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Original Article

Serine/threonine phosphorylation associated with hamster sperm hyperactivation

MASAKATSU FUJINOKI,[1]* TADASHI ISHIMODA-TAKAGI[2] and HIDEKI OHTAKE[1]

[1]*Department of Physiology, Dokkyo University School of Medicine, Mibu, Tochigi and* [2]*Department of Biology, Tokyo Gakugei University, Koganei, Tokyo, Japan*

Background and Aims: Mammalian sperm activation and hyperactivation is regulated by protein phosphorylation. Although tyrosine phosphorylation is considered very important, several studies have investigated whether serine and threonine phosphorylation are also associated with sperm activation and hyperactivation, and that was also the aim of the present study.

Methods: Protein phosphorylation of hamster spermatozoa was detected by Western blotting using antiphospho-amino acid monoclonal antibodies after tricine sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Amino acid sequences were analyzed using a peptide sequencer.

Results: Four proteins were phosphorylated at serine residues during hyperactivation via activation and their approximate molecular weights were 90, 38, 32 and 10 kDa, respectively. Five proteins were phosphorylated or dephosphorylated at threonine residues and their approximate molecular weights were 90, 70, 65, 35 and 10 kDa, respectively. The 10-kDa protein corresponded to a previously reported 10-kDa tyrosine phosphoprotein. N-terminal sequences of the 10-kDa protein were similar to carcinustatin, which is a neuropeptide.

Conclusions: During hyperactivation, four serine phosphorylation and five threonine phospho- or dephosphorylations occurred, which suggested that the 10-kDa protein was phosphorylated at tyrosine residues when spermatozoa were activated and then dual-phosphorylated at the serine and threonine residues during hyperactivation. (Reprod Med Biol 2004; **3**: 223–230)

Key words: dephosphorylation, hamsters, hyperactivation, phosphorylation, spermatozoa.

INTRODUCTION

EJACULATED MAMMALIAN SPERMATOZOA are not able to fertilize eggs[1] until they become capacitated, which occurs after activation. Activated spermatozoa can be prepared *in vitro* by adding activating factors, such as calcium and/or bicarbonate, and are moved progressively with a high-beat frequency.[1–3] Capacitated spermatozoa are prepared *in vitro* by adding calcium, albumin, glucose, etc., and capacitation involves an acrosomal reaction in the sperm head, which is very important for fertilization, and hyperactivation of motility,[1] which comprises a large bend amplitude, whiplash and frenzied flagellar movements.[4–6]

The most recent understanding is that the changes in sperm motility are closely associated with phosphorylation of flagellar proteins,[3] particularly serine, threonine and tyrosine; for example, 175-, 93-, 44-, 40-, 38-, 20-kDa proteins have been detected in boar sperm,[7] 90-, 80-, 62-, 48-kDa proteins in hamsters[8,9] and 190-, 110-, 94-, 43–55-, 35-, 18-kDa proteins in humans.[10] From these results, it has been assumed that tyrosine phosphorylation plays an especially important role in the hyperactivation of mammalian spermatozoa.[1,7,8,11–13] The phosphorylation at the tyrosine residues of a flagellar protein, A-kinase anchor protein (AKAP), has also been reported in detail.[14] AKAP binds to the RII subunit of A-kinase[15,16] and is a component of the fibrous sheath.[17,18] It has been suggested that the phosphorylation of AKAP is regulated through calcium-calmodulin dependent signal transduction,[14] and it has been demonstrated that the phosphorylation of AKAP is also regulated by protein phosphatase.[8] However, the regulatory mechanism of capacitation is still unclear.

Because we had investigated tyrosine phosphorylation in a previous study,[9] in the present experiment we investigated serine and threonine phosphorylation associated with motility activation and hyperactivation in hamster spermatozoa.

*Correspondence: Dr Masakatsu Fujinoki, Department of Physiology, Dokkyo University School of Medicine, Mibu, Tochigi 321-0293, Japan. Email: fujinoki@dokkyomed.ac.jp
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MATERIALS AND METHODS

Reagents

ADENOSINE TRIPHOSPHATE, CYCLIC adenosine monophosphate (cAMP), antiphosphoserine monoclonal antibody and antiphosphothreonine monoclonal antibody were purchased from Sigma (St Louis, MO, USA). A Histofine SAB-PO (M) kit was purchased from Nichirei (Tokyo, Japan). Other chemicals of reagent grade were obtained from Wako Pure Chemical Industries (Osaka, Japan).

Preparation of spermatozoa

Spermatozoa were obtained from the cauda epididymis of sexually mature (12–16 week old) male golden hamsters (*Mesocricetus auratus*) as described previously.[19] Immotile spermatozoa were prepared according to our previous method.[19] In brief, one volume of cauda epididymal spermatozoa were suspended directly in 10 volumes of ice-cold pure water, and then homogenized in 50 volumes of homogenizing buffer (200 mmol/L sucrose, 25 mmol/L glutamic acid, 25 mmol/L KOH and 20 mmol/L Tris-HCl [pH 7.9]) using a Teflon homogenizer.

Activated and hyperactivated spermatozoa were prepared according to the methods described previously,[9,19] using modified Tyrode's albumin lactate pyruvate (m-TALP) medium.[20] An aliquot of cauda epididymal spermatozoa was placed in a test tube, several milliliters of m-TALP medium was then carefully added and the mixture was overlayed with mineral oil. The tubes were incubated for 10 min to allow the activated spermatozoa to swim up. The supernatant containing activated spermatozoa was collected and homogenized in 50 volumes of the homogenizing buffer using a Teflon homogenizer. Spermatozoa exhibiting approximately 90% motility were used in the experiment (Table 1).

For hyperactivation, the activated sperm suspension was placed on a culture plate, covered with mineral oil and incubated for 3 h at 37°C under 5% CO_2 in air. The hyperactivated spermatozoa were then collected and homogenized in 50 volumes of the homogenizing buffer using a Teflon homogenizer. Spermatozoa exhibiting approximately 80% hyperactivated motility were used in the experiment (Table 1).

Table 1 Motility, activation and hyperactivation of hamster spermatozoa

	Motility (%)	Activation (%)	Hyperactivation (%)
Activated	95	93	0
Hyperactivated	91	12	83

Spermatozoa suspended in the m-TALP medium were diluted 10-fold and placed on a glass slide coated with 5% bovine serum albumin (BSA). Sperm hyperactivity was recorded on a videotape via a CCD camera (Sony, Tokyo, Japan) attached to a microscope (Olympus, Tokyo, Japan) with phase contrast illumination and warm stage (Kitazato Supply Co. Ltd, Shizuoka, Japan). Each observation was performed at 37°C, lasted for 2 min and involved analyzing the number of motile sperm or hyperactivated sperm in 10 different fields. Percent motility was defined as the number of motile spermatozoa/number of total spermatozoa, percent activation as the number of activated spermatozoa/number of motile spermatozoa, and percent hyperactivation as the number of hyperactivated spermatozoa/number of motile spermatozoa.

Preparation of demembranated sperm flagella

Demembranated sperm flagella were prepared from immotile, activated and hyperactivated spermatozoa according to the method described previously.[19] Each sperm suspension prepared as described above was centrifuged at 5500 *g* for 5 min at 4°C. The pelleted spermatozoa were suspended in 100-fold of the homogenizing buffer and after centrifugation at 750 *g* for 5 min at 4°C, the precipitate was resuspended in a 20-fold volume of the homogenizing buffer supplemented with 2 mmol/L phenylmethanesulfonyl fluoride (PMSF) and 20 μg/mL leupeptine. The suspension was homogenized with 100 strokes of a Teflon homogenizer in order to separate flagella from heads. The homogenate was then diluted in a fourfold volume of the homogenizing buffer supplemented with 0.5 mmol/L PMSF and 5 μg/mL leupeptine. After centrifugation at 750 *g* for 5 min at 4°C, the supernatant was collected and centrifuged at 5500 *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 mmol/L sucrose, 25 mmol/L glutamic acid, 25 mmol/L KOH, 1 mmol/L dithiothreitol, 0.1% (w/v) Triton X-100 and 20 mmol/L Tris-HCl (pH 7.9), and incubated for 30 s at room temperature. The flagellar suspension was centrifuged at 5500 g for 5 min at 4°C. The protein concentration of the pelleted flagella was determined by the method of Bradford[21] and adjusted to a final protein concentration of 1 mg/mL with homogenization buffer.

Dissolution of demembranated sperm flagella

Dissolution of demembranated sperm flagella was performed according to the method described previously.[19] Tri-chloroacetic acid was added to the suspension of demembranated sperm flagella to a final concentration of 10% (w/v) and it was centrifuged at 15 000 *g* for 20 min at 4°C. The precipitate was rinsed three times with a 10-fold volume of ice-cold acetone and then suspended in 1 mg/mL guanidine solution containing 8 mol/L guanidine hydrochroride, 10 mmol/L sodium pyrophosphate, 10% (v/v) 2-mercaptoethanol, 2% (v/v) Nonidet P-40 and 500 mmol/L Tris-HCl (pH 7.5). After centrifugation at 15 000 *g* for 20 min at 4°C, the suspension was dialyzed against a urea solution containing 7 mol/L urea and 1% (v/v) 2-mercaptoethanol.

Gel electrophoresis

Tricine sodium dodecyl sulfate-polyacrylamide gel electrophoresis (tricine SDS-PAGE) was carried out according to the method of Schagger and Jagow.[22] The separating gel was 10% polyacrylamide containing 0.1% SDS.

Western blotting

Western blotting was performed according to the method of Towbin *et al*.[23] The blotted membrane was blocked with 5% (w/v) BSA in Tris-buffered saline (TBS) containing 0.15 mol/L NaCl and 20 mmol/L Tris-HCl (pH 7.5) for 1 h at 20°C, and incubated with first antibodies (1:1000 dilution with 5% [w/v] BSA in TBS) for 1 h at 20°C. Color reaction was carried out with the Histofine SAB-PO (M) kit and H_2O_2 and 3,3′-diaminobenzidine tetrahydrochloride as the substrate for peroxidase.

Amino acid sequence analysis with a peptide sequencer

Amino-acid sequence analysis with a peptide sequencer (PPSQ-21, Shimadzu Co. Ltd, Kyoto, Japan) was carried out according to the method used in our previous study.[24] After tricine SDS-PAGE, proteins were transferred to a polyvinylidene fluoride membrane (Immobilone P^{sq}; Millipore Corp., Bedford, MA, USA) with 10 mmol/L Caps buffer containing 10 mmol/L Caps-NaOH, pH 8.0. After the transfer, the membrane was stained with a Coomassie Brilliant Blue (CBB) solution containing 0.025% CBB and 40% methanol, and destained with a destaining solution containing 40% methanol and 10% acetic acid. Bands were cut out of the membrane and subjected to amino-acid sequence analysis, from which the 10-kDa protein was identified by means of searching the FASTA database (http://fasta.genome.ad.jp/).

RESULTS

IN ORDER TO examine the successive changes in serine and threonine phosphorylation that are related to sperm motility, we prepared three suspensions of spermatozoa: immotile, activated and hyperactivated spermatozoa. The immotile spermatozoa were obtained by dilution of mature epididymal spermatozoa in pure water,[19] activation occurred after dilution of spermatozoa in m-TALP (they moved progressively with a high beat frequency of approximately 10 Hz)[8,19] and hyperactivated spermatozoa were obtained after 3 h incubation in m-TALP; they exhibited a beat frequency of approximately 10 Hz, large bend amplitude, whiplash and frenzied flagellar movements.[4–6,8] We therefore redefined the activation of spermatozoa as the change from immotile to active and hyperactivation as the change from active to hyperactive.

Serine and threonine phosphorylation associated with hyperactivation via activation

The first aim was to detect serine phosphorylation of flagellar proteins, particularly during activation and hyperactivation. As shown in Figure 1, we detected four proteins that were phosphorylated at serine residues during either activation or hyperactivation, and these were designated as 90-, 38-, 32- and 10-kDa proteins from their apparent molecular weights. Phosphorylation of the 90-kDa protein occurred on activation and was maintained or increased during the change in sperm motility from activation to hyperactivation. Conversely, phosphorylation of the 38-, 32- and 10-kDa proteins occurred when the activated spermatozoa were hyperactivated.

We then investigated threonine phosphorylation of flagellar proteins and as shown in Figure 2, we detected three proteins that were phosphorylated at threonine residues, during either activation or hyperactivation and these were designated as 90-, 65- and 10-kDa proteins from their apparent molecular weights. Moreover, two proteins were dephosphorylated at threonine residues on activation and these were designated as 70- and 35-kDa proteins from their apparent molecular weights. Phosphorylation of the 10-kDa protein

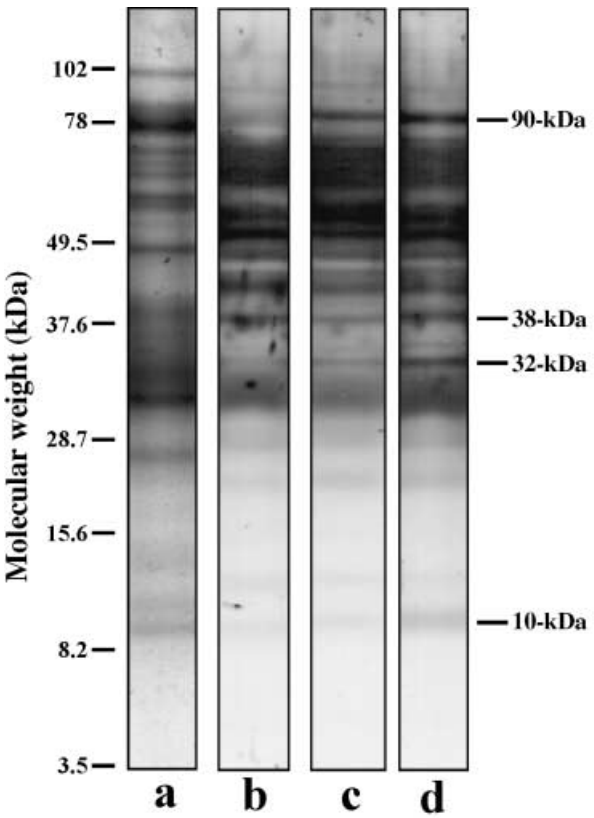

Figure 1 Phosphorylation of serine in immotile, activated and hyperactivated sperm flagella. Protein stain (lane a), Western blotting of immotile spermatozoa (lane b), activated spermatozoa (lane c) and hyperactivated spermatozoa (lane d) are shown. Bars on the right side indicate phosphoproteins associated with activation and hyperactivation. Bars on the left side show molecular weight standards.

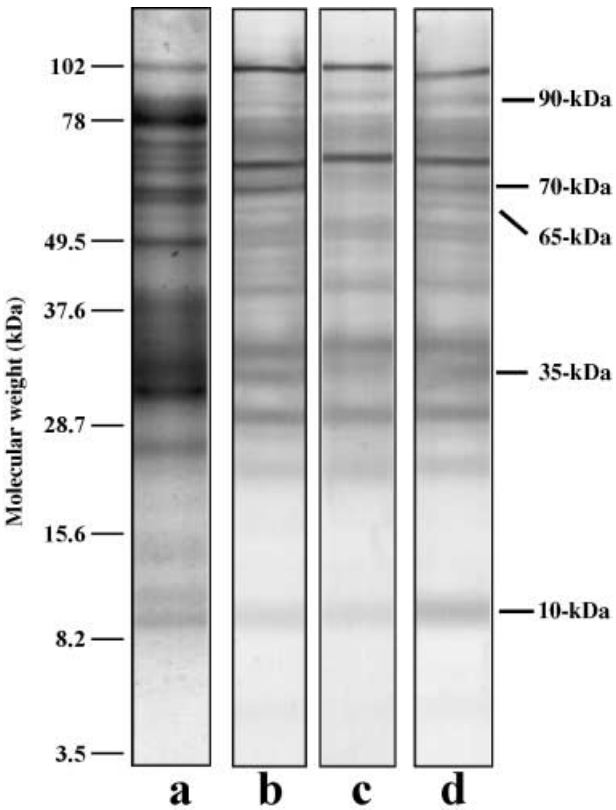

Figure 2 Phosphorylation of threonine in immotile, activated and hyperactivated sperm flagella. Protein stain (lane a), Western blotting of immotile spermatozoa (lane b), activated spermatozoa (lane c) and hyperactivated spermatozoa (lane d) are shown. Bars on the right side indicate phosphorylated or dephosphorylated proteins associated with activation and hyperactivation. Bars on the left side show molecular weight standards.

occurred when the activated spermatozoa were hyperactivated, whereas dephosphorylation of the 70- and 35-kDa proteins occurred on activation. Moreover, the 90- and 10-kDa proteins were dual-phosphorylated at serine and threonine residues when the spermatozoa were activated or hyperactivated (Figs 1, 2).

Phosphorylation of the 10-kDa protein

When activated spermatozoa were hyperactivated, the 10-kDa protein was phosphorylated at both serine and threonine residues (Figs 1, 2). In our previous study,[9] we found that the 10-kDa protein was phosphorylated at tyrosine residues on activation and hyperactivation, and as shown in Figure 3, this also occurred in the present experiment. Because the same protein band of 10-kDa was phosphorylated at the tyrosine, serine and threonine residues (Fig. 3), it is likely that multiphosphorylation of the 10-kDa protein is associated with sperm motility.

Identification of the 10-kDa protein

In order to identify the 10-kDa protein, its N-terminal sequence was analyzed by peptide sequencer and was found to be MYNRRRPQLYA. A search of the FASTA database revealed that the N-terminal sequence of the 10-kDa protein was similar to the N-terminal sequence (5th to 15th) of carcinustatin[25] (Fig. 4).

DISCUSSION

IT HAS BEEN suggested that protein phosphorylation is a very important event in the regulation of sperm

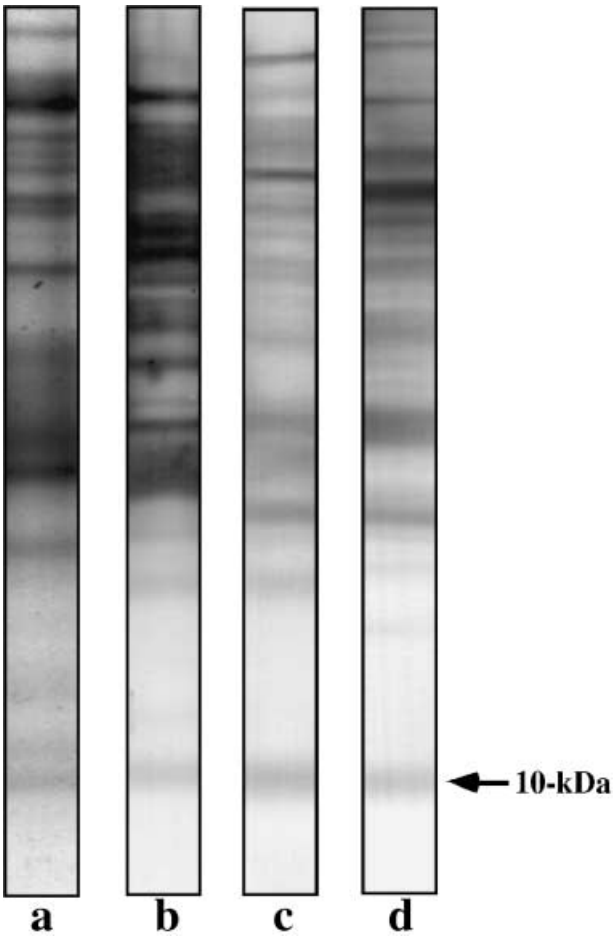

Figure 3 Phosphorylation of the 10-kDa protein. Protein stain (lane a), phosphoserine (lane b), phosphothreonine (lane c) and phosphotyrosine (lane d) are shown. Bars on the right side indicate the 10-kDa protein.

10-kDa protein	MYNRRRPQLYA
Carcinustatin	EYDDMYTEKRPKVYAFGL

Figure 4 N-terminal amino acid sequences of the 10-kDa protein and carcinustatin.

motility,[1–3] and many protein phosphorylations associated with sperm motility have been detected in several species of animals, for example, bovine,[26,27] dog,[28] hamster,[8,9,19,29,30] human,[10] mouse,[11–13,31] ascidian,[32] salmon and trout.[33–36] As for capacitation, it has been suggested that protein phosphorylation is also a very important event,[1] particularly tyrosine phosphorylation.[9,12,13,29,30] Although many tyrosine phosphorylations have been detected, it is now accepted that tyrosine phosphorylation of AKAP, a major fibrous sheath protein, is the most essential event associated with sperm capacitation.[14,16–18,29,30]

Recently, it was shown that serine and threonine phosphorylation are also associated with sperm capacitation.[10] Several previous studies have shown that the tyrosine phosphorylation of AKAP is regulated through a serine/threonine phosphorylation cascade,[13,14,29] and it is likely that the same mechanism regulates sperm capacitation. Moreover, it has been shown that sperm capacitation is regulated through the classical MAP kinase cascade,[37,38] and that tyrosine phosphorylation of AKAP is also associated with this cascade.[38] Although the dual serine/tyrosine kinase is associated with the MAP kinase cascade, the cascade is basically one of serine/threonine phosphorylation.

Capacitated spermatozoa show an acrosomal reaction in the head and hyperactivation in the flagellum.[1] Many protein phosphorylations have been detected in the acrosomal reaction when it is used as a capacitation event,[10,37–39] and several have been detected during hyperactivation as a capacitation event.[9,29,30] The results of these studies suggest that tyrosine phosphorylation of AKAP is also associated with sperm hyperactivation and therefore, it is speculated that tyrosine phosphorylation of AKAP is not only related to the acrosomal reaction, but also to hyperactivation.

In the present experiments, we detected four serine phosphorylations (Fig. 1) and five threonine phosphorylations (Fig. 2) associated with sperm motility activation and hyperactivation. Although we detected four serine phosphoproteins associated with sperm motility initiation and activation in a previous study,[19] the four serine phosphoproteins detected in the present experiment did not correspond to the previous ones[19] because their approximate molecular weights differed.

Serine and threonine phosphorylations of hamster sperm proteins during capacitation have been reported on the basis of detecting four serine phosphoproteins and five threonine phosphoproteins,[39] but the protein phosphorylations detected by us in the present experiments using hamster sperm flagella differed from those detected by Kula Nand and Shivaji.[39] We think the discrepancies are caused by sample preparation and electrophoresis. We used a lysate of isolated sperm flagella, whereas Kula Nand and Shivaji used a whole sperm lysate.[39] We carried out tricine SDS-PAGE according to the method of Schagger and Jagow,[22] whereas Kula Nand and Shivaji carried out SDS-PAGE according to the method of Laemmli.[40] However, it is likely that the 65-kDa protein detected in the present experiment

(Fig. 2) corresponds to the 61-kDa protein detected by Kula Nand and Shivaji,[39] according to the molecular weights and threonine phosphorylation of both proteins.

The 90-kDa phosphoprotein detected in the present experiments (Figs 1, 2) was dual-phosphorylated at the serine and threonine residues during sperm activation and maintained during hyperactivation. Therefore, it is likely that phosphorylation of the 90-kDa protein is associated with motility activation. In human spermatozoa, it has been shown that the 94-kDa protein is dual-phosphorylated at the serine and threonine residues during capacitation.[10] Therefore, the 90-kDa protein in hamster spermatozoa may correspond to the 94-kDa protein from human spermatozoa, according to the molecular weights and dual-phosphorylations of both proteins.[10]

The 10-kDa protein detected in the present experiments (Figs 1, 2) was also dual-phosphorylated at the serine and threonine residues during hyperactivation (Figs 1, 2). In our previous study,[9] the 10-kDa protein was identified as the protein phosphorylated at tyrosine residues in a cAMP-dependent manner when hamster spermatozoa were activated. By its molecular weight, the 10-kDa protein detected in the present experiments corresponds to that previous 10-kDa protein[9] (Fig. 3). Serine phosphorylation and threonine phosphorylation of the 10-kDa protein occurred during hyperactivation, whereas tyrosine phosphorylation occurred during activation and was maintained during hyperactivation.[9] Therefore, it seems that the 10-kDa protein was triple-phosphorylated at the serine, threonine and tyrosine residues in association with sperm activation and hyperactivation. Because the 10-kDa protein is phosphorylated at the tyrosine residues in a cAMP-dependent manner,[9] we first considered that it corresponded to the 15-kDa protein detected in salmonid spermatozoa by Morisawa *et al.*,[33,41] but the present study showed that the 10-kDa protein included carcinustatin-like sequences[25] (Fig. 4), rather than the tubulin-like sequences of the 15-kDa protein.[42]

In the present study, two proteins, the 70-kDa and 35-kDa proteins, were dephosphorylated at threonine residues (Fig. 2) when hamster spermatozoa were activated. Protein phosphorylation is proposed as one of the events regulating mammalian sperm motility.[2,43] In mammalian spermatozoa, protein phosphatase activity is regulated by calcium via calmodulin.[43,44] Because an activation factor of hamster spermatozoa is calcium iron,[29] it seems that the dephosphorylation of the 70-kDa and 35-kDa proteins depends on calmodulin-dependent protein phosphatase. In our previous study,[9] we detected dephosphorylation of a 20-kDa protein at the tyrosine residues when hamster spermatozoa were activated and moreover, protein tyrosine phosphatase activity has been detected in hamster spermatozoa.[45] Therefore, it is likely that three proteins are dephosphorylated at threonine or tyrosine residues when hamster spermatozoa are activated, and this is regulated by calmodulin-dependent protein phosphatase. Recently, it was demonstrated that the dynein light chain is phosphorylated in a cAMP-dependent manner during activation of salmonid fish sperm motility,[46] and dephosphorylated by a type 2A protein phosphatase.[47] Moreover, it has been also suggested that the regulatory subunit of cAMP-dependent protein kinase is phosphorylated in a cAMP-dependent manner[34] and dephosphorylated by other types of protein phosphatase.[47] Therefore, it is likely that sperm motility activation is regulated not only by protein phosphorylation, but also by protein dephosphorylation.

Although we did not always identify and characterize the proteins detected in the present experiments, they were phosphorylated and/or dephosphorylated at serine and/or threonine residues in association with hamster sperm activation and hyperactivation. Future studies should investigate the role of those proteins in motility.

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