Molecular Cloning of Vps26a, Vps26b, Vps29, and Vps35 and Expression Analysis of Retromer Complex in Micro Pig

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ABSTRACT

Members of the Vps (Vacuolar protein sorting) protein family involved in the formation of the retromer complex have been discovered in a variety of species such as yeast, mouse, and human. A mammalian retromer complex is composed of Vps26, Vps29, and Vps35 proteins and plays an important role in cation-independent mannose-6-phosphate receptor retrieval from the endosome to the trans-Golgi network. In this study, we have identified the full-length sequences of the retromer components of Vps26, Vps29, and Vps35 in micro pigs. The cDNA sequences of these retromer components have been determined and the result showed there is 99% homology among the component counterparts from mouse, micro pigs, and humans. In addition, the retromer complexes formed with hetero-components were found in the brain of micro pigs. Based on above results, we suggest mammalian Vps components are well conserved in micro pigs.

(Key words : Vps, Retromer complex, Micro pig)

INTRODUCTION

The sorting and transport of membrane proteins in the eukaryotic cell is mediated by cytoplasmic coat molecules called retromer. This complex was originally identified by S. Emr in the yeast *Saccharomyces cerevisiae* (Emr *et al.*, 1986), where it was found to be consisted of vacuolar protein sorting proteins (Vps26, Vps29, and Vps35). The retromer complex formed with these Vps proteins plays a role in cation-independent mannose-6-phosphate receptor (CI-MPR) retrieval from the endosome to the trans-Golgi network (Seaman *et al.*, 1998; Bionifacino *et al.*, 2003; Nothweth *et al.*, 2003; Seaman, 2004). Although at least 60 *Vps* genes have been identified in a variety of species, the Vps protein function was not fully elucidated. In particular, there are many isoforms of the Vps proteins that are exclusively or predominantly expressed in a tissue-specific manner, but their functions remain uncharacterized yet.

Among these retromer components, Vps35 has been extensively studied; Vps35 is able to bind with both Vps26 and Vps29 through the N-terminal domain and the C-terminal domain of Vps35 as suggested by the experimental data obtained from yeast two-hybrid system. Moreover, the Vps35 motif is essentially capable of binding to the cargo protein, Vps17 (Haft *et al.*, 2000; Reddy and Seaman, 2001). Recently it was shown that Vps26 is present as two distinct subtypes (Vps26a and Vps26b) that share approximately 70% identity (Kerr *et al.*, 2005; Colllins *et al.*, 2008), which are each localized on mouse chromosomes 10 and 9 and human chromosomes 10 and 11, respectively. Besides the phenotype of Vps26a-deficient mouse exhibited growth arrest on 7.5 d.p.c. during embryogenesis, the double mutant mouse for Snx1 and Snx2 known as retromer sub-components showed similar results to that of Vps26 (Lee *et al.*, 1992; Bachhawat *et al.*, 1994; Schwarz *et al.*, 2002). Thus, mouse retromer complex plays an important role during the embryogenesis. On the other hand, mouse Vps26b was recently found to be predominantly expressed in the brain tissues (unpublished data) and Vps13, as known to be responsible for chorea-acanthocytosis (ChAc), likewise possesses brain-specific variant form (Mizuno et al., 2007). Although studies on the retromer complex have so far been mainly carried out in yeasts, little is known about the molecular aspects of the retromer complex formed in other mammalian animals.

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To elucidate the role(s) of the retromer complex in micro pigs, we have attempted to identify and characterize the retromer components and the interactions among Vps26a, Vps26b, Vps29, and Vps35 proteins from micro pig brain tissue. As results, we have found these retromer components are well conserved in miniature pigs, and for the first time we provided evidences supporting for the formation of the retromer complex within the brain of micro pigs.

MATERIALS AND METHODS

Animal

The micro pigs, two years of age, were purchased from PWG Genetics Korea, Ltd. (Korea). The micro pigs were housed in case and maintained under a specific pathogen-free environment in light-controlled and air-conditioned rooms (temperature, 24℃; humidity, 50%). The animals used in this study were cared for and used under the Guidelines for Animal Care and Use Committee of KRIBB.

Total RNA Extraction and cDNA Cloning of Vps26a, Vps26b, Vps29, and Vps35 Genes from Micro Pigs

Total cellular RNA was extracted from various tissues of micro pig using ISOGEN (Nippon Gene) and subsequently, cDNA was synthesized by oligo (dT) priming using SuperScript III First-Strand Synthesis System (Invitrogen). To amplify miniature pig's cDNA fragments encoding *Vps26a, Vps26b, Vps29,* and *Vps35,* eight oligonucleotide primers were designed based on a mouse EST (GenBank accession number NM-133672; *Vps26a,* NM-0178027; *Vps26b,* NM-019780; *Vps29,* NM-022997; *Vps35*) The oligonucleotide sequences for primers are listed in Table 1. PCR was performed for 35 cycles of 94℃ for 60 sec, 60℃ for 60 sec, and 72℃ for 120 sec, with a PCR thermal cycler (Takara). The RT-PCR products corresponding to *Vps26a, Vps26b, Vps29,* and *Vps35* genes obtained from brain tissue of micro pig was cloned and sequenced. Following electrophoresis on a 1% agarose gel, the desired PCR bands excised with a razor blade. Gel fragments were purified using a gel extraction kit (Qiagen) in accordance with the manufacturer's guideline. Purified DNA fragments were cloned in DH5 α using the pGEM-T Easy kit (Promega). After isolation of plasmid DNA, inserts were sequenced with the vector-specific T7 and SP6 sequencing primers (Promega) by an ABI 3100 DNA sequencer (Applied Biosystems).

Preparation of Protein Extracts

Brain tissue of micro pigs was suspended in a lysis buffer consisting of 20 mM Tris-HCl, pH 7.4, 1% Triton X-100, 150 mM NaCl, and 1% protease inhibitor cocktail (Sigma-Aldrich) and kept on ice for 2 hr. After centrifugation at 10,000 × g for 10 min at 4℃, proteins in the supernatant solution were analyzed by Western blotting, as described below. Protein concentration was determined by the method of Bradford (Bradford, 1976).

Table 1. Oligonucleotide primers used for amplification of the Vps cDNAs

Primers for PCR of Vps26a	
Vps42	5'-AAGAATTCATTAAAATGGAAGTGGGCATT-3'
Vps8	5'-TTCTCGAGTTACATCTCAGGCTGCTCC-3'
Primers for PCR of Vps29	
Vps21	5'-AAGAACATGTTGGTGTTGGTACTAGGA-3'
Vps22	5'-AACTCGAGTTACGACTTTTTATACTCAATTCG-3'
Primers for PCR of Vps35	
Vps18	5'-GAATTCAGTGAAGAGAATCATGAACCCT-3'
Vps26	5'-TTGGGCCCTTAAAGGATGAGACCTTCATAG-3'

Western Blot Analysis

Proteins were denatured by boiling for 3min in the presence of 1% SDS and 1% 2-mercatoethanol (2ME), and transferred onto Immobilon-P membranes (Millipore). Immunoreactive proteins were detected by an ECL Western blotting detection kit (Amersham Biosciences). When the complex formation between the retromer components was examined, the protein samples were kept on ice for 5 min in the presence of 0.3% SDS, separated by SDS-PAGE under non-reducing condition, and transferred onto Immobilon-P membrane as described previously (Kim *et al.*, 2006).

Recombinant DNA Constructs

The full-length genes for *Vps26a, Vps26b, Vps29,* and *Vps35* of miniature pig were cloned into the yeast two-hybrid "prey" vector, GAL4 transcription activation domain (AD) vector (pACT2), by PCR which introduced a *Bam*HI-*Xho*I site at the 5′ and 3′ ends. To obtain the "bait" vectors, the micro pig full-length cDNAs for *Vps26a, Vps26b* (NCBI Accession No. NM0178027), *Vps29* (NCBI Accession No. NM019780), and *Vps35* were ligated into the pGBT0 vector (Clontech), respectively. The oligonucleotide sequences for primers are listed in Table 2.

Yeast Two-Hybrid Assay

The prey and bait vectors were co-transformed into the AH109 yeast strain (*MATa, trp1-901, leu2-3,112, ura3-52, his3-200, gal4, gal80, LYS2::GAL1UAS-GAL1TATA*-HIS

Table 2. Oligonucleotide primers used for amplification of the Vps cDNAs used yeast two hybrid assay

Primers of Vps26a for prey vector	
Vps26aPF	5'-GGATCCGATGTTGCCCTTAATGATG-3'
Vps26aPR	5'-CTCGAGCATCTCAGGCTGCTCCGCA-3'
Primers of Vps26b for prey vector	
Vps26bPF	5'-GGATCCGATGAGCTTCTTCGGCTTCG-3'
Vps26bPR	5'-CTCGAGCTGCCTGCTGTTGTTGTCAG-3'

Primers of Vps26a for bait vector	
Vps26aBF	5'-AAGAATTCATGAGTTTTCTTGGAGGCT-3'
Vps26aPR	5'-CTCGAGCATCTCAGGCTGCTCCGCA-3'
Primers of Vps26b for bait vector	
Vps26bBF	5'-GAATTCATGAGCTTCTTCGGCTTCG-3'
Vps26bPR	5'CTCGAGCTGCCTGCTGTTGTTGTCAG-3'

Primers of Vps29 for prey vector	
Vps29PF	5'-GGATCCGATGTTGGTGTTGGTACTAGGA-3'
Vps29PR	5'-CTCGAGACTTTTTATACTCAATTCGTTC-3'
Primers of Vps35 for prey vector	
Vps35PF	5'-GGATCCCATGCCTACAACACAGCAGTCAC-3'
Vps35PR	5'-CTCGAGTTAAAGGATGAGACCTTCATA-3'

Primers of Vps29 for bait vector	
Vps29BF	5'-GAATTCATGTTGGTGTTGGTACTAGGA-3'
Vps29PR	5'-CTCGAGACTTTTTATACTCAATTCGTTC-3'
Primers of Vps35 for bait vector	
Vps35BPF	5'-GAATTCATGCCTACAACACAGCAGTCAC-3'
Vps35BBR	5'-CTCGAGTTAAAGGATGAGACCTTCATA-3'

3, GAL2*UAS-GAL2TATA-ADE2, ura3*::*MEL1UAS- MEL1-TATA-lacZ)* by the lithium acetate method as previously described (Gietz *et al.*, 1992). The transformants were selected for growth on the -Leu, -Trp, -His, and -Ade solid SD media containing 25 mM 3-aminotriazole (3-AT). β -galactosidase production was assayed by incubating freeze-fractured colonies on nitrocellulose in the Z-buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 0.03 mM β-mercaptoethanol and 2.5 μM 5-bromo 4-chloro-3-indolyl-β-D-galactopyranoside (X-gal)) at 30℃, and the blue colonies were selected as positive candidates. As negative controls, the AH-109 strain was co-transformed with pGBT9 (empty vector) and *Vps-26a/b/35*-pACT2.

RESULTS

Isolation and Characterization of the Retromer Components Vps26a, Vps26b, Vps29, and Vps35 from Micro Pigs

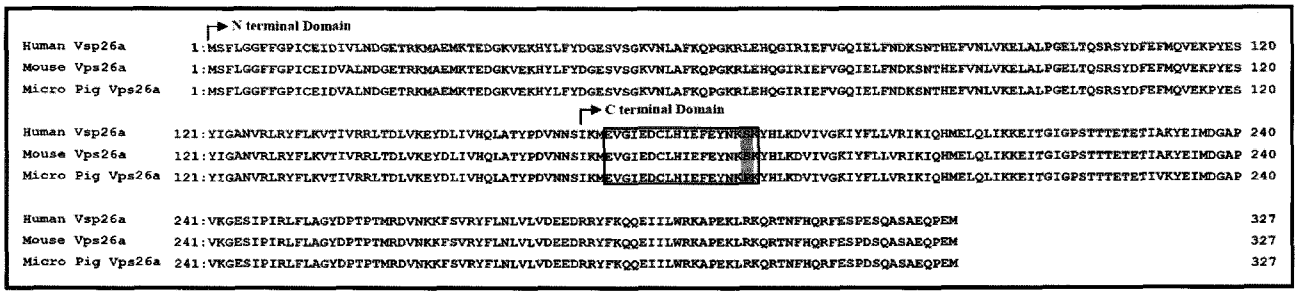
In micro pigs, the full length of cDNA clones encoding entire region of the *Vps26a, 26b,* and *Vps35* have not yet been identified. The mouse retromer component cDNA sequences information is available for the micro pig's *Vps* genes. By use of 3'-RACE and RT-PCR analysis from the micro pig brain cDNA, we isolated 4 retromer complex factors. Sequence analysis predicted that four *Vps* genes (*Vps26a, Vps26b, Vps29,* and *Vps35*) were matched to encode a 327, 336, 182, and 796-amino acid polypeptide, respectively. As shown in Fig. 1, the ORF regions were compared with the reported mouse and human genes. These *Vps* genes share a high sequence homology at nucleotide level (≈98% identity) in the coding region among the mouse, miniature pigs, and humans. The comparison of amino acid sequences of the deduced Vps proteins indicated that only one or two amino acid residues were changed among the mammalians (Fig. 1).

Retromer Complex Formations among Vps26a, Vps26b, Vps29, and Vps35

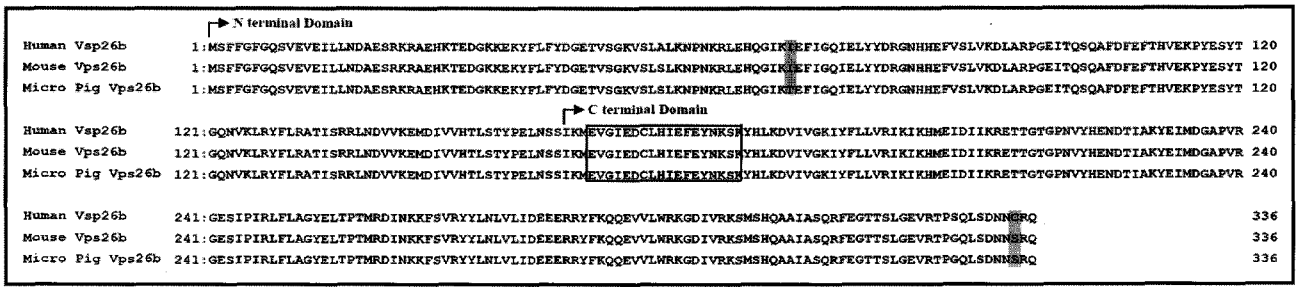
To examine whether the Vps35 interacts with Vps26a, Vps26b, and Vps29 in the formation of the retromer complex, the extracts from micro pig brain were denatured by SDS under the mildly non-reducing condition and analyzed by Western blotting (Fig. 2). Antibodies raised against Vps26a, Vps26b, Vps29, and Vps35, respectively, immunoreacted with 140-kDa. These data suggest that the retromer complex formed in the micro pig brain can be consisted of Vps26a, Vps29, and Vps35, and/or Vps-26b, Vps29, and Vps35.

Micro Pig Vps35 Interacts with Micro Pig Vps26a, Vps26b, and Vps29 in Yeast Two-Hybrid System

To determine whether micro pig Vps35 interacts with each component of the retromer complex directly, we used yeast two-hybrid system to see the interaction between the two components matched one by one. The full-length cDNAs of *Vps26a, Vps26b, Vps29,* and *Vps35* were subcloned into the prey and bait vectors. Then β -galactosidase assay was performed using thirteen independent colonies co-transformed with the prey and bait vectors. Colony growth and the β-galactosidase assay showed that the Vps35 of miniature pigs do interact with Vps26a, Vps26b, and Vps29. No self-interaction was observed in Vps26a, Vps26b, and Vps35, respectively (Fig. 3).



(A)



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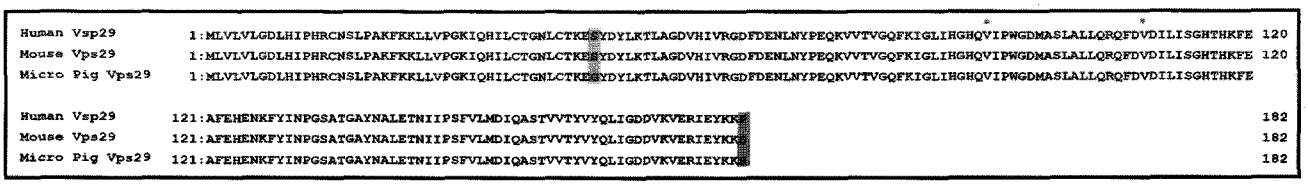

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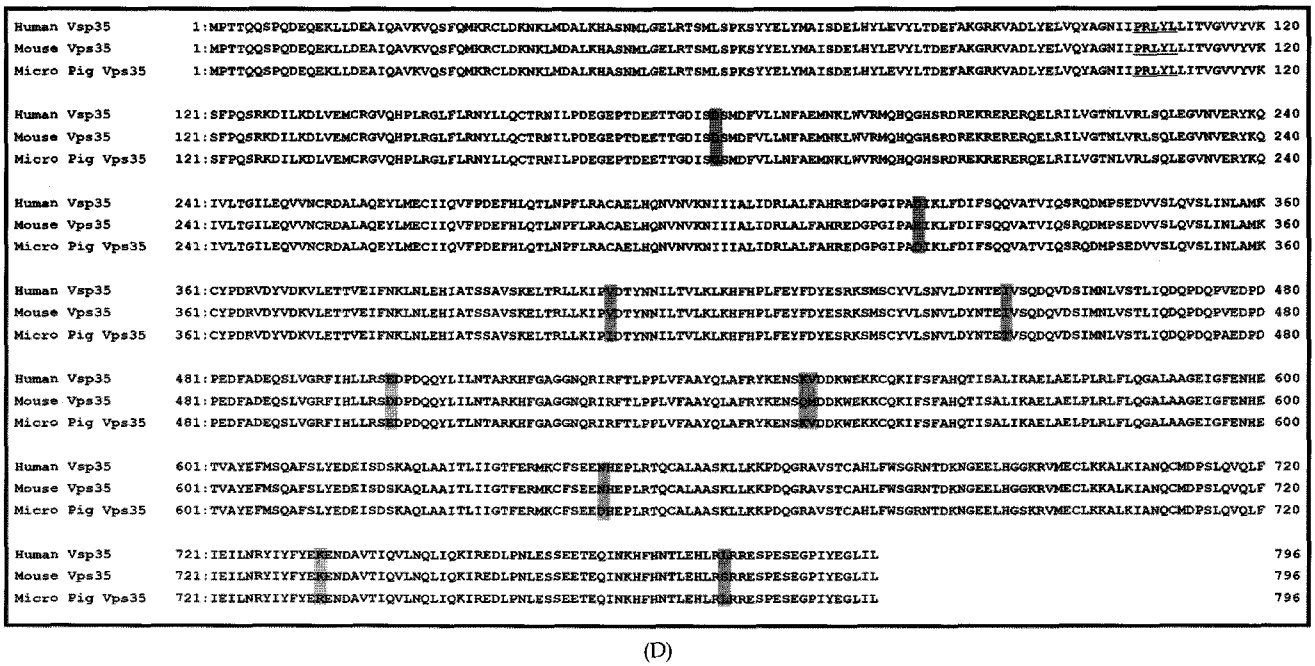

(D)

Fig. 1. Comparison of amino acid sequences for the orthologs of mouse, micro pig, and human of the retromer components Vps26a (A), Vps26b (B), Vps29 (C), and Vps35 (D). The binding motif involved in the interaction of Vps35 with Vps26 is shown by open boxes. Any amino acid changes among the three proteins are indicated by shaded boxes. The residues of Valine[90, 109] of Vps29 were known to be essential for the interaction with Vps35 is marked by asterisks. The P-R-L-Y-L motif in Vps35 is underlined.

DISCUSSION

In the present study, we have identified and characterized four genes encoding different *Vps* (26a, 26b, 29, and 35) proteins in micro pigs. Those Vps proteins have been proposed to do sorting of growth factor receptors (Kurten *et al.*, 1996), transcytosis of polymeric immunoglobulin (Verges *et al.*, 2007), and formation of Wnt gradients (Prasad and Clark, 2006). The best characterized Vps is Vps35 which plays a crucial role in the formation of the retromer complex. Vps35 interacts with Vps26 through its N terminus and with Vps29 through its C terminus (Gokool *et al.*, 2007). Vps35 also interacts with the cargo proteins such as Vps10 or the CI-MPR. It is known that Snx1 and Snx2 dimerize through their C-terminal (Bar) domains (Carlton *et al.*, 2004). The best characterized binding motif is PRLYL sites in Vps35. This motif was originally identified by using yeast two-hybrid system as a role of sorting carboxypeptidase Y (CPY). In addition, it is suggested that valine residue at 109 in the Vps29 polypeptide is essential for its binding to Vps35 (Collins *et al.*, 2005). Moreover, Vps26a has

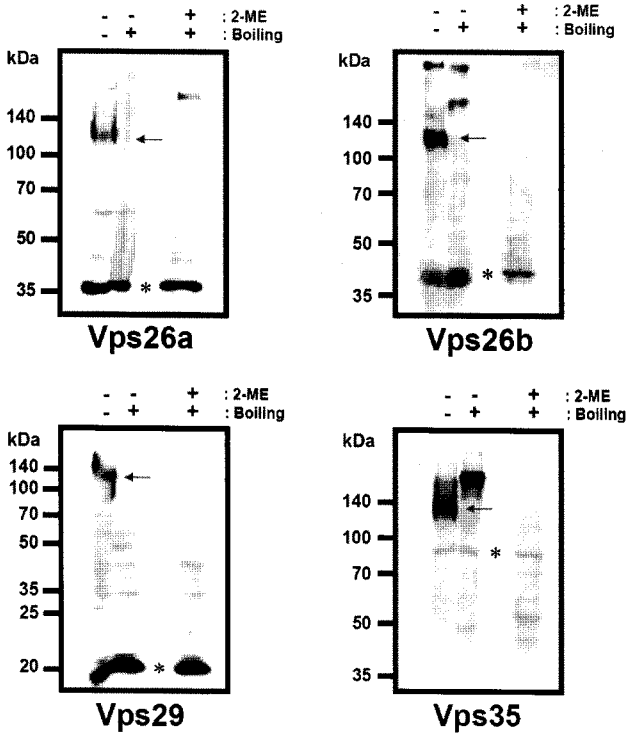

Fig. 2. Formation of the retromer complexes consisted of either Vps26a or Vps26b, and Vps29 and Vps35 in micro pigs. The protein extracts from the brain of micro pig were separated by SDS-PAGE under the mildly non-reducing condition and subjected to Western blot analysis using antibodies raised against Vps26a, Vps26b, Vps-29, and Vps35. Arrows and asterisks indicate the retromer complex and monomeric forms of Vps26a, Vps26b, Vps29, and Vps35, respectively.

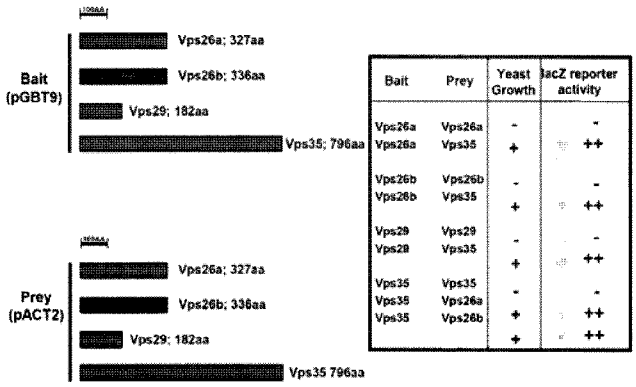

Fig. 3. Yeast two-hybrid analysis for the interactions between the retromer components Vps26a, Vps26b, Vps29, and Vps35. (A) Schematic representation of the retromer components. Retromer components cloned into the pGBT9 vector were co-expressed in the yeast strain with full-length genes of *Vps26a, Vps26b, Vps29,* and *Vps35* that cloned into the pACT2 vector. The abbreviations used are: Vps, Vacuolar protein sorting, aa, amino acid. (B) The results of yeast two-hybrid analyses expressed as β-galactosidase activity. Bait vectors were co-expressed in the yeast strain with the Prey vectors containing genes for Vps26a, Vps26b, and Vps35. In every case, yeast cells were selected on medium deficient in tryptophan and leucine. The β-galactosidase activity was scored as ++ (deep blue), + (blue), − (white).

been reported to interact directly with Vps35 through the Vps35 binding motif in the C-terminal domain. In micro pigs, the binding motifs of these Vps proteins are well conserved, suggesting potential activity of the retromer complex (Fig. 1).

Considering the ubiquitous expressions and the housekeeping functions of the retromer components in mammalian cells, the distribution of each retromer component is expected to be similar with each other in a tissue of micro pigs. However, there has been not much evidences supporting the interactions among the retromer components *in vivo*. To test the presence of the retromer complex (es) in the brain protein extracts from micro pigs, we used western blot analysis with non-reducing SDS-PAGE system. By conducting western blot under the mildly-denatured condition, we identified 140 kDa bands suggesting the Vps interaction among the retromer components in the brain of miniature pigs (Fig. 2). These results indicate that there is the retromer complex formed in the brain of micro pigs by interactions among the Vps proteins. We also found that Vps35 interacts with either Vps26a or Vps26b, as well as Vps29 (Fig. 3). Thus, Vps35 would be a key molecule involved in the functional formation of the retromer complexes in micro pigs (Fig. 3). The propensity of retromer complex to associate, particularly Vps35 with other Vps components and with cargo proteins, will continue to provide clues about protein trafficking on embryogenesis within the endosomal system.

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