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Identification of 36-kDa Flagellar Phosphoproteins Associated with Hamster Sperm Motility

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In our previous paper [M. Fujinoki *et al.* (2001) *Biomed. Res.* 22, 45–58], we reported that two types of 36-kDa protein, which were designated as 36K-A protein and 36K-B protein, obtained from hamster sperm flagella were phosphorylated at serine residues associated with the regulation of motility activation. In the present experiments, it was suggested that these two types of 36-kDa protein were phosphorylated in a cAMP-dependent manner associated with motility activation of hamster spermatozoa. Because the 36K-B protein was the most intensely phosphorylated in a cAMP-dependent manner, attempts were made to further characterize it. The 36K-B protein was assumed to be localized in the middle piece. The localization of the 36K-B protein was the same as that of the 36-kDa protein reported in our previous paper [Y. Si *et al.* (1999) *Mol. Reprod. Dev.* 52, 328–334]. In order to identify the 36K-B protein, it was analyzed by peptide mass finger printing and amino acid sequencing. The results suggested that the 36K-B protein was a pyruvate dehydrogenase E1 component β subunit and a component of the mitochondrial sheath of the middle piece.

Key words: cAMP, hamster, mitochondrial sheath, pyruvate dehydrogenase, spermatozoa.

Cyclic AMP is an important component in the activation of sperm motility (*1–4*). In the signal pathway that regulates the activation of sperm motility, cAMP is produced through activation of adenylate cyclase and activates cAMP-dependent protein kinase, resulting in protein phosphorylation (*5–11*). As a result of these processes, the sperm flagellum moves (*12–14*).

In previous studies, several flagellar proteins were found to be phosphorylated in a cAMP-dependent manner. For example, 45, 31, 20, and 15-kDa proteins in salmonid spermatozoa (*6*, *10*), a 58-kDa protein, named axokinin, in dog spermatozoa (*5*), 65-kDa proteins in mouse spermatozoa (*7*), and 36-, 30-, and 10-kDa proteins in hamster spermatozoa (*11*, *15*) were reported to be phosphorylated in a cAMP-dependent manner. It was also suggested that all these proteins were phosphorylated in relation to the activation of sperm motility. Although these phosphoproteins may be substrates for cAMP-dependent protein kinase, a protein kinase that phosphorylates them has not been detected.

In our previous studies, it was shown that 36-, 30-, and 10-kDa proteins obtained from hamster sperm flagella (*11*, *15*), and a 65-kDa protein obtained from mouse sperm flagella (*7*) were phosphorylated in a cAMP-dependent manner. These four phosphoproteins were related to the activation of sperm motility. It was suggested that among them, the 36-kDa protein is localized in the middle piece and regulates the sliding of microtubules (*15*). We recently proposed that sperm motility should be regulated through two types of phosphorylation cascade in hamster spermatozoa (*16*) because hamster spermatozoa moved slowly even when extracellular calcium was chelated, and the spermatozoa swam vigorously when calcium was present in the medium (*15*). The former slow movement was triggered independently of extracellular activation factors such as calcium and bicarbonate, and was essential for sperm motility to start. We defined the start of motility as "initiation." The latter vigorous movement was extracellular activation factor–dependent. We defined the activation of motility as "activation." Activated spermatozoa moved progressively with a high beat frequency (*15*). We detected four flagellar phosphoproteins associated with sperm initiation and activation (*16*). At the start of motility (initiation), both 66- and 58-kDa proteins were phosphorylated at serine residues. In the activation, two types of 36-kDa proteins, which were designated as the 36K-A protein and 36K-B protein, were phosphorylated at serine residues, but it was not clear whether they were phosphorylated in a cAMP-dependent manner or not. The 36K-A protein and 36K-B protein have different pI values, although their molecular weights are roughly the same.

In this study, we focused on the 36-kDa protein. It appeared that two types of 36-kDa protein were phosphorylated in a cAMP-dependent manner. Furthermore, we examined the 36K-B protein in more detail. It was also

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revealed that the 36K-B protein was localized in the middle piece and was identified as the pyruvate dehydrogenase E1 component β subunit, which is a mitochondrial component.

MATERIALS AND METHODS

Reagents—ATP and cAMP were purchased from Sigma Chemical Company (St. Louis, MO, USA). [γ-^{32}P]ATP (3,000 Ci/mmol) was from Daiichi Pure Chemical (Tokyo). The HISTOFINE SAB-PO (M) KIT was from NICHIREI (Tokyo). Agarose IEF and ampholine were from Amersham-Biosciences (Buckingham, UK). Trypsin was from Promega (Madison, WI, USA). Other chemicals were of reagent grade from Wako Pure Chemical Industries (Osaka).

Preparation of Spermatozoa—Sexually mature male golden hamsters (*Mesocricetus auratus*) were used. Spermatozoa were obtained from the cauda epididymis.

Initiated and activated spermatozoa were prepared according to the method described previously (*15*, *16*). Initiated spermatozoa were prepared by suspending one volume of cauda epididymal spermatozoa in 100 volumes of an isotonic sucrose-EGTA solution containing 310 mM sucrose, 1 mM EGTA, and 10 mM HEPES-NaOH (pH 7.4 at 37°C). Activated spermatozoa were prepared by suspending one volume of cauda epididymal spermatozoa in 100 volumes of an isotonic sucrose–calcium solution containing 310 mM sucrose, 2 mM $CaCl_2$, and 10 mM HEPES-NaOH (pH 7.4 at 37°C). The initiated and activated sperm suspensions were incubated for 10 min at 37°C. Spermatozoa that exhibited approximately 90% motility after the incubation were used in the experiments.

Preparation of Demembranated Sperm Flagella for Biochemical Analysis—Demembranated flagella were prepared from the initiated and activated spermatozoa according to the method described previously (*15*, *16*). The initiated and activated sperm suspensions were centrifuged at 5,500 ×*g* for 5 min at 4°C. The pelleted spermatozoa were suspended in a 100-fold volume of homogenization buffer containing 200 mM sucrose, 25 mM glutamic acid, 25 mM KOH, and 20 mM Tris-HCl (pH 7.9). After centrifugation at 750 ×*g* for 5 min at 4°C, the precipitate was resuspended in a 20-fold volume of the homogenization buffer supplemented with 2 mM PMSF and 20 μg/ml leupeptine. The suspension was homogenized with a Teflon homogenizer for 100 strokes to remove the flagella from the heads. The resultant homogenate was then diluted with a 4-fold volume of the homogenization buffer supplemented with 0.5 mM PMSF and 5 μg/ml leupeptine. After centrifugation at 750 ×*g* for 5 min at 4°C, the supernatant was collected and centrifuged again at 5,500 ×*g* for 5 min at 4°C. The precipitate, which contained isolated flagella, was suspended in a 20-fold volume of demembranation buffer containing 200 mM sucrose, 25 mM glutamic acid, 25 mM KOH, 1 mM DTT, 0.1% (w/v) Triton X-100, and 20 mM Tris-HCl (pH 7.9), and then incubated for 30 s at ambient temperature. The flagellar suspension was centrifuged at 5,500 ×*g* for 5 min at 4°C. The protein concentration of pelleted flagella was determined by the method of Bradford (*17*), followed by adjustment to a final protein concentration of 1 mg/ml with the homogenization buffer.

Preparation of Sperm Specimens for Reactivation Assays—A demembranated sperm model was prepared according to the method of Ishida *et al*. (*3*) with some modifications. Spermatozoa obtained from the cauda epididymis were covered with mineral oil in a test tube until use. A drop (approximately 10 μl) of spermatozoa was suspended in 1 ml of the demembranation buffer (see "Preparation of demembranated sperm flagella for biochemical analysis") and then incubated for 30 s at 37°C. Demembranated spermatozoa were transferred to reactivation buffer containing 200 mM sucrose, 25 mM glutamic acid, 25 mM KOH, 1 mM ATP, 50 μM cAMP, and 20 mM Tris-HCl (pH 7.9), and then incubated for 1 min at 37°C. The sperm suspension was placed on a glass slide and then observed by videomicroscopy. The percentage of motile reactivated spermatozoa was determined in 100 different fields.

Isolation of Outer Dense Fibers (ODFs) from Hamster Spermatozoa—Outer dense fibers were isolated according to the method of Brito and Burzio (*18*). Spermatozoa obtained from the cauda epididymis were suspended in cold PBS containing 150 mM NaCl, 10 mM sodium phosphate, 0.2 mM EGTA, and 0.5 mM PMSF. The sperm suspension was centrifuged at 3,000 ×*g* for 10 min at 4°C and the pellet was washed two times with the PBS. The final sperm pellet was resuspended in 10 mM Tris-HCl (pH 8.0) containing 0.5 mM PMSF to a final concentration of about 2×10^8 sperm/ml. About 2×10^8 spermatozoa were diluted to 75 ml with 10mM Tris-HCl (pH 8.0) containing 0.2 mM PMSF. The suspension was then mixed with an equal volume of an ODF-extract solution containing 1.6 M sucrose, 0.1% CTAB (cetyltrimethylammonium bromide), 10 mM Tris-HCl (pH 8.0), 60 mM 2-mercaptoethanol, and 0.2 mM PMSF, and then was incubated at 18°C for 60 min with occasional shaking. Dissociation of the sperm structures was monitored by phase contrast microscopy until the only visible structures were the sperm heads and the complex of outer dense fibers. Twenty-five milliliters of this suspension was layered over 10 ml of the ODF-extract solution and then centrifuged at 9,000 ×*g* for 30 min at 15°C. On centrifugation the outer dense fibers–connecting piece complexes were accumulated as a layer at the interface between the 0.8 and 1.6 M sucrose layers. The dense fiber fraction was obtained with a disposable pipet, diluted with 3 volumes of 10 mM Tris-HCl (pH 8.0), centrifuged at 10,000 ×*g* for 15 min, and then resuspended in the same buffer solution.

Dissolution of Demembranated Flagella—The dissolution of demembranated flagella and outer dense fibers was performed according to the method used in our previous study (*16*). Tri-chloroacetic acid (TCA) was added to the suspension of demembranated flagella or accessory structures to a final concentration of 10% (w/v). The suspension was centrifuged at 15,000 ×*g* for 20 min at 4°C. The precipitate was rinsed with a 10-fold volume of ice-cold acetone three times, resuspended at 1mg/ml in a guanidine solution containing 8 M guanidine hydrochroride, 10 mM sodium pyrophosphate, 10% (v/v) 2-mercaptoethanol, 2% (v/v) Nonidet P-40 (NP-40), and 0.5 M Tris-HCl (pH 7.5), and then dialyzed against a urea solution containing 7 M urea and 1% (v/v) 2-mercaptoethanol.

Gel Electrophoresis—SDS–PAGE was carried out according to the method of Laemmli (*19*). The separating gel used was 10% (w/v) polyacrylamide containing 0.1% (w/v) SDS.

Two-dimensional gel electrophoresis involving agarose isoelectric focusing (agarose IEF) and SDS–PAGE was carried out according to the method of Hirabayashi (*20*) with some modifications (*16*). A pre-blended ampholine (pH 3.5–9.5) or a mixture of two types of ampholine (pH 4–6 and 3.5–10 at 2:1) were used as a carrier ampholite. SDS–PAGE, in the second dimension, was carried out as described above.

Autoradiography—The procedure for investigating the incorporation of inorganic phosphate into the flagellar proteins was based on the method described previously (*7*, *16*). Demembranated sperm flagella were incubated for 30 sec at ambient temperature in a reactivation solution containing 200 mM sucrose, 25 mM glutamic acid, 25 mM KOH, 1 mM $MgSO_4$, 20 mM Tris-HCl (pH 7.9), 0.5 μM cAMP, 1 mM ATP, and 3.7 MBq [γ-^{32}P]ATP (6,000 Ci/mM). The protein concentration was adjusted to 400 μg/ml. The reactivation was terminated by the addition of TCA to a final concentration of 10% (w/v). After centrifugation at 15,000 ×*g* for 10 min at 4°C, the precipitate was washed with a 10-fold volume of ice-cold methanol three times. After drying, the precipitate was suspended in 20 μl of 2× SDS sample buffer containing 2.5% SDS, 0.1% 2-mercaptoethanol, and 78 mM Tris-HCl (pH 6.8) for SDS–PAGE or 40 μl of a urea-NP-40 solution containing 5 M urea, 1 M thiourea, 10 mM sodium pyrophosphate, and 2% (v/v) NP-40 for two-dimensional gel electrophoresis. After sonication for a total of 30 min on ice, the resuspension was centrifuged at 15,000 ×*g* for 5 min at 4°C. The supernatant was subjected to two-dimensional gel electrophoresis. The stained gel was exposed in a BAS-2000 Image Analyzer (Fuji Film, Tokyo) for 1 day at ambient temperature.

Sucrose Density Gradient Isoelectric Focusing (Sucrose Density Gradient IEF)—Sucrose density gradient IEF was carried out according to the method of Vesterberg (*21*) with some modifications (*16*). The sucrose density gradient was from 0 to 50% (v/v), with an ampholine (pH 3.5–10) as the carrier ampholite. To all solutions was added 3 M urea as a final concentration. After electrophoresis, the sucrose density gradient solution was collected in fractions of 2 ml. The absorbance at 580nm and the pH of each fraction were determined.

Preparation of Anti 36K-B Protein Antiserum—After sucrose density gradient IEF, the pH 4.5 to 5.5 fractions were subjected to two-dimensional gel electrophoresis. For two-dimensional gel electrophoresis, a mixture of two types of ampholine (pH 4–6 and 3.5–10, at 2:1) was used as a carrier ampholite. After electrophoresis, the CBB-stained polyacrylamide gels were washed with pure water. The spots of the 36K-B protein were excised from the gels, and homogenized in PBS containing 145 mM NaCl, 7.4 mM Na_2HPO_4, and 2.6mM NaH_2PO_4 (pH 7.1). Mixtures of spot homogenates and an equal volume of Freund's complete adjuvant were injected into 6-week-old mice. Subsequent injections were performed at 1-week intervals in the same way as for the first injection. After checking the reactivity, the antiserum was collected.

Western Blotting—Western blotting was based on the method of Towbin *et al*. (*22*). A blotted membrane was blocked with 5% BSA in TBS (Tris buffered saline) containing 0.15 M NaCl and 20 mM Tris-HCl (pH 7.5) for 1 h at 20°C, and the incubated with anti 36K-B protein antiserum (1:500 dilution) for 1 h at 20°C. The color reaction was carried out with a HISTOFINE SAB-PO (M) KIT, DAB (3, 3′-diaminobenzidine tetrahydrochloride) and H_2O_2 being used as substrates for peroxidase.

Immunohistochemistry—Spermatozoa were demembranated with a mitochondria sheath extract solution containing 200 mM sucrose, 25 mM glutamic acid, 25 mM KOH, 1 mM DTT, 0.1% (w/v) Triton X-100, and 20 mM Tris-HCl (pH 9.0) (*23*) by incubation for 30 s at ambient temperature. Demembranated spermatozoa were fixed with acetone on glass slides for 10 min, washed three times with TBS (see "Western blotting"), and then incubated for 1 h in 1% H_2O_2 and MeOH. After washing three times with TBS, the spermatozoa were reacted with anti 36K-B protein antiserum (1:10 dilution) for 12 h. The color reaction was carried out with a HISTOFINE SAB-PO (M) KIT, DAB, and H_2O_2 being used as substrates for peroxidase.

Peptide Mass Finger Printing—After two-dimensional gel electrophoresis with a mixture of two ampholines (pH 4–6 and 3.5–10), spot was cut out from the polyacrylamide gel and washed five times with pure water. The neutralized spot was dried with acetonitrile and then soaked in 100 mM ammonium bicarbonate containing 10 μg/ml trypsin, 50 μl of 100 mM ammonium bicarbonate was added, and then the mixture was incubated for 15 h at 37°C for in-gel digestion. After the incubation, sample was centrifuged at 15,000 ×*g* for 5 min at 4°C. The supernatant was subjected to mass spectrometry with a MALDI-TOF-MASS (M@LDI. Micromass, Manchester, U.K.) in order to determine the molecular masses of all peptides digested. Based on the results obtained on mass spectrometry, the 36K-B protein was identified by means of MS-Fit (http://prospector.ucsf.edu/ucsfhtml4.0/msfit.htm).

Amino Acid Sequence Analysis by LC-MS/MS—After the spots obtained on two-dimensional gel electrophoresis with a mixture of two ampholines (pH 4–6 and 3.5–10) had been washed five times with 500 μl of pure water for 10 min at 37°C, the washed spots were destained twice with 100 μl of a destaining solution containing 50% acetonitrile and 100 mM ammonium bicarbonate for 10 min at 37°C. The spots were dehydrated with 100 μl of acetonitrile for 10 min at 37°C and then dried with a Speed Vac® System (Savant, NY, USA). In order to reduce cysteine residues, the spots were incubated in a solution containing 10 mM DTT and 100 mM ammonium bicarbonate for 15 min at 50°C. The spots were then incubated in a solution containing 10 mM iodoacetamide and 100 mM ammonium bicarbonate for 15 min at ambient temperature. After drying with a Speed Vac®, the spots were soaked in 40 μl of 100 mM ammonium bicarbonate containing 10 μg/ml trypsin. After the spots had been incubated for 15 h at 37°C, the digested peptides were extracted from the spots for 10 min at 37°C with 40 μl of a solution containing 50% acetonitrile and 0.1% TFA. After the extracts had been collected with a pipet, the digested peptides were re-extracted from the spots for 15 min at 37°C with 40 μl of a solution containing 20% HCOOH,

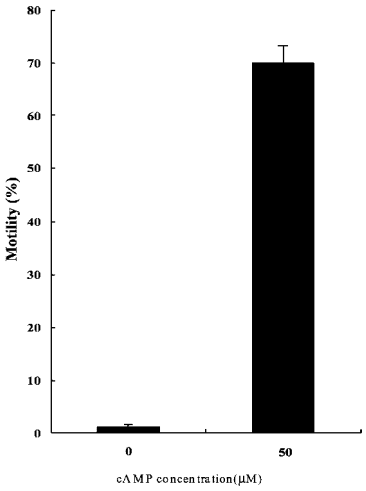


Fig. 1. **Effect of cAMP on flagellar movement of demembranated spermatozoa.** The rate of reactivation of demembranated spermatozoa is shown in the absence (left) and presence (right) of cAMP.

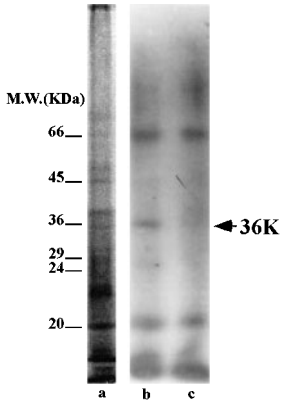


Fig. 2. **Detection of cAMP-dependent protein phosphorylation on SDS–PAGE.** Protein staining (lane a), and the ^{32}P-labeled patterns in the presence (lane b) and absence (lane c) of cAMP are shown. The arrow on right indicates the protein phosphorylated in the presence of cAMP. The bars on the left indicate molecular weight standards. "kDa" represents molecular mass.

25% acetonitrile, and 15% iso-propanol. After the re-extracts had been collected with a pipet, the digested peptides were re-re-extracted from the spots for 2 min at 37°C with 40 μl of 80% acetonitrile. After the re-re-extracts had been collected with a pipet, all the peptide extracts were mixed and dried for 60 min at 50°C with a Speed Vac® System. After the peptides had been solubilized in 20 μl of 2% acetonitrile and 0.1% HCOOH, they were subjected to mass spectrometry with an LC-MS/MS (MAGIC2002 HPLC., Michrom Bioresources, Auburn, CA / LC-Q ion-trap mass spectrometer, Thermo Finnigan, San Jose, CA, USA). Based on the results obtained on mass spectrometry, the 36K-B protein was identified with a TurboSequest (Thermo Finnigan, San Jose, CA, USA).

Amino Acid Sequence Analysis with a Peptide Sequencer—Amino acid sequence analysis of prepared peptides was performed with a peptide sequencer (PPSQ-21., Shimazu, Kyoto). After two-dimensional gel electrophoresis, for which a mixture of two types of ampholine (pH 4–6 and 3.5–10, at 2:1) was used as the carrier ampholite, spots were transferred to a PVDF membrane (Immobilone P^{sq}, Millipore, Bedford, MA, USA) using 10 mM Caps buffer containing 10 mM Caps-NaOH, pH 8.0. After the transfer, the membrane was stained with a CBB solution containing 0.025% CBB and 40% methanol and destained with a destaining solution containing 40% methanol and 10% acetic acid. Spots were cut out of the membrane and subjected to amino acid sequence analysis.

RESULTS

Two 36-kDa Proteins Phosphorylated in a cAMP-Dependent Manner—It has been demonstrated that cAMP is one of the essential components for the activation of sperm motility, and the demembranated sperm model was reactivated through cAMP-dependent protein phosphorylation (*1–15*, *25*). Since the demembranated sperm model was reactivated in a cAMP-dependent manner, as shown in Fig. 1, we tried to detect proteins phosphorylated in the presence of cAMP. As shown in Fig. 2, one protein

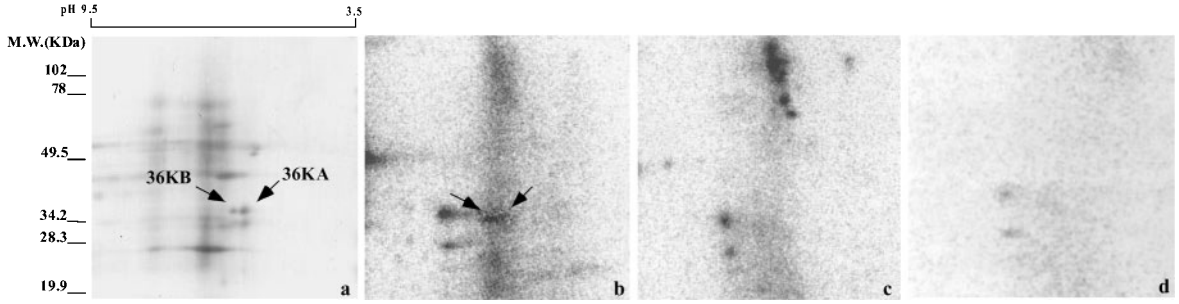


Fig. 3. **Protein phosphorylation in relation to cAMP or motility activation on two-dimensional gel electrophoresis.** Protein staining in the presence of cAMP (a), and the ^{32}P-labeled patterns in the presence of cAMP (b), the absence of cAMP (c) and the presence of cAMP in activated spermatozoa (d) are shown. No difference was observed among the patterns on protein staining in (b), (c), and (d). The arrows in (a) and (b) indicate the two types of 36-kDa protein, 36K-A protein (acidic side) and 36K-B protein (basic side). These 36-kDa proteins were not detected in (c) or (d). The bars on the left of (a) indicate molecular weight standards. "kDa" represents molecular mass. The bar at the top of (a) shows the pI range.

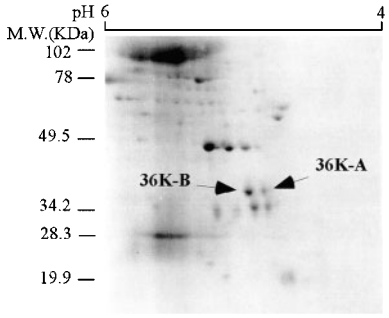


Fig. 4. **Two-dimensional gel electrophoresis of the pH 5 fraction obtained on sucrose density gradient IEF.** The arrows in the photograph indicate the two types of 36-kDa protein, 36K-A protein (acidic side) and 36K-B protein (basic side). The bars on the left of the photograph indicate molecular weight standards. "kDa" represents molecular mass. The bar at the top of the photograph shows the pI range.

band phosphorylated in a cAMP-dependent manner was detected on autoradiography after SDS–PAGE. The apparent molecular weight of the phosphoprotein was estimated to be approximately 36-kDa from its mobility on SDS–PAGE, as shown in previous study (*15*). In the present study, we further investigated the protein by means of two-dimensional electrophoresis. The 36-kDa protein was separated into two components on two-dimensional electrophoresis, as shown in Fig. 3. The two 36-kDa proteins were designated as 36K-A protein (acidic side) and 36K-B protein (basic side) based on the protein patterns on two-dimensional electrophoresis (Fig. 3a and Ref. *1*). These two types of 36-kDa protein were phosphorylated in a cAMP-dependent manner (Fig. 3, b and c) since ^{32}P was incorporated in the presence of cAMP. To determine whether or not the phosphorylation of these 36-kDa proteins is associated with motility activation, activated sperm flagella were demembranated and subjected to autoradiography with [γ-^{32}P]ATP. As shown in Fig. 3, b and d, phosphorylation of the two 36-kDa proteins was not detected in activated spermatozoa. This observation suggested that they were phosphorylated when hamster spermatozoa were activated.

Localization of 36-kDa Proteins in Hamster Sperm Flagella—Two types of 36-kDa proteins were purified on sucrose density gradient IEF and two-dimensional gel electrophoresis. Since a large amount of 36K-B protein was obtained (Fig. 4), we examined it. In order to examine the localization of 36K-B protein in sperm flagella,

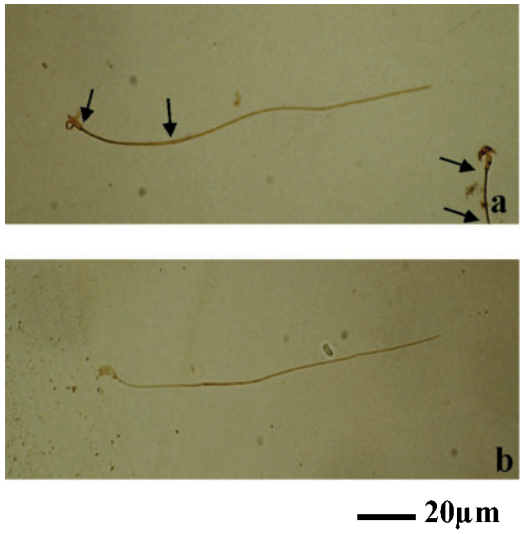


Fig. 6. **Localization of 36K-B protein in spermatozoa.** The immunostaining patterns with antiserum to 36K-B protein (a) and pre-immuno antiserum (b) are shown. 36K-B protein was localized in the area the sperm flagellum between the two arrows. Scale bar 20 μm.

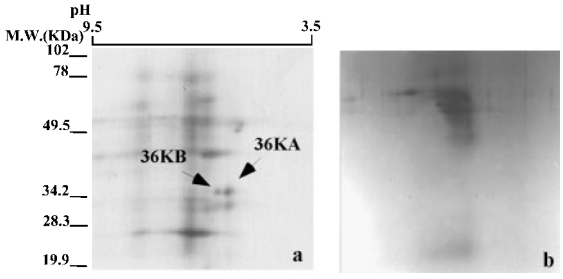
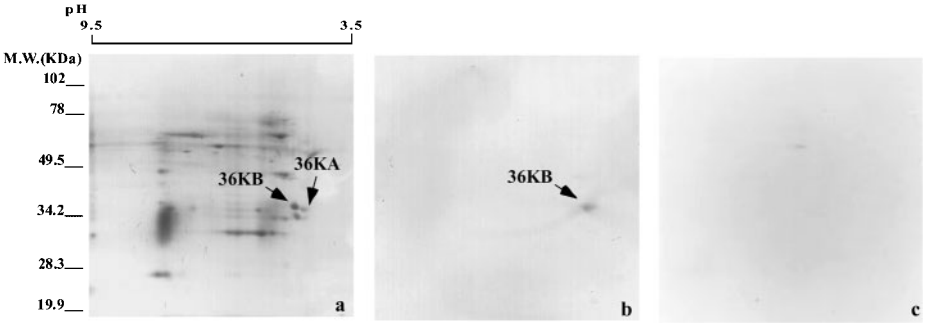


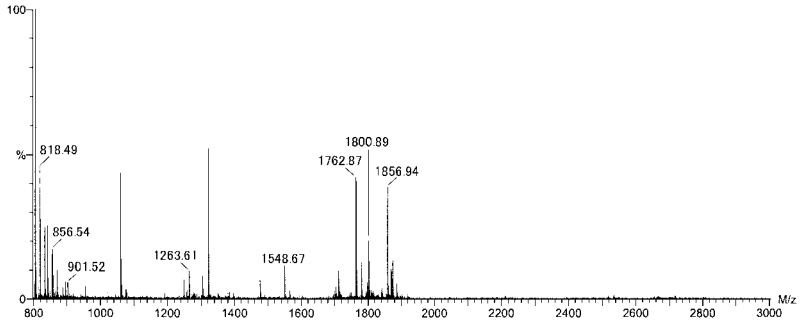
Fig. 7. **Two-dimensional gel electrophoresis of outer dense fibers.** Protein staining of flagella (a) and outer dense fibers (b) is shown. The arrows in (a) indicate 36K-A protein and 36-B protein. Both 36K-A protein and 36K-B protein were not detected in outer dense fibers, as shown in (b). The bars on the left of (a) indicate molecular weight standards. "kDa" represents molecular mass. The bar at the top of (a) shows the pI range.

antiserum to 36K-B protein was raised. As shown in Fig. 5, the antiserum reacted only to 36K-B protein, *i.e.* not to 36K-A protein or other proteins. The localization of 36K-B protein in hamster spermatozoa was examined by means of this antiserum (Fig. 6). 36K-B protein was localized in the middle piece of sperm flagella (Fig. 6a). In this

Fig. 5. **Western blotting with anti 36K-B antiserum.** Protein staining (a) and western blotting with anti 36K-B antiserum (b) and pre-immuno antiserum (c) are shown. The arrows in (a) indicate 36K-A protein and 36K-B protein. The arrow in (b) indicates 36K-B protein. The bars on the left of (a) indicate molecular weight standards. "kDa" represents molecular mass. The bar at the top of (a) shows the pI range.



A



B

Matching peptides:

	MW	Delta	Start	End	Sequence
	818.4902	-0.05	348	354	(K)DIIPA IK(K)
*	856.5494	0.08	185	192	(K)GLIKS AIR(D)
	901.5273	0.00	220	227	(K)DFLIP IGK(A)
	1800.8934	0.06	53	68	(K)VFLLG EEVAQ YDGAY K(V)
	1263.6169	-0.07	325	336	(R)VTGAD VPMPY AK(I) + Methionine Sulfoxide
	1548.6726	0.01	37	49	(R)EAINQ GMDEE LER(D) + Methionine Sulfoxide
	1762.8712	0.04	309	324	(R)IMEGP AFNFL DAPAV R(V) + Methionine Sulfoxide
	1856.9495	0.06	130	145	(K)TYYMS AGLQP VPIVF R(G) + Methionine Sulfoxide

C

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1    MAGVAGLVRG LCGRLSGLLK RLFHCSAPAA VQLTVREAIN QGMDEELERD EKVFLLGEEV AQYDGAYKVS
71   RGLWKKYGDK RIIDTPISEM GFAGIAVGAA MAGLRPICEF MTFNFSMQAI DQVINSAAKT YYMSAGLQPV
141  PIVFRGPNGA SAGVAAQHSQ CFAAWYGHCP GLKVVSPWNS EDAKGLIKSA IRDDNPVVML ENELMYGVAF
211  ELPTEAQSKD FLITIGSAKI ERQGTHINVV CYSRPVGHCL EAAAVLSKGG IECEVINLRT IRPMDIEAIE
281  ASVMKTNHLV TVEGGWPQFG VGAEICARIM ESPAFNFLDA PAVRVTGADV PMPYAKILED NSIPQVKDII
351  FAIKKTLNI
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middle piece of an intact sperm flagellum, there are the mitochondrial sheath, outer dense fibers and the axoneme. In order to determine the localization of 36K-B protein more precisely, the outer dense fibers were extracted and analyzed by two-dimensional gel electrophoresis, but no 36K-B protein was found in the outer dense fibers (Fig. 7).

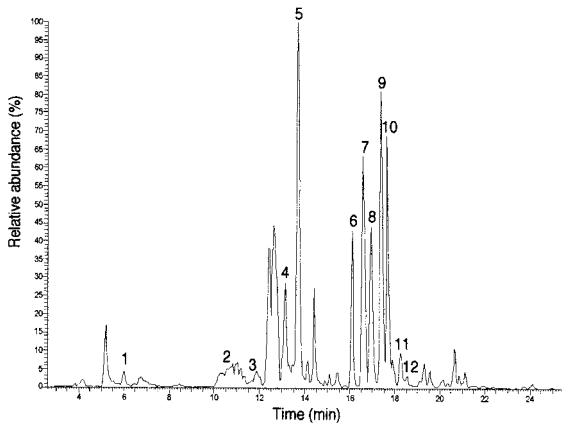
Identification of 36K-B Protein—As mentioned above, 36K-B protein was found to be a component of the middle piece and was phosphorylated in a cAMP-dependent manner associated with sperm motility. In order to understand the mechanism regulating sperm motility, we studied 36K-B protein carefully. The 36K-B protein spot was digested with trypsin and then analyzed by peptide mass finger printing (Fig. 8). As shown in Fig. 8A, many peptide peaks of 36K-B protein and trypsin were detected. Among them, eight peaks matched ones of the pyruvate dehydrogenase E1 component β subunit, as judged on a data base search (Fig. 8, B and C). Coverage of the eight peaks was 26.7% and 18.7% in the pyruvate dehydrogenase E1 component β subunit of rat and human, respectively. For precise analysis, 36K-B protein was analyzed by means of LC-MS/MS (Fig. 9). Twelve peaks originating from 36K-B protein were detected (Fig. 9A). As shown in Fig. 8B, the amino acid sequences of the materials were GLIK (peak 1), EAINQGMDEELERDEK (peak 2), EAINQGMDEELER (peak 3), VVSPWNSEDAK (peak 4), ILEDNSIPQVK (peak 5), DIIFAIK (peak 6), DFLIPIGK (peak 7), TYYMSAGLQPVPIVFR (peak 8), IMEGPAFNFLDAPAVR (peak 9), VFLLGEEVAQYDGAYK (peak 10), IMEGPAFNFLDAPAVR (peak 11), and ILEDNSIPQVKDIIFAIK (peak 12). The data base search revealed that the amino acid sequences of the materials also matched ones of the pyruvate dehydrogenase E1 component β subunit (Fig. 9C). Coverage of the eight peaks was 32% as to amino acid count. On both peptide mass finger printing and LC-MS/MS, several areas of amino acid sequences of the pyruvate dehydrogenase E1 component β subunit could not be analyzed, for example, N-terminal sequence. Therefore, N-terminal amino acid sequences of 36K-B protein were analyzed with an amino acid sequencer (Fig. 10). From the results, the N-terminal amino acid sequence of 36K-B protein was found to be LQLTVREAINQGMDEELERD. The homology of this sequence was 85% with the human pyruvate dehydrogenase E1 component β subunit and 95% with that of rat. This sequence was equal to the sequence between the 31st to 50th residues of the rat and human pyruvate dehydrogenase E1 component β subunits.

DISCUSSION

Cyclic AMP is one of the major second messengers in cell signaling. In spermatozoa, cAMP is assumed to regulate the motility activation of flagella and capacitation, which includes the acrosomal reaction of the head and hyperactivation of the flagellum (*12–14*, *24*, *25*). It has been established that cyclic AMP activates cAMP-dependent protein kinase, resulting in protein phosphorylation. In many previous studies it was found that many proteins

Fig. 8. **Peptide mass finger printing of 36K-B protein.** A: Mass spectrogram of 36K-B protein digested with trypsin. The numbers in the spectrogram indicate the molecular masses of the eight peaks originating from 36K-B protein. B: Results of a data base search with MS-fit. The amino acid sequences of the eight materials originating from 36K-B protein were estimated. All estimated amino acid sequences are included in the pyruvate dehydrogenase E1 component β subunit. C: Amino acid sequences of the rat pyruvate dehydrogenase E1 component β subunit. The highlighted sequences indicate the area covered by the results of peptide mass finger printing.

A



B

No.	ion observed	molecular weight experiment	molecular weight calculation	Resiues	peptide
1	430.83(M+H)+	429.82	429.30	185-188	GLIK
2	961.52(M+2H)2+	1921.03	1920.84	37-52	EAINQGMDEELERDEK + Oxidation (M)
3	775.44(M+2H)2+	1548.86	1548.67	37-49	EAINQGMDEELER + Oxidation (M)
4	616.53(M+2H)2+	1231.04	1230.59	174-184	VVSPWNSEDAK
5	628.39(M+2H)2+	1254.77	1254.68	337-347	ILEDNSIPQVK
6	410.58(M+2H)2+	819.14	818.49	348-354	DIIFAIK
7	902.37(M+H)+	901.36	901.53	220-227	DFLIPIGK
	452.11(M+2H)2+	902.20	901.53	220-227	DFLIPIGK
8	929.68(M+2H)2+	1857.35	1856.95	130-145	TYYMSAGLQPVPIVFR + Oxidation (M)
9	882.45(M+2H)2+	1762.88	1762.87	309-324	IMEGPAFNFLDAPAVR + Oxidation (M)
10	901.8(M+2H)2+	1801.59	1800.89	53-68	VFLLGEEVAQYDGAYK
	1802.09(M+H)+	1801.08	1800.89	53-68	VFLLGEEVAQYDGAYK
11	874.48(M+2H)2+	1746.95	1746.88	309-324	IMEGPAFNFLDAPAVR
12	686.32(M+3H)3+	2055.93	2055.16	337-354	ILEDNSIPQVKDIIFAIK

C

MAGVAGLVRG LCGRLSGLLK RLFHCSAPAA VQLTVREAIN QGMDEELERD
EKVFLLGEEV AQYDGAYKVS RGLWKKYGDK RIIDTPISEM GFAGIAVGAA
MAGLRPICEF MTFNFSMQAI DQVINSAAKT YYMSAGLQPV PIVFRGPNGA
SAGVAAQHSQ CFAAWYGHCP GLKVVSPWNS EDAKGLIKSA IRDDNPVVML
ENELMYGVAF ELPTEAQSKD FLIPIGKAKI ERQGTHINVV CYSRPVGHCL
EAAAVLSKGG IECEVINLRT IRPMDIEAIE ASVMKTNHLV TVEGGWPQFG
VGAEICARIM EGPAFNFLDA PAVRVTGADV PMPYAKILED NSIPQVKDII
FAIKKTLNI

Fig. 9. **LC-MS/MS analysis of 36K-B protein.** A: Chromatogram of 36K-B protein digested with trypsin. Many peaks originating from 36K-B protein and trypsin were detected. Twelve of the peaks originated from 36K-B protein. The numbers in the spectrogram indicate the peaks originating from 36K-B protein. B: Molecular mass and amino acid sequence of each material originating from 36K-B protein. All determined amino acid sequences were identical to ones of the pyruvate dehydrogenase E1 component β subunit, as judged from the results of the data base search. C: Amino acid sequence of the rat pyruvate dehydrogenase E1 component β subunit. The highlighted sequences indicate the area covered by results of LC-MS/MS analysis in hamster.

	0	20
36K-B protein	LQLTVREAINQGMDEELERD	
Pyruvate dehydrogenase E1 component beta subunit	VQLTVREAINQGMDEELERD	
	31	50

Fig. 10. **N-terminal amino acid sequence of 36K-B protein.** Twenty amino acids of the N-terminal sequence of 36K-B protein are shown. Under this sequence, the homologous sequence of rat pyruvate dehydrogenase E1 component β subunit is indicated. The N-terminal 20 amino acid sequence of 36K-B protein corresponds to the sequences of the rat pyruvate dehydrogenase E1 component β subunit between the 31st and 50th amino acid.

were phosphorylated in association with motility activation or capacitation of mammalian spermatozoa, for example, 175, 93, 44, 40, 38, and 20-kDa proteins in boar spermatozoa (*9*), 45.0, 31.5, 17.2, 14.7, and 13.3-kDa proteins in rat spermatozoa (*26*), a 58-kDa protein, named axokinin, in dog spermatozoa (*5*), 120, 82, and 66 or 65 -kDa proteins in mouse spermatozoa (*7*, *27*), 190, 120, 110, 105, 94, 81, 35, and 18-kDa proteins in human spermatozoa (*8*, *27*, *28*) and 80-, 66-, 58-, 36-k, 30-, and 10-kDa proteins in hamster spermatozoa (*11*, *15*, *16*, *29*).

In our previous studies, it was suggested that 65-kDa protein obtained from mouse sperm flagella (*7*) and 36-kDa protein obtained from hamster sperm flagella (*15*) were phosphorylated in a cAMP-dependent manner associated with motility activation of spermatozoa. It was also demonstrated that 36-kDa protein regulated the sliding of microtubules (*15*). In the present experiments, this 36-kDa protein was found to consist of two components designated as 36K-A protein and 36K-B protein (see Figs. 2 and 3). 36K-A protein and 36K-B protein differed in pI values, although their molecular weights were roughly the same. 36K-A protein and 36K-B protein were both phosphorylated at serine residues (*16*). In the present study, we focused on 36K-B protein since it was more intense than 36K-A protein (see Figs. 4 and 5).

In a previous study (*15*), it was shown that 36-kDa protein is localized in the middle piece of sperm flagella. In the present study, we prepared anti 36K-B protein antiserum and examined the localization of 36K-B protein. The results showed that 36K-B protein is also localized in the middle piece of the sperm flagellum (see Fig. 6). Both 36-kDa protein in the previous study (*15*) and 36K-B protein in the present study were phosphorylated in a cAMP-dependent manner and localized in the middle piece of the sperm flagellum. Therefore, it is likely that the 36K-B protein corresponds to the 36-kDa protein described by Si and Okuno (*15*). In the middle piece of mammalian sperm flagella, there are the mitochondrial sheath, outer dense fibers and the axoneme. Since 36K-B protein was not included in the fraction of outer dense fibers (see Fig. 7), it is likely that this protein is a component of the mitochondrial sheath or axoneme, not outer dense fibers (*30*).

We often found positive staining in the tip of a sperm head, as observed in Fig. 6a. However, we presumed that it was nonspecific since it is amorphous and was not always observed. It looked like membranes debris resulting from demembranation.

Although we did not determine the localization of 36K-A protein in the present experiment, it is likely that 36K-A protein is a component of the fibrous sheath since the protein was detected in fibrous sheath extracts (*30*). Therefore, it is assumed that the 36K-A protein does not correspond to the 36-kDa protein described by Si and Okuno (*15*).

As the next step we tried to identify the 36K-B protein. The results of peptide-mass finger printing, LC/MS-MS and amino acid sequencing showed the 36K-B protein to be the pyruvate dehydrogenase E1 component β subunit (see Figs. 8, 9, and 10). The subunit is one of the components of the pyruvate dehydrogenase complex that converts pyruvate to acetyl coenzyme A. The enzyme exists in the mitochondrial matrix, constituting an essential

step in aerobic glucose oxidation. The pyruvate dehydrogenase complex consists of seven subunits: E1α, E1β, E2, E3, protein X, a pyruvate dehydrogenase–specific kinase, and a pyruvate dehydrogenase–specific phosphatase (*31*, *32*). Pyruvate dehydrogenase activity is tightly controlled *via* the cyclic phosphorylation and dephosphorylation of serine residues located on the α subunit of the E1 component (*33*). In several studies (*34–38*), the testis-specific isoforms of E1 component α subunit were cloned and examined as to the pattern of their expression during spermatogenesis. The different action of pyruvate dehydrogenase on l-malate suggested that they were sperm-specific isoenzymes (*39*). Although we could not determine whether 36K-B protein was a testis or sperm-specific isoform of the pyruvate dehydrogenase, it seemed that 36K-B protein was a different type from the pyruvate dehydrogenase E1 component β subunit of the somatic cell since it lacked 30 amino acids of the N-terminal sequence. Testis-specific isoforms that lack N-terminal sequences have been found in several experiments. The pyruvate dehydrogenase E1 component α subunit in human (*34*) lacks 3 amino acids of N-terminal sequence. Similar results were reported for the catalytic subunits of cAMP-dependent protein kinase in sheep (*40*), mouse (*41*), human (*41*), and rainbow trout (*42*).

It is quite reasonable that pyruvate dehydrogenase E1 β is localized in the middle piece of flagella since it is a component necessary for ATP production in mitochondria. ATP production in mitochondria is important for the generation of flagellar motility via active sliding of microtubules caused by dynein ATPase (*43*). This indicated to us the importance of regulation of the metabolic pathway. Does the phosphorylation of 36K-B play a role in the regulation of flagellar motility *via* ATP production? As to this, however, we only have negative pieces of evidence. One was obtained in the experiment involving demembranated spermatozoa. The reactivation of demembranated spermatozoa requires cAMP (*1–4*) even though a sufficient amount of ATP is present. Although 36K-B protein was phosphorylated in a cAMP-dependent manner when demembranated spermatozoa were cAMP-dependently reactivated, ATP was not produced in the demembranated spermatozoa. All ATP required for the reactivation of demembranated spermatozoa was present beforehand in the medium, however, they were not reactivated without cAMP. Therefore, it is unlikely that the phosphorylation of 36K-B regulates the motility by controlling ATP production. Another piece of evidence concerns the properties of pyruvate dehydrogenase E1 β. The phosphorylation related to enzyme activation occurs in pyruvate dehydrogenase E1 α rather than in E1 β. We do not have any information on the phosphorylation of E1 β at present. What is the role of phosphorylation of 36K-B protein pyruvate dehydrogenase E1 β? We have no idea at present.

In the present experiments, it was suggested that two types of 36-kDa protein, which were detected as phosphoproteins associated with sperm motility activation (*1*), were phosphorylated in a cAMP-dependent manner. Furthermore, we also demonstrated that 36K-B protein, one of the two 36-kDa proteins, was localized in the middle piece of sperm flagellum, and was pyruvate dehydrogenase E1 β, which is a component of mitochondria. But we failed to determine the role of phosphorylation in 36K-B protein in sperm motility activation. In order to understand the role of phosphorylation in 36K-B protein associated with sperm motility activation, detailed characterization of the 36K-B protein is needed, for example, determination of the full length of the gene of the 36K-B protein.

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