Mouse VAP33 is associated with the endoplasmic reticulum and microtubules

P. A. Skehel*, R. Fabian-Fine†, and E. R. Kandel§

*Division of Neurophysiology, National Institute for Medical Research, The Ridgeway, Mill Hill, London, NW7 1AA, United Kingdom; †Department of Biological Sciences, The Open University, Milton Keynes, MK7 6AA United Kingdom; and §Center for Neurobiology and Behavior, Howard Hughes Medical Institute, Columbia University, New York, New York 10032

Contributed by E. R. Kandel, November 9, 1999

VAMP/synaptobrevin is a synaptic vesicle protein that is essential for neurotransmitter release. Intracellular injection of antibodies against the Aplysia californica VAMP/synaptobrevin-binding protein ApVAP33 inhibited evoked excitatory postsynaptic potentials (EPSPs) in cultured cells, suggesting that this association may regulate the function of VAMP/synaptobrevin. We have identified and characterized a mouse homologue of ApVAP33, mVAP33. The overall domain structure of the proteins is conserved, and they have similar biochemical properties. mVAP33 mRNA is detectable in all mouse tissues examined, in contrast to the more restricted expression seen in the mouse. We analyzed the cellular distribution of mVAP33 protein in brain slices and cultured cortical cells by light and electron microscopy. Although present at higher levels in neurons, immunoreactivity was detected throughout both neurons and glia in a reticular pattern similar to that of endoplasmic reticulum-resident proteins. mVAP33 does not colocalize with VAMP/synaptobrevin at synaptic structures, but expression overlaps with lower levels of VAMP/synaptobrevin in the soma. Ultrastructural analysis revealed mVAP33 associated with microtubules and intracellular vesicles of heterogeneous size. In primary neuronal cultures, large aggregates of mVAP33 are also detected in short filamentous structures, which are occasionally associated with intracellular membranes. There is no evidence for accumulation of mVAP33 on synaptic vesicles or at the plasma membrane. These data suggest that mVAP33 is an endoplasmic-reticulum–resident protein that associates with components of the cytoskeleton. Any functional interaction between mVAP33 and VAMP/synaptobrevin, therefore, most likely involves the delivery of components to synaptic terminals rather than a direct participation in synaptic vesicle exocytosis.

Eukaryotic cells contain elaborate and dynamic systems of membrane-organellar and transport vesicles. The trafficking and integrity of these structures rely on a complex series of protein–protein and protein–lipid interactions that are generally conserved in all eukaryotes (1–3). Neurons contain specialized organelles, the synaptic vesicles, that facilitate the sustained, rapid, and coordinated release of neurotransmitter into the synaptic cleft (4, 5). The SNAP receptor (SNARE) hypothesis was developed to describe the mechanism whereby a synaptic vesicle interacts with and subsequently fuses with a target membrane. This complex formed between the v-SNARE synaptobrevin and the t-SNAREs syntaxin and SNAP-25 accumulate on different membranes, and a number of additional proteins have been identified that are capable of interacting with synaptobrevin, syntaxin, and SNAP-25 (10–19). These interactions may function to regulate the formation of the SNARE complex or the intracellular targeting of its constituents. Alternatively, such interactions may be part of regulatory or biosynthetic mechanisms quite distinct from vesicle exocytosis.

ApVAP33 (Aplysia VAMP/synaptobrevin-associated protein of 33 kDa) was originally identified by its ability to interact with VAMP/synaptobrevin in a yeast two-hybrid assay (18). In Aplysia sensory-motorneuron cocultures, it was shown that presynaptic injection of ApVAP33-specific antisera inhibited postsynaptically measured excitatory postsynaptic potentials (EPSPs) suggesting that an interaction between ApVAP33 and VAMP/synaptobrevin was required for efficient synaptic transmission in this culture. Homologous genes have been identified in humans, rats, and Saccharomyces cerevisiae (20–22). To analyze the function of the protein in a more genetically amenable vertebrate context, we have characterized a mouse homologue of ApVAP33.

Experimental Procedures

Cloning of Mouse VAP33. The similarity between ApVAP33 and a human expressed sequence tag was used to design two degenerate oligonucleotides for a PCR generating a DNA fragment of about 210 bp from mouse brain cDNA: 5'-GAGTTAAGAT-TCAA(GATC)GG(GATC)CC(GATC)TTCA-3' and 5'-CTTGTCTTTTCTCCTTGGATCATA-3'. This DNA was then used as a probe to screen a placmid-based mouse brain cDNA library by hybridization (CLONTECH).

RNA Isolation and Northern Blot Hybridization Analysis. Samples of the indicated tissues were dissected from adult male C57BL6 mice. Total RNA was then purified by using the TRIzol Reagent (GIBCO/BRL) as directed by the manufacturer. RNA (10 μg) from each tissue was denatured and separated on a 1% agarose gel, transferred to Hybond N+ (Amersham Pharmacia), and hybridized to DNA probes radiolabeled with the Prime-It II reagents as directed (Stratagene). Filters were washed twice in 2× SSC at room temperature and twice in 0.5× SSC at 65°C and then were analyzed by autoradiography.

Antisera Production. The coding sequence of mVAP33 was cloned into the bacterial expression vector pET-30a(+) as an EcoR1/ 

Abbreviations: EPSP, excitatory postsynaptic potential; ER, endoplasmic reticulum.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AF157497).

To whom reprint requests should be addressed. E-mail: pskehel@nimr.mrc.ac.uk.

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NotI fragment generated by a Pfu-based PCR with the mutagenic primer 5'-CGAATTCACCATGGCTAGCCAGAACAA-3', and the T7 primer on a cDNA clone in pCDNA1 (CLON-TECH). The His-tagged recombinant protein was expressed in Escherichia coli BL21-DE3 and purified by Ni-nitriotropic acid (NTA)-agarose affinity chromatography (Qiagen, Chatsworth, CA). Rabbit antisera were prepared by Murex Bioservices, Dartford, U.K.

**Protein Fractionation and Western Blot Analysis.** Two brains from adult male mice were homogenized with a dounce Tecflon/glass homogenizer in 20 ml of ice-cold 20 mM Hepes, pH 7.4/320 mM sucrose/Complete protease inhibitors (Roche Molecular Biochemicals). The homogenate was then centrifuged at 1800 × g for 10 min at 4°C in a JA-20 Beckman rotor. The supernatant was removed, leaving a pellet, P1, and centrifuged again under the same conditions to remove traces of P1. P2 and S2 were then removed, leaving a pellet, P1, and centrifuged under the same conditions to remove traces of P1. P2 and S2 were then re-suspended in 5 ml of cold 20 mM Hepes, pH 7.4/100 mM KCl. Aliquots (1 ml) were taken, and Triton X-100 was added to 1% (vol/vol). After incubation at 37°C or 4°C for 1h, the samples were centrifuged at 356,000 × g in a TL100.2 at 4°C for 30 min. to generate P2–Triton X-100 and S2–Triton X-100.

Triton X-114 extracts were produced from P2 by using 1.5% detergent as described by Bordier (23).

Equivalent amounts of extract were analyzed by Western blotting. mVAP33 and syntaxin (AutogenBiochlear ABB36)-specific antisera were used at dilutions of 1:2000 and 1:5000, respectively. Bound antibody was detected using horseradish peroxidase-conjugated donkey anti-rabbit or anti-mouse serum (Jackson ImmunoResearch) and enhanced chemiluminescence peroxidase-conjugated donkey anti-rabbit IgG antibody conjugated to 1.0-nm gold particles (GAR1; BB International, Cardiiff, U.K.; 1:80 in IB). After rinsing in PBS (5 times for 15 min per rinse) gold particles were enlarged with a silver enhancement solution (SEKL15; BB International). For this, the preparations were washed in distilled water (twice for 5 min each) and incubated for 6 min in silver enhancement solution in the dark at room temperature. Preparations were then washed (twice for 5 min each time in distilled water; twice for 10 min each time in PBS) and postfixed in 0.5% OsO4/PBS for 6 min at room temperature. After subsequent rinsing in PBS, preparations were dehydrated in a graded series of acetone solutions before embedding in Araldite (1030; Agar Scientific Ltd.). The resin was polymerized in embedding moulds at 60°C overnight. Ultrathin sections (75 nm) were cut with a Reichert Ultracut and collected on pioloform-coated single-slot nickel grids. Sections were contrasted with aqueous 1.5% uranyl acetate and Reynolds’ lead citrate before examination in a JEOL JEM-100CX transmission electron microscope.

**Electron-Microscopic Immunolabeling of Organotypic Hippocampal Tissue Slices.** The method used for electron-microscopic immunolabeling was a modified version of that described by Stirling and Graff (25). Hippocampal tissue slices (11 wk old) were fixed in 3% paraformaldehyde/0.3% glutaraldehyde in PBS for 20 min. After rinsing in PBS (twice for 15 min each) preparations were postfixed in 0.5% OsO4, washed, dehydrated, embedded, and cut as described above. The ultrathin sections were then etched in 2% sodium m-periodate (S-1878; Sigma) for 25 min. After thorough rinsing in distilled water, preparations were incubated in 1% glycine/PBS (30 min), rinsed briefly in PBS, and heated in small dishes for 10 min in phosphate-citrate buffer (0.1 M; pH 6.0) at 95°C in a small oven to unmask the epitopes. Preparations were allowed to cool for 15 min, rinsed in PBS, and preincubated in IB for 30 min at room temperature. Incubation with the primary antibody was at a dilution of 1:5000 in IB overnight at 4°C. After thorough washing in PBS and preincubation in IB (30 min), preparations were incubated for 4 h with a secondary antibody anti-rabbit IgG antibody conjugated to 10-nm gold particles (Sigma G-7402, 1:80 in IB). After final washing in PBS (three times for 5 min each), the preparations were postfixed in 0.1% glutaraldehyde, rinsed in distilled water (5 min), contrasted in uranyl acetate and lead citrate, and examined as described above.

**Results**

**Cloning and Structure of mVAP33.** Comparison of the ApVAP33 cDNA to the National Center for Biotechnology Information database by using a BLAST search identified a human expressed sequence tag that showed significant homology to the 3' portion of the *Aplysia* cDNA. This sequence was used to design degenerate oligonucleotides for a PCR generating a DNA probe from mouse brain cDNA. This DNA fragment was then used to screen a mouse brain cDNA library by hybridization. Three clones were identified, and the full sequence of the largest insert was submitted to the database (accession no. AF157497). The 1.6-kilobase cDNA contains a long ORF encoding 242 amino acids of predicted molecular mass 27.3 kDa and pI 8.79. Although there are two possible ATG initiation codons, only the down-
by 1% (vol/vol) Triton X-100 at 37°C (33). This extraction, a C-terminal hydrophobic domain that may anchor the protein to membranes.

In nematodes the major sperm protein assumes the function of actin in the motile spermatozoa (27) and aggregates into membrane-associated filaments by a process that is thought to underlie amoeba motility (28). The crystal structure of the major sperm protein has been solved, and residues critical for homophilic interaction have been identified by mutagenesis (29, 30). These critical residues are poorly conserved in the VAP33 protein and the human and rat sequences that were recently identified (refs. 20 and 21; Fig. 1).

The similarity of the biochemical characteristics of ApVAP33 and mVAP33 and the primary structure homology of the two proteins indicate that mVAP33 is also a C-terminally anchored integral membrane protein.

Cellular Distribution of mVAP33. Indirect immunofluorescence microscopy was used to analyze the cellular distribution of mVAP33 in primary cultures from rat neocortex (Fig. 4). Both neurons and glia express mVAP33 immunoreactivity. In glia, the immunoreactivity accumulates in the perinuclear area and extends throughout the cytoplasm in a diffuse reticular pattern.

Expression of mVAP33. By Northern blot analysis, we found mVAP33 to be expressed in all mouse tissues examined (Fig. 2). This is in contrast to A. californica, in which gene expression was detected primarily in the central nervous system (18).

Antisera were raised in rabbits to bacterially expressed mVAP33. A major immunoreactive protein of 33 kDa was identified by immunoblot analysis of mouse brain homogenates (Fig. 3A i). These results suggest that mVAP33 also interacts with the cytoskeleton, possibly via the major sperm protein-homologous domain, because this protein forms cytoskeletal-like filaments (28). When solubilized with Triton X-114, mVAP33 partitions entirely in the detergent phase. Notably, after Triton X-114 extraction mVAP33 appears to form an SDS-stable complex of 66 kDa. This may be the result of a homotypic interaction between hydrophobic C termini, as suggested by Nishimura et al. (20), perhaps involving the conserved GXXXG motif.

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Fig. 1. Comparison of deduced VAP33 protein sequences from mouse, human, rat, and Aplysia. The regions of the major sperm protein homology and the predicted coiled-coil domains are indicated by thin and thick underscores, respectively. Identical and similar residues are shaded. Asterisks mark the positions of two conserved glycines that may function in coiled-coil interactions between transmembrane domains. mVAP33 GenBank accession no. AF157497.

Fig. 2. Tissue distribution of mVAP33 mRNA expression. Total RNA from the tissues indicated was analyzed by Northern blot hybridization. Parallel blots were hybridized with radiolabeled mVAP33 or mouse β-actin cDNAs. mVAP33 transcripts of ~2 kilobases are detectable in all tissues analyzed.
neurons also show a perinuclear accumulation. In addition, the signal extends throughout most of the cell processes. This distribution is distinct from that of VAMP/synaptobrevin (Fig. 4). Processes of all calibers appear to be labeled. A similar distribution is seen for calnexin (Fig. 4; ref. 34). More detailed inspection of neuronal processes reveals mVAP33 immunoreactivity in a punctate distribution that may reflect larger complexes or aggregates of protein. These results suggest that mVAP33 is present in the endoplasmic reticulum (ER) membrane. This is consistent with the relatively short hydrophobic C-terminal domain of the protein because transmembrane domains of 17 amino acids have been shown to retain membrane proteins in the ER/Golgi (32). The yeast protein SCS2 shows extensive identity with mVAP33 and has been localized to the ER (22). This report also analyzed the topology of the protein in relation to the ER membrane, demonstrating that the amino terminus of the protein is cytoplasmic. ApVAP33 was originally identified by its ability to interact with VAMP/synaptobrevin in a yeast two-hybrid assay, and the mammalian protein has a similar activity (21; data not shown). However, unlike VAMP/synaptobrevin, mVAP33 is not enriched at synaptic sites (Fig. 4). There is no indication of immunoreactivity at the plasma membrane. Moreover, although mVAP33 often appears microscopically as aggregates, these structures do not colocalize with VAMP/synaptobrevin (Fig. 4). This suggests that mVAP33 is retained in the ER and that any interaction with VAMP/synaptobrevin is transient and before this protein’s accumulation on mature synaptic vesicles.

Ultrastructural Analysis of mVAP33. Immunogold electron microscopic analysis of organotypic hippocampal slice cultures and dissociated cortical cell cultures identified mVAP33-specific immunoreactivity associated with three types of structures. First, in organotypic cultures, there was a low level of immunoreactivity that was mainly associated with microtubules (Fig. 5A). There were multiple gold particles often clustered on a single microtube (Fig. 5B and C). Second, multiple sites of immunoreactivity were also seen associated with the membranes of vesicular structures (Fig. 5D and E). Third, occasionally the
aggregates of mVAP33 immunoreactivity were occasionally detected in short filamentous structures that were also frequently localized with membranes (Fig. 6 E and F).

Discussion

Since the initial isolation of *Aplysia* VAP33, homologous proteins have been described from humans, rats, yeasts, and now mice. In addition there are similar sequences in the databases for *Caenorhabditis elegans* and *Arabidopsis*. In contrast to the restricted expression pattern seen in *A. californica* (18), the VAP33 homologues are more ubiquitously expressed, which suggests a general rather than a neuron-specific function.

Ultrastructural and biochemical analysis of mVAP33 indicates several features of the molecule that can be related to its primary structure. mVAP33 purifies as an integral membrane protein that is present on intracellular membranes in a reticular distribution. In addition to its membrane localization, mVAP33 also seems to associate with microtubules, and can form larger aggregates that may be associated with cytoskeletal elements. The major sperm protein forms a cytoskeletal structure in the amoeboid spermatozoa (28), and although the residues critical for major sperm protein oligomerization are not well conserved in the homologous N-terminal domain of mVAP33, this region could contribute to this aggregated distribution. Whereas coiled coils are a common structural motif found in some microtubule-binding proteins (35, 36), mVAP33 contains no primary structural features with homology to known microtubule-binding motifs. Another type II integral membrane protein, p63, was recently shown to interact directly with microtubules (37).

Microinjection of ApVAP33-specific antisera into the presynaptic cell of a sensorimotor-cell coculture leads to a reduction in the evoked EPSP when measured several hours later (18). The gross structural similarity between syntaxin and ApVAP33 originally suggested that both molecules might have similar functions in mediating synaptic-vesicle interaction with the presynaptic plasma membrane. This is unlikely to be the case for mVAP33. Although there may be additional members of a VAP33 gene family, our immunocytochemical and ultrastructural analysis suggests that mVAP33 associates mainly with ER membranes and microtubules, consistent with a function in intracellular transport, because vesicle and organelle trafficking requires interaction with the microtubule network (1). Thus, the simplest idea for the function of mVAP33 might be that it has a role in trafficking or chaperoning vesicular components such as VAMP/synaptobrevin through the ER and on to the synaptic terminals. A similar function has been suggested for another VAMP/synaptobrevin-binding protein BAP31 (12). Based on this view, intracellular injection of mVAP33 antisera could reduce evoked EPSP by inhibiting vesicular transport, leading to a depletion of limiting components at the synaptic terminal. In this case, mVAP33 would have a general function in intracellular transport. Unlike VAMP/synaptobrevin, mVAP33 does not accumulate at synaptic sites. Any functional interaction that may exist between the two molecules, therefore, is probably within the ER. VAMP/synaptobrevin is inserted into the ER membrane post-translationally by a saturable ATP-dependent mechanism (38). It is possible that mVAP33 might function in the process directly or act as a chaperonelike protein, facilitating VAMP/synaptobrevin’s passage through the ER system. In either case, inhibiting the function of mVAP33 by antibody injection could lead to reductions in the levels of VAMP/synaptobrevin on synaptic vesicles, resulting in a reduction in the efficiency of exocytosis. This would be consistent with mini-EPSP analysis of the inhibited *Aplysia* cocultures, which showed no overall reduction in amplitude or frequency of spontaneous or osmotically evoked release, but a 50% decrease in electrically evoked EPSP (P.A.S., M. Giarardi, and E.R.K., unpublished work).
Another possibility is suggested by the knock-out of a VAP33 homologue, SCS2, in yeasts, that causes a perturbation of an ER-to-nucleus signaling system. Nikawa et al. isolated SCS2 as a high copy suppressor of an inositol auxotrophic mutation (39) and subsequently showed that deletion of the gene resulted in inositol auxotrophy (22). Notably, deletion of SCS2 did not result in a detectable secretion phenotype. This may indicate that the inhibition of evoked EPSP in the *Aplysia* coeruleus is a secondary consequence of disrupting a process that is distinct from synaptic vesicle and plasma membrane fusion.

In yeasts, the inositol biosynthesis pathway shares much of the same regulatory pathway with the Unfolded Protein Response (UPR; 40). The central regulator of the yeast UPR, IRE1, has recently been identified in humans, indicating that a similar regulatory pathway with the Unfolded Protein Response is present in higher eukaryotes (41). The UPR has recently been identified in humans, indicating that a similar signaling system is present in higher eukaryotes (41). It has been suggested that the UPR and inositol biosynthesis pathways are coregulated to coordinate the protein modification and chaperone capacity of the ER with its size. Kagiwada et al. have proposed that SCS2 may act as a transcription factor signaling a heat shock response by proteolytic activation (22). Although a possible proteolyzed derivative of mVAP33 was detected by Western blot (Fig. 3), there was no evidence for nuclear localization of mVAP33 immunoreactivity by fluorescence or electron microscopy. The phenotype of the SCS2 mutation was more pronounced at higher temperatures, and it was argued that this may reflect an involvement with the heat shock response (22). A structural homologue of VAP33 has been identified in *Arabidopsis* after its induction by osmotic shock (43). Although in this case the protein appears to be localized to the plasma membrane, it is consistent with VAP33 proteins having some function in membrane metabolism.

The structure of the ER is maintained by interactions between the ER membrane and the microtubules. Perhaps mVAP33, which faces the cytoplasm from the ER membrane, may have a signaling activity to coordinate the levels of type II membrane proteins with the size or capacity of the ER. A striking feature from the ultrastructural localization analysis of mVAP33 is its association with vesicular membranes and microtubules, frequently at a point of close juxtaposition between these structures. The hydrophobic C terminus of mVAP33 most likely mediates a direct interaction with the membrane, but the nature of the microtubule association is not clear. In this way, in addition to a possible signaling activity, mVAP33 may have a more direct function in maintaining or regulating distribution of the ER or ER-like organelles within the cell.

We thank Drs. Vincent O’Connor, Tim Bliss, Michael Stewart, and Dusan Bartsch for their help during the course of this work and the preparation of the manuscript.

References