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Marlin-1 Is Expressed in Testis and Associates to the Cytoskeleton and $GABA_B$ Receptors

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Abstract Marlin-1 is a $GABA_B$ receptor and Jak tyrosine kinase-binding protein that also associates with RNA and microtubules. In humans and rodents, expression of Marlin-1 is predominantly restricted to the brain, but expression in lymphoid cells has also been reported. Here, we have studied the distribution of Marlin-1 in testis and spermatozoa. Our results indicate that Marlin-1 is highly expressed in testis. The protein is abundant in spermatogonia, spermatocytes, spermatozoa, and Sertoli cells. We also have studied the subcellular distribution in spermatozoa. Marlin-1 is present in the tail and to a lesser degree in the head of the sperm cell. Finally, we have explored two protein interactions. Our findings demonstrate that Marlin-1 associates with a microtubule fraction and with $GABA_B$ receptors in testis suggesting that the set of protein interactions of Marlin-1 are conserved in different tissues. J. Cell. Biochem. 103: 886–895, 2008.

Key words: testis; spermatozoa; Marlin-1; microtubules; $GABA_B$

Protein–protein interactions are responsible for the formation and maintenance of the signaling platforms that incorporate neurotransmitter receptors into local transduction units [Alberts, 1998]. In addition, protein–protein interactions participate in the trafficking of neurotransmitter receptors [Collingridge et al., 2004]. Marlin-1 was recently identified in a yeast two-hybrid screen with $GABA_B$ receptors [Couve et al., 2004a]. It belongs to a new family of three structurally related proteins which are highly conserved in vertebrates. Marlin-1 contains a putative nuclear localization signal, but it is predominantly localized to the cytoplasm in neurons of the central nervous system (CNS). It contains multiple coiled-coil domains (CCDs) arranged along the entire length of the protein with two leucine zippers in the central and carboxyl terminal regions. Marlin-1 also binds nucleic acids, in particular single stranded RNA [Couve et al., 2004a]. Furthermore, studies in lymphoid cell lines indicate that Marlin-1 associates with Jaks, a family of non-receptor tyrosine kinases, and microtubules [Steindler et al., 2004]. In this respect, it is interesting to note that the different CCDs of Marlin-1 display binding specificity to different partners. This partner selection based on repeated modules is reminiscent of adaptor proteins [Flynn, 2001].

In cortical and hippocampal neurons, the absence of Marlin-1 results in the accumulation of the $GABA_BR2$ subunit of the $GABA_B$ receptor heterodimer [Couve et al., 2004a]. Other studies in lymphoid cell lines have shown that Marlin-1 may be involved in microtubule

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Abbreviations used: CNS, central nervous system; CCD, coiled-coil domains.

René L. Vidal and Alfredo Ramírez contributed equally to this study.

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stability [Steindler et al., 2004]. These observations suggest that Marlin-1 may associate with cytoskeletal elements and participate in the transport of cargo vesicles or cargo particles and affect the abundance of target proteins.

The tissue distribution of Marlin-1 is not ubiquitous. Northern blot analyses from human, mouse, and rat tissues have shown that the transcript for Marlin-1 occurs primarily in the brain [Couve et al., 2004a; Steindler et al., 2004]. In addition, RNA species have been observed in mouse and human testis [Couve et al., 2004a; Steindler et al., 2004]. Importantly, the expression of Marlin-1 in testis and the profile of its cellular distribution have not been studied in detail. In this report, we provide the initial characterization of the expression of Marlin-1 in testis and germ cells. We also explore the association to microtubules and $GABA_B$ receptors.

METHODS

Animals

Male BALB/c mice or Sprague-Dawley rats were purchased from the Central Animal Facility at the Faculty of Medicine at Universidad de Chile and killed by asphyxia in a CO_2 chamber according to the Guide for Care and Use of Laboratory Animals (copyright 1996, National Academy of Science). We refer to 60-day-old rats as adults.

Reagents

Paclitaxel (Taxol^{TM}), Nocodazole, propidium iodide, and Histochoice were purchased from Sigma (St. Louis, MO). Sytox Green was purchased from Molecular Probes (Invitrogen, Carlsbad, CA).

Plasmids

The construct containing FLAG-Marlin-1 in the pRK5 mammalian expression vector has been described previously [Couve et al., 2004a].

Antibodies

Marlin-1 antibodies have been described previously [Couve et al., 2004a]. Chicken $GABA_BR1$ antibodies were kindly provided by S.J. Moss (University of Pennsylvania) and have been characterized and described previously [Kuramoto et al., 2007]. β-tubulin antibodies were purchased from Sigma. S6 ribosomal protein antibodies were purchased from Cell Signaling (Danvers, MA). The secondary anti-mouse, anti-rabbit, and anti-chicken antibodies conjugated to Texas Red (TR), fluorescein isothiocyanate (FITC), and horseradish peroxidase (HRP) were purchased from Jackson Immuno Research Laboratories (West Grove, PA).

Reverse Transcriptase (RT) and Polymerase Chain Reaction (PCR)

Total brain, testis, and liver RNA from mice were extracted using the Trizol reagent according to the manufacturer's instructions (GIBCO, Rockville, MD) and cDNA was obtained using oligo-dT primers. Primers were designed to anneal to conserved regions of Marlin-1 (forward, 5′-GAACTCAAGGCCAAGCTGCAC-3′; reverse, 5′-AGCCTCAGGAACTCTATCTC-3′). PCR was performed by denaturation at 95°C for 30 s, annealing at 55°C for 45 s, and extension at 72°C for 90 s in a total of 25 cycles. Amplified fragments were resolved on 0.8% agarose gels and photographed.

Fractionation

Tissue from adult mice or rats was homogenized in 10 volumes of 5 mM Tris-Cl (pH 7.4) containing 0.32 M sucrose using 50 strokes in a glass–teflon homogenizer. The homogenate was centrifuged at 1,400*g* for 10 min and the supernatant was saved (S1.1). The pellet was homogenized again in the same buffer and centrifuged at 1,400*g* for 10 min at 4°C (S1.2). The resulting supernatants were pooled (S1) and centrifuged at 16,000*g* for 30 min at 4°C to obtain a crude membrane preparation (P16) and a post-mitochondrial fraction (S16). The crude membrane fraction (P16) was washed three times in 50 mM Tris-Cl (pH 7.4), 0.1% phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 10 μg/ml antipain and resuspended at a concentration of 5 mg/ml. The soluble fraction was spun at 100,000*g* for 30 min at 4°C. The resulting supernatant (S100) was saved and the pellet (P100) was resuspended in an equivalent volume of 50 mM Tris-Cl (pH 7.4). Samples were denatured, separated by SDS–PAGE, and transferred to nitrocellulose membranes.

Preparation of a Fraction Enriched in Microtubules

Tissue from adult rats was homogenized in 5 volumes of 5 mM Tris-Cl (pH 7.4) containing 0.32 M sucrose using 50 strokes in a glass–teflon

homogenizer. The homogenate was centrifuged at 1,400*g* for 10 min at 4°C and the supernatant was saved (S1). S1 was centrifuged at 16,000*g* for 30 min at 4°C to obtain a crude membrane preparation (P16) and a post-mitochondrial fraction (S16). The S16 fraction was centrifuged at 100,000*g* for 30 min at 4°C. The resulting supernatant (S100) was saved and the pellet (P100) was resuspended in an equivalent volume of 50 mM Tris-Cl (pH 7.4) buffer. The resuspended P100 fraction was divided in three. Aliquots were left untreated or treated with 20 μM Taxol (plus 1 mM GTP) or 10 μM Nocodazole for 40 min at room temperature. Aliquots were then centrifuged at 100,000*g* for 30 min at 4°C. The supernatants were saved and the resulting pellets containing polymerized microtubules were resuspended in an equal volume of 50 mM Tris-Cl (pH 7.4) buffer. Samples were denatured, separated by SDS–PAGE, and transferred to nitrocellulose membranes.

Immunoblot Analysis

Immunoblots were performed as described previously [Couve et al., 2002]. Reactions were visualized using enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech, Buckinghamshire, UK).

Coimmunoprecipitation

Descapsulated testis, brain, or liver were washed with ice-cold phosphate buffer saline (PBS) and lysed with immunoprecipitation buffer containing 150 mM NaCl, 2 mM $MgCl_2$, 10 mM Tris (pH 7.4), 1 mM EDTA, 1 mM EGTA, protease inhibitors, 0.5% Nonidet P-40, and 1 mM dithiotreitol. Lysates were precleared with protein G-Sepharose beads (Sigma) for 1 h at 4°C. For immunoprecipitation, 150 μg of total cell lysate were incubated overnight with 5–10 μg of Marlin-1 antibodies, followed by the addition of protein G-Sepharose beads for a further 2 h, at 4°C. Bound immune complexes were washed three times with immunoprecipitation buffer containing protease inhibitors and detergents. The pellet was eluted by boiling in sample buffer. Supernatant proteins were separated by SDS–PAGE as above, transferred to nitrocellulose filters and immunoblotted.

Immunohistochemistry

Tissue was rapidly removed from animals and fixed in 4% (v/v) Bouin's fluid for 24 h at room temperature. After dehydration in ethanol series, tissues were embedded in Paraplast Plus (Monoject Scientific, St. Louis, MO) or Hiscosec (Merck, Darmstadt Germany). Sections were processed for deparaffinization with alcohol series and washed in 10% H_2O_2 for 15 min. Immunostaining was performed using the Universal ICQ LSAB plus kit (DAKO, Glostrup, Denmark). Sections were rinsed in H_2O and 1× PBS for 10 min and incubated with primary antibody at 22°C overnight. Sections were washed three times in 1× PBS and incubated with anti-rabbit IgG-biotin-conjugated secondary antibody for 15 min at room temperature. Sections were then washed three times in 1× PBS and incubated with streptavidin/PAP complex for 15 min at room temperature. Sections were washed three times in 1× PBS and developed with diaminobenzidine for 5 min. Sections were then rinsed with H_2O to stop the reaction and counterstained with hematoxylin for 30 s at room temperature. Finally, sections were incubated with borate and dehydrated with a series of alcohols before mounting. Coverslips were examined using a Zeiss Axioskope II microscope equipped with a digital video camera (NikonDXM1200).

Spermatozoa Preparation

An incision was made on the cauda epididymis of adult mice and rats and low pressure was applied to expel spermatozoa. Cells were resuspended in 1× PBS for 30 min at 37°C then washed twice by centrifugation at room temperature. The pellet of spermatozoa was resuspended in 10 mM HEPES (pH 7.2) containing 150 mM NaCl.

Immunofluorescence

Cells were washed twice with PBS, fixed for 10 min in 1:1 Histochoice/ethanol and blocked for 10 min in blocking solution (0.25% BSA, 10% horse serum in PBS). Cells were permeabilized in blocking solution containing 0.5% NP-40 for 10 min at room temperature and blocked for another 10 min in immunofluorescence solution containing 0.1% NP-40. Samples were incubated sequentially with primary and secondary antibodies for 1 h at room temperature in blocking solution. Coverslips were examined using an Olympus Fluoview FV1000 laser scanning confocal microscope.

RESULTS

Marlin-1 Is Expressed in Testis

The expression of Marlin-1 has been well documented in brain preparations, cultured neurons, and lymphoid cells [Couve et al., 2004a; Steindler et al., 2004]. To determine whether Marlin-1 is also expressed in testis, the existence of the transcript was evaluated by PCR. RNA was extracted from testes of adult rats and total cDNA was prepared using RT. Specific primers were designed to amplify a conserved region of 990 bp in the central region of the Marlin-1 cDNA (bp 210–1,200). Analysis of the PCR products clearly indicated that the same set of primers amplified an identical ~1 kb band from a control plasmid containing Marlin-1, from rat brain cDNA and rat testis cDNA (Fig. 1A, lanes 2–4). Importantly, the transcript was absent from a control tissue such as liver (Fig. 1A, lane 5). Additionally, no product was obtained when one of the specific primers was omitted (Fig. 1A, lane 1).

To determine whether Marlin-1 is appropriately translated in testis, tissue samples were homogenized and fractionated to obtain a post-mitochondrial fraction (S16). Protein samples were analyzed by immunoblots using a specific Marlin-1 antibody. As expected, a band of approximately 80 kDa was recognized by the antibody in the S16 fraction derived from rat brain (Fig. 1B, top panel, lane 1). A band of identical molecular weight was observed in the S16 fraction from rat testis (Fig. 1B, top panel, lane 2), but was absent when a liver preparation was used as control (Fig. 1B, top panel, lane 3). A control with the ribosomal S6 protein confirmed the integrity of the protein samples (Fig. 1B, bottom panel). Identical results were obtained from mouse testis cDNA and protein (not shown). Together these results indicate that Marlin-1 is transcribed and translated in brain and testis, and suggests that the same isoform of the Marlin-1 protein, containing at least amino acids 70–399 (bp 210–1,200) is present in both organs. The lower abundance of Marlin-1 in testis is consistent with previous Northern blots, but the smaller transcripts observed before remain to be explored [Couve et al., 2004a; Steindler et al., 2004].

Marlin-1 Is Localized in Somatic and Germ Cells in Testis

To confirm the expression of Marlin-1 in testis and determine its cellular distribution, slices were prepared from adult rats and were subjected to immunohistochemistry using specific Marlin-1 antibodies. The results show that Marlin-1 is differentially expressed according to the seminiferous epithelial cycle. A strong reaction product was observed in the cytoplasm of Sertoli cells in the basal compartment (Fig. 2A′–D′, Sr). Immunoreaction was also present in the cytoplasm of spermatogonia (Fig. 2C′, D′, stars). Likewise, the medial region of the seminiferous tubule also showed

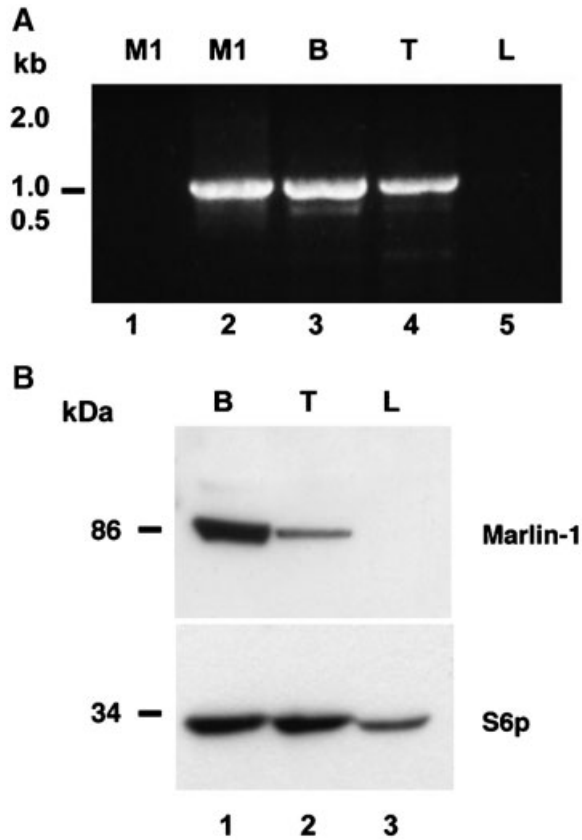

Fig. 1. Marlin-1 is expressed in testis. **A**: Marlin-1 transcripts are present in testis. Negative control PCR from a plasmid encoding Marlin-1 (M1, **lane 1**) with a single 5′-primer. Positive control PCR from a plasmid encoding Marlin-1 with 5′ and 3′ primers (M1, **lane 2**). PCR from total cDNA derived from adult rat brain (B, **lane 3**), adult rat testis (T, **lane 4**), or adult rat liver (L, **lane 5**) using specific 5′ and 3′ Marlin-1 primers. Positions of relevant size standards are indicated on the left. **B**: Marlin-1 protein is present in rat testis. Analysis of the distribution of Marlin-1 protein after fractionation of brain (B), testis (T), and liver (L). One hundred microgram of a post-mitochondrial fraction (S16) from adult rat brain (lane 1), adult rat testis (lane 2), or adult rat liver (lane 3) were separated by SDS–PAGE and transferred to a nitrocellulose membrane. The membrane was used for immunoblots with Marlin-1 (**top panel**) or S6 ribosomal protein antibodies (**bottom panel**). Positions of relevant molecular weight standards are indicated on the left.

significant staining (Fig. 2A′, B′, D′, arrows). This immunoreaction may correspond to Sertoli cells or Sertoli cells associated with germ cells. In addition, a strong staining was observed in the cytosol of primary spermatocytes (Fig. 2, SPI in A′), but not in the cytoplasm of round spermatids (Fig. 2B′, C′, Sd). The densely stained regions in the lumen of the seminiferous epithelium (Fig. 2C′, arrows) correspond to the apical processes of Sertoli cells encapsulating the heads of late step spermatids (Fig. 2C′, arrowheads). Spermatids revealed a moderate and variable staining according to the stage of spermiogenesis (Fig. 2A′–D′) and a significant staining in the tail of spermatids located near the luminal surface (Fig. 2C, C′, arrowheads). Marlin-1 was also present in the interstitial compartment, possibly in Leydig cells (not shown). Control panels utilizing pre-immune antibodies demonstrated that the patterns for Marlin-1 are specific (Fig. 2A, inset).

To complement these findings and correlate the expression of Marlin-1 to differentiation, we performed indirect immunofluorescence using Marlin-1 antibodies and counterstaining for DNA to establish the specific maturation stage of germ cells. Marlin-1 localization was prominent in the seminiferous tubule (Fig. 3A, B). It showed a cytosolic and granular localization in Sertoli cells (Fig. 3C, arrows). The distribution of Marlin-1 was also evident in Sertoli cells of two adjacent tubules (Fig. 3C, arrowheads showing nuclei). In primary spermatocytes (pachytene), Marlin-1 displayed a strong granular pattern in the cytosolic region (Fig. 3E, arrows) and at several spots possibly associated to the synaptonemal complex (Fig. 3B, E, arrowheads). It also revealed a prominent staining in round spermatids in an early stage with a granular staining pattern in cytosol (Fig. 3D, arrow) and nuclei (Fig. 3D, arrowheads).

Marlin-1 Is Expressed in Spermatozoa

To confirm whether Marlin-1 is expressed in spermatozoa and to determine its precise subcellular localization, a confocal microscope analysis was carried out using Marlin-1 antibodies. A granular distribution was observed in

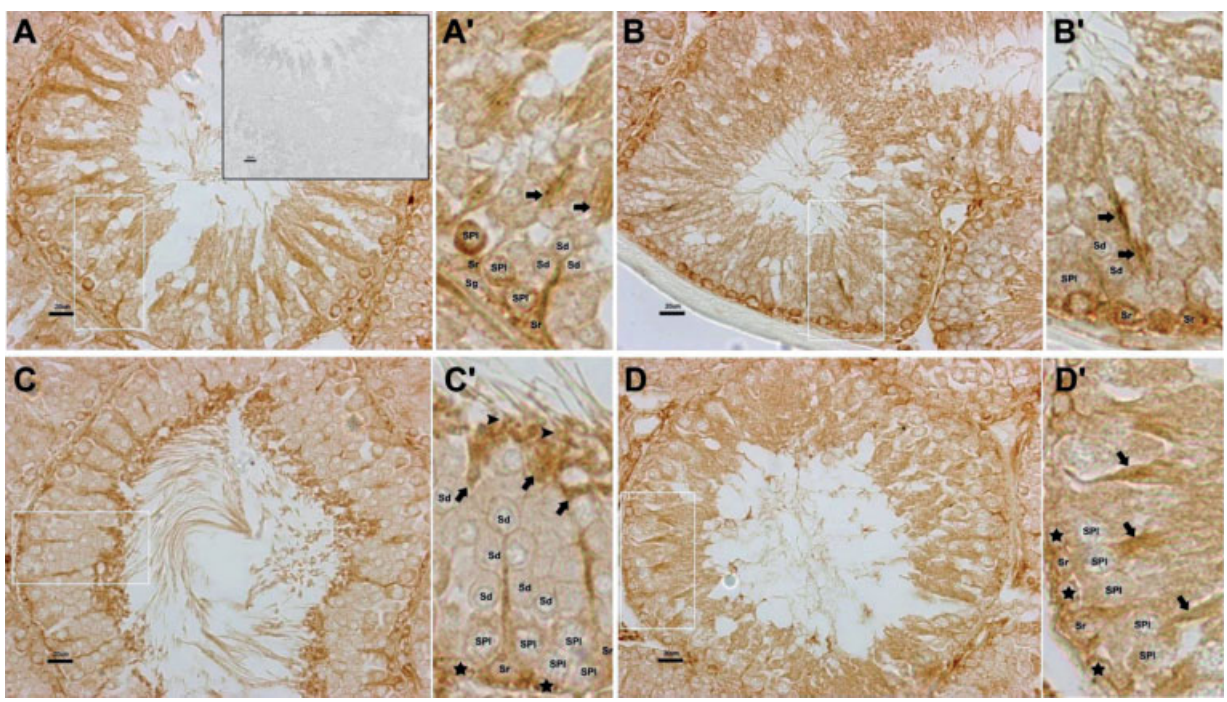

Fig. 2. Marlin-1 is present in different stages of the seminiferous epithelial cycle. Light micrographs of seminiferous tubules showing general and specific patterns of immunoperoxidase staining obtained with antibodies against Marlin-1. **A**, **B**, **C**, and **D**: Correspond to apparent stages I–III, V–VI, VII, and XII of the seminiferous epithelial cycle, respectively. **A′**–**D′**: Correspond to high magnification images of the regions boxed in the corresponding panel. Sd, round spermatids; SP1, primary spermatocytes; Sr, Sertoli cells; scale bar, 20 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

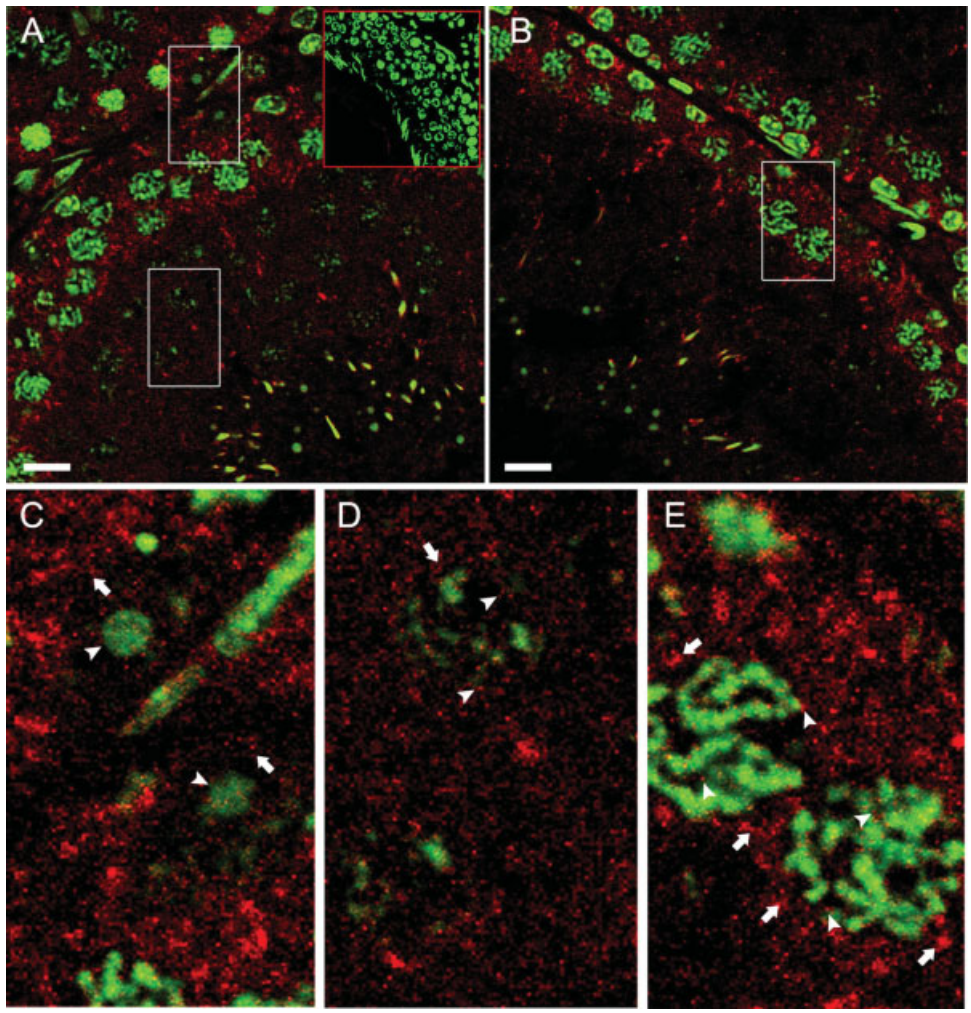

Fig. 3. Marlin-1 localizes to somatic and germ cells. **A**: Immunofluorescence of Marlin-1 from testis. Marlin-1 was detected using specific antibodies and red secondary antibodies. Green staining for DNA was achieved with Sytox Green. A control without primary antibodies is shown in the red inset. **B**: Immunofluorescence of Marlin-1 from testis. **C**, **D**: correspond to high magnification images of regions boxed in (A). **E**: Corresponds to high magnification image of the region boxed in (B); scale bar, 10 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

rat spermatozoa (Fig. 4A–F). The staining was prominent in the acrosomal region and also concentrated in the proximity of the midpiece of the spermatozoa (Fig. 4D–F). Counterstaining with propidium iodide indicated that the signal was excluded from the nucleus (Fig. 4D–F). A similar pattern was observed for mouse and human spermatozoa (Supplementary Fig. 1A–C). It is interesting to note that in human spermatozoa the signal showed a greater concentration in the midpiece (Supplementary Fig. 1A). These observations indicate that Marlin-1 is expressed in sperm cells across mammalian species and that its subcellular distribution shows a high degree of conservation.

Marlin-1 Associates to Microtubules in Testis

Recently, we have reported the association of Marlin-1 and microtubules in the brain [Vidal et al., 2007]. In addition, a separate study has demonstrated the interaction in Jurkat cells [Steindler et al., 2004]. This evidence led us to explore whether the association of Marlin-1 to microtubules is a general occurrence. To this end, we performed a microtubule association assay in a preparation from rat testis. Marlin-1 distributed to a fraction enriched in polymerized microtubules under control conditions and was absent from a soluble fraction (Fig. 5, lanes 1 and 2). Treating the extract with Taxol, a drug

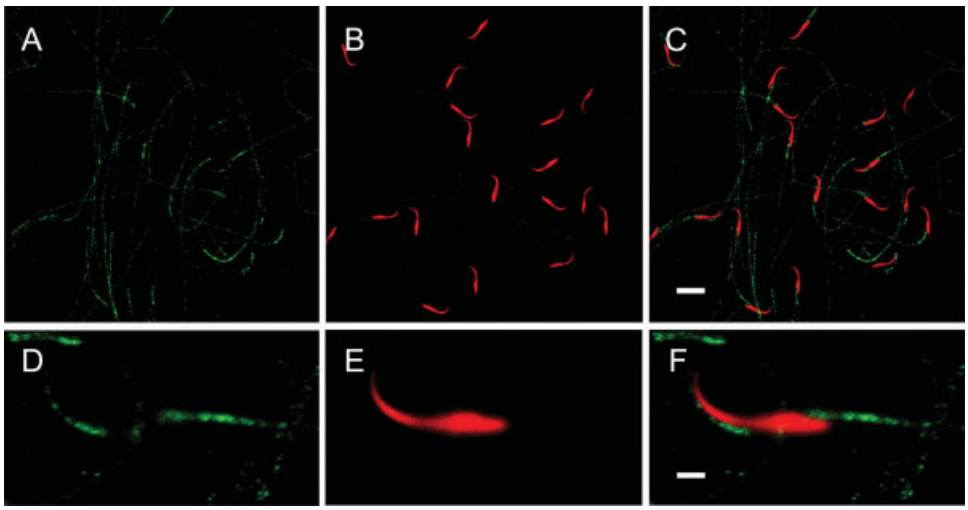

Fig. 4. Marlin-1 is expressed in spermatozoa. **A**: Rat spermatozoa were fixed and stained with Marlin-1 antibodies and FITC-conjugated secondary antibodies. **B**: Propidium iodide was used to visualize the nucleus. **C**: Images were merged to evaluate colocalization. **D**–**F**: Same as above at higher magnification. Scale bars: (A–C), 10 μm; (D–F), 2 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

that stabilizes microtubules, did not change the distribution of Marlin-1 and it remained in the polymerized fraction (Fig. 5, lanes 3 and 4). Importantly, treating the sample with Nocodazole, an agent that destabilizes microtubules, caused a shift of Marlin-1 to the depolymerized fraction of soluble tubulin (Fig. 5, lanes 5 and 6). These results indicate that Marlin-1 and microtubules associate in testis. Although our evidence does not show that Marlin-1 and microtubules interact directly, it suggests that the association to microtubules is an intrinsic property of Marlin-1.

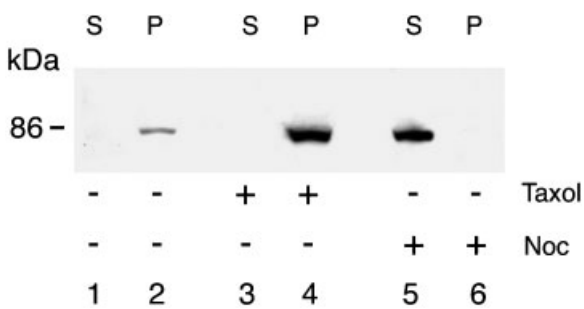

Fig. 5. Marlin-1 associates to microtubules. Rat testes were homogenized and a microtubule-enriched fraction was prepared (P). A soluble pool was also collected (S). Samples were left untreated (**lanes 1** and **2**), treated with 20 μM Taxol for 40 min at room temperature (**lanes 3** and **4**), or treated with 10 μM Nocodazole (Noc) for 40 min at room temperature (**lanes 5** and **6**). Samples were separated by SDS–PAGE and transferred to a nitrocellulose membrane. The membrane was used for immunoblots with Marlin-1 antibodies and HRP-conjugated secondary antibodies. Positions of relevant molecular weight standards are indicated on the left.

Marlin-1 and $GABA_BR1$ Coimmunoprecipitate in Testis

$GABA_B$ receptors are heterodimeric G protein-coupled receptors that control the slow and prolonged phase of synaptic inhibition in the CNS [Mott and Lewis, 1994; Couve et al., 2004b]. Their physiological role in epilepsy and pain has been confirmed by the production of $GABA_BR1$ and $GABA_BR2$ knockout mice [Prosser et al., 2001; Schuler et al., 2001; Gassmann et al., 2004; Thuault et al., 2004]. They also have been implicated in depression, neuroprotection, cognition, and addiction [Roberts and Andrews, 1997; Couve et al., 2004b]. In neurons of the CNS, $GABA_B$ receptors associate with several proteins that regulate their function, biosynthesis, trafficking, and degradation [Bettler and Tiao, 2006]. Marlin-1 directly interacts with the carboxyl terminal domain of the $GABA_BR1$ subunit of the receptor heterodimer [Couve et al., 2004a]. Earlier studies have shown that Marlin-1 and $GABA_BR1$ are expressed in a subpopulation of cortical and hippocampal neurons, and analysis of their subcellular localization has revealed that they partially overlap in the soma and dendritic projections of cultured hippocampal neurons [Couve et al., 2004a]. Like many other neurotransmitter receptors, $GABA_BR1$ and $GABA_B$

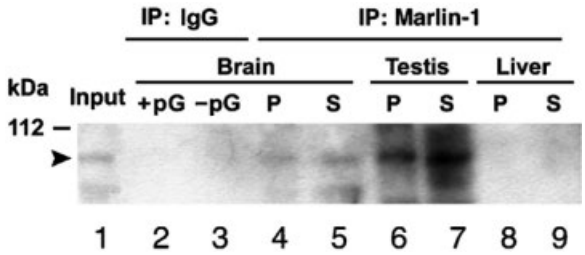

Fig. 6. Marlin-1 associates to $GABA_B$ receptors. Extracts from adult rat brain (**lanes 1–5**), testis (**lanes 6** and **7**), or liver (**lanes 8** and **9**) were immunoprecipitated with non-specific IgG in the presence or absence of protein G-Sepharose (pG, lanes 2 and 3), or Marlin-1 antibodies (lanes 4–9). Samples were resolved by SDS–PAGE, transferred to PVDF membrane and immunoblotted with $GABA_BR1$ antibodies. Brain input (lane 1). Immunoprecipitated material is shown in pellets (P). Unbound material is shown in supernatants (S). Positions of relevant molecular weight standards are indicated on the left.

R2 are abundantly expressed in spermatozoa [Meizel, 2004]. To determine whether Marlin-1 and $GABA_B$ receptors associate in testis, we performed a coimmunoprecipitation assay. Importantly, a clear association of the two proteins was observed in extracts from brain and testis (Fig. 6, lanes 4–7). To determine the specificity of the association, we included some negative controls in our assay. As expected, negative reactions were observed for immunoprecipitations carried out with non-specific IgG (Fig. 6, lanes 2 and 3) or liver extracts (Fig. 6, lanes 8 and 9). These observations indicate that Marlin-1 and $GABA_B$ receptors associate in testis. They also suggest that the interaction between neurotransmitter receptors and their associated proteins may extend beyond populations of neurons in the CNS to other highly polarized cells.

DISCUSSION

The Gabaergic System in Testis

The presence of a gabaergic system in the spermatozoa has been reported recently. Ionotropic $GABA_A$ receptors, metabotropic $GABA_BR1$ and $GABA_BR2$ receptors, and GABA transporter 1 (GAT1) are all present in sperm cells [Hu et al., 2000; He et al., 2001; Hu et al., 2002a; He et al., 2003] where activation of $GABA_AR$ and $GABA_BR$ alters the rate of acrosomal reaction [Hu et al., 2002b]. We have now extended these observations to Marlin-1, a $GABA_B$ receptor-associated protein. We have conclusively shown that Marlin-1 is expressed in mammalian testis. We also have shown that Marlin-1 associates to $GABA_B$ receptors in testis even though the expression levels are lower than in the brain. These findings indicate that the presence of neurotransmitter receptors of the $GABA_B$ type in testis extends to their interacting proteins that modulate their function in neurons. Thus, they argue in favor of conserved functional neurotransmitter receptor complexes in spermatozoa. Given the critical role of $GABA_B$ receptors in synaptic transmission and their current use as drug targets, it will be interesting to evaluate the function of Marlin-1 in the processes of sperm motility, acrosomal reaction, or fertilization.

Several other $GABA_B$-associated proteins such as PKA, 14-3-3, ATF4/CREB2, and Mupp1 have been described in the CNS [Nehring et al., 2000; White et al., 2000; Couve et al., 2001; Milligan and White, 2001; Vernon et al., 2001; Couve et al., 2002]. Whether these are also expressed in spermatozoa and whether they interact with the receptor remains to be evaluated.

A number of other neurotransmitter receptors also have been described in spermatozoa [Hu et al., 2002a; Otth et al., 2007]. Although the significance of this phenomenon is still unclear, it is likely that paracrine secretion of neurotransmitters will affect the function of spermatozoa by acting on local receptors. It remains to be demonstrated whether neurotransmitter receptor complexes, which have been shown to be the functional units of signaling in neurons [Alberts, 1998], are also the functional units in testis.

Cellular and Subcellular Distribution

During spermatogenesis, the staining pattern of Marlin-1 depends on the stage of the seminiferous epithelial cycle and cell type. The cytoplasmic and granular distribution, for example in Sertoli cells, is similar to the cytoplasmic staining observed in hippocampal neurons [Couve et al., 2004a]. Furthermore, our microscopic analysis indicates that Marlin-1 is also excluded from the nucleus in somatic cells and partially excluded from the nucleus in germ cells in testis. This is consistent with our hypothesis, which maintains that although Marlin-1 is capable of binding different kinds of nucleic acids, it is preferentially associated with single stranded RNA. It is not surprising to find RNA-binding proteins in spermatozoa. Local metabolism of mRNAs has been identified as a key element in the organization of the

embryo [Palacios et al., 2001]. Furthermore, other RNA-binding proteins such as FXR1P [Huot et al., 2001], TIAR [Beck et al., 1998], Staufen [Bateman et al., 2004], and TB-RBP [Gu et al., 1998] are found in spermatozoa and some play critical roles during the early stages of development and associate to microtubules. A recent study has pointed out that the presence of these proteins in the flagella of the spermatozoa is puzzling [Huot et al., 2001]. However, our data regarding Marlin-1 confirm that it may be a more common occurrence than originally thought. The interaction between Marlin-1 and RNA will need further exploration in testis. If binding does occur, it will be of interest to study its significance for early development as there is yet no direct evidence for the role of Marlin-1 in RNA metabolism and the regulation of $GABA_B$ receptor targeting and stability.

Marlin-1 and Microtubules

In addition to its role in binding $GABA_B$ receptors and Jaks, Marlin-1 has been shown to associate with the cytoskeleton through microtubules [Steindler et al., 2004; Vidal et al., 2007]. The presence of Marlin-1 in a highly polarized cell like the sperm may be indicative of its role in modulating intracellular trafficking along cytoskeletal elements. In this respect, it is interesting to note that in Sertoli cells Marlin-1 is preferentially localized to regions where these cells closely associate with germ cells in translocation process to the luminal compartment. Also, it is interesting to point out the discrete localization of Marlin-1 in the midpiece of the sperm. As centrioles are present in the neck and the midpiece of spermatozoa, it is conceivable that Marlin-1 may also affect the polarity or stability of the cytoskeleton in this cell type. This is compatible with the Marlin-1-mediated stabilization of microtubules in tissue culture cells [Steindler et al., 2004].

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