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Delivery of ferric ion to mouse spermatozoa is mediated by lipocalin internalization

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Abstract

The aim of this study was to illustrate the further process of 24p3 protein after association with epididymal spermatozoa. We have previously identified a caput-initiated 24p3 protein, which interacts with the spermatozoa surface in vitro. In the present study, we investigate another role of the 24p3 protein with spermatozoa. Mouse epididymal spermatozoa exhibit the ability to bind spontaneously with exogenous 24p3 protein, a part of which is further internalized into the spermatozoa in epididymal caput. We have now focused on this issue using freshly prepared spermatozoa from caudal region of epididymis. First, the cytosolic fractionation of spermatozoa has revealed that biotinylated 24p3 protein signal could be detected by supplying biotinylated protein under 37 °C incubation after 30 min at this experiment. Further, flow cytometric analysis of FITC-protein containing spermatozoa has revealed two distinct types of fluorescent spermatozoa, and microscopical experimentation with fluorescent FITC-24p3 protein has shown that the 24p3 protein did accumulate in the cytosolic portion of spermatozoa. All of these events, which showed protein uptake into the cell, demonstrated time- and temperature-dependence of endocytotic characteristics, these constituting the critical points in the process of endocytosis for spermatozoa as for other cells. Using a fluorometric method, the binding affinities of ferrous ion and ferric ion to 24p3 protein were shown to be $(1.5 \pm 0.2) \times 10^6$ and $(3.0 \pm 0.4) \times 10^7\ M^{-1}$, respectively. We have also determined the internalization of this protein in the transition of iron into spermatozoa. We report here that spermatozoa, from the caudal epididymis, demonstrate the ability to bind with 24p3 protein and further internalize it and deliver the ferric ion to the spermatozoa via protein internalization. We suggest that the 24p3 protein plays a physiological role in spermatozoa in the context of protein–ligand complex internalization.

Keywords: Epididymis; Lipocalin; 24p3 Protein; Secretory protein

Epididymal sperm modification appears to be the result of a series of successive sequential events most of which occur only in different regions of the epididymis [1]. The main change that occurs in the spermatozoa located within the epididymis is the transition to the ability to move and undergo intense changes during epididymal transit in protein compositions. There are several ways in which epididymal proteins may be involved in sperm modification [1]. Several epididymal proteins can be anchored by linking to GPI, an example being a glycoprotein HE5 (CD52) inserted into a matured human spermatozon [2,3], although the exact role and function of these epididymal proteins is difficult to demonstrate. Studies of those proteins the roles of which are directly related to epididymal spermatozoa activity are not easy to conduct. Our first aim as regards epididymal investigation was the identification and characterization of the epididymal secretory proteins.

During passage through the epididymis, spermatozoa modification events are believed to be dependent upon the microenvironment created by the absorptive and secretory functions of the epididymis [1,4,5]. The principal components of this environment are specific proteins that are synthesized and secreted in certain regions (caput, corpus or cauda) of the epididymis and may play

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some role in spermatozoa viability [6–8]. These above-mentioned proteins may be important in eliciting/participating in spermatozoal changes that occur in the different regions of the epididymis during postnatal development and may also be involved in regulating the functional integrity of spermatozoa [1,7]. Although the regional-specific expression of epididymal proteins has been established for some time, the identity and function of these proteins in spermatozoa have not yet been clearly elucidated. A number of secretory proteins deriving from the epididymal epithelium, and the hormonal regulation of their synthesis, have been previously studied and reported on [9–11], although the biological function of many of these proteins remains unclear. Several proteins may contribute to be lipid transporters, such as cholesterol-binding protein (CTP), they being principally involved in hydrophobic binding activities. Lipocalins are a large and diverse group of small, extracellular, and hydrophobic molecules responsible for transporting proteins [12]. Several lipocalins have been implicated in the modulation of cell growth, metabolism, and cell regulation [12]. Indeed, for many lipocalins, their biological functions remain unclear and need to be elucidated, especially as regards the reproductive system.

We have previously characterized a secreted mouse lipocalin, named the 24p3 protein, the gene expression of which is caput-initiated and unique to the epididymis in the male reproductive tract then associates with spermatozoa [13,14]. Analysis of the fluorescence data in our previous report [15] suggests that the 24p3 protein has a binding site for a hydrophobic ligand to form a (24p3) protein–ligand complex since 24p3 protein is considered to be a lipocalin [15]. Some evidences suggest that lipocalins undergo internalization into the cell by receptor-mediated endocytosis [16] whilst others refer to a specific iron-delivery pathway also mediated by lipocalin [17]. Another plausible mechanism for lipocalin might be that the lipocalin interaction with receptor creates a direct signal inducing various physiological processes of cell activity [18]. Based upon our observations with freshly prepared mouse spermatozoa from caudal epididymis, the enhancement of spermatozoa motility by the elevation of the intracellular pH and accumulation of the cytosolic cAMP of spermatozoa upon being associated with 24p3 protein [19] might trigger a biological event. Cyclic AMP-induced desensitization of a protein receptor induces a rapid internalization of the endothelin-A receptor, i.e., the trafficking of a receptor from the extracellular to the intracellular environment coincides with an increase in cAMP level of intra-hepatic cells [20]. As a lipocalin, the protein 24p3 is reasonably suggested to be an internalized-transporter for transporting hydrophobic molecules into the cell via an elevation of the cAMP level within cytosol, which needs further approval. In our experimental approach, we tested the possibility of the 24p3 protein being responsible for the internalization into the spermatozoa, and further, whether iron might be able to be delivered to the cytosol via such protein internalization.

Materials and methods

Chemicals, biochemicals, and bio-assay kits. An Enhanced Chemiluminescence plus kit (ECL) was purchased from Amersham–Pharmacia Biotech (Buckinghamshire, UK). Anti-rabbit IgG-horseradish peroxidase conjugate (HRP-conjugated anti-rabbit IgG) prepared from goats and fluorescein isothiocyanate (FITC) were obtained from Sigma (St. Louis, MO, USA). Desferrioxamine mestylate (DFO) and diethylene triamine pentaacetic acid (DTPA) were purchased from Sigma (St. Louis, MO, USA). A permeable ester form of the calcein fluorescent probe was purchased from Molecular Probe (Eugene, Oregon, USA). The 24p3 protein antibody was prepared from rabbits as has been previously described [14]. All chemicals were of reagent grade quality.

Experimental Animals. Outbred ICR mice were purchased from Charles River Laboratories (Wilmington, MA, USA). They were bred in the animal center at the College of Medicine (National Taiwan University, Taipei, Taiwan). Animals were treated in accordance with the institutional guidelines for care and use of experimental animals. The test animals were kept under controlled lighting (14 h light/10 h dark) at a constant temperature (23 ± 2 °C) with water and NIH 31 laboratory mouse chow supplied ad libitum. The 24p3 protein was purified from the uterine fluid of DES (diethylstilbestrol)-stimulated 3-week-old female mice as previously described [13]. Adult male mice (12–16 weeks) were killed by cervical dislocation in order to provide the spermatozoa.

Preparation of spermatozoa. Modified Hepes medium (HM) [21] was used for sperm preparation. The medium consisted of NaCl (120 mM), KCl (2 mM), $MgSO_4 \cdot 7H_2O$ (1.2 mM), NaH_2PO_4 (0.36 mM), $NaHCO_3$ (25 mM), Hepes (10 mM), glucose (5.6 mM), sodium pyruvate (1.1 mM), penicillin (100 IU/ml), and streptomycin (100 μg/ml). The pH of the media was adjusted to 7.3–7.4 by aeration with humidified air/CO_2 (19:1) in an incubator at 37 °C for 24 h prior to the media's use. The caudal portion of the sperm from this epididymis was used since it is recognized that sperm from this region lacks the 24p3 protein [14]. Sperm were extruded into the medium from the distal portion of the connective tissue and incubated at 37 °C for a period of 10 min. The sperm were then filtered through nylon gauze, layered over 0.8 ml of 75% Percoll medium, and centrifuged for 30 min at 275*g* to harvest the motile sperm. The harvested spermatozoa were washed twice with HM and the motile percentage was estimated by CASA equipment. The motile spermatozoa were kept in 80–90% of total spermatozoa under CO_2 incubator at 37 °C and were ready for further use.

Protein extraction from spermatozoa. Spermatozoa from the caput epididymis (10^6 cells) were suspended in 200 μl HM containing 1× Protease Inhibitor Cocktail (PI Cocktail, Cat No. 1697498, Roche Molecular Biochemicals, Germany) at room temperature for 30 min and then centrifuged at 80*g* for a period of 10 min to remove the cell debris. The supernatant was collected as the first spermatozoa washing solution (C1). The pellet was further washed twice with HM containing PI Cocktail (200 μl/each wash). After each washing step had been completed, the supernatant was collected as C2 and C3 samples and the pellet was resuspended in hypotonic buffer in order to extract the cytosolic proteins. Prior to protein extraction, spermatozoa were washed with 5× volume hypotonic buffer [Hepes (10 mM), KCl (10 mM), and $MgCl_2$ (1.5 mM), pH 7.9]. Subsequent to washing, the spermatozoa were resuspended in 200 μl hypotonic buffer and

extracted by sonication for 10 min at 10 °C, following which the spermatozoa were centrifuged at 500*g* for 15 min, the supernatant was collected as cytosolic proteins, and the pellet was finally extracted with 200 μl of 2% SDS solution for membrane proteins. Ten microliters of each protein extract was added to an equal volume of double-strength Laemmli buffer and subjected to SDS/PAGE and Western blot analysis.

Protein labeling. FITC-labeled 24p3 protein (FITC-24p3 protein) was prepared by incubating purified 24p3 protein (5 mg/ml) with fluorescein isothiocyanate (FITC; 5 mg/ml) in sodium bicarbonate buffer (200 mM), pH 9.0, for a period of 20 h at 10 °C. Unconjugated FITC was removed by gel filtration on a PD-10 column (Amersham Biosciences, Germany).

Biotinylated 24p3 protein was prepared with D-biotinamidocarproate *N*-hydroxysuccinimide ester (Sigma). The 24p3 protein was dissolved at 1 mg/ml in sodium borate buffer (100 mM), pH 8.3, and 2.5 μl biotin reagent (20 mM as a stock solution) was added into the solution which was then mixed and incubated at room temperature for 2 h. The biotin to 24p3 protein ratio was selected to be a 1 to 1 molar ratio for the performance of this assay. Further, biotinylated 24p3 protein was separated from free biotin on a PD-10 column equilibrated in PBS (50 mM sodium phosphate–150 mM NaCl, pH 7.4). The labeled protein concentration was determined using BCA protein assay reagent (Pierce).

Western blot analysis. The dissolved proteins in the sample buffer were resolved by use of an SDS/PAGE technique [4–20% (w/v) gradient acrylamide] on a gel slab. Proteins were transferred from the gel to a nitrocellulose (NC) membrane by semi-dry electrotransfer (1 mA/cm^2) at room temperature for 2 h. The transferred proteins were detected using 24p3 protein antibody (diluted to 2 μg/15 ml), followed by HRP-conjugated anti-rabbit IgG diluted to 1:10,000 and subsequent fluorography. The biotinylated 24p3 protein in the NC membrane was detected by HRP-conjugated strepavidin diluted to 1:10,000. The reactive bands were visualized using an enhanced chemiluminescence plus (ECL) kit and exposed on X-ray film, and the biotinylated 24p3 protein was detected by use of HRP-conjugated strepavidin (1 μg/ml) in the assay solution.

Flow cytometric analysis. The freshly prepared spermatozoa (10^6 cells/ml) were incubated for 30 min in HM at 37 °C, following which, FITC-24p3 protein was added (final concentration; 12.5 μg/ml) and incubated for various times at 37 °C. To analyze the FITC-24p3 protein interacting with the spermatozoa flow cytometry was conducted. Analysis was performed on a COULTER EPICS XL flow cytometer (Beckman–Coulter, FL, USA). Fluorescence was initiated by excitation at a wavelength of 488 nm and measured via a 525 nm filter. PMT voltages and gains were set to attempt to maximize the dynamic range of the signal. The fluorescence intensity of the FITC and protein complex was quantified for 10,000 individual cells.

Detection of FITC-24p3 protein internalization by spermatozoa via fluorescence microscopy. The freshly prepared spermatozoa (10^7 cells/ml) were incubated for 30 min in HM at 37 °C prior to use. FITC-24p3 protein was added into the spermatozoa solution (final concentration, 100 μg/ml) and incubated for various times at either 10 or 37 °C. In order to remove free ligand that had not been associated with spermatozoa specifically, cells were washed with glycine buffer (5 mM; pH 3.0), fixed in 100% ice-cold acetone for 5 min, and then analyzed with a microscope equipped for epifluorescence detection (AH3-RFCA, Olympus, Tokyo, Japan).

Iron-binding assay by spectrofluorometry. The fluorescence intensity of the 24p3 protein in Tris-buffered saline (TBS), expressed in arbitrary units, was measured at room temperature using a fluorescence spectrophotometer (Hitachi F-4000, Tokyo, Japan). Both the excitation and emission slit widths were set at 10 nm. It took no more than 5 min to scan a spectrum, such alacrity thus avoiding protein denaturation. A modified Scatchard plot [22] was constructed in order to analyze the fluorescence data of a complex formed by 24p3 protein and iron, with the following algebraic linkage:

$$|\Delta F|/[\mathrm{L}]_{\mathrm{free}} = F_\infty/K_\mathrm{d} - |\Delta F|K_\mathrm{d}, \quad (1)$$

where ΔF is the change in protein fluorescence on the addition of ligand L, F_∞ is the protein fluorescence in the absence of ligand, and K_d is the dissociation constant of the complex. Throughout the titration, $|\Delta F|/[\mathrm{L}]_{\mathrm{total}}$ was plotted against $|\Delta F|$, because $[\mathrm{L}]_{\mathrm{free}}$ was close to $[\mathrm{L}]_{\mathrm{total}}$.

Detection of iron uptake by means of 24p3 protein internalization. The spermatozoa intracellular iron concentration was measured using calcein, a fluorescent metal-sensitive probe. In cell-free systems, calcein binds to iron and results in quenching its fluorescence [23,24]. After cellular uptake of calcein-acetoxymethyl ester (calcein-AM), intracellular esterase converts the molecule into an impermeable acid form of calcein [24]. It is generally accepted that the observed decrease in calcein fluorescence occurring as a consequence of binding with iron is due to iron binding if such a species is present [24]. Calcein-AM feeding of spermatozoa was performed on HM at a concentration of 5 μM in an atmosphere of 5% CO_2-in-air at 37 °C. Following incubation for a period of 30 min, spermatozoa were washed twice to remove unincorporated dye, resuspended in HM, to which 24p3 protein (4 μM) and/or $Fe(NO_3)_3$ (5 μM) was then added into the medium that was then allowed to incubate for a further 30 min. The iron chelators, cell impermeable form of DFO and cell permeable form of DTPA, were used in this current investigation. Fluorescence intensity was measured using a Twinkle LB970 spectrofluorimeter (excitation, 490 nm; emission, 535 nm). The continuous observation of ferric ion uptake by spermatozoa was detected by spectrofluorimetry (Hitach F-4000, Tokyo, Japan) for a serial of time.

Results

24p3 protein accumulation in the spermatozoa cytosol

As described previously, the epididymal caput was found to produce a large quantity of 24p3 protein in luminal fluid and was also found to be associated with caudal spermatozoa, for which the 24p3 protein had virtually vanished [14]. To test whether there was any further processing of the spermatozoa in the epididymal caput after protein association, we extracted the proteins from the different portions of the caput spermatozoa. Using Western blotting, we detected the location of 24p3 protein via the use of 24p3 protein antibody. As shown in Fig. 1, large amounts of 24p3 protein were

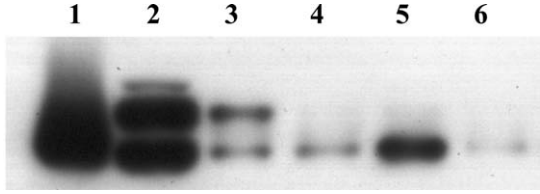

Fig. 1. 24p3 protein detected by Western blot analysis using 24p3 antibody. Proteins extracted from epididymal caput spermatozoa under different conditions as described in the text, and subjected to SDS/PAGE. Following this, the proteins were transferred to NC membrane, and the 24p3 protein was detected by 24p3 protein antibody (2 μg/15 ml) followed by HRP-conjugated anti-rabbit IgG (1:10,000 dilution) exposure, the details of which are described in the text. Lane 1, 24p3 (20 ng) protein was purified from uterine fluid as a standard protein; lane 2, C1-extracted proteins; lane 3, C2-extracted proteins; lane 4, C3-extracted proteins; lane 5, cytosolic proteins; and lane 6, membrane proteins.

detected in the first washing solution (Fig. 1, lane 2), the protein being secreted from the caput and associated with the spermatozoa surface, and were washed by HM gradually (Fig. 1, lanes 3 and 4). By the third washing, almost all of the surface 24p3 protein was washed away from the spermatozoa surface. After hypotonic buffer extraction of the washed spermatozoa, a significant amount of 24p3 protein was found in the spermatozoa cytosol portion as compared with the membrane extracted portion (Fig. 1, lanes 5 and 6). This result gave us the first indication that there was an accumulation of 24p3 protein in the cytoplasm of caput spermatozoa, this probably being associated with the protein internalization.

Temperature-dependent biotinylated 24p3 protein internalization

The process of cell endocytosis may reflect a temperature-dependent pathway, such that a reduced temperature may block or slow such transcytosis [25]. In order to investigate whether the 24p3 protein is involved in the internalization of spermatozoa, we obtained biotinylated 24p3 protein, which can be detected by HRP-strepavidin, in order to distinguish this protein from other proteins present. Spermatozoa were incubated in the presence of biotinylated 24p3 protein for a period of 30 min at 37 °C in order to allow for binding of labeled protein to internalize. Subsequent to washing and extraction of the spermatozoa with hypotonic buffer, the biotinylated 24p3 protein was detected using a Western blotting assay technique. The data shown in Fig. 2, the biotinylated 24p3 protein, were able to be found in the cytoplasmic portion of spermatozoa (lane 1). Using 10-fold unlabeled 24p3 protein as a competitor, a reduced

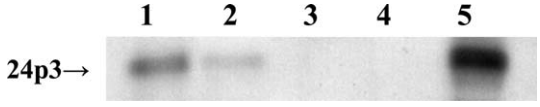

Fig. 2. Uptake of biotinylated 24p3 protein by caudal spermatozoa at different temperatures. Freshly prepared caudal spermatozoa (10^5/ml) were incubated with biotinylated 24p3 (2 μg) protein for 30 min at 10 °C or at 37 °C. Thirty micrograms of unlabeled 24p3 protein was added into the medium as a competitor for the specific interaction assay. Subsequent to the completion of the reaction, any free 24p3 protein was removed by washing twice with PBS [sodium phosphate (50 mM), NaCl (150 mM), pH 7.4], followed by extraction of the cytosolic proteins using 100 μl of hypotonic buffer. Ten microliters of each medium was subjected to SDS/PAGE (4–20% gradient acrylamide) and immunoblotting. Lane 1, biotinylated 24p3 protein extracted from 37 °C-incubated spermatozoa; lane 2, biotinylated 24p3 protein extracted from 37 °C-incubated spermatozoa in the presence of unlabeled 24p3 protein; lane 3, biotinylated 24p3 protein extracted from 4 °C-incubated spermatozoa; lane 4, biotinylated 24p3 protein extracted from 37 °C incubated spermatozoa in the presence of unlabeled 24p3 protein; and lane 5, 2 μg of biotinylated 24p3 protein used as a standard.

level of biotinylated 24p3 protein was able to be internalized into the spermatozoa within a period of 30 min incubation at 37 °C as compared to the then on-competitor situation (Fig. 2, lane 2). Such a result may suggest the specific binding of 24p3 protein to spermatozoa and that the unlabeled protein could interrupt the internalization of labeled 24p3 protein. At 10 °C incubation for 30 min, we were not able to detect any biotinylated 24p3 protein uptake by spermatozoa (Fig. 2, lanes 3 and 4), the internalization of the 24p3 protein into the spermatozoa were appearing to have been blocked at the reduced temperature.

Time-dependent FITC-24p3 protein internalization

The internalization of protein sample of spermatozoa that had been exposed to FITC-24p3 protein at 37 °C for various time periods was assessed by the use of a flow cytometer. The basal fluorescence level was obtained for the spermatozoa at zero time by incubation with FITC-24p3 protein at 37 °C, such a base level demonstrating a low level of fluorescence intensity (Fig. 3A, peak I). The mean of fluorescence intensity of total counting up control spermatozoa at zero time was 3.8 ± 1.8 (in arbitrary unit), which indicated a low level of labeled protein interacting with spermatozoa at the zero time. Ten minutes subsequent to the incubation with FITC-24p3 protein, the level of fluorescence emitted by spermatozoa was noted to increase substantially (Fig. 3B, peak II) and the mean of fluorescence intensity was 44 ± 7 (in arbitrary unit). Such a response was deemed to have been directly related to the interacting of 24p3 protein with spermatozoa. Two different fluorescence intensity peaks would appear to arise following the further incubation of FITC-24p3 protein (Figs. 3C and D). Subsequent to the incubation of spermatozoa with FITC-24p3 protein for a period of 20 min, spermatozoa were noted to be able to exhibit two emission fluorescence peaks. One of these two peaks (Fig. 3C, peak II) was similar in fluorescence intensity to that displayed in Fig. 3B (peak II) but it did demonstrate a lower level of counts than was the case illustrated in Fig. 3B (peak II), whilst the other peak (Fig. 3C, peak III) revealed a greater emission fluorescence intensity but a lower level of counts than that was the case for peak II. The mean fluorescence intensity of peak II and peak III as depicted in Fig. 3C would be extended to 65 ± 6 (in arbitrary unit), this suggested that the more FITC-24p3 protein interacted with spermatozoa comparing to Fig. 3B. Subsequent to incubation of FITC-24p3 protein with spermatozoa for a further 10 min, peak II was noted to reveal a lower level of counts than 10 min previously, and peak III the opposite, (Fig. 3D, peaks II and III) with mean of fluorescence intensity increasing to 85 ± 5 (in arbitrary unit) under FITC-24p3 protein incubation with spermatozoa for 30 min. Such a

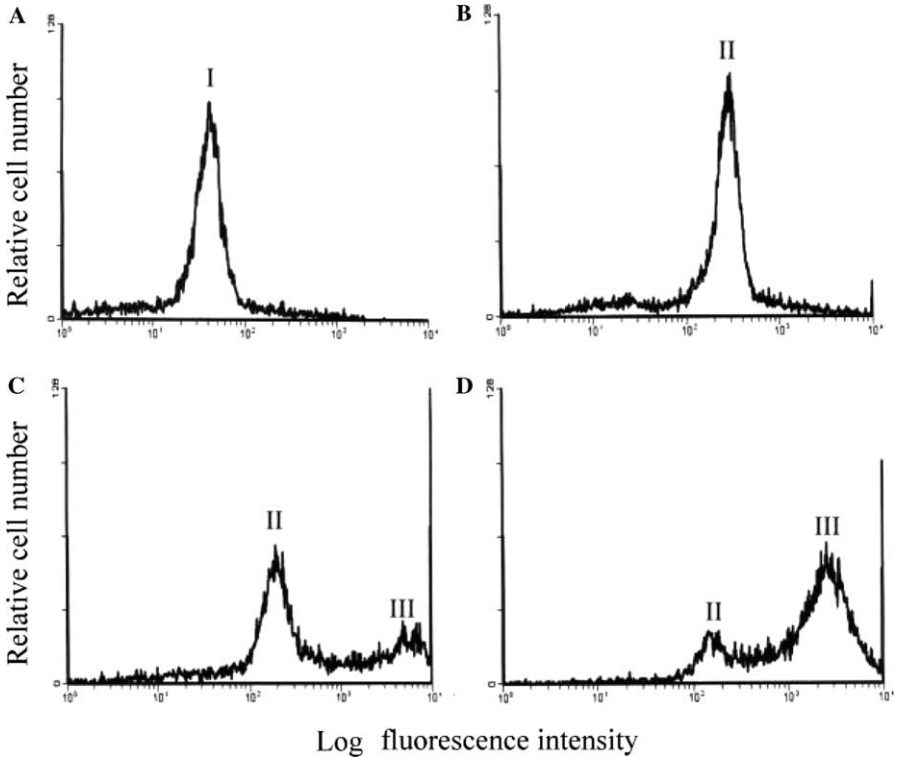

Fig. 3. Flow cytometry analysis of FITC-24p3 protein binding and internalization. Use of flow cytometry to detect the status of spermatozoa associated with FITC-labeled 24p3 protein. The details of the procedure are described in the text. (A) Spermatozoa treated with HM alone as a control; (B) spermatozoa incubated with FITC-24p3 protein for 10 min at 37 °C; (C) spermatozoa incubated with FITC-24p3 protein for 20 min at 37 °C; and (D) spermatozoa incubated with FITC-24p3 protein for 30 min at 37 °C. I, II, and III represent different fluorescent statuses of caudal spermatozoa.

result reflected that fluorescence intensity increased proportionally with increasing incubation time for spermatozoa and FITC-24p3 protein, and it would appear that two different fluorescence statuses existed at the same time for the spermatozoa under continuous incubation with FITC-24p3 protein. These data suggested that two separate different locations for the accumulation of FITC-24p3 protein would likely occur within spermatozoa. When FITC-24p3 protein incubation with spermatozoa was carried out on ice, under which conditions the appearance of a peak in emission fluorescence intensity is prevented, there is no evidence of protein internalization (data not shown).

Detection of FITC-24p3 protein internalization by fluorescence microscopy

To confirm the internalization of 24p3 protein by spermatozoa, observing the FITC-24p3 protein fluorescence response under microscopy, it was clearly apparent that spermatozoa revealed a low level of fluorescence intensity following incubation for both 15- and 30-min periods at 10 °C (Figs. 4E and F). Subsequent to incubation at 10 °C for a period of 30 min, the data demonstrated less than $10 \pm 2\%$ of spermatozoa in the population were fluorescent with 24p3 protein being bound to the spermatozoa head. For incubation of FITC-24p3 protein with spermatozoa at 37 °C for 15 min, the FITC-24p3 protein internalized into the spermatozoa was represented by $28 \pm 7\%$ of examined spermatozoa. Subsequent to the incubation of FITC-24p3 protein with spermatozoa for 30 min (Fig. 4G) the majority were bound to the spermatozoa head, a large proportion of the spermatozoa examined appeared to reveal a fluorescent head and showed strong fluorescence intensity ($85 \pm 8\%$ of total spermatozoa) (Fig. 4H). These observations under the fluorescence microscope coincided with the flow cytometric data, and suggested that the two different intensities of the fluorescent peaks presented in Fig. 3 (peak II and III) represented two different types of fluorescent spermatozoa. These experiments further confirm that 24p3 protein is involved in the internalization of 24p3 protein by the spermatozoa.

Characterization of 24p3 protein–iron binding

We speculated here that the protein internalization by spermatozoa might be correlated to some form of protein–ligand complex transition, so we attempted to locate the ligand for the 24p3 protein. According to the 2002 report of Yang et al. [17] as regards the delivery of iron to cells by lipocalin, it seemed logical that we should demonstrate the binding ability of iron to 24p3

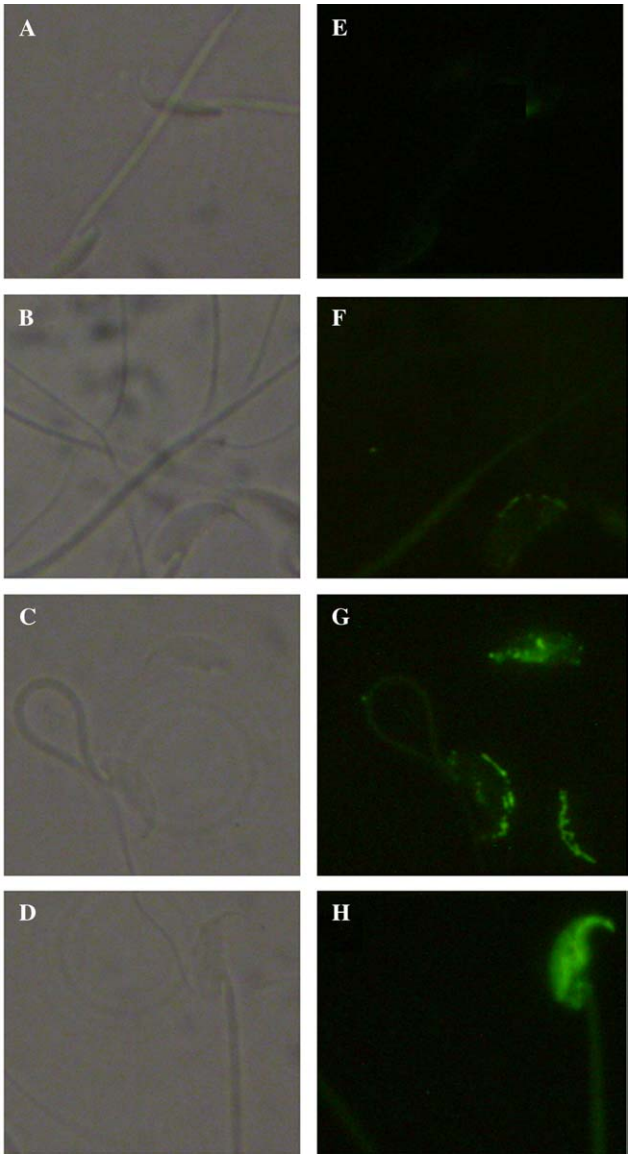

Fig. 4. Internalization of FITC-24p3 protein by spermatozoa. Plates (A–D) are bright field micrographs of spermatozoa. (E) Micrograph of spermatozoa incubated with FITC-24p3 protein for 15 min at 10 °C; (F) micrograph of spermatozoa incubated with FITC-24p3 protein for 30 min at 10 °C; (G) micrograph of spermatozoa incubated with FITC-24p3 protein for 15 min at 37 °C; and (H) micrograph of spermatozoa incubated with FITC-24p3 protein for 30 min at 37 °C. The unbound FITC-24p3 protein was removed with glycine buffer (50 mM glycine, 150 mM NaCl, pH 3.0). Control cells incubated with unconjugated FITC showed no fluorescence (data not shown). The fluorescence slides were observed under a 100× objective on an Olympus microscope (AH3-RFCA, Olympus, Tokyo, Japan).

protein. Using a fluorometric method, we analyzed the binding affinity of the ferric ion and the ferrous ion to the 24p3 protein, Fig. 5 displays the emission spectrum of 24p3 protein under different irons containing media. The excitation wavelength was set to 275 nm in order to elicit the fluorescence of the tyrosine residues in the 24p3 protein. The protein in TBS exhibits a λ_{max} peak at 335 nm (Fig. 5, solid line), suggesting that the tyrosine residues on the protein are excited by irradiation at 275 nm. The presence of $FeCl_2$(1 mM) in the protein solution would likely quench the protein fluorescence intensity that would otherwise have been produced in the absence of $FeCl_2$ (Fig. 5, dot-dashed line). In the presence of $Fe(NO_3)_3$ (1 mM) in the protein solution, the protein emission intensity would be considerably quenched, more than what would be expected to be the case for the ferrous ion quenching efficiency (Fig. 5, dotted line). It indicated iron was interacting with the 24p3 protein and acting to suppress protein fluorescence. For this reason, the result of iron–24p3 binding was probed in this study by means of assessing the ability of iron to perturb the protein-fluorescence pattern. We fitted the emission fluorescence values, obtained by titrating 24p3 protein with iron, according to the constraints described in Eq. (1) above. As shown in the inset of Fig. 5, the modified Scatchard plot of ΔF vs. $\Delta F/\mu M$ is linear, supporting the notion that there is only one iron-binding site on the 24p3 protein molecule. The apparent K_a of ferrous–24p3 protein binding and ferric–24p3 protein binding were estimated as being of the order of $(1.5 \pm 0.2) \times 10^6\ M^{-1}$ (Fig. 5, ▲) and $(3.0 \pm 0.4) \times 10^7\ M^{-1}$ (Fig. 5, ■), respectively. Such a result indicates that the 24p3 protein is more favorably disposed to binding with the ferric ion than is the case for the ferrous ion.

Determination of ferric ion transport via 24p3 protein

To determine whether iron would be carried into the spermatozoa by way of the 24p3 protein, we measured the intracellular calcein fluorescence status by using a quenching assay. Spermatozoa were incubated in various media for periods of 30 min under 5% CO_2-in-air, following which the fluorescence intensity was measured, the data shown in Fig. 6A. Exposure of the spermatozoa to $Fe(NO_3)_3$ (5 μM) alone had no significant impact upon the uptake of ferric ion by spermatozoa, although an increase in the uptake of ferric ion by the spermatozoa was noted subsequent to the addition of 24p3 protein (4 μM) into the solution. Spermatozoa that were exposed to ferric ions and DFO (1 mM), a membrane-permeable iron chelator, were able to recover the level of calcein fluorescence that they displayed in the absence of exposure to ferric ions. Based upon such an observation, we conclude that DFO chelates the ferric ion present in solution, which in the absence of DFO would be delivered by the 24p3 protein into the cytosol of spermatozoa. The calcein fluorescence quenching elicited by the ferric ion does not appear to arise in spermatozoa maintained at a temperature under 10 °C and incubated for a period of 30 min (data not shown). The fluorescence-based method has enabled the

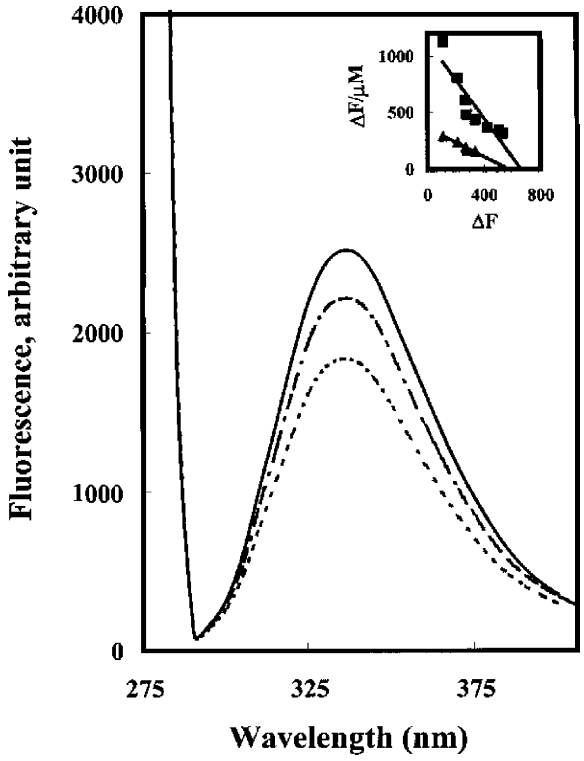

Fig. 5. Binding assay of iron to 24p3 protein. The emission spectra indicated here were scanned with an excitation wavelength of 275 nm. The protein concentration was 10 μM in TBS, at a pH of 7.0. Solid line, protein alone; dot-dashed line, $FeCl_2$ (1 mM) in protein solution; and broken line, $Fe(NO_3)_3$ (1 mM) in protein solution. The inset shows the modified Scatchard plot of the fit of the fluorescence data from the titration of ferrous and ferric ions to the protein solution according to the conditions imposed by Eq. (1). (▲) Indicates ferrous-ion titration; (■) indicates ferric-ion titration.

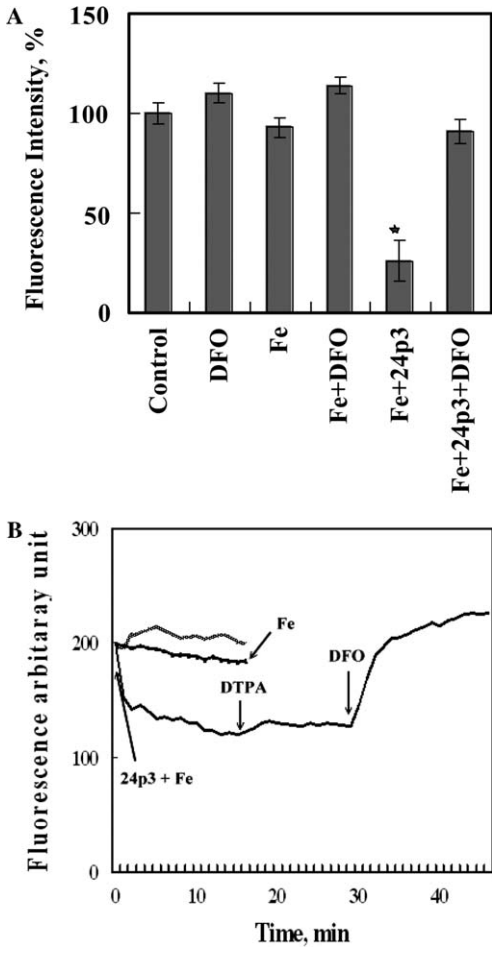

Fig. 6. Histogram illustrating ferric ion uptake by spermatozoa. (A) Using calcein as a fluorescent probe to measure the intracellular ferric ion concentration after the treatment of spermatozoa with ferric ion (5 μM) or/and 24p3 protein (4 μM). One millimolar DFO was used as an iron chelator to recover the calcein fluorescence. The detail was described in the text. The data are expressed as means ± SD, $n = 4$. *$p < 0.005$. (B) The fluorescence intensity for the treatment of spermatozoa would change is relative to reference. A stable line is established in control spermatozoa and the other slightly decreased line is ferric ion treatment of spermatozoa as reference. When supplemented with 24p3 protein (4 μM) and ferric ion (5 μM), the fluorescence associated with spermatozoa decrease, 200 μM DTPA added into the solution after 15 min later to chelate the extracellular ferric ion. All ferric ion can be chelated by addition of the DFO in further 15 min.

online monitoring of changes in the ferric ion in living spermatozoa. Fig. 6B showed the detection of ferric ion influx in spermatozoa exposed to 24p3 protein and ferric ion. A stable line is established in control spermatozoa (Fig. 6B, upper line) and fluorescence signal decreased slightly by addition of ferric ion into spermatozoa suspension (Fig. 6B, middle line). DTPA is an impermeable iron chelator that stops ferric ion import. The fluorescence signal can be decreased significantly by supplying 24p3 protein and ferric ion, which can be stopped by addition of DTPA and restored by addition of DFO. The data indicated that the ferric ion could be taken up by mediating 24p3 protein. According to the data, we suggest that the 24p3 protein would be a ferric ion transporter for spermatozoa, the 24p3 protein seemingly carrying the ferric ion into the cell via endocytosis.

Discussion

A prerequisite for understanding the physiological function of lipocalins is some knowledge pertaining to the mode of delivery of the bound ligands into the cells. Evidence suggests that lipocalin binds to certain cells and internalizes the protein into these cells [16], whereas others suggest that the lipocalin present creates a direct signal that induces various physiological processes to commence [26]. The interaction of 24p3 protein with murine spermatozoa resulting in an increase in the spermatozoa's motility and a suppression of the spermatozoa acrosome reaction has been previously characterized [18]. To comprehend the complicated processes we need to clarify some details regarding the process of the binding of the 24p3 protein to spermatozoa. Considering that 24p3 protein binds hydrophobic molecules [15], the cellular

internalization of the whole protein–ligand complex would appear to be in high possibility, but none has demonstrated the translocation of a lipocalin across the spermatozoa membrane. Previous studies have revealed an increased cAMP accumulation in spermatozoa in association with the presence of 24p3 protein in solution in vitro [18]. Such an association possibly demonstrates a connection with physiological change which occurs in spermatozoa. It may be that cAMP-mediated vesicle exocytosis/endocytosis constitutes a mechanism for the regulation of normal cell function [27]. In the present study, the appearance of 24p3 protein in the caput spermatozoa indicates that protein internalization by spermatozoa does occur in vivo, and further, the data have also revealed the uptake of 24p3 protein by caudal spermatozoa in vitro, thus it may be that such processes are triggered by intracellular cAMP accumulation contributing to an increase in spermatozoa motility [18]. The cAMP accumulation in spermatozoa was suggested to trigger an appropriate response for physiological change within spermatozoa, thus conducting an investigation of the 24p3 protein internalization by spermatozoa became our first priority in future study. Dunn et al. [28] were the first to report, in 1980, that reduced temperature prevents the delivery of endocytosis-transferred materials into various cells, such low-temperature blocking of endocytosis has also been documented for many cell types. Here, we have demonstrated that at reduced temperature, namely below 10 °C, this transport process is blocked. Microscopical experimentation with FITC-24p3 protein has supported this result in Fig. 4. A summary of our findings would suggest that the 24p3 protein can be internalized into the spermatozoa.

Iron is an essential element to cells in suitable concentration [29]. In plasma, this is mediated mostly by transferrin, but some reports suggest that there is an alternate pathway for entry of iron in cells [30,31]. In our laboratory, we have measured the ferric ion concentration in mouse epididymal fluid (7–12 μM) and less lactoferrin (also for iron transition) in caput epididymis (data not shown here). The ferric ion might be transited via 24p3 protein in an alternate pathway. The use of calcein as a fluorescent probe for cellular iron has been shown by Breuer et al. [24] to reflect the status of iron in mammalian cells. In 1999, Thomas et al. [32] concluded that calcein is a good chemosensor for ferric ion within a cell, but not so for the ferrous ion. The molar affinity of calcein for ferric ions would appear to be considerably stronger than it is for the ferrous ion in buffered-saline at a neutral pH [32]. Using the calcein fluorescent probe, we could detect ferric ion to be more effective than the ferrous ion. Subsequent to the ferric–24p3 protein complex internalization into the spermatozoa, it would appear likely that ferric ions would be released from the protein–ferric ion complex, albeit by an unknown mechanism. This release of ferric ion facilitates the binding of the ions with the calcein present intracellularly, this resulting in a quenching of the fluorescence intensity normally arising in the absence of ferric ion in solution. Such an investigation as conducted herein should shed some light upon the transportation of ferric ion into spermatozoa by the 24p3 protein; the results presented appearing to coincide with the data reported in 2002 by Yang et al. [17], namely that the 24p3 protein is an iron-trafficking protein in ureteric bud cell lines. This alternate pathway may serve as an important way for entry of ferric ion into spermatozoa in caput epididymis. One key step in the process of internalization of a protein–ligand complex is the association between such a complex and the appropriate receptor located on the cell surface. Based upon this concept and our previous and current results, our ongoing projects include the further clarification of the 24p3 protein receptor's nature and function, and an attempt to further illustrate the internalization mechanism of the 24p3–ferric ion complex, and the physiological function of ferric ion within spermatozoa.

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