Characterization of an alternatively spliced isoform of rat vesicle associated membrane protein-2 (VAMP-2)

Robert Mandic, Anson W. Lowe*

Department of Medicine and the Digestive Disease Center, MSLS Building, Rm. P-308, Stanford University, Stanford, CA 94305-5487, USA

Received 1 March 1999; received in revised form 6 April 1999

Abstract VAMPs are vesicle associated membrane proteins that are essential for secretion. A spliced isoform of rat VAMP-2, called VAMP-2B, is characterized in this study. The VAMP-2B transcript is the result of alternative RNA splicing in which an intron is retained. The predicted amino acid sequence of VAMP-2B differs from VAMP-2 at its carboxy-terminal end. Because recent studies have shown that VAMP's carboxy-terminal end influences the protein's sorting, the location of myc-epitope tagged VAMP-2B in PC12 cells was determined. Subcellular fractionation showed colocalization of myc-VAMP-2B and endogenous VAMP-2. Thus alternative RNA splicing does not affect VAMP-2 sorting in PC12 cells.

Key words: Membrane protein; Secretory pathway; RNA splicing; Secretion

1. Introduction

Vesicle associated membrane proteins (VAMPs), also known as synaptobrevins, were initially characterized in synaptic vesicles [1,2] and represent a conserved protein family from yeast to mammals [3–5]. The VAMPs are type II membrane proteins that are essential for the vesicle mediated transport of proteins [3,6,7]. As described in the SNARE hypothesis, VAMPs that are present on transport vesicles will bind to t-SNAREs represented by syntaxin and SNAP-25 on the target membrane [8]. Potential functions proposed for the VAMP protein family include a role in the targeting, docking, or fusion of transport vesicles with the target membrane [7,9–11]. Although initially characterized in synaptic vesicles, the VAMPs have also been detected in non-neural tissues [12–14]. The recent characterization of several new mammalian VAMP isoforms that each occupy a different subcellular compartment provides support for VAMP's role in mediating transport in different intracellular pathways [14].

Several studies have focused on the identification of VAMP domains responsible for targeting the VAMPs to different subcellular compartments. Data supporting the presence of a sorting signal within the cytoplasmic domain of VAMP-2 was obtained when mutations introduced into the protein's cytoplasmic domain disrupted its sorting into a synaptic vesicle compartment in the neuroendocrine cell line, PC12 [15]. Ossig et al. demonstrated that the transmembrane and carboxy-terminal end of the yeast VAMP homologue, Snc1p, may serve as a sorting domain for VAMP homologues in yeast [16].

Alternative RNA splicing has been demonstrated in *Drosophila* and mammalian cells as one means of expanding the diversity of VAMP isoforms [17–19]. The alternative RNA splicings described to date for the VAMPs all affect the carboxy-terminal end of the protein, specifically the domain that putatively extends into the vesicle lumen. We have previously characterized VAMP-1B, an isoform of rat VAMP-1 that is the result of alternative RNA splicing [18]. Recent studies by Isenmann et al. demonstrated that the carboxy-terminal domain affected by the alternative RNA splicing of human VAMP-1 influences the protein's sorting. When epitope-tagged VAMP-1B is expressed in endothelial cells, it is targeted to mitochondria whereas VAMP-1A was targeted to the plasma membrane and endosomes [19].

This study describes the identification and characterization of rat VAMP-2B, an alternatively spliced isoform of VAMP-2. The predicted amino acid sequence of VAMP-2B results in a change at the carboxy-terminal end of the protein.

2. Materials and methods

PC12 and AR42J cells were obtained from ATCC (American Type Culture Collection, Manassas, VA). All materials were obtained from Sigma Chemical Company unless otherwise indicated.

2.1. Antibodies

Hybridomas producing the mouse monoclonal anti-c-myc antibodies (9E10) were purchased from ATCC. Rabbit polyclonal anti-human chromogranin-A antiserum was kindly provided by Dr. Daniel T. O'Connor (University of California, San Diego, CA). Monoclonal anti-synaptophysin antibodies (SVP-38) were purchased from Sigma.

2.2. PCR cloning and sequencing

A PCR approach as described by Frohman [20] was used to amplify cDNAs at the 3′-end (3′-RACE) to search for novel isoforms of VAMP-2. An oligo-dT primer (5′-CCAGTGAGCAGAGTGACGAGGACTCGAGCTCAAGCTTTTTTTTTTTTTTTT-3′) and rat pancreatic poly-A RNA were used to create a cDNA library. The polymerase chain reaction was then performed using a primer representing a highly conserved sequence present in rat VAMP-2 (5′-TGAGGTGGTGGACATCATGAGGGTGAAT-3′) (nt 140–167 [5]) and another based on the oligo-dT primer (5′-CCAGTGAGCAGAGTGACG-3′). A 495 nt PCR product was obtained and the nucleotide sequence was determined. The nucleotide sequence revealed differences from the VAMP-2 sequence that would predict a change in the amino acid sequence at the carboxy-terminal end. Oligonucleotide primers based on the unique sequences were then used to obtain the 5′-end of the cDNA using the 5′-RACE approach [20]. The cDNA sequence of the PCR product derived from the 5′-RACE approach was determined. Finally, oligonucleotides 5′-GGGAGCCAGCGCGAGCGGCCGCCGCTGTCA-3′ (nt 1–30, Fig. 1) and 5′-CTCTACGTGAGTCAGAACTGCCAATAGCA-3′ (nt 548–520, Fig. 1) based on the 5′- and 3′-RACE approaches were used to obtain a single PCR product. The entire cloning process including the produc-

*Corresponding author. Fax: (1) (650) 723-5488.
E-mail: lowe@stanford.edu

Abbreviations: nt, nucleotides; PCR, polymerase chain reaction; VAMP, vesicle associated membrane protein

0014-5793/99/$20.00
PII: S0014-5793(99)00551-7

tion of rat pancreatic cDNA and the generation of PCR products as described above was repeated to ensure reproducibility of the results. All PCR products were initially subcloned into the *Eco*RV site of the pBluescript KS II(+) plasmid vector (Stratagene) [21]. Dideoxy sequencing was performed on both DNA strands as described by Sanger [22] (Sequenase kit, United States Biochemical). Sequencing was also performed at the Stanford University Protein and Nucleic Acid Facility using an automated DNA sequencer (Model 373A, PE Applied Biosystems).

2.3. RNA purification

Poly-A RNA from tissues for the Northern blots and RACE reactions was prepared as described [23]. Total RNA from tissue culture cells was prepared utilizing the RNeasy Midi Kit (Qiagen Inc.) according to the manufacturer's instructions. Four confluent 15 cm dishes of each respective cell line were used for each preparation. The quality of the purified RNA was verified on a formaldehyde gel showing clear 28S and 18S bands in the expected ratio of 2:1.

2.4. Ribonuclease protection assay

The ribonuclease protection assay was performed using the RPA II Kit (Ambion Inc.) according to the manufacturer's instructions. [^{32}P]UTP-labeled antisense RNA was generated from nt 55 to 548 of VAMP-2B, which included the open reading frame and part of the 3′-untranslated region (Fig. 1). An antisense β-actin probe was generated utilizing a template provided by the manufacturer and was used as a positive control.

2.5. Protein immunoblotting

Protein immunoblots were prepared as described by Towbin et al. [24]. Detection was performed using antibodies raised against the different VAMP isoforms or the myc-epitope tag. Buffer containing 5% non-fat milk, PBS, 0.05% Tween 20 was used in all steps as described [25]. Detection of the primary antibodies used goat anti-rabbit or goat anti-mouse antibodies conjugated to horseradish peroxidase (Bio-Rad Laboratories) at a dilution of 1:2000, followed by visualization using enhanced chemiluminescence (ECL, Amersham Corp.) and Kodak XAR5, X-OMAT film.

2.6. Transfection of PC12 cells

Myc-epitope tagged VAMP-2 and -2B constructs were made using the PJ3M vector that was kindly provided by Dr. J. Chernoff (Fox Chase Cancer Center, Philadelphia, PA) [26]. The entire open reading frame for each VAMP isoform was subcloned from the pBluescript KS II+ vector into PJ3M using the restriction enzymes *Eco*RI and *Cla*I. DNA sequencing confirmed the VAMP inserts to be in frame with the sequence encoding the myc-epitope and resulted in the addition of 25 amino acids [NH2-MEQKLISEEDLSRGSPGELEFAT-PA-COOH] at the amino-terminal end. PC12 cells grown to 50% confluence were cotransfected with 1–2 μg of the PJ3M construct along with 0.5 μg of a plasmid containing the neomycin resistance gene using Lipofectamine (Life Technologies, Inc.) according to the manufacturer's instructions. Resistant clones were selected using 0.5 mg/ml of G-418 (Life Technologies Inc.). Positive clones were identified using protein immunoblotting. In addition to the stably transfected cell lines, transiently transfected PC12 and COS-7 cells were also used.

2.7. Subcellular fractionation

Subcellular fractionation was performed as previously described [27]. The cells were grown in a 15 cm plate until nearly confluent, rinsed, and washed off the plate with PBS. The cells were then collected by centrifugation for 5 min at $600\times g$. The cell pellet was resuspended in 0.8 ml homogenization buffer and homogenized with 8 strokes in a custom made ball bearing cell cracker with a clearance of 0.0004 inch (10 μm). The homogenate was centrifuged for 10 min at $700\times g$. 200–400 μl of the supernatant was layered over the following gradients.

Glycerol (velocity) gradient: Sample preparation was performed as described above using a homogenization buffer consisting of 150 mM NaCl, 1 mM EGTA, 1 mM $MgCl_2$, 10 mM HEPES, pH 7.4. A continuous 5–25% glycerol gradient was generated utilizing the Auto-Densi Flow IIC (Buchler Instruments). The gradients were centrifuged in a SW65 Ti rotor (Beckman Instruments) for 30 min at 54 000 rpm, 4°C. 0.5 ml fractions were collected from the top of the tube using the Auto-Densi Flow IIC.

Sucrose (equilibrium density) gradients: The sample preparation was performed as described above using a homogenization buffer consisting of 0.25 M sucrose, 4 mM HEPES pH 7.4 and 1 mM $MgCl_2$. A continuous 0.5–1.7 M sucrose gradient was generated as described for the glycerol gradient. The samples were centrifuged in a SW41 rotor (Beckman Instruments) for 20 h at 25 000 rpm, 4°C, followed by the collection of 1 ml fractions. 45 μl of each fraction was loaded on a 12% SDS polyacrylamide gel and prepared for protein immunoblotting as described above.

3. Results

3.1. Cloning and sequencing of VAMP-2B

Low stringency Northern blots using a probe derived from rat VAMP-2 cDNA suggested the presence of other related VAMP isoforms (data not shown). A PCR based approach produced a product with a nucleotide sequence that differed from VAMP-2 at the 3′-end of the open reading frame. The entire open reading frame of the new isoform, named VAMP-2B, could be obtained in a single polymerase chain reaction from a rat pancreatic cDNA library (Fig. 1, nt 1–548, underlined). Comparisons with the published rat and human VAMP-2 sequence [5,28] revealed that the predicted amino acid sequence for VAMP-2B is identical to VAMP-2 through exon 4, whereupon the protein terminates with 24 novel amino acids at the carboxy-terminal end (Fig. 1). We have previously demonstrated that VAMP-1 isoforms are produced by alternative RNA splicing which results in the retention of an intron and predicts changes at the carboxy-terminal end [18]. Examination of the nucleotide sequence specific for VAMP-2B revealed a potential 5′-donor splice site [29,30]. To determine whether the VAMP-2B specific sequence is derived from a retained intron, PCR analysis of the rat genomic VAMP-2 gene was performed. Oligonucleotide primers derived from the novel sequence of VAMP-2B and exon 5 of the VAMP-2 gene were used to obtain a 597 nt PCR product from rat genomic DNA. Analysis of the nucleotide sequence for known intron/exon boundary sequences revealed that the VAMP-2B specific sequence was indeed a retained intron (Fig. 1, nt 401–1001) [29,30].

3.2. Ribonuclease protection assays

Northern blots of RNA derived from a variety of rat tissues were performed using a VAMP-2B specific probe (data not shown). A 3000 nt transcript is seen in pancreas, heart, kidney, liver, spleen, parotid, and brain. Compared to the previously described 2400 nt transcript for VAMP-2, the observed VAMP-2B transcript was consistent with that of a retained intron of 601 nt. The presence of VAMP-2B transcripts was examined using ribonuclease protection assays of pancreatic and brain RNA (Fig. 2). The ribonuclease protection assay used an antisense probe that is able to distinguish between VAMP-2 and -2B transcripts. Two dominant protected fragments were observed in the pancreas, a 346 nt fragment consistent with the VAMP-2 transcript and a 494 nt fragment consistent with that of VAMP-2B. In contrast, brain RNA produced only a single protected fragment consistent with VAMP-2 (Fig. 2). This demonstrated that the relative amounts of VAMP-2B to VAMP-2 transcripts is significantly higher in the pancreas compared to the brain.

Ribonuclease protection assays were also used to assess the presence of the VAMP-2B transcript in PC12 and AR42J cells. AR42J cells are a rat cell line derived from the acinar

A

```
  1          GGG AGC CAG CGC GAG CGG CCG CCG CTG TCA CTG CCT CTG CCA AGT CCT CTG   51
                         M   S   A   T   A   A   T   V   P   P   A   A   P   A
 52  CCC GCT ACC CCC GCC ATG TCG GCT ACC GCT GCC ACC GTC CCG CCT GCC GCC CCG GCC  108
     G   E   G   G   P   P   A   P   P   P   N   L   T   S   N   R   R   L   Q
109  GGC GAG GGT GGC CCC CCT GCA CCT CCT CCA AAT CTT ACC AGT AAC AGG AGA CTG CAG  165
     Q   T   Q   A   Q   V   D   E   V   V   D   I   M   R   V   N   V   D   K
166  CAG ACC CAG GCC CAG GTG GAT GAG GTG GTG GAC ATC ATG AGG GTG AAT GTG GAC AAG  222
     V   L   E   R   D   Q   K   L   S   E   L   D   D   R   A   D   A   L   Q
223  GTC CTG GAG CGG GAC CAG AAG CTA TCG GAA CTG GAT GAT CGC GCA GAT GCC CTC CAG  279
     A   G   A   S   Q   F   E   T   S   A   A   K   L   K   R   K   Y   W   W
280  GCA GGG GCC TCC CAG TTT GAA ACA AGT GCA GCC AAG CTC AAG CGC AAA TAC TGG TGG  336
     K   N   L   K   M   M   I   I   L   G   V   I   C   A   I   I   L   I   I
337  AAA AAC CTC AAG ATG ATG ATC ATC TTG GGA GTG ATT TGC GCC ATC ATC CTC ATC ATC  393
     I   I   G   E   W   S   R   S   G   Q   G   P   F   P   G   E   V   E   G
394  ATC ATC Ggt gag tgg agc agg agt ggc cag ggc cct ttc cct gga gaa gta gag ggt  450
     F   P   V   G   S   G   L   .
451  ttc cct gtg ggg tcg ggg ctt tga agg tca ttg acc tag ttt tta ctc ttc tac caa  507
508  aac aca ttt tgc tgc tat tgg cag ttc tga ctc acg tag agc ccc aac ttg agt gtt  564
565  ata gcc tta ttt tcc ctt gtt atg ggt agt tct ggg taa gat ttt ggg gat agg aat  621
622  act ggg tag tcc ctg gtg cct ttc tag gaa ccc ttt gag cct aaa agc tca gtc tgg  678
679  ggt ttc taa aac tgg aga gta gaa aac agg tca ggc tga ggg tct tga act cca atc  735
736  aca gga tac act tac tcc act att tga aag cta tcg tca cat gcc agt gcc tta aag  792
793  gtt tga tac ccc ttt gtg cga tcg tgg cgt tta agg gat ctc aat acc cac ttg tgc  849
850  agt ccc ttt gaa gaa act gag gca gag agg gaa ggg atg tca gtg ggg gac tgg gga  906
907  agt ttg gga aca ggg cca ggc ctg ata cac tgc tgt ctt tct ctt cct tcc ctt ccc  963
                                                 (V)  Y   F   S   T   .
964  cca ccc cct acc cct cct cat gcc ctc tct ctc cac agT TTA CTT CAG CAC TTA AGT 1020
1021 CCC TGA GGA GTC TGC CCT GCC T                                             1042
```

B

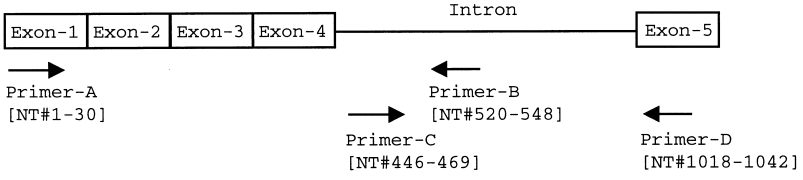

Fig. 1. The VAMP-2B cDNA clone represented by nt 1–548 (A, underlined) was derived from a pancreatic cDNA library using primers A and B (B) and includes the whole open reading frame. The remaining part of the sequence was derived from a rat kidney genomic DNA library using primers C and D (B) and is represented by nt 446–1042. Differences in the amino acid sequence compared to VAMP-2 are on the carboxy-terminal end where the VAMP-2 cDNA sequence normally encoded by exon-5 is interrupted by a novel sequence that replaces the carboxy-terminal five amino acids of VAMP-2 (VYFST) with 24 amino acids (GEWSRSGQGPFPGEVEGFPVGSGL). The first and last two nucleotides of the intron sequence are shown in bold. The intron sequence is depicted in lower case letters. The sequence has been deposited in the EMBL sequence database under accession number AJ133104.

cell of the pancreas [31]. PC12 cells are a neuroendocrine cell line derived from the rat adrenal medulla and have been commonly used to study VAMP sorting. RNA antisense probes representing VAMP-2B resulted in protected fragments consistent with endogenous VAMP-2 and VAMP-2B in AR42J and PC12 cells. Similar to the pancreas, ribonuclease protected transcripts consistent with VAMP-2 were much more abundant than VAMP-2B. In contrast to the brain, PC12 cells exhibited protected fragments consistent with both VAMP-2 isoforms (Fig. 2B).

3.3. Subcellular distribution of VAMPs-2 and -2B

The subcellular distribution of VAMP-2 and -2B was examined to determine whether differences in sorting existed between the alternatively spliced isoforms. PC12 cells were used because these cells have often been used as a model system to examine VAMP sorting [15,32–34]. In addition, ribonuclease protection assays also established the presence of the VAMP-2B transcript in PC12 cells. Thus it is likely that PC12 cells would possess the necessary factors for sorting VAMP-2B. A myc-epitope was appended to the amino-terminal end of VAMP-2B and expressed in PC12 cells using both stable and transient transfections. The subcellular distribution of myc-VAMP-2B was compared to that of endogenous VAMP-2 using equilibrium and velocity sedimentation approaches for subcellular fractionation. Both of these approaches were previously used to assess the distribution of VAMPs in endosomes, secretory granules, and synaptic vesicles [27,32]. Equilibrium gradients using sucrose are able to resolve the endosomal and synaptic vesicle compartments from the secretory granules. Myc-VAMP-2B colocalized with the endosomal and synaptic vesicle fractions. No difference in the distribution of myc-VAMP-2B from endogenous VAMP-2 was seen (Fig. 3A). Glycerol velocity gradients were also employed to obtain better resolution between endosomes and synaptic vesicles. Again, myc-VAMP-2B exhibited the same distribution as endogenous VAMP-2 (Fig. 3B). The resolution of the sucrose and glycerol gradients was validated when the expected distribution for chromogranin A (secretory granules), transferrin receptor (endosomes), and synaptophysin (synaptic vesicles) was obtained (Fig. 3A,B).

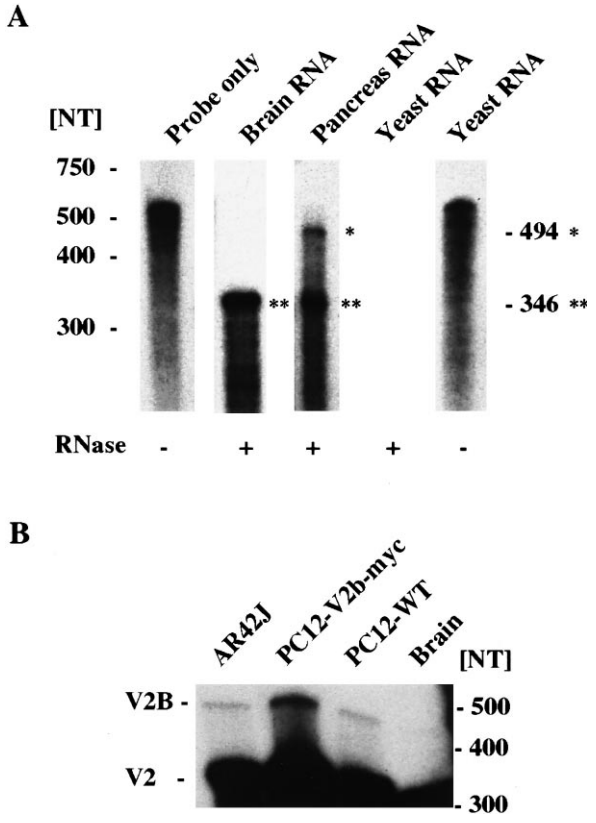

Fig. 2. A: Ribonuclease protection assays using a complementary antisense RNA derived from a VAMP-2B clone (Fig. 1, nt 55–548) were performed with brain and pancreatic poly-A RNA. A 346 nt protected fragment expected for the VAMP-2 transcript is present in brain and pancreas (**). A 494 nt protected fragment consistent with VAMP-2B is seen only in the pancreas (*). Sequences derived from the vector account for the larger size of the probe. 0.3 μg of brain and 10 μg of pancreatic poly-A RNA were used. B: Ribonuclease protection assays of RNA derived from AR42J cells, PC12 cells transfected with myc-VAMP-2B cDNA, wild-type PC12 cells, and rat brain were performed using the VAMP-2B specific antisense probe. The V2B protected fragment in PC12-V2B-myc cells represents the endogenous VAMP-2B and the transfected myc-VAMP-2B. The mobility of the protected fragments are VAMP-2 = V2; VAMP-2B = V2B.

4. Discussion

This study characterizes VAMP-2B, an isoform of VAMP-2 that is generated by alternative RNA splicing. Alternative RNA splicing resulting in a transcript with a retained intron often occurs at the 3′-end and has also been described for bovine growth hormone [35], effector cell protease receptor-1 [36], myelin oligodendrocyte glycoprotein [37] and tau [38]. Intron retention was also recently reported for VAMP-1B, where a transcript with a retained intron was expressed in the spleen, liver, kidney, pancreas, and parotid, whereas in the brain and heart, only the fully spliced transcript of VAMP-1 is present [18]. In contrast to VAMP-1 and -1B, in which only one of the isoforms was expressed in each tissue, ribonuclease protection assays demonstrated the presence of VAMP-2 and -2B in the AR42J and PC12 cell lines and the pancreas.

The carboxy-terminal end of VAMP-2B is predicted to result in the replacement of the five uncharged carboxy-terminal amino acids with 24 amino acids. The additional amino acids provided by VAMP-2B includes four glutamic acid residues and one arginine, which would produce a significant change in the charge and structure of the carboxy-terminal end. In comparison, VAMP-1B differs from VAMP-1A in the shortening of the hydrophobic domain by four amino acids and the addition of two basic residues.

Because the previously published work by Ossig et al. suggested a role for VAMP sorting at the carboxy-terminal end, we examined whether alternative RNA splicing of VAMP-2s influences the protein's sorting. In addition, studies published by Isenmann et al. [19] while this work was in progress provided further support that VAMP-2B may be sorted differently from VAMP-2. Because untransfected PC12 cells express VAMP-2B RNA as determined by the ribonuclease protection assays, they were chosen to study the effects of the alternative RNA splicing. When myc-VAMP-2B was expressed from PC12 cells, it colocalized with the endogenous VAMP-2 isoform on equilibrium and velocity gradients. Controls for these experiments indicated that a significant difference in the subcellular distribution of VAMP-2 and -2B should have been detected. Clearly, the sorting of myc-VAMP-2B to a compartment as large as mitochondria as seen for VAMP-1B would have been detected. These results indicate that the VAMP-2B transcript does not encode a protein that is sorted differently from VAMP-2 in PC12 cells.

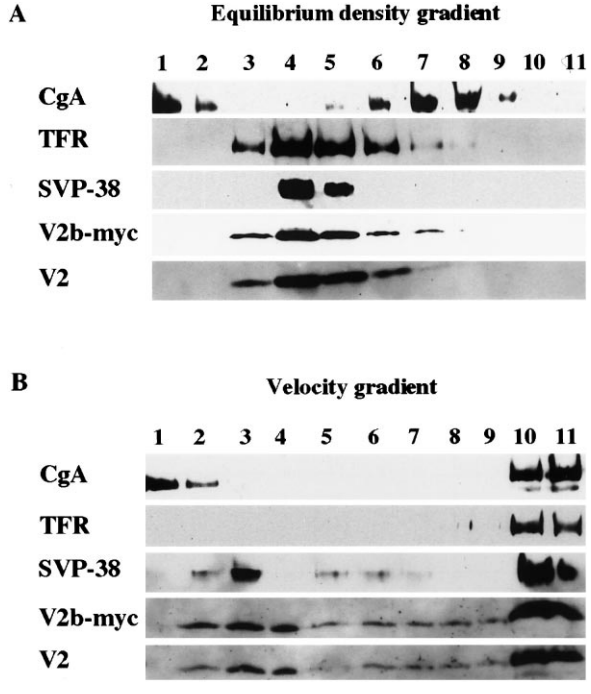

Fig. 3. Protein immunoblots of subcellular fractions derived from equilibrium sucrose density (A) and glycerol velocity gradients (B). Colocalization of the transfected tagged VAMP-2B isoform (V2B-myc) with the endogenous VAMP-2 protein (V2) is seen. Chromogranin A (CgA), synaptophysin (SVP-38) and transferrin receptor (TFR). The indicated fractions represent the lowest (1) to the highest (11) density.

Acknowledgements: The authors appreciate the contributions of Shirley M.E. Wong and Erin Wheatley to the early phases of this work. A.W.L. was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grant DK-43294 and Digestive Disease Center Grant DK-38707.

References

[1] Baumert, M., Take, K., Hartinger, J., Burger, P.M., von Mollard, G.F., Maycox, P.R., De Camilli, P. and Jahn, R. (1990) J. Cell Biol. 110, 1285–1294.
[2] Trimble, W.S., Cowan, D.M. and Scheller, R.H. (1988) Proc. Natl. Acad. Sci. USA 85, 4538–4542.
[3] Protopopov, V., Govindan, B., Novick, P. and Gerst, J.E. (1993) Cell 74, 855–861.
[4] Sudhof, T.C., Baumert, M., Perin, M.S. and Jahn, R. (1989) Neuron 2, 1475–1481.
[5] Elferink, L.A., Trimble, W.S. and Scheller, R.H. (1989) J. Biol. Chem. 264, 11061–11064.
[6] Schiavo, G., Benfenati, F., Poulain, B., Rossetto, O., Polverino de Laureto, P., DasGupta, B.R. and Montecucco, C. (1992) Nature 359, 832–835.
[7] Nichols, B.J., Ungermann, C., Pelham, H.R.B., Wickner, W.T. and Haas, A. (1997) Nature 387, 199–202.
[8] Sutton, R.B., Fasshauer, D., Jahn, R. and Brunger, A.T. (1998) Nature 395, 347–353.
[9] Sollner, T., Whiteheart, S.W., Brunner, M., Erdjument-Bromage, H., Geromanos, S., Tempst, P. and Rothman, J.E. (1993) Nature 362, 318–324.
[10] Ungermann, C., Sato, K. and Wickner, W. (1998) Nature 396, 543–548.
[11] Weber, T., Zemelman, B.V., McNew, J.A., Westermann, B., Gmachl, M., Parlati, F., Sollner, T.H. and Rothman, J.E. (1998) Cell 92, 759–772.
[12] Rossetto, O., Gorza, L., Schiavo, G., Schiavo, N., Scheller, R.H. and Montecucco, C. (1996) J. Cell Biol. 132, 167–179.
[13] Braun, J.E.A., Fritz, B., Wong, S.M.E. and Lowe, A.W. (1994) J. Biol. Chem. 269, 5328–5335.
[14] Advani, R.J., Bae, H.-R., Bock, J.B., Chao, D.S., Doung, Y.-C., Prekeris, R., Yoo, J.-S. and Scheller, R.H. (1998) J. Biol. Chem. 273, 10317–10324.
[15] Grote, E., Hao, J.C., Bennett, M.K. and Kelly, R.B. (1995) Cell 81, 581–589.
[16] Ossig, R., Laufer, W., Schmitt, H.D. and Gallwitz, D. (1995) EMBO J. 14, 3645–3653.
[17] Chin, A.C., Burgess, R.W., Wong, B.R., Schwarz, T.L. and Scheller, R.H. (1993) Gene 131, 175–181.
[18] Mandic, R., Trimble, W.S. and Lowe, A.W. (1997) Gene 199, 173–179.
[19] Isenmann, S., Khew-Goodall, Y., Gamble, J., Vadas, M. and Wattenberg, B.W. (1998) Mol. Biol. Cell 9, 1649–1660.
[20] Frohman, M.A. (1993) Methods Enzymol. 218, 340–356.
[21] Marchuk, D., Drumm, M., Saulino, A. and Collins, F.S. (1991) Nucleic Acids Res. 19, 1154.
[22] Sanger, F. and Coulson, R. (1975) J. Mol. Biol. 94, 441–448.
[23] Han, J.H., Stratowa, C. and Rutter, W.J. (1987) Biochemistry 26, 1617–1625.
[24] Towbin, H., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350–4354.
[25] Batteiger, B., Newhall, W.J. and Jones, R.B. (1982) J. Immunol. Methods 55, 297–307.
[26] Chernoff, J. and Sells, M.A. (1995) Gene 152, 187–189.
[27] Linstedt, A.D. and Kelly, R.B. (1991) Neuron 7, 309–317.
[28] Archer, B.T., Ozcelik, T., Jahn, R., Francke, U. and Sudhof, T.C. (1990) J. Biol. Chem. 265, 17267–17273.
[29] Mount, S.M. (1982) Nucleic Acids Res. 10, 459–472.
[30] Shapiro, M.B. and Senapathy, P. (1987) Nucleic Acids Res. 15, 7155–7175.
[31] Longnecker, D.S., Lilja, H.S., French, J., Kuhlmann, E. and Noll, W. (1979) Cancer Lett. 7, 197–202.
[32] Chilcote, T.J., Galli, T., Mundigl, O., Edelmann, L., McPherson, P.S., Takei, K. and De Camilli, P. (1995) J. Cell Biol. 129, 219–231.
[33] Papini, E., Rossetto, O. and Cutler, D.F. (1995) J. Biol. Chem. 270, 1332–1336.
[34] Grote, E. and Kelly, R.B. (1996) J. Cell Biol. 132, 537–547.
[35] Hampson, K.H. and Rottman, R.M. (1987) Proc. Natl. Acad. Sci. USA 84, 2673–2677.
[36] Altieri, D.C. (1994) Biochemistry 33, 13848–13855.
[37] Hilton, A.A., Slavin, A.J., Hilton, D.J. and Bernard, C.C.A. (1995) J. Neurochem. 65, 309–318.
[38] Sadot, E., Marx, R., Barg, J., Behar, L. and Ginzburg, I. (1994) J. Mol. Biol. 241, 325–331.