RESEARCH COMMUNICATION

Identification of a human homologue of the vesicle-associated membrane protein (VAMP)-associated protein of 33 kDa (VAP-33): a broadly expressed protein that binds to VAMP

M. Lynn WEIR, Amira KLIP and William S. TRIMBLE[1]

Program in Cell Biology, Hospital for Sick Children, 555 University Avenue, Toronto, Ontario, Canada M5G 1X8

We report the identification of a human homologue of the vesicle-associated membrane protein (VAMP)-associated protein (hVAP-33) that has been implicated in neuronal exocytosis in *Aplysia californica*. This hVAP-33 shared 50% amino acid identity with the *A. californica* form and had similar length, structural organization and VAMP-binding abilities. However, in contrast with the neuron-specific expression seen in *A. californica*, hVAP-33 was broadly expressed, suggesting possible roles in vesicle fusion in both neuronal and non-neuronal cells.

INTRODUCTION

Intracellular protein transport in eukaryotes is mediated by vesicle intermediates, which shuttle molecules from one cell compartment to another via membrane-fusion events [1]. One example is neuronal exocytosis, which involves the calcium-triggered fusion of cytosolic synaptic vesicles with the presynaptic plasma membrane in order to release neurotransmitter [2]. Although components of the protein machinery involved have been identified [2,3], the exact mechanism by which exocytosis occurs has yet to be elucidated. The SNARE [soluble NSF (*N*-ethylmaleimide-sensitive fusion protein) attachment protein (SNAP) receptor] hypothesis has been proposed to explain some features of synaptic-vesicle fusion [3]. Proteins localized on the synaptic-vesicle membrane (v-SNAREs) interact with complementary proteins situated on the target or plasma membrane (t-SNAREs) to allow vesicle docking [3]. Neuronal v-SNAREs include the synaptobrevin/VAMP (vesicle-associated membrane protein) family [4] and synaptotagmin I, the latter perhaps acting as the calcium sensor for triggering vesicle fusion [2,5]. Neuronal t-SNAREs include syntaxin-1A [6] and SNAP-25 (synaptosome-associated protein of 25 kDa) [7]. These interacting SNAREs serve as a receptor for cytosolic SNAPs, which in turn bind to cytosolic NSF [3,8,9]. ATP hydrolysis by NSF leads to partial disassembly of the docked proteins and formation of a fusion-competent intermediate that responds to a calcium signal [3].

The identification of SNARE protein homologues in non-neuronal tissues, at a variety of transport steps throughout the cell, and in simpler organisms, including yeast, suggests that the protein machinery for secretion is conserved among species and may be common to various steps in the secretory pathway [10]. However, it is unlikely that the SNARE hypothesis fully explains the process of membrane fusion and it is probable that many other proteins are involved. One such protein, VAP-33 (VAMP-associated protein of 33 kDa), was identified in *Aplysia californica* through a yeast two-hybrid screen and shown to bind to VAMP [11]. Furthermore, antibodies specific to VAP-33 blocked neurotransmission when injected into cultured neurons, indicating that VAP-33 plays an essential role in this process [11]. Given the high degree of conservation between SNARE proteins among species [10], it seemed plausible that a mammalian VAP-33 might also exist and analysis of such a molecule would contribute to the understanding of vesicle fusion at the molecular level in all eukaryotes. This study describes the first evidence for a human homologue of VAP-33 (hVAP-33), which was found to be similar to the *A. californica* form in terms of its structure and ability to bind to VAMP. Surprisingly, hVAP-33 showed a wider tissue distribution, indicating that its role may not be limited to neurotransmission, but may include regulation of membrane fusion in many cell types.

EXPERIMENTAL

hVAP-33 cDNA sequence analysis

The GenBank database of expressed sequence tags was examined for sequences similar to *A. californica* VAP-33 cDNA [11]. From this search, an I.M.A.G.E. Consortium (LLNL) cDNA clone (Id# 328880) [12] appeared to contain a full-length insert and was purchased from Research Genetics Inc. (Huntsville, AL, U.S.A.). This cDNA in pBluescript SK− had already been cloned unidirectionally from a human pancreatic-islet cell library using an oligo dT primer [13]. An *Nco*I–*Sph*I fragment from this cDNA (see Northern blotting section) was subcloned and sequenced on both strands (Acgt Corp., Toronto, Ontario, Canada). The longest open reading frame was analysed and the predicted amino acid sequence was compiled.

Northern blotting

Total RNA from mouse tissues was extracted using the TRIzol method (Gibco-BRL, Burlington, Ontario, Canada), separated on a 1.1% (w/v) agarose gel containing 2.2 M formaldehyde (10 μg per lane) and transferred to Genescreen Plus nylon membrane (NEN Research Products Inc., Boston, MA, U.S.A.). Blots were prehybridized at 42 °C for 1 h in 50% (v/v) formamide/1% (w/v) SDS/1 M NaCl/10% (w/v) dextran sul-

Abbreviations used: GST, glutathione S-transferase; MSP, major sperm protein; NSF, *N*-ethylmaleimide-sensitive fusion protein; SNAP, soluble NSF attachment protein; SNAP-25, synaptosome-associated protein of 25 kDa; SNARE, SNAP receptor; VAMP, vesicle-associated membrane protein; VAP-33, VAMP-associated protein of 33 kDa; hVAP-33, human homologue of VAP-33.

[1] To whom correspondence should be addressed (e-mail wtrimble@sickkids.on.ca).

The nucleotide sequence reported in this paper can be found in GenBank's database using accession number AF057358.

phate. Hybridization was done in the same solution, but with 200 μg/ml denatured salmon sperm DNA and [α-^{32}P]dCTP-labelled probe, prepared by random priming (Stratagene, La Jolla, CA, U.S.A.). The probe consisted of the *Nco*I–*Sph*I fragment of hVAP-33 cDNA (full coding region with 1 and 10 additional nts at the 5′ and 3′ ends, respectively). After washing 2×5 min in $2 \times$ SSC [$20 \times$ SSC: 3 M NaCl/0.3 M sodium citrate (pH 7)] at room temperature, 2×30 min in $2 \times$ SSC/1 % (w/v) SDS at 50 °C and 2×30 min in $0.1 \times$ SSC/0.1 % (w/v) SDS at room temperature, blots were exposed to autoradiography film and intensifying screen at −80 °C. Blots were stripped of probe and rehybridized with ^{32}P-labelled 18 S rRNA cDNA, as described for hVAP-33.

Preparation of recombinant proteins

Full-length hVAP-33 (*Nco*I–*Sph*I cDNA fragment; see Northern blotting section) was expressed in pGEX-KG (modified pGEX-2T; Pharmacia, Baie d'Urfe, PQ, Canada) with glutathione S-transferase (GST) at the N-terminal [14]. Rat syntaxin-1A cDNA encoding amino acids 4–267 was provided in vector pGEX-KG by Dr. R. Scheller (Stanford University, Stanford, CA, U.S.A.). GST and GST-fusion proteins were purified using glutathione–agarose beads, as described by the manufacturer (Sigma, St. Louis, MO, U.S.A.). Recombinant protein left on the beads for binding assays was measured by boiling an aliquot in SDS sample buffer [2 % (w/v) SDS/5 % (v/v) glycerol/62.6 mM Tris/HCl (pH 6.8)/0.13 % (v/v) Bromophenol Blue/5 % (v/v) β-mercaptoethanol] and quantificating eluted protein by the Lowry method [15]. Purity of recombinant proteins was monitored by SDS/PAGE [12 % (w/v) acrylamide].

Full-length rat VAMP-1 and VAMP-2 were expressed in pET32a (Novagen, Madison, WI, U.S.A.) with an N-terminal histidine/thioredoxin tag. Mouse SNAP-25 cDNA (a gift from Dr. M. Wilson, University of New Mexico, Alberquerque, NM, U.S.A.) was expressed in pQE30 (Qiagen, Santa Clarita, CA, U.S.A.) as a full-length protein with an N-terminal histidine tag. Cell extracts were prepared from VAMP-1-, VAMP-2- or SNAP-25-expressing bacteria by sonication at 4 °C in 10 mM Tris/HCl (pH 8)/100 mM NaCl/0.05 % (v/v) Tween-20/5 μg/ml leupeptin/5 μg/ml pepstatin/0.5 mM PMSF (latter used for SNAP-25 only). Total protein was measured by the Lowry method [15]. The histidine/thioredoxin tag was removed from recombinant VAMP-1 and VAMP-2 by incubation of cell extract with thrombin (1 μg of enzyme per 100 μg of total protein), followed by inactivation with 2 mM PMSF.

Binding assay

Glutathione–agarose (50 μl of packed beads) bound with 0.5 nmol of immobilized GST or GST-fusion protein was incubated, with agitation, for 30 min at room temperature with 1, 15 or 70 μg of cell extract prepared from bacteria expressing VAMP-1, SNAP-25 or VAMP-2, respectively. The buffer used was 10 mM Tris/HCl (pH 8)/100 mM NaCl/0.05 % (v/v) Tween-20 in a total volume of 200 μl. Beads were washed 5 times with 5 volumes of the same buffer at room temperature and the proteins were denatured by boiling for 5 min in 50 μl of SDS sample buffer. Equal volumes were analysed by SDS/PAGE [12 % (w/v) acrylamide] and transferred electrophoretically to a PVDF membrane (Millipore, Bedford, MA, U.S.A.). Blots were incubated with the appropriate antibodies and proteins visualized using enhanced chemiluminescence (Pierce, Rockford, IL, U.S.A.) and BioMax MR film (Kodak, Rochester, NY, U.S.A.). VAMP-1 and VAMP-2 were detected using polyclonal rabbit antibodies specific for VAMP-1 (1/250) or VAMP-2 (1/200), respectively, followed by goat anti-rabbit horseradish peroxidase-conjugated IgG (Bio-Rad, Mississauga, Ontario, Canada; 1/5000). SNAP-25 was visualized using a monoclonal mouse anti-SNAP-25 antibody (Sternberger Monoclonals Inc., Baltimore, MA, U.S.A.; 1/1000) and goat anti-mouse horseradish peroxidase-conjugated IgG (Bio-Rad; 1/5000).

RESULTS AND DISCUSSION

Structural features of hVAP-33

In order to determine if a mammalian homologue of VAP-33 existed, a search of the GenBank database of expressed sequence tags was carried out using *A. californica* VAP-33 cDNA [11]. This scan revealed numerous partial sequences of cDNAs from various species and tissues, which were similar to *A. californica* VAP-33 cDNA, suggesting the existence of VAP-33 homologues. The human clones could be classified into at least three distinct groups, differing by more than 4 % at the nt level. Since only partial sequences of these human cDNAs have been reported, the full extent of these nt differences and whether they would alter hVAP-33 function in the encoded protein is not clear. Such differences may reflect polymorphisms in the human population. It is also possible that isoforms of hVAP-33 exist, since other proteins involved in membrane fusion also have multiple family members [4]. Whether *A. californica* VAP-33 exists in several forms is not known, although only one type of VAMP has been identified in this species [16].

A human clone, showing 68 % nt identity compared with the first 83 bp of coding region of *A. californica* VAP-33 cDNA, was

```
1    atggcgaacgacgagcagatcctggtcctcgatccgcccacagacctcaaattcaaaggc  60
      M  A  N  D  E  Q  I  L  V  L  D  P  P  T  D  L  K  F  K  G

61   cccttcacagatgtagtcactacaaatcttaaattgcgaaatccatcggatagaaaagtg  120
      P  F  T  D  V  V  T  T  N  L  K  L  R  N  P  S  D  R  K  V
                                                 ▲
121  tgtttcaaagtgaagactacagcacctcgccggtactgtgtgaggcccaacagtggaatt  180
      C  F  K  V  K  T  T  A  P  R  R  Y  C  V  R  P  N  S  G  I

181  attgacccagggtcaactgtgactgtttcagtaatgctacagccctttgactatgatccg  240
      I  D  P  G  S  T  V  T  V  S  V  M  L  Q  P  F  D  Y  D  P

241  aatgaaaagagtaaacacaagtttatggtacagacaatttttgctccaccaaacacttca  300
      N  E  K  S  K  H  K  F  M  V  Q  T  I  F  A  P  P  N  T  S
                                                                 *
301  gatatggaagctgtgtggaaagaggcaaaacctgatgaattaatggattccaaattgaga  360
      D  M  E  A  V  W  K  E  A  K  P  D  E  L  M  D  S  K  L  R

361  tgcgtatttgaaatgcccaatgaaaatgataaattgaatgatatggaacctagcaaagct  420
      C  V  F  E  M  P  N  E  N  D  K  L  N  D  M  E  P  S  K  A

421  gttccactgaatgcatctaagcaagatggacctatgccaaaaccacacagtgtttcactt  480
      V  P  L  N  A  S  K  Q  D  G  P  M  P  K  P  H  S  V  S  L
                  *                                          *
481  aatgataccgaaacaaggaaactaatggaagagtgtaaaagacttcagggagaaatgatg  540
      N  D  T  E  T  R  K  L  M  E  E  C  K  R  L  Q  G  E  M  M
                  ▲
541  aagctatcagaagaaaatcggcacctgagagatgaaggtttaaggctcagaaaggtagca  600
      K  L  S  E  E  N  R  H  L  R  D  E  G  L  R  L  R  K  V  A

601  cattcggataaacctggatcaacctcaactgcatccttcagagataatgtcaccagtcct  660
      H  S  D  K  P  G  S  T  S  T  A  S  F  R  D  N  V  T  S  P
         ▲                          ▲*
661  cttccttcacttcttgttgtaattgcagccatttttcattggattctttctagggaaattc  720
      L  P  S  L  L  V  V  I  A  A  I  F  I  G  F  F  L  G  K  F

721  atcttgtag
      I  L  -
```

Figure 1 Nucleotide and predicted amino acid sequence of hVAP-33

The top and bottom sequences of each set of lines represent the nt (numbering for coding region only) and protein sequences, respectively. The stop codon is denoted by a dash. The hydrophobic C-terminal is underlined and a possible α-helical domain capable of forming coiled-coils is boxed. Potential protein kinase C and casein kinase II phosphorylation sites are shown by arrowheads and asterisks, respectively.

```
hVAP   MANDEQI..LVLDPPTDLKFKGPFTDVVTTNLKLRNPSDRKVCFKVKTTA 48
aVAP   **SH**A..*I*E*AGE*R**********AD***S**T**RI******** 48
SCS2   *SAV*I.......S*DV*VY*S*L*EQS*EYASIS*N**QTIA******* 43
MSP    **QSVPPGDIQTQ*NAKIV*NA*YD*KH*YHI*VI*S*A*RIGYGT***N 50

hVAP   PRRYCVRPNSGIIDPGSTVTVSVMLQPFDYDPNE..KSKHKFMVQTIFAP 96
aVAP   *K**********LE*KTSIA*A******N*****..*N*******SMY** 96
SCS2   *KF******AAVVA**E*IQ*Q*IFLGLTEE*AADF*CRD**L*I*LPS* 93
MSP    MK*LG*D*PC*VL**KEA*LLA*SCDA*AFGQED..TNNDRIT*EWTNT* 98

hVAP   .PNTSD.MEAVWKEAKPD...ELMDSKLRCVFEMPNEND..KLNDMEPSK 139
aVAP   .DHVVESQ*LL**D*P*E...S***T*********DGSH..QAPASDA*R 140
SCS2   YDLNGKAVAD**SDLEAEFKQQAISK*IKVKYLISPDVHPAQNQNIQEN* 143
MSP    .DGAAKQFRRE*......FQGDG*VRRKN....L*I*YNP          127

hVAP   AV.............PLNASKQDGPMPKPHS.VSLNDTETRKLMEECKR 174
aVAP   *TDAGAHFSESALEDPTVASR*TETQS**RVQA*GSAGEDVK**QH*L*K 190
SCS2   ET.............VEPVVQDSE*KEV*AV.*NEKEVPAEPETQPPVQ 178

hVAP   LQGEMMKLSEENRHLRDEGLRLRKVAHSDK..PGSTSTASFRDNVTSPLP 222
aVAP   A*S*ITS*KG**SQ*K***I****MT*TVS*TPLNPSPAPAAAVRAF* 240
SCS2   VKK*EVPPVVQKTVPHENEKQTSNSTPAPQ..NQIKEA*TVPAENE*SSM 226

hVAP   SLLVVIAAIFIGFFLGKFIL 242
aVAP   PVVY*V***IL*LII***L* 260
SCS2   GIFILV*LLILV..**W*YR 244
```

Figure 2 Comparison of hVAP-33 amino acid sequence with related proteins

The hVAP-33 protein sequence was aligned with that of *A. californica* VAP-33 (aVAP), *S. cerevisiae* SCS2 protein and *C. elegans* major sperm protein (MSP) using MULTALIN 5.0 [20]. Numbering of amino acids is shown to the right. Dots represent gaps introduced for optimal alignment. Asterisks replace residues that are identical to hVAP-33. Putative transmembrane regions are underlined and possible coiled coils are boxed.

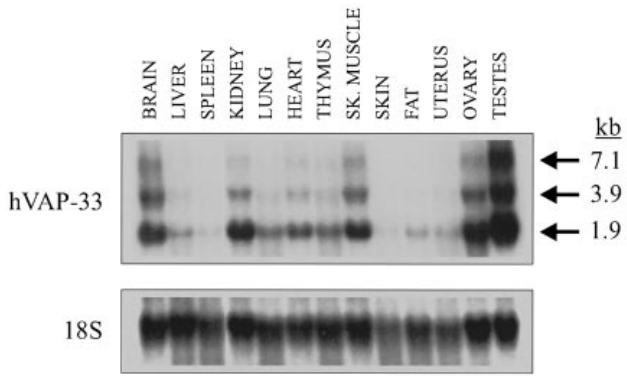

Figure 3 Northern blot of mouse tissues using VAP-33 cDNA probe

Total RNA was extracted from mouse tissues, separated on an agarose gel (10 μg per lane) and transferred to nylon membrane, as described in the Experimental section. The blot was probed with the full coding region of hVAP-33 cDNA (top panel), followed by stripping and reprobing with 18 S rRNA cDNA (bottom panel). Autoradiograms were scanned with a Hewlett Packard ScanJet 5p and the resulting images were manipulated in Adobe Photoshop 4.0 and CorelDRAW 7. The sizes of VAP-33 RNA species detected are shown in kb (arrows). SK, skeletal.

chosen for further analysis, since it was thought to be full length. This cDNA had previously been cloned unidirectionally from a human pancreatic-islet cell library using an oligo dT primer [12,13]. Based on the partial sequence of this clone reported in GenBank, the translation start site was localized to a methionine codon (bp 235) within a Kozak consensus sequence [17]. Further sequencing of this clone (Figure 1) showed an open reading frame of 726 nt flanked by a stop codon. This hVAP-33 cDNA encoded a predicted protein of 242 amino acids, molecular mass 27.3 kDa and a calculated pI of 8.3 (Figure 1). Distinguishing structural features included a hydrophobic C-terminal, suggesting a possible transmembrane region, determined using HELIXMEM (PC/GENE), and a potential α-helical domain capable of participating in coiled coils (residues 162–198), as predicted by COILS 2.1 using the MTIDK matrix and equal weightings of all positions [18,19]. Four possible phosphorylation sites were found each for protein kinase C and casein kinase II, although their significance with respect to hVAP-33 function is not yet known.

Comparison of hVAP-33 with related proteins

Human and *A. californica* VAP-33 showed 50 % identity at the amino acid level (Figure 2), with a higher level of similarity at the N-termini (65 % identical over 121 residues) than the C-termini (35 % identical over 121 residues). Conservation of the N-terminal may reflect a critical role for this region in the function of VAP-33. Although less well conserved, the C-terminal halves shared structural similarities, including the hydrophobic C-termini and the predicted coiled-coil domains (Figure 2). Since *A. californica* VAP-33 has been shown to be an integral membrane protein in which the C-terminal serves as a transmembrane anchor [11], this region may play a similar role in the human form. Coiled-coil domains are a common motif in many SNARE proteins [21] and appear to be important for protein–protein interactions. It has been shown that association of VAMP, SNAP-25 and syntaxin induces secondary structures within each protein, presumably through the formation of coiled coils, in the resulting tripartite complex [22]. Hence, the coiled-coil domain in VAP-33 may mediate the observed binding to VAMP [11] and potentially interact with other SNARE proteins.

Search of the GenBank protein database using the BLASTp algorithm [23] revealed that hVAP-33 showed limited similarity with several proteins. *Saccharomyces cerevisiae* SCS2 protein [24] was 22 % identical to hVAP-33, with the N-termini being more alike (33 % identical over 121 residues) than the C-termini (10 % identical over 121 residues) (Figure 2). The *SCS2* gene was isolated by its ability to suppress mutants with defects in the inositol uptake and synthesis pathways [24]. *Caenorhabditis elegans* MSP (major sperm protein) [25] was half the size of hVAP-33 and showed 22 % identity compared with the human protein (first 128 amino acids only) (Figure 2). Numerous MSP isoforms with similar sequences exist, only one of which is shown in Figure 2. MSP contributes to sperm pseudopodial movement, where it forms a cytosolic filament network that translocates vesicles to the plasma membrane [26]. Two other proteins that shared limited similarities with hVAP-33 were found by the BLASTp search, but are not shown in Figure 2. One was a 40.3 kDa protein in *Schizosaccharomyces pombe* that was similar to SCS2 and has yet to be characterized. The second one, found in *Arabidopsis thaliana*, shared 26 % identity with hVAP-33 (first 121 residues) and has been suggested to be a plant VAP-33 homologue [27].

Tissue distribution of VAP-33 RNA

The search in GenBank for mammalian cDNAs similar to *A. californica* VAP-33 showed human clones from various tissues, suggesting a widespread distribution. To determine the extent of hVAP-33 expression, Northern blotting was done to examine RNA levels using mouse tissues. Using the coding region of the human clone, the resulting Northern blot showed that VAP-33

RNA was detectable in all tissues examined, but at different levels (Figure 3). In contrast to *A. californica*, in which a single 3 kb VAP-33 RNA species was detected [11], three RNA bands were seen in all lanes (Figure 3), including a major species of 1.9 kb, whose size corresponded well with that of the hVAP-33 cDNA (1.7 kb), and two minor ones of 3.9 and 7.1 kb. These latter two bands most likely represent heteronuclear RNAs of varying splice sizes, since total RNA was examined and the relative ratio of band intensities was the same for all tissues. However, it cannot be ruled out that these bands may also represent RNAs encoding additional VAP-33 isoforms, since two of the classes of hVAP-33 cDNAs found in GenBank also appeared to exist in the mouse database. Due to the lack of full-length sequences for the mouse clones, it was not clear whether additional classes existed or if the human cDNA probe used for Northern blotting hybridized to all types of VAP-33 RNAs under the conditions used.

VAP-33 RNA was detected in all mouse tissues examined and levels were highest in brain, testes, ovary, kidney and skeletal muscle (Figure 3). This widespread distribution differs from that seen for *A. californica* VAP-33, which showed neuron-specific expression [11], suggesting that hVAP-33 may play a role not only in neuronal exocytosis in the brain, but also in vesicle-fusion events in many non-neuronal tissues. In fact, SNARE proteins have been localized in many of the non-neuronal tissues in which VAP-33 was found to be expressed [28,29]. In the kidney, both syntaxin and VAMP are present, with VAMP-2 co-localizing with vesicles of Aquaporin-2 water channels and perhaps contributing to their translocation to the plasma membrane [29,30]. In skeletal muscle, syntaxin and VAMP-2 may function in the exocytosis of vesicles containing glucose transporters [29,31]. Syntaxin and VAMP homologues have also been detected in sea-urchin egg and sperm, where they may be important for cortical granule exocytosis in eggs and the fusion of acrosomal vesicles with the plasma membrane of sperm [32,33]. Such SNARE proteins may also be found in mammalian gametes and, since VAP-33 was expressed in ovary and testes, this molecule may participate in the membrane-fusion events associated with mammalian fertilization.

Interaction of hVAP-33 with SNARE proteins

Since *A. californica* VAP-33 was initially isolated due to its VAMP-binding ability [11], it was important to determine whether the human form could also mediate this interaction. An *in vitro* binding assay was used to test whether recombinant VAMP-1 or VAMP-2 in a bacterial cell extract could associate with immobilized GST–hVAP-33. Western blotting, using a VAMP-1 antibody (Figure 4, top panel), showed that VAMP-1 bound to GST-syntaxin-1A, as expected, and also interacted with GST-hVAP-33. Some non-specific binding of VAMP-1 to glutathione–agarose beads alone and to GST immobilized on beads was observed, but was much less than GST–syntaxin-1A and GST–hVAP-33. VAMP-1 detected in cell extract that was not incubated with beads and/or GST-fusion proteins (last lane), co-migrated with VAMP-1 seen in the other four lanes. Similar results were obtained when these binding studies were repeated with recombinant VAMP-2 protein (Figure 4, middle panel). These data revealed a general VAMP-binding ability of hVAP-33 and demonstrated that it is an authentic homologue of the VAMP-associated *A. californica* VAP-33. Binding experiments with SNAP-25 (Figure 4, bottom panel) revealed that this SNARE protein did not interact with GST–hVAP-33 under conditions where the expected association with GST–syntaxin-1A was detected. This result suggested that hVAP-33 may be an exclusive VAMP-binding partner, although other SNARE proteins of the fusion complex need to be tested for hVAP-33 binding to confirm this idea.

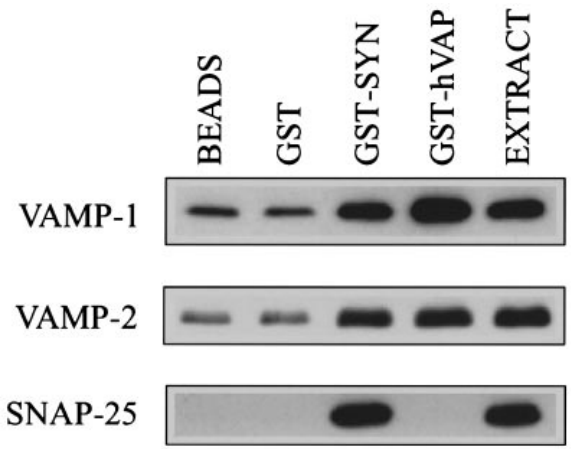

Figure 4 Binding assay to test recombinant hVAP-33 interaction with VAMPs and SNAP-25

Bacterial cell extracts containing recombinant VAMP-1, SNAP-25 or VAMP-2 (1, 15 or 70 μg of total protein, respectively) were incubated with glutathione–agarose beads only or 0.5 nmol of bead-immobilized GST, GST–syntaxin-1A (GST-SYN) or GST–hVAP-33. Bound proteins were eluted and equal volumes analysed by Western blotting using anti-VAMP-1 (top panel), anti-VAMP-2 (middle panel) or anti-SNAP-25 (bottom panel) antibodies, as described in the Experimental section. Cell extract that was not incubated with beads and/or GST-fusion proteins is shown in the last lane (5 μg of total protein from bacteria expressing SNAP-25 or VAMP-2, or 50 ng from bacteria expressing VAMP-1). Data shown are representative of experiments done twice. Blots were scanned with a Hewlett Packard ScanJet 5p and the resulting images were manipulated in Adobe Photoshop 4.0 and CorelDRAW 7.

It has been shown previously that injection of VAP-33 antibodies into *A. californica* presynaptic neurons blocks neurotransmission [11]. Although the exact step at which this protein acts is not clear, the fact that it is plasma membrane-associated and can bind to VAMP, has led to the suggestion that VAP-33 may play a role prior to SNARE complex assembly in exocytosis [11], such as tethering and/or localizing synaptic vesicles to the cell membrane prior to fusion. Given that hVAP-33 was found to be similar to the *A. californica* form in terms of structure and VAMP-binding ability, and given the widespread expression patterns of hVAP-33 (shown here), VAMP-1 and VAMP-2 [28], hVAP-33 may serve an analogous role in plasma membrane fusion events in neuronal and non-neuronal cells.

Conclusions

Identification for the first time of hVAP-33 that bound to VAMP-1 and VAMP-2 and had such widespread expression has important implications for the mammalian secretory pathway. This molecule may be found in all eukaryotic cells and may contribute to general vesicle fusion. Further analysis of hVAP-33 will aid understanding membrane fusion at the molecular level. Subcellular localization of hVAP-33, its potential interactions with other SNARE proteins and functional studies will be critical for determining the exact step at which this protein acts within the fusion process.

We thank J. Fata and Dr. R. Khokha for assistance with Northern blots, M. Mohtashami for subcloning SNAP-25, Dr. R. Scheller for pGEX-syntaxin-1A and Dr. M. Wilson for SNAP-25 cDNA. This work was supported by a grant to A.K. and W.S.T. from the Juvenile Diabetes Foundation International (JDFI).

REFERENCES

1 Rothman, J. E. (1994) Nature (London) **372**, 55–63
2 Sudhof, T. C. (1995) Nature (London) **375**, 645–653
3 Sollner, T., Whiteheart, S. W., Brunner, M., Erdjument-Bromage, H., Geromanos, S., Tempst, P. and Rothman, J. E. (1993) Nature (London) **362**, 318–324
4 Elferink, L. A., Trimble, W. S. and Scheller, R. H. (1989) J. Biol. Chem. **264**, 11061–11064
5 Matthew, W. D., Tsavaler, L. and Reichardt, L. F. (1981) J. Cell Biol. **91**, 257–269
6 Bennett, M. K., Calakos, N. and Scheller, R. H. (1992) Science **257**, 255–259
7 Oyler, G. A., Higgins, G. A., Hart, R. A., Battenberg, E., Billingsley, M., Bloom, F. E. and Wilson, M. C. (1989) J. Cell Biol. **109**, 3039–3052
8 Clary, D. O. and Rothman, J. E. (1990) J. Biol. Chem. **265**, 10109–10117
9 Block, M. R., Glick, B. S., Wilcox, C. A., Wieland, F. T. and Rothman, J. E. (1988) Proc. Natl. Acad. Sci. U.S.A. **85**, 7852–7856
10 Bennett, M. K. and Scheller, R. H. (1993) Proc. Natl. Acad. Sci. U.S.A. **90**, 2559–2563
11 Skehel, P. A., Martin, K. C., Kandel, E. R. and Bartsch, D. (1995) Science **269**, 1580–1583
12 Lennon, G., Auffray, C., Polymeropoulos, M. and Soares, M. B. (1996) Genomics **33**, 151–152
13 Takeda, J., Yano, H., Eng, S., Zeng, Y. and Bell, G. I. (1993) Hum. Mol. Genet. **2**, 1793–1798
14 Frangioni, J. V. and Neel, B. G. (1993) Anal. Biochem. **210**, 179–187
15 Peterson, G. L. (1977) Anal. Biochem. **83**, 346–356
16 Yamasaki, S., Hu, Y., Binz, T., Kalkuhl, A., Kurazono, H., Tamura, T., Jahn, R., Kandel, E. and Niemann, H. (1994) Proc. Natl. Acad. Sci. U.S.A. **91**, 4688–4692
17 Kozak, M. (1986) Cell **44**, 283–292
18 Lupas, A., Van Dyke, M. and Stock, J. (1991) Science **252**, 1162–1164
19 Lupas, A. (1996) Methods Enzymol. **266**, 513–524
20 Corpet, F. (1988) Nucleic Acids Res. **16**, 10881–10890
21 Weimbs, T., Low, S. H., Chapin, S. J., Mostov, K. E., Bucher, P. and Hofmann, K. (1977) Proc. Natl. Acad. Sci. U.S.A. **94**, 3046–3051
22 Fasshauer, D., Otto, H., Eliason, W. K., Jahn, R. and Brunger, A. T. (1997) J. Biol. Chem. **272**, 28036–28041
23 Altschul, S. F., Gish, W., Miller, W., Myers, E. W. and Lipman, D. J. (1990) J. Mol. Biol. **215**, 403–410
24 Nikawa, J., Murakami, A., Esumi, E. and Hosaka, K. (1995) J. Biochem. (Tokyo) **118**, 39–45
25 Klass, M. R., Kinsley, S. and Lopez, L. C. (1984) Mol. Cell. Biol. **4**, 529–537
26 Italiano, J. E., Roberts, T. M., Stewart, M. and Fontana, C. A. (1996) Cell **84**, 105–114
27 Galaud, J.-P., Laval, V., Carriere, M., Barre, A., Canut, H., Rouge, P. and Pont-Lezica, R. (1997) Biochim. Biophys. Acta **1341**, 79–86
28 Rossetto, O., Gorza, L., Schiavo, G., Schiavo, N., Scheller, R. H. and Montecucco, C. (1996) J. Cell Biol. **132**, 167–179
29 Bennett, M. K., Garcia-Arraras, J. E., Elferink, L. A., Peterson, K., Fleming, A. M., Hazuka, C. D. and Scheller, R. H. (1993) Cell **74**, 863–873
30 Nielsen, S., Marples, D., Birn, H., Mohtashami, M., Dalby, N. O., Trimble, W. and Knepper, M. (1995) J. Clin. Invest. **96**, 1834–1844
31 Volchuk, A., Mitsumoto, Y., He, L., Liu, Z., Habermann, E., Trimble, W. and Klip, A. (1994) Biochem. J. **304**, 139–145
32 Conner, S., Leaf, D. and Wessel, G. (1997) Mol. Reprod. Dev. **48**, 106–118
33 Schulz, J. R., Wessel, G. M. and Vacquier, V. D. (1997) Dev. Biol. **191**, 80–87

Received 9 April 1998/8 May 1998; accepted 28 May 1998