Characterization of mouse sperm TMEM190, a small transmembrane protein with the trefoil domain: evidence for co-localization with IZUMO1 and complex formation with other sperm proteins

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Abstract

TMEM190, a small transmembrane protein containing the trefoil domain, was previously identified by our proteomic analysis of mouse sperm. Two structural features of TMEM190, ‘trefoil domain’ and ‘small transmembrane protein’, led us to hypothesize that this protein forms a protein–protein complex required during fertilization, and we characterized TMEM190 by biochemical, cytological, and genetic approaches. We showed in this study that the mouse Tmem190 gene exhibits testis-specific mRNA expression and that the encoded RNA is translated into a 19-kDa protein found in both testicular germ cells and cauda epididymal sperm. Treatment of the cell surface with proteinase K, subcellular fractionation, and immunofluorescence assay all revealed that mouse TMEM190 is an inner-acrosomal membrane protein of cauda epididymal sperm. During the acrosome reaction, TMEM190 partly relocated onto the surface of the equatorial segment, on which sperm–oocyte fusion occurs. Moreover, TMEM190 and IZUMO1, which is an immunoglobulin-like protein required for gamete fusion, co-localized in mouse sperm both before and after the acrosome reaction. However, immunoprecipitates of TMEM190 contained several sperm proteins, but did not include IZUMO1. These findings suggest that a mouse sperm protein complex(es) including TMEM190 plays an indirect role(s) in sperm–oocyte fusion. The role(s), if any, is probably dispensable since Tmem190-null male mice were normally fertile.

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Introduction

Fertilization is a reproductive strategy to create a diploid zygote from two haploid gametes, which are usually a sperm and an oocyte. In mammals (Yanagimachi 1994), fertilization is initiated by sperm binding to the zona pellucida (ZP), an extracellular glycoprotein matrix surrounding the oocyte cell. The ZP-bound sperm penetrates through the ZP, reaches the perivitelline space between the ZP and the oocyte, and binds to and fuses with the oocyte plasma membrane. To complete this process, the sperm acrosome reaction plays two major roles: to release acrosomal contents into the extracellular space, which possibly facilitate sperm penetration through the ZP, and to remodel the sperm surface so that it can participate in sperm–oocyte fusion.

We do not currently understand mammalian fertilization at the molecular level. For example, ZP3, which is a component glycoprotein of the oocyte ZP, had been long believed to act as a sperm receptor that triggers the acrosome reaction (Litscher et al. 2009, Wassarman & Litscher 2009). However, there are recent data that contradict this interpretation (Baibakov et al. 2007, Dean 2007, Gahlay et al. 2010). As another example, both the sperm immunoglobulin-like protein IZUMO1 (Inoue et al. 2005) and the oocyte tetraspanin protein CD9 (Kaji et al. 2000, Le Naour et al. 2000, Miyado et al. 2000) appear to be required for sperm–oocyte fusion in mice, yet the fusogenic mechanism including how sperm IZUMO1 and oocyte CD9 are involved is still mysterious.

Currently, there are too many gaps in the available data to allow a comprehensive and coherent model of fertilization in animals. A fundamental problem is that sperm and oocytes are specialized, terminally differentiated cell types that are haploid. Consequently, there are few useful tools, such as RNA-mediated interference using tissue-cultured cells, which can be applied to explore gene functions in vitro. Analysis of genetically manipulated animals is currently the only rigorous way to investigate the functions of fertilization-related genes.
Indeed, much recent progress in fertilization research has resulted from studies of gene-targeted mice (Inoue et al. 2007, Okabe & Cummins 2007, Ikawa et al. 2008).

We have recently evaluated the mouse sperm proteome (Stein et al. 2006), in order to identify candidate genes that would be investigated by gene targeting. Among the identified mouse sperm proteins, there was a single-pass transmembrane protein (166 amino acids) encoded by the Tmem190 gene (also known as 4930572D21Rik or UniGene Ms.160043). Tmem190 contains five exons (Fig. 1A, upper) and is a single-copy gene located on the chromosome 7. This gene is likely testis-specific, since mouse expressed sequence tag (EST) clones of Tmem190 were isolated exclusively from the testis. As for the human ortholog gene TMEM190 (also known as MDAC1 or UniGene Hs.590943), its EST clones were isolated from the pharynx and the ovary. However, it is not yet clear whether TMEM190 is expressed in the human testis.

Mouse and human TMEM190 proteins (68% identity to each other) possess a single trefoil domain (also called the P-domain or TFF domain) in the extracellular region (Fig. 1A, lower), although the entire sequences of these two proteins show no significant homology to those of other proteins. The trefoil domain consists of six conserved Cys residues that form three disulfide linkages in the pattern of Cys1–Cys5, Cys2–Cys4, and Cys3–Cys6 (Fig. 1A, lower). This domain has been implicated in binding, probably to carbohydrates and/or proteins (Hoffmann & Hauser 1993, Otto & Wright 1994, Kjellev 2009). Indeed, the trefoil domain is contained in a variety of proteins that participate in protein–protein and/or protein–carbohydrate interactions, such as sucrase-isomaltase (Alfalah et al. 1999, Spodsberg et al. 2001), the ZP-glycoprotein ZP1 (Conner et al. 2005), the human ZP-glycoprotein ZP4 (Conner et al. 2005), and trefoil factors 1–3 (Hoffmann & Hauser 1993, Otto & Wright 1994, Kjellev 2009).

Moreover, small single-pass transmembrane proteins from various species and tissues have been known to associate with self and/or non-self proteins to play roles in regulation of Na/K-ATPase (Rivard et al. 2005), mitochondrial oxidative phosphorylation (Zickermann et al. 2010), ligand-independent activation of receptors (Freeman-Cook & Dimaio 2005, Lai et al. 2005), polarization of epithelial cells (Makarova et al. 2003,
Roh et al. 2003), synthesis of poly-phosphate chains (Fang et al. 2007), and organization of the Golgi apparatus (Rojo et al. 2000).

Based on these lines of evidence, we hypothesized that TMEM190 acts during fertilization by creating a protein complex with itself and/or other mouse sperm proteins. In this study, mouse TMEM190 was characterized by biochemical and cytological approaches, and a mouse line that carries a null-mutated allele of the Tmem190 gene was created. Our data demonstrated that TMEM190 is co-localized with IZUMO1 in mouse sperm before and after the acrosome reaction. TMEM190 was also found to associate with several sperm proteins, but not with IZUMO1. Since male mice lacking TMEM190 were normally fertile, the function(s) of this mouse sperm protein is probably dispensable during fertilization.

Results

The mouse Tmem190 gene shows testis-specific transcription and encodes a 19-kDa protein found in male germ cells and gametes

The only EST clones of mouse Tmem190 were isolated from testis, suggesting that this mouse gene has testis-specific transcription. To confirm this possibility, we examined expression of the Tmem190 gene in various mouse tissues by RT-PCR. As shown in Fig. 1B, a PCR product for Tmem190 was found only in first-strand cDNA samples created from mRNA of mouse testis or testicular germ cells (TGCs, a mixed population of spermatogenic cells at various stages). This pattern of testis-specific expression was also observed for the Adam2 or Adam3 genes, but the ubiquitously expressed glyceraldehyde-3-phosphate dehydrogenase gene Gapdh was amplified in all tested samples. These data show that the mouse Tmem190 gene is expressed specifically in the testis.

Next, we investigated expression of the Tmem190-encoded protein during spermatogenesis. Protein extracts of TGCs or sperm were examined by immunoblotting using affinity-purified rabbit anti-mouse Tmem190 antibody (α-TMEM190; Fig. 1C). In TGC lysates, a prominent 19-kDa protein was detected with α-TMEM190 under the non-reducing and reducing conditions. A 14-kDa immunoreactive signal was detected in non-reduced TGC proteins, but not in reduced samples. These results indicate that TGCs contain TMEM190, which exists mostly as a 19-kDa single-chain protein. The 14-kDa variant is probably a multi-chain form of the TMEM190 protein where two or more chains are linked together by disulfide bonds.

Non-reduced sperm extracts contained 19-, 15-, and 14-kDa proteins that reacted strongly and 37- and 32-kDa proteins that reacted weakly with α-TMEM190. Sperm extracts after reduction lacked the two largest variants but still contained prominent 19-, 15-, and faint 14-kDa proteins. These data suggest that most of the 19- and 15-kDa bands represent single-chain forms, whereas the 14-kDa band corresponds to a multi-chain protein. The 37- and 32-kDa proteins observed under non-reducing conditions are likely to be multimeric forms consisting of some combination of the 19-, 15-, and 14-kDa TMEM190 variants.

TMEM190 mostly resides within TGCs, although this protein is partly present on the cell surface

To gain clues about the TMEM190 function(s) during fertilization, we determined the localization of this protein in mouse TGCs, acrosome-intact sperm (AI-sperm), and acrosome-reacted sperm (AR-sperm). Firstly, TGCs were prepared from the mouse line B6F1SJ1, in which soluble green fluorescent protein (GFP) accumulates exclusively in the sperm acrosomes (Kim & Gerton 2003). Therefore, TGCs, which are progenitor cells of sperm, contain GFP as an intracellular protein. Cell suspensions (Fig. 2A, left upper panel) and lysates (Fig. 2A, right upper panel) of TGCs were digested with proteinase K, followed by immunoblotting for the TGC-surface protein SPAM1 (formerly PH-20; Nishimura et al. 2007). This protein almost or completely disappeared in cell suspensions and lysates at any tested concentration of proteinase K, demonstrating that most SPAM1 resides on the TGC surface. On the other hand, both 29- and 27-kDa proteins of GFP, which presumably correspond to precursor and mature forms respectively, were not affected by up to 100 µg/ml proteinase K in intact TGCs (Fig. 2A, left middle panel). In TGC lysates (Fig. 2A, right middle panel), proteinase K degraded 29-kDa GFP at all tested concentrations and 27-kDa GFP at 20 µg/ml or higher concentrations. These results expectedly show that GFP is intracellularly localized in TGCs.

By treatment of intact TGCs with 10–100 µg/ml proteinase K, small but significant amounts of 19-kDa TMEM190 were degraded into a 15-kDa protein (Fig. 2A, left lower panel). As lysed TGCs were used (Fig. 2A, right lower panel), the 19-kDa immunoreactive signal was completely missing even at the lowest concentration (10 µg/ml) of proteinase K, and the 15-kDa signal was mostly erased by the addition of 100 µg/ml proteinase K. Therefore, testicular TMEM190 seems to be an intracellular protein, although a small fraction of this protein is present on the cell surface.

We also examined the TMEM190 localization in mouse TGCs by immunofluorescence (Fig. 2B). Methanol-fixed TGCs were treated with mock or 0.1% (v/v) Triton X-100 for permeabilization, followed by staining with α-TMEM190. To monitor the acrosome formation during spermatogenesis, we counter-stained the same TGCs with peanut agglutinin (PNA), which is a probe to detect the sperm acrosomes. Among mock-permeabilized TGCs,
round spermatids contained TMEM190 on the cell surface (Fig. 2Bd and Be), whereas this protein was barely found on the testicular sperm surface (Fig. 2Bi and Bj). As TGCs were permeabilized with TX100, immunoreactive signals of TMEM190 were unevenly distributed within the intracellular space of primary spermatocytes (Fig. 2Bn and Bo). In round spermatids, TMEM190 was localized both on the cell surface and in the ring-shaped organelle, which probably corresponds to an intermediate structure of the acrosome whose formation processes during spermatogenesis (Fig. 2Br and Bt). We carried out similar experiments using normal rabbit IgG (rbIgG) instead of α-TMEM190, but no significant immunoreactive signal was detected in any tested sample (data not shown). Three independent experiments confirmed the data shown here. DIC, differential interference contrast.

These findings indicate that primary spermatocytes pre-meiotically contain intracellular TMEM190 protein. It was also shown that, in post-meiotic round spermatids, TMEM190 resides mostly in the intracellular space and partly on the cell surface, whereas the surface of testicular sperm barely contains TMEM190. Moreover, these immunofluorescence-based data (Fig. 2B) were in good agreement with the results obtained by the proteinase K digestion assay of TGCs (Fig. 2A).

**TMEM190 relocates from the inner-acrosomal membrane onto the equatorial segment surface during the acrosome reaction**

We next determined the localization of mouse TMEM190 in Al- and AR-sperm. For the proteinase K digestion assay, Al-sperm (cauda epididymal sperm) were prepared from B6F1SJ mice. Intact (Fig. 3A, left upper panel) and lysed (Fig. 3A, right upper panel)
AI-sperm were treated with proteinase K and then analyzed by immunoblotting for the sperm-surface protein ADAM2 (Kim et al. 2003, 2004). The amounts of this protein were reduced as a function of the proteinase K concentration, and similar proteolytic rates were observed irrespective of whether or not the AI-sperm were lysed prior to proteinase K addition; as expected, our results confirmed that ADAM2 is a sperm-surface protein. In contrast, intracellular GFP was not obviously affected by up to 100 \( \mu \text{g/ml} \) proteinase K (Fig. 3A, left middle panel), but definite proteolysis of GFP was observed in AI-sperm lysates at either 50 or 100 \( \mu \text{g/ml} \) (Fig. 3A, right middle panel).

The 19-, 15-, and 14-kDa TMEM190-derived immunoreactive signals were detected in intact sperm at all tested concentrations of proteinase K (Fig. 3A, left lower panel). However, in sperm lysates, proteinase K digested the 19-kDa TMEM190 at any tested concentration and the 15- and 14-kDa proteins at 50 or 100 \( \mu \text{g/ml} \) (Fig. 3A, right lower panel). These data indicate that most TMEM190 is intracellular in AI-sperm. However, TMEM190 might be partly located on the surface of intact sperm, since 19-kDa TMEM190 seemed to be slightly degraded into a 15- or a 14-kDa protein by increasing the proteinase K concentration (Fig. 3A, left lower panel).

We also determined the subcellular fraction(s) of mouse sperm that contained TMEM190 (Fig. 3B). Cauda epididymal sperm were prepared from B6SJF1 mice, incubated with the calcium ionophore A23187 to induce the acrosome reaction, and fractionated into subcellular components (Fig. 3B, left). We prepared two fractions that were analyzed by immunoblotting (Fig. 3B, right): the soluble acrosomal contents plus the membrane proteins released during the acrosome reaction (RPA fraction) and mixed vesicles of the plasma and outer-acrosomal membranes (PM/OAM fraction). As previously reported (Kim et al. 2003, 2004), ADAM2 was 44 kDa. GFP was 27 kDa. TMEM190 was 19, 15, and 14 kDa.
was found in PM/OAM fraction and unfractionated AR-sperm (Fig. 3B, right upper panel). GFP, a marker protein of the sperm acrosome, was exclusively present in RPA fraction after the acrosome reaction (Fig. 3B, right middle panel). Therefore, previously characterized subcellular components of mouse sperm were fractionated in the expected manner. We found that the TMEM190 protein is present in Al- and AR-sperm, but not in RPA and PM/OAM fractions (Fig. 3B, right lower panel). Furthermore, TMEM190 was contained in the acrosome-reacted sperm head (ARH) fraction (data not shown). These results show that most TMEM190 stays at the sperm heads even after the acrosome reaction.

Immunofluorescence was then conducted to further examine the subcellular location of TMEM190 in mouse sperm (Fig. 4). In mock-permeabilized Al-sperm, any significant immunoreactive signal was not detected with rbIgG (data not shown) or α-TMEM190. However, Al-sperm permeabilized with 0.1% (v/v) TX100 showed an immunoreactive signal with α-TMEM190, but not with rbIgG (data not shown), around the rim of the acrosomes. These data indicate that TMEM190 is mostly localized in the inner-acrosomal membrane of Al-sperm. In contrast, regardless of whether or not AR-sperm were permeabilized, TMEM190 seemed to partly relocate during the acrosome reaction from the inner-acrosomal membrane onto the equatorial segment surface. Interestingly, this relocation pattern was similar to that of mouse IZUMO1 (Kawai et al. 1989, Yamashita et al. 2007, Fujihara et al. 2010), an essential protein for sperm–oocyte fusion (Inoue et al. 2005).

**TMEM190 is co-localized with IZUMO1 in mouse sperm before and after the acrosome reaction**

To our knowledge, IZUMO1 is the only protein to satisfy both of that it resides in sperm similarly to TMEM190 and that its role during fertilization is clear. Therefore, we examined whether TMEM190 and IZUMO1 are co-localized together in permeabilized Al- or AR-sperm (Fig. 5), to access the TMEM190 function(s). TMEM190 was found to co-localize with IZUMO1 at the inner-acrosomal membrane of Al-sperm. In AR-sperm, both TMEM190 and IZUMO1 were present at the inner-acrosomal membrane and partly at the equatorial segment. These data demonstrate that TMEM190 and IZUMO1 co-localize in mouse sperm both before and after the acrosome reaction.

**TMEM190 forms a protein complex(es) with other sperm proteins, but not with IZUMO1**

We immunoprecipitated TMEM190 to investigate whether this protein associates with IZUMO1 (Fig. 6A). In all tested cell lysates (total), immunoprecipitates with neither rbIgG- nor α-TMEM190-beads included
IZUMO1 (Fig. 6A, left panel), whereas TMEM190 clearly formed an immune complex specifically with α-TMEM190 (Fig. 6A, right panel). These data reveal that TMEM190 is physically independent of IZUMO1 in mouse TGCs, AI-sperm, and AR-sperm under immunoprecipitating conditions. However, it remains possible that TMEM190 and IZUMO1 are loosely associated and do not withstand the methods used for immunoprecipitation.

We also explored the possibility that TMEM190 associates with other sperm proteins (Fig. 6B). Cell-surface proteins on AR-sperm were biotinylated, bound to monomeric avidin-beads, washed, and eluted to prepare a fraction containing sperm-surface proteins (surface). To check whether biotinylation is restricted to cell-surface proteins, ‘total’ and ‘surface’ fractions of AR-sperm before or after biotinylation were examined by immunoblotting for the sperm-surface protein ADAM2 (Fig. 6B, left upper panel) or the intracellular protein α-tubulin (Fig. 6B, left lower panel). ADAM2 exhibited a slower migration in biotinylated ‘total’ fractions than control samples, indicating that ADAM2 was modified with biotin. Indeed, this protein was found in ‘surface’ fractions of biotinylated, but not mock-biotinylated, AR-sperm. On the other hand, α-tubulin was indistinguishable in size between mock- and actually biotinylated ‘total’ samples. Moreover, ‘surface’ samples of biotinylated AR-sperm did not contain this protein.
Finally, we created mice lacking TMEM190 to elucidate the function(s) of this protein. The entire region of the Tmem190 gene was replaced by the neomycin-resistance gene cassette in the mouse genome by homologous recombination (Fig. 7A). This gene targeting was confirmed by PCR genotyping of tail DNA samples that had been prepared from Tmem190<sup>+/+</sup>, Tmem190<sup>−/−</sup>, or Tmem190<sup>−/+</sup> mice (Fig. 7B). Moreover, Tmem190 protein was completely missing in TGCs and cauda epididymal sperm of Tmem190-null mice, whereas IZUMO1, a co-localized protein with TMEM190, was of normal amounts in the same TGC or sperm samples (Fig. 7C). Protein levels of ADAM2, ADAM3, and SPAM1, of which functions are presumably independent of the Tmem190 function(s), were similar between Tmem190<sup>+/+</sup> and Tmem190<sup>−/−</sup> cell lysates of TGCs or cauda epididymal sperm (data not shown). These data show that a mouse line lacking Tmem190 was established.

By crossing of Tmem190<sup>+/−</sup> male and female mice, Tmem190<sup>−/−</sup> mice were produced according to the expected Mendelian frequency (Tmem190<sup>+/−</sup>: Tmem190<sup>−/−</sup>:Tmem190<sup>−/+</sup> = 32 (24%):68 (50%):36 (26%)), and a ratio of Tmem190<sup>−/−</sup> male and female progeny was also as expected (male:female = 17 (47%):19 (53%)). There was no significant abnormality affecting the health, body size, and behavior in Tmem190<sup>−/−</sup> mice.

Therefore, ‘surface’ fractions of AR-sperm were appropriately prepared. TMEM190 was then immunoprecipitated with α-TMEM190-beads in ‘surface’ fractions. After co-precipitated proteins were separated by SDS-PAGE, they were transferred onto membranes and captured by incubation of the blotted membranes with NeutrAvidin (Fig. 6B, middle panel) or α-TMEM190 (Fig. 6B, right panel). TMEM190 and its co-precipitated proteins were contained only in the immunoprecipitates with α-TMEM190-beads. Particularly, 65-, 43-, 40-, and 19-kDa proteins showed clear association with TMEM190. It is not yet clear whether the biotinylated 19-kDa protein is TMEM190, since a 15-kDa surface protein was not found in the immune complexes including both 19- and 15-kDa TMEM190. Efficiency of biotinylation might be different between these two TMEM190-derived proteins. At any rate, these combined results demonstrate that TMEM190 forms a protein complex(es) on the AR-sperm surface with, at least, 65-, 43-, and 40-kDa proteins.

**Tmem190-null mice are fertile**

Finally, we created mice lacking TMEM190 to elucidate the *in vivo* function(s) of this protein. The entire region of the *Tmem190* gene was replaced by the neomycin-resistance gene cassette in the mouse genome by homologous recombination (Fig. 7A). This gene targeting was confirmed by PCR genotyping of tail DNA samples that had been prepared from *Tmem190<sup>+/+</sup>* (26%), *Tmem190<sup>−/−</sup>* (50%), and *Tmem190<sup>−/+</sup>* (24%) mice (Fig. 7B). Moreover, *Tmem190* protein was completely missing in TGCs and cauda epididymal sperm of *Tmem190-null* mice, whereas IZUMO1, a co-localized protein with TMEM190, was of normal amounts in the same TGC or sperm samples (Fig. 7C). Protein levels of ADAM2, ADAM3, and SPAM1, of which functions are presumably independent of the *Tmem190* function(s), were similar between *Tmem190<sup>+/+</sup>* and *Tmem190<sup>−/−</sup>* cell lysates of TGCs or cauda epididymal sperm (data not shown). These data show that a mouse line lacking TMEM190 was established.

By crossing of *Tmem190<sup>+/−</sup>* male and female mice, *Tmem190<sup>−/−</sup>* mice were produced according to the expected Mendelian frequency (*Tmem190<sup>+/−</sup>*: *Tmem190<sup>−/−</sup>*:*Tmem190<sup>−/+</sup> = 32 (24%):68 (50%):36 (26%)), and a ratio of *Tmem190<sup>−/−</sup>* male and female progeny was also as expected (male:female = 17 (47%):19 (53%)). There was no significant abnormality affecting the health, body size, and behavior in *Tmem190<sup>−/−</sup>* mice.
Figure 7Tmem190-null mice are fertile. (A) The targeting strategy used for the mouse Tmem190 gene. Exons and introns of Tmem190 are shown by black boxes and intervening horizontal lines respectively. White boxes represent the neomycin-resistance gene (neo) cassette. A targeting vector was linearized at the KpnI site (not shown) before electroporation into mouse ES cells. FW, WTRV, and NeoRV were PCR primers used for genotyping of ES cell clones or mouse tails. WT, wild-type; KO, knock-out; Sc, SacI; X, XhoI; and Sl, SalI. (B) PCR genotyping of mice carrying the targeted mutation in the Tmem190 gene. Genomic DNA was prepared from tails of Tmem190+/+ (+/+), Tmem190+/− (+/−), or Tmem190−− (−/−) mice and used for PCR genotyping. This result is a representative of three independent experiments. (C) Immunoblotting of Tmem190-null TGCs and sperm. Protein extracts were prepared from TGCs or cauda epididymal sperm of Tmem190+/+ (+/+) or 56-kDa IZUMO1. These data were confirmed by three independent experiments. (D) In vivo fertility test of Tmem190-null mice. Tmem190+/+ (+/+) or Tmem190−− (−/−) male mice were individually placed with two C57BL/6 (B6) female mice in a cage. Each female that had a vaginal plug was transferred into a new cage, and the numbers of pups born were counted. The same experiments were also conducted in mating pairs of B6 males and +/+ or −/− females. Data are expressed as ‘mean±s.d.’.

Figure 7D showed the data on in vivo fertility of Tmem190-null mice. As male mice lacking TMEM190 were crossed to C57BL/6 female mice, an average litter size (mean±s.d.) of the males (8.7±0.90, n=6) was similar to that of Tmem190+/+ males (8.5±0.65, n=7). Breeding of C57BL/6 males and tested females resulted in similar average litter sizes between Tmem190+/+ and Tmem190−− females (8.0±0.65, n=6 vs 8.5±0.71, n=6). Hence, the mouse Tmem190 gene is not essential for in vivo fertilization.

Discussion

We previously identified TMEM190 in the mouse sperm proteome as a small transmembrane protein containing the trefoil domain (Stein et al. 2006). From two keywords, ‘trefoil domain’ and ‘small transmembrane protein’, we hypothesized that TMEM190 acts during fertilization through the association with self and/or non-self proteins. We found in this study that mouse TMEM190 resides at the inner-acrosomal membrane of AI-sperm and relocates onto the plasma membrane of the equatorial segment during the acrosome reaction. We also found that mouse sperm contain a protein complex(es) including TMEM190. Lastly, mice lacking TMEM190 were normally fertile.

We observed 19-, 15-, and 14-kDa proteins in both non-reduced and reduced sperm extracts by immunoblotting using α-TMEM190. Therefore, the 19-kDa protein probably corresponds to intact sperm TMEM190 and is degraded into the 15- or the 14-kDa proteins. A calculated size (16 476 kDa) of the predicted TMEM190 polypeptide lacking a 21-mer signal sequence is phosphorylation, since the cytoplasmic tail of TMEM190 contains seven Ser (residues 124, 129, 131,
sperm are defective in both the relocation of IZUMO1 and sperm–oocyte fusion. Since we were not able to show that TMEM190 and IZUMO1 interact with each other and Tmem190-null mice are fertile, the fusogenic role(s) of TMEM190, if any, is probably indirect and dispensable.

The second clue is a recent study on sperm CD9 (Ito et al. 2010); this protein also exhibits essentially the same subcellular localization pattern in mouse sperm as those of TMEM190 and IZUMO1. While oocytes from mice lacking CD9 are incapable of being fused with wild-type sperm, CD9-deficient sperm are normal in all tested sperm functions that include the ability to fertilize oocytes (Kaji et al. 2000, Le Naour et al. 2000, Miyado et al. 2000). This shows that the function(s) of mouse sperm CD9 is dispensable during fertilization, like mouse sperm TMEM190 (Fig. 7D). Therefore, CD9 might be functionally related to TMEM190 in mouse sperm with both indirectly regulating sperm–oocyte fusion.

There are two facts about TMEM190 that are puzzling. First, the human Tmem190 gene seems to be expressed predominantly in the pharynx and the ovary unlike the testis-specific expression of the mouse Tmem190 gene. One currently unexplored possibility is that the human gene might be expressed in the testis at low but enough levels to produce the functional Tmem190 protein. If human sperm do not contain TMEM190, another protein(s) might play the same role(s) as that of mouse TMEM190. This possibility is based on the following findings: the mouse testis-specific genes Adam1a (Nishimura et al. 2004) and Adam3 (Shamsadin et al. 1999, Nishimura et al. 2001) are essentially required for male fertility, even though these two genes are both pseudogenes in the human genome (Jury et al. 1997, Grzmil et al. 2001). Currently, there is no follow-up evidence yet in this case, but other human testis/sperm-specific ADAM proteins presumably compensate for the functions of mouse ADAM1a and ADAM3.

Second, Tmem190-null mice show normal fertility. Tmem190 might be a mouse gene that requires a two or more multiple-gene knockout within the same animal to cause a profound defect(s) (Shastry 1995). Mouse Tmem190 is found in a multi-protein complex(es) and perhaps if one or more of other components for the sperm protein complex(es) are targeted, the mutant males would be sterile or subfertile.

As a summary, we propose an indirect role(s) of mouse TMEM190 in gamete fusion by comprising a sperm protein complex(es). Alternatively, the protein complex(es) is possibly involved in regulation of the acrosome reaction, since the protein dynamics of TMEM190 is clearly linked to the acrosome reaction. At any rate, the Tmem190 role(s) within the mouse sperm protein complex(es) requires to be examined further. Particularly, Tmem190 and CD9 should be investigated to determine whether they are functionally linked to each other in mouse sperm. It would also...
be worthwhile to clarify whether human sperm contain a protein complex(es) that is functionally equivalent to the TMEM190-bearing mouse sperm protein complex(es). This would lead to understanding the molecular basis of human male infertility.

Materials and Methods

Animals

CD1 and C57BL/6 mice were purchased from Charles River Laboratories International, Inc. (Wilmington, MA, USA) and the Jackson Laboratory (Bar Harbor, ME, USA) respectively. The transgenic mouse line B6SJF1 (Kim & Gerton 2003), in which GFP is localized in the sperm acrosomes, was a kind gift from Dr G L Gerton (University of Pennsylvania, Philadelphia, PA, USA). Tmem190-null mice were created in this study (for details, see ‘Creation of Tmem190-null mice’). All these mouse lines were maintained in an animal facility at the University of California Davis (UCD). Rabbits used for immunization to raise α-TMEM190 were maintained in the UCD Comparative Pathology Laboratory. All experiments using animals were carried out under approval by the UCD Institutional Animals Care and Use Committee.

Isolation of TGCs and mature sperm

TGCs (Phelps et al. 1990, Nishimura et al. 2007) and mature sperm (Claassens et al. 1998, Nishimura et al. 2007) were isolated from ~16-week-old male mice. Briefly, for TGC preparation, cell suspensions containing all testicular cell types were obtained by thoroughly mincing testicles with razor blades, and TGCs were separated from the testicular cell suspensions on a 52% (v/v) gradient of Percoll (GE Healthcare). Mature sperm were obtained by thoroughly mincing testicles with razor blades, and TGCs were separated from the testicular cell suspensions on a 52% (v/v) gradient of Percoll (GE Healthcare). Mature sperm were released from the cauda epididymis and subsequently purified by using OptiPrep Density Gradient Medium (Sigma–Aldrich). To prepare protein extracts of TGCs or mature sperm, cells were collected by centrifugation of the suspensions at 20 000 g for 10 min at 4°C, the supernatants were used for experiments. We also used PBS/OG containing the EDTA-free Complete Protease Inhibitor Cocktail Tablets (Roche Applied Science) for extraction of TGC or sperm proteins. However, the protease inhibitor cocktail showed no significant effect on blocking the degradation of TMEM190 protein, which presumably occurred during the cellular protein extractions (data not shown). Therefore, all cell lysates used in this study were prepared in PBS/OG containing no protease inhibitors.

RT-PCR

Gene expression of Tmem190 was examined in various mouse tissues by RT-PCR, in which first-strand cDNAs of the tested tissues were used as templates for conventional PCR. Except for TGCs, all tested mouse tissues could be evaluated using various first-strand cDNAs that were contained in the Mouse Multiple Tissue cDNA Panel I Kit (Clontech Laboratories, Inc.).

To prepare first-strand cDNA of mouse TGCs, poly(A)+ RNA was purified from 1 × 10⁸ TGCs with the FastTrack 2.0 mRNA Isolation Kit (Invitrogen Life Technologies). First-strand cDNA was then synthesized from 100 ng of the mouse TGC poly(A)+ RNA with random primers in 20-μl reaction mixtures by using the StrataScript First-Strand cDNA Synthesis Kit (Agilent Technology, Inc., formerly Stratagene, Santa Clara, CA, USA), according to the manufacturer’s protocol. PCR was carried out in 10-μl reaction mixtures composed of 1× PCR buffer, 200 μmol/l dNTP mixture, 0.2 μmol/l forward primer, 0.2 μmol/l reverse primer, 1 μl of first-strand cDNA, and 1× Advantage cDNA Polymerase Mix (Clontech). Primer sets were designed to target N-TMEM190 (435 bp), 5′-ATCGTCTCTCATATTGGGAAATTGCAT-3′ (forward) and 5′-CTATTTCTCACCTTGGTTGTC-3′ (reverse) for Tmem190 (Kim et al. 2006), 5′-TAACAAAGAATTCTGGAGAATGG-3′ (forward) and 5′-AAGCCCTCACAGCTAACGCTTTGG-3′ (reverse) for Adam2 (Kim et al. 2006), 5′-GGTTAGGAGTCGAGTCAACGGATTTGGT-3′ (forward) and 5′-CAGTGGGC-CATGAGGTCCACCACAGCT-3′ (reverse) for Gapdh (Kim et al. 2006). Target cDNAs were amplified by one cycle of 94°C for 3 min, followed by 30 cycles of 94°C for 1 min, 63°C for 1 min, and 72°C for 1 min, and one cycle of 72°C for 10 min. The resulting PCR products were separated by 0.8% (w/v) agarose gel electrophoresis.

Antibodies

Mouse MAB against mouse IZUMO1 (α-IZUMO1; IgG, clone OBF13; Okabe et al. 1997) was a gift of Dr M Okabe (Osaka University, Osaka, Japan). Affinity-purified mouse polyclonal antibody against mouse SPAM1/PH-20 was purchased from Chemicon/Millipore Corporation (Temecula, CA, USA). A mixture of two mouse MABs against GAPDH (IgG1, clones 7.1 and 13.1) was purchased from Invitrogen Life Technologies. Alexa Fluor 488-conjugated goat anti-mouse IgM (m chain) antibody was from Thermo Scientific (formerly Pierce Biotechnology; Rockford, IL, USA), and HRP-conjugated goat antibodies against rabbit IgG and mouse IgG were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Alexa Fluor 488-conjugated goat anti-mouse IgG (m chain) antibody and Alexa Fluor 568-conjugated donkey anti-rabbit IgG antibody, which were the secondary antibodies used for immunofluorescence, were purchased from Molecular Probes/Invitrogen (San Diego, CA, USA).

We prepared α-TMEM190 as follows: a DNA fragment encoding the N-terminal region of mouse TMEM190 (residues 22–80, N-TMEM190) was ligated into a pGEX-4T-1 vector (GE Healthcare). By using this expression vector, a glutathione S-transferase (GST)-tagged recombinant protein of N-TMEM190 (GST-N-TMEM190) was produced in the Escherichia coli strain BL21. GST-N-TMEM190 was purified on a column of GST-Bind Resin (Novagen/EMD Chemicals, Inc., Gibbstown, NJ, USA) and used for immunization.
of rabbits. For affinity purification of α-TMEM190, the DNA fragment coding for N-TMEM190 was ligated into pET-23d (Novagen), and this vector was used to produce a recombinant protein of N-TMEM190 that is tagged at its C-terminal 6 × His (N-TMEM190/His6) in the E. coli strain BL21 (DE3). The N-TMEM190/His6 protein was immobilized onto Sepharose 4B (GE Healthcare), incubated with raw α-TMEM190 sera, and washed with PBS containing 500 mmol/l NaCl. α-TMEM190 was eluted from the beads with 0.1 mol/l glycine-HCl, pH 2.5, and immediately neutralized with one-ninth volume of 2 mol/l Tris–HCl, pH 9.0. The purified antibody was dialyzed against PBS prior to using in experiments.

**Immunoblotting**

SDS-PAGE using 14% (w/v) Novex Tris-Glycine Gels (Invtrogen) was carried out under the reducing conditions, unless otherwise noted. Separated proteins were transferred onto Immobilon-P Transfer Membranes (Millipore, Bedford, MA, USA), followed by incubation of the membranes at room temperature for 1.5 h with 1 µg/ml primary antibodies and subsequently for 30 min with 50 ng/ml HRP-conjugated secondary antibodies. To capture biotinylated proteins, blots were incubated for 1.5 h at room temperature with 0.5 µg/ml High Sensitivity NeutrAvidin–HRP (Thermo Scientific). Immune or biotin–NeutrAvidin complexes were then detected by using the SuperSignal West Dura Extended Duration Substrate (Thermo Scientific) according to the manufacturer’s instruction.

**Proteinase K digestion of cell-surface proteins**

TGCs or cauda epididymal sperm of B6SJF1 mice were suspended in PBS (1 × 10⁸ cells/ml), and 90-µl aliquots from each cell suspension were incubated with 10 µl of various concentrations of proteinase K for 30 min at 37°C. To terminate the proteinase K digestion, 2 µl of 100 mmol/l phenylmethylsulfonyl fluoride (PMSF) was added, followed by additional incubation for 15 min at room temperature. The digested cell samples were washed three times with PBS by centrifugation at 800 g for 5 min at 4°C, suspended in 2 µl of 100 mmol/l PMSF plus 100 µl of PBS/OG, and incubated for 30 min on ice. After centrifugation at 20 000 g for 10 min at 4°C, the supernatants were used as ‘cell suspension’. Similar experiments were also carried out using TGC or sperm protein extracts derived from B6SJF1 mice (equivalent to 1 × 10⁸ cells/ml, see ‘Isolation of TGCs and mature sperm’) for preparation, instead of cell suspensions. In this case, reaction mixtures after the PMSF treatment to terminate proteinase K digestion were used as ‘cell lysate’. Proteins that were contained in aliquots (5.7 µl for TGCs and 11.3 µl for sperm) from ‘cell suspension’ or ‘cell lysate’ samples were analyzed by immunoblotting for SPAM1, ADAM2, GFP, or TMEM190.

**Subcellular fractionation of mature sperm**

Subcellular components of mature sperm were prepared according to the published methods (Honda et al. 2002, Kim et al. 2003, 2004) with slight modifications. Briefly, Al-sperm of B6SJF1 mice (1 × 10⁸ sperm/ml) were incubated for 1 h at 37°C with 10 µmol/l calcium ionophore A23187 in Human Tubal Fluid (HTF) medium (without BSA, Irvine Scientific, Santa Ana, CA, USA). The sperm suspensions after the acrosome reaction were centrifuged at 800 g for 5 min at room temperature to remove AR-sperm, and the supernatants were again centrifuged at 20 000 g for 5 min at room temperature. The low-speed supernatants were subjected to ultracentrifugation at 100 000 g for 90 min at 4°C, and the resulting high-speed supernatants included proteins released during the acrosome reaction plus soluble acrosomal contents (RPA). The pellets obtained by ultracentrifugation were washed once with PBS and suspended in PBS/OG to prepare protein extracts (for preparation, see ‘Isolation of TGCs and mature sperm’). The extracts were then used as a fraction of the PM and OAM that had been fused to each other during the acrosome reaction (PM/OAM). AR-sperm, obtained by centrifugation after the incubation of Al-sperm with A23187, were washed three times with PBS, resuspended in PBS (1 × 10⁸ cells/ml), and sonicated to detach their tails from the sperm heads. Fractions of the AR-sperm heads (ARH) and tails (ART) were subsequently separated on a sucrose density gradient. Proteins in each subcellular fraction (equivalent to 1 × 10⁶ sperm) were analyzed by immunoblotting for ADAM2, GFP, or TMEM190.

**Immunofluorescence**

TGCs were isolated from CD1 mice and suspended (0.5 × 10⁷ cells/ml) in HEPES-buffered saline containing Mg²⁺ (Phelps et al. 1990, Nishimura et al. 2007). Ten-µl aliquots from the TGC suspensions were placed as ~1-cm spots onto coverslips that had been coated by poly-l-lysine and allowed to stand at room temperature for ~10 min until the edge of each spot became dried. The cell samples were then fixed in methanol for 15 min at −20°C, followed by washing three times with PBS. As for sperm samples, Al- or AR-sperm (for preparation, see ‘Subcellular fractionation of mature sperm’) of CD1 mice were washed three times with PBS at 800 g for 5 min at room temperature, suspended (1 × 10⁷ sperm/ml) in PBS containing 4% (w/v) paraformaldehyde (PBS/PFA), and incubated for 30 min on ice to allow these sperm samples to be fixed. After washing three times with PBS, sperm were resuspended in PBS (0.5 × 10⁷ sperm/ml), placed onto poly-l-lysine-coated coverslips (5-µl sperm suspension/spot), and kept for ~15 min at room temperature until the sperm became completely dried. The sperm spots were then washed three times with PBS. The cell spots of TGCs, Al-sperm, or AR-sperm that had been fixed followed by washing with PBS were incubated for 30 min at room temperature in 100 µl of PBS containing 3% (w/v) BSA (PBSA) or PBSA containing 0.1% (v/v) TX100 (PBSAT) and then washed once with PBS. The cells were subsequently incubated for 1 h at room temperature in 100 µl of PBSA containing 10 µg/ml Alexa Fluor 488-conjugated PNA (Molecular Probes) and/or 5 µg/ml rhbG or α-TMEM190 and/or 5 µg/ml moIgM or α-IZUMO1. After washing three times with PBS containing 0.1% (v/v) TX100 (PBST), the cells were incubated for 30 min at room temperature in 100 µl of PBSAT containing 5 µg/ml 4’,6-diamino-2-phenylindole (DAPI) and 10 µg/ml Alexa...
Fluor 568-labeled donkey anti-rbIgG antibody and/or 10 µg/ml Alexa Fluor 488-labeled goat anti-molG antibodies. After the cell-bearing coverslips were washed three times with PBS and twice with PBS, one drop of the ProLong anti-fade reagent (Invitrogen) was put onto each cell spot. The cell samples were then observed under the FluoView FV1000 Confocal Microscope (Olympus America, Inc., Center Valley, PA, USA).

**Immunoprecipitation**

Protein extracts of TGCs, Al-sperm, and AR-sperm from CD1 mice (total) were prepared as described in ‘isolation of TGCs and mature sperm’. Cell-surface protein fractions of AR-sperm (surface) were prepared as previously reported (Nishimura et al. 2007). Briefly, AR-sperm (0.5 × 10⁸ sperm/ml) were incubated for 30 min at room temperature with 1 mmol/l EZ-Link Sulfo-NHS-LC-Biotin (Thermo Scientific) in 2 ml of HTF medium. After washing three times with PBS, the biotinylated AR-sperm were suspended in 1 ml of PBS/OG to prepare protein extracts. To isolate biotinylated proteins, the cell lysates (equivalent to 1 × 10⁹ sperm) were incubated for 30 min at room temperature with 100 µl of 50% (v/v) slurry of Monomeric Avidin Agarose (Thermo Scientific) in 2 ml of PBS/OG containing 500 mmol/l NaCl (Binding Buffer). The beads were washed three times with Binding Buffer, suspended in 200 µl of 5 mol/l NaCl in the same buffer, and incubated for 30 min at room temperature to allow biotinylated proteins to be eluted out from the monomeric avidin-beads. Following centrifugation at 500 g for 5 min at room temperature, the supernatants containing AR-sperm surface proteins were used as ‘surface’ fraction. To confirm that only cell-surface proteins are biotinylated, ‘total’ and ‘surface’ fractions of AR-sperm with or without biotinylation were subjected to immunoblotting for ADAM2 or α-tubulin.

For immunoprecipitation, ‘total’ or ‘surface’ samples (equivalent to 2.5 × 10⁷ cells) were incubated for 2 h on ice in 1 ml of Binding Buffer containing 25 µl of 50% (v/v) slurry of Sepharose 4B-beads where rbIgG or α-TMEM190 was immobilized (~2 mg IgG/ml beads). After washing three times with Binding Buffer, the beads were suspended in 62.5 µl of 0.2 mol/l glycin-HCl, pH 2.5, containing 1% (w/v) OG, and incubated for 15 min at room temperature. The suspensions were then centrifuged at 500 g for 5 min at room temperature, and their supernatants containing mouse TMEM190 and its associated proteins were mixed with one-fourth volume of 2 mol/l Tris–HCl, pH 9.0, to neutralize the supernatant solutions. Proteins in the immunoprecipitated fractions were analyzed by immunoblotting for TMEM190, IZUMO1, or biotinylated proteins.

**Creation of Tmem190-null mice**

The mouse genomic clone bMQ-202C5, including the Tmem190 gene, was obtained from the Wellcome Trust Sanger Institute (Cambridge, UK). To construct a targeting vector, 2.2- and 6.3-kbp DNA fragments that correspond to 5'- and 3'-flanking regions of Tmem190 respectively were excised from the bMQ-202C5 DNA. These two fragments and an expression cassette of the neomycin-resistance gene (neo) were then ligated together into a pBluescript II SK(+) vector (Agilent Technology). After the targeting vector that had been linearized by KpnI digestion was electroporated into mouse embryonic stem (ES) cells, homologous recombinants were selected by using G418. By PCR screening (for details, see ‘PCR screening’), seven ES cell clones carrying the targeted mutation in the Tmem190 gene were identified among 96 G418-resistant clones and subsequently injected into C57BL/6 mouse blastocysts. The resulting chimeric male mice were crossed to C57BL/6 female mice to obtain heterozygous mutant progeny for the Tmem190 gene. Homozygous mutant mice were obtained by mating of heterozygous males and females.

**PCR screening**

Tmem190 genotypes of ES cell clones or mice were determined by PCR. Primer sets used were as follows: 5’-TTCCCCCTCCCATTTTCACACT-3’ (FW, forward) and 5’-TCCTGCTTTAGGGAACACAGACCAG-3’ (WTRV, reverse) for the wild-type locus, and the FW primer (forward) and 5’-GACCCGCTCTCGTGAGTTCGATTTCCG-3’ (NeoRV, reverse) for the mutant locus. Template genomic DNA samples were prepared from ES cells or mouse tails, according to the published method (Aljanabi & Martinez 1997). PCR mixtures (20 µl) were composed of 1× PCR buffer, 200 µmol/l dNTP mixture, 0.2 µmol/l forward primer, 0.2 µmol/l reverse primer, ~100 ng of genomic DNA, and 1× Advantage cDNA Polymerase Mix. The PCRs were subjected to one cycle of 94 °C for 3 min, followed by 32 cycles of 94 °C for 1 min, 65 °C for 1 min, and 72 °C for 3 min, and one cycle of 72 °C for 10 min. Amplified DNAs were then subjected to 0.8% (w/v) agarose gel electrophoresis.

**In vivo fertility test**

Each tested male (8–16 weeks old) was placed with two C57BL/6 females (6–8 weeks old) in a cage, while two tested females were crossed to one C57BL/6 male. Once a day, every female was checked for the vaginal plug formation, and plug-positive females were individually transferred into new cages. After ~20 days, the numbers of pups born were counted to compare in vivo fertility of Tmem190-null mice with that of wild-type mice.

**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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