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Molecular Cloning and Characterization of Mammalian Homologues of Vesicle-Associated Membrane Protein-Associated (VAMP-Associated) Proteins

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We have identified human and rat homologues of the VAMP-associated protein (VAP) of 33 kDa of *Aplysia californica* (aVAP-33), which we designated VAP-A, VAP-B, and VAP-C. Human VAP-A (hVAP-A) was found to be identical to the recently reported protein hVAP-33, with the exception of two amino acid residues. VAP-B contained a coiled-coil domain and a transmembrane domain (TMD). Human VAP-B (hVAP-B) was 46 and 60% homologous of the amino acid level to aVAP-33 and hVAP-A, respectively. Human VAP-C was a splicing variant of hVAP-B, lacking both the coiled-coil domain and the TMD. hVAP-B had VAMP-binding ability. Moreover, hVAP-A and hVAP-B associated with each other through their respective TMDs. These results suggest that complex formation by VAPs might be important in the trafficking of mammalian vesicle. © 1999 Academic Press

It is now widely accepted that SNARE proteins, a family of compartmentally specific and cytoplasmically oriented integral membrane proteins, provide the fundamental machinery that is required for fusion of membranes [1, 2]. The molecules that are believed to be involved in the core recognition between a vesicle and its target membrane are known as v-SNAREs and t-SNAREs, respectively [1]. Distinct sets of these proteins participate at each step in membrane transport and, therefore, it has been proposed that they determine the specificity and fidelity of membrane-trafficking reactions [3]. This proposal leads to the prediction that large numbers of different SNAREs must exist.

Recently, a 33-kDa nerve-specific VAMP-associated protein was cloned from *Aplysia californica* and designated VAP-33 [4]. Presynaptic injection of antibodies against *Aplysia* VAP-33 (aVAP-33) inhibited synaptic transmission and, therefore, it was proposed that aVAP-33 might be required for the exocytosis of neurotransmitters [4]. Many homologues of SNARE proteins have been isolated from mammalian cells and it is, thus, likely that homologues of aVAP-33 exist in mammalian cells and participate in the regulation of protein trafficking.

In the present study, we identified mammalian homologues of aVAP-33, which we designated VAP-A, VAP-B and VAP-C. The human VAP-A (hVAP-A) that we identified was almost identical to another recently reported human homologue of aVAP-33 [5], but both VAP-B and VAP-C appear to be novel mammalian homologues of aVAP-33. We also investigated tissue distribution of these VAPs and binding activity of human VAP-B (hVAP-B).

MATERIALS AND METHODS

Cloning of mammalian VAPs. Several expressed sequence tag (EST) clones that appeared to encode human homologues of aVAP-33 were identified in the EST database at the National Center for Biotechnology Information in a search of the database using the BLAST method and the sequence of aVAP-33. Two clones (IMAGE Consortium: IMAGE 685423 and 724209) encoded hVAP-A. One clone (IMAGE Consortium: 347040) encoded human VAP-C (hVAP-C). The inserts were sequenced using an automated DNA sequencer (Applied Biosystems Inc.).

For cloning of hVAP-B, rat VAP-A (rVAP-A) and rat VAP-B (rVAP-B), we performed the rapid amplification of cDNA ends (RACE) [6] using primers 5′-ATATTTCAAGAGGTAACAGCAACAGAT -3′ and 5′-GCCATGGCGAACGACGAGCAGAT-3′ for rVAP-A and 5′-TAAT-

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The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL, and NCBI nucleotide sequence databases with the following accession numbers: AF086627 (hVAP-A), AF086628 (hVAP-B), AF086629 (hVAP-C), AF086630 (rVAP-A), and AF086631 (rVAP-B).

Abbreviations used: VAMP, vesicle-associated membrane protein; VAP, VAMP-associated protein; TMD, transmembrane domain; SNARE, soluble NSF attachment protein receptor; EST, expressed sequence tag; RACE, rapid amplification of cDNA ends; PCR, polymerase chain reaction; bp, base pair; kb, kilo base; GST, glutathione *S*-transferase.

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KACACCAACGATAAAGAACA-3′ and 5′-AATTGCCAGCAGARAA-TGMTAAAC-3′ for hVAP-B and rVAP-B, a Marathon cDNA amplification kit (Clontech), and human brain and rat heart RACE cDNA libraries (Clontech).

To confirm the deletion in hVAP-C, we performed the PCR with the human heart cDNA library (Clontech) as template and oligonucleotides 5′-GCTCCGCCGCTAAGGAACATG-3′ and 5′-TCCTGTGCAT-GCTACCTCTACAAG-3′ as forward and reverse primers, respectively.

Northern blotting analysis. We performed Northern blotting analysis using Human Multiple Tissue Northern Blots I, III, and IV and a Rat Multiple Tissue Northern Blot (Clontech). Random-primed cDNA probes were synthesized with Strip-EZ DNA (Ambion). Each heat-denatured cDNA probe was added at 2×10^6 cpm/ml to ExpressHyb hybridization solution (Clontech). The bands of RNA on membranes were allowed to hybridize with probes for 1 h at 68°C. The membranes were washed according to the manufacture's protocol and exposed to X-ray film at −80°C. The VAP-A cDNA probe did not hybridize to VAP-B and VAP-C cDNAs and *vice versa* under these conditions. The hVAP-B cDNA probe was designed to correspond to the spliced domain that was absent from hVAP-C cDNA. The hVAP-C cDNA probe was generated using clone 347040 as template and it cross-reacted with hVAP-B cDNA. After autoradiography, membranes were stripped according to the manufacture's protocol and hybridization was repeated with another probe.

Binding assay in vitro. The cDNAs for hVAP-A [amino acids (aa) 1–242] and hVAP-B (aa 1–243); their truncated forms hVAP-A (-TMD) (aa 1–218) and hVAP-B (-TMD) (aa 1–219); human VAMP1 (aa 1–100); and human VAMP2 (aa 1–95) were subcloned into the pGEX-2T expression vector (Amersham Pharmacia Biotech) using fragments that had been generated by PCR. All constructs were verified by sequence analysis to ensure that no mutations had been introduced during their construction. Fusion proteins with glutathione *S*-transferase (GST) were generated and purified according to the manufacture's protocol. [^{35}S]Methionine-labeled VAP-B was generated using the mMESSAGE mMACHINE (Ambion) and an *In Vitro* Express Translation Kit (Stratagene). [^{35}S]Methionine-labeled hVAP-B or truncated hVAP-B (-TMD) was incubated for 1 h at 4°C with 40 μl of a 50% slurry of glutathione-agarose to which had been coupled 1 pmol of GST, 0.5 pmol of GST-VAMP1, 2 pmol of GST-VAMP2, 0.5 pmol of GST-hVAP, or 1 pmol of GST-hVAP (-TMD). Samples were washed three times in 0.5 ml of 10 mM Hepes/KOH (pH 7.4) that contained 150 mM NaCl with brief centrifugation. The beads were boiled in 20 μl of sample buffer for SDS–PAGE and proteins were fractionated by electrophoresis in SDS/12.5% polyacrylamide gels. Gels were stained with Coomassie brilliant blue, dried, and subjected to autoradiography.

Analysis of protein sequences. TMDs were predicted by use of the SOSUI server [7]. Predictions of sites of coiled-coils were made by the COILS server using the MTIDK matrix and equal weighting of all positions [8].

RESULTS

Identification of mammalian VAPs. To find human homologues of aVAP-33, we searched the EST database using amino acid (aa) and nucleotide (nt) sequences of aVAP-33 as probes. We found a number of EST sequences derived from two species of cDNA that encoded homologues of aVAP-33.

One set of cDNA clones predicted the existence of a membrane protein of 242 aa with overall structural organization similar to that of aVAP-33 and 48% homology of the amino acid level. This protein was named hVAP-A and was identical, with the exception of two amino acid residues, to hVAP-33 [5], a recently reported human homologue of aVAP-33.

The second set of cDNA clones predicted the existence of a protein of 243 aa, which we named hVAP-B. To clone the cDNA for hVAP-B, we performed RACE using a human brain RACE cDNA library as template. The complete nucleotide sequence and predicted amino acid sequence of hVAP-B are shown in Fig. 1A. The coding region of the cDNA contained 732 nt, including an ATG initiation codon within a Kozak consensus sequence [9]. The 3′-noncoding sequence of the cDNA for hVAP-B contained a polyadenylation signal and several copies of ATTTA that might be a signal for destabilization of the mRNA [10]. The encoded hVAP-B included three potential sites for phosphorylation by protein kinase C and it exhibited 48 and 60% homology of the amino acid level to aVAP-33 and hVAP-A, respectively.

In our search of the database, we identified some human ESTs that seemed to encode the same sequences as the cDNA for hVAP-B. Among these cDNA clones, we chose clone 347040 for sequencing since it appeared to contain a long insert. The sequence from clone 347040 was almost same as that of hVAP-B cDNA except for a deletion of 362 nt. To confirm the deletion, we performed an analysis by PCR using primers based on common sequences in hVAP-B and clone 347040 cDNAs. Two products of PCR were generated when the human heart cDNA library was used as template. One product was 767 bp long and contained the entire open reading frame of the cDNA for hVAP-B, and the other product was 405 bp long and had the same sequence as clone 347040. These results suggested that clone 347040 encoded a splice variant of the gene for hVAP-B, which we designated human VAP-C (hVAP-C). The spliced domain of 362 nt (nt 358–719) that was missing from hVAP-C cDNA is shown in Fig. 1A.

In the deduced amino acid sequence of hVAP-C, the first 70 residues were identical to those of hVAP-B. This region was followed by 29 unrelated amino acids as a consequence of a frame shift mutation so that hVAP-C lacked both the coiled-coil domain and the carboxyl-terminal TMD of hVAP-B (Fig. 1B).

We also cloned cDNAs for rat homologues of aVAP-33, which we named rVAP-A and rVAP-B. The encoded rVAP-A was 95% homologous of the amino acid level to hVAP-A, while the encoded rVAP-B was 88% homologous of the amino acid level to hVAP-B (88%). In addition, we identified the cDNA for a putative homologue of aVAP-33 in *Caenorhabditis elegans* (F33D11.11) in our search of databases.

Comparison of the deduced sequences of the putative VAPs (Fig. 2) revealed the presence of three characteristic regions in these proteins. The first is a well-conserved amino-terminal domain, with striking identity within the amino acid sequence CFKVKTTAP-

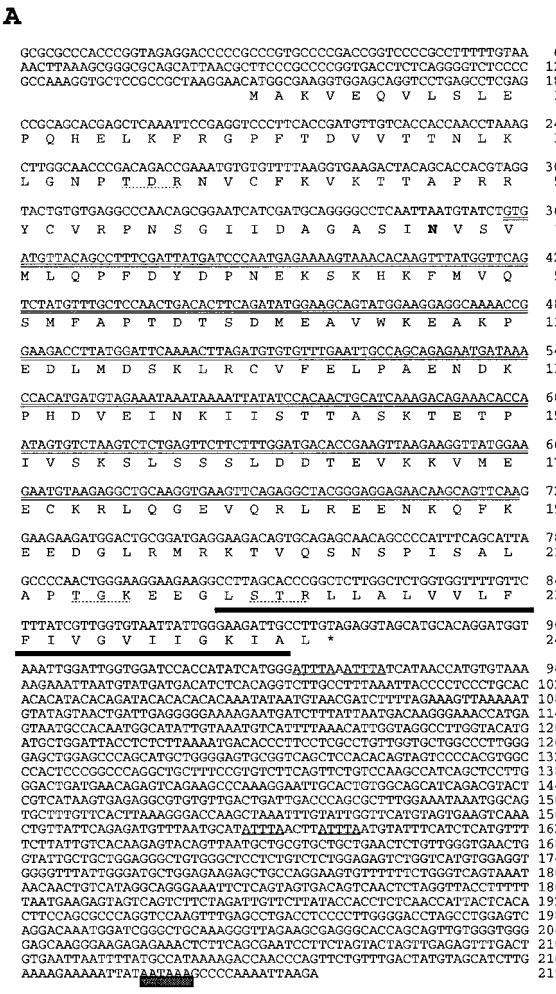

FIG. 1. (A) Nucleotide and predicted amino acid sequences of hVAP-B cDNA. The amino acid sequence of hVAP-B is shown in the single-letter code below the nucleotide sequence. Putative sites of phosphorylation by protein kinase C ([ST]-X-[RK], . . .), the transmembrane domain (—) and the spliced domain (=) that is absent from hVAP-C are underlined. The ATTTA motif (–) and a polyadenylation signal (■) are also indicated. hVAP-B has a consensus site for N-glycosylation at position 68. (B) Alignment of the amino acid sequences of hVAP-B and hVAP-C. The asterisks indicate the amino acid residues that are identical in the two sequences.

XXYCVRPNSG (aa 41–59). The second is a coiled-coil domain which is a common motif in many t-SNARE proteins [11]. The third is a carboxyl-terminal TMD, which is characteristic of a class of integral membrane proteins.

Tissue distribution of mammalian VAPs. We performed Northern blotting analysis to determine the tissue distribution of mammalian VAPs. It was reported previously that aVAP-33 is expressed only in neural tissues [4]. By contrast, all the VAPs that we examined were not associated with nerve-specific expression. In good agreement with results in a previous report [5], we found that the hVAP-A and rVAP-A transcripts were 1.7 and 1.8 kb long, respectively, and both mRNAs for hVAP-A and rVAP-A were expressed ubiquitously (Fig. 3A).

On blots probed with cDNA for hVAP-B (Fig. 3B), we detected a major hVAP-B transcript of 2.5 kb; the length of this transcript was in good agreement with the length of hVAP-B cDNA (2.2 kb). We also detected two minor transcripts of 1.1 and 8.7 kb, respectively. The origins and significance of these minor transcripts remain to be determined. Hybridization with rVAP-B cDNA revealed a transcript of 2.5 kb (Fig. 3B). Transcripts for both hVAP-B and rVAP-B were expressed ubiquitously, although levels of expression were lower than those of the transcripts for VAP-A.

In blots probed with cDNA for hVAP-C (Fig. 3C), transcripts for both hVAP-B and hVAP-C were detected. The hVAP-C mRNA was 1.9 kb long, reflecting the size of the insert in clone 347040 (1.8 kb).

Interaction of hVAP-B with VAMPs and VAPs. It has been demonstrated that aVAP-33 binds to VAMP. Therefore, we examined whether hVAP-B could also bind VAMPs. We used GST fusion proteins to detect binding to hVAP-B that had been generated by translation *in vitro.* hVAP-B bound to GST-VAMP1 and also to GST-VAMP2, albeit to a lesser extent than to GST-VAMP1 (Fig. 4A).

We then examined the ability of hVAP-B to interact with hVAP-A and hVAP-B. Figure 4B shows that hVAP-B bound to intact GST-hVAP-A and GST-hVAP-B. hVAP-A that had been translated *in vitro* also bound to both GST-hVAPs (data not shown). To determine whether the TMD of each hVAP was required for binding to hVAP-B, we removed the carboxyl-terminal TMDs of hVAP-A and hVAP-B. ^{35}S-labeled hVAP-B failed to bind to GST-hVAPs (-TMD) and truncated ^{35}S-labeled hVAP-B (-TMD) failed to bind to intact GST-hVAPs. Thus, each TMD appeared to be required for association with hVAPs.

DISCUSSION

In this study, we identified mammalian homologues of aVAP-33, which we named VAP-A, VAP-B and VAP-C. Although hVAP-A was almost identical to the recently reported human homologue of aVAP-33 that was designated hVAP-33 [5], VAP-B and VAP-C appear to be novel mammalian homologues.

To investigate the function of VAP-B, we studied the binding properties of hVAP-B *in vitro.* hVAP-B bound to both GST-VAMP1 and GST-VAMP2. This result

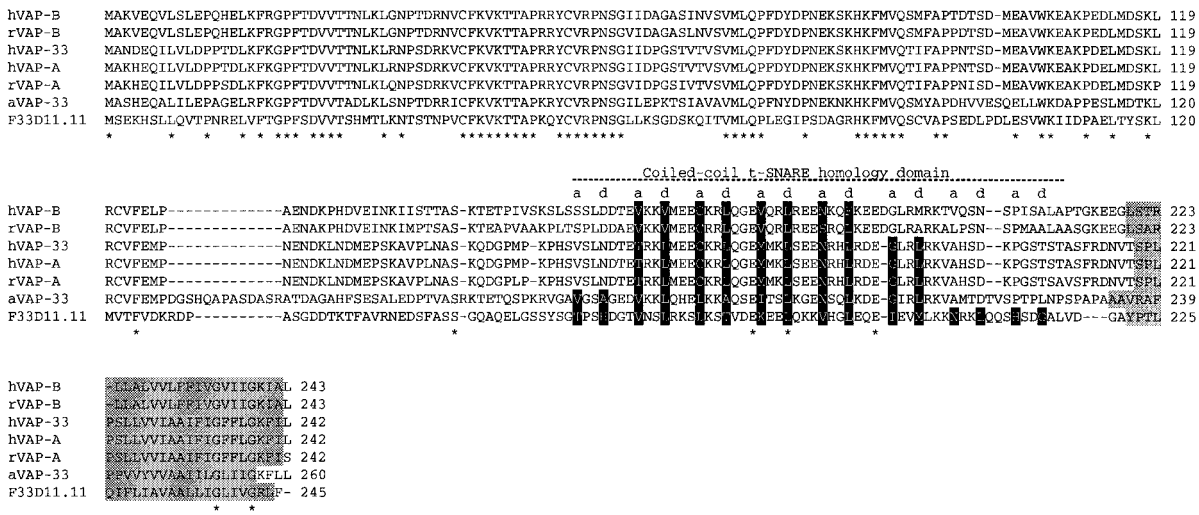

FIG. 2. Comparison of the deduced amino acid sequences of various VAPs and F33D11.11. The asterisks indicate amino acid residues that are identical in all seven sequences. The domains with a predicted coiled-coil structure and homology to t-SNAREs are indicated. The conserved hydrophobic residues in the coiled-coil domains are enclosed in black boxes. The putative transmembrane domains are shaded. Dashes represent gaps introduced to optimize the alignment of the various sequences.

suggests that VAP-B is an authentic mammalian homologue of aVAP-33. Since the tissue distribution of VAP-A and VAP-B was apparently similar (Fig. 3), we examined whether hVAP-B could associate with hVAP-A and, using appropriate fusion proteins, we found that hVAP-B associated with both hVAP-A and

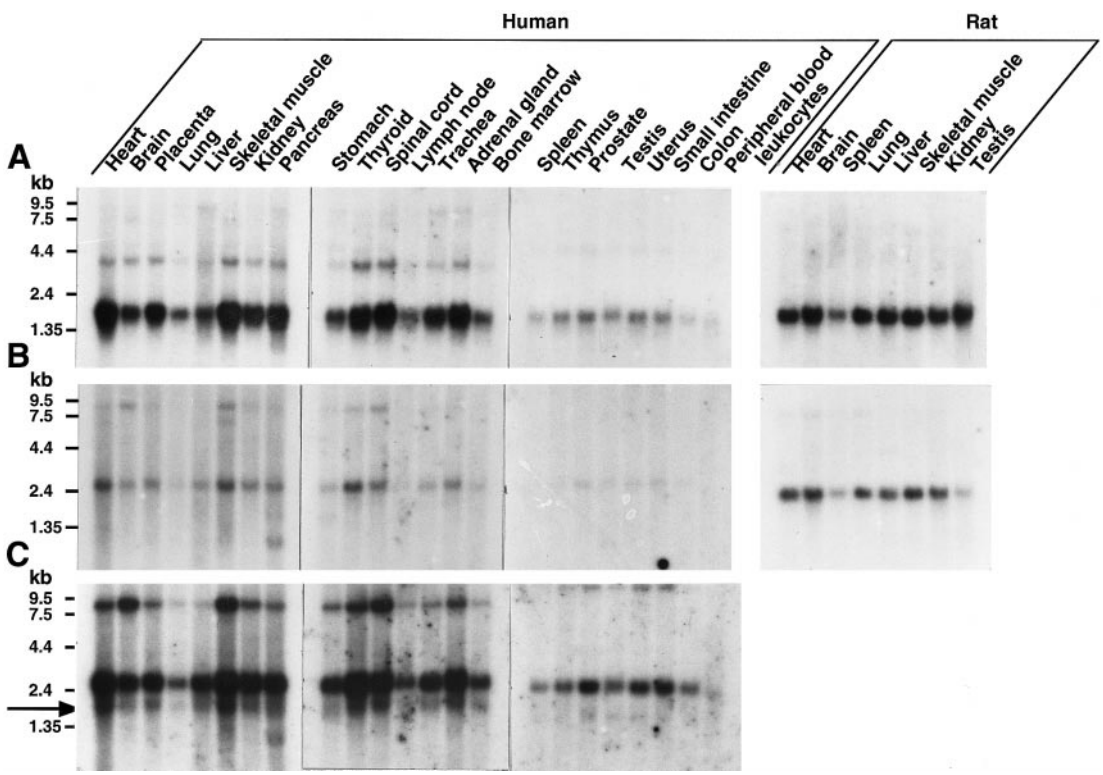

FIG. 3. Tissue distribution of mammalian VAPs. Two μg per lane of poly(A)$^+$ RNA, prepared from the indicated human and rat tissues, were analyzed by Northern blotting using probes derived from cDNAs for VAP-A (A), VAP-B (B) and VAP-C (C). The arrow in C indicates the band that corresponds to the transcript of hVAP-C. The autoradiograms for VAP-A and VAP-B were exposed for 1 day, whereas the autoradiograms for hVAP-C were exposed for 8 days.

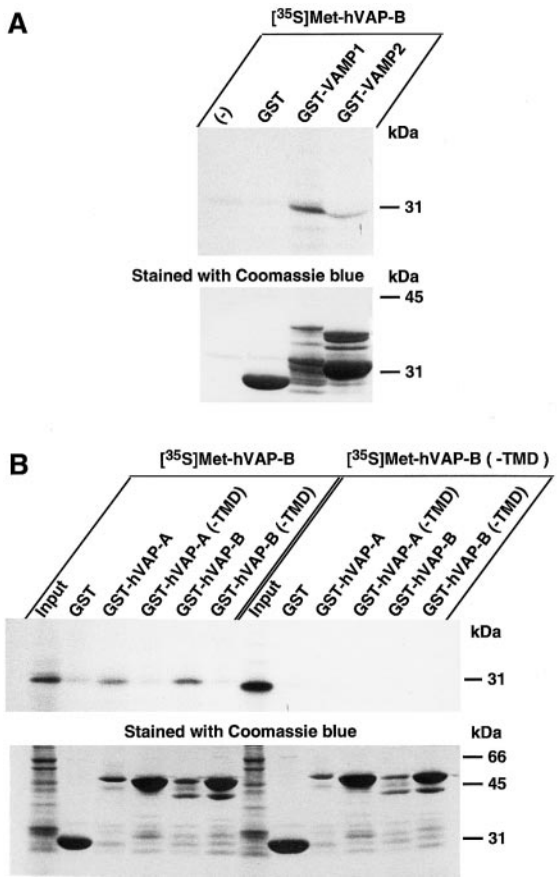

FIG. 4. Binding of hVAP-B to VAMPs and VAPs *in vitro.* Bacterially expressed GST or a GST fusion protein was incubated with ^{35}S-methionine-labeled hVAP-B that had been translated *in vitro.* After washing, bound proteins were analyzed by SDS–polyacrylamide gel electrophoresis. The gels were stained with Coomassie Blue and subjected to autoradiography. (A) Buffer alone (−), GST, GST-VAMP1, or GST-VAMP2 was incubated with ^{35}S-methionine-labeled hVAP-B. (B) GST, GST-hVAP-A, GST-hVAP-A (-TMD), GST-hVAP-B or GST-hVAP-B (-TMD) was incubated with ^{35}S-methionine-labeled hVAP-B or truncated hVAP-B (-TMD).

hVAP-B. We next investigated the role of the carboxyl-terminal TMD in the binding ability of hVAP since it has been reported that the membrane protein glycophorin A forms noncovalently bounded dimers via reversible association of its single hydrophobic TMD [12, 13]. As shown Fig. 4B, hVAP-B generated by translation *in vitro* did not bind to truncated GST-hVAPs and similarly translated but truncated hVAP-B did not bind to GST-hVAPs. These results suggest that hVAP-A and hVAP-B associate with each other through their TMDs. In a previous study, it was demonstrated that the homologue in *Drosophila* of syntaxin 5, Sed5, probably forms membrane-associated dimers through the interaction of coiled-coil regions [14]. Our results are the first to indicate that it is the TMD that is critical for both the homo- and the heterooligomerization of hVAP-A and hVAP-B. Although removal of the TMD of hVAP-B reduced its affinity for VAMPs, truncated hVAP-B retained VAMP-binding ability, a result that suggests that the TMD is not the VAMP-binding site (data not shown).

Both VAP-A and VAP-B have a coiled-coil domain near the carboxyl-terminal TMD. The coiled-coil domain has been found in almost all t-SNAREs and has been referred to as the t-SNARE homology domain [11]. In agreement with results in a previous report [11], we found that the conserved hydrophobic positions in the coiled-coils of VAP-A and VAP-B showed preference for leucine at the d position (see Fig. 2). This observation suggests that these VAPs might belong to the t-SNARE superfamily.

A comparison of the amino acid sequences of VAPs revealed that the amino-terminal regions have been well conserved. In particular, the peptide CFKVKTTAPXXYCVRPNSG (aa 41–59) was found in all the VAPs. The function of this sequence is unknown [15]. We searched databases using the sequence CFKVKTTAPXXYCVRPNSG as a probe and found some gene products in which the sequence was relatively well conserved. The gene products (with their GenBank accession numbers in parenthesis) included SCS2 (D44493) in *Saccharomyces cerevisiae;* AC17C9.12 (Z73099) and BC16G.05c (AL023554) in *Schizosaccharomyces pombe;* F33D11.11 (AF039720) and F42G2.5 (AF024499) in *C. elegans;* and F6N15.21 (AF069299) and F20O13.33 (AC003981) in *Arabidopsis thaliana.* Among these gene products, only F33D11.11 was found to have both a coiled-coil domain and a carboxyl-terminal TMD. It is possibly that F33D11.11 might be a homologue in *C. elegans* of aVAP-33. F6N15.21 of *A. thaliana* has a coiled-coil domain, but it also has two TMDs. In our search of databases, we failed to find any gene product from yeast that had all three of the following domains; the well-conserved amino-terminal peptide, a coiled-coil domain and a carboxyl-terminal TMD.

We also identified hVAP-C, a splicing variant of hVAP-B. hVAP-C resembled syntaxin 3D and syntaxin 16C in that each of these proteins lacks a coiled-coil domain and a TMD [16, 17]. The function of such carboxyl-terminally truncated molecule remains unknown.

In this study, we identified several VAPs in mammals only and demonstrated the association of VAP-B with VAP-A. Our results suggest that formation of complexes composed of VAPs might be a mammal-specific mechanism for vesicle trafficking. Further studies are required to define the localization and roles in intracellular trafficking of these proteins.

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