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Identification of novel retromer complexes in the mouse testis ☆

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ABSTRACT

A family of vacuolar protein sorting (Vps) proteins, which are components of mammalian retromer complex, has been studied in the mouse. Vps26a is known as a retromer component that plays an important role in embryonic development: however, its cell-type expression and precise role remain to be elucidated. In this study, we identified a new isoform of Vps26a, called Vps26aT, which was expressed specifically in the mouse testis. Diverse expression patterns of Vps26 variants in mouse tissues were determined by Western blot and RT-PCR analyses, and the direct interaction of Vps26aT with Vps35 was also demonstrated by immunoprecipitation and pull-down assay using antibodies raised against each Vps component. Our results revealed that the retromer complex could be formed from different Vps26 isoforms in a tissue-specific manner, resulting in more than two types of the retromer complex, including the Vps26a-Vps29-Vps35, Vps26aT-Vps29-Vps35, and Vps26b-Vps29-Vps35 complexes in mouse tissues.

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The vacuolar protein sorting (Vps) family of proteins was discovered in yeast and has been shown to be involved in the delivery of proteins to the proper destinations. Vps proteins are therefore reported to have vital functions in various biological events including early embryonic development [1], vacuolar hydrolase transportation [2], and transcytosis of cell membrane proteins. In addition to these functions in the yeast, Vps proteins are major constituents of the mammalian retromer complexes that were first characterized in the mouse. Retromers are thought to be involved in delivering not only polymeric IgG receptor, which transcytoses polymeric IgA from the basolateral to the apical surface of epithelial cells and hepatocytes [3–5], but also cation-independent mannose 6-phosphate receptor, which carries hydrolases from the endosome to the trans-Golgi network [6].

Mammalian retromer complexes have five components: Vps26, Vps29, Vps35, SNX1, and SNX2 [7]. Vps35 is known as a key molecule in the formation of the retromer complex that binds with Vps29 and Vps26, and acts as a receptor for the binding with Vps10 [8–11]. In yeast, the localization of Vps10 is affected by the mutations in the Vps35 molecule, implying that Vps35 plays a central role in the localization of Vps10 [12]. Vps35 in yeast cells may exert a direct effect on the recycling of Vps10 to the Golgi apparatus from the prevacuolar endosome. It has been proposed that Vps29, a metallo-phosphoesterase for a CI-MPR substrate peptide [13], plays an important role in the formation and/or stability of the retromer complex because a retromer containing Vps35 becomes unstable when Vps29 is mutated or deleted. Vps26, also known as H-β-58, is the best characterized of the molecules in the mouse retromer complex. It was demonstrated that embryonic development in Vps26 mutant mice cease at the post-implantation stage [1]. Previous results have shown that Vps26 interacts with Vps35 through its C-terminal domain, which contains predicted coiled-coil regions [14]. Recently, two different isoforms of Vps26, termed Vps26a and Vps26b, have been reported in mouse tissues [8]. Unlike Vps26a, which is localized in the endosome, Vps26b is primarily localized in the plasma membrane, suggesting that the two isoforms may play different role(s) in intra-cellular sorting and/or transporting of various proteins. However, the precise role played by each of these Vps26 isoforms within the context of the formation and functioning of the retromer complex has not been fully characterized in vivo.

In this study, we identified the presence of a 40-kDa Vps26a-related protein in the mouse testis. Western blot and RT-PCR analyses revealed that the novel protein is a new Vps26a isoform, Vps26aT, specifically expressed in the mouse testis. Moreover, the results suggest that Vps26aT is capable of interacting with Vps35 directly. The presence of two types of retromer complex in the mouse testis may imply a distinct function of complexes containing Vps26aT in spermatogenesis.

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Materials and methods

Total RNA extraction and RT-PCR. Total RNAs were obtained from various mouse tissues using Isogen (Nippon Gene, Toyama, Japan), as described previously [15]. Total RNA (5 g) was reverse-transcribed to cDNA with SuperScript III First-Strand Synthesis System (invitrogen). 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 in PCR as a template. The specific PCR primers were designed for amplification of the mouse genes for Vps26a, Vps26aT, and Vps35 (GenBank Accession Nos. NM133672, AK028096, and NM022997, respectively). Oligonucleotide sequences of all primers used in this study are as follows: primers for PCR of Vps26a were Vps42 (5′-AAGAATTCATTAAAATGGAAGTGGGCATT-3′) and Vps8 (5′-TTCTCGAGTTACATCTCAGGCTGCTCC-3′); primers for Vps26aT were Vps9 (5′-AAGAATTCATGTCGGAGCCTCTGCCCGCCT-3′) and Vps2 (5′-TTCTCGAGGACATCAGGATAGGTGGCTA-3′); primers for Vps26aN were Vps19 (5′-AAGAATTCATGAGTTTTCTTGGAGGCT-3′) and Vps50 (5′-AACTCGAGCTGTAATTCCATGTGTTGTA-3′); primers for Vps29 were Vps21 (5′-AAGAACATGTTGGTGTTGGTACTAGG A-3′) and Vsp22 (5′-AACTCGAGTTACGACTTTTTATACTCAATTC G-3′); and primers for Vps35 were Vsp18 (5′-GAATTCAGTGAAG AGAATCATGAACCCT-3′) and Vsp26 (5′-TTGGGCCCTTAAAGGATG AGACCTTCATAG-3′).

Antibodies. Two DNA fragments encoding the C-terminus and the residues (269–336) of the Vps26b protein and residues (637–796) of the Vps35 protein were amplified by PCR, then introduced into pET-23d (Novagen), and expressed in *Escherichia coli* BL21 (DE3). The recombinant His-tagged proteins were emulsified with Freund's complete adjuvant (Sigma–Aldrich) and injected intradermally into female New Zealand white rabbits. Following fractionation of the antisera with ammonium sulfate (0–40% saturation), anti-Vps26b and anti-Vps35 antibodies were affinity-purified on a sepharose 4B column that had been coupled with the C-terminal 37 amino acids of Vps26b and the C-terminal 45 amino acids of Vps35 proteins fused to glutathione *S*-transferase (GST), as described previously [16]. Antibodies against Vps26a and Vps29 were purchased from Abcom.

Preparation of protein extracts. Various mouse tissues 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) for the extraction of proteins, after chilling on ice for 2 h. After centrifugation at 10,000*g* for 10 min at 4 °C, proteins in the supernatant solution were analyzed.

Western blot analysis. Denatured proteins were separated by SDS–polyacrylamide gel electrophoresis (PAGE) and transferred onto Immobilon-P membranes (Millipore). After blocking with 2% skim milk, the blots were incubated with primary antibodies for 2 h, and subsequently with horseradish peroxidase-conjugated secondary antibodies for 1 h. Then, the immunoreactive proteins were detected by an ECL Western blotting detection kit (Amersham Biosciences) [17].

Immunoprecipitation (IP). An aliquot (500 g) of protein extracts was kept on ice for 2 h with 20 g of anti-Vps26a, -Vps26b, and -Vps35 antibodies in 500 l of PBS/500 M NaCl/0.5% TX-100. Immune complexes that formed in the reaction mixtures were captured with protein G (Amersham Biosciences) during 2 h incubation at 4 °C, followed by washing five times with the same buffer. The beads carrying the immune complexes of Vps26a, Vps26b, and Vps35 were boiled for 3 min in 100 l of reducing SDS-sample buffer. Then, the proteins were analyzed by immunoblotting [17].

Production of recombinant proteins. To express His-tagged proteins, genes for Vps26a, Vps26aT, Vps26b, Vps29, and Vps35 were cloned into pET23a (Novagen) and transformed into *E. coli* BL21 (DE3). The resulting transformants were cultured and protein expression was induced by the addition of IPTG (final concentration 0.5 mM) over 3 h at 22 °C. The cells were pelleted and washed with 1× PBS. The cell pellets were resuspended in 0.3% TX-100 in 1× 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). The beads were washed with the binding buffer, followed by elution steps. The eluted samples were dialyzed at 4 °C by 1× PBS.

In vitro translation. For Vps35, the DNA construct was prepared by PCR amplification of the *vps35* gene from mouse testis cDNA using the gene-specific primers encoding the N-terminal FLAG epitope within the 5′-primer. The amplified Vps35-coding region was cloned into pcDNA3.1 (Invitrogen). The PCR reaction with the Vps35 template DNA was performed with LA Taq polymerase (Takara) and the primers (5′-GGATCCTAATACGACTCACTATAG-3′, 5′-TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTAAAGGATGAGACCTTC ATAG-3′). In vitro translation was carried out using TNT Quick for PCR DNA system in the Promega technical bulletin (Promega).

Pull-down assays. The anti-Vps26a, -Vps26b, and -Vps29 antibodies immobilized on protein G were mixed with 100 μg of each purified His-tagged protein, in a buffer containing 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, and the protein inhibitor cocktail for 2 h. The gel was washed five times with the same buffer to remove unbound antibodies, and then mixed with the in vitro translated Vps35 protein at 4 °C overnight. After centrifugation, the pellet was washed five times with the binding buffer, and the proteins on the gel were denatured and analyzed by SDS–PAGE and Western blots using anti-Vps35 and anti-FLAG antibodies.

Results

Immunodetection of Vps proteins expressed in various tissues of the mouse

To examine whether the retromer components are present in various mouse tissues, Western blot analyses were carried out with antibodies raised against Vps proteins. The presence of Vps26a, Vps26b, Vps29, SNX1, and Vps35 proteins was consistent with the specific detection of 38, 39, 20, 70, and 90 kDa protein bands, respectively (Fig. 1). As expected, the retromer components were found in all mouse tissues tested, although the VPS26b protein was expressed predominantly in the brain, as shown in Fig. 1. In addition to the 38-kDa Vps26a band that is commonly found among the various tissues, interestingly, another band (42 kDa) was detected in the testis extract (Fig. 1). Because the 42-kDa band was specifically detected by anti-Vps26a antibody, this novel form was designated as a testis-specific Vps26a (Vps26aT).

Tissue-specific expression of Vps26a variants formed by alternative splicing

Since an additional Vps26a variant protein was identified in the mouse as shown in Fig. 1, we analyzed the genomic organization of the testis-specific Vps26a variant through a BLAST search that was carried out with the Vps26a gene sequence already characterized. The National Center for Biotechnology Information (NCBI) database provides a total of 26 Vps26 mRNAs that exist in mouse tissues (http://www.ncbi.nlm.nih.gov). We identified three variants of the Vps26a mRNA sequence in the GenBank that are formed by alternative splicing during the Vps26a gene transcription. The Vps26a variant (mRNA), Vps26aT was specifically expressed in the mouse testis, and the other variant form derived from neonatal

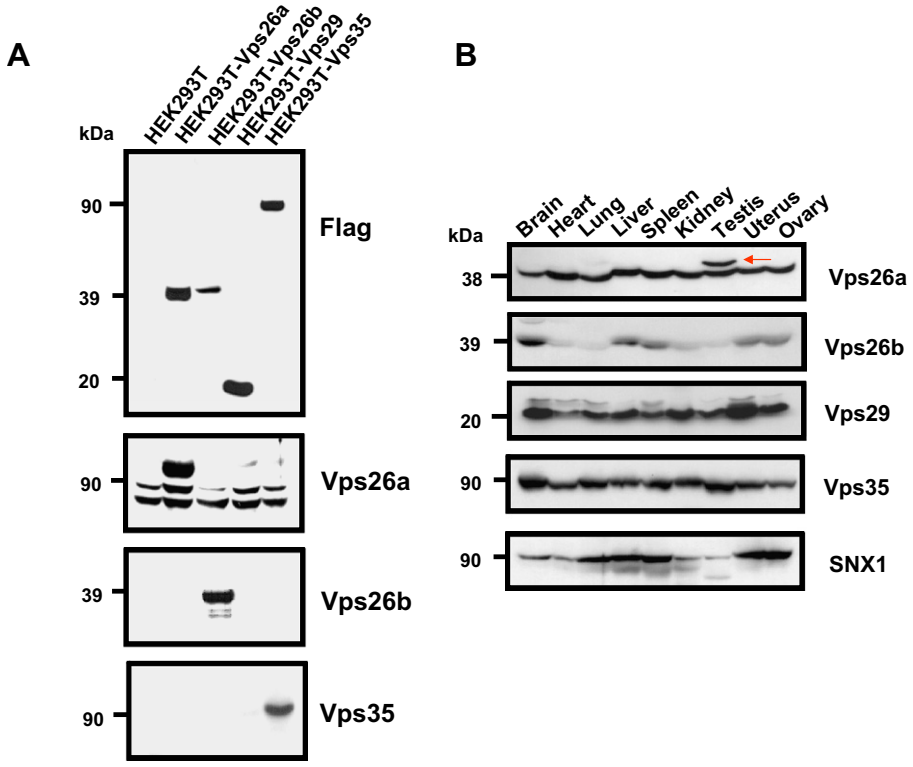

Fig. 1. Expression of retromer components in various mouse tissues. (A) Specificity of antibodies raised against Vps26a, Vps26b, Vps29, and Vps35 proteins. (B) Tissue distribution of the retromer components. Proteins in Triton X-100 extracts from various mouse tissues and HEK293T cells were separated by SDS–PAGE under reducing conditions and subjected to Western blot analysis using antibodies raised against Vps26a, Vps26b, Vps29, Vps35, and SNX1. Arrow indicates the testis-specific form of Vps26a (Vps26aT).

mouse tissue and named Vps26aN was also identified (Fig. 2B). The difference between Vps26aT and the known Vps26a is in the presence and absence of the first and second exons. The genomic analysis for the Vps26a gene revealed that the Vps26a gene spans about 32 kbp and consists of a total of 10 exons (Fig. 2B). The Vps26aT variant has 9 exons comprising exon-2 to exon-10, and is thus only missing the first exon, whereas the Vps26a gene is missing the second exon only (Fig. 2B). Compared with the mouse genome, the transcription start site for Vps26aT mRNA is located 59-bp nucleotides downstream from the first exon.

In addition, we found another Vps26a variant, Vps26aN, in the NCBI database that was reported in neonatal and olfactory tissues of the mouse. This Vps26aN mRNA was composed of seven exons (1, 3, 5, 7, 8, 9, and 10); however, the ATG start codon was positioned in exon-5. Collectively, our findings suggest that there are at least three Vps26a isoforms (the known Vps26a plus two additional variants, Vps26aT and Vps26aN) in the mouse (Fig. 2). These Vps26a isoforms contain a putative Vps35-binding domain, consisting of isoleucine (235)-methionine (236) and the amino acid residues of 238–246 in the C-terminal domain of Vps26a.

Expression of each component of the retromer complex was identified by RT-PCR with mRNA isolated from the ten tissues of mouse tested (Fig. 2C). The results showed that mouse Vps26aT was expressed exclusively in the testis, in contrast to Vps26a which was ubiquitously expressed in all mouse tissues tested. To confirm the presence of the Vps26aN isoform in tissues, PCR was performed using a primer set with sequences from the first to fifth exons of the Vps26a gene. The bands that were approximately 470 and 400 bp in size were ubiquitously observed in all tissues tested, but the intensity of the 400-bp band comprising the exons 1, 3, and 5 was negligibly low in all tissues tested, in spite of the abundance of mRNAs for the Vps26a gene. Interestingly, a low level of Vps26aT expression was detected in the uterus (Fig. 2C).

Association of Vps26a isoforms with other Vps proteins in vivo

The existence of the retromer complex formed from Vps proteins in the mouse brain led us to postulate that a unique retromer complex may be formed with Vps26aT in the testis. IP analyses were carried out with protein extracts from mouse brain and testis using antibodies raised against Vps26a, Vps26b, and Vps35, respectively. The results showed that Vps35 was co-immunoprecipitated with antibodies raised against Vps26a and Vps26b. In the IPs with anti-Vps26a and anti-Vps26b antibodies for the brain lysates, both Vps29 and Vps35 were co-immunoprecipitated (Fig. 3A). SNX1 was not co-precipitated in the immune complexes of Vps26a, Vps26b, and Vps35, despite the presence of SNX1 molecules in the brain. However, there is a possibility that SNX1 could be involved in the formation of a complex with Vps35 but the amount of the complex was too small to be detected by our IP using the above-mentioned antibodies.

Since we have identified a testis-specific Vps26a (Vps26aT), we tested whether the two Vps26a molecules (Vps26a and Vps26aT) could be co-immunoprecipitated with anti-Vps35 antibody in the testis, because these two molecules have a Vps35 binding site (Figs. 2A and 3B). IP analysis revealed that, in addition to the association with Vps29, Vps35 was associated with either Vps26a or Vps26aT. However, Vps26b from the Vps26b–Vps35 immune complex was not quantified because the amount of endogenous Vps26b was negligibly low in the lung and testis (Fig. 3B).

Vps26aT interacts with Vps35 in vitro

The results shown in Fig. 3 imply that Vps26aT is involved in an interaction with Vps35 in the mouse testis. The in vitro interaction between Vps26aT and Vps35 was investigated by using His-tagged Vps26aT pull-down assay. The recombinant His-tagged Vps26a,

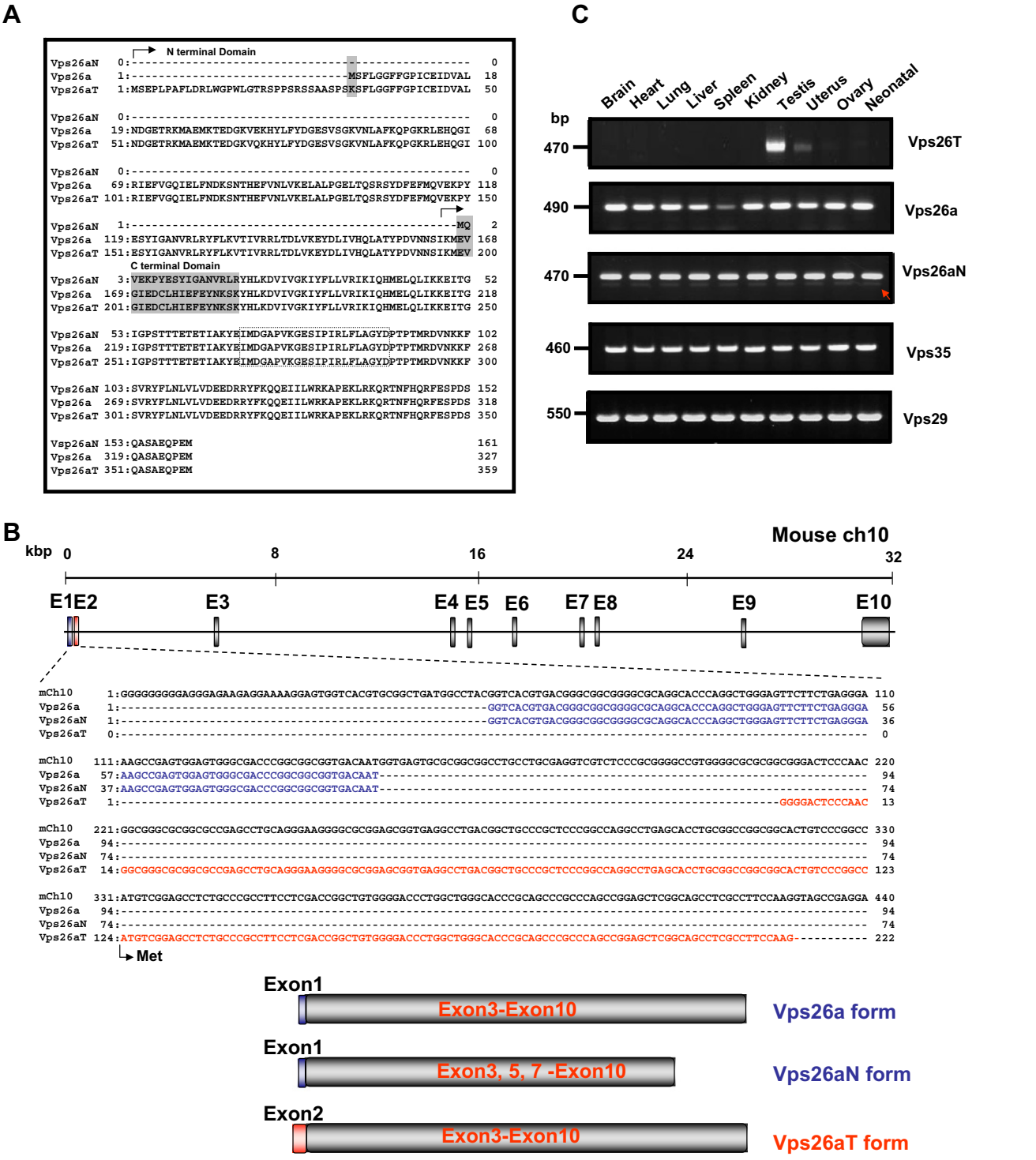

Fig. 2. Alignment of amino acid sequences of mouse Vps26a variants and RT-PCR detection of transcripts for Vps proteins in various mouse tissues. (A) The amino acid sequences of Vps26a and its isoforms were compared. The box with dotted lines indicates the predicted Vps35 binding motif located in the C-terminal domain. (B) Genomic organization of mouse Vps26a gene. The top panel shows the genomic organization of all the exons for Vps26a and its isoforms. The lower panel depicts Vps26a variants formed by alternative splicing. (C) Detection of transcripts for various Vps genes by RT-PCR with mRNA isolated from the mouse tissues tested. Arrow indicates Vps26aN form, which is the faint band that appears below the thick band of Vps26a gene.

Vps26aT, Vps26b, and Vps29 proteins were synthesized in *E. coli* (Fig. 4), purified, immobilized on Ni–NTA beads, and incubated with in vitro translated FLAG-tagged Vps35. The asterisk in Fig. 3B indicates that Vps35 modified by FLAG tagging was slightly higher in molecular weights, compared with testis-derived Vps35. Analysis of the bound proteins eluted from the Ni–NTA beads revealed that Vps26aT binds to Vp35 directly.

Discussion

A retromer is a polymeric protein complex mainly composed of Vps26, Vps29, and Vps35. Because most studies regarding Vps26, Vps29, and Vps35 and their retromer formations have been performed using cultured cells, the actual distribution of these components in vivo has not been well characterized. Considering the

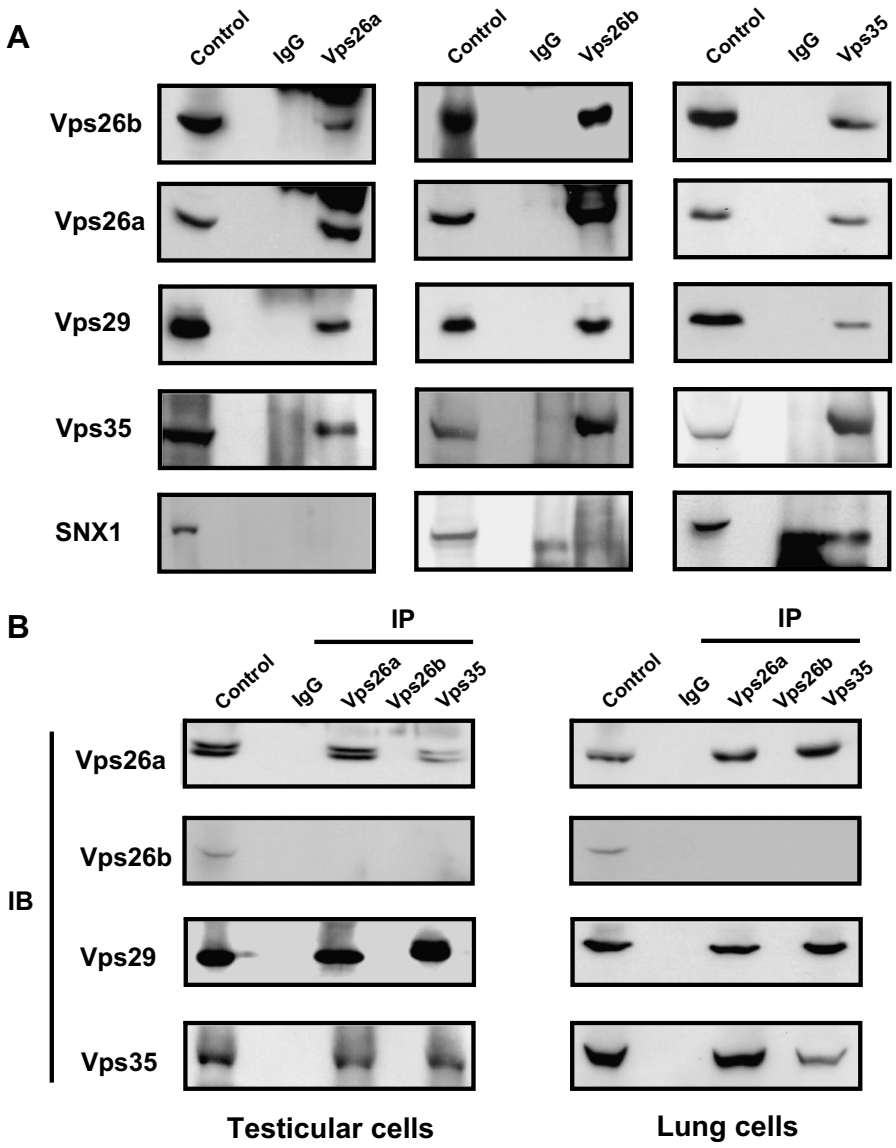

Fig. 3. Immunoprecipitation analysis of retromer components in mouse tissues. (A) The protein extracts from mouse brain were immunoprecipitated with anti-Vps26a, -Vps26b, and -Vps35 antibodies and subjected to Western blot analysis using the antibodies indicated on the left side of the picture. (B) Proteins extracted from testicular germ cells and lung cells were immunoprecipitated using the antibodies indicated as IP, and the subsequent Western blot analysis was performed using the antibodies indicated as IB. As noted, a doublet band was only detected in the mouse testicular cells by anti-Vps26a antibody, in which the upper band was found to be the testis-specific Vps26aT and the lower band is Vps26a, which is ubiquitously expressed.

ubiquitous house-keeping functions played by the retromer complexes in mammalian cells, the tissue distribution of each retromer component is expected to be equivalent to that of the others. However, in this study, we report for the first time the distinctive distribution of Vps26a and Vps26b in mouse tissues, compared to the ubiquitous tissue distribution of Vps29 and Vps35. Among them, Vps26 is considered to be an important component of the retromer complex, and is essential for its physiological function during the period of mouse embryonic development since embryonic death occurs in Vps26-null mice [1]. The amino acid residues 238–246 in the C-terminal domain of Vps26a have been reported to function as the binding motif for the interaction with Vps35. The Vps35-binding motif is well conserved in the two proteins Vps26a and Vps26b, although identity in the C-terminal domain sequences of Vps26a and Vps26b is only 70%. Moreover, the two sequences of Vps26a and the newly identified Vps26aT are more than 99% identical, which may also imply that Vps26aT could result in the formation of a retromer complex by direct binding to Vps35.

In the present study, we demonstrated the difference in expression patterns among the retromer subunits (Vps26a, Vps26b, Vps29, and Vps35) in various mouse tissues. Notably, Vps26b appeared to be distinguished from the other proteins by its distinct expression confined to the brain (Fig. 1). These data suggest that Vps26b is likely to play a more important role in brain cells than in the other tissues tested, and that the role played by Vps26b might differ from the role of Vps26a. Interestingly, we also identified a novel testis-specific Vps26a (Vps26aT) expressed specifically in the mouse testis (Figs. 1 and 2C). We analyzed possible alternative splicing of the Vps26a mRNA and expression of the consequent spliced mRNA of Vps26a in detail. We found the Vps26a transcripts encoding an extra peptide consisting of 32 amino acids in the N-terminus are mostly testis-specific, whereas the other transcript without the extra N-terminal peptide is expressed in various tissues at variable levels (Fig. 2A). Overall, the presence of retromer complex containing Vps26aT in the testis led us to speculate that the Vps26aT subunit in the mouse retromer may be necessary for

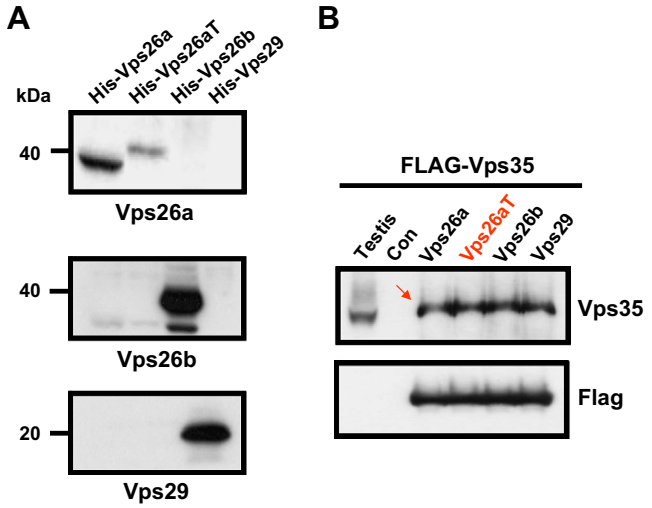

Fig. 4. Direct interaction of the testis-specific Vps26aT and Vps35. (A) Confirmation of Vps proteins expressed and purified using a His-tagging system by specific antibodies indicated below the image. (B) Interactions of Vps35 with His-tagged Vps proteins, demonstrating the direct binding with Vps26aT. Pull-down assays were carried out using anti-FLAG antibodies with the protein complex formed with the FLAG-tagged Vps35.

spermatogenesis, although no direct evidence has been provided to support this possibility.

Mouse Vps13A and Vps13C, which are known to have the largest molecular weights among the Vps proteins, were reported to possess various transcript variants. Interestingly, like the case of Vps26, these Vps13 variants are expressed in a tissue-specific manner in mouse brain and testis [18]. On the other hand, it was reported that VPS16, a well known component of class C Vps, is highly expressed in various brain areas, suggesting that it may function in neurons [19]. Here we found that Vps26b is expressed predominantly in brain cells. The reason why these molecules are expressed specifically in the brain is unknown at present. Upon disruption of the Vps26a gene, the association among the components of the retromer complex could be changed structurally, which may result in impaired retromer function [20]. Indeed, we have found that the level of expression of Vps35 was approximately 50% in the Vps26a-deficient mouse ($Vps26a^{-/-}$), compared with wild type controls (data not shown). The distinct reduction of Vps35 expressed in the $Vps26a^{-/-}$ mouse may be due to instability in Vps35 in the absence of Vps26a.

Vps26 derivatives are of importance in the formation of retromer complexes in that there are two differentially expressed subtypes, Vps26a and Vps26b. Furthermore, Vps26a has two isoforms, Vps26a and Vps26aT. The former is expressed ubiquitously in tissues, whereas the latter is expressed specifically in the testis. The diverse aspects of Vps26 may imply that each of the Vps26 proteins plays an important role in sorting and/or transporting specific proteins during retrograde vacuolar trafficking within the cell. In this context, our findings that Vps26aT binds to Vps35 directly (Fig. 4) suggest that Vps26a plays an important role in the formation of a retromer complex. Also, our findings of the differential expression of Vps26b in mouse brain and the testis-confined expression of Vps26aT provide some insights into the functional association of specific subunits in the formation of the retromer complexes in specialized tissues of the mouse.

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