A novel terminal ampullae peptide is involved in the proteolytic activity of sperm in the prawn, *Macrobrachium rosenbergii*

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Abstract

As the distal part of the crustacean male reproductive tract, terminal ampullae play important roles in sperm development and storage of mature spermatophores. In the present study, the novel gene terminal ampullae peptide (TAP) was cloned from terminal ampullae of the prawn, *Macrobrachium rosenbergii*. The cDNA sequence consists of 768 nucleotides, with an open-reading frame of 264 nucleotides which encodes a putative 88-amino acid precursor protein with a 17-amino acid residue signal peptide. Western blotting and immunohistochemical analysis revealed that TAP was distributed on terminal ampullae and sperm, and its expression was related to gonad development. To elucidate the functional role of TAP *in vivo*, we disrupted the TAP gene by RNA interference (RNAi) and evaluated the effect on fertility and several sperm parameters. Although there was no difference in fertility between RNAi-induced prawns and controls, RNAi treatment decreased the sperm gelatinolytic activity and blocked proteolytic activity on the vitelline coat. These data provide evidence that TAP participates in regulating sperm proteolytic activity, and performs a crucial role in sperm maturation and degradation of the vitelline coat during fertilization.

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Introduction

Fertilization is a complex physiological process that involves a precisely ordered set of molecular and cellular events, including generation and maturation of haploid gametes, sperm penetration of the egg coat, and final fusion of oocyte–sperm cell membranes (Vacquier 1998, Wassarman 1999, Stein *et al.* 2006). In most fertilization models, motile male gamete systems have been well studied. In general, testicular sperm become competent to fertilize only after undergoing a series of changes during their transit through the male and female reproductive tracts; in mammals, these changes are known as maturation and capacitation respectively (Yanagimachi 1994). In the male reproductive tract, the epididymis synthesizes a large numbers of products and releases them into the lumen of the tract (Kinura *et al.* 1998, Gatti *et al.* 2004, Dacheux *et al.* 2005, Sipilä *et al.* 2009). These secretions are arranged around or on the different parts of the sperm, and affect sperm motility and proteolytic ability (Kinura *et al.* 1998, Cornwall 2009, Sipilä *et al.* 2009). Many epididymis tissue and development-specific genes that potentially contribute to specialized functions in sperm maturation or oocyte–sperm fusion have been identified and characterized (Penttinen *et al.* 2003, Gatti *et al.* 2004, Dacheux *et al.* 2005, Thimon *et al.* 2007). For example, P34H expressed in the epididymis progressively accumulates in the

sperm acrosomal region, and participates in zona pellucida recognition and binding (Saez *et al.* 2003). Furthermore, TESP5, a gelatin-hydrolyzing serine protease, is involved in the acrosome reaction and sperm penetration of the zona pellucida in the mouse (Honda *et al.* 2002). TESP5 is initially synthesized in the testis, then is converted into active enzymes during sperm transport in the epididymis and localized on the sperm plasma membrane (Honda *et al.* 2002). In addition, several protease inhibitor families present in the epididymis, including the cystatin (Syntin & Coenwall 1999, Wassler *et al.* 2002, Hsia & Cornwall 2003), Kunitz (Penttinen *et al.* 2003), Kazal (Jalkanen *et al.* 2006), WAP (Jalkanen *et al.* 2006), and serpin families (Laurell *et al.* 1992, Law *et al.* 2006), interfere with the activity of enzymes on sperm and block fertilization.

In contrast, nonmotile male gamete systems are poorly understood, particularly in decapod crustaceans such as crayfish, crab, lobster, and prawn. Spermiogenesis, sperm maturation, and fertilization in decapods greatly differ from those of motile male gamete systems (Lynn & Clark 1983a, Shigekawa & Clark 1986, Bauer & Min 1993, Alfaro *et al.* 2007, Kang *et al.* 2008). First, decapod sperm are atypical and nonflagellate. A decapod sperm cell consists of a spherical main body with a variable number of radiating appendages (Talbot & Summers 1978, Medina 1994, Medina *et al.* 1994,

Tudge *et al.* 1998, Kim *et al.* 2003, Kang *et al.* 2008). They are nonmotile and invariably enclosed in sperm packets called spermatophores before being transferred to the female during mating. A spermatophore is an uninterrupted column that is formed by secretory epithelium products in the terminal ampullae of the male reproductive tract (Lynn & Clark 1983a, Bauer & Min 1993, Tudge *et al.* 1998). Secondly, crustacean fertilization is external, occurring as the eggs are released from the female gonopores and pass posteriorly over the spermatophore mass (Lynn & Clark 1983b). However, few data are available about the genes and proteins that participate in sperm maturation of crustacean decapods.

In recent studies, some male reproductive tract peptides from the giant fresh water prawn, *Macrobrachium rosenbergii*, were examined. For instance, MRPINK, a Kazal-type peptidase inhibitor that is uniquely expressed in the male reproductive tract (Cao *et al.* 2007), was found to have an inhibitory effect on the gelatinolytic activity of prawn sperm and to specifically block the activity of *M. rosenbergii* sperm gelatinase (MSG), a sperm gelatinase (Li *et al.* 2008). Mr-IAG, an insulin-like peptide, is the androgenic gland (AG)-specific peptide in *M. rosenbergii*. Silencing of Mr-IAG led to the arrest of testicular spermatogenesis and spermatophore development in the terminal ampullae of the sperm duct (Ventura *et al.* 2009). Much more research is needed to understand the molecular mechanisms of sperm maturation and fertilization in crustaceans. The terminal ampullae are greatly dilated portions of the distal male reproductive tract in decapods, and they perform important functions on sperm maturation, such as secretion of epithelium products surrounding the sperm and storage of the mature spermatophore in a thick layer of muscle (Lynn & Clark 1983b, Pochon-Masson 1983). Consequently, identifying and characterizing the functions of terminal ampullae-specific proteins are important research goals.

In the present study, we identified a novel terminal ampullae peptide (*TAP*) gene in *M. rosenbergii* by screening a subtracted library. The mRNA expression and protein distribution were well illuminated. Furthermore, *in vivo* knockdown of the *TAP* gene by RNA interference (RNAi) was performed, and changes in various parameters related to reproductive function were evaluated. The influence of this knockdown on sperm gelatinolytic activity and proteolytic activity on the vitelline coat were detected.

Results

Gene screening and identification of *TAP*

To study the molecular mechanism of sperm maturation regulated by terminal ampullae in *M. rosenbergii*, a suppression subtractive hybridization (SSH) library was constructed using mRNA from the adult male

reproductive tract. The dsDNA from terminal ampullae and vas deferens of *M. rosenbergii* were used as tester and driver respectively. Forty-seven clones were obtained from the SSH library enriched for the terminal ampullae-specific transcripts. One of them was found to be terminal ampullae-specific and was named *TAP*. The cDNA sequence contains 768 nucleotides, including an open-reading frame of 264 nucleotides which encodes an 88-amino acid peptide with a molecular weight of 10.165 kDa, pI 7.00. The deduced peptide consists of a putative 17-amino acid residue signal peptide and a 71-amino acid residue mature peptide. The amino acid sequence contains two predicted casein kinase II phosphorylation sites and a relatively high percentage of Leu and Cys (14.9 and 9.2% respectively; Fig. 1). The nucleotide sequence of *TAP* has been submitted to the GenBank database (accession number FJ595507).

TAP expression characterization and localization

As no signal was detected in northern blotting analysis, semi-quantitative RT-PCR was performed to characterize the tissue-specific expression pattern of the *TAP* gene using primers F2 and R1 (Table 1). The male reproductive tract of *M. rosenbergii* (Fig. 2A-a) is divided morphologically into three parts: testis, vas deferens, and terminal ampullae (Pochon-Masson 1983, Ro *et al.* 1990). Significant accumulations of mRNA were observed in terminal ampullae of the male reproductive tract, and fewer were observed in testis (Fig. 2A-b); mRNA expression was not detected in any other tissues, including vas deferens, ovary, hepatopancreas, heart, muscle, and thoracic ganglia. Thus, the results showed that the *TAP* gene was dominantly expressed in the terminal ampullae of the male reproductive tract.

In *M. rosenbergii*, there are three morphotypically differentiated developmental stages (small males, orange-claw males, and blue-claw males; Fig. 2B-a), which have been reported to be under the control of the AG hormone (AGH; Kuris *et al.* 1987, Okumura & Hara 2004). Our results revealed that the *TAP* transcript level was low in immature prawns, and a steady increase was observed as gonad development progressed (Fig. 2B-b).

In situ hybridization was used to determine the cellular location of *TAP* mRNA in testis and terminal ampullae. *M. rosenbergii* testis (Fig. 2C-a) consists of a large number of testicular lobules or cylinders compactly held together by connective tissues (Sagi *et al.* 1988). During spermatogenesis, the testicular lobule (Fig. 2C-a') contains a spermatogenic zone formed by germinal and sustentacular cells, and a large amount of sperm confined to a blind-ending cyst (Dougherty & Sandifer 1983, Sagi *et al.* 1988). *In situ* hybridization results showed that the distinct signals were observed in spermatogenic cells of testicular lobules (Fig. 2C-b, b'), and no signal was detected in the testis hybridized with a sense control probe (Fig. 2C-c, c').

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nt 1   GTATGCTGCCGGAATTTAGCTGAGAAGTCATCTTGGTTGTGCCCATCCCCGGACGAGG
nt 61  ACGAAGAAGAAGAAACGTAACATGAAGTGGTTCTCTCTTTTCTTGTGATGGCACTGGT
aa 1   M K W F S L F L L M A L V
nt 121 CTACGTGTCCGGTCAACAGAGCGTCTATGACAAGAGTGAAGAAGAACAAATAGAGATTCT
aa 14  Y V S G Q Q S V Y D K S E E E Q I E I L
nt 181 TGGTCCTCTGCTCAGAAAATTGCTGATGCCAGACCAGTCCCGTCTACCAGATCCACCA
aa 34  G P L L R K L L M P R P R P V Y Q I H Q
nt 241 GCTTCTGAGGGTGCACGTGCAGAAATGCTGCAACTCGCAGCCTATGATGGACTGCTGCAC
aa 54  L L R V H V Q K C C N S Q P M M D C C T
nt 301 ACCTGCTCTCTGCTGCGACATGAACCTGGAATGCTGCGAGAAATAGAGAAAACCTTTTGT
aa 74  P A L C C D M N L E C C E K *
nt 361 CATTAACACCCGAAGAATGCTGTGTGCTGTAGACCTATAACAGCTTGCTTAAGACGAAAT
nt 421 GATAATATTACGGGATATTATTTGAAAAGTGCAGAAAGTTTAAACGACGAATTAGTTTCA
nt 481 AATGTTCTTTTGCATCTTTAACACCTTTCTGTCTCTTACTACAGTAATAATTTTCCT
nt 541 AAGAATTTTAAGAATTGATTTTAACTTGAAAGCAGTTGAAAAGTCCTTTAATAAGTAAAT
nt 601 TTTTAAATGAATGTTTACGAAGATATATCGTAATTGGGTACGAGGAACTAAATTTAACA
nt 661 CTTCGCTTCCAATTTTCAGTTTCCATCATGATATTGGAACTGTTTAAATACCTTTTTC
nt 721 CAAGCGGCACAGTTACATTAAACATCTTATAACAGTACAAAAA

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Figure 1 Complete cDNA of *TAP* and its deduced amino acid sequence. The putative signal peptide sequence is marked in bold. The open boxes indicate the predicted casein kinase II phosphorylation sites. The asterisk indicates the position of the stop codon.

Terminal ampullae are the end dilated part of male reproductive tract, and the AG adhibits on the outer surface of terminal ampullae (Fig. 2D-a). The epithelial cells synthesize secretions to form spermatophore wall (Pochon-Masson 1983), and the whole spermatophore was stored and packaged inside the muscle layer (Fig. 2D-b). *In situ* hybridization results showed that clear signals were observed in the epithelial cells of terminal ampullae and in AG cells (Fig. 2D-c, c'). The signal was absent in muscle layer. Hybridization with a sense control probe produced no significant signal in the tissues (Fig. 2D-d, d').

In conclusion, our data showed that *TAP* mRNA was located specifically in the spermatogenic cells of testis, the epithelial cells of terminal ampullae, and AG cells. *TAP* was a terminal ampullae-dominating gene, and its transcription increased remarkably with AG maturation.

Western blotting and immunohistochemical analysis

Western blotting was performed to identify the distribution of *TAP* protein in the male reproductive tract. *TAP* protein was distributed exclusively on sperm and terminal ampullae; no signal was detected in other tissues (Fig. 3A). Analysis of the levels of *TAP* protein at different prawn developmental stages showed that along with gonad development, *TAP* protein increased steadily from the immature stage to the mature stage, and was abundantly accumulated in blue-claw prawns (Fig. 3B) in accordance with the trend of *TAP* mRNA in terminal ampullae. Immunohistochemical analysis further illustrated that *TAP* was located in secretory epithelial cells of terminal ampullae (Fig. 3C-a) and spermatophore sperm (Fig. 3C-a, a'). The signal was absent in the spermatophore wall. No hybridization signal was observed in sections incubated without anti-*TAP* antibody (Fig. 3C-b, b').

Table 1 Nucleotide sequences and positions of primers used in PCRs.

Primer	Length (bp)	Position	Direction	Sequence (5'–3')
F1	20	54–70	F	GACGAGGACGAAGAAGAAGA
F2	20	131–150	F	GGTCAACAGAGCGTCTATGA
R1	20	719–738	R	ATGTAAGTGTGCCGCTTGGA
R2	20	327–346	R	CTATTTCTCGCAGCATTC
RNAiF	20	98–117	F	GCTCTAGACTTTTCTGTTGATGGCACT
RNAiR	20	314–333	R	GGAATTCATTCCAGGTTTCATGTCGCA
GFPF	30	122–144	F	GGAATTCAACTACCTTAATTTTATTGTC
GFPR	28	461–480	R	GCTCTAGAGCCATTCCTTGGTTTGCTC
ISHF	20	101–121	F	CCCAAGCTTCTTGTGATGGCACTGGTC
ISHR	20	315–335	R	GGAATTCAGCATTCAGGTTTCATGTCG
18SF	22		F	GGTAGTGACGAAAAATAACAAT
18SR	21		R	CCCACCCAGTCCGGAAGTGA

F and R indicate the forward and reverse directions respectively. The underlined regions represent the adscititious recognition sequences of restriction endonucleases.

Figure 2 Expression characterization and localization of *TAP* mRNA. (A) *TAP* mRNA expression in different tissues.

(a) Diagram of the male reproductive tract. The tract was divided into three parts marked by the diagonal denotations. (b) Semi-quantitative RT-PCR. *18S* was used as an inner control: testis (T), vas deferens (VD), terminal ampullae (TA), ovary (OV), hepatopancreas (HP), heart (HT), muscle (M), and thoracic ganglia (TG). (B) The expression of *TAP* mRNA in the three morphotypically differentiated developmental stages.

(a) Examples of the three morphotypically differentiated developmental stages. Scale bar, 20 cm. (b) Semi-quantitative RT-PCR: small males (SM), orange-claw males (OC), and blue-claw males (BC). (C) *In situ* hybridization analysis of testis. (a, a') A general cross-section: testicular lobules (TL), sperm (S), spermatogenic cells (SC). (b, b') Antisense probe. The black arrows indicate positive signals. (c, c') Sense probe. Scale bars, 30 µm. (D) *In situ* hybridization analysis of terminal ampullae.

(a) A photograph of terminal ampullae with the adhesive androgenic gland (AG). (b) A hand drawn illustration of cross-section: muscle layer (M), spermatophore wall (SW), sperm (S), epithelial cell layer (E). (c, c') Antisense probe. The black arrows indicate positive signals. (d, d') Sense probe. Scale bars, 30 µm.

Thus, we concluded that TAP protein was secreted from the epididymal cells, and then transferred and localized on sperm in the terminal ampullae, and its expression increased steadily with gonad development.

Localization of *TAP* on sperm

The mature sperm of *M. rosenbergii* is a nonmotile, aflagellate, atypical cell that resembles an everted umbrella consisting of a base and a single spike. The main body (base) of the sperm is slightly cupped with a convex surface, and has a ruffled or irregular appearance of peripheral edges (Lynn & Clark 1983b). To determine the localization of TAP on sperm, individual sperm cells were taken from terminal ampullae and mounted on glass slides for immunoreactivity analysis. The immunofluorescence results showed distinct signals on the convex surface of the sperm base and spike (Fig. 4A-a) compared with control sperm (i.e. those not incubated with anti-TAP antibody; Fig. 4A-b). Moreover, transmission electron microscope immunohistochemical analysis revealed that vivid gold nanoparticles were exiguously scattered on the cap-like area of the convex surface of the base (Fig. 4B-a) and the spike of the sperm (Fig. 4B-b). No signal was observed on sperm incubated without anti-TAP antibody (Fig. 4B-c, d, e).

In vivo knockdown of *TAP* by RNAi in *M. rosenbergii*

RNAi was performed by dsRNA injection into mature male *M. rosenbergii* prawns. Both the *TAP* mRNA and protein levels were reduced to <10% compared with control samples (Fig. 5A-a, b). Moreover, immunohistochemical analysis further illustrated that TAP protein was greatly decreased in RNAi-induced prawns. Only a weak signal was detected on sperm of RNAi-induced spermatophores, whereas a distinct intensity was visible in control sperm (Fig. 5A-c).

Although RNAi-induced and control sperm showed neither significant abnormal phenotypes nor distinct changes in fertilization ability, the sperm gelatinolytic activity of RNAi treatment was remarkably decreased. On the gelatin zymographs, three major bands with gelatinolytic activity were observed at ~120, 20, and 16 kDa in control sperm extracts (Fig. 5B-control). In contrast, these bands were not present in RNAi sperm extracts (Fig. 5B-RNAi). This result illustrates that the gelatinolytic activity of RNAi-induced sperm was distinctly inhibited.

To further explain the influence of *TAP* knockdown on sperm activity, the proteolytic activity of sperm extracts on the vitelline coat was analyzed using 10% SDS-PAGE. When the vitelline coat was incubated with control sperm extracts for 24 h at 28 °C, the two major bands (90 and 95 kDa) of vitelline coat components were sharply

Figure 3 Western blotting and immunohistochemical analysis. (A) The distribution of TAP protein in different tissues. Equal amounts of tissue proteins (50 μ g) were hybridized with anti-TAP antibody. For the control protein, α -tubulin was detected with anti- α -tubulin antibody: sperm (S), testis (T), vas deferens (VD), terminal ampullae (TA), ovary (OV), hepatopancreas (HP), heart (HT), muscle (M), thoracic ganglia (TG), abdominal ganglia (AG), gill (GL), stomach (SH), and gut (GT). (B) The distribution of TAP protein in the three morphotypically differentiated developmental stages: small males (SM), orange-claw males (OC), and blue-claw males (BC). (C) Immunohistochemical analysis. Cross-sections of terminal ampullae (a) and spermatophore (a') were hybridized with anti-TAP antibody: epithelial cell (E), spermatophore wall (SW), and sperm (S). The black arrows indicate positive signals. No hybridization signal was observed in sections incubated without anti-TAP antibody (b, b'). Scale bars, 40 μ m.

degraded (Fig. 5C-1, 2, 3). However, these bands were intact, and degradation of the vitelline coat was inhibited when incubated with RNAi sperm extracts (Fig. 5C-4). The proteolytic activity of sperm extracts on the vitelline coat was obviously blocked. These results indicate that TAP likely plays a crucial role in regulating sperm proteolytic activity during sperm maturation and degradation of vitelline coat.

Discussion

The male reproductive tract and spermiogenesis of crustaceans are extraordinarily different from those of vertebrates (Lynn & Clark 1983a, Pochon-Masson 1983, Shigekawa & Clark 1986, Bauer & Min 1993, Alfaro *et al.* 2007). To date, only a few sperm proteins have been reported to be associated with reproduction in decapods. In the present study, we successfully isolated a novel terminal ampullae gene and named it *TAP*. We found that *TAP* mRNA was transcribed dominantly in terminal ampullae of the male reproductive tract, and that TAP protein was distributed exclusively on terminal ampullae and sperm.

In situ hybridization revealed that *TAP* was located in the spermatogenic cells of testis, the epithelial cells of terminal ampullae, and in AG cells. The AG is a peculiar and unique gland in crustaceans, and principally

governs male sexual differentiation. In the isopod, *Armadillidium vulgare*, the gonads of genetic females were transformed into testes by implantations of AGs during the sex differentiation period (Suzuki 1999). AG is also essential for inhibiting female differentiation, and controlling male primary (e.g. spermatogenesis) and secondary (e.g. external morphology) sexual characteristics (Sagi *et al.* 1990, 1997, Suzuki 1999, Khalaila *et al.* 2001, Ventura *et al.* 2009). In the red claw crayfish, *Cherax quadricarinatus*, most immature females with AG implanted developed male-like propodi and inhibited female secondary sex characteristics and vitellogenesis (Khalaila *et al.* 2001). At present, AGs have been isolated in the terrestrial isopod species *A. vulgare* (Okuno *et al.* 1999), *Porcellio scaber*, and *P. dilatatus* (Ohira *et al.* 2003). However, decapod AGs have not been identified, and only two AG-specific genes have been characterized: the *C. quadricarinatus* insulin-like AG factor (*Cq*-IAG) gene (Manor *et al.* 2007), and the *M. rosenbergii* insulin-like AG (*Mr*-IAG) gene (Ventura *et al.* 2009). In our study, the distribution of *TAP* mRNA in AG cells indicated that TAP was one component of the AG, and that it may be a peptide involved in sexual function. AG seems to synthesize several proteins (e.g. TAP and *Mr*-IAG) to cooperate and regulate sexual functions in *M. rosenbergii*.

Moreover, *TAP* was located in the spermatogenic cell zone of testicular lobules in testis. In general, testicular

Figure 4 Immunolocalization of TAP on sperm. (A) Immunofluorescence analysis. Sperm from terminal ampullae were immunostained with anti-TAP antibody: base (b), spike (s). The immunofluorescence signal appeared on the convex surface of the sperm base as a green fluorescent zona and spike (a). White arrows indicate the positive signals. No fluorescence was detected on sperm incubated without anti-TAP antibody (b). The bright field images of sperm (a', b'). Scale bars, 5 μ m. (B) Transmission electron micrographs of immunohistochemical analysis. The prawn sperm were incubated with anti-TAP antibody and gold nanoparticles (a, b). Positive signals (white gold nanoparticles) were observed on the convex surface of the base (a) and the spike of sperm (b). No signal was detected on the base (c) and spike (d) of sperm (e) incubated without anti-TAP antibody. Scale bars, 100 nm.

lobules are full with spermatocytes during the sexually differentiated period. During spermatogenesis, testicular lobules always contain the spermatogenic zone and sperm, whereas the testicular lobules of mature males contain mature spermatozoa almost to the exclusion of other cell types, and the spermatogenic zone is barely observable (Sagi *et al.* 1988). In RNAi-induced small males, both spermatogenic zone and sperm were present in comparison with only spermatocytes present in control groups (data not shown). Silencing of *TAP* led to the express development of testicular spermatogenesis. These results clearly indicate that TAP participates in controlling spermatogenesis.

On the other hand, *TAP* mRNA was also found in the epithelial cells of terminal ampullae. Generally, the epithelial cells secrete numerous and complex regulatory elements into the lumen to modify pre-existing proteins or maintain a particular environment for sperm activity. These epididymis secretion processes are highly regulated, and half of the epididymal proteins are under androgen control (Dacheux *et al.* 2005). In our study, TAP likely was a secreted protein, as indicated by the existence of a 17-amino acid signal peptide. It was originally synthesized from epithelial cells, transferred on sperm, and then participated in controlling or inducing the final sperm changes. Moreover, this process likely was under AG control, as TAP expression increased progressively in the three morphotypically differentiated developmental stages.

The RNAi technique was applied to reveal the potential function of TAP in fertilization and sperm maturation. The mature male prawns underwent targeted gene disruption to elucidate the functional role of TAP. When RNAi-induced male prawns were bred with females of proven fertility, they were found to be fertile.

The fertilization ratio and fertilized egg cell development showed no difference between RNAi-induced and control groups (data not shown). RNAi-induced male prawns also were used to evaluate different parameters related to reproductive function. No difference between treated and control prawns was observed in terminal ampullae at the histological level, and no apparent changes were detected in sperm morphology (data not shown). In other words, *TAP* gene knockdown had no influence on the process and capability of fertilization. Hence, TAP does not seem to be essential for *M. rosenbergii* fertilization.

Considering that gene knockdown might influence sperm maturation, we evaluated the proteolytic activity of RNAi-induced sperm and their ability to penetrate the egg coat. Routine investigation of sperm viability, motility, and incidence of spontaneous acrosome reactions are not suitable for prawn sperm because they are nonmotile, aflagellate, and atypical. Instead, we used other methods to test sperm activity. Previously, the gelatin SDS-PAGE assay was routinely used to determine acrosin activity of crustacean sperm (Rios & Barros 1997, Honda *et al.* 2002, Li *et al.* 2008). In this study, gelatinolytic activity of sperm extracts was reduced in RNAi-induced prawns. The knockdown of the *TAP* gene caused a significant inhibitory effect on the gelatinolytic activity of prawn sperm. More interestingly, the activity of MSG was also severely impaired in RNAi-induced prawns (data not shown). These results indicate that the TAP protein may play an important role in regulating sperm proteolytic activity in terminal ampullae.

Such gelatinolytic activity has also been suggested to play a role in nonmotile decapod sperm penetration of the vitelline coat (Rios & Barros 1997, Honda *et al.* 2002, Li *et al.* 2008). Several reports have indicated that sperm

Figure 5 Differences between *TAP* RNAi-induced and control sperm extracts. (A-a) Detection of the relative *TAP* mRNA level in RNAi-induced and control groups. (A-b) Western blotting of RNAi-induced and control sperm extracts. (A-c) Immunohistochemical analysis of RNAi-induced and control spermatophores. Scale bars, 50 μm. (B) Gelatin SDS-PAGE zymographs. Three major bands with gelatinolytic activity were visible in the control sperm extract zymograph (black arrows), but the bands were not present in the RNAi-induced sperm extracts. (C) The proteolytic effect of sperm extracts on the vitelline coat: 1) sperm extracts, 2) vitelline coat, 3) vitelline coat incubated with control sperm extracts, and 4) vitelline coat incubated with RNAi-induced sperm extracts. Two bands (90 and 95 kDa) (arrows) were degraded when vitelline coat was incubated with control sperm extracts (3), but the degradation was blocked when vitelline coat was incubated with RNAi-induced sperm extracts (4).

extracts possess proteolytic activity on vitelline coat components. For instance, in ascidians, enzymes released from the sperm acrosome degraded a sperm receptor, HrVC70 (70 kDa), on the surface of the vitelline coat during fertilization (Lambert *et al.* 2002, Sawada *et al.* 2002). Likewise, in *M. rosenbergii*, two major components (90 and 95 kDa) of the vitelline coat were hydrolyzed by the sperm extracts (Li *et al.* 2008). In the present study, sperm proteolytic activity on the vitelline coat was severely impaired in RNAi-induced

prawn. Moreover, the vitelline coat degradation of the fertilized eggs of females that mated with the RNAi males was distinctly different from wild-type group. The vitelline coat of wild-type fertilized eggs was immediately degraded into several various sized proteins. Whereas, only few degraded proteins were observed in RNAi group (data not shown). There are likely to other fungible pathways to help sperm penetration vitelline coat in the fertilization of *TAP* silencing group. In other words, silencing of *TAP* mRNA interfered with prawn sperm's ability to degrade the vitelline coat. Thus, *TAP* likely performs a pivotal role in regulating the proteolytic activity of the sperm.

In general, a sperm cell contains a differentiated organelle, the acrosome, which packages digestive enzymes (including hyaluronidase and acrosin). Many proteinases and protein inhibitors are located or bound specifically to the acrosomal region (Stein *et al.* 2006), and perform specific functions (Vacquier 1998, Sawada & Yokota 2007, Kashiwabara & Baba 2009). In most decapods (e.g. *Sicyonia ingentis* and *Penaeus aztecus*), the spike of the sperm is part of an elaborate acrosomal complex (Medina *et al.* 1994). However, the structure of the *M. rosenbergii* spike is dissimilar, and its possible role in the acrosome remains uncertain (Lynn & Clark 1983b). The spike is composed primarily of protein, projects from the convex surface of the base, is formed by radial fibril anastomosis, and undergoes a dramatic bending process during fertilization (Lynn & Clark 1983a). In our study, the immunolocalization assay provided evidence that *TAP* was located on the convex surface of the sperm base and the spike. This peculiar distribution of *TAP* further implied the crucial function of *TAP* in the sperm–egg interaction. In a previous study, MRPINK was also illustrated to bond to the lateral edge of the sperm base (Li *et al.* 2008). Thus, the convex surface of the sperm base and the spike may have a function similar to that of the acrosome, as they contain proteins involved in the sperm–egg interaction. We conclude that *TAP* affects the activity of the proteinases or protease inhibitors on the sperm spike during sperm maturation process of terminal ampullae. Further studies are needed to identify its target peptide and regulatory elements in sperm maturation and degradation vitelline coat.

Materials and Methods

Animals

Giant fresh water prawns, *M. rosenbergii*, were obtained from Yuanpu Prawn Culture located in Hangzhou, China. They were reared in aquaria with circulating freshwater at 25 °C with an artificial photoperiod of 14-h light:10-h dark per day. Prawns were fed with compound foods twice daily.

Total RNA extraction, cDNA synthesis, and SSH

Mature male prawns were placed in an ice bath for 1–2 min until they were lightly anesthetized. Vas deferens and terminal ampullae of the male reproductive tract were removed, snap-frozen in liquid nitrogen, and stored at -80°C until RNA preparation. Tissues were homogenized in Trizol Reagent (Invitrogen), and total RNA was prepared according to the manufacturer's instructions.

Next, 2 μg of each total RNA were used to synthesize the first-strand cDNA using PowerScript reverse transcriptase (Takara Bio Inc., Otsu, Kyoto, Japan) at 42°C for 90 min, and two adaptors were added to both ends using the SMART 3' CDS Primer II A and SMART II Oligonucleotide (SMART PCR cDNA Synthesis Kit; Takara Bio Inc.). The first-strand cDNAs were purified by NucleoSpin columns (Takara Bio Inc.), and then were amplified using 5' PCR Primer II A for optimized cycles. The dscDNAs were purified using NucleoSpin columns.

For SSH, the dscDNAs of terminal ampullae samples were used as the tester, and the dscDNAs of vas deferens samples were used as the driver. The subtractive library was generated using the Clontech PCR-Select cDNA Subtraction Kit (Takara Bio Inc.), according to established protocols. After two cycles of hybridization and two cycles of PCR amplification, a total of 192 individual recombinant clones in pUCm-T vector (Bio Basic Inc., Markham, ON, Canada) were chosen and used as templates for PCR amplification using the vector primers M13F and M13R. Each PCR product (1 μl) was spotted onto a nylon membrane (Millipore Corporation, Billerica, MA, USA), hybridized with the tester and driver cDNA probes in order, and detected using the digoxigenin (DIG) chemiluminescent detection system (DIG High Prime DNA Labeling and Detection Starter Kit II, Roche Diagnostics GmbH).

3'- and 5'-RACE

Forty-seven clones that exhibited distinct expression differences according to the SSH results were sequenced (Shanghai Sangon Biological Engineering Technology & Service Co. Ltd, Shanghai, China). One full-length cDNA (*TAP*) of these clones was obtained using the RACE method. The gene-specific primers F1 and F2 for 3'-RACE, and R1 and R2 for 5'-RACE (Table 1) were designed based on the nucleotide sequences of *TAP*, and the cDNAs for RACE were synthesized from the terminal ampullae of the male reproductive tract. All processes of 3'- and 5'-RACE were conducted following the manufacturer's protocol for the FirstChoice RLMRACE Kit (Ambion; Austin, TX, USA). The sequenced cDNA was analyzed using Lasergene software (DNASTar Inc., Madison, WI, USA), the deduced amino acid sequence of the peptide was predicted by the PredictProtein (<http://www.predictprotein.org/>) and Scratch Protein Predictor (<http://www.ics.uci.edu/~baldis/scratch/>) websites, and the potential signal sequences were identified using SignalP V3.0 at the Center for Biological Sequence Analysis (<http://www.cbs.dtu.dk/services/SignalP>). Otherwise, Blast (blastx and blastn) program was performed for protein analysis using the NCBI website (<http://www.ncbi.nlm.nih.gov/blast>). The nucleotide sequence of *TAP* was submitted to GenBank under the accession number FJ595507.

Semi-quantitative RT-PCR

All the tissues, including muscle, hepatopancreas, heart, thoracic ganglia, gut, ovary, and all three parts of the male reproductive tract, were removed from mature prawns. Tissues were homogenized in Trizol reagent (Invitrogen), and total RNA was prepared according to the manufacturer's instructions.

First-strand cDNAs of each sample were synthesized from 2 μg of total RNA using the SuperScript/First-Strand cDNA Synthesis Kit (Invitrogen). Next, 2 μl of RT products were used as the PCR templates, and both the *TAP* cDNA fragment and the constitutively expressed *18S* cDNA fragment were simultaneously amplified in a 25- μl reaction containing 10 pmol of F2 and R1 (Table 1) and 5 pmol of 18SF and 18SR (Table 1). After PCR amplification, an aliquot of the PCR products was fractionated in a 1.5% agarose gel.

Relative abundances were expressed as the ratio of *TAP* transcript levels to those of *18S* (GenBank accession number DQ642856). In each case, peak values were set to 100, and the rest of the values were normalized. All data are from three separate RNA pools.

In situ hybridization

The DIG-labeled sense and antisense RNA probes corresponding to nucleotides 101–335 of *M. rosenbergii TAP* cDNA cloned in the plasmid vector pSPT18 were transcribed *in vitro* using the DIG RNA Labeling kit SP6/T7 (Roche Diagnostics GmbH) from the EcoRI- and HindIII-linearized templates respectively.

For tissue slice preparation, terminal ampullae and testis were removed from the specimens and immediately snap-frozen in liquid nitrogen. The samples were embedded in a TissueTek OCT-compound (Sakura Finetechnical Co. Ltd, Chuo-ku, Tokyo, Japan), and cut into 8- μm thick section using a frozen ultramicrotome. Dry sections were fixed with 4% (w/v) paraformaldehyde, digested with proteinase K, and hybridized at 42°C overnight. These slices were then blocked by 1% blocking solution (Roche Diagnostics GmbH) and treated with the anti-DIG-alkaline phosphatase (AP) conjugate fab fragments (1:500, Roche Diagnostics GmbH) and visualized with the colorimetric substrates nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate toluidine salt (NBT/BCIP; 1:50, Roche Diagnostics GmbH) according to the manufacturer's instructions. Finally, photographs were taken using an inverted microscope equipped with a camera (Nikon Eclipse TE2000-S; Nikon Instruments Inc., Kyoto, Japan).

SDS-PAGE and western blotting

Proteins were extracted from each tissue using protein loading buffer (0.2 mmol/l dithiothreitol, 4% (w/v) SDS, 0.2% (w/v) bromophenol blue, 20% (v/v) glycerol in 0.1 mmol/l Tris-HCl, pH 6.8). Rabbit anti-TAP antibody was produced by immunization with TAP polypeptide (CSVYDKSEEEQIEL; HuaAn Biotechnology Co. Ltd, Hangzhou, China). Then, 50 μg of protein of each sample were separated on 12.5% SDS-PAGE gels and transferred to PVDF membranes (Millipore Corporation) for western blotting analysis. The membranes

were incubated with anti-TAP antibody (1:600, HuaAn Biotechnology Co. Ltd) and anti- α -tubulin antibody (1:10 000; HuaAn Biotechnology Co. Ltd) overnight at 4 °C, and detected using the BM Chemiluminescence Western Blotting Kit (Roche Diagnostics GmbH).

Immunohistochemistry

The terminal ampullae and spermatophores were removed from the specimens and cut into 8- μ m thick sections for tissue slice preparation according to the above instructions. Frozen sections were alcohol-gradient dehydrated and incubated with the anti-TAP antibody (1:60) overnight at 4 °C, then incubated with the goat anti-rabbit IgG (Fc) AP-conjugate secondary antibody (1:250; Promega Corporation). The staining was performed using the NBT/BCIP solution in the darkness.

Immunolocalization of TAP on sperm

Spermatophores from terminal ampullae were fragmented and resuspended in PBS. Sperm suspensions were mounted on glass slides and dried up. All samples were fixed in 4% (w/v) paraformaldehyde/PBS for 10 min. After washing with PBS, slides were blocked with 0.5% BSA (w/v)/PBS for 1 h and incubated with anti-TAP antibody (1:60) at 4 °C overnight. After washing with 0.1% (v/v) Tween-20/PBS, the slides were incubated with anti-rabbit IgG labeled with Alexa Fluor 488 (1:250; Biotechnology Co. Ltd) for 30 min at 37 °C. After washing, the slides were observed under a fluorescence microscope (Nikon Eclipse TE2000-S; Nikon Instruments Inc).

For transmission electron microscopy (TEM) immunohistochemical analysis, sperm were fixed in 2.5% (v/v) glutaraldehyde/PBS at 4 °C overnight. Then, they were washed and postfixed in 1% (w/v) osmium tetroxide, dehydrated in a graded acetone series, and embedded in spurr resin. Sections of 70 nm were cut with a microtome (Leica EM UC6; Leica, Nussloch, Germany), incubated with the anti-TAP antibody (1:100), and coated with 15 nm gold nanoparticle-conjugated secondary antibody (1:50; HuaAn Biotechnology Co. Ltd). Sperm were viewed under the TEM (JEM-1230; JEOL Ltd, Tokyo, Japan) and photographed at a voltage of 80 kV.

RNA interference

A pair of primers (RNAiF and RNAiR in Table 1) was designed for the preparation of TAP dsRNA. A 236-bp cDNA fragment was subcloned into the plasmid pET-T7 vector at the XbaI and EcoRI restriction sites. For a negative control, a 359-bp GFP cDNA fragment was amplified using the primers GFPF and GFP R (Table 1), and then subcloned into the pET-T7 vector at the same restriction sites. The recombinant plasmids were transformed into *Escherichia coli* HT115, and the dsRNAs were produced and purified as described by Yodmuang *et al.* (2006).

For RNAi experiments, mature male prawns (25–35 g) were purchased from the prawn culture facility. In total, 40 *M. rosenbergii* males were selected according to the presence of genital papillae on the fifth walking legs, and were acclimated in a culture tank 1 week prior to injection. Then, 2 μ l of TAP dsRNA (10 μ g in 0.9% (w/v) physiological saline) were injected into the prawns ($n=20$) through the arthrodial

membrane at the basis of the fifth walking leg using microinjection needles. The controls ($n=20$) received an equal amounts of GFP dsRNA injection. Prawns were returned to the culture tanks for 4 weeks before being killed to obtain samples for further analysis.

Gelatin SDS-PAGE

A 10% SDS-PAGE containing 0.1% (w/v) gelatin was prepared according to the technique described previously (Rios & Barros 1997). Spermatophores from terminal ampullae were fragmented, resuspended in PBS, and treated with ultrasound for periods of 3 s on an ice bath for a total of 10 min in 50 mmol/l Tris-HCl, pH 7.4. The suspension was centrifuged at 12 000 *g* for 10 min at 4 °C. The supernatant was stored at –80 °C until use. Sperm extracts (30 μ g) were loaded and ran in nonreducing conditions at 20 mA at 4 °C. After electrophoresis, the gels were equilibrated in 0.1 mol/l Tris-HCl, pH 8.0, containing 2.5% (v/v) Triton X-100 for 1.5 h at room temperature. After being washed several times with 0.1 mol/l Tris-HCl, pH 8.0, the gels were incubated for 20 h at 37 °C in the same buffer without Triton X-100, but containing 5 mmol/l CaCl₂ and 0.2 mol/l NaCl. The gels were stained with Coomassie blue.

Extraction of vitelline coat

The mature ovary was dissected from the cephalothorax of three reproductive ecdysis females. The membrane surrounding the ovary was gently peeled off and discarded. Oocytes were suspended in PBS containing 0.5% (v/v) Triton X-100. After stirring for 30 min at 4 °C, the suspension was centrifuged at 10 000 *g* for 30 min to obtain the supernatant (i.e. the solubilized vitelline coat). The proteolytic effect of sperm extracts on the vitelline coat was evaluated by coincubation of the vitelline coat with sperm extracts for 24 h at 28 °C, and then analysis by 10% SDS-PAGE in reducing conditions.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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