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MSAP, the *Meichroacidin* Homolog of Carp (*Cyprinus carpio*), Differs from the Rodent Counterpart in Germline Expression and Involves Flagellar Differentiation

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ABSTRACT

To gain access to the molecular mechanisms of spermatogenesis, the genes from a subtractive screen of the carp testis cDNA library were investigated. In this study, a male-specific homolog of the *meichroacidin* gene, called *MSAP* (MORN motif-containing sperm-specific axonemal protein), was isolated and further characterized. Database search and zoo-Western blot analyses revealed that MSAP homologs might be widespread in a variety of phyla but divergent in their C-terminal length and sequences. Carp *MSAP* is exclusively transcribed in testis, while mouse *meichroacidin* message is present in gonads of both sexes, although especially enriched in testis. In mouse, meichroacidin is expressed in male germ cells of meiotic stages, while carp MSAP is expressed during late spermiogenesis and accumulated in mature spermatozoa, in which MSAP is localized to the basal body and flagellum. Contrary to mouse meichroacidin revealed previously, existence of multiple pI variants of MSAP in two-dimensional electrophoresis suggested regulatory differences of the homologous molecules between mammal and teleost. These results indicate that *MSAP* homologs may play different roles in male germline development between vertebrates. Proteomic analysis and immunolocalization disclosed that MSAP is associated with septin7, a conserved GTPase that may participate in cellular morphogenesis, in the basal body of carp sperm. These findings suggest the involvement of carp MSAP in flagellar differentiation during spermiogenesis.

gamete biology, sperm, spermatid, spermatogenesis, testis

INTRODUCTION

Spermatogenesis is a complex process including mitotic renewal of spermatogonia, meiosis of spermatocytes, and morphogenesis of spermatozoa (spermiogenesis). The cellular compositions and meiotic processes in testis are generally conserved among vertebrates. However, sperm morphology and testicular anatomy differ greatly between vertebrates [1, 2].

In mammals, spermatogenesis occurs in a permanent germinal epithelium, a tripartite compartment consisting of Sertoli cells, germ cells, and basement membrane. The germinal epithelium appears as a tubular structure, dubbed the seminiferous tubule. Spermatogonia and Sertoli cells reside near the periphery of the tubule. During spermatogenesis, maturing germ cells sequentially align and continuously move toward the central lumen of the tubule. The Sertoli cell-germ cell association persists during spermatogenic progression until mature spermatozoa are released into the lumen. After spermiation, Sertoli cells remain associated with immature germ cells and spermatogonia to support the next spermatogenic cycles [2]. In teleosts, however, spermatogenesis is discontinuous and the compartmentalization of germinal epithelia is highly different from that of mammals. Teleost spermatogenesis commences in spermatocyst (or cyst), which is formed by association of a Sertoli cell with a primary spermatogonium in the periphery of the anastomosing or lobular seminiferous tubule [3, 4]. Each cyst contains synchronized and syncytial germ cell clones. Following spermiogenic maturation, the mature cyst ruptures and a cohort of spermatozoa are released into the lumen of the seminiferous tubule. Thereafter, the Sertoli cells that enclose the mature cysts degenerate or are incorporated into the epithelium of vas deferens. Unlike mammals, there is no permanent germinal epithelium in teleosts because matured cysts are shed in each spermatogenic cycle [2, 5].

Besides differences in testicular morphology, the sperm morphology is also greatly different between vertebrates. The most prominent discrepancy is the acrosomal complex, which is present in most animal species but is generally lacking in teleosts. Other diversifications between teleost and tetrapod spermatozoa include the number of flagella (some fish spermatozoa are biflagellate or aflagellate), the perforatoria, the accessory filaments of the flagellum, the distribution of mitochondria, the constituents of the axoneme, and the axonemal configurations [6]. Their evolutionary significances have been extensively discussed [7].

The varied sperm morphology underlies the difference in spermatogenic mechanisms between teleost and tetrapod. However, little molecular information about teleost spermatogenesis is available now. Therefore, we attempted to find genes that are involved in teleost spermatogenesis as a basis for future studies. Here, the homolog of mouse *meichroacidin* (*Tsga2*) gene [8, 9] had been cloned from the common carp. The homolog encodes a flagellar protein that contains seven newly proposed MORN (membrane occupation and recognition nexus) motifs [10], thus, named *MSAP* (MORN motif-containing sperm-specific axonemal protein). Mouse *meichroacidin* is gonad specific, especially prominent in testis, and expressed from pachytene spermatocytes to round spermatids. The association of meichroacidin with metaphase chromosome and meiotic spindle in mouse germ cells implicates its role in meiotic segregation [8, 9]. However, the expression patterns of carp *MSAP* are spatiotemporally different from those of mouse *meichroacidin.* The difference indicates the regulatory and functional divergence of the homologous genes in the evolution of male germline development. In addition, the MSAP-interacting candidates in carp sperm were also in-

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vestigated, and one of them had been identified to be septin7. Based on the potential functions of the septin proteins in cellular morphogenesis [11–13], the roles played by MSAP and septin in sperm differentiation are discussed here.

MATERIALS AND METHODS

All animal experiments were conducted in accordance with the guidelines of the Academia Sinica Institutional Animal Care and Utilization Committee.

Subtractive Screen of cDNA Library and Cloning of Testis-Specific cDNA

RNA was extracted from various tissues of the mature common carp by using Ultraspec-II RNA Isolation System (Biotecx Laboratories Inc., East Houston, TX). The poly(A)$^+$ RNA was isolated by two cycles of oligo(dT)-cellulose chromatography. The testicular poly(A)$^+$ RNA was used to synthesize the double-stranded cDNA and then the cDNA was ligated with the λZAPII arms to construct a phage library using the Gigapack Packaging kit (Stratagene, La Jolla, CA). To prepare testis-specific cDNA probe, the testicular poly(A)$^+$ RNA was transcribed to single-stranded cDNA and the resulting cDNA-RNA hybrid was treated by alkali. An excess of driver poly(A)$^+$ RNA from ovary and liver, which had been photobiotinylated, was used to subtract the single-stranded testis cDNA following the manufacture's instructions (Invitrogen, Carlsbad, CA). The second strand of the subtracted testicular cDNA was synthesized by Klenow fragment and the product was used to generate digoxigenin (DIG)-labeled probe according to the random-primed protocol (Boehringer Mannheim, Germany). Poly(A)$^+$ RNA from ovary and liver was also used as template to generate DIG-labeled cDNA probe.

By screening testis cDNA library with the subtracted testicular probe, there were several positive plaques isolated. These plaques were individually transfected into *Escherichia coli* following the in vivo excision procedures (Stratagene). To identify the tissue specificity, the plasmids prepared from transfected *E. coli* were examined by dot-blot analysis using the tissue-specific DIG-labeled probes. Clones that hybridized with the subtracted testicular probe but not with either ovary nor liver cDNA probes were selected. Two of them were further identified to be testis-specific by Northern analysis. The inserts of the clones were sequenced in both directions by vector-derived primers and gene-specific primers. One clone, denoted as p196, contained a cDNA (then denoted as *MSAP*) with an open reading frame that is homologous to mouse *Tsga2* (*meichroacidin*) gene [8, 9] and a complete 3′ untranslated region. To obtain the full-length *MSAP* cDNA, 5′RACE (rapid amplification of cDNA ends) was performed using Smart RACE kit (Clontech, Palo Alto, CA). The 5′RACE product was subcloned into pGEM-T Easy (Promega, Madison, WI) for sequencing.

Northern Hybridization

MSAP cDNA was gel purified for generating DIG-labeled probe. Twenty micrograms of RNA from various carp tissues were fractionated by agarose-formaldehyde gel electophoresis. After separation, the gels were rinsed with 20× SSC (3 M NaCl and 0.3 M sodium citrate, pH 7.0). RNA was transferred to the nylon membrane, cross-linked by ultraviolet light, and then hybridized with the *MSAP* probe. Following hybridization (18 h at 50°C), the blot was washed by 2× SSC/0.1% SDS twice (15 min each at room temperature), 0.5× SSC/0.1% SDS twice (15 min each at 68°C), and finally 0.1× SSC/0.1% SDS once (15 min at 50°C). Signals were detected by using CDP-Star chemiluminescent substrate (Tropix Inc, Bedford, MA) following the manufacture's instructions (Boehringer Mannheim). Carp *ferritinH* cDNA was used as a control probe.

Sequence Analysis

The coding region and hydropathy plot of *MSAP* were predicted via software DNASIS2.5 (Hitachi Software Engineering, Tokyo, Japan). The database search and alignment were performed via the BLAST search and Clustal W program, respectively. To construct the phylogenetic tree, the nucleotide sequences of the conserved coding regions from the deduced *MSAP*/*meichroacidin* cDNAs (corresponding to the N-terminal 220 residues of mouse meichroacidin) were aligned and computed using the MEGA2 software [14]. The distance matrix was generated using the Kimura two-parameter method and only transversional substitutions were considered. The tree was created by the neighbor-joining method and bootstrapped 1000 times to confirm the branch relationship between the species.

Reverse Transcription-PCR Analysis

Single-stranded cDNA was synthesized from total RNA of various tissues with an *MSAP*-specific primer (5′AGAGGCCATGATAATGTGC AGTC3′) using Ready-To-Go You-Primed First-Strand Beads (Amersham Biosciences, Piscataway, NJ). For PCR amplification, another gene-specific primer (5′ATCAGAGACATGGGCAGGGC3′) was added and the reaction was performed under the conditions of 94°C/45 sec, 59°C/45 sec, and 72°C/1 min for 35 cycles.

Recombinant Protein and Antibody Production

The coding region of *MSAP* was PCR amplified, ligated to expression vector pET-22b$^+$ (Novagen, Madison, WI), and then transfected into the expression host BL21/λDE3. The host was cultured at 37°C overnight in supplemented LB broth with appropriate antibiotics and refreshed for 2.5 h. Once isopropylthio-βD-galactoside (1 mM) was added, the culture was shifted to 28°C. After a 5-h induction, the cells were collected and lysed by 8 M urea containing 1 mM dithiothreitol (DTT), 10 mM Na_2HPO_4, and 0.1 M Tris, pH 8.0. Recombinant MSAP (rMSAP) was purified by Ni-NTA resin following the manufacture's protocol (Qiagen GmbH, Germany). Two forms of rMSAP (31 and 27 kDa) were obtained. After separation by SDS-PAGE, the rMSAP of 27 kDa was sliced and used as an antigen to induce antibody in female New Zealand white rabbits. Pooled antisera were further affinity purified using the rMSAP-coupled Sepharose 4B (Amersham Biosciences). The concentration of the purified antibody (anti-MSAP) was determined by the bicinchoninic acid method (BCA reagent; Pierce, Rockford, IL).

Protein Extraction and Immunoblotting

Tissues from carp, goldfish (*Carassius auratus*), loach (*Paramisgurnus dabryanus*), domestic pigeon (*Columba livia*), mouse (*Mus musculus*), and rat (*Rattus norvegicus*) were homogenized in ice-cold Nonidet P-40 (NP-40) lysis buffer (150 mM NaCl, 1% NP-40, 1 mM DTT, 1 mM EDTA, 1 mM EGTA, and 25 mM Tris, pH 7.6, 0.2 mM PMSF, 20 μM leupeptin, and 1 mM benzamidine). Homogenates were sonicated (6-sec pulse 10 times) at 0°C with a sonicator (Vibra Cell X; Sonics and Materials Inc., Newtown, CT). Sonicated homogenates were centrifuged at 12 000 × *g* for 15 min at 4°C to collect the supernatant. To prepare sperm extracts, carp semen was first washed with TBS (25 mM Tris, pH 7.6, 2.7 mM KCl, and 138 mM NaCl) and centrifuged (1000 × *g* for 3 min at 4°C) to collect sperm cells. Cells were lysed and sonicated in NP-40 lysis buffer (in 1.0×10^{10} sperms/ml) to obtain total sperm extract. To fractionate sperm proteins, the NP-40-lysed sperm was first centrifuged (12 000 × *g* at 4°C for 15 min) to separate the soluble extract (NP-soluble fraction) from the insoluble pellet. The pellet was further sonicated in NP-40 lysis buffer, then centrifuged to harvest sonication-resolubilized proteins (NP-insoluble fraction).

Proteins were separated by SDS-PAGE and then blotted onto the nitrocellulose membrane using a semidry electroblotter (Hoefer, San Francisco, CA). Following blocking by 5% nonfat milk in PBST (PBS with 0.1% Tween-20), the blot was first incubated with anti-MSAP in blocking solution overnight (4°C). The blot was washed with PBST and followed by incubation with peroxidase-conjugated anti-rabbit IgG (ICN Biomedicals Inc., Costa Mesa, CA). After extensive washing with PBST, the blot was visualized by the 3,3′-diaminobenzidine tetrahydrochloride/$NiCl_2$ method or SuperSignal substrate (Pierce). Duplicate blots were probed with monoclonal antibodies against α-tubulin (NeoMarkers Inc., Fremont, CA), β-tubulin, dynein IC, or β-actin (Sigma, St. Louis, MO), followed by incubation with anti-mouse IgG-alkaline phosphatase conjugates (ICN Biomedicals Inc.). These blots were developed using the BCIP/NBT method.

Immunofluorescence Microscopy

Sliced carp testis was fixed in PBS-buffered paraformaldehyde (4%) at 4°C for 4 h and then dehydrated in the graded series of ethanol, cleared in xylene, embedded in paraffin, and sectioned (5 μm in thickness). After deparaffinization and rehydration, sections were permeabilized with 1% Triton X-100 in PBS, preincubated with blocking solution (PBST containing 5% normal goat serum) for 1 h, and incubated with either anti-MSAP (1.66 μg/ml) or normal rabbit γ-globulin (2 μg/ml) in blocking solution

overnight (4°C). After being washed by PBST, sections were probed with Alexa Fluor 568-conjugated anti-rabbit IgG (2 μg/ml; Molecular Probes, Eugene, OR) and then counterstained with 4′,6-diamidino-2-phenylindole (DAPI; 1 μg/ml in PBS; Sigma). For double labeling, anti-β-tubulin (1:500 dilution) and Alexa Fluor 488-conjugated anti-mouse antibody (2 μg/ml; Molecular Probes) were also included for the primary and secondary antibody incubations, respectively. To immunolabel carp spermatozoa, the semen was diluted 1×10^4-fold with PBS-buffered paraformaldehyde and then spotted on the slides. After air drying, slides were immersed in 0.1 M glycine (in PBS) to block the unreacted aldehyde groups. The procedures for permeabilization, blocking, and dual immunolabeling with anti-MSAP or polyclonal septin7 antibody (1:50 dilution; Santa Cruz Biotech, Santa Cruz, CA) and β-tubulin monoclonal antibody were the same as described above.

Observations were done on the ApoAxioplan II microscope (Zeiss, Germany). Images with individual fluorescence colors were captured by the laser scanning confocal microscopy (Multiphoton Microsystem; Leica, Germany) and processed using Photoshop software. The size of the DAPI-stained nuclei of germ cells was estimated using the Sigma ScanPro software (Sigma) to sort the developmental stages of the spermatocysts.

Two-Dimensional Electrophoresis

The first dimension of two-dimensional (2D) electrophoresis was performed using immobilized pH-gradient (IPG) isoelectric focusing (IEF) method [15]. Briefly, the NP-insoluble sperm pellet (see above) was washed with TE buffer (1 mM EDTA and 25 mM Tris, pH 8.0) and then extracted (in 10^{10} sperm/ml) using urea lysis solution (9 M urea, 4% CHAPS, 0.5% NP-40, and 2% Pharmalyte, pH 3–10). Before IEF, the extract was diluted in rehydration solution (8 M urea, 2% CHAPS and 0.01% bromophenol blue) and supplemented with DeStreak reagent (Amersham Pharmacia Biotech, final 1.2%) and IPG buffer (Amersham Biosciences) with appropriate pH range (final 0.5%). Both IEF and SDS-PAGE were resolved following manufacture's instructions (IPGphor IEF system; Amersham Biosciences). Silver or Coomassie blue staining was used to visualize protein spots in 2D gel. To identify proteins of interest, 2D gel blots were probed with the antibodies described above.

2D-Blot Overlay Analysis and Tandem Mass Spectrometry

The 2D blot was first rinsed with MEM buffer (1 mM $MgCl_2$, 2 mM EGTA, 1 mM DTT, and 50 mM MES, pH 6.8) and then preincubated with overlay buffer (MEM buffer containing 0.2% Tween-20 and 3% BSA) for 30 min. After preincubation, rMSAP (0.67 mg/ml in 20 mM Tris, pH 7.6) was added (finally 0.1 μg/ml in overlay buffer) and incubated for 2 h. The blot was rinsed twice with MEM buffer containing 0.5 M NaCl and 0.2% Tween-20 and then twice with PBST. The washed blot was blocked and then probed with anti-MSAP. The rMSAP-reactive proteins were detected using SuperSignal substrate. After visualization, the membrane was stripped and reprobed with various antibodies to identify the known proteins described above. Some rMSAP-interacting spots in preparative 2D gel ($180 \times 160 \times 1$ mm^3) were picked and treated with sequence-grade trypsin (Promega). Digested peptides were analyzed by liquid chromatography-coupled tandem mass spectrometry (LC-MS/MS; Micromass Q-Tof Ultima API system; Waters Corp., Milford, MA). The raw MS/MS data was analyzed using the Mascot program [16].

RESULTS

Homology of Carp MSAP *to Mammalian* Meichroacidin

A testis-specific cDNA, called *MSAP* (GenBank AY_327474), was isolated from a subtractive screen of the carp testis cDNA library. It encodes an acidic polypeptide (theoretical pI 5.0) consisting of 218 amino acids (Fig. 1A, Cypr) with a theoretical mass of 24 565 Da. A high proportion of polar amino acids (60%) confer on the protein a highly hydrophilic property (Fig. 1B). Like meichroacidin [9], MSAP possesses seven MORN tandem repeats [10] that are separated by spacers with even length (usually 9 residues, Fig. 1A). Similarity between MSAP and mammalian meichroacidins is up to 75%. Resemblance in acidity, hydropathy, and MORN motifs indicates the homology of MSAP to the mammalian meichroacidins. Search of the EST database revealed that *MSAP* homologs might be conserved across various chordates (Fig. 1A), including ascidian (*Ciona intestinalis*), teleosts (*Oncorhynchus mykiss* and *Danio rerio*), amphibian (*Xenopus laevis*), and chicken (*Gallus gallus*), as well as species of other phyla, including insects, plants, and *Chlamydomonas* (see Supplemental Table 1, available at: http://cell.lifescience.ntu.edu.tw/faculty/Supp_Huang/supplementaltable1.htm). To date, neither *meichroacidin*- nor *MSAP*-like genes could be detected except *meichroacidin* itself in mouse or human genome.

Although the chordate homologs are highly varied in C-terminal length and sequences (Fig. 1A), analysis of the MORN-containing regions strongly suggests the structural conservation and the common origin of the *MSAP*/*meichroacidin* gene (Fig. 1, B and C). Comparison of MORN motifs between these chordate species revealed a glycine-rich consensus sequence of YEG×W××NG×RHGQG (Fig. 1A), where × may be any amino acid.

Testicular Specificity of Carp MSAP *Expression*

Northern blot analysis showed that the *MSAP* mRNA of 1.2 kilobases is exclusively present in carp testis. Other tissues, such as ovary, liver, brain, head kidney, or kidney, gave no positive signals (Fig. 2A). On the contrary, the *meichroacidin* message is present in mouse gonads of both sexes although predominantly in testis [8, 9]. To clarify whether the levels of *MSAP* gene expression in ovary and other tissues are too low to be detected by Northern blotting, reverse transcription (RT)-PCR experiment was performed. RT-PCR analysis revealed that the expression of *MSAP* is exactly testis specific (Fig. 2B, arrow). Tissue specificity of *MSAP* expression was further confirmed by immunoblotting, in which a 25-kDa protein was specifically detected in testis but not in the other tested tissues (Fig. 2C).

Polymorphism of MSAP/Meichroacidin Homologs

A database search revealed that the primary structures of MSAP/meichroacidin homologs are conserved between chordate species (Fig. 1A). The result suggests that the antibody against MSAP (anti-MSAP) may also recognize the homologs from other vertebrates. As seen in Figure 3, the anti-MSAP did react to the putative MSAP/meichroacidin homologs of various sizes from other species, including goldfish (24 kDa), loach (57 kDa), pigeon (47 kDa), mouse (44 kDa), and rat (44 kDa). Size polymorphism could be accounted for by the variations in the length of the nonrepeated C-terminal part of the homologs (Fig. 1A). The polymorphism follows the phylogenetic trend, i.e., the difference of the molecular size between closely related groups (e.g., carp/goldfish or mouse/rat) is much less than that between distantly related groups. The data suggested that the *MSAP*/*meichroacidin* genes have rapidly and divergently evolved during phylogenesis of vertebrates. In this experiment, the reproductive accessory organs, epididymides, of rodents were also examined. No meichroacidin signal was detected in both rodent epididymides (Fig. 3, Epi), confirming that the expression of meichroacidin in rodent is restricted to testicular germ cells but not mature spermatozoa [9].

Immunolocalization of MSAP in Carp Testis and Sperm

The testis-specific expression of carp MSAP suggests that the molecule may take part in the process unique for male germline development. To probe this, carp testis was

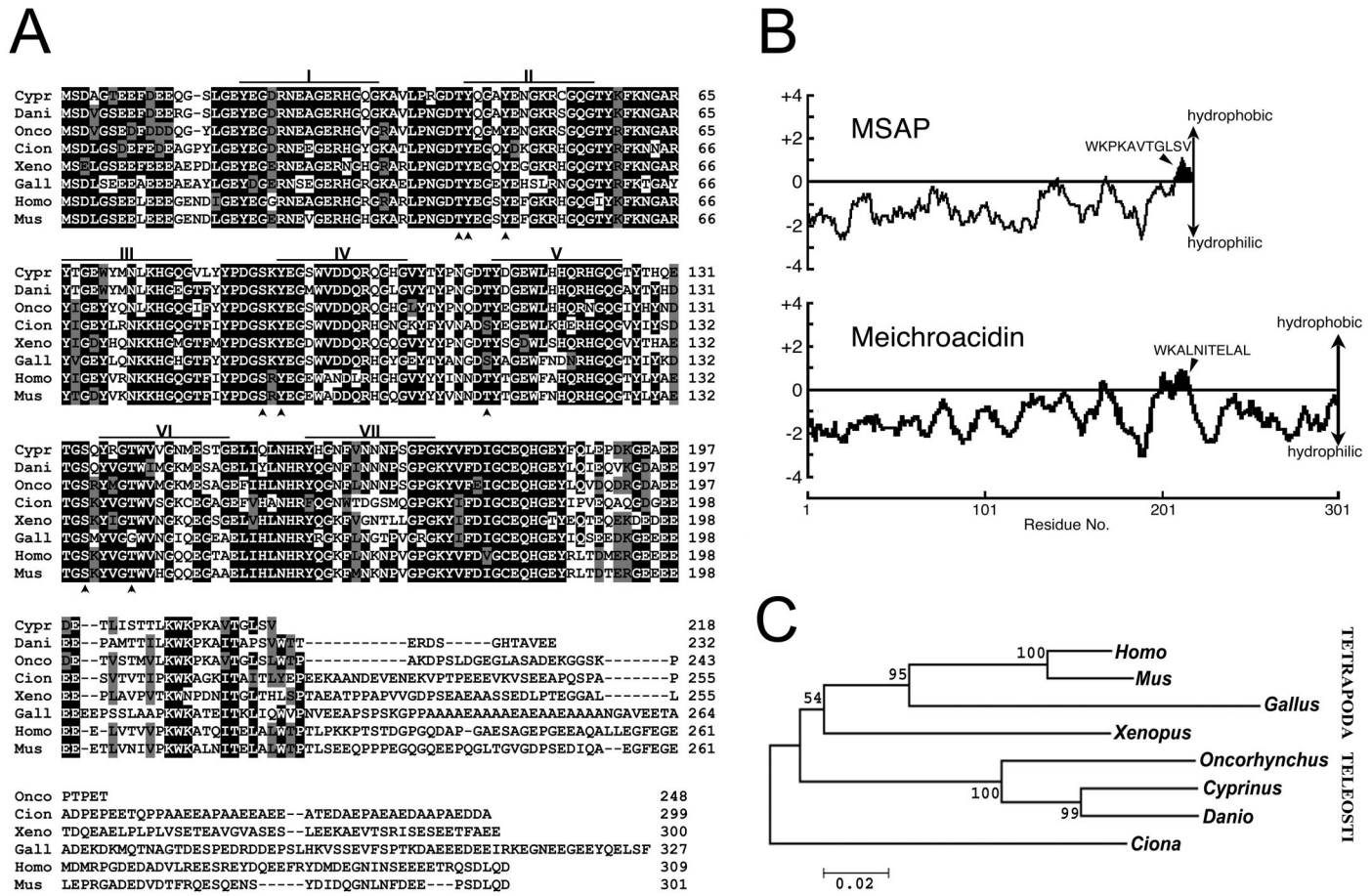

FIG. 1. Sequence analysis of MSAP/meichroacidin homologs. **A**) Alignment of MSAP/meichroacidin amino acid sequences of common carp (Cypr), zebrafish (Dani), rainbow trout (Onco), ascidian (Cion), mouse (Mus), human (Homo), chicken (Gall), and clawed frog (Xeno). Identical residues and residues with conserved substitutions (>60%) are shaded in black and gray, respectively. MORN motifs are lined and numbered above. Potential phosphorylation sites that conserved among these species were highlighted with arrowheads. Gaps marked with dashes were introduced to maximize the alignment. Some sequence data are available under GenBank AY_327474 (Cypr), NM_025290 (Mus), and NM_080860 (Homo). The other sequences without accession numbers were derived from the combination of partial cDNAs deposited in the EST database (see Supplemental Table 1, available at: http://cell.lifescience.ntu.edu.tw/faculty/Supp_Huang/supplementaltable1.htm). **B**) Hydropathy plots of carp MSAP (upper) and mouse meichroacidin (lower). Hydrophobic regions in the plots are filled and the corresponding amino acids are indicated. **C**) Phylogenetic analysis of MSAP/meichroacidin homologs. The branch values refer to the bootstrapped frequencies (percentage) of a given branching pattern. The bar listed below indicates the relative distance.

scrutinized by immunofluorescence microscopy. In carp, the seminiferous tubule of testis consists of a large number of cysts. The germ cells within each cyst are synchronous in developmental stage [2, 17], and hence the developmental stage of germ cells can be easily followed. During the breeding season of carp, the late spermatids and spermatozoa are predominant whereas the germ cells of earlier stages, such as primary spermatocytes, secondary spermatocytes, and early spermatids, are sparsely distributed in the seminiferous tubule [18, 19] (Figs. 4 and 5A). Spermatogonia could be occasionally found near the periphery of the seminiferous tubule (Fig. 4C). The nuclei of germ cells became more condensed and smaller as development proceeded (see Supplemental Table 2, available at: http://cell.lifescience.ntu.edu.tw/faculty/Supp_Huang/supplementaltable2.htm).

Immunofluorescence data showed that MSAP was especially enriched in the cysts filled with late spermatids (Fig. 4G) and spermatozoa (Fig. 4, A and C–H). No signal was detected by using normal rabbit IgG (Fig. 4B). On the other hand, the germ cells of earlier stages, including spermatogonia, primary spermatocytes (Fig. 4C), secondary spermatocytes (Fig. 4D), metaphase spermatocytes (Fig. 4E), and early spermatids (Fig. 4F), had no significant signal detected. MSAP was also not present in somatic cells, such as peritubular cells, blood cells, interstitial cells, and Sertoli cells (Fig. 4A, and data not shown).

MSAP appeared as filamentous distributions in late spermatids and spermatozoa (Fig. 4, G and H), suggesting that the molecule may be localized to the differentiating flagellum. This possibility was examined by double labeling the testis with antibodies against MSAP and β-tubulin. As shown in Figure 5, the β-tubulin signal also appeared as filamentous patterns in late spermatids and spermatozoa that overlapped with the signal of MSAP, although some β-tubulin was also detected in the periphery of the nuclei of spermatocytes. Note that such filamentous signals were especially prominent in the spermiating lumen (Fig. 5A, Lm), strengthening the notion that MSAP is localized to the flagellum of spermatozoa. Immunostaining of isolated spermatozoa further demonstrated that both β-tubulin and MSAP were colocalized to the flagellum (Fig. 5B). It is noteworthy that MSAP, but not β-tubulin, was also detected in the basal bodies (Fig. 5B, arrows).

Immunolocalization data showed that carp MSAP started to express in late spermatids and accumulated in sperm fla-

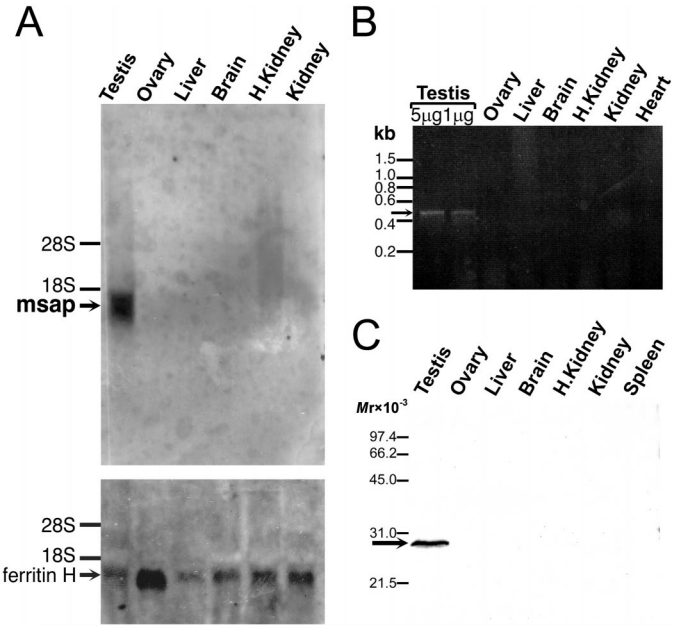

FIG. 2. Tissue specificity of *MSAP* expression. **A**) Northern blotting of RNA from indicated carp tissues with *MSAP* cDNA probe. The lower panel shows a duplicate blot probed with *ferritinH* cDNA probe. The positions of 28S and 18S ribosomal RNAs are indicated on the left. **B**) RT-PCR. Total RNA from the indicated tissues were reverse transcribed and then PCR amplified with *MSAP*-specific primers. A predicted product of 461 bp is indicated by an arrow. Five micrograms of RNA was used except for one sample of testis, wherein 1 μg of RNA was also analyzed. **C**) Immunoblot analysis of various carp tissues with anti-MSAP antibody. Proteins (75 μg) from indicated tissues were loaded. The position of MSAP is indicated by an arrow. Protein standards are shown on the left.

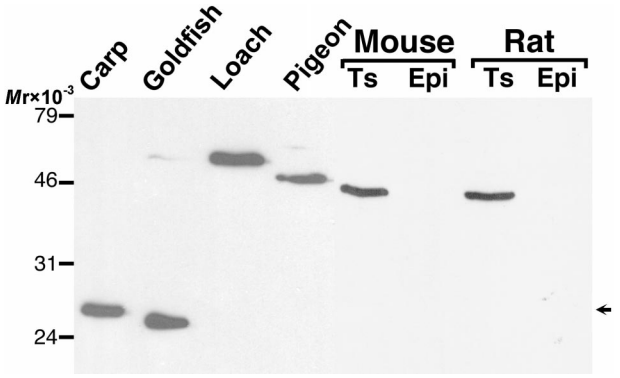

FIG. 3. Zoo-Western blot of vertebrate MSAP/meichroacidin homologs. Testicular proteins from carp (10 μg), goldfish (20 μg), loach (80 μg), pigeon (80 μg), rodents (Ts, 80μg each), and proteins of rodent caudal epididymes (Epi, 80 μg each) were immunoblotted with anti-MSAP. The position of carp MSAP is indicated by an arrow. Protein standards are shown on the left.

gella. This expression pattern is completely different from that in mouse testis, where the expression of meichroacidin, as noted in Figure 3, is limited to germ cells of the earlier spermatogenic stage and associated with meiotic chromosomes and spindles [9]. The discrepancy of the expression pattern between MSAP and meichroacidin strongly suggests functional divergence of the homolog in vertebrate spermatogenesis.

Biochemical Characterization of Sperm MSAP

Because carp MSAP is especially abundant in sperm flagella (Fig. 4), a variety of fractionation procedures were designed to explore the possible relationship of MSAP with the sperm flagellum. Spermatozoa were extracted by NP-40 and the residual pellets were further extracted by NP-40 under sonication. These two extracts were denoted as NP-soluble and NP-insoluble fractions, respectively. As shown in Figure 6, MSAP was not present in the NP-soluble fraction, yet a large quantity of MSAP, comparable with those from the total sperm extract, was found in the NP-insoluble fraction (Fig. 6, A and B). This detergent-resistant property strongly suggests that MSAP is not associated with plasma membrane and raises the possibility that MSAP may be a structural component of axoneme. A similar situation was also observed in α- and β-tubulins, the major components of axoneme. Both α- and β-tubulins were especially enriched in the NP-insoluble pellet, but only a few of them were present in the NP-soluble fraction (Fig. 6, C and D). Another cytoskeletal protein, β-actin, was also examined. However, no actin was detected in any fraction (data not shown), indicating that tubulin may be the only cytoskeletal component coextracted with MSAP in carp sperm. Coextraction of MSAP with tubulin implies that MSAP may associate with the flagellar microtubule. In contrast with MSAP, a considerable amount of the axonemal motor subunit, dynein intermediate chain (dyneinIC), could be easily extracted by NP-40 without sonication (Fig. 6E).

Because sperm MSAP is exclusively present in the NP-insoluble pellet, this pellet was thus extracted by urea and analyzed using 2D electrophoresis. In combining with immunoblotting, four MSAP spots were identified. They have a similar size of 25 kDa, yet they differ in isoelectric points (pI), ranging from 4.8 to 5.3 (Fig. 7). Due to little size difference between these pI variants in the 2D gel, an explanation for this pI variation is that sperm MSAP may be differentially modified by small charged groups, such as phosphates. In fact, a number of potential phosphorylation sites, which were predicted on the basis of the protein sequence and structure [20], are present in MSAP (Fig. 1A). Therefore, the pI variations of MSAP may be attributed to the result of differential phosphorylation. Note that mouse meichroacidin appeared as a single spot (pI 4.9) in the 2D blot [9], suggesting different regulations of the homologous molecules between fish and mammal.

Identification of Septin7 as A MSAP-Interacting Partner in Carp Sperm

Due to the MSAP association with the detergent-resistant, tubulin-enriched sperm pellet (NP-insoluble fraction), it is speculated that MSAP may complex with some proteins within the pellet. To test this speculation, an experiment that combines 2D electophoresis and blot overlay with rMSAP was adopted to analyze the proteins that interact with MSAP in carp sperm. Numerous spots were positively stained in the 2D-blot overlay analysis, but tubulins were not among them (Fig. 8, A and B). The results indicate that the association of MSAP with the flagellar microtubule might be indirect. Most of the rMSAP-interacting proteins are basic (pI 6–10), suggesting that sperm MSAP may complex with some basic proteins to associate with the flagellar microtubule. To characterize the nature of these MSAP-interacting candidates, some positive spots (Fig. 8, numbered spots) were selected and digested by trypsin and then analyzed by LC MS/MS. The data of the LC MS/MS revealed two spots (spots 5 and 6 shown in Fig. 8) that contained sequences (Fig. 8C) matching hCDC10/septin7 [21],

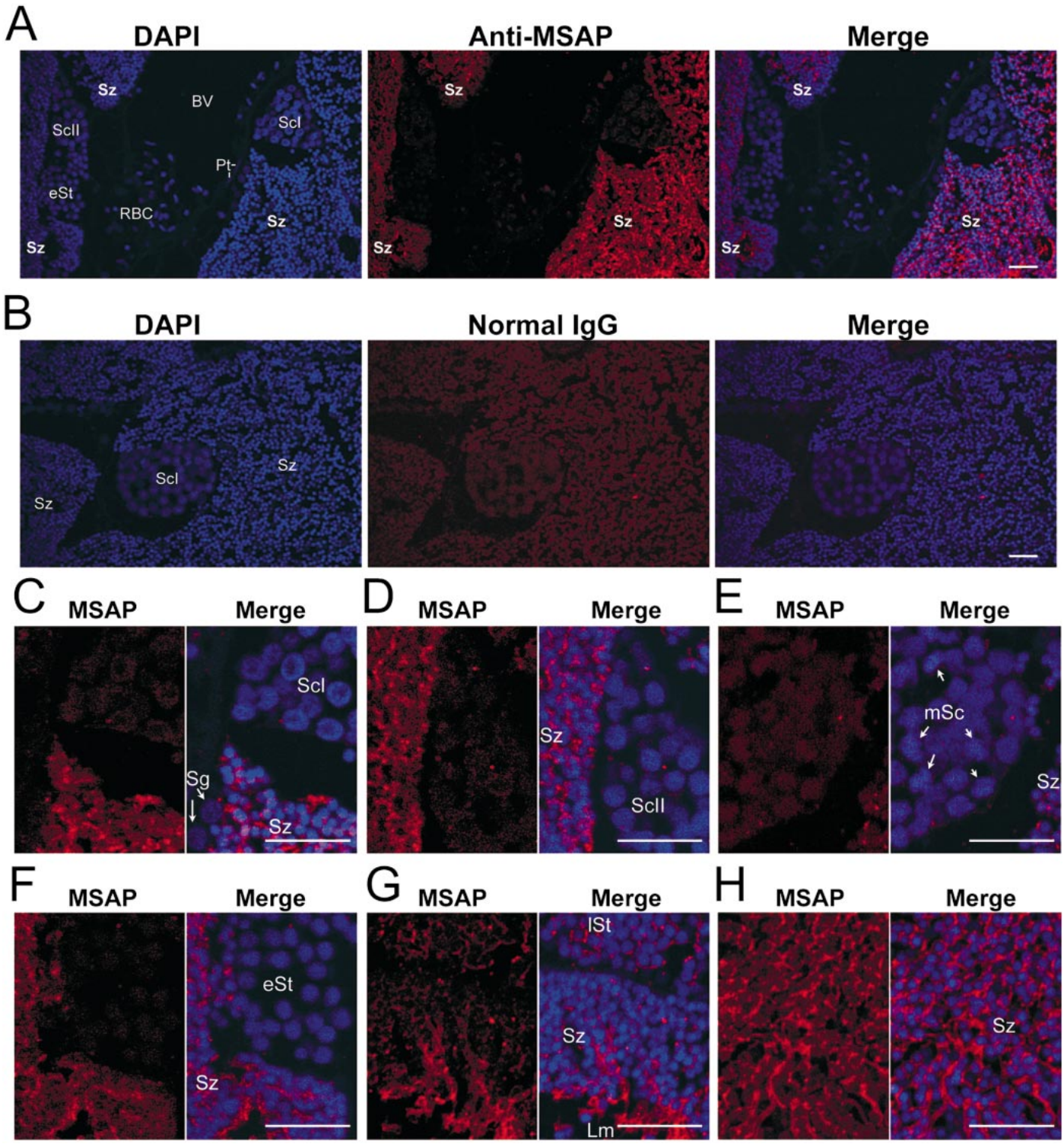

FIG. 4. Immunolocalization of MSAP in carp testis. **A** and **B**) Sections labeled by anti-MSAP (**A**) or by normal rabbit IgG (**B**) counterstained with DAPI. **C**–**H**) MSAP immunostaining patterns of germ cells of various developmental stages: (**C**) Spermatogonia (Sg) and primary spermatocytes (ScI); (**D**) secondary spermatocytes (ScII); (**E**) meiotic spermatocytes (mSc); (**F**) early spermatids (eSt); (**G**) late spermatids (lSt); (**H**) spermatozoa (Sz) in the lumen of seminiferous tubule. DAPI-merged images are shown as indicated. Lm, Lumen of seminiferous tubule; Pt, peritubular cell; RBC, red blood cell. Bar = 20 μm.

while the deduced peptides of other spots (numbers 1–4 and 7–10) did not significantly match any known protein.

To verify whether the two proteins that interacted with rMSAP were indeed septin7, immunoblotting using human septin7 antibody (anti-hSEPT7) was performed. As shown in Figure 8D, a cluster of 10 spots with discrete sizes (48, 50, and 52 kDa) and pI values (8.4~8.9) that included spots 5 and 6 (Fig. 8B) were stained. Hence, septin7 is considered to be an interacting partner of MSAP in carp sperm. To test whether MSAP and septin7 did actually colocalize in vivo, isolated spermatozoa were labeled with anti-hSEPT7. Unlike the staining pattern of anti-MSAP, sperm flagella were

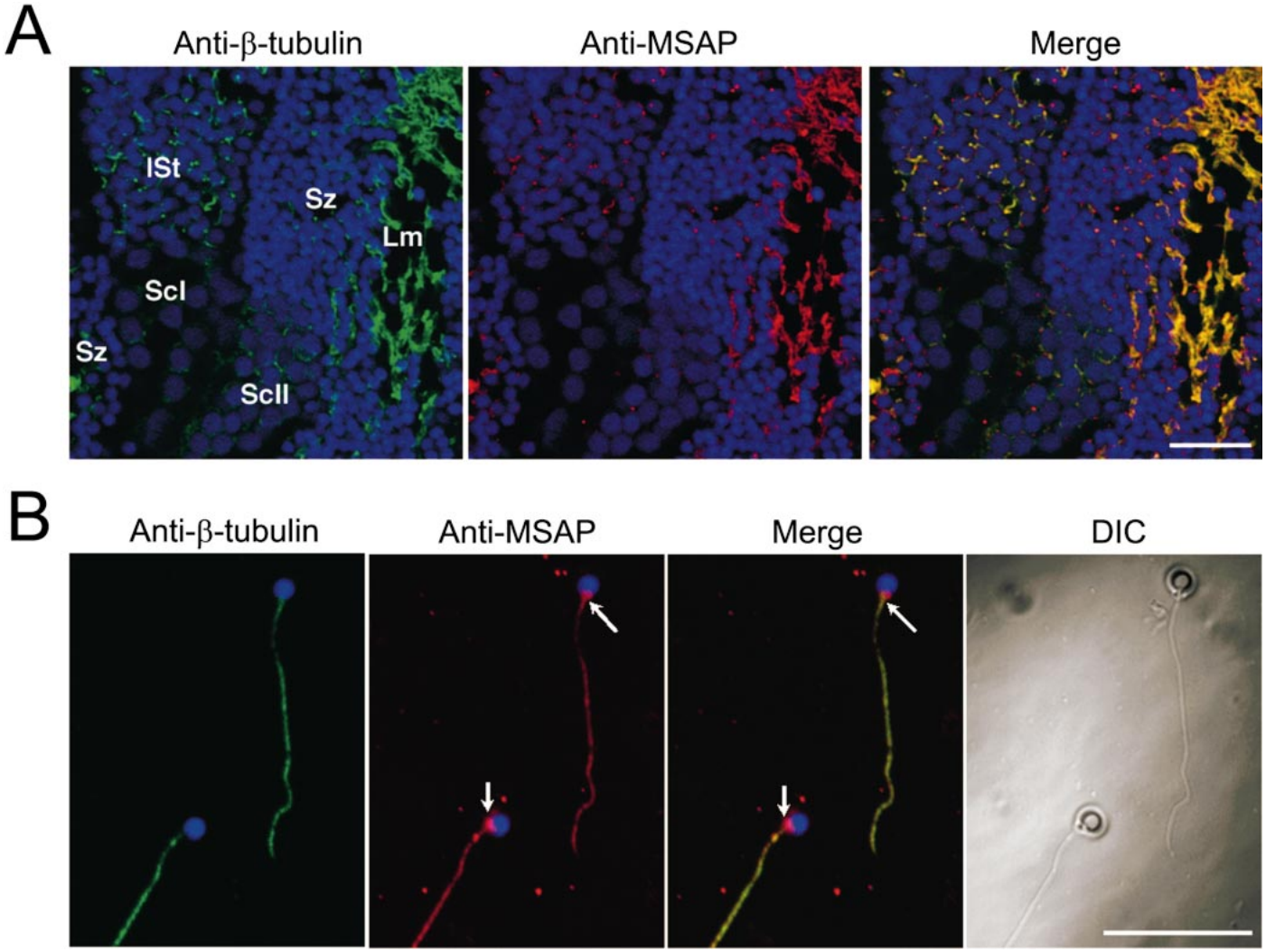

FIG. 5. Dual immunofluorescence localization of MSAP (red) and β-tubulin (green) in carp testis (**A**) and spermatozoa (**B**). Nuclei were stained by DAPI (blue). Merged and differential interference contrast (DIC) images are shown as indicated. Arrows indicate MSAP-specific signals in the basal body. ScI, primary spermatocytes; ScII, secondary spermatocytes; lSt, late spermatids; Sz, spermatozoa; and Lm, lumen. Bar = 15 μm.

not stained by anti-hSEPT7. However, septin7 antigen is exclusively present in the basal body (Fig. 8E, arrows), which is consistent with the partial MSAP staining in carp spermatozoa (Fig. 5B, arrows). Taken together, these data suggest that septin7 might associate with a subset of MSAP in the basal body of carp spermatozoa.

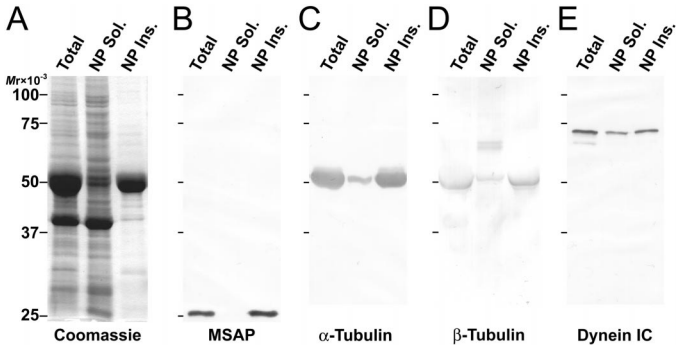

FIG. 6. Immunoblot analysis of fractionated sperm proteins. **A**) Coomassie blue staining. **B**–**E**) Immunoblotting with antibodies against MSAP (**B**), α-tubulin (**C**), β-tubulin (**D**), and dyneinIC (**E**), respectively. The total lysate (Total), NP-soluble (NP Sol), and NP-insoluble (NP Ins) fractions (see *Materials and Methods*) from 8×10^7 spermatozoa per lane were analyzed. Protein standards are shown on the left.

DISCUSSION

In this study, the carp *meichroacidin* homolog, *MSAP*, has been cloned and further characterized. *MSAP*/*meichroacidin* homologs are widely found in organisms ranging from *Chlamydomonas* to mammals, suggesting that the *MSAP*/*meichroacidin* gene may evolve from a common ancestor. In carp, MSAP starts to appear in late spermatid and is retained in the basal body and the flagellum of sperm. This fact implies that MSAP may be involved in the metamorphosis of the spermatid or flagellar activity, which are completely different from the functional implication of mouse meichroacidin in spermatogenesis [9]. Furthermore, different 2D patterns between MSAP and meichroacidin suggest the differential regulation of the homologous molecules in germ cell development between fish and mammal. Differences between MSAP/meichroacidin homologs also raise questions about genetic and functional divergence of the gene in vertebrates.

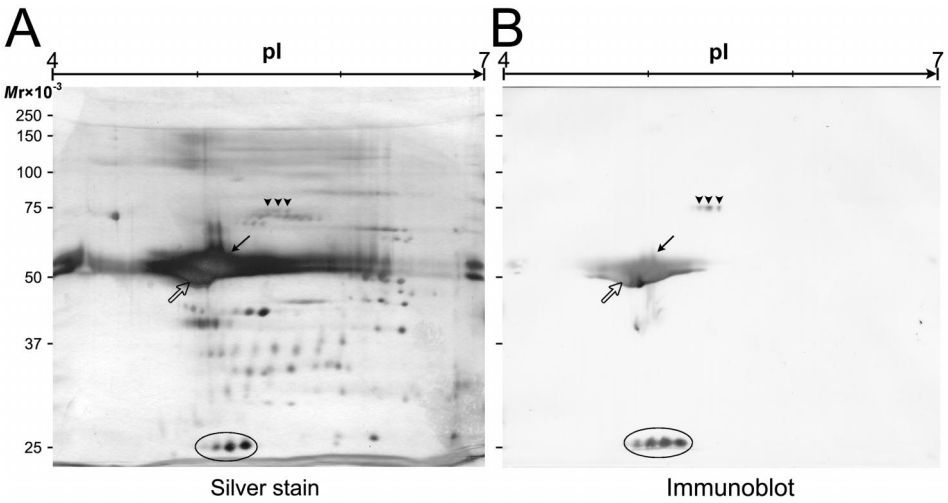

FIG. 7. Identification of sperm MSAP in 2D gel electrophoresis. **A**) Silver staining. **B**) Immunoblotting with antibodies against MSAP, α-tubulin, β-tubulin, and dyneinIC. The NP-insoluble proteins from 2×10^8 spermatozoa were analyzed. The locations of MSAP (encircled), β-tubulin (open arrow), α-tubulin (arrow), and dyneinIC (arrowheads) are indicated. The pH range of the first dimensional IEF is indicated above and protein standards are shown on the left.

Evolutionary Significance of MSAP/Meichroacidin *Genes*

Carp MSAP appears during late spermiogenesis and is assembled into sperm flagellum. Does *MSAP*/*meichroacidin* family function in sperm flagellum originally and then shift to another function in more advanced vertebrates? Sequence analysis revealed that carp MSAP shares a higher identity than mammalian *meichroacidins* to urochordate (*Ciona*) homolog (see Supplemental Table 1, available at: http://cell.lifescience.ntu.edu.tw/faculty/Supp_Huang/supplementaltable1.htm), indicating that *MSAP* may retain more ancient characters (pleisiomorphs) than do the mammalian meichroacidin. By comparison, the flagellar structure of carp spermatozoa resembles more those of the remote vertebrate relatives, such as urochordate and cephalochordate, than do those of mammalian spermatozoa [1, 6, 22]. These lines of evidence favor the notion that the *MSAP*/*meichroacidin* family participates in the flagellar function in primitive vertebrates, then shifts to other functions in higher vertebrates. The present data imply that *MSAP*/*meichroacidin* family has diversified in both size and function during evolutionary radiation. Such divergence also occurs within closely related groups, such as carp (Cyprinidae) and loach (Cobitidae). Both of them belong to Cypriniformes, yet carp MSAP was much smaller than the loach counterpart (Fig. 3). In fact, a fast-evolving trend in sex-related genes, especially in male reproductive genes, had been proposed elsewhere [23–25]. Here, we provide a new example to support this hypothesis. Although the impact of the size difference on the function of these homologs remains to be investigated, the molecular evolution of *MSAP* genes may serve as an important reference for exploring the mechanisms of sperm ontogeny, the processes of speciation, or the evolution of reproductive strategies.

Alternatively, the evolutionary divergence between *MSAP* and *meichroacidin* may also originate from gene duplication of an ancestral gene. Whether a duplication of carp *MSAP* gene exists or not remains unclear. However, in this study, no other *MSAP*/*meichroacidin*-related gene product could be detected in carp or other vertebrates tested (Figs. 2 and 3). In addition, GenBank database searching revealed that *meichroacidin* might be derived from a single gene locus in mouse (chromosome 17A3.3) and human (chromosome 21q22.3). These results supported the speculation that the functional difference between *MSAP* and *meichroacidin* might originate from divergent evolution, but not duplication, of an orthologous gene.

Possible Roles of MSAP and Septin in Carp Sperm

Immunofluorescence studies showed that MSAP is colocalized with tubulin in the flagellum of spermatozoa (Fig. 5), suggesting that MSAP is an axonemal component. A similar conclusion was reached by biochemical approaches. Sperm MSAP is hardly extracted by nonionic detergent, indicating that MSAP is neither a membranous nor a cytosolic protein. On the other hand, MSAP could be solubilized under much harsher conditions, such as sonication or urea extraction. A similar situation also occurred in α- and β-tubulins, the major components of the flagellar axoneme (Figs. 6 and 7). Note that the flagellar axoneme is a very stable protein complex that is highly resistant to detergent extraction [26, 27]. The detergent-resistant property of MSAP also suggests that the protein is a structural component of the axoneme.

Because MSAP expression coincides with the timing of late spermiogenesis, MSAP may play a role in flagellar assembly. MSAP/meichroacidin homologs contain seven MORN repeats, which are also present in the members of the junctophilin (JP) family from a wide range of organisms [10, 28, 29]. MORN motifs had been demonstrated to be required for targeting JP to plasma membrane and for the formation of the intracellular membrane junction [10]. Inferring from these functional implications, a model for MSAP-mediated flagellar assembly is thus proposed. In spermatids and spermatozoa of certain teleost species, a large amount of vesicles were found between the posterior portion of the nucleus and the proximal end of the flagellum that were thought to be responsible for the formation of the flagellar membrane [22, 30, 31]. We assume that MSAP may associate these vesicles during late spermiogenesis. At the beginning of flagellogenesis, these vesicles may fuse with the membrane of the flagellum growing site via MORN repeats and anchor to the axoneme via the C-terminal region of MSAP. Hence, MSAP may act as a bridge or adaptor spanning between the membrane and the axonemal machinery as the flagellum elongates.

In addition to the involvement of MSAP in flagellar differentiation, other possible functions of MSAP in flagellar activity should also be considered. For example, it has been proposed that JP proteins may mediate Ca^{2+} channel-dependent excitation-contraction coupling in muscle cells [10]. Whether a similar pathway mediated by MSAP in carp sperm remains elusive; however, Ca^{2+}-mediated initiation of carp sperm motility had been demonstrated [32].

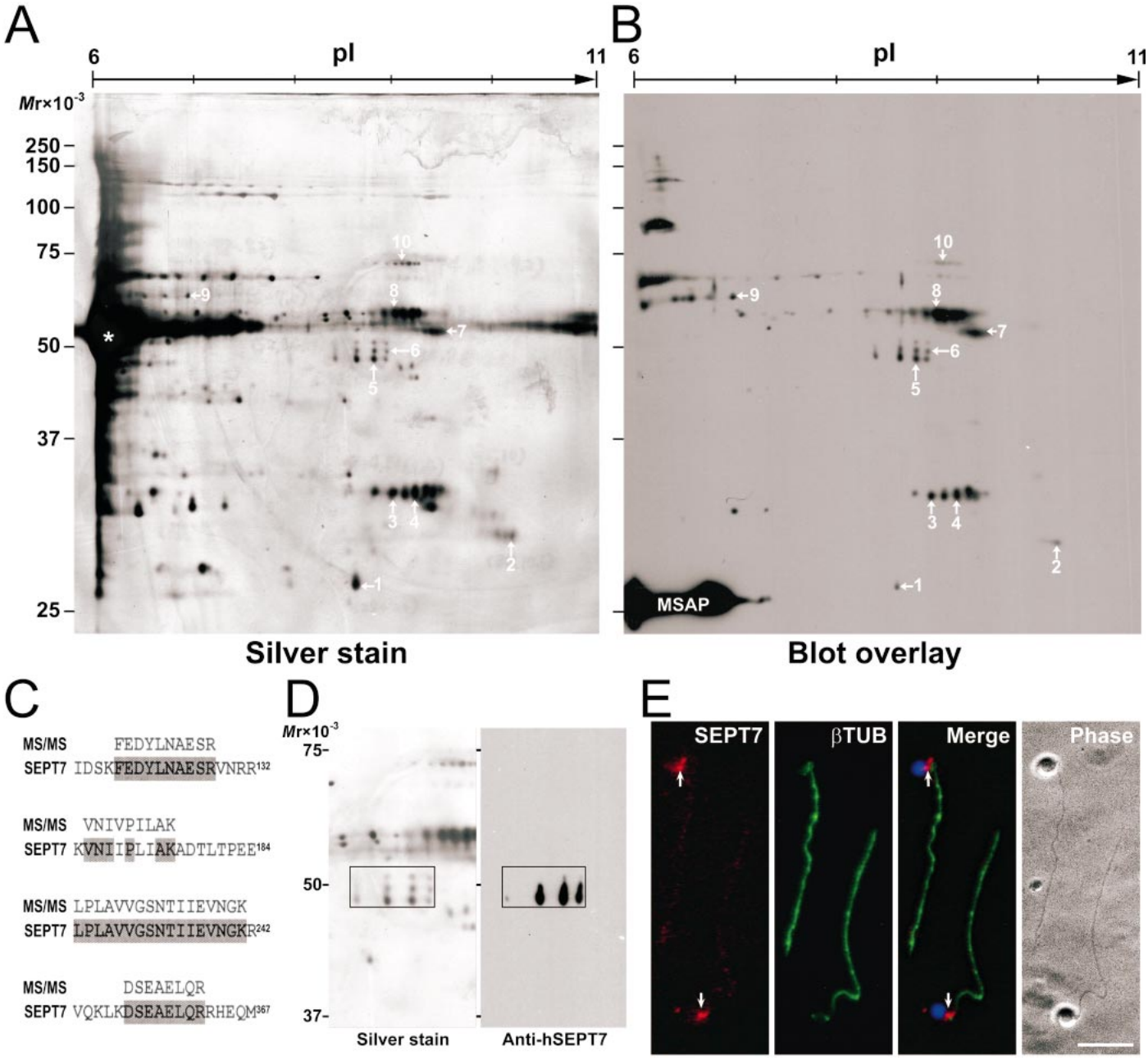

FIG. 8. Proteomic analysis of the rMSAP-interacting proteins and immunolocalization of septin7 in carp sperm. **A** and **B**) The NP-insoluble proteins from 4×10^8 spermatozoa were analyzed. **A**) Silver staining. **B**) Two-dimensional blot overlay analysis. Note that rMSAP did not interact with tubulins (asterisk in **A**). The smear in the lower-left corner (MSAP) of the blot could be the residual MSAP that did not run out of first dimensional IEF (pH 6–11), because an identical situation was also found in the mock control blot. Ten spots (indicated numerically) were selected for trypsin digestion and subjected to LC MS/MS analysis. **C**) The tryptic peptides (MS/MS) of spots 5 and 6 match the sequences of human septin7 (SEPT7; GenBank NP_001779). Matched residues are shaded in gray. **D**) Immunoblot of sperm protein using an antibody against human septin7 (right panel). Silver staining is shown in the left panel. The positive spots are boxed. The sperm protein used for analysis is the same as in **A**. **E**) Localization of septin7 in carp sperm. Spermatozoa that counterstained with DAPI were double labeled by anti-hSEPT7 (SEPT7) and anti-β-tubulin (βTUB). Merged and phase contrast images are shown as indicated. Arrows indicate the locations of septin7. Bar = 10 μm.

As noted above, the vesicular structures in the proximal portion of the flagellum of carp sperm may possibly act as Ca^{2+} reservoirs as well. Because the overall structures of MSAP and JP proteins share some extent of similarity [10](Fig. 1A), whether MSAP has functions similar to JP protein that are involved in the formation of the junctional membrane complex or Ca^{2+}-dependent signaling in carp sperm deserves further investigation.

Septin7 was found to interact with MSAP and colocalize with MSAP in the basal body of carp sperm (Figs. 5B and 8). The protein is a member of the septin GTPase family that has a ubiquitous role in cytokinesis in organisms ranging from yeast to metazoa [11, 33]. Accumulating lines of evidence indicate that septins may also participate in other cellular events, such as vesicle trafficking, exocytosis, membrane remodeling, and polarity establishment, all of which are related to cellular differentiation [34–36]. These findings lead to a prediction that septins may be involved in the MSAP-mediated vesicle targeting or flagellar assembly as inferred from the following lines of evidence. In

nervous system, a septin GTPase activity-dependent regulation of synaptic vesicle fusion had been proposed from the study of septin5 [37]. The conserved polybasic motif of septins required to interact with phosphoinositide lipids had been demonstrated [38]. Next, several septins, including septin7, were associated with the exocyst complex and displayed a highly polarized distribution in differentiating neurons, reflecting that the septin complex might be involved in local secretion or membrane fusion/addition during neurite outgrowth [39, 40]. Interestingly, exocyst and septin complexes were colocalized within the microtubule-organizing center [13], the organelle that is structurally similar to the basal body. As found in the present study, septin7 is localized in the basal body of carp sperm. Taken together, these facts point out that septin may be involved in the formation of the microtubule-based processes, such as neurite and flagellum, during neuronal and germline differentiations. Consistent with this prediction, a branched vesicular structure, termed the fusome, was actually found in *Drosophila* to associate with the septin-ring structure (ring canal) throughout spermatogenesis. Associations of the ring canal and fusome were retained in the distal tips of elongating spermatids, suggesting the relationship of septins with flagellar organization [12]. Although the correlation of septins with the basal body remains to be established in this animal model, the data more or less strengthen our functional prediction of septins in local membrane addition during flagellar formation.

The molecular mechanisms of septin function remain poorly understood. However, based on their heteropolymerizing [39, 41, 42] and membrane-associating properties [38, 43], septins have been thought to act as a mechanical scaffold for protein recruitment and a diffusion barrier to prevent discrete membrane domains or their associating components from free lateral movement [11, 33]. Thus, the localization of septin7 in the sperm basal body suggests that the molecule or its protein complex may act as a gatekeeper on separating flagellar components, such as MSAP or other axonemal proteins, from the sperm head or to specify the growing site of flagellum. In addition, numerous septin-associating proteins, such as the components of kinase cascades, the phosphatase, and the microtubule orientation protein, have been identified in yeast [35, 44–47], indicating that septins may form a regulatory center for coordinating cellular morphogenesis. As resolved by 2D electrophoresis analyses, both septin7 and MSAP have several pI variants (Figs. 7 and 8), suggesting that both of them may be regulated by posttranslational modification. The modifications may have important functions for targeting of flagellar components, such as MSAP or other axonemal proteins, or for oriented growth of the flagellum.

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