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ORIGINAL ARTICLE

Mutual adaptation between mouse transglutaminase 4 and its native substrates in the formation of copulatory plug

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Abstract Formation of copulatory plugs by male animals is a common means of reducing competition with rival males. In mice, copulatory plugs are formed by the coagulation of seminal vesicle secretion (SVS), which is a very viscous and self-clotting fluid containing high concentration of proteins. In its native state, mouse SVS contains a variety of disulfide-linked high-molecular-weight complexes (HMWCs) composed of mouse SVS I–III, which are the major components of mouse SVS. Further, mouse SVS I–III are the substrates for transglutaminase 4 (TGM4), a cross-linking enzyme secreted from the anterior prostate. According to activity assays, mouse TGM4 prefers a mild reducing and alkaline environment. However, under these conditions, the activity of mouse TGM4 toward SVS I–III was much lower than that of a common tissue-type TGM, TGM2. On the other hand, mouse TGM4 exhibited much higher cross-linking activity than TGM2 when native HMWCs containing SVS I–III were used as substrates under non-reducing condition. By the action of TGM4, the clot of SVS became more resistant to proteolysis. This indicates that the activity of TGM4 can further rigidify the copulatory plug and extend its presence in the female reproductive tract. Together with the properties of TGM4 and the nature of its disulfide-linked SVS protein substrates, male mice can easily transform the semen into a rigid and durable copulatory plug, which is an important advantage in sperm competition.

Keywords Copulatory plug · Mouse · Prostate · Seminal vesicle secretion · Transglutaminase

Abbreviations

CGS	Coagulating gland secretion
DTT	Dithiothreitol
GST	Glutathione S-transferase
HMWCs	High-molecular-weight complexes
REST	Rapidly evolving substrates for TGM
SVS	Seminal vesicle secretion
TGM	Transglutaminase

Introduction

Since the existence of sexual reproduction in animals, there has been competition among the males for access of their sperms to oocytes. Preventing female partners from re-mating with rival males by forming copulatory plugs is a common fitness measure to reduce such competition

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(Timmermeyer et al. 2010). From nematodes (Palopoli et al. 2008), insects (Wolfner 2002; Chapman and Davies 2004), spiders (Fromhage and Schneider 2006), and stingrays (Piercy et al. 2005) to mammals (Clark and Swanson 2005; Ramm et al. 2009; Parga 2010), different classes of animals form different kinds of copulatory plugs, which act as physical barriers blocking the path to a female's oocytes, although the compositions and formation mechanisms of copulatory plugs may greatly differ.

In mammals, the formation of copulatory plug is a complex biochemical reaction, in which various seminal proteins from accessory reproductive glands clot to form a coagulum (Williams-Ashman 1984). It has been demonstrated that surgical or genetic impairment of the secretory function of the seminal vesicle and coagulating gland (anterior prostate) in rodents not only influences the formation of copulatory plug but also greatly reduces fertility (Pang et al. 1979; Peitz and Olds-Clarke 1986; Simanainen et al. 2008). Therefore, the importance of copulatory plug in mammalian reproduction should not be overlooked.

To understand the compositions and formation mechanisms of copulatory plugs is one of the best ways to elucidate their physiological significance. In most mammals, such structures are formed from seminal vesicle secretion (SVS). Considerable progress has been made in the systematic identification and functional studies of SVS proteins, which has made mouse a great model in this respect. Mouse SVS is a viscous fluid consisting of seven major proteins, designated SVS I–VII (Chen et al. 1987; Luo et al. 2001), and several minor proteins such as seminal vesicle autoantigen (SVA) (Yu et al. 1993; Huang et al. 1995; Yu et al. 1997), P12 (Lai et al. 1991; Chen et al. 1998), and Ceacam10 (Li et al. 2005). The functions of mouse SVS proteins include inhibition of female immunological challenges to sperms (Metafora et al. 2007), regulation of sperm motility or capacitation (Huang et al. 2000; Luo et al. 2004; Huang et al. 2005; Lin et al. 2006b; Huang et al. 2007), and semen coagulation (Lundwall et al. 1997; Lin et al. 2002; Tseng et al. 2009). It is believed that the formation of ε-(γ-glutamyl) lysine cross-bridges between SVS proteins by the action of transglutaminase (TGM) is the cause of semen coagulation and copulatory plug formation, since mouse SVS I (Tseng et al. 2009), SVS II (Lundwall et al. 1997), SVS III (Lin et al. 2002), and SVS IV (Metafora et al. 2007) have been identified as substrates of TGM, which is also present in coagulating gland secretion (CGS) (Tseng et al. 2008). Meanwhile, mouse SVS itself clots easily even without CGS. Therefore, the formation mechanism of mouse copulatory plug could be more complicated.

In this study, we focused on the native state of mouse SVS and found that mouse SVS I–III are cross-linked by disulfide bridges to produce a variety of high-molecular-weight complexes (HMWCs) within the tissue prior to their secretion into the lumen of the seminal vesicle. In addition, the formation of HMWCs might be a consequence of fitness, which makes mouse SVS I–III better substrates for mouse prostate-type TGM (TGM4).

Materials and methods

General materials

All chemicals, including phenylmethylsulfonyl fluoride (PMSF), isopropyl β-D-1-thiogalactopyranoside (IPTG), trypsin, and guinea pig liver TGM (type 2 TGM, TGM2) were purchased from Sigma-Aldrich and were of the highest purity available. HisTrap FF column, GSTrap FF column, goat anti-rabbit immunoglobulin G (IgG) conjugated with alkaline phosphatase or horseradish peroxidase, Ready-To-Go™ reverse transcriptase-polymerase chain reaction (RT-PCR) beads, expression vector pGEX-6P-1, and enhanced chemiluminescence (ECL) kit were obtained from GE Healthcare. Expression vector pET-21a was procured from Novagen, anti-TGM2 antibody from Abcam, 5-(biotinamido) pentylamine (EZ-link) from Thermo Scientific, streptavidin-β-galactosidase and *o*-nitrophenyl-β-D-galactopyranoside from Calbiochem. The A25 peptide was synthesized according to the previous studies (Ruoppolo et al. 2003).

Preparation of mouse seminal vesicle homogenate; collection of SVS, CGS, uterine fluid, vaginal fluid, and copulatory plugs; and purification of TGM4

Normal adult mice (8–12 week old, Institute of Cancer Research [ICR] strain) were killed by cervical dislocation. Seminal vesicles were dissected out through a longitudinal incision, blotted onto a filter paper to remove the adhering SVS, and washed thoroughly with ice-cold 5% acetic acid containing 1 mM PMSF. The tissue was further washed with ice-cold wash buffer consisting of 10 mM Tris–HCl, pH 7.5, with 1 mM PMSF. The washed tissue was immediately homogenized with M-PER mammalian protein extraction reagent (Thermo Scientific) in the presence of 1 mM PMSF by grinding with a blue stick in a 1.5-ml microcentrifuge tube. The mixture was incubated on ice for 30 min, centrifuged at 14,000 $\times$ *g* for 10 min at 4°C, and the supernatant was collected as seminal vesicle tissue homogenate.

SVS and CGS were squeezed individually into ice-cold 10 mM Tris–HCl, pH 8.0, in the presence of 1 mM PMSF. CGS was centrifuged at 8,000 $\times$ *g* for 20 min at 4°C to remove the precipitate, and TGM4 was purified from CGS according to a previously described procedure (Tseng et al. 2008).

The estrous cycle of sexually mature female mice (6–8 week old, ICR strain) was screened by examining vaginal smears to identify the stage. The mice in the estrus stage were killed by cervical dislocation, and the uterine and vaginal fluids were collected. The mice in the pre-estrus stage were caged with adult stud mice for one night. In the morning, copulatory plugs were collected once formed.

Protein blotting and immunochemical staining

Simultaneously, mouse SVS proteins were resolved by 14% reducing sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) or 6% non-reducing SDS–PAGE (Lin et al. 2006a). The proteins on the gel were stained with Coomassie brilliant blue or transferred to a nitrocellulose membrane. After transfer, the protein blots were detected by using the antisera against SVS I, SVS II, and SVS III (diluted to 1:10,000 with 5% non-fat skimmed milk in phosphate-buffered saline [PBS]) as the primary antibody and goat anti-rabbit IgG conjugated with horseradish peroxidase (diluted to 1:10,000 with 5% non-fat skimmed milk in PBS) as the secondary antibody. The bands detected by Western blotting were visualized using an ECL kit according to the manufacturer's instructions.

TGM activity assay

The activity of TGM was measured either by the solid-phase microtiter assay or by the cross-linked percentage of SVS substrates (Tseng et al. 2008). A recombinant GST-fused polypeptide comprising residues 116–145 of SVS III was used as the protein substrate in the solid-phase microtitle assay. The soluble substrate, 0.5 mM EZ-link or A25 peptide (Ruoppolo et al. 2003), and 2.5 μg of TGM2 or TGM4 in 100 μl of 50 mM Tris–HCl (pH 7.5) containing 10 mM $CaCl_2$ and 10 mM DTT were incubated at 37°C for 60 min in individual wells of a microtiter plate pre-coated with 10 μg of protein substrate. Incorporation of EZ-link or A25 peptide into the protein substrate was measured by ELISA using streptavidin-β-galactosidase. The enzyme activity in the hydrolysis of O-nitrophenyl-β-D-galactopyranoside was determined by measuring the absorbance at 412 nm.

Freshly collected SVS was diluted with 50 mM Tris–HCl (pH 7.5) containing or devoid of 10 mM dithiothreitol (DTT). The reduced or non-reduced SVS (1 mg/ml) was incubated with 1.6–25 μg/ml of TGM4 for 60 min at 37°C. The reaction solution was then mixed with an equal volume of SDS–PAGE sample buffer containing 10 mM DTT and boiled before performing electrophoresis to detect the unreacted protein components by resolving the proteins on a 14% reducing SDS–PAGE gel (6.5 × 10.5 × 0.075 cm). On the basis of the protein staining pattern on the polyacrylamide gel, the amount of each unreacted SVS protein component in a reaction was normalized to yield a percentage (P) of the total amount present in the control. With this approach, the value 1–*P* determined for each protein component reflected its percentage cross-linked by the enzyme.

Protease resistance assay of SVS clots

To prepare clots of self-clotted SVS or in vitro copulatory plugs, 1 mg net weight of freshly collected SVS alone or of freshly collected SVS mixed with purified mouse TGM4 (100 μg) was incubated at room temperature until it rigidified into a clot. Each SVS clot was trimmed into a 5-mm^3 cube and incubated with 10 ml of trypsin solution (10 μg/ml in 50 mM Tris–HCl, pH 7.5) at 37°C. The time elapsed until the clots became invisible was recorded.

Results

Formation of HMWCs via disulfide links between SVS I–III in the seminal vesicle

By using reducing SDS–PAGE, three of the major plug-forming proteins, namely SVS I, SVS II, and SVS III, in mouse SVS could be clearly identified as 91-, 38-, and 34-kDa proteins, respectively (Fig. S1b). On the other hand, analysis of mouse SVS by non-reducing SDS–PAGE showed quite different patterns of protein bands. The major protein bands were detected at 72 – 212 kDa, and some protein bands were also observed at a relative molecular mass (M_r) even higher than 212 kDa (Fig. 1a, lane 7).

In our previous study, we proved that these protein bands were complexes composed of mouse SVS I–III and linked by disulfide bridges (Lin et al. 2006a). To further understand the composition and formation of each protein complex, we prepared antibodies specific to mouse SVS I, SVS II, and SVS III (see detail in Supplementary material and Fig. S1). By using these antibodies and from the molecular masses of the proteins of native mouse SVS on 6% non-reducing SDS–PAGE gel (Fig. 1a, lanes 1, 3, 5, 7), we could clearly assign 6 of the major complexes, which are shown as bands *i–vi* (Fig. 1b). The complexes were formed by interpolypeptide disulfide linkages between the monomers of SVS I–III. The following major HMWCs were detected at M_r 72–170 kDa: homopolypeptide dimers of SVS I (band *i*) and SVS II (band *vi*) and heteropolypeptide complexes formed by (1) an SVS I monomer and an SVS III monomer (band *iv*); (2) an SVS I monomer, an SVS II monomer, and an SVS III monomer (band *ii*); and (3) 2 SVS II monomers and 2 SVS III monomers (band *iii*).

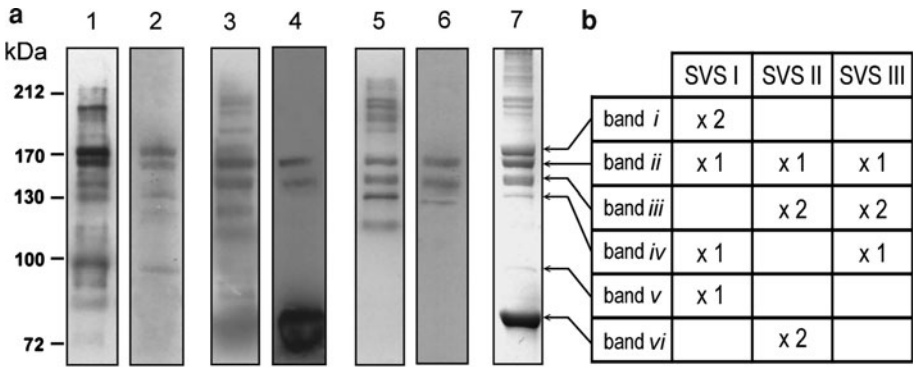

Fig. 1 Determination of the composition of HMWCs from SVS and seminal vesicle homogenate. **a** Protein samples from freshly prepared SVS without disulfide reduction (*lanes* 1, 3, and 5) or from the soluble fraction of seminal vesicle homogenate (*lanes* 2, 4, and 6) were subjected to 6% non-reducing SDS–PAGE. The proteins on each gel were transferred to nitrocellulose membranes for Western blot analysis by using the antiserum against SVS I (*lanes* 1 and 2), SVS II (*lanes* 3 and 4), or SVS III (*lanes* 5 and 6). The non-reduced SVS proteins on the gel were also stained with Coomassie brilliant blue to detect their positions (*lane* 7). **b** Stoichiometric composition of 6 major HMWCs, shown as bands *i*–*vi*, was determined from the molecular masses and Western blotting results

Besides, a very small amount of SVS I monomer (band *v*) was detected at 91 kDa, and several complexes with uncertain ratios of SVS I, SVS II, and SVS III were observed in the region of M_r greater than 212 kDa.

The soluble proteins in the mouse seminal vesicle homogenate were also resolved by 6% non-reducing SDS–PAGE, and the occurrence and composition of HMWCs were determined by using antibodies specific to mouse SVS I, SVS II, and SVS III (Fig. 1a, lanes 2, 4, 6). The aforementioned 6 major bands were also detected for the seminal vesicle homogenate, but HMWCs with M_r greater than 212 kDa were not observed. Apparently, the majority of HMWCs with M_r lower than 180 kDa are formed in the tissue of the seminal vesicle prior to their secretion into the lumen. The trace amounts of HMWCs with M_r greater than 212 kDa are possibly because of further cross-linking of HMWCs after their secretion into the lumen of the seminal vesicle.

Further cross-linking of HMWCs in copulatory plug

It is believed that the formation of mouse copulatory plug is the result of the cross-linking of SVS proteins by TGM (Williams-Ashman 1984). However, mouse SVS has the innate tendency to clot after extraction from the seminal vesicle. The existence of sulfhydryl oxidase-2 (SOx-2) (Benayoun et al. 2001), which may further cross-link HMWCs via disulfide bridges, also provides a potential mechanism of plug formation. Therefore, freshly formed copulatory plugs and self-clotted SVS were collected and extracted with SDS sample buffers with or without 10 mM DTT. The extractions were subjected to 14% SDS–PAGE, and the proteins released from the clots were detected by staining with Coomassie brilliant blue or by using specific antibodies. When self-clotted SVS was extracted with non-reducing SDS sample buffer to break down the non-covalent protein–protein interactions in the SVS clots, the extraction contained low-M_r SVS proteins, i.e., SVS IV–VII, and HMWCs (Fig. 2a, lane 1), which are composed of SVS I–III (Fig. 2a, lanes 2–4). On the other hand, when self-clotted SVS was extracted with SDS sample buffer with 10 mM DTT, the protein content was very similar to that of freshly collected SVS (Fig. 2a, lane 5), and only monomeric forms of SVS I–III could be detected (Fig. 2a, lanes 6–8). This indicated that disulfide cross-links might be the cause of the self-clotting of SVS and that reducing SDS sample buffer could break down the protein–protein interactions.

When copulatory plugs were extracted with the same extraction buffers, the protein content was very different from that of the self-clotted SVS. Although the low-M_r SVS proteins and some HMWCs were also released from the copulatory plugs under non-reducing condition, there was only one major protein located at 34 kDa (Fig. 2b, lane 1). Reaction with specific antibodies showed that the 34-kDa protein was not SVS III and the HMWCs did not consist of SVS I–III (Fig. 2b, lanes 2–4). This indicates that minor SVS protein components may form other types of HMWCs through disulfide bridges in native mouse copulatory plugs. The SVS III antibody did recognize a protein around 20 kDa, which may be the degraded product of SVS III. Under reducing condition, the HMWCs disappeared and the intensity of the 34-kDa protein band increased (Fig. 2b, lane 5). Western blot analysis showed that trace amounts of SVS I and SVS III, but not SVS II, were released from the copulatory plugs (Fig. 2b, lanes 6–8). In addition to the degraded or monomeric form of SVS III, the antibody also detected a dimeric form of SVS III, which was not dissociated by DTT (Fig. 2b, lane 8). Thus, compared with the self-clotted SVS, there should be

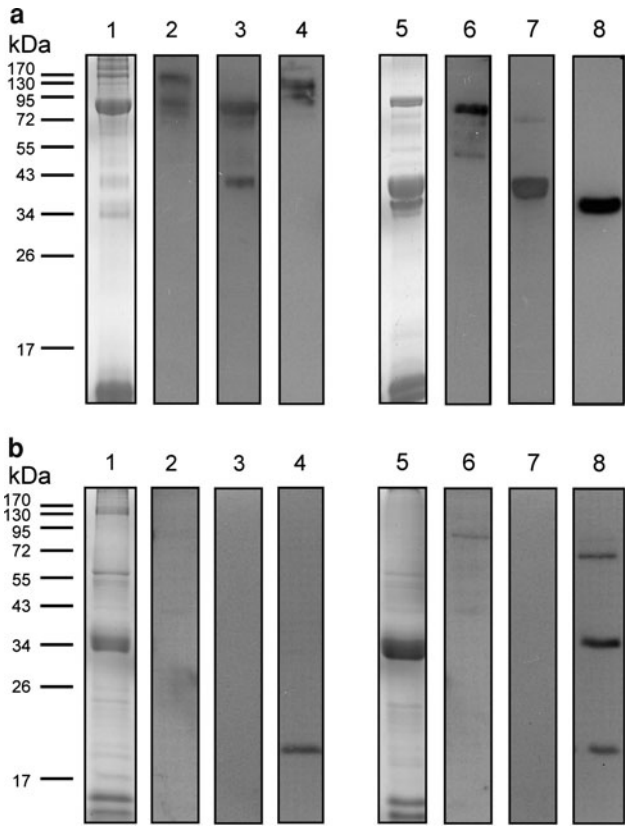

Fig. 2 Identification of the protein–protein interactions between SVS I–III in self-clotted SVS and copulatory plug. **a** Self-clotted SVS was extracted with SDS sample buffer without DTT (non-reducing condition) to dissociate the non-covalent protein–protein interactions (*lanes* 1–4) or with 10 mM DTT to break down the intermolecular disulfide bridges (*lanes* 5–8). **b** Copulatory plugs were extracted with SDS sample buffer without DTT (*lanes* 1–4) or with DTT (*lanes* 5–8). The extractions were subjected to 14% SDS–PAGE, and the proteins were detected by Coomassie *brilliant blue* staining (**a** and **b**, *lanes* 1 and 5) or Western blotting using the antisera against SVS I (a and b, *lanes* 2 and 6), SVS II (**a** and **b**, *lanes* 3 and 7), and SVS III (**a** and **b**, *lanes* 4 and 8). Each lane contains 20 μg of the total protein

additional cross-links besides disulfide bridges between SVS proteins in the copulatory plugs.

TGM4, but not TGM2, is detectable in the fluids of reproductive tracts

According to the previous studies, SVS I–III are good substrates for TGM (Lundwall et al. 1997; Lin et al. 2002; Tseng et al. 2009). Therefore, it is likely that the additional covalent cross-links in copulatory plugs were formed by TGM. However, most of the in vitro studies have been conducted using commercially available TGM, which is usually TGM2, instead of prostate-type TGM4. Since TGM2, also known as tissue-type TGM, essentially exists in every tissue (Beninati and Piacentini 2004; Park et al. 2010), it may also participate in plug formation. In order to find out which TGM actually participates in the formation of copulatory plug, fluids from the reproductive glands of male or female mice were collected and analyzed by Western blotting. TGM2 was not detected in CGS, SVS, and uterine and vaginal fluids (Fig. 3a). On the other hand, TGM4 was detected in CGS but not in SVS and uterine and vaginal fluids (Fig. 3b).

Reaction properties of mouse TGM4

Although TGM4 seems to be the cross-linking enzyme responsible for plug formation, previous observations indicated that the enzyme activity of TGM4 is much lower than that of other TGMs (Esposito et al. 1996; Esposito et al. 1999; Tseng et al. 2008). Since different TGMs have their own substrate preference (Csosz et al. 2009; Hitomi et al. 2009), the lower enzymatic activity of TGM4 in the previous studies may be due to the use of unsuitable substrates. To clarify this, a glutathione S-transferase (GST) fusion protein containing residues 116–145 of mouse SVS III (GST-SVS $III_{116\text{-}145}$), which has been demonstrated to be a good cross-linking target region for TGM4 (Lin et al. 2002), was used as a substrate in the modified solid-phase microtiter activity assay (Tseng et al. 2008). By measuring the incorporation efficiency of two biotinylated compounds, EZ-link as an acyl acceptor or A25 peptide as an acyl donor, into GST-SVS $III_{116\text{-}145}$, it was found that the activity of TGM4 was only about 40% of that of control TGM2, isolated from guinea pig liver (Fig. 4).

There is still a possibility that such a condition (50 mM Tris–HCl/pH 7.5, 10 mM DTT and 10 mM $CaCl_2$) is not suitable for mouse TGM4. Therefore, a series of solid-phase microtiter assays, using EZ-link and GST-SVS $III_{116\text{-}145}$ as substrates, were applied to determine the best reaction condition for mouse TGM4 under pH 5–10 in the

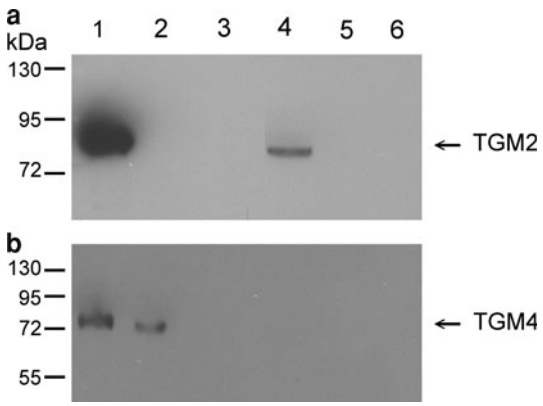

Fig. 3 Reproductive tract fluids, including CGS (*lane* 2), SVS (*lane* 3), uterine fluid (*lane* 5), and vaginal fluid (*lane* 6), together with positive controls of purified TGM2 (**a**, *lane* 1), TGM4 (**b**, *lane* 1), and mouse liver extract (*lane* 4) were subjected to 14% SDS–PAGE and analyzed using antibodies specific to TGM2 (**a**) and TGM4 (**b**)

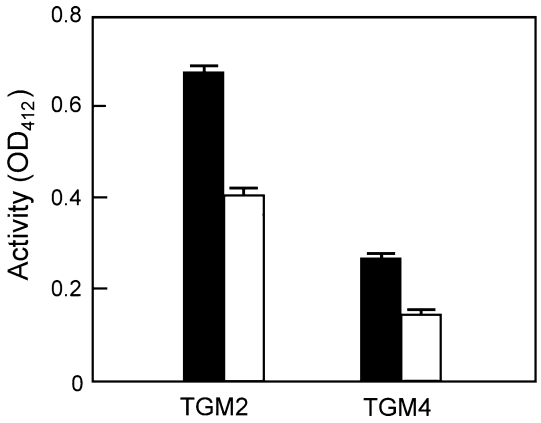

Fig. 4 The enzymatic activities of TGM2 and TGM4 were assayed by the incorporation efficiency of biotinylated substrates, EZ-link (*solid columns*) or A25 peptide (*open columns*), into the immobile protein substrate, GST-SVS $III_{116\text{-}145}$. The incorporation efficiency was measured by ELISA using streptavidin-β-galactosidase (see "Materials and Methods"). The enzyme activity in the hydrolysis of O-nitrophenyl-β-D-galactopyranoside was determined by measuring the optical density at 412 nm (OD_{412}). The data represent the average of three determinations, and *error bars,* the SD

presence or absence of 10 mM DTT. Like other TGMs, mouse TGM4 exhibited maximum reactivity in a mild alkaline environment. Addition of a reductant such as DTT could further enhance its activity about 60% at pH 8.0 (Fig. 5).

The semen itself provides a mild alkaline environment, which is a suitable condition for TGM4 activity. On the other hand, the formation of HMWCs indicates that there is no reductant in the semen. Therefore, we compared the cross-linking ability of both the native forms of SVS I–III in HMWCs and their free reduced forms by treatment with TGM4 or TGM2 as a control (Fig. 6). Freshly prepared

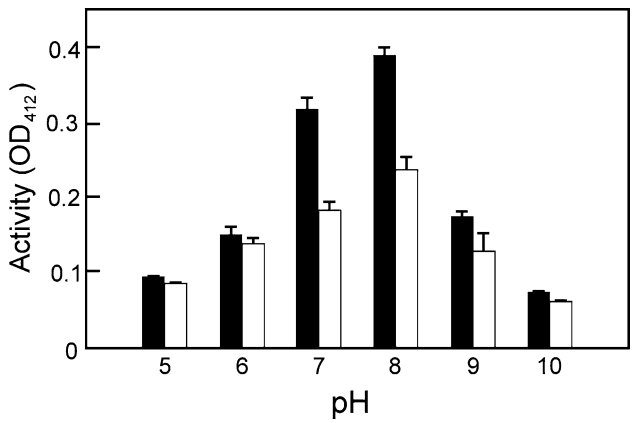

Fig. 5 The activity of TGM4 at pH 5–10 was determined by a modified solid-phase microtiter assay (see "Materials and methods"), in reaction buffers with 10 mM DTT (*solid columns*) or without 10 mM DTT (*open columns*). *Error bars* indicate standard deviations of the means from 3 independent experiments. OD_{412}, optical density at 412 nm

SVS with or without disulfide reduction by DTT was incubated with either enzyme. Following the reaction, further cleavage of protein disulfide bonds by DTT was tracked on a reducing SDS–PAGE gel in order to detect the residual unreacted monomer proteins in the reaction solution. In the absence of enzyme, the intensity of each monomer protein band on the polyacrylamide gel (Fig. 6a, c, lane C) represented its total amount available for enzyme catalysis. Since an ε-(γ-glutamyl) lysine-cross-linked complex can be distinguished from the unreacted monomer proteins on the basis of molecular size, a high level of enzyme-catalyzed cross-links with increased enzyme concentration can lead to the detection of a decreased amount of unreacted monomer proteins by SDS–PAGE. Precisely the same result was observed for each of the native SVS I–III and their free forms after incubation with either of the enzymes (Fig. 6a, c; lanes 1–3 for TGM2 catalysis and lanes 4–6 for TGM4 catalysis). On the basis of the protein staining pattern, we could estimate a value of *P*, which represented the percentage of unreacted protein by normalization of its staining intensity after one reaction to the total amount present in the control. With this approach, the value 1–*P* determined for each protein component reflected its percentage cross-linked by the enzyme. Similar with the result of solid-phase microtiter assay (Fig. 4), TGM2 exhibited higher cross-linking activity than TGM4 toward SVS I–III in the presence of 10 mM DTT (Fig. 6a, b). The cross-linking activity of TGM4 toward SVS I–III in the presence of 10 mM DTT was only 50, 52, and 20% of that of TGM2, respectively (Fig. 6a, b, 25 μg/ml of enzyme). The cross-linking activities of TGM2 toward SVS I–III were decreased in the absence of DTT (Fig. 6b, d). In contrast, TGM4 exhibited higher cross-linking activity toward SVS I–III in the absence of DTT and was about 0.2-, 0.4-, and 5-fold higher than its own activity in the presence of 10 mM DTT, respectively (Fig. 6b, d, 25 μg/ml of enzyme). The cross-linking activity of TGM4 toward SVS I–III in the absence of DTT was almost equal to that of TGM2 in the presence of 10 mM DTT. It is interesting to note that SVS IV could be cross-linked by TGM2 in the presence of a reductant but could not be cross-linked by either enzyme in the absence of a reductant (Fig. 6a, c).

TGM4-catalyzed cross-linking of HMWCs rigidifies the plug and protects it from proteolysis

One of the major functions of TGMs is to rigidify the substrates and protect them from proteolysis, similar to the action of factor XIII on a blood clot (Bruner-Lorand et al. 1966; Lorand 2001; Ariëns et al. 2002). Therefore, it is possible that TGM4 could increase the resistance of copulatory plug against proteases in vaginal or uterine fluid. Since TGM4-targeted male mice are unavailable,

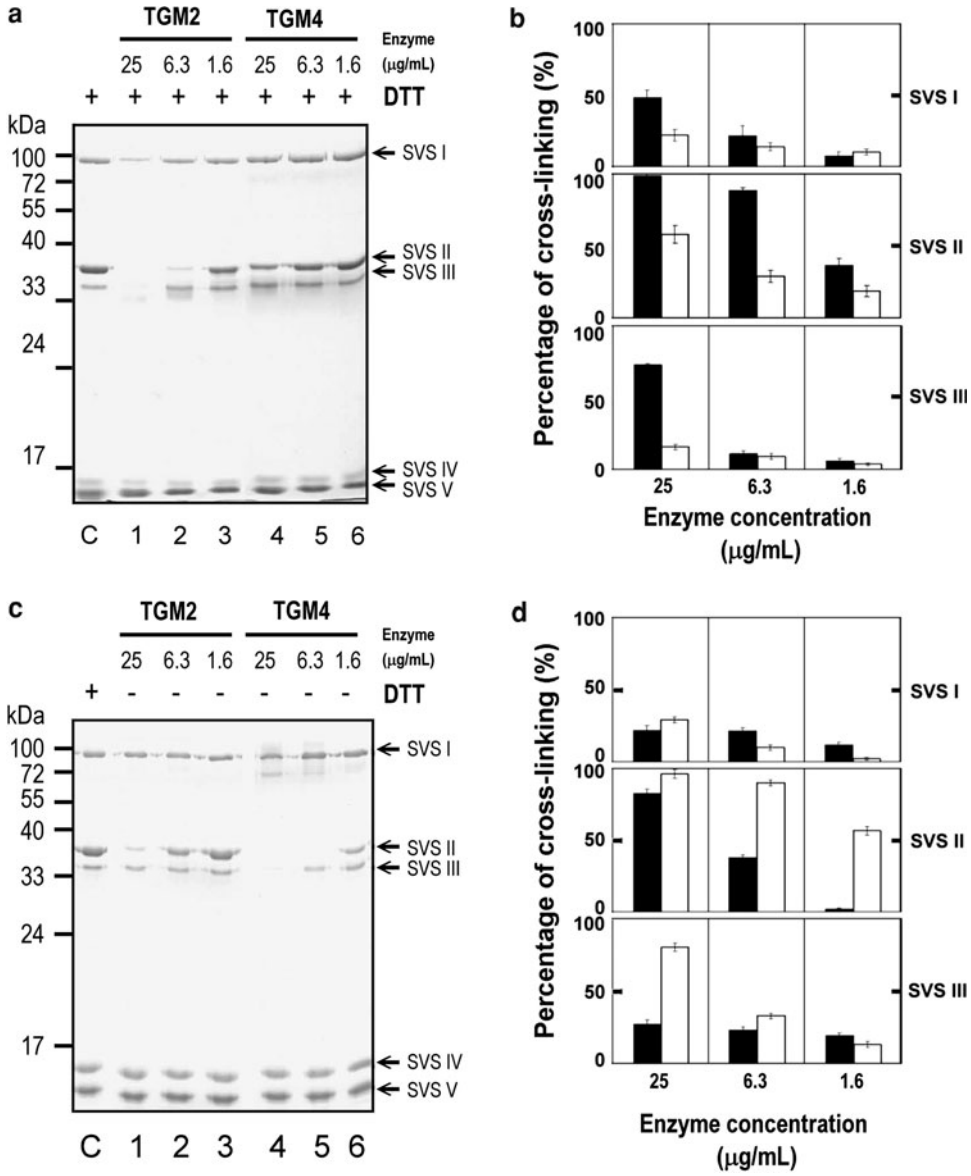

Fig. 6 Cross-linking of SVS proteins by TGM2 or TGM4. Freshly prepared SVS was diluted in the reaction buffer with or without 10 mM DTT and used as the substrate for 1.6–25 μg/ml of TGM2 (**a** and **c**, *lanes* 1–3) or TGM4 (**a** and **c**, *lanes* 4–6) under reducing (**a**) or non-reducing (**c**) condition. After 60 min of the reaction, all the protein samples were again reduced by treating with SDS sample buffer containing 10 mM DTT and subjected to 14% SDS–PAGE to yield the cross-linking results. As described in "Materials and methods", the percentage of cross-linking of each protein component by 1.6–25 μg/ml of TGM2 (*solid columns*) or TGM4 (*open columns*) under reducing (**b**) or non-reducing (**d**) condition was estimated. *Error bars* indicate standard deviations of the means from 3 independent experiments. *Lane* C in **a** and **c** represents the control for reduced mouse SVS

we adopted an alternative approach to prove the theory. Artificial in vitro copulatory plugs were prepared by mixing purified TGM4 with freshly collected SVS. Equal sizes of the clots of self-clotted SVS and in vitro copulatory plugs were then incubated in trypsin solution, which mimicked the proteolytic attacks in the female reproductive tract. The protease evidently took a longer time to completely disintegrate the TGM4-treated in vitro copulatory plugs (Fig. 7).

Discussion

In the native state of mouse SVS, most SVS I–III proteins form HMWCs through disulfide bridges. By using specific antibodies, the composition of each HMWC was clearly identified (Fig. 1). According to the primary sequence, one molecule of SVS I, SVS II, and SVS III contains 6, 1, and 3 cysteine residues, respectively. This implies that SVS I and SVS III, which contain more than one cysteine, might be interlinked with each other or themselves to form a framework, which may further establish linkages with SVS II. Such basic frameworks were built inside the tissue of the seminal vesicle (Fig. 1a, lanes 2, 4, 6); thus, it is unlikely that the oxygen-dependent enzyme SOx-2 takes part in the formation of HMWCs. However, we cannot rule out the possibility that SOx-2 enhances the cross-links between HMWCs and other SVS proteins in mouse copulatory plug (Fig. 2b).

Besides the formation of HMWCs, mouse SVS I, SVS II, and SVS III have also been proved as good TGM substrates (Lundwall et al. 1997; Lin et al. 2002; Tseng

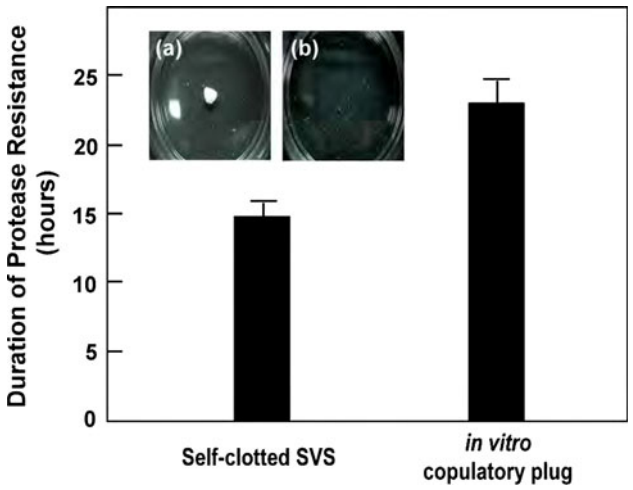

Fig. 7 TGM4-rigidified in vitro copulatory plug was more resistant to protease degradation. Clots of self-clotted SVS and in vitro copulatory plugs were prepared as described in "Materials and methods". Both types of clots were incubated with 10 ml of trypsin solution (10 μg/ml) at 37°C (inset **a**). The time taken for complete disintegration of the clot (inset **b**) was recorded. *Error bars* indicate standard deviations of the means from three determinations

et al. 2009). In fact, SVS II and SVS III share the same evolutionary origin, which also relates to the major SVS proteins in many other mammals. The genes for these proteins are grouped into the rapidly evolving substrates for TGM (REST) gene superfamily (Lin et al. 2005), which usually contains tandem repeat sequences of the substrates for TGM; these proteins are exclusively expressed in the seminal vesicle. Although the function and expression pattern of SVS I is similar to those of the REST family proteins, it is not encoded by the REST genes and is only present in mouse and rat genomes (Lundwall et al. 2003). Thus, SVS I might have evolved functionally to meet the requirements for mouse reproduction, for example, the formation of HMWCs.

We confirmed that TGM4 and not TGM2 was the major TGM involved in the formation of mouse copulatory plug (Fig. 3). Although, in a study, some amount of factor XIIIa was also detected in uterine and vaginal fluids (Lin et al., 2011, unpublished data), the fact that factor XIIIa-knock-out mice could get pregnant normally implies that factor XIII may not play any role in plug formation (Ariëns et al. 2002). Like other TGMs, mouse TGM4 prefers a mild alkaline environment, which is provided by the pH of the semen. Although the addition of 10 mM DTT could increase mouse TGM4 activity about 60% (Fig. 5), the activity remained much lower than that of TGM2. Rat TGM4 is a closely homologous enzyme, which has been characterized thoroughly. In previous studies, rat TGM4 also exhibited considerably low enzyme activity, but addition of 1.5 mM SDS could increase the enzyme activity 11 times (Esposito et al. 1996; Esposito et al. 1999). We did not examine whether mouse TGM4 also has such unusual property, since there is no SDS in the semen under native condition. However, this led us to consider that mouse TGM4 might have special requirements to enhance its activity for plug formation.

It is clear that the native substrates of TGM4 are SVS I–III in the form of disulfide-linked HMWCs. To maintain the native structure of HMWCs, a reaction buffer without a reductant was used for the cross-linking reaction. Unusually, TGM4 exhibited up to 5-fold reactivity toward native SVS I–III under the non-reducing condition (Fig. 6), in which TGM4 was only 60% active. Thus, the formation of HMWCs could make SVS I–III better substrates. Even though TGM4 was not in the best reactive condition, the total cross-linking rate was increased anyway. It is noteworthy that the cross-linking reaction rate of SVS II was the highest followed by that of SVS III and SVS I (Fig. 6). Apparently, SVS II is the best TGM substrate and the most abundant protein in SVS (Lundwall et al. 1997; Lin et al. 2002). According to our result, SVS II is linked with SVS I, SVS III, or itself to form the major components of HMWCs, shown as bands *ii*, *iii*, and *vi*, respectively (Fig. 1b). By forming disulfide linkages with SVS II, SVS I and SVS III come close to the best TGM substrate. Thus, the ε-(γ-glutamyl) lysine cross-bridges between them could be readily established by TGM4.

Semen contains not only high concentration of TGM protein substrates but also plentiful polyamines. Since polyamines are small-molecule acyl acceptors of TGM (Lentini et al. 2004; Lentini et al. 2009), they are potential competitive inhibitors to the TGM protein substrates. The result of Fig. 4 illustrated that mouse TGM4 is less active than TGM2 toward small-molecule substrates. On the other hand, the activity of TGM4 was much stronger when native HMWCs were substrates (Fig. 6). Therefore, the reactivity of TGM4 toward polyamine might be similar with the small-molecule substrates used in this study, such as A25 peptides and EZ-link. In the copulatory plug from natural coitus (Fig. 2), that the cross-linking of HMWCs proceeded even in the presence of polyamines might be ascribed to the substrate preference of mouse TGM4.

Even without TGM4, mouse SVS can self-clot into a plug, but the proteases from female reproductive glands may easily decompose it. Our result showed that the in vitro copulatory plug formed after reaction with TGM4 was more resistant to proteolysis (Fig. 7). Thus, TGM4 might play an important role in extending the presence of copulatory plug in the female, which might be a consequence of fitness for male mice to deter their competitors. A similar theory in primates indicates that the degree of seminal coagulation can be associated with predicted levels of sperm competition (Dixson and Anderson 2002).

Gorillas may be an opposite example. They live in social groups containing a single dominant male and several females with their dependent offspring. Therefore, male gorilla does not face sperm competition. It is interesting to note that a genetic study indicates that the TGM4 gene in gorillas has consequently evolved into a nonfunctional one (Carnahan and Jensen-Seaman 2008).

Male mice produce high concentration of SVS containing SVS I–III self-linked in HMWCs, which are apparently the best mouse TGM4 substrates. In this way, male mice can quickly form rigid and endurable copulatory plugs, which provide them with an advantage in sperm competition. In summary, the present study revealed the detailed mechanism involved in the formation of mouse copulatory plug by investigating the reaction properties of mouse TGM4 and its native substrates. Moreover, the coevolutionary relationship of mouse TGM4 and its substrates may shed some light in future studies on other TGMs and their substrates.

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