Mol. Cells *30*, 443-448, November 30, 2010
DOI/10.1007/s10059-010-0138-4

Molecules and Cells

Extracellular Domain of V-Set and Immunoglobulin Domain Containing 1 (VSIG1) Interacts with Sertoli Cell Membrane Protein, while Its PDZ-Binding Motif Forms a Complex with ZO-1

Ekyune Kim[1,4], Youngjeon Lee[1,2,4], Ji-Su Kim[1], Bong-Seok Song[1], Sun-Uk Kim[1], Jae-Won Huh[1], Sang-Rae Lee[1], Sang-Hyun Kim[1], Yonggeun Hong[2,3], and Kyu-Tae Chang[1,*]

V-set and immunoglobulin domain containing 1 (VSIG1) is a newly discovered member of the junctional adhesion molecule (JAM) family; it is encoded by a gene located on human chromosome X and preferentially expressed in a variety of cancers in humans. Little is known about its physiological function. To determine the role(s) of VSIG1 in mammalian spermatogenesis, we first generated a specific antibody against mouse VSIG1 and examined the presence and localization of the protein in tissues. RT-RCR and Western blot analysis of the mouse tissues indicated that VSIG1 was specifically expressed in the testis. Furthermore, the results of our trypsinization and biotinylation assays strongly support the assumption that VSIG1 is localized on the testicular germ cell surface. In order to determine whether VSIG1 is capable of participation in homotypic interactions, we performed a GST-pull down assay by using recombinant GST-fusion and His-tagging proteins. The pull-down assay revealed that each GST-fusion Ig-like domain shows homotypic binding. We further show that mVSIG1 can adhere to the Sertoli cells through its first Ig-like domain. To identify the protein that interacted with cytoplasmic domain, we next performed co-immunoprecipitation analysis. This analysis showed that ZO-1, which is the central structural protein of the tight junction, is the binding partner of the cytoplasmic domain of mouse VSIG1. Our findings suggest that mouse VSIG1 interacts with Sertoli cells by heterophilic adhesion via its first Ig-like domain. In addition, its cytoplasmic domain is critical for binding to ZO-1.

INTRODUCTION

Mammalian spermatogenesis occurs in the seminiferous epithelium, which is composed of Sertoli cells and germ cells (Wang and Cheng, 2007; Yan et al., 2007). The interaction between spermatogenic and Sertoli cells as well as that between elongated spermatids and Sertoli cells is tightly regulated by junctional adhesion molecules (JAMs) which are involved in various biological processes such as fertilization, neurogenesis, tumorigenesis, and spermatogenesis (Inoue et al., 2005; Takai et al., 2003; Urase et al., 2001; Wakayama et al., 2001).

Members of the JAM family have a unique structure: they contain an N-terminal signal peptide domain, immunoglobulin (Ig)-like domains, transmembrane and cytoplasmic tail domains, each of which has distinct functions (Arrate et al., 2001). The extracellular Ig-like domains interact with neighboring cells in a homophilic or heterophilic manner. In addition, the PDZ-binding motif of their cytoplasmic tail domain has been reported to be important in the tight junction (TJ) assembly (Wakayama and Iseki, 2009). Although members of the JAM family are exclusively present in the testis, their precise roles in spermatogenesis and fertilization have not yet been completely explored.

Recently, the Scanlan research group has classified human VSIG1 (hVSIG1), which was originally known as A34, as a member of the JAM family; VSIG1 is composed of two extracellular Ig-like domains, a transmembrane domain, and a cytoplasmic domain (Scanlan et al., 2006). VSIG1 is expressed in a variety of cancers and in the testis; hence, it seems to be a candidate for antibody-based diagnostics and therapeutics (Scanlan et al., 2006). Interestingly, the C-terminal peptide of mouse VSIG1 (mVSIG1), unlike hVSIG1, is similar to peptide motifs known to interact with PDZ protein domains, such as those contained in zonula occludens-1 (ZO-1) and other membrane-associated guanylate kinase (MAGUK) proteins (Gonzalez-Mariscal et al., 2000).

On the basis of the structure of the mVSIG1 protein, we predicted that its Ig-like domains play an important role in cell-cell interaction, whereas its C-terminal domain is needed for TJ assembly. In the present study, we generated a specific poly

[1]National Primate Research Center, Korea Research Institute of Bioscience and Biotechnology, Ochang 363-883, Korea, [2]Department of Rehabilitation Science in Interdisciplinary PhD Program, College of Biomedical Science and Engineering, Inje University, Gimhae 621-749, Korea, [3]Department of Physical Therapy, Cardiovascular & Metabolic Disease Center, College of Biomedical Science and Engineering, Inje University, Gimhae 621-749, Korea, [4]These authors contributed equally to this work.
*Correspondence: changkt@kribb.re.kr

Received May 28, 2010; revised August 17, 2010; accepted August 19, 2010; published online October 14, 2010

Keywords: adhesion molecules, JAM family, spermatogenesis, tight junction

clonal antibody against mVSIG1 to order identify the expression pattern and subcellular localization of this protein and thereby gain insights into its biological function. In addition, by using biotinylated Sertoli cell membrane proteins, we showed that mVSIG1 binds to Sertoli membrane protein. Furthermore, our co-immunoprecipitation experiments confirmed the direct interaction between mVSIG1 and ZO-1 though a PDZ binding motif in the C-terminal domain of mVSIG1.

MATERIALS AND METHODS

Total RNA extraction and reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was extracted from various mouse tissues by using Isogen (Nippon Gene, Japan), as described before (Nishimura et al., 2002). Five micrograms of total RNA was reverse-transcribed to cDNA with the SuperScript III First-Strand Synthesis System (Invitrogen). Polymerase chain reaction (PCR) amplification was carried out using EX Taq DNA polymerase (Takara, Japan) according to the manufacturer's instructions. A tenth of the first-strand cDNA reaction mixture was used as PCR template. PCR primers were designed for amplification of the mouse *VSIG1* and *Vps35* genes (GenBank Accession nos. NM_030081 and NM_022997, respectively). The primers for *VSIG1* were 5′-AGTATGGTGCAAGTGACC ATC-3′ and 5′-TTGACTAAGACAGTGACGTC-3′ and the primers for Vps35 were 5′-GAATTCAGTGAAGAGAATCATGAA CCCT-3′ and 5′-TTGGGCCCTTAAAGGATGAGACCTTCAT AG-3′ (Kim et al., 2008).

Antibodies

The fragment encoding the first Ig-like domain of VSIG1 (residues 269-336) was amplified by PCR, introduced into the pCold (Takara, Japan) vector, and expressed in *Escherichia coli* BL21 (DE3) cells. The recombinant His-tagged proteins were emulsified with Freund's complete adjuvant (Sigma-Aldrich), and injected intradermally into female New Zealand white rabbit. After fractionation of antisera with ammonium sulfate (0-40% saturation), anti-VSIG1 was affinity-purified on a sepharose 4B column coupled with the first Ig-like domain of VSIG1 proteins fused to glutathione *S*-transferase (GST), as described previously (Kim et al., 2003). Polyclonal anti-JAM-C and monoclonal anti-mouse ADAM2 antibodies were purchased from Santa Cruz and Chemicon (Oh et al., 2009).

Purification of testicular germ cells

Testicular tissues from 3- to 4-month old mice were minced with a razor blade in 4 mM Hepes-NaOH, pH 7.4, containing 140 mM NaCl, 4 mM KCl, 10 mM glucose, and 2 mM $MgCl_2$, filtered through a nylon mesh, and centrifuged at 1,200 × *g* for 10 min at 4°C as described (Kim et al., 2003). The cell pellet was suspended in the same buffer, and the suspension was put on a 52% Percoll gradient (Amersham Biosciences) in the above buffer and centrifuged at 11,000 × *g* for 10 min at 4°C. Testicular germ cells (TGC) was then recovered from a white band near the top of the gradient and washed three times with phosphate-buffered saline.

Preparation of protein extracts

Various mouse tissues, TGC, cauda epididymal sperm and testis (aged 10, 15, 20, 25, 40 and 90 days) were subjected to a lysis buffer consisting of 20 mM Tris-HCl, pH 7.4, 1% Triton X-100 (TX-100), 150 mM NaCl, and 1% protease inhibitor cocktail (Sigma-Aldrich, USA) for the extraction of proteins, after keeping on ice for 2 h. After centrifugation at 10,000 × *g* for 10 min at 4°C, proteins in the supernatant solution were determined using a Coomassie protein assay reagent kit (Pierce, USA) by the Bradford method (Bradford, 1976).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis

Proteins were denatured by boiling for 3 min in the presence of 1% sodium dodecyl sulfate; these denatured proteins were separated by SDS-PAGE, and transferred onto Immobilon-P membranes. After the blots were blocked with 2% skim milk, they were incubated with primary antibodies for 2 h, and subsequently with horseradish peroxidase-conjugated secondary antibodies for 1 h. Then, immunoreactive proteins were detected with an ECL Western blotting detection kit (Amersham Biosciences) (Kim et al., 2010).

Trypsinization and Biotinylation of mouse testicular germ cells

Spermatogenic cells were washed 3 times with Phosphate-buffered saline (PBS) and kept on ice for 0, 10, 20, 30 and 40 min in PBS containing 500 μg/ml trypsin (Sigma-Aldrich). The trypsinized spermatogenic cells were immediately washed 5 times with PBS containing 1% protease inhibitor cocktail (Sigma-Aldrich) and lysed as described above. The spermatogenic cells (2.5×10^7/ml) were kept at room temperature for 30 min in PBS containing 1 mM sulfo-NHS-LC-biotin (Pierce). The biotinylated spermatogenic samples were washed 3 times with PBS and lysed with the above lysis buffer. Dialysis was subsequently carried out to remove unbound-biotin from the biotinylated spermatogenic cell extracts. Biotin-labeled proteins were subjected to SDS-PAGE under reducing conditions and then Western bolt analysis (Kim et al., 2010).

Recombinant VSIG1 domains

Expression vectors of each domain of mouse VSIG were constructed in pCold (TaKaRa, Japan) or pGEX4T1 (Amersham Biosciences) vectors, which were tagged 6x His or GST. PCR was conducted using cDNA clones encoding *VSIG1* as a template with the following 3 sets of primers: the first Ig-like domain, 5′-AAGAATTCACTGTTGGATCTAATGTTACTCTTCT-3′, and 5′-AACTCGAGGGTGCCAACTTTTTTCTCAGT-3′; the second Ig-like domain, 5′-AAGAATTCGGATCCTATTTCCTTGTC-3′ and 5′-AACTCCAGTGGCCGTGTTGAA-3′; cytoplasmic tail domain, 5′-TTGAATTCAAATCCAAGCAGCAGAAGAAT-3′ and 5′-AACTCGAGTGCCTTAACTGTATCCTCTC-3′.

Each GST-fused or His-tagged construct of the first Ig-like, second Ig-like and cytoplasmic tail domains were sequenced correctly and were expressed in *E. coli* BL21 (DE3). The resulting transformants were cultured, and protein expression was induced by addition of IPTG (final concentration, 0.5 mM) for 3 h at 22°C. The cells were pelleted and washed with PBS. The cell pellets were resuspended in 0.3% TX-100 in PBS containing 1% protein inhibitor cocktail (Sigma-Aldrich) and sonicated with VCX 130 (Sonics & Materials) on ice. After centrifugation at 16,000 × *g* for 10 min, the supernatant solutions were applied to Ni-NTA agarose (Novagen, USA) and GST- sepharose 4B (GE Healthcare). The beads were washed with the binding buffer and subjected to elution. The eluted samples were dialyzed at 4°C by PBS.

Immunoprecipitation assay

TM4 cells (2.5×10^7/ml) were kept at room temperature for 30 min in PBS containing 1 mM sulfo-NHS-LC-biotin (Pierce). The biotinylated TM4 cells were washed 3 times with PBS and lysed with the above lysis buffer. Dialysis was subsequently

Table 1. Primer sequences for GFP -fused VSIG domains

	Sense primer (5′ to 3′)	Anti sense primer (5′ to 3′)
VSIG1	AACTCGAGATGATGGTGTTTGCATTTTGGA	AAGAATTCTGCCTTAACTGTATCCTCTC
Cyto	AACTCGAGAAATCCAAGCAGCAGAAGAAT	AAGAATTCTGCCTTAACTGTATCCTCTC
D-Cyto	AACTCGAGAAATCCAAGCAGCAGAAGAAT	AAGAATTCCTGGGGATCAGGCTCAAGCTC

Cyto, Cytoplasmic tail domain; D-Cyto, deletion of PDZ binding motif in cytoplasmic tail domain

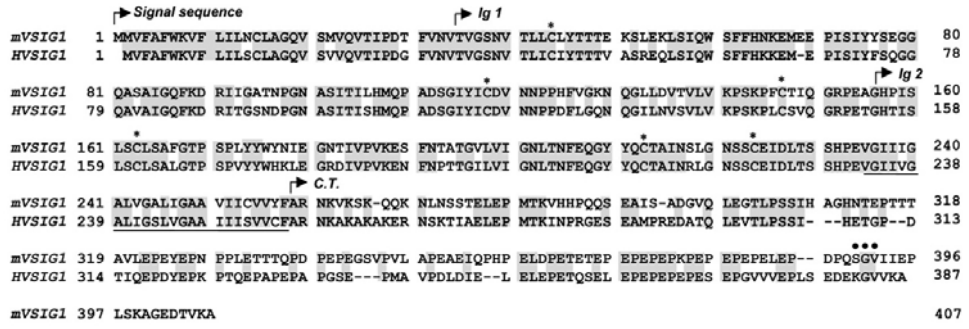

Fig. 1. Amino acid comparison between human and mouse VSIG1. Amino acids identical between the two proteins are represented by shaded boxes. Arrows indicate putative starting points of the signal sequence, the first Ig-like domain (Ig 1), the second Ig-like domain (Ig 2), and cytoplasmic tail domain (C.T.). The transmembrane domain is underlined. The possible motif of PDZ-binding is represented by closed circles. The asterisks indicate cysteine residues, which are predicted to form a disulfide bond.

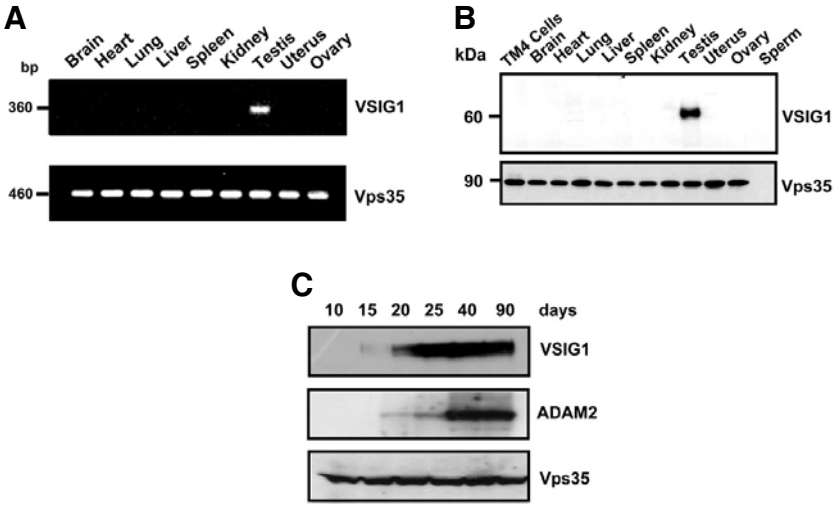

Fig. 2. Characterization of VSIG1. (A) RT-PCR analysis. Total RNA was isolated from various mouse tissues and subject to RT-PCR analysis with primers specific for mouse VSIG1 and VPS35. (B) Western blot analysis. Proteins in Triton X-100 extracts from various mouse tissues were separated by SDS-PAGE under reducing conditions and subjected to Western blot analysis using mVSIG1 and VPS35 antibodies. (C) The expression pattern of mVSIG1 during spermatogenesis was examined by Western blot analysis using protein extracts from testis samples of various ages (10, 15, 20, 25, 40, and 90 days).

carried out to remove unbound biotin from the biotinylated TM4 cell extracts. Biotin-labeled proteins were precipitated with immobilized streptavidin beads (Pierce) and then green fluorescent protein (GFP) -fused VSIG1 was added. The resulting pellet extracts were denatured and analyzed by SDS-PAGE and Western bolts using aiti-mVSIG1 and anti-GFP (Santa Cruz) antibodies. For analysis of the binding motif of the cytoplasmic tail domain in VSIG1, GFP-fused proteins were amplified by PCR using primers (Table 1). These fragments were cloned into the pEGFP vector. To express GFP-fused protein, NIH3T3 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum.

RESULTS

Sequence comparison between mouse VSIG1 and human VSIG1

On the basis of our *in silico* analysis using gene information from NCBI, the DNA sequence indicated that mouse VSIG1 is synthesized as single-chain proteins comprising 407 residues with a calculated molecular mass of 44,013 Da, which is predicted to contain multi-domains, including a signal peptide; an extracellular domain; a transmembrane domain; and cytoplasmic tail domain. Compared with human VSIG1, mouse VSIG1 possessed an approximately 20- residue extra sequence at the cytoplasmic tail domain (Fig. 1). In particular, the Ser/Thr-X-Val/Ile motif known to be mediated by ZO-1, the central structural protein of the tight junction, is present only in mouse VSIG1. The sequences of the first and second Ig-like domains showed 81.3% and 79.7% similarity, respectively, between mouse and human VSIG1, while the sequence of the cytoplasmic tail domain showed 45.3% similarity, which was relatively low compared to the similarity for the Ig-like domains. The overall homology was 68% between human and mouse VSIG1.

Expression and subcellular localization of VSIG1 in mouse spermatogenic cells

Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis indicated that mouse *VSIG1* is expressed specifically in the testis (Fig. 2A). To further examine the expression pattern

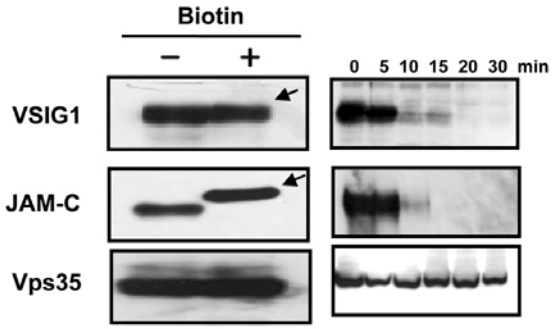

Fig. 3. Subcellular localization of mVSIG1. Testicular germ cells were (+) or were not (-) treated with biotin (left) or trypsine (right) and subjected to Western blot analysis using mVSIG1, JAM-C, and Vps35 antibodies. Arrows indicate the biotinylated mVSIG1 and JAM-C forms which are localized on the cell surface.

of mVSIG1 in testicular germ cells (TGC), we generated a polyclonal antibody against the first Ig-like domain of mVSIG1. The mVSIG1 antibody immunoreacted with the 60-kDa protein in TGC and no immunoreactive signal for mVSIG1 was found in the cauda epididymal sperm extracts and mouse Sertoli cell line TM4 (Fig. 2B). Further Western blot analysis was carried out using the mouse testis extracts obtained at different times after birth. mVSIG1 was detected in the postnatal mouse testis at the 15th day, and the protein level gradually increased during germ cell development (Fig. 2C).

The cellular localization of mVSIG1 in mouse testicular germ cells was examined by biotinylation and trypsinization assay using the mVSIG1 antibody. Biotinylation of surface proteins on testicular germ cells resulted in slow migration of the mVSIG1 and JAM-C on SDS-PAGE, and the intracellular protein VPS35 was not biotinylated (Fig. 3). When the trypsinization assay was carried out using testicular germ cells, the levels of 60-kDa mVSIG1 were negligibly low in the sample in 10 min, but not the level of VPS35 was affected (Fig. 3). Therefore, mouse VSIG1 is localized on the testicular germ cell surface.

mVSIG1 mediates homophilic adhesion

To determine whether mVSIG1 forms complex, we generated a glutathione *S*-transferase (GST)- recombinant protein of each Ig-like domain and cytoplasmic tail domain (Fig. 4A). The purified proteins were denatured by SDS under mild non-reducing conditions. Coomassie brilliant blue (CBB) staining clearly showed that each Ig-like domain formed homo-complex (Fig. 4B). To further examine whether these Ig-like domains crossreact with between the first Ig-like domain and the second Ig-like domain, we performed a pull-down assays using GST-fused and His-tagged Ig-like domains. The His-tagged first Ig-like domain was incubated with Ni-NTA agarose beads in the presence of GST-fused first Ig-like domain or GST-fused second Ig-like domain, and the pellets were then subjected to immunoblotting with and antibody specific to GST (Fig. 4C). The GST-fused first Ig-like domain was not detected in the His-tagged second Ig-like sample. However, the GST-fused first Ig-like domain was detected in the His-tagged first sample. The results of molecular interactions showed that the first Ig-like domain could interact with itself but not with the second Ig-like domain and vice versa.

Interaction between spermatogenic and Sertoli cell is mediated by the first Ig-like domain

To examine whether mVSIG1 can interact with a Sertoli cell membrane protein to mediate adhesion between the spermatogenic and Sertoli cell, we carried out immunoprecipitation (IP) assay using biotinylated-TM4 surface proteins. After the biotinylated TM4 cell surface proteins were precipitated with streptavidin beads, mVSIG1 blotting was observed in the pellet (Fig. 5A). This shows that mVSIG1 did interact with a Sertoli cell membrane protein. To further confirm whether the first Ig-like domain mediates interaction between spermatogenic cells and Sertoli cells, we carried out IP analysis from biotinylated-TM4 surface proteins. As shown in Fig. 5B, biotinylated protein(s) only binds to the first Ig-like domain. These results suggested that the first Ig-like domain is required for Sertoli cell interaction.

The cytoplasmic tail domain of mVSIG1 associates with ZO-1

In order to identify the possible PDZ binding motif of mVSIG1 that interacts with ZO-1, we generated a cytoplasmic tail domain expression vector encoding a deletion of the Ser-Gly-Val motif as well as wild-type VSIG1. NIH3T3 cells were trans-

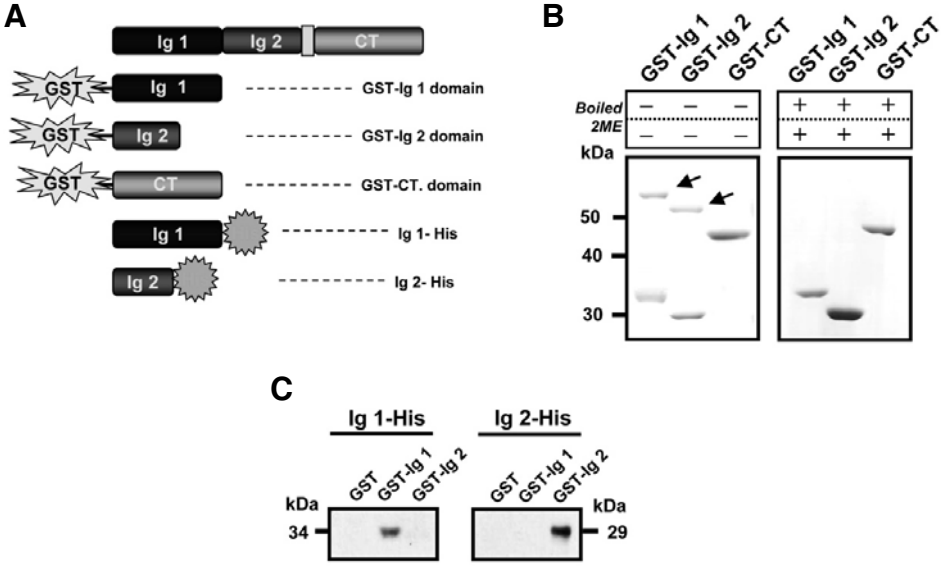

Fig. 4. mVSIG1 mediates homophilic adhesion and interaction between Sertoli cell membrane proteins. (A) Structure of VSIG1 constructs. (B) GST-fused recombinant domains. The GST-fused proteins were subjected to SDS-PAGE under non-reducing conditions followed by CBB staining. (C) GST-pull down analysis. Arrows indicate homodimer forms for each domain. GST-F. Ig, GST- the first Ig-like domain; GST-S. Ig, GST- the second Ig-like domain; GST-CT, GST- cytoplasmic tail domain; Ig 1-His, His tagged the first Ig-like domain; Ig 2-His, His tagged the second Ig-like domain.

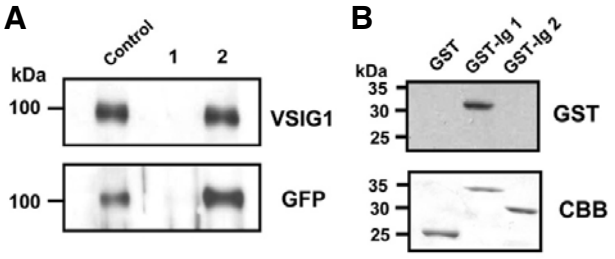

Fig. 5. Protein complex between GFP-fused mVSIG1 and Sertoli cells. (A) The biotinylated Sertoli cell (TM4) surface proteins co-precipitated with immobilized streptavidin beads; the resulting extracts were subjected to immunoblot analysis. Control, GFP-fused mVSIG1; lane 1, unbiotinlyated fraction; lane 2, biotinylated fraction. (B) Interaction between GST fusion the first Ig-like domain biotinylated Sertoli cell surface protein. GST-Ig 1, GST- the first Ig-like domain; GST-Ig 2, GST- the second Ig-like domain.

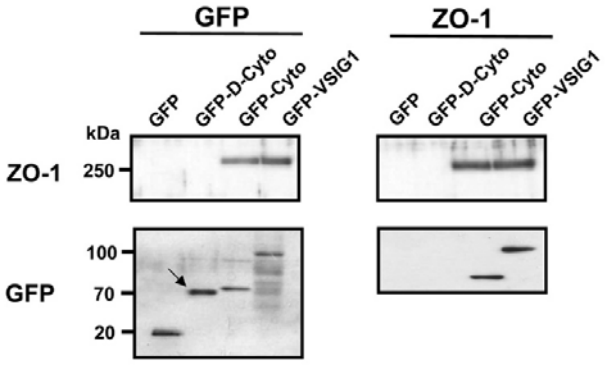

Fig. 6. Immunoprecipitation analysis of the interaction between mVSIG1 and ZO-1. The GFP-fused protein extracts from NIH3T3 cells were immunoprecipitated with anti-GFP and ZO-1 antibodies and subjected to Western blot analysis using the antibodies indicated on the left. Arrow indicates the mVSIG1 lacking PDZ binding motif in C- terminus.

fected with expression vectors encoding both pEGFP-cytoplasmic tail, EGFP-deletion of Ser-Gly-Val in cytoplasmic tail domain and pEGFP-mVSIG1. Protein extracts from transfected cells were immunoprecipitated with antibodies to both GFP and ZO-1, and the immunoprecipitates were subjected to Western blot analysis (Fig. 6). The asterisk in Fig. 6 indicates that PDZ-binding motif in mVSIG1 was slightly lower in molecular weights, compared with non-deletion form. GFP-mVSIG1 and the GFP-cytoplasmic tail domain co-precipitated with the ZO-1, but neither the GFP nor GFP-mutant cytoplasmic domain co-precipitate with ZO-1 under the same conditions. These results show that the PDZ-binding motif in the C-terminal of mVSIG1 is critical for binding to ZO-1.

DISCUSSION

Knock-out studies of JAM family members in mice have revealed that the loss of spermatogenic cell-Sertoli cell interactions leads to defects in spermatogenesis (Wang and Cheng, 2007). Despite the abundant expression of VSIG1 in the human testis, its role in spermatogenesis remains unknown. In the present study, we characterized mVSIG1, which contains two immunoglobulin-like domains, one trans-membrane domain and one cytoplasmic domain. To elucidate the functions of this protein, we first raised a rabbit polyclonal antibody against the first Ig-like domain of mVSIG1. We found that mVSIG1 protein was specifically expressed in the mouse testis, but not in cauda epididymal sperm extracts and mouse Sertoli cell line TM4, indicating that mVSIG1 is probably involved in spermatogenesis rather than fertilization (Fig. 2B). Recent studies on mice with targeted null alleles of JAM family members have suggested that these proteins function as cell adhesion molecules during spermatogenesis (Gliki et al., 2004; Inagaki et al., 2006; Yamada et al., 2006) and mediate homophilic or heterophilic interactions between spermatogenic and Sertoli cells by forming homodimers on cell surface (Bazzoni, 2003). Consistent with these results, we present direct evidence of homotypic VSIG1 binding through its extracellular domains. Owing to its localization pattern in spermatogenic cells, mVSIG1 has been postulated to interact with Sertoli cells (Figs. 3 and 5). We have now shown that only the first Ig-like domain can bind with Sertoli cells. In contrast, the second Ig-like domain was found not to affect the binding of Sertoli cell. Since no expression of mVSIG1 in Sertoli cells, these results suggest that the first Ig-like domain of mVSIG1 associates with Sertoli cells by heterophilic adhesion (Fig. 4). Indeed, cell adhesion molecule-1 (CADM1) is one of the best-characterized members of the JAM family and is composed of three Ig-like domains, a transmembrane domain and a short cytoplasmic tail domain (Bazzoni, 2003). Among the three Ig-like domains of CADM1, only the first Ig-like domain binds to Sertoli cells as a heterophilic manner as well as a homophilic manner because CADM1 was detected both Sertoli and spermatogenic cells, suggesting that its second Ig-like domain forms homodimer (Wakayama and Iseki, 2009). mVSIG1 also may not only bind to another molecule on the Sertoli cells in a heterophilic manner, but also bind to molecules on neighboring spermatogenic cells in homophilic manner via its first Ig-like domain. In agreement with the findings of previous studies, we suggest that the second Ig-like domain of VSIG1 may be important in forming homodimer.

Proteins containing PDZ domains mediate protein-protein interactions by interacting with short consensus motifs found at the free carboxyl terminus of transmembrane proteins, are predominantly present in the plasma membrane and have been reported to act as motifs required for TJ assembly (Songyang et al., 1997). The amino acid sequence of Ser/Thr-X-Val/Ile, which is found in the C-terminus of mVSIG1, is predicted to interact with PDZ domains. The cytoplasmic domain of JAM family members binds to the TJ-associated protein ZO-1, suggesting that this domain may be connected to the actin cytoskeleton (Fanning and Anderson, 2009). Thus, mVSIG1 is highly likely to possess similar binding activities. To further characterize the interaction between mVSIG1 and TJ assembly molecules, we generated a deletion form of Ser-Gly-Val in the C-terminus and confirmed that the mVSIG1-ZO-1 interaction depends on the Ser-Gly-Val motif. We believe that this motif may also participate in TJ assembly and sustain the adhesion established by the extracellular Ig domain.

The physiological role of mVSIG1 in the interactions between spermatogenic cells and Sertoli cells or between spermatogenic cells should be determined; this requires further research not only identify heterophilic interaction partner(s) but also produce mice that lack this gene, since these mice will be useful for elucidating the mechanism of spermatogenesis.

ACKNOWLEDGMENT

This research was supported, in part, by a Research Grant from the Ministry of Education, Science and Technology

(KHM4311022 and NBM 2300812), Republic of Korea.

REFERENCES

Arrate, M.P., Rodriguez, J.M., Tran, T.M., Brock, T.A., and Cunningham, S.A. (2001). Cloning of human junctional adhesion molecule 3 (JAM3) and its identification as the JAM2 counter-receptor. J. Biol. Chem. *276*, 45826-45832.

Bazzoni, G. (2003). The JAM family of junctional adhesion molecules. Curr. Opin. Cell Biol. *15*, 525-530.

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. *72*, 248-254.

Fanning, A.S., and Anderson, J.M. (2009). Zonula occludens-1 and -2 are cytosolic scaffolds that regulate the assembly of cellular junctions. Ann. N Y Acad. Sci. *1165*, 113-120.

Gliki, G., Ebnet, K., Aurrand-Lions, M., Imhof, B.A., and Adams, R.H. (2004). Spermatid differentiation requires the assembly of a cell polarity complex downstream of junctional adhesion molecule-C. Nature *431*, 320-324.

Gonzalez-Mariscal, L., Betanzos, A., and Avila-Flores, A. (2000). MAGUK proteins: structure and role in the tight junction. Semin. Cell Dev. Biol. *11*, 315-324.

Inagaki, M., Irie, K., Ishizaki, H., Tanaka-Okamoto, M., Miyoshi, J., and Takai, Y. (2006). Role of cell adhesion molecule nectin-3 in spermatid development. Genes Cells *11*, 1125-1132.

Inoue, N., Ikawa, M., Isotani, A., and Okabe, M. (2005). The immunoglobulin superfamily protein Izumo is required for sperm to fuse with eggs. Nature *434*, 234-238.

Kim, E., Nishimura, H., and Baba, T. (2003). Differential localization of ADAM1a and ADAM1b in the endoplasmic reticulum of testicular germ cells and on the surface of epididymal sperm. Biochem. Biophys. Res. Commun. *304*, 313-319.

Kim, E., Lee, J.W., Baek, D.C., Lee, S.R., Kim, M.S., Kim, S.H., Imakawa, K., and Chang, K.T. (2008). Identification of novel retromer complexes in the mouse testis. Biochem. Biophys. Res. Commun. *375*, 16-21.

Kim, E., Lee, J.W., Baek, D.C., Lee, S.R., Kim, M.S., Kim, S.H., Kim, C.S., Ryoo, Z.Y., Kang, H.S., and Chang, K.T. (2010). Processing and subcellular localization of ADAM2 in the Macaca fascicularis testis and sperm. Anim. Reprod. Sci. *117*, 155-159.

Nishimura, H., Kim, E., Fujimori, T., Kashiwabara, S., Kuroiwa, A., Matsuda, Y., and Baba, T. (2002). The ADAM1a and ADAM1b genes, instead of the ADAM1 (fertilin alpha) gene, are localized on mouse chromosome 5. Gene *291*, 67-76.

Oh, J.S., Han, C., and Cho, C. (2009). ADAM7 is associated with epididymosomes and integrated into sperm plasma membrane. Mol. Cells *28*, 441-446.

Scanlan, M.J., Ritter, G., Yin, B.W., Williams, C., Jr., Cohen, L.S., Coplan, K.A., Fortunato, S.R., Frosina, D., Lee, S.Y., Murray, A.E., et al. (2006). Glycoprotein A34, a novel target for antibody-based cancer immunotherapy. Cancer Immun. *6*, 2.

Songyang, Z., Fanning, A.S., Fu, C., Xu, J., Marfatia, S.M., Chishti, A.H., Crompton, A., Chan, A.C., Anderson, J.M., and Cantley, L.C. (1997). Recognition of unique carboxyl-terminal motifs by distinct PDZ domains. Science *275*, 73-77.

Takai, Y., Irie, K., Shimizu, K., Sakisaka, T., and Ikeda, W. (2003). Nectins and nectin-like molecules: roles in cell adhesion, migration, and polarization. Cancer Sci. *94*, 655-667.

Urase, K., Soyama, A., Fujita, E., and Momoi, T. (2001). Expression of RA175 mRNA, a new member of the immunoglobulin superfamily, in developing mouse brain. Neuroreport *12*, 3217-3221.

Wakayama, T., and Iseki, S. (2009). Role of the spermatogenic-Sertoli cell interaction through cell adhesion molecule-1 (CADM1) in spermatogenesis. Anat. Sci. Int. *84*, 112-121.

Wakayama, T., Ohashi, K., Mizuno, K., and Iseki, S. (2001). Cloning and characterization of a novel mouse immunoglobulin superfamily gene expressed in early spermatogenic cells. Mol. Reprod. Dev. *60*, 158-164.

Wang, C.Q., and Cheng, C.Y. (2007). A seamless trespass: germ cell migration across the seminiferous epithelium during spermatogenesis. J. Cell Biol. *178*, 549-556.

Yamada, D., Yoshida, M., Williams, Y.N., Fukami, T., Kikuchi, S., Masuda, M., Maruyama, T., Ohta, T., Nakae, D., Maekawa, A., et al. (2006). Disruption of spermatogenic cell adhesion and male infertility in mice lacking TSLC1/IGSF4, an immunoglobulin superfamily cell adhesion molecule. Mol. Cell. Biol. *26*, 3610-3624.

Yan, H.H., Mruk, D.D., Lee, W.M., and Cheng, C.Y. (2007). Ectoplasmic specialization: a friend or a foe of spermatogenesis? Bioessays *29*, 36-48.