed with PBS and suspended to a final concentration of 4×10^6 cells/ml in PBS. Sperm suspension was placed on the MAS-coated slide (Matsunami Glass Ind. Ltd., Osaka, Japan) and dried at 50°C. After fixation with 4% paraformaldehyde in PBS on ice for 30 min, sperm were washed with PBS three times and blocked with 5% skim milk in PBS (blocking buffer) at room temperature for 30 min. Sperm were then exposed to the anti-CABS1 serum diluted with blocking buffer at 37°C for 1 h, followed by washing with PBS three times. Sperm were incubated with the fluorescein isothiocyanate-conjugated anti-rabbit IgG dissolved in blocking buffer at room temperature for 30 min. After washing with PBS, sperm were observed under a microscope. Specificity control was done as mentioned above.

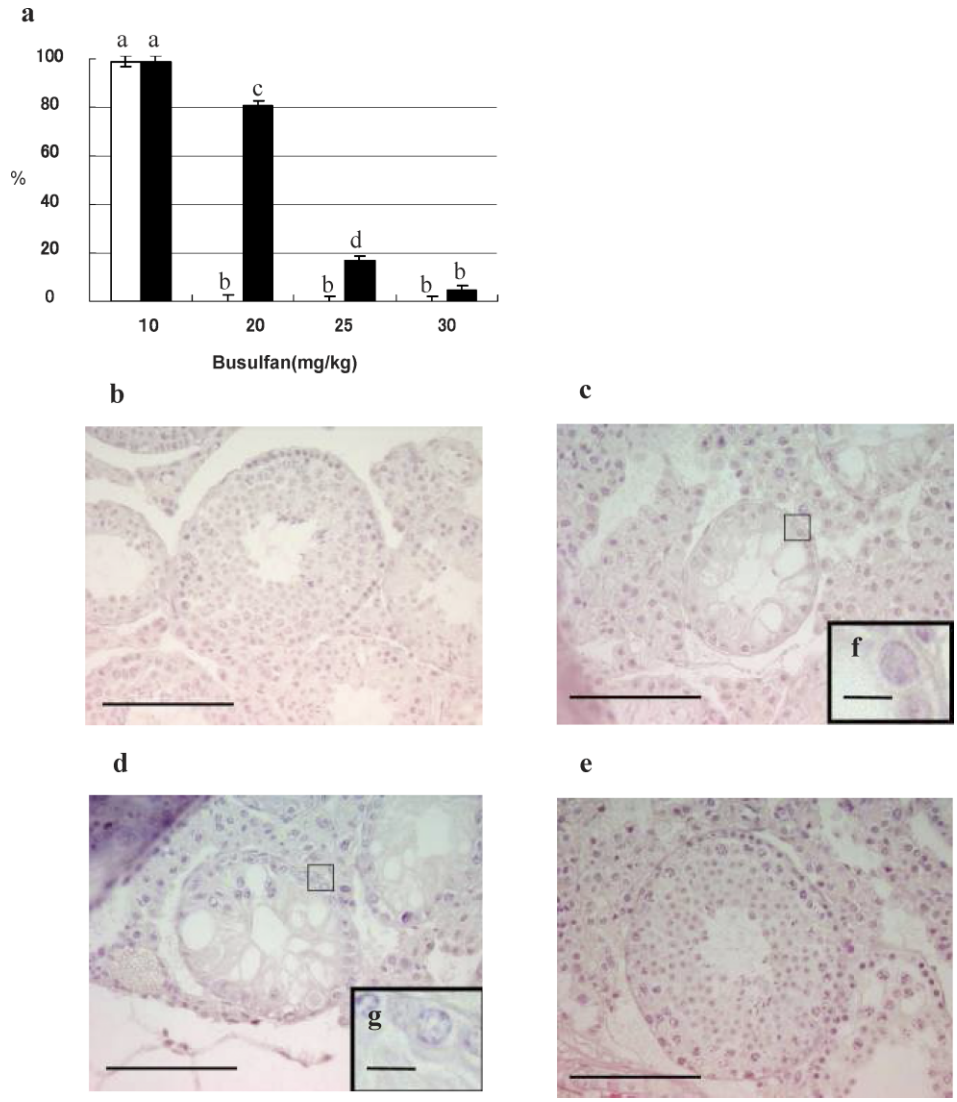

FIG. 1. Effects of busulfan on spermatogenesis in mice. **a**) The adult mice were injected i.p. with various concentrations of busulfan. At 4 wk (open bars) and 9 wk (closed bar) after the treatment, mice were dissected, and 4-μm sections of testes were used for assessment of spermatogenesis. The results are expressed as a percentage of the seminiferous tubules containing spermatocytes per 200 seminiferous tubules. Data are mean ± SEM from three different experiments, each with three sets of determinations of 200 seminiferous tubules in the different regions of the testis. Statistical analysis was conducted by *t* test with the significance level at $P < 0.05$. Groups with different lowercase letters are significantly different. On 0 (**b**), 32 (**c**), 41 (**d**), and 77 (**e**) days after the treatment with 20 mg/kg busulfan, mice were dissected, and 4-μm sections of testes stained by hematoxylin and eosin were observed under the microscope. Insets are undifferentiated (**f**) and differentiated (**g**) spermatogonia nuclei. Bars = 50 μm (**b**–**e**) and 5 μm (**f** and **g**).

Analysis of Calcium-Binding Activities

The calcium-binding proteins in the testicular protein extracts were detected by two methods. First, calcium-binding proteins were stained with Stains-all (1-ethyl-2-{3-(1-ethyl-naphtol [1,2-d] thiazoline-2-ylidine)-2-methylpropenyl}-naphtho [1,2-d] thiazolium bromide) in 2-DE gel according to the method of Campbell et al. [20]. Briefly, 2-DE gel was fixed with 25% isopropanol overnight and washed extensively with the same medium. Then, the gel was stained with 0.0025% Stains-all, 25% isopropanol, 7.5% formamide, and 30 mM Tris, pH 8.8, for 48 h in the dark. The calcium-binding proteins were stained in blue, whereas most other proteins were stained in red or pink.

Second, the calcium-binding proteins were stained with ruthenium red [21]. The PVDF transfers of testis proteins separated by 2-DE were incubated with or without 50 mM $CaCl_2$ in 60 mM KCl, 5 mM $MgCl_2$, and 10 mM Tris-HCl, pH 7.5 (buffer A) at 20°C for 30 min. The membranes were washed twice with buffer A for 10 min and then were stained with 25 mg/l ruthenium red in buffer A.

The calcium-binding activity was studied using a recombinant CABS1 protein, which covered the full length of the deduced CABS1 sequence except the N-terminal five amino acids, as shown in Supplemental Fig. S1. The purified recombinant CABS1 was incubated with 5 μM Stains-all in 30% (v/v) ethylene glycol, 2 mM MOPS-KOH, pH 7.2, in the dark for 30 min, and then the absorption spectra were obtained with a spectrophotometer as described by Caday and Steiner [22].

RESULTS

Effects of a Low Dose of Busulfan on Mouse Spermatogenesis

In order to determine the proteins expressed at the specific stages of spermatogenesis, busulfan was used to deplete the adult mouse testis of spermatogenic germ cells, followed by the recovery of spermatogenesis by the surviving spermatogonia. The effects of a single peritoneal injection of 10~40 mg/kg busulfan on the mouse testis were determined. It was found that the least amount of busulfan that induces both the complete deletion of spermatogenic germ cells and the ensuing recovery of spermatogenesis was 20 mg/kg (Fig. 1a). As shown in Figure 1c, 32 days after the injection of 20 mg/kg busulfan, only undifferentiated spermatogonia were detected in the seminiferous tubules in addition to Sertoli cells. Reproliferation of the surviving germ cells was first observed 41 days after the injection (Fig. 1d). The first cycle of the resumed spermatogenesis proceeded synchronously in almost all seminiferous tubules. Spermatids appeared 7 wk after the busulfan treatment, and the spermiation occurred in the 11th wk (Fig. 1e).

Analysis of Proteins in the Busulfan-Treated Testis by 2-DE and TOF-MS

Proteins were extracted from the testes 32, 41, 56, and 77 days after the busulfan treatment and were separated by 2-DE. As shown in Figure 2 and Table 1 [23–36], the testicular proteins identified by TOF-MS were categorized into three groups according to the pattern of the changes in the contents and using actin (spot no. 3) as an unchanged control: unchanged (I), temporarily increased (II), and temporarily

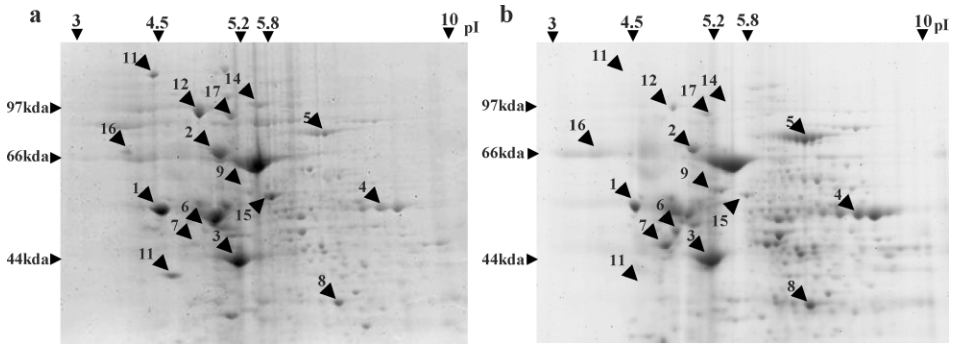

FIG. 2. Profiles of testiscular proteins separated by 2-DE. The proteins extracted from testes 32 days after the injection of 200 μl of 50% DMSO with (**b**) or without (**a**) busulfan (20 mg/kg body weight) were separated by 2-DE. The 10% SDS-polyacrylamide gels were stained with CBB. The protein spots identified by mass spectrometry are indicated by the arrows and numbered from 1 to 17, which correspond to "Spot No." in Table 1. The pIs 4.5, 5.2, and 5.8 are representative values of the identified spots for well-known proteins calreticulin, actin, and protein disulfide isomerase-associated 3, respectively. kda, kilodaltons.

decreased (III) proteins. Most of the proteins in group I had been known to be ubiquitously present in the testis. Because actin has been used generally as an internal control for the comparative experiments of testicular mRNAs and proteins [37–39], the levels of actin remained constant throughout the processes examined in this study (Fig. 3f). All of the identified proteins that had already been reported to be localized either in the Sertoli cells or Leydig cells were shown to belong to group II. The proteins known to be present in the differentiated spermatogenic cells belonged to group III. The proteins specifically expressed in the undifferentiated spermatogonia were shown to be contained in group I or II.

In this study, we newly identified two proteins whose localization and function in the mouse testis had not been reported (Table 1, see row IV). Here, we focused on an acidic protein (isoelectric point [pI] = 4.1, molecular mass = 66 kDa) whose contents were temporarily decreased after the busulfan treatment and recovered along with the resumption of spermatogenesis, as shown in Figure 3. It was strongly suggested that such a protein was localized in the spermatogenic cells to be categorized into group III. This protein was identified as CABS1 (testis development protein NYD-SP26: 4931407G18Rik) by TOF-MS. *CABS1* was originally identified as one of the testis development- and spermatogenesis-related genes both in human and mouse testes using cDNA microarrays, but neither the localization nor the physiological function of CABS1 in testis has been characterized yet [40].

The complete sequence of *Cabs1* mRNA was determined in combination with RT-PCR and 5′-RACE and 3′-RACE methods using the total RNA fraction prepared from the adult

TABLE 1. List of spots identified by MALDI-TOF-MS and bioinformatic analysis.

Spot no.	Protein name	pI theoretical	Molecular weight theoretical	pI observed	Molecular weight observed	Sequence coverage (%)	Score >59	Localization	Reference
Group I									
1	Calreticulin	4.6	42.12	4.5	54	24	67	Spermatocyte	[23]
								Spermatid	[23]
								Sertoli cell	[23]
2	Heat shock 70kD protein 5	5.1	72.38	5.2	73	24	86	Spermatocyte	[24]
								Spermatid	[24]
3	β-Actin	5.2	42.04	5.2	43	32	95	Spermatocyte	[25]
								Spermatid	[25]
								Sertoli cell	[25]
								Leydig cell	[25]
Group II									
4	Aldehyde dehydrogenase family 1 member A1	7.9	54.3	7.8	54	13	70	Leydig cell	[26]
5	Transferrin	7	78.83	7	78	22	61	Sertoli cell	[27]
6	Vimentin	5.1	53.79	4.9	48	18	83	Sertoli cell	[28]
7	Vimentin	5.1	53.79	5	46	12	60	Sertoli cell	[28]
8	Aldo-ketoreductase	8.8	58.86	7	34	20	72	Sertoli cell	[29]
Group III									
9	Heat shock protein 1 (chaperonin)	5.7	61.11	5.6	62	18	80	Spermatogonium	[30]
10	Testis specific histone binding protein NASP	4.4	84.01	4.5	140	23	83	Spermatid	[31]
11	Meichroacidin	4.4	34.23	4.6	42	22	68	Spermatocyte	[32]
12	Transitional endoplasmic reticulum ATPase	5.1	89.21	5.3	89	16	65	Spermatocyte	[33]
13	Phosphoglycerate kinase 2	6.4	45.23	6.4	44	25	70	Spermatocyte	[34]
14	APG-1/94 kDa osmotic stress protein	5.5	95.22	5.7	95	11	72	Spermatocyte	[35]
								Spermatid	[35]
15	Protein disulfide isomerase associated 3	5.9	57.12	5.8	60	34	112	Spermatid	[36]
Group IV									
16	Testis development protein NYD-SP26	4.1	42.28	4.1	66	37	97	Unknown	–
17	Superkiller viralicidic activity 2-like-2	5.6	69.56	5.2	87	16	63	Unknown	–

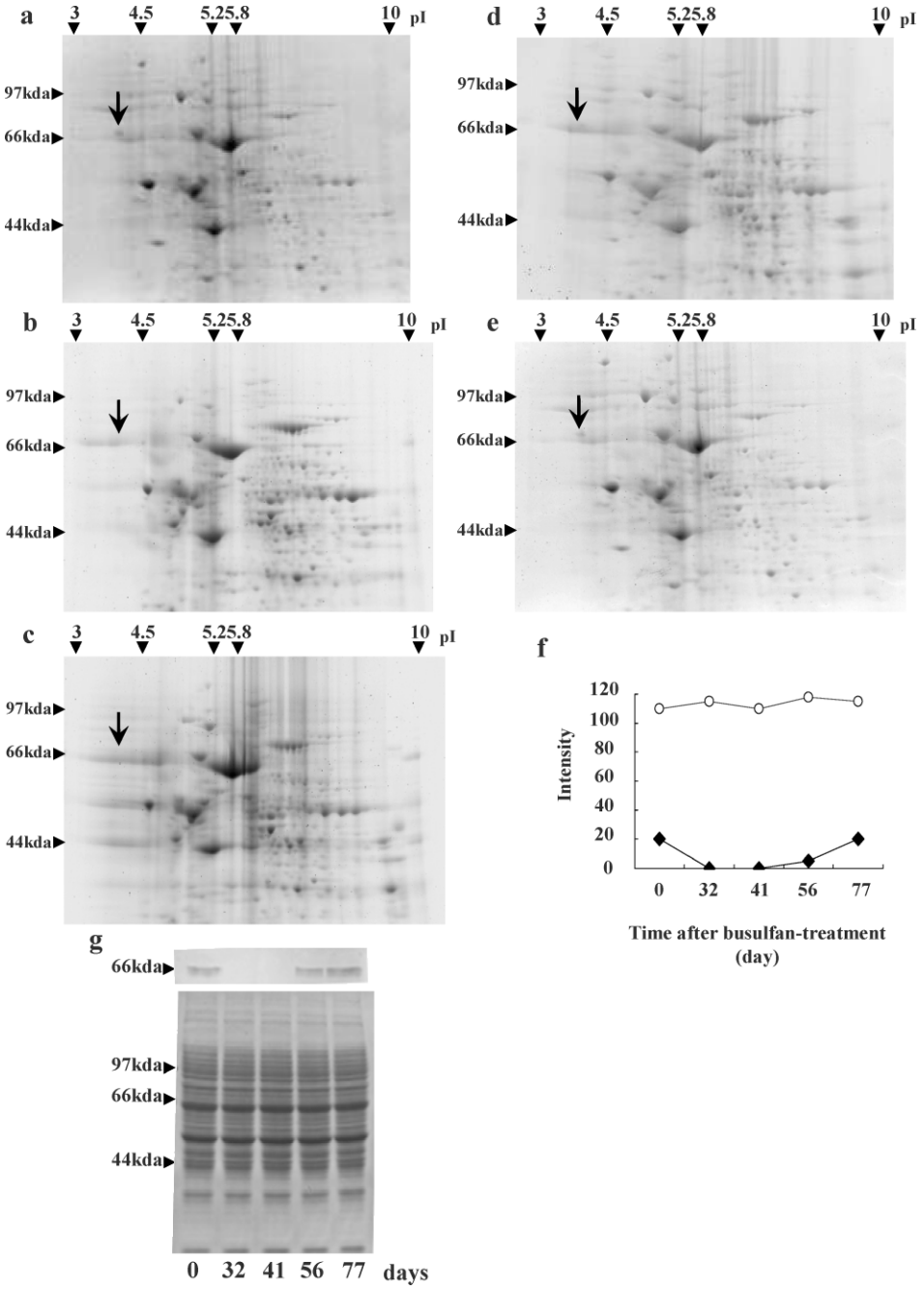

FIG. 3. Changes of the CABS1 contents in testis after the busulfan treatment. The testicular proteins were extracted at various intervals of time—0 (**a**), 32 (**b**), 41 (**c**), 56 (**d**), and 77 (**e**) days—after busulfan treatment and separated by 2-DE. CABS1 is indicated by the arrows. **f**) The intensity of the CABS1 spot (closed diamonds) on the 10% SDS-polyacrylamide gel was analyzed with the software PDQuest Version 6.2. The intensity of the actin spot is shown by open circles as an unchanged protein control. **g**) Western blot analysis of CABS1 protein in the testicular proteins separated by 10% SDS-polyacrylamide gel. Upper: Western blot analysis. Lower: CBB staining. kda, kilodaltons.

mouse testis (Supplemental Fig. S1). The nucleotide sequence of *Cabs1* thus obtained was the same as that of "BC 049750," except the addition of nine nucleotides, ACTAAGCCC, to its 5′ terminus.

Expression and Localization of Cabs1 *in Testis*

The distribution of the *Cabs1* mRNA among various mouse tissues was examined by RT-PCR and Northern blot analyses as described in *Materials and Methods*. As shown in Figure 4, a–c, the *Cabs1* mRNA was detected only in testis. It was also found that the contents of the *Cabs1* mRNA in testis were changed exactly with the same tendency as those of CABS1 after the treatment with busulfan (Fig. 4d).

The localization of the *Cabs1* mRNA and CABS1 in testis were examined by in situ hybridization and immunohistochemical analyses, respectively. As shown in Figure 5, b and c, *Cabs1* mRNA was specifically expressed in germ cells, which was first detected in the elongate spermatids at step 10 in stage X of the seminiferous epithelium cycle of the mouse. The maximal intensity of the hybridization signals was observed in the elongate spermatids at steps 13 and 14 during the stages from I to III.

The antisera against CABS1, which were prepared as described in *Materials and Methods*, specifically reacted with CABS1 among the proteins extracted from mouse testis. Figure 5, e and f, shows that CABS1 was localized in the elongate spermatids at steps 13 to 16 in the seminiferous epithelium of stages I to VIII. The expression patterns of *Cabs1* mRNA and CABS1 in spermatogenesis were summarized in Figure 5g.

Furthermore, although some portions of immunoreactivity were thrown away in the residual body during spermiation, it was found that CABS1 was still present, specifically in the

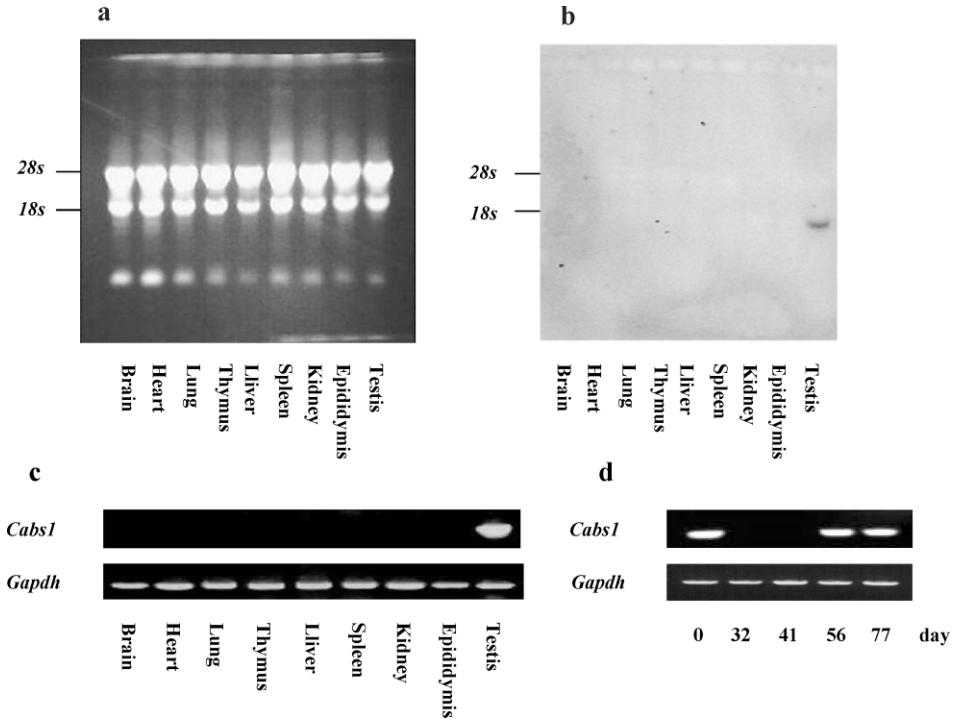

FIG. 4. Northern blot analysis and RT-PCR of *Cabs1*. The total RNAs (10 μg each) prepared from mouse brain, heart, lung, thymus , liver, spleen, kidney, epididymis, and testis were electrophoresed (**a**), transferred to a nylon membrane, and probed with DIG-labeled cRNA probe (**b**). **c**) The RT-PCR analysis of *Cabs1* mRNA expression in the various mouse organs as in **a**. *Gapdh* was used as a loading control. **d**) The RT-PCR analysis of *Cabs1* mRNA in testis at various intervals of time after busulfan (20 mg/kg body weight) treatment.

principal piece of flagellum of mature sperm in the cauda epididymis (Fig. 6).

Analysis of Calcium-Binding Activity of CABS1

It has been reported that the gene of the human homolog of *Cabs1* is located within the gene cluster of the secretory calcium-binding phosphoproteins, such as casein, statherin, histatin, and enamel matrix proteins [41]. This report prompted us to analyze the calcium-binding activity of CABS1. As shown in Figure 7, both methods to detect the calcium-binding proteins, Stains-all staining and ruthenium red binding, proved that CABS1 bound calcium in a specific manner. These results strongly suggest that CABS1 is a new member of sperm-specific calcium-binding protein.

As shown in Figure 8, the recombinant CABS1 protein induced only the J band at 600–650 nm of Stains-all in a dose-dependent manner, but not the γ band at 500–510 nm. The addition of 10 mM $CaCl_2$ reduced the J band by displacing the dye from the recombinant CABS1.

Furthermore, it was found that CABS1 in the mature sperm migrated to more a basic pH area (pI = 5.8) than the testicular protein on 2-DE, as shown in Figure 9. Preincubation of the proteins extracted from testis with 5 mM $CaCl_2$ prior to the first-dimensional electrofocusing resulted in the translocation of CABS1 to the position of the sperm CABS1 on 2-DE. In turn, when the protein extracts of the cauda epididymal sperm were preincubated with 5 mM EDTA and separated by 2-DE, CABS1 migrated to the position of testicular CABS1 (pI = 4.1). These results indicate that the calcium binding to CABS1 occurs during the process of sperm maturation in the epididymis.

DISCUSSION

In the present study, spermatogenesis was temporarily ceased by a single intraperitoneal injection of a lower dose of busulfan (20 mg/kg) than that used for the experiments of germ cell transplantation [9–13]. At this dose, busulfan should act only on the proliferating germ cells, because the round spermatids normally completed spermiogenesis and then were spermiated. The testes at the various stages of the first cycle of the recovered spermatogenesis provide good materials to analyze the genes showing the spatiotemporally specific expression during the spermatogenesis.

In mouse, it takes about 5 wk from birth to complete the first cycle of spermatogenesis. These processes have been extensively used for the analysis of stage-specific gene expression in spermatogenesis. Most of those studies have been done at the transcriptional level by Northern blot analysis, RT-PCR, or cDNA microarray, but not at the protein level because the weight of testis is too small. Furthermore, the postnatal differentiation of Leydig cells into the adult type occurs by puberty, which makes the analysis of those processes complicated [42, 43]. Here, we used the sexually mature mice, which made it possible to extract enough materials for the proteome analysis and to exclude the changes involved in the differentiation of the somatic cells in testis throughout the first cycle of the recovered spermatogenesis induced by busulfan. Although it is known that busulfan selectively acts on the differentiated spermatogonia and spermatocytes even at 40 mg/kg, one must be careful to distinguish the changes in spermatogenesis from the pharmacological side effects of busulfan.

Profiling the testicular protein spots on 2-DE into three groups as shown in Table 1 is useful to select proteins that are expressed in the spermatogenic cells. Furthermore, the duration necessary for the recovery of a protein into the busulfan-treated testis was shown to depend on the stage of the spermatogenesis where the protein is expressed. For example, we identified a protein that once disappeared from testis and turned up again prior to CABS1 as superkiller viralicidic activity 2-like 2, official symbol SKIV2L2, a predicted DEAD-box RNA helicase (accession no. NP_082427 in GenPept database). It was for the first time shown to be expressed specifically in spermatocytes in the mouse testis by in situ hybridization (data not shown).

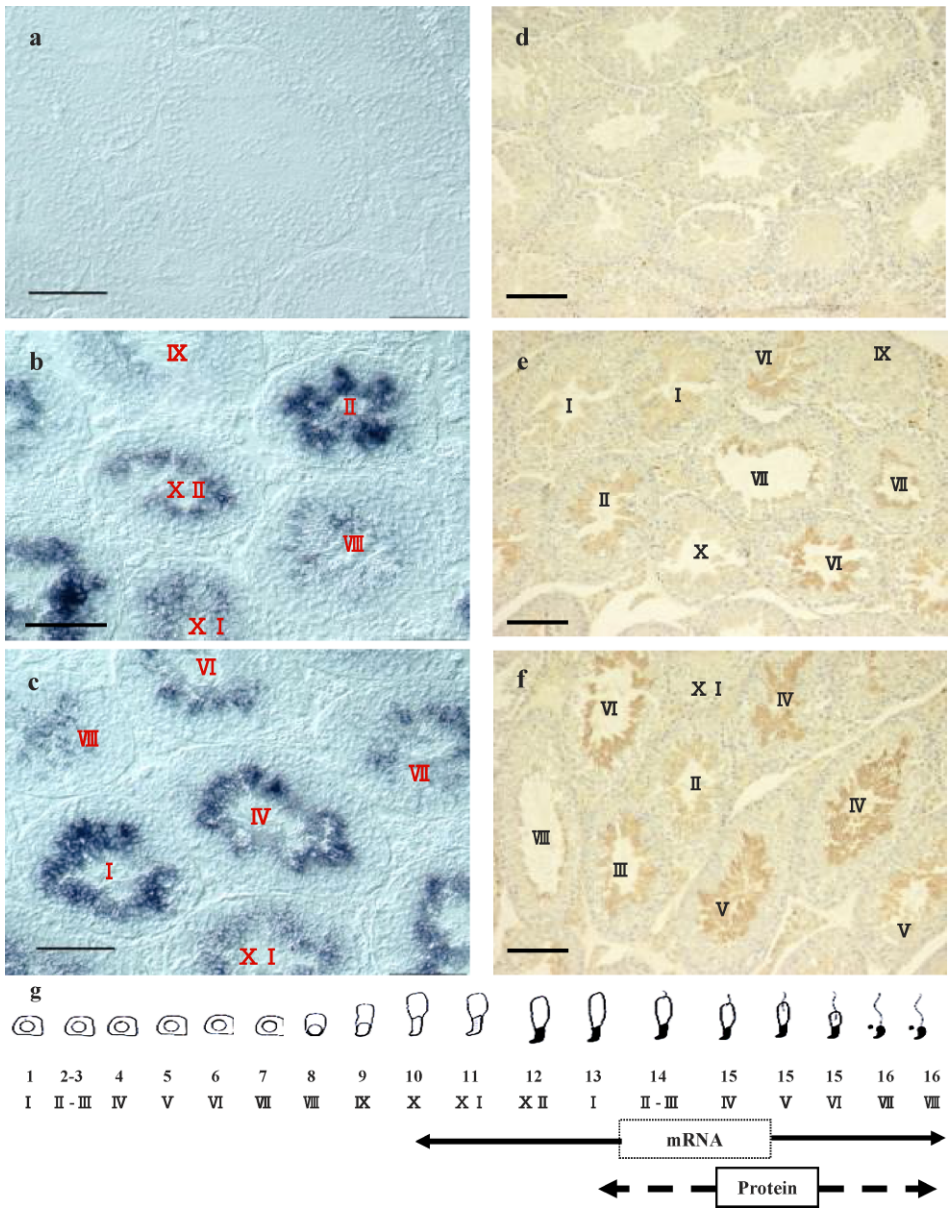

FIG. 5. Localization of *Cabs1* mRNA and protein in the mouse testis. **a**–**c**) Localization of *Cabs1* mRNA analyzed by in situ hybridization. Frozen sections were hybridized with a DIG-labeled antisense (**b** and **c**) or sense (**a**) probe. **d**–**f**) Immunohistochemical localization of CABS1 protein in the mouse testis analyzed with anti-CABS1 antisera (**e** and **f**). Replacement of the antisera with the antisera previously neutralized with CABS1 antigen gives no specific staining (**d**). **g**) Schematic summary of the localization of *Cabs1* mRNA and protein during spermiogenesis. Solid line and broken line represent the expression windows of *Cabs1* mRNA and protein, respectively. Roman numerals in **b**, **c**, **e**, **f**, and **g** represent stages of the seminiferous epithelial cycles, and Arabic numbers in **g** represent steps of spermatid development. Bars = 50 μm.

Cabs1 mRNA was found to be specifically expressed in the elongate spermatids at steps 10–16. No signals were observed in either spermatogonia, spermatocytes, round spermatids, or the somatic cells. Although postmeiotic expression has been reported in number of genes, some of those transcriptions, such as LDH-C4, acrosin, RT-7, and c-mos, have begun before meiosis, which continues into the elongate spermatids [44–47]. On the other hand, protamine (*Prm*) and SP-10 (*Acrv*) genes are transcribed in the haploid cells during the steps of round spermatids [48, 49]. It has been suggested that mRNAs can hardly be transcribed after the condensation of chromatin. So, the onset of *Cabs1* transcription seems to occur at the latest periods in spermatogenesis.

Furthermore, immunohistochemical analysis revealed the restricted expression of CABS1 protein to the elongate spermatids at steps 13–16, which is slightly later than the transcription of mRNA. Such a delay of protein expression during spermiogenesis is well known as a posttranscriptional control, which is very important for the completion of the final processes of spermiogenesis, including acrosome formation, tail formation, chromatin condensation, elongation of nucleus, cytoplasm removal, and spermiation [45, 48, 50–52]. It is very interesting to explore the regulatory mechanisms of such highly spatiotemporally specific gene expression in the haploid germ cells.

CABS1 was first reported as one of the genes highly expressed in human testis by cDNA microarray analysis, although its function was not known [18]. Then, its gene was shown to locate within the secretory calcium-binding phosphoprotein gene cluster on human chromosome 4q13, where the genes for caseins, enamel matrix proteins, and some salivary proteins were localized [41]. In mouse, *Cabs1* gene was also found to reside conservatively within the similar gene cluster on chromosome 5. In the present study, the physiological function of CABS1 has been for the first time identified to be a calcium-binding protein.

Along with the morphological changes, the signal transduction system is also built up during spermiogenesis. It is well accepted that Ca^{2+} plays central roles in the regulation of sperm cell function. Although sperm do not have endoplasmic reticulum, which is an essential component in somatic cells to control the intracellular concentrations of calcium ($[Ca^{2+}]_i$), it

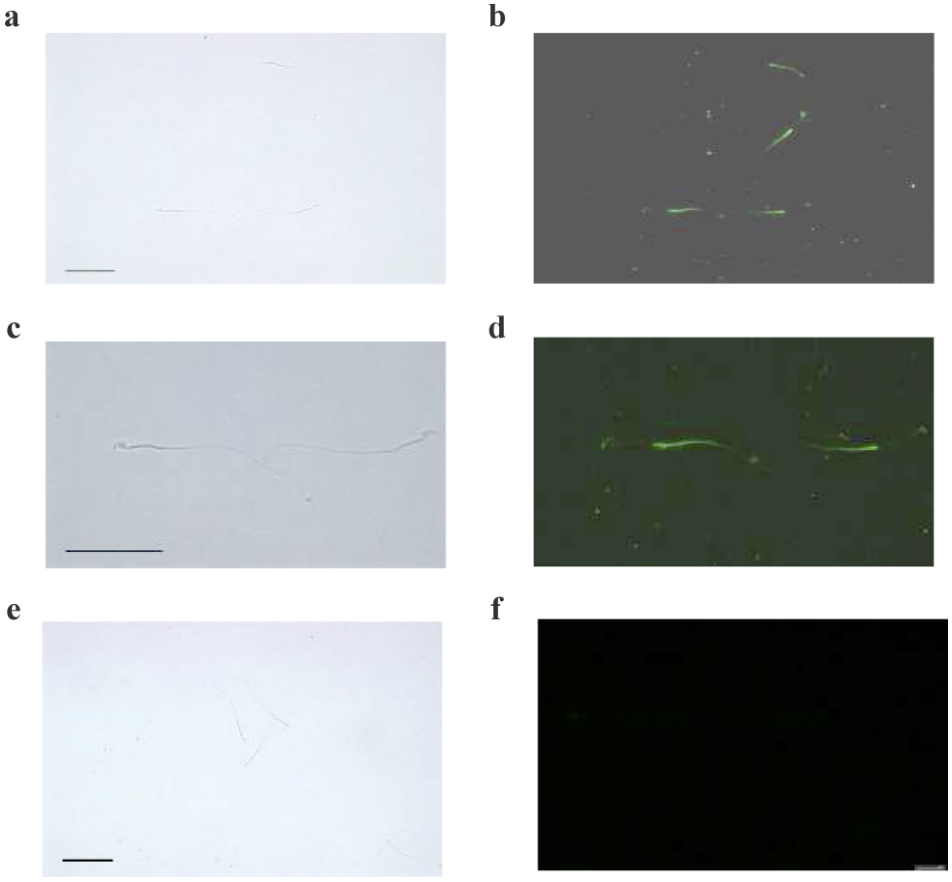

FIG. 6. Immunofluorescent detection of CABS1 protein in the cauda epididymal sperm. **a**, **c**, and **e**) Phase-contrast microscopic analysis of the cauda epididymal sperm. **b** and **d**) The localization of CABS1 in the cauda epididymal sperm was analyzed with anti-CABS1 antisera. **f**) Epididymal sperm incubated with antisera previously neutralized with CABS1 antigen were used as a negative control. Bars = 50 μm.

is suggested that mammalian sperm store Ca^{2+} at least both in the acrosome and neck regions, where $InsP_3$ receptor, ryanodine receptor, secretory pathway Ca^{2+}-ATPase 1 (SPCA 1, official symbol ATP2C1), and sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase 2 (SERCA 2, official symbol ATP2A2) mediate Ca^{2+} mobilization [53–57]. It is also reported that sperm possess Ca^{2+}-mobilizing components in the plasma membranes; Ca^{2+}-permeable channels, such as CATSPERs and voltage-operated Ca^{2+} channels, and Ca^{2+}-clearance agents, such as ATP-driven Ca^{2+} pumps (PMCAs, official symbol ATP2Bs) and Na^{+}/Ca^{2+} exchangers [58–62]. These proteins must be localized to their proper sites during spermiogenesis to exert their activities in responding to the signals that control sperm motility and fertilizing activity.

Furthermore, several calcium-binding proteins as well as calmodulin are also suggested to play important roles in the

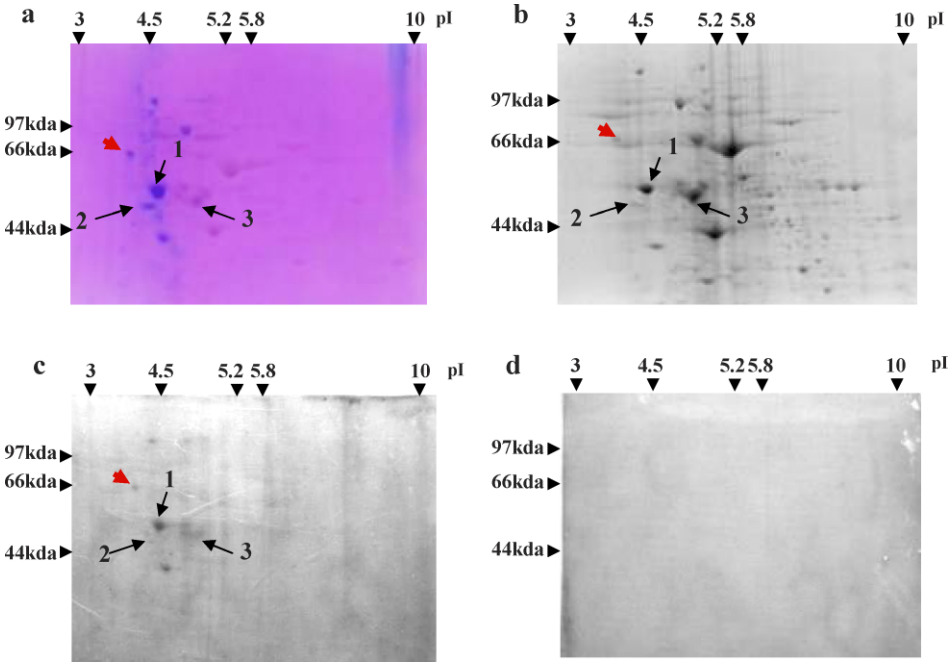

FIG. 7. Detection of CABS1 protein by Stains-all and ruthenium red. Mouse testicular proteins were separated by 2-DE using 10% SDS-polyacrylamide gels and stained either with Stains-all (**a**) or CBB (**b**). Mouse testicular proteins separated by 2-DE were electroblotted onto PVDF membranes, which were incubated with (**c**) or without (**d**) 50 mM $CaCl_2$ and then overlaid with ruthenium red solutions. Red arrows indicate the CABS1 spot. Spot 1 in **a** was identified as calreticulin, and spots 2 and 3 were identified as beta- and alpha-tubulin, respectively. kda, kilodaltons.

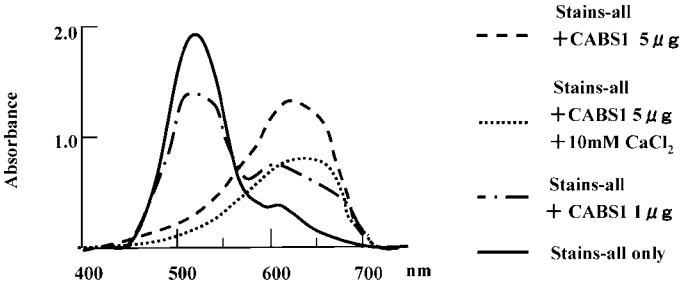

FIG. 8. Absorption spectra of the complexes of Stains-all with the recombinant CABS1 protein. Various concentrations of the recombinant CABS1 were incubated with Stains-all as described in *Materials and Methods,* and absorption spectra were taken with a spectrophotometer. Solid line, dashed line, and broken line indicate Stains-all spectra in the presence of 0, 1, and 5 μg of the recombinant CABS1, respectively. Dotted line indicates Stains-all spectrum in the presence of 5 μg of the recombinant CABS1 and 10 mM $CaCl_2$.

calcium signaling in sperm. Calreticulin has been shown to colocalize with $InsP_3$ receptor in the acrosome, in the equatorial segment, and in vesicular structures in the cytoplasmic droplets of the neck region and regulate $[Ca^{2+}]_i$ in sperm during capacitation and the acrosome reaction [63]. Calcium-binding proteins localizing to the principal piece of flagellum in association with the fibrous sheath, CABYR/FSCB, are also suggested to regulate hyperactivation and capacitation [64, 65]. In this connection, it is very interesting that CABS1 has been proven to be a novel member of the calcium-binding proteins and to be present in the mature sperm in the cauda epididymis. CABS1 was shown to localize to the principal piece of flagellum in mature sperm, which is a localization similar to CABYR/FSCB. Although the physiological importance of CABS1 in sperm remains to be concretely demonstrated, these results lead to the hypothesis that CABS1 is involved in the control of sperm motility and fertility.

By the analyses of nucleotide and deduced amino acid sequences, it was found that CABS1 did not contain any known calcium-binding motifs, such as EF-hand. So, the calcium-binding activity in CABS1 seems to be due to its high contents of acidic residues in the same manner as calsequestrin, calreticulin, and CABYR/FSCB [63–66]. In this connection, it is interesting that CABS1 was stained blue by Stains-all. The metachromatic cationic carbocyanine dye Stains-all stains several calcium-binding proteins blue or purple, and other proteins red or pink, suggesting that this dye might be able to identify potential calcium-binding proteins [67]. In the present study, well-known calcium-binding proteins, such as tubulin and calreticulin, in addition to CABS1 were also identified by TOF-MS analysis directly with the spots on the 2-DE gel stained by the Stains-all, as shown in Figure 7a. Calreticulin

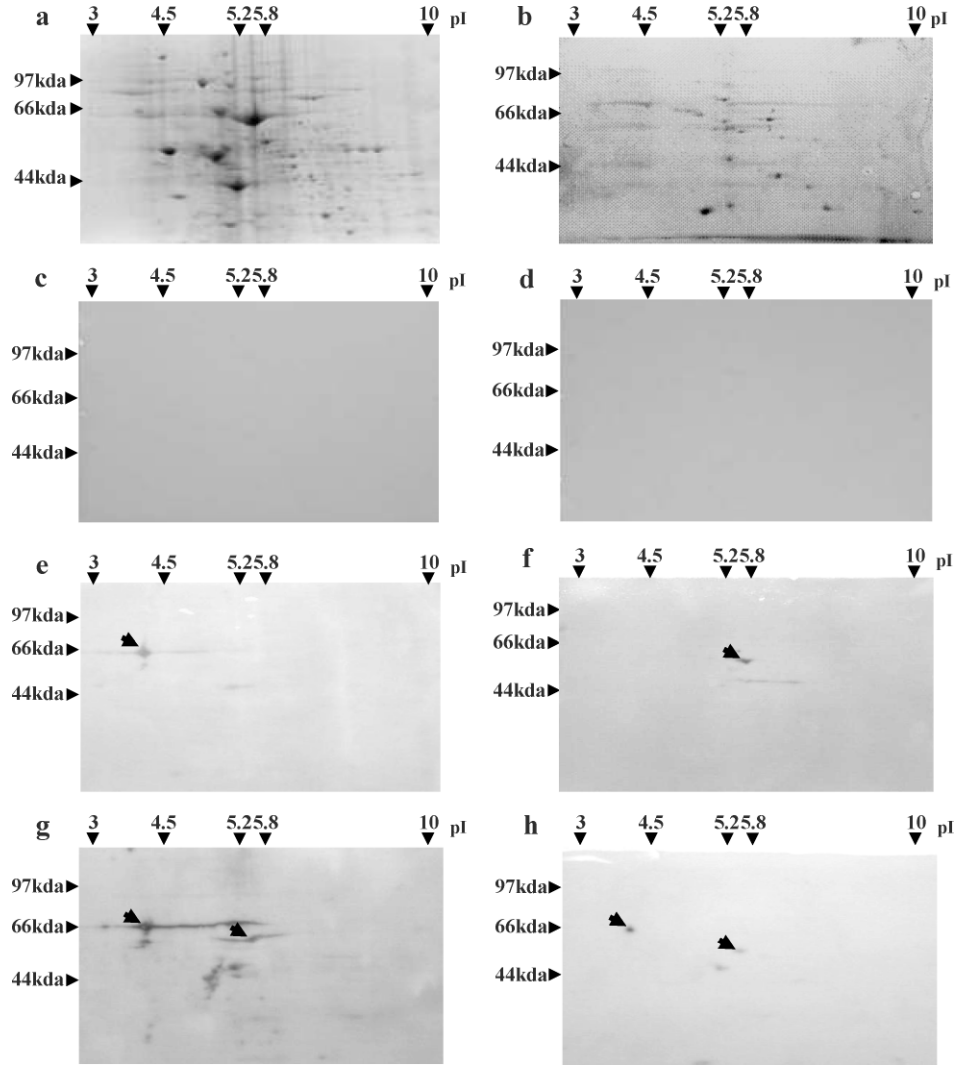

FIG. 9. Differences in the behavior of CABS1 protein on the 2-DE gel between the testis and the cauda epididymal sperm. Proteins extracted from the mouse testis (**a**, **c**, **e**, and **g**) and the cauda epididymal sperm (**b**, **d**, **f**, and **h**) were separated by 2-DE. Western blot analyses of CABS1 protein both in the testis (**e** and **g**) and the cauda epididymal sperm (**f** and **h**) were done. The testicular protein extracts were incubated at 25°C for 30 min with (**g**) or without (**e**) 5 mM $CaCl_2$ before 2-DE. The protein extracts of the mature sperm were also preincubated with (**h**) or without (**f**) 5 mM EDTA. **a** and **b**) CBB staining. **c** and **d**) Negative control of Western blot analysis with the neutralized antisera against CABS1. CABS1 is indicated by the arrow. kda, kilodaltons.

was identified as an abundant protein in spermatozoa [63], whose contents were similar to the sum of alpha- and beta-tubulins as shown in Figure 7b. Although both CABS1 and beta-tubulin (spot 2) are less sensitive to CBB staining, Stains-all clearly recognizes them. These results indicate that Stains-all staining is a useful tool for electrophoretic analysis of calcium-binding proteins. As shown in Figure 7a, although beta-tubulin was stained blue by Stains-all, alpha-tubulin (spot 3) was stained red. In accordance with this, it has been reported that Stains-all selectively stains isoforms of axonemal beta-tubulin blue following isoelectric focusing, whereas those of alpha-tubulin are stained red [68].

It is known that different calcium-binding proteins interact differently with the dye, yielding different spectral bands, either the J band (600–650 nm) or the γ band (500–520 nm) or both [69, 70]. This enables the distinction of the possible structural differences among calcium-binding proteins. Binding of Stains-all to CABS1 was shown to induce only the J band dose dependently, which was similar in spectral properties to the complexes of Stains-all with parvalbumin [22], β-crystallin [71], and RVCaB [72]. It has been suggested that the J band is induced by dye binding to anionic sites in the globular or compact conformations of proteins [69]. It is very interesting that acidic amino acid residues are abundantly present in CABS1.

Furthermore, it was found that CABS1 in the cauda epididymal mature sperm migrated on 2-DE to a more basic area than that in testis. Such translocation indicates the differences of the net charge of acidic residues in CABS1 between testicular immature sperm and epididymal mature sperm, which are caused by calcium binding, as reported in the calcium binding to calreticulin [63]. It is well known that some highly acidic proteins show anomalous behavior in SDS-PAGE, migrating to the position with higher molecular mass than expected and restoring normal electrophoretic migration by neutralization of the negative charges [73, 74]. The testicular CABS1 migrated as a 66-kDa protein on 2-DE, although its true molecular mass is close to 42 kDa (Table 1). It was found that CABS1 in the cauda epididymal mature sperm shifted downward, 58 kDa, as shown in Figure 9f, to approach its true molecular mass, suggesting that the calcium bound to the protein in the mature sperm. This was confirmed by the results that preincubation of testicular extract with 10 mM $CaCl_2$ shifted the position of CABS1 on 2-DE to that of the mature sperm. On the other hand, when the mature sperm extract was preincubated with 5 mM EDTA, CABS1 was migrated to the acidic position of testicular CABS1 on 2-DE. CABS1 reversibly shifted the electrophoretic migration between the two positions (pI = 4.1; 66 kDa) and (pI = 5.8; 58 kDa) in dependence on the calcium binding. These results strongly suggest that calcium binding to CABS1 occurs or greatly increases in sperm after spermiation and during its transit through the epididymis, and that CABS1 plays important roles in the control of the calcium signaling as a Ca^{2+} storage protein in the mature sperm.

ACKNOWLEDGMENTS

We would like to thank Dr. Jun Yanagisawa and Ms. Mitsue Arimoto (University of Tsukuba) for assistance with mass spectrometry.

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