J. Biochem. **134**, 559–565 (2003)
DOI: 10.1093/jb/mvg174

Identification of the 58-kDa Phosphoprotein Associated with Motility Initiation of Hamster Spermatozoa

Masakatsu Fujinoki*[,1], Takeshi Kawamura[2], Toshifusa Toda[3], Hideki Ohtake[1], Nobuyoshi Shimizu[2], Sadao Yamaoka[1] and Makoto Okuno[4]

[1]Department of Physiology, Dokkyo University School of Medicine, Mibu, Tochigi 321-0293; [2]Department of Molecular Biology, Keio University School of Medicine, Shinanomachi, Shinjuku-ku, Tokyo 160-8582; [3]TMIG Proteomics Collaboration Center, Tokyo Metropolitan Institute of Gerontology, Sakaecho, Itabashi-ku, Tokyo 173-0015; and [4]Department of Biology, Graduate School of Arts and Science, University of Tokyo, Komaba, Meguro-ku, Tokyo 153-8902

Received June 6, 2003; accepted July 29, 2003

In our previous paper [M. Fujinoki *et al.* (2001) *Biomed. Res.* 22, 45–58], we reported that the 58-kDa protein obtained from hamster sperm flagella was phosphorylated at serine residues in association with the start of motility. In the present experiments, we identified and localized the 58-kDa protein. The 58-kDa protein was assumed to exist in the acrosomal region domain of the sperm head and the whole sperm flagellum. In particular, a large amount of 58-kDa protein was localized in the equatorial segment of the acrosomal region domain of the sperm head and the middle piece of the sperm flagellum. In the next step, the 58-kDa protein was identified by peptide mass finger printing and LC-MS/MS analysis. The results suggested that the 58-kDa protein was ATP synthase H^+ transporting F1 β, which is one of the mitochondrial components. Therefore, it is likely that the 58-kDa protein is associated with ATP production in the mitochondrial sheath in the middle piece of the sperm flagellum, and H^+ transport in the sperm head and the sperm flagellum except for the middle piece, since ATP synthase also acts as an H^+ pump.

Key words: ATP synthase, hamster, LC-MS/MS, peptide mass finger printing, phosphorylation, spermatozoa.

Extracellular calcium and/or bicarbonate are essential components for the activation of mammalian spermatozoa (*1–3*). They stimulate adenylate cyclase, which results in cAMP production (*4*, *5*). Cyclic AMP activates a cAMP-dependent protein kinase and causes protein phosphorylation and/or dephosphorylation (*6–13*). Finally, the interaction between dynein ATPase and microtubules is initiated and the motility of spermatozoa is triggered. Since it seems that sperm activation is regulated through protein phosphorylation and/or dephosphorylation, they have often been examined in several mammalian species.

In hamster spermatozoa, the extracellular activation factor is calcium (*14*). It is assumed that the pathways for intracellular signal transduction related to motility activation in hamster spermatozoa are basically the same as described above. In our previous studies (*11–13*, *15–17*), we detected many phosphoproteins associated with hamster sperm motility. We recently proposed that sperm motility is regulated through two types of phosphorylation cascade in hamster spermatozoa (*17*), because hamster spermatozoa moved slowly even when extracellular calcium was chelated, and the spermatozoa swam vigorously when calcium was present in the medium (*11*). The former slow movement started independently of extracellular activation factors such as calcium and bicarbonate. We called the start of motility "initiation." The latter vigorous movement was extracellular activation factor-dependent. We called the triggering of motility "activation." Activated spermatozoa moved progressively with a high beat frequency (*11*). We detected four flagellar phosphoproteins associated with sperm initiation and activation (*13*, *17*). At motility initiation, the 66-kDa protein and 58-kDa protein were phosphorylated at serine residues. At activation, two 36-kDa proteins, which were designated as the 36K-A and 36K-B proteins, were phosphorylated at serine residues in a cAMP-dependent manner. The 36K-A and 36K-B proteins differ in the pI value, although their molecular weights are roughly the same. The 36K-B protein was localized in the middle piece of the sperm flagellum and was identified as the pyruvate dehydrogenase E1 component β subunit (*13*).

In this study, we focused on the 58-kDa protein that is phosphorylated in hamster motility initiation (*17*). It appeared that the 58-kDa protein was localized in the acrosomal region domain of the sperm head and the whole sperm flagellum, and was identified as the ATP synthase H^+ transporting F1 complex β subunit, which is one of the mitochondrial components.

MATERIALS AND METHODS

Reagents—The HISTOFINE SAB-PO (M) KIT was purchased from NICHIREI (Tokyo). Agarose IEF and ampholine were purchased from Amersham-Biosciences (Buckingham shire, UK). Trypsin was purchased from

*To whom correspondence should be addressed. Tel: +81-282-87-2125, Fax: +81-282-86-7835, E-mail: fujinoki@dokkyomed.ac.jp

Promega (Madison, WI, USA). Other chemicals were of reagent grade from Wako Pure Chemical Industries (Osaka).

Preparation of Demembranated Sperm Flagella—Demembranated sperm flagella were prepared according to the method described in our previous papers (*11*, *17*). Spermatozoa obtained from the cauda epididymis of sexually mature male golden hamsters (*Mesocricetus auratus*) were suspended in a 100-fold volume of homogenization buffer comprising 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 10 mM PMSF and 20 μg/ml leupeptine. The suspension was homogenized with 100 strokes up and down in a Teflon homogenizer to remove flagella from the heads. The homogenate was then diluted with a 4-fold volume of the homogenization buffer supplemented with 2.5 mM PMSF and 5 μg/ml leupeptine. After centrifugation at 750 ×g for 5 min at 4°C, the supernatant was collected and centrifuged 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 comprising 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 sec at ambient temperature. The flagellar suspension was centrifuged at 5,500 ×g for 5 min at 4°C. After the flagella had been resuspended in homogenization buffer, the protein concentration was determined by the method of Bradford (*18*), and then adjusted to a final concentration of 1 mg/ml with the homogenization buffer.

Dissolution of Demembranated Sperm Flagella—Dissolution of the demembranated sperm flagella was performed according to the method described in our previous report (*17*). Trichloroacetic acid (TCA) was added to the suspension of demembranated flagella 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 1 mg/ml in a guanidine solution comprising 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 comprising 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 comprised 10% (w/v) polyacrylamide containing 0.1% (w/v) SDS.

Two-dimensional gel electrophoresis, combined with agarose isoelectric focusing (agarose IEF) and SDS-PAGE, was carried out according to the method of Hirabayashi (*20*) with some modifications (*17*, *21*). First-dimension IEF was performed on cylindrical agarose gels at 4°C with a mixture of two types of ampholines (pH 4–6 and pH 3.5–10, used at 2:1) or pre-blended ampholine (pH 3.5–9.5) as the carrier ampholite. SDS-PAGE as the second-dimension was carried out as described above.

Sucrose Density Gradient Isoelectric Focusing (Sucrose Density Gradient IEF)—Sucrose density gradient IEF was carried out according to the method of Vesterberg (*22*) with some modifications (*17*). A sucrose density gradient, from 0 to 50% (v/v), was made together with an ampholine (pH 3.5–10) as the carrier ampholite. To all solutions urea was added to a final concentration of 3M. After electrophoresis, the sucrose density gradient solution was collected in 2 ml fractions. The absorbance at 580 nm and the pH of each fraction were determined.

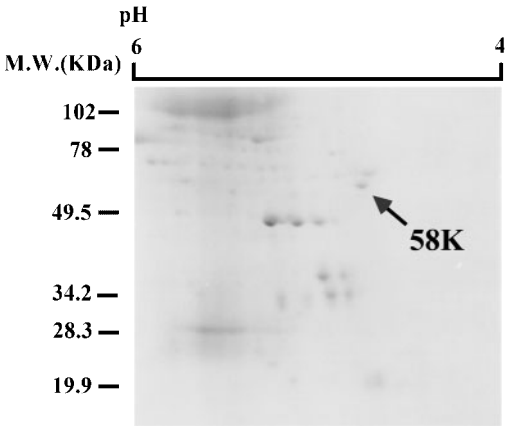
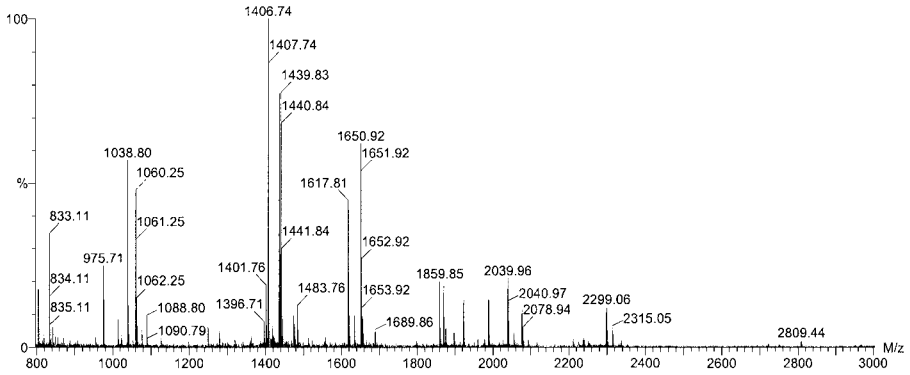


Fig. 1. **Two-dimensional gel electrophoresis of the pH 4–6 fraction obtained on sucrose density gradient IEF.** The arrow indicates the 58-kDa protein. The bars on the left side show the molecular weights of standard samples. The bracket at the top of the photograph shows the pI range.

Peptide Mass Finger Printing—Peptide mass finger printing was carried out according to our previous paper (*13*). After two-dimensional gel electrophoresis with a mixture of two ampholines (pH 4–6 and pH 3.5–10, used at 2:1), a spot was cut from the polyacrylamide gel and then washed five times with pure water. The neutralized spot was dehydrated with acetonitrile for 10 min at ambient temperature. After the acetonitrile had been evaporated off with a Speed Vac® System (Savant, N.Y., USA) and then soaking 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, a 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, UK) in order to determine the molecular masses of all peptides digested. Based on the results of the mass spectrometry, the 58-kDa protein was identified by means of MS-Fit (http://prospector.ucsf.edu/ucsfhtml4.0/msfit.htm).

Amino Acid Sequence Analysis with LC-MS/MS—Amino acid sequence analysis by LC-MS/MS was carried out according to our previous paper (*13*). After the spots obtained on two-dimensional gel electrophoresis with a mixture of two ampholines (pH 4–6 and pH 3.5–10, used at 2:1) 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 comprising 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 the Speed Vac® System. In order to reduce cysteine residues, the spots were incubated in a solution

A



B

MW	Delta	Start	End	Sequence
974.5549	-0.15	202	212	(K)IGLFG GAGVG K(T)
1405.6738	-0.05	226	239	(K)AHGGY SVFAG VGER(T)
1434.7467	-0.04	311	324	(R)FTQAG SEVSA LLGR(I)
1438.7820	-0.04	282	294	(R)VALTG LTVAE YFR(D)
1649.9100	-0.00	95	109	(R)LVLEV AQHLG ESTVR(T)
1920.9581	0.03	295	310	(R)DQEGQ DVLLF IDNIF R(F)
1987.0262	0.04	388	406	(R)AIAEL GIYPA VDPLD STSR(I)
1400.6969	-0.05	144	155	(R)IMNVI GEPID ER(G) + Methionine Sulfoxide
1472.8272	-0.02	213	225	(K)TVLIM ELINN VAK(A) + Methionine Sulfoxide
1616.7980	-0.00	265	279	(K)VALVY GQMNE PPGAR(A) + Methionine Sulfoxide
1857.8679	0.03	407	422	(R)IMDPN IVGNE HYDVA R(G) + Methionine Sulfoxide
2037.9982	0.04	463	480	(R)FLSQP FQVAE VFTGH MGK(L) + Methionine Sulfoxide
2075.9833	0.05	242	259	(R)EGNDL YHEMI ESGVI NLK(D) + Methionine Sulfoxide
2297.0667	0.02	325	345	(R)IPSAV GYQPT LATDM GTMQE R(I) + Methionine Sulfoxide + Methionine Sulfoxide

C

Q9CWX7
(Q9CWX7) ATP SYNTHASE, H+ TRANSPORTING MITOCHONDRIAL F1 COMPLEX, BETA

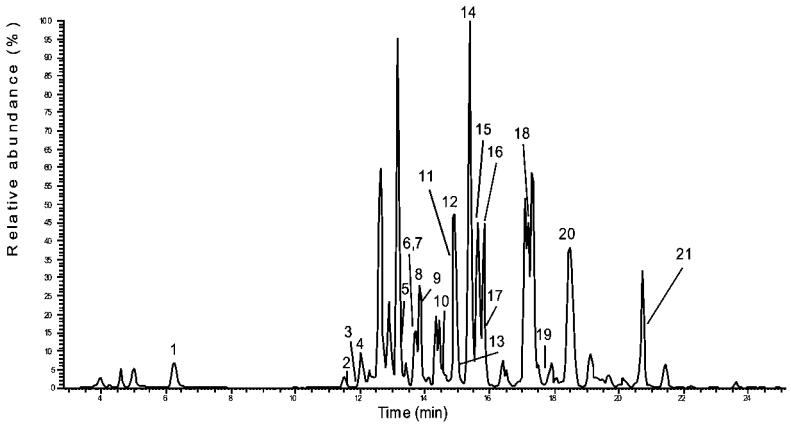
```
1       MLSLVGRVAS ASASGALRGL SPSAALPQAQ LLLRAAPAGV HPARDYAAQA SAAPKAGTAT GRIVAVIGAV
71      VDVQFDEGLP PILNALEVQG RDSRLVLEVA QHLGESTVRT IAMDGTEGLV RGQKVLDSGA PIKIPVGPET
141     LGRIMNVIGE PIDERGPIKT KQFAPIHAEA PEFIEMSVEQ EILVTGIKVV DLLAPYAKGG KIGLFGGAGV
211     GKTVLIMELI NNVAKAHGGY SVFAGVGERT REGNDLYHEM IESGVINLKD ATSKVALVYG QMNEPPGARA
281     RVALTGLTVA EYFRDQEGQD VLLFIDNIFR FTQAGSEVSA LLGRIPSAVG YQPTLATDMG TMQERITTTK
351     KGSITSVQAI YVPADDLTDP APATTFAHLD ATTVLSRAIA ELGIYPAVDP LDSTSRIMDP NIVGNEHYDV
421     ARGVQKILQD YKSLQDIIAI LGMDELSEED KLTVSRARKI QRFLSQPFQV AEVFTGHMGK LVPLKETIKG
491     FQQILAGEYD HLPEQAFYMV GPIEEAVAKA DKLAEEHGS
```

comprising 10 mM DTT and 100 mM ammonium bicarbonate for 15 min at 50°C, and then incubated in a solution comprising 10 mM iodoacetamide and 100 mM ammonium bicarbonate for 15 min at ambient temperature. After drying with the 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 comprising 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 comprising 20% HCOOH, 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 the Speed Vac® System. After the peptides were 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 58-kDa protein was identified with a TurboSequest (Thermo Finnigan, San Jose, CA, USA). Furthermore, several of the amino acid sequences obtained on mass spectrometry were subjected to a blast search (http://blast.genome.ad.jp/).

Preparation of Anti 58-kDa Protein Antiserum—Anti 58-kDa protein antiserum was prepared according to the method described previously (*13*). After sucrose density gradient IEF, the pH 4.5 to 5.5 fractions were subjected to two-dimensional gel electrophoresis. For the two-dimensional gel electrophoresis, a mixture of two types of

Fig. 2. **Peptide mass finger printing of the 58-kDa protein.** A: Mass spectrogram of the 58-kDa protein digested with trypsin. The numbers on the spectrogram indicate the molecular masses for fourteen peaks originating from the 58-kDa protein. B: Results of a data-base search with MS-fit. The amino acid sequences of the fourteen materials originating from the 58-kDa protein were estimated. All the estimated amino acid sequences are present in the ATP synthase H^+ transporting mitochondrial F1 complex β. C: Amino acid sequence of mouse ATP synthase H^+ transporting mitochondrial F1 complex β. The shadowed 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	779.3(M+H)+	778.29	778.42	437-442	ILQDYK
	390.47(M+2H)2+	778.93	778.42	437-442	ILQDYK
2	796.29(M+2H)2+	1590.57	1590.80	120-134	TIAMDGTEGLVRGQK + Oxidation (M)
3	639.98(M+2H)2+	1277.95	1277.63	120-131	TIAMDGTEGLVR + Oxidation (M)
4	520.84(M+2H)2+	1039.67	1039.66	491-499	LVPLKETIK
5	676.41(M+3H)3+	2026.20	2025.09	154-171	IMNVIGEPIDERGPIKTK + Oxidation (M)
6	701.65(M+2H)2+	1401.29	1400.70	154-163	IMNVIGEPIDER + Oxidation (M)
7	704.11(M+2H)2+	1406.21	1405.67	236-249	AHGGYSVFAGVGER
8	1149.63(M+2H)2+	2297.25	2297.07	335-355	IPSAVGYQPTLATDMGTMQER + 2 Oxidation (M)
9	599.99(M+3H)3+	1796.94	1795.95	154-169	IMNVIGEPIDERGPIK + Oxidation (M)
10	946.51(M+3H)3+	2836.50	2835.37	250-274	TREGNDLYHEMIESGVINLKDATSK + Oxidation (M)
11	744.86(M+3H)3+	2231.54	2231.26	132-153	GQKVLDSGAPIKIPVGPETLGR
12	826.17(M+2H)2+	1650.32	1649.91	105-119	LVLEVAQHLGESTVR
	551.24(M+2H)2+	1650.69	1649.91	105-119	LVLEVAQHLGESTVR
13	488.67(M+2H)2+	975.33	974.55	212-222	IGLFGGAGVGK
14	960.21(M+2H)2+	1918.40	1918.09	135-153	VLDSGAPIKIPVGPETLGR
	640.89(M+3H)3+	1919.63	1918.09	135-153	VLDSGAPIKIPVGPETLGR
15	544.98(M+2H)2+	1087.95	1087.63	199-208	VVDLLAPYAK
16	1435.54(M+H)+	1434.53	1434.75	321-334	FTQAGSEVSALLGR
	718.61(M+2H)2+	1435.20	1434.75	321-334	FTQAGSEVSALLGR
17	679.99(M+3H)3+	2036.93	2036.10	102-119	ETRLVLEVAQHLGESTVR
18	1020.61(M+2H)2+	2039.20	2038.00	473-490	FLSQPFQVAEVFTGHMGK + Oxidation (M)
19	720.59(M+2H)2+	1439.17	1438.78	292-304	VALTGLTVAEYFR
20	737.61(M+2H)2+	1473.21	1472.83	223-235	TVLIMELINNVAK + Oxidation (M)
21	1346.28(M+2H)2+	2690.55	2690.37	443-466	SLQDIIAILGMDELSEEDKLTVSR + Oxidation (M)

C

```
  1 MTSLWGKGTG CKLFKFRVAA APASGALRRL TPSASLPPAQ LLLRAVRRRS HPVRDYAAQT SPSPKAGAAT GRIVAVIGAV VDVQFDEGLP PILNALEVQG
101 RETRLVLEVA QHLGESTVRT IAMDGTEGLV RGQKVLDSGA PIKIPVGPET LGRIMNVIGE PIDERGPIKT KQFAPIHAEA PEFMEMSVEQ EILVTGIKVV
201 DLLAPYAKGG KIGLFGGAGV GKTVLIMELI NNVAKAHGGY SVFAGVGERT REGNDLYHEM IESGVINLKD ATSKVALVYG QMNEPPGARA RVALTGLTVA
301 EYFRDQEGQD VLLFIDNIFR FTQAGSEVSA LLGRIPSAVG YQPTLATDMG TMQERITTTK KGSITSVQAI YVPADDLTDP APATTFAHLD ATTVLSRAIA
401 ELGIYPAVDP LDSTSRIMDP NIVGSEHYDV ARGVQKILQD YKSLQDIIAI LGMDELSEED KLTVSRARKI QRFLSQPFQV AEVFTGHMGK LVPLKETIKG
```

ampholine (pH 4–6 and pH 3.5–10 used at 2:1) was used as the carrier ampholite. After electrophoresis, the CBB-stained polyacrylamide gel was washed with pure water. The spot of the 58-kDa protein was excised from the gel, and then homogenized in PBS containing 145 mM NaCl, 7.4 mM Na_2HPO_4 and 2.6 mM NaH_2PO_4 (pH 7.1). A Mixture of spot homogenate and an equal volume of Freund's complete adjuvant was 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.* (*23*). The 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 then incubated with anti 58-kDa protein antiserum (1: 500 dilution) for 1 h at 20°C. The color reaction was carried out with the HISTOFINE SAB-PO (M) KIT, DAB (3,3′-diaminobenzidine tetrahydrochloride) and H_2O_2 being used as substrates for peroxidase.

Immunohistochemistry—Immunohistochemical analysis was performed according to the method described in our previous paper (*13*). Spermatozoa were demembranated with a mitochondrial sheath extract solution comprising 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) (*24*) by incubation for 30 sec at ambient temperature. The demembranated spermatozoa were fixed with acetone on a glass slide 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 the anti 58-kDa protein antiserum (1:10 dilution) for 12 h at 4°C. The color reaction was carried

Fig. 3. **LC-MS/MS analysis of the 58-kDa protein.** A: Chromatogram of the 58-kDa protein digested with trypsin. Many peaks originating from the 58-kDa protein and trypsin were detected. Twenty one of the peaks originated from the 58-kDa protein. The numbers on the spectrogram indicate peaks originating from the 58-kDa protein. B: Molecular mass and amino acid sequence of each peak originating from the 58-kDa protein. All the determined amino acid sequences were identical to that of the ATP synthase H^+ transporting mitochondrial F1 complex β based on the results of the data-base search. C: Amino acid sequence of human ATP synthase H^+ transporting mitochondrial F1 complex β. Red letters indicate sequences covered by the results of LC-MS/MS analysis.

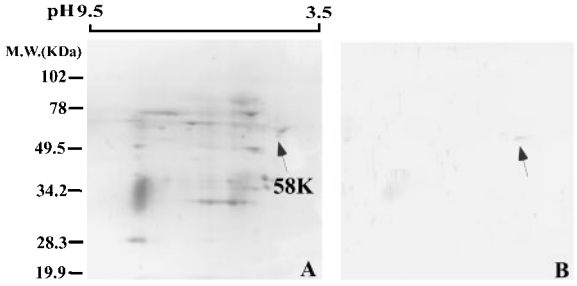


Fig. 4. **Western blotting with anti 58-kDa antiserum.** Protein staining (A) and Western blotting with anti 58-kDa antiserum (B) are shown. The arrow in (A) indicates the 58-kDa protein. The arrow in (B) indicates the 58-kDa protein. The bars on the left side of (A) show the molecular weights of standard samples. The bracket at the top of (A) shows the pI range.

out with the HISTOFINE SAB-PO (M) KIT, DAB and H_2O_2 being used as substrates for peroxidase.

RESULTS

Identification of the 58-kDa Protein—We recently suggested that the motility of hamster spermatozoa was regulated through two protein phosphorylation pathways, calcium dependent or independent pathways (*17*). In the calcium independent pathway, the 58-kDa protein is phosphorylated in association with the initiation of motility of hamster spermatozoa (*17*). In order to characterize the 58-kDa protein, it was purified by sucrose density gradient IEF and two-dimensional gel electrophoresis (Fig. 1), and then identified. After the spot of the 58-kDa protein had been digested with trypsin, it was analyzed by peptide mass finger printing (Fig. 2). As shown in Fig. 2A, many peptids of the 58-kDa protein and trypsin were detected. Fourteen peptides obtained matched the ATP synthase H^+ transporting mitochondrial F1 complex β subunit found as a result of a database search (Fig. 2, B and C). The coverage of the fourteen peaks was 40.64% in mouse, 37.62% in rat, 37.62% in human, and 34.28% in bovine. For precision, the 58-kDa protein was analyzed by means of LC-MS/MS (Fig. 3). As shown in Fig. 3A, many peptides of the 58-kDa protein and trypsin were detected. Twenty-one peaks originating from the 58-kDa protein were detected and examined (Fig. 3, A and B). Judging from the results of the data-base search, the amino acid sequences of these peptides also matched the human ATP synthase H^+ transporting mitochondrial F1 complex β subunit (Fig. 3C). The coverage of the eight peaks was 48% as to the amino acid count.

Localization of the 58-kDa Protein in Hamster Sperm—In the next step, antiserum to the 58-kDa protein was raised. As shown in Fig. 4, anti 58-kDa protein antiserum reacted to the 58-kDa protein only. By means of this antiserum, localization of the 58-kDa protein in hamster spermatozoa was examined (Fig. 5). The 58-kDa protein was localized in the acrosomal region domain of the sperm head and the whole sperm flagellum (Fig. 5a). An especially large amount of 58-kDa protein was localized in the equatorial segment of the acrosomal region domain of the sperm head and the middle piece of the sperm flagellum rather than in the principal piece and end piece of the sperm flagellum.

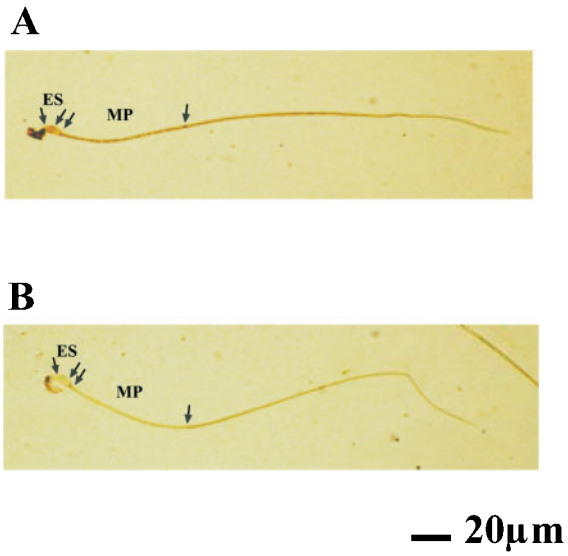


Fig. 5. **Localization of the 58-kDa protein in spermatozoa.** The results with antiserum to the 58-kDa protein (A) and pre-immuno serum (B) are shown. The 58-kDa protein was localized in the acrosomal region domain of the sperm head and the whole sperm flagellum. In the acrosomal region domain of the sperm head, the 58-kDa protein was localized in the equatorial segment (ES). A large amount of 58-kDa protein was localized in the middle piece (MP) of the sperm flagellum. The equatorial segment is the area between the two arrows for the sperm head. The middle piece was the area between the two arrows for the sperm flagellum. The scale bar shows 20 μm.

DISCUSSION

Protein phosphorylation is the most major event in cell signaling. In spermatozoa, it is widely accepted that motility activation and capacitation, which includes acrosomal reaction and hyperactivation, are regulated though protein phosphorylation pathways (*1–3*, *25*, *26*). Protein phosphorylation occurs in species-specific factors and in a cAMP-dependent manner. In the signal regulated motility activation of mammalian spermatozoa, species-specific factors, which include calcium, potassium, bicarbonate, *etc*., activate an adenylate cyclase and cause the production of cAMP. Cyclic AMP activates a cAMP-dependent protein kinase and causes protein phosphorylation. Sperm motility is triggered by such protein phosphorylation (*1*, *2*). In many previous studies, it was found that many proteins are phosphorylated in association with motility activation or capacitation of mammalian spermatozoa. In hamster spermatozoa, the species-specific factor is calcium (*14*). It is assumed that the pathways which regulate motility activation in hamster spermatozoa are basically the same as described above, although an adenylate cyclase is activated through protein phosphorylation by calmodulin-dependent protein kinase (*2*).

In the present study, we identified the 58-kDa protein and examined its localization. The 58-kDa protein was localized mainly in the equatorial segment of the acro-

somal region domain of the sperm head and the middle piece of the sperm flagellum (see Fig. 5). From the results of peptide mass finger printing and LC/MS-MS analysis, the 58-kDa protein was demonstrated to be the ATP synthase H^+ transporting mitochondrial F1 complex β subunit (see Figs. 2 and 3). In our previous study (*17*), several partial amino acid sequences of the 58-kDa protein were obtained, *i.e.* MQQTEYCCCCCATDQ, which is the N-terminal sequence of the 58-kDa protein, AYPVAPNAGKAVPNE and APNYDNIALKAPK. The N-terminal sequence was slightly similar that of human tyrosine kinase (TXK) (*17*). TXK is a member of the Tec subfamily of Src type (non-receptor) tyrosine kinases and has been reported to be expressed in T cells (*27*). The other two sequences, AYPVAPNAGKAVPNE and APNYDNIALKAPK, were shown not to be similar at all by the results of the data-base search. The sequences reported in our previous paper (*13*) were not included in the sequence of the ATP synthase H^+ transporting mitochondrial F1 complex β subunit obtained from the data-base. However, we could not examine several parts of the ATP synthase H^+ transporting mitochondrial F1 complex β subunit such as its N-terminal sequence. These sequences reported in our previous paper (*13*) may be the sequences of the 58-kDa protein that we could not examine in the present experiment.

Axokinin is well-known as a 58-kDa phosphoprotein associated with sperm motility (*6*, *7*). Is the 58-kDa protein that was detected in our previous study (*17*) and was identified in the present experiment, axokinin? We consider that the 58-kDa protein is not axokinin because the 58-kDa protein is a detergent-insoluble protein (*17*) and axokinin is a detergent-soluble protein (*6*, *7*).

The 58-kDa protein that was detected as a phosphoprotein associated with the motility of sperm flagella (*17*) was identified as the ATP synthase H^+ transporting mitochondrial F1 complex β subunit. The ATP synthase H^+ transporting mitochondrial F1 complex β subunit is one of the enzymes associated with ATP production in mitochondria, and changes ADP to ATP. In the present experiments (see Fig. 5), the 58-kDa protein was found to exist in the acrosomal region domain of the sperm head and the whole sperm flagellum. In the acrosomal region domain of the sperm head, the 58-kDa protein was especially localized in the equatorial segment. In the acrosomal region domain of the sperm head, an ATPase and a H^+ pump exist, which are associated with the acrosomal reaction (*28*). It is likely that the 58-kDa protein localized in the equatorial segment of the acrosomal region domain of the sperm head acts as an ATPase or H^+ pump associated with the acrosomal reaction since ATP synthase also functions as an ATPase or H^+ pump. On the other hand, the 58-kDa protein was also localized in the sperm flagellum. An especially large amount of the 58-kDa protein was localized in the middle piece of the sperm flagellum. The middle piece of the sperm flagellum consists of three structures, the mitochondrial sheath, the outer dense fibers and the axoneme. The mitochondrial sheath contains many mitochondria. Since the 58-kDa protein was identified as the ATP synthase H^+ transporting mitochondrial F1 complex β subunit, it seems that the 58-kDa protein acts as one of the subunits of ATP synthase in the mitochondria in the mitochondrial sheath and is associated with ATP production. What is the role of phosphorylation of the ATP synthase H^+ transporting mitochondrial F1 complex β subunit? Recently, it was suggested that phosphorylation of the ATP synthase H^+ transporting mitochondrial F1 complex β subunit regulated ATP synthesis in skeletal muscle (*29*). Therefore, phosphorylation of the 58-kDa protein may also be related to the regulation of ATP synthesis in sperm mitochondria, although we have no evidences of this.

Furthermore, a small amount of the 58-kDa protein is localized in the principal piece and in the end piece of the sperm flagellum. In the previous studies (*30*, *31*), it was suggested that the motility of spermatozoa was regulated through changes in intercellular pH. Therefore, it also seems that the 58-kDa protein acts as one of the subunits of the H^+-pump associated with the regulation of the intercellular pH in the sperm flagellum, but we do not yet have any evidence as to the function of the 58-kDa protein. Further study on characterization of the 58-kDa protein in more detail may lead to a clearer understanding of ATP production and the regulation of sperm motility.

We wish to thank Ms. N. Fujinoki and Ms. Y. Imaizumi (Department of Physiology, Dokkyo University School of Medicine) for their excellent assistance. We also thank the staff of the Laboratory Animal Research Center of Dokkyo University School of Medicine for their support. This work was partly supported by the MEXT. KAKENHI (15790860).

REFERENCES

1. Morisawa, M. (1994) Cell signaling mechanisms for sperm motility. *Zool. Sci.* **11**, 647–662
2. Yanagimachi, R. (1994) Mammalian fertilization in *The Physiology of Reproduction* (Knobil, E. and Neill, J.D., eds.) Vol. **1**, pp. 189–317, Raven Press, New York
3. Lindemann, C.B. and Kanous, K.S. (1997) A model for flagellar motility. *Int. Rev. Cytol.* **173**, 1–72
4. Hyne, R.V. and Garbers, D.L. (1979) Regulation of guinea pig sperm adenylate cyclase by calcium. *Biol. Reprod.* **21**, 1135–1142
5. Okamura, N. and Sugita, Y. (1983) Activation of spermatozoan adenylate cyclase by a low molecular weight factor in porcine seminal plasma. *J. Biol. Chem.* **258**, 13056–13062
6. Tash, J.S., Kakar, S.S., and Means, A.R. (1984) Flagellar motility requires the cAMP-dependent phosphorylation of a heat-stable NP-40-soluble 56 kd protein, axokinin. *Cell* **38**, 551–559
7. Tash, J.S., Hidaka, H., and Means, A.R. (1986) Axokinin phosphorylation by cAMP-dependent protein kinase is sufficient for activation of sperm flagellr motility. *J. Cell Biol.* **103**, 649–655
8. Tash, J.S., Krinks, M., Patel, J., Means, R.L., Klee, C.B., and Means, A.R. (1988) Identification, characterization, and functional correlation of calmodulin-dependent protein phosphatase in sperm. *J. Cell Biol.* **106**, 1625–1633
9. Tash, J.S. and Bracho, G.E. (1994) Regulation of sperm motility: emerging evidence for a major role for protein phosphatases. *J. Androl.* **15**, 505–509
10. Si, Y. and Okuno, M. (1995) Activation of mammalian sperm motility by regulation of microtubule sliding via cyclic adenosine 5′-monophosphate-dependent phosphorylation. *Biol. Reprod.* **53**, 1081–1087
11. Si, Y. and Okuno, M. (1999) Regulation of microtubule sliding by a 36-kDa phosphoprotein in hamster sperm flagella. *Mol. Reprod. Dev.* **52**, 328–334
12. Fujinoki, M., Ohtake, H., and Okuno, M. (2001) Tyrosine phosphorylation and dephosphorylation associated with motility of hamster spermatozoa. *Biomed. Res.* **22**, 147–155

13. Fujinoki, M., Kawamura, T., Toda, T., Ohtake, H., Ishimoda-Takagi, T., Shimizu, N., Yamaoka, S., and Okuno, M. (2003) Identification of 36k-Da flagellar phosphoproteins associated with hamster sperm motility. *J. Biochem.* **133**, 361–369
14. Feng, B., Bhattacharyya, A., and Yanagimachi, R. (1987) Ca^{2+} is essential for the motility of plasma membrane-intact, but not of demembranated, hamster spermatozoa. *Andrologia* **20**, 155–162
15. Si, Y. and Okuno, M. (1999) Role of tyrosine phosphorylation of flagellar proteins in hamster sperm hyperactivation. *Biol. Reprod.* **61**, 240–246
16. Si, Y. (1999) Hyperactivation of hamster sperm motility by temperature-dependent tyrosine phosphorylation of an 80-kDa protein. *Biol. Reprod.* **61**, 247–252
17. Fujinoki, M., Ohtake, H., and Okuno, M. (2001) Serine phosphorylation of flagellar proteins associated with the motility activation of hamster spermatozoa. *Biomed. Res.* **22**, 45–58
18. Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248–254
19. Laemmli, U.K. (1970) Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature* **227**, 680–685
20. Hirabayashi, T. (1981) Two-dimensional gel electrophoresis of chicken skeletal muscle proteins with agarose gels in the first dimension. *Anal. Biochem.* **117**, 443–451
21. Fujinoki, M., Tomiyama, T., and Ishimoda-Takagi, T. (2002) Tropomyosin isoforms presents in the sea anemone, *Anthopleura japonica* (Anthozoan, Cnidaria). *J. Exp. Zool.* **293**, 649–663
22. Vesterberg, O. (1971) Isoelectric focusing of proteins. In *Methods Enzymol.* Vol. **22**, pp. 399–412, Academic Press, New York
23. Towbin, H., Staehelin, T., and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl Acad. Sci. USA* **76**, 4350–4354
24. Si, Y. and Okuno, M. (1993) The sliding of the fibrous sheath through the axoneme proximally together with microtubule extrusion. *Exp. Cell Res.* **208**, 170–174
25. Visconti, P.E., Bailey, J.L., Moore, G.D., Pan, D., Olds-Clarke, P., and Kopf, G.S. (1995) Capacitation of mouse spermatozoa. I. Correlation between the capacitation state and protein tyrosine phosphorylation. *Development* **121**, 1129–1137
26. Visconti, P.E., Moore, G.D., Bailey, J.L., Leclerc, P., Connors, S.A., Pan, D., Olds-Clarke, P., and Kopf, G.S. (1995) Capacitation of mouse spermatozoa. II. Protein tyrosine phosphorylation and capacitation are regulated by a cAMP-dependent pathway. *Development* **121**, 1149–1150
27. Harie, R.N., Ohta, Y., Lewis, J.E., Fu, S.M., Kroisel, P., and Litman, G.W. (1994) TXK, a novel human tyrosine kinase expressed in T cells shares sequence identity with Tec family kinases and maps to 4p12. *Hum. Mol. Genet.* **3**, 897–901
28. Tulsiani, D.R.P., Abou-Haila, A., Loeser, C.R., and Pereira, B.M.J. (1998) The biological and functional significance of the sperm acrosome and acrosomal enzymes in mammalian fertilization. *Exp. Cell Res.* **240**, 151–164
29. Højund, K., Wrzesinski, K., Larsen, P.M., Fey, S.J., Roepstorff, P., Handberg, A., Dela, F., , Vinten, J., McCormack, J.G., Reynet, C., and Beck-Nielsen, H. (2003) Proteome analysis reveals phosphorylation of ATP synthase b-subunit in human skeletal muscle and proteins with potential roles in type 2 diabetes. *J. Biol. Chem.* **278**, 10436–10442
30. Wong, P.Y.D., Lee, W.M., and Tsang, A.Y.F. (1981) The effects of extracellular sodium on acid release and motility initiation in rat caudal epididymal spermatozoa in vitro. *Exp. Cell Res.* **131**, 97–104
31. Babcock, D.F., Rufo Jr, G.A., and Lardy, H.A. (1983) Potassium-dependent increases in cytosolic pH stimulate metabolism and motility of mammalian sperm. *Proc. Natl. Acad. Sci. USA* **80**, 1327–1331