Rhophilin, a small GTPase Rho-binding protein, is abundantly expressed in the mouse testis and localized in the principal piece of the sperm tail

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Abstract Tissue distribution and cellular localization of rhophilin, a 71 kDa Rho-binding protein, were examined in mice. Rhophilin mRNA was highly expressed in adult testis, but was absent in the testis of WlW^V mice deficient in germ cells. An anti-rhophilin antibody detected a band of an expected size in sperm extracts, which was enriched in the tail fraction. Immunofluorescence analysis revealed two lines of striated staining running in parallel in the principal piece of the sperm tail. These results suggest that rhophilin is expressed in germ cells and localized in the fibrous sheath of the sperm tail.
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Key words: Rho; Rhophilin; Testis; Sperm; Sperm tail; Fibrous sheath

1. Introduction

The small GTPase Rho shuttles between the inactive GDP-bound form and the active GTP-bound form and works as a molecular switch in various cellular processes. The Rho actions are examined by inactivating endogenous Rho using botulinum C3 exoenzyme which specifically ADP ribosylates Rho or by expressing dominant active Rho mutants in cultured cells [1,2]. These analyses have revealed that Rho regulates cell to substrate adhesion, motility and cytokinesis through the reorganization of the actin cytoskeleton. Rho also regulates serum response factor-mediated transcription and the G1-S progression in the cell cycle. Rho elicits these actions presumably by acting on multiple downstream effector molecules.

Recently, several putative downstream targets for Rho that show selective binding to the GTP-bound form of Rho have been isolated. These include two splice isoforms of citron: citron-N [3] and citron-K [4], the ROCK family of serine/threonine kinases: p160ROCK (ROCK-I) [5] and ROK/Rho-kinase/ROCK-II [6–8], p140mDia [9] and three molecules containing a homologous Rho-binding motif of 70 amino acid stretch, protein serine/threonine kinase PKN [10], rhophilin [10] and rhotekin [11]. Among them, the ROCK family of kinases and p140mDia mediate the Rho-induced actomyosin-based contractility and actin polymerization, re-

spectively [9,12,13], and citron-K is involved in the contractile process of cytokinesis [4]. However, the cellular functions of the other molecules including PKN, rhotekin and rhophilin, remain unknown. Rhophilin was isolated by the yeast two-hybrid system using Val[14]-Rho, an active mutant, as a bait [10]. Because rhophilin, unlike PKN, has no catalytic domain, it presumably acts as an adaptor protein linking Rho to other protein(s). To elucidate the function of rhophilin, we have examined the tissue distribution and cellular localization of this molecule. Our study shows that rhophilin is highly expressed in germ cells of the mouse testis and localized in the principal piece of the sperm tail.

2. Materials and methods

2.1. Animals and sperm preparation and fractionation
ICR strain mice were purchased from SLC (Hamamatsu, Japan). They were mated, and testes were isolated from neonates at postnatal days 6, 9, 12, 15, 18, 24, 30, 35 or 40. They were immediately frozen on dry ice and stored at −80°C until use for RNA isolation. Sperm was collected as follows. Adult mice were anesthetized by ether inhalation. Caudae epididymides were isolated and minced with scissors in PBS(−) containing 5 mM EDTA, 5 mM EGTA, 1 mM dithiothreitol, 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml pepstatin A and 1 μg/ml leupeptin (PBS-PI). Debris was removed by filtration through 80 μm pore size nylon membranes and the passed fraction was centrifuged at $1000 \times g$ for 10 min at 4°C. The pellets were resuspended in PBS-PI, and used for immunochemistry and immunofluorescence studies. Microscopically, cells in this suspension consisted mostly of spermatozoa. The sperm head and tail fractions were prepared from this suspension by the use of sonication and Percoll gradient separation as described previously [14]. The tail was further subjected to solubilization with urea. The tail fraction isolated above was resuspended in 10 mM Tris-Cl, pH 8.0, containing 150 mM KCl, 5 mM MgCl$_2$, 1 mM EDTA, 1 mM ATP, 1 mM dithiothreitol and 6 M urea, and incubated for 2 h at room temperature. The sample was then centrifuged at $10\,000 \times g$ for 10 min and the supernatant and pellet were collected separately. The pellet was treated with 10% TCA containing 150 mM NaCl before solubilization in Laemmli's sample buffer [15].

2.2. Northern blot analysis
Poly(A)$^+$ RNAs were prepared from mouse testes using QuickPrep mRNA purification kit (Pharmacia Biotech, Sweden) according to the manufacturer's protocol. The RNAs were separated by electrophoresis in a 1.0% agarose gel containing 0.66 M formaldehyde, and transferred to nylon filters (Biodyne A, Pall Biosupport, New York) in $20 \times$ SSC. ^{32}P-labelled probes were made by the random priming method with the full length mouse rhophilin cDNA as a template. Probes for mouse citron and mouse PKN were made with the respective cDNA without kinase domains and that for mouse RhoA was made with the full-length cDNA as templates. Hybridization was performed in $5 \times$SSPE containing $10 \times$Denhardt's, 2% SDS, 100 μg/ml denatured salmon sperm DNA and 50% formamide at 42°C for 16 h. Filters were washed in $0.1 \times$SSC containing 0.1% SDS at 50°C,

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Abbreviations: FITC, fluorescein isothiocyanate; TCA, trichloroacetic acid

and subjected to autoradiography. The filter used for tissue distribution and the filter of testis RNA obtained from W/W^V mutant mice were as described [9,16].

2.3. Antibodies

A polyclonal anti-rhophilin antibody was prepared as follows. Rhophilin cDNA was excised with BamHI and EcoRI from pBluescript SK(+)-rhophilin [10] and ligated into pQE-9 (Qiagen, Germany) and pGEX-2T (Pharmacia, Sweden) vectors. His_6-tagged rhophilin was expressed in the Escherichia coli JM-109 strain and purified with Ni-NTA resin (QIAexpressionist, Qiagen) according to the manufacturer's instructions. GST-rhophilin fusion was obtained by expression in the E. coli DH-5α strain. Purified His_6-rhophilin was emulsified with Freund complete adjuvant, and injected into guinea pigs at a dose of 100 μg protein/guinea pig, followed by three booster injections of the same amount of the protein in incomplete adjuvant. Antisera were collected and the polyclonal antibody was affinity-purified using GST-rhophilin fusion immobilized on nitrocellulose membranes as described previously [17].

2.4. Western blot analysis

The sperm suspension was directly mixed with 0.1 volume of 100% TCA, and chilled on ice for 30 min. After sonication with 3×3 s bursts (Handysonic UR20P, Tomy Seiko, Japan), the homogenate was centrifuged at $10\,000 \times g$ for 10 min. The precipitates were boiled in Laemmli buffer for 10 min. The solubilized proteins were subjected to SDS-PAGE and separated proteins were transferred onto nitrocellulose membranes. The membranes were blocked in Tris-buffered saline (TBS) containing 5% skim milk (Difco, MI, USA) for 2 h and were incubated at room temperature with an anti-rhophilin antiserum (1:500) in TBS plus 3% skim milk for 2 h. After three washes in TBS containing 0.05% Tween-20 (TBS-T) each for 5 min, the membranes were incubated at room temperature with a horseradish peroxidase-conjugated anti-guinea pig IgG antibody (1:5000) (DAKO, Denmark) in TBS-T with 3% skim milk for 1 h. After the membranes were

washed again in TBS-T, bound antibodies were visualized using an ECL Western blotting analysis system (Amersham, UK).

2.5. Immunofluorescence microscopy

Mouse spermatozoa isolated as described above were allowed to attach onto a silanized glass slide for 10 h at 4°C. The cells were fixed in PBS containing 4% paraformaldehyde for 20 min, and then permeabilized in PBS containing 0.2% Triton X-100 for 10 min. After blocking in PBS plus 5% skim milk for 1 h, they were incubated with either an anti-rhophilin antiserum (1:300) or an affinity-purified anti-rhophilin antibody (100 ng/ml) in PBS containing 3% skim milk for 2 h. They were then washed in PBS containing 0.05% Tween-20 (PBS-T), and incubated with FITC-conjugated rabbit anti-guinea pig IgG (DAKO) in PBS-T with 3% skim milk for 1 h. After four washes in PBS-T each for 5 min, samples were enclosed with Citifluor antifader (UKC, UK), and examined under a Zeiss Axiophot epifluorescence microscope.

3. Results

3.1. Rhophilin is developmentally induced and highly expressed in the testis

The distribution of rhophilin mRNA was examined in various adult mouse tissues by Northern blot analysis (Fig. 1A). A major mRNA species was detected at 3.2 kb and was most highly expressed in the testis, followed by the kidney, brain and stomach. In addition to various types of somatic cells including tubular epithelia, Sertoli cells and Leydig cells, adult testis contains various stages of germ cells which differentiate during development. In the embryonic testis, there are only primordial germ cells and gonocytes. During the first few weeks after birth, the gonocytes differentiate successively
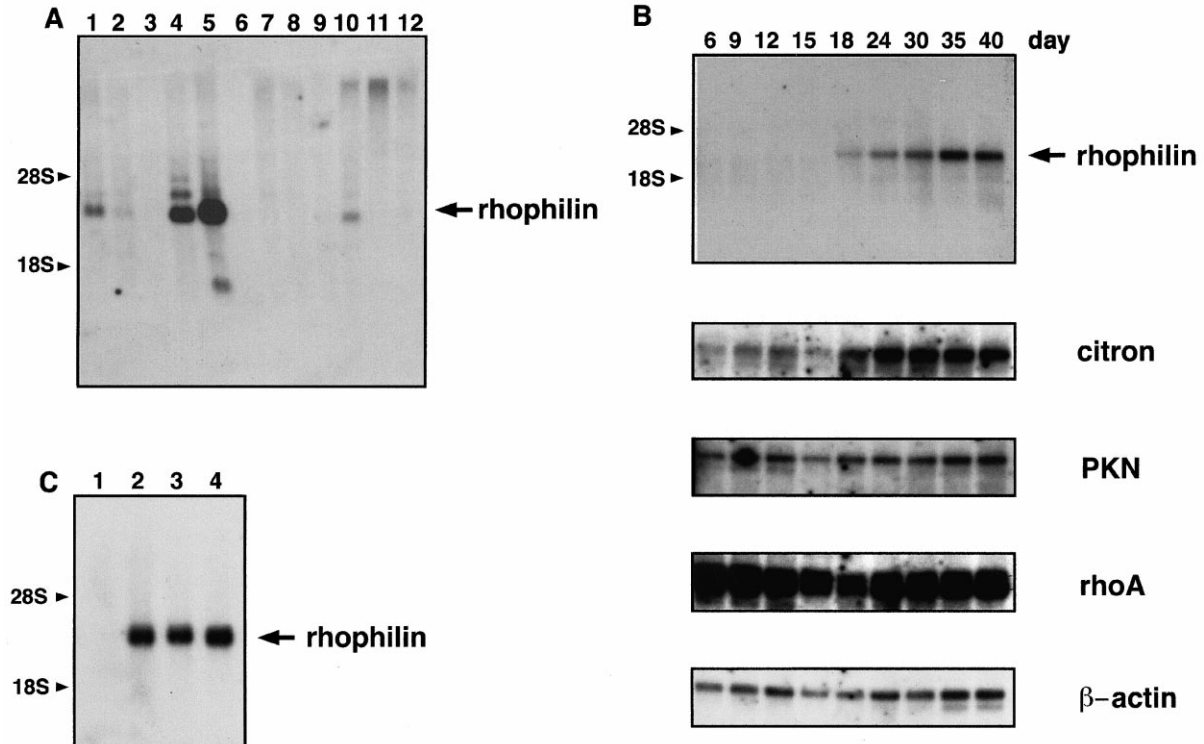

Fig. 1. Northern blot analyses of rhophilin expression. A: Tissue distribution. Six μg of poly(A)$^+$ RNA prepared from various mouse organs were used. 1, brain; 2, lung; 3, heart; 4, kidney; 5, testis; 6, skeletal muscle; 7, thymus; 8, spleen; 9, liver; 10, stomach; 11, small intestine; 12, colon. B: Expression of rhophilin, citron, PKN and rhoA mRNAs during mouse testis development. One μg of poly(A)$^+$ RNA from testes of mice of indicated postnatal days were used in each lane. C: Expression of rhophilin mRNA in germ cells. Each lane contained 4 μg of poly(A)$^+$ RNA extracted from testes of W/W^V mutant mice (lane 1), from testes of wild-type mice (lane 2), from the enriched fraction of pachytene spermatocytes (lane 3) and from the enriched fraction of spermatids (lane 4). Arrows indicate the 3.2 kb rhophilin transcript.
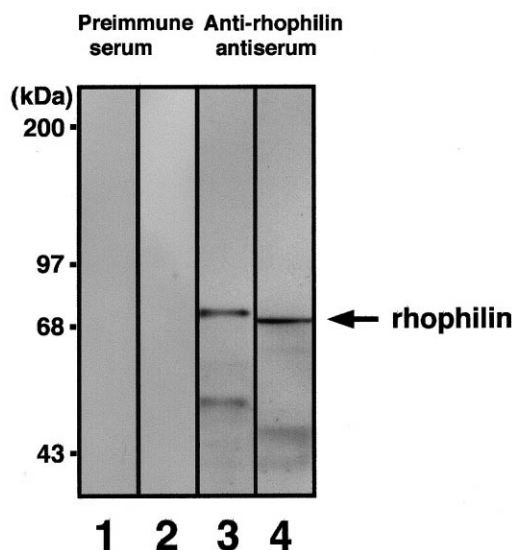
Fig. 2. Specificity of guinea pig anti-rhophilin antiserum. Recombinant His6-rhophilin (10 ng protein) and mouse sperm lysates (30 μg protein) were loaded on lanes 1 and 3, and on lanes 2 and 4, respectively, and probed with a pre-immune guinea pig serum (1:500) (lanes 1 and 2), or with an anti-rhophilin antiserum (1:500) (lanes 3 and 4).

into spermatogonia, spermatocytes, spermatids and spermatozoa, and the testis contains the full stages of germ cells 30 days postnatum [18,19]. To examine if rhophilin mRNA is expressed in germ cells and if so, to identify the stage of

germ cells expressing rhophilin, we isolated testes from mice at various postnatal days, and determined rhophilin mRNA expression. As shown in Fig. 1B, rhophilin mRNA was not detected in the testis of mice of postnatal day 15. It first appeared in the testis at day 18 and increased in amount from then onwards. Because the late pachytene spermatocytes undergo the first meiosis and the spermiogenesis starts around day 18 postnatum, these results indicate that rhophilin is not expressed in somatic cells but in germ cells undergoing and after meiosis. Using the same filter, we also analyzed the expression of other putative Rho effectors during testis development (Fig. 1B). Citron mRNA showed an increased expression from day 18 on, as was found for rhophilin, whereas the expression of PKN and rhoA was relatively constant throughout testis development.

To confirm the above results on the rhophilin expression, we used W/W^V mutant mice, the testis of which lacks germ cells but contains normal populations of somatic cells [20]. As shown in Fig. 1C, no rhophilin mRNA signal was detected in the testis of W/W^V mice. On the other hand, a parallel experiment showed that rhophilin mRNA was present in pachytene spermatocytes and spermatids prepared from wild type mice.

3.2. Rhophilin is localized in the principal piece of the sperm tail

Using recombinant His6-rhophilin expressed in *E. coli*, we raised an anti-rhophilin antiserum in guinea pigs. This antiserum detected not only the recombinant rhophilin expressed in *E. coli* but also reacted with a single protein of an expected size, 71 kDa, in mouse sperm lysates (Fig. 2, lanes 3 and 4),
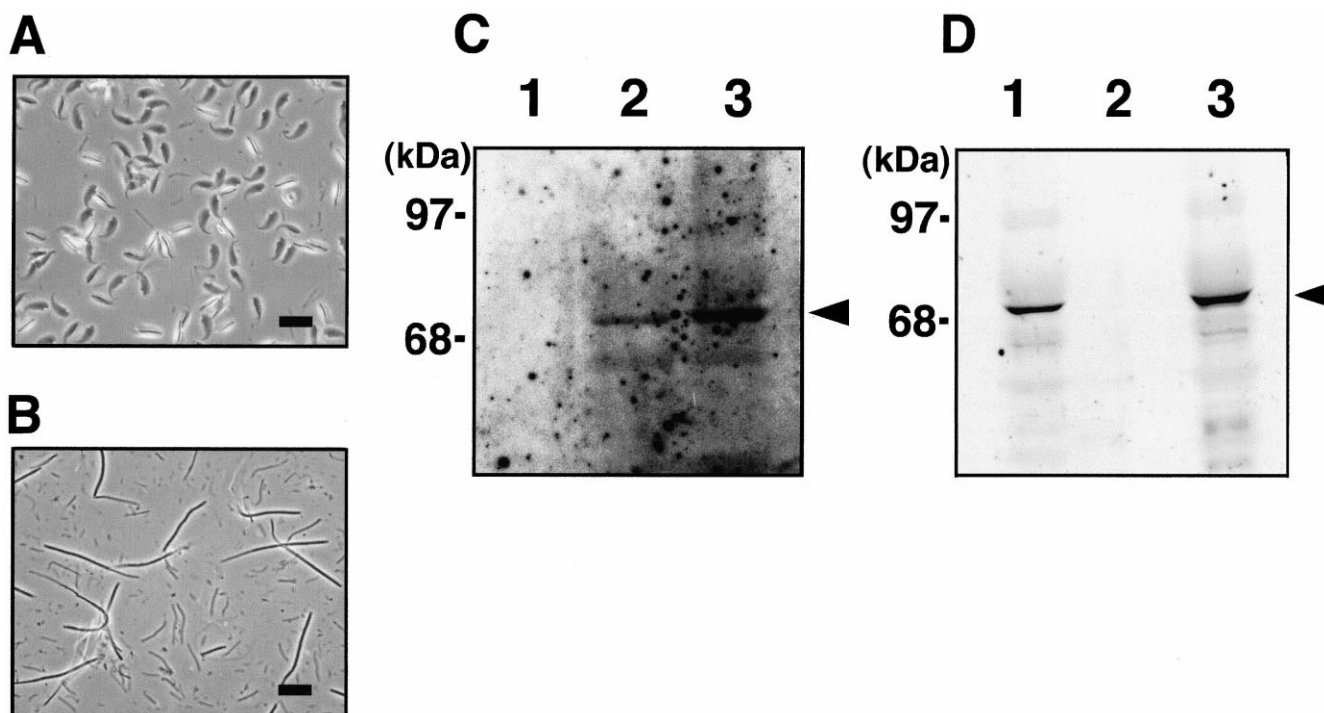

Fig. 3. Presence of rhophilin in the sperm tail. Spermatozoa were disrupted by sonication and fractions enriched in sperm heads (A) and sperm tails (B) were obtained. Bars in A and B: 10 μm. C: Western blot analysis with anti-rhophilin antiserum for proteins from fractionated sperm heads and tails. Proteins extracted from 2×10^5 sperm heads and sperm tails were loaded on lanes 1 and 2, respectively. Proteins extracted from an equivalent number of intact sperm were applied on lane 3. Anti-rhophilin antiserum was used at 1:500. D: Presence of the rhophilin immunoreactivity in the 6 M urea residues of the sperm tail. The tail fraction was subjected to the urea extraction as described in Section 2, and the urea extracts and residues were used for Western blot analysis with an anti-rhophilin antiserum. Lane 1, the tail fraction used for extraction; 2, the 6 M urea extracts; 3, the 6 M urea residues.
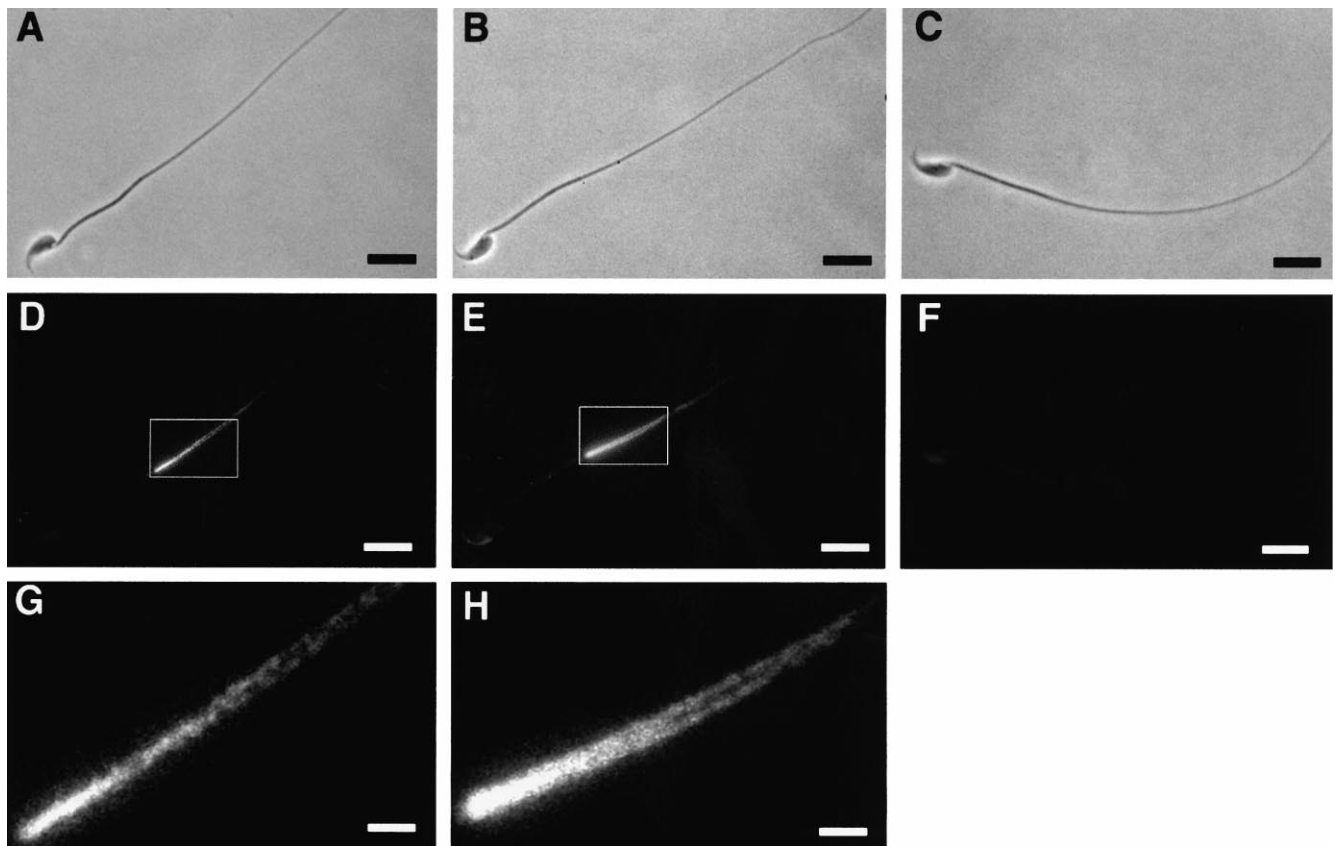
Fig. 4. Localization of rhophilin in the principal piece of the sperm tail. Sperms fixed and permeabilized on glass slides were incubated with a guinea pig anti-rhophilin antiserum (1:300) (A, D and G), with an affinity-purified anti-rhophilin antibody (100 ng/μl) (B, E and H) or with a pre-immune serum (1:300) (C and F), and stained with FITC-conjugated anti-guinea pig IgG antibody (D, E and F). Upper panels (A, B and C) show the corresponding phase contrast images. Insets in D and E are magnified in G and H, respectively. Bars: 10 μm in A to F, 2 μm in G and H.

while neither sample showed a positive signal with pre-immune serum (lanes 1 and 2). The immunoreactivity for the 71 kDa protein was abolished by a prior incubation of the antiserum with an excess amount of the recombinant protein (data not shown). These results strongly indicate that the antiserum detected endogenous rhophilin in sperm. Interestingly, this band was hardly visible when the sperm was extracted only with the Laemmli buffer and required TCA denaturation for reliable detection, suggesting that rhophilin may be associated very tightly with detergent-resistant structure(s) or may be highly sensitive to proteolysis. Using this antiserum, we examined the subcellular distribution of rhophilin in mouse sperm. Spermatozoa were disrupted and separated into the head and tail, and fractions enriched in either component were used for immunoblot analysis (Fig. 3A,B). The 71 kDa band was detected in the tail fraction but not in the head fraction (Fig. 3C), suggesting that rhophilin is present selectively in the sperm tail.

Finally we examined the localization of rhophilin in mouse sperm by the immunofluorescence method. The anti-rhophilin antiserum specifically stained the sperm tail (Fig. 4A,D). A strong tapering staining was seen in the principal piece. No specific staining was seen in either the middle piece or the end piece of the tail. An identical pattern was obtained with an affinity-purified anti-rhophilin antibody (Fig. 4B,E), but not with a pre-immune serum (Fig. 4C,F). At a higher magnification, the staining could be resolved into two parallel lines of striated structures running along the head to tail axis to the end of the principal piece (Fig. 4G,H). As discussed below, this staining pattern is reminiscent of the fibrous sheath, a structure restricted to the principal piece of the sperm tail. Since the fibrous sheath is resistant to solubilization with 6 M urea [21], we examined if the rhophilin immunoreactivity was extracted from the tail by this procedure. As shown in Fig. 3D, the immunoreactivity was not recovered in the 6 M urea extracts, but remained associated with the 6 M urea residues (lanes 2 and 3).

4. Discussion

In this paper we have shown that rhophilin, a binding partner of the small GTPase Rho, is highly expressed in germ cells in the mouse testis and is localized in the principal piece of the spermatozoa. The tail of mammalian spermatozoon is morphologically divided into three parts, the middle, principal and end pieces, with distinct structures covering the axoneme. The axoneme in the middle and principal pieces is covered by outer dense fibers, which is further coated by a mitochondrial sheath in the middle piece and by the fibrous sheath in the principal piece. The fibrous sheath is a structure unique to the sperm tail without any counterpart in somatic cells. It consists of a tapering cylinder formed by two longitudinal columns connected by numerous semicircular ribs [22]. The limited localization of rhophilin to the principal piece and its taper-

ing, striated staining pattern strongly indicate that rhophilin is associated with the fibrous sheath. The poor solubility of rhophilin in 6 M urea solution is also consistent with the reported property of the fibrous sheath [21]. As rhophilin appears to be a minor component of this structure compared to its major components such as A-kinase anchoring protein-82 [23], we presume that rhophilin may act as a regulator of its assembly and/or function. The fibrous sheath has been suggested to play a role in regulating sperm motility by placing a constraint to the plane of bending of the sperm tail [24,25].

Rhophilin presumably acts downstream of Rho. A previous study using botulinum C3 exoenzyme identified Rho in the tail membrane of bovine sperm [26]. This study further reported that inactivation of endogenous Rho in bovine sperm decreased its motility. In a variety of cells other than sperm, Rho elicits its actions through the reorganization of the actin cytoskeleton. However, there is little F-actin stained with phallacidin in the sperm of various mammalian species [27] and the sperm motility is driven by the dynamics of the axoneme, thus questioning an active role of Rho in the sperm motility. Recently, however, the involvement of Rho in the stabilization of the microtubules was shown in migrating NIH 3T3 fibroblasts [28]. The microtubules stabilized by the action of Rho then become detyrosinated. It is interesting to note that detyrosinated tubulins are enriched in the microtubules of the sperm tail axoneme [29]. Rhophilin contains a consensus sequence for a PDZ domain in its C-terminus (residues 500–573). PDZ domains have been identified in many signalling molecules, and appear to mediate protein to protein interactions [30]. It is, therefore, likely that rhophilin may work as an adaptor protein linking the GTP-bound form of Rho to other protein(s) through its PDZ domain.

Here we report that rhophilin is localized in the principal piece of mouse sperm, and suggest that rhophilin may have a role in the sperm motility. Dissection of the Rho-rhophilin pathway may help to reveal transduction mechanisms of sperm motility and may give an insight into the pathology of some cases of asthenozoospermia, a clinical syndrome characterized by defects in sperm motility.

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