The SNARE Vti1a-β Is Localized to Small Synaptic Vesicles and Participates in a Novel SNARE Complex

Wolfram Antonin,[2] Dietmar Riedel,[2] and Gabriele Fischer von Mollard[1]

[1]*Zentrum Biochemie und Molekulare Zellbiologie, Abteilung Biochemie II, Universität Göttingen, 37073 Göttingen, Germany, and* [2]*Abteilung Neurobiologie, Max-Planck Institut für Biophysikalische Chemie, 37077 Göttingen, Germany*

Specific soluble *N*-ethylmaleimide-sensitive factor attachment protein (SNAP) receptor (SNARE) proteins are required for different membrane transport steps. The SNARE Vti1a has been colocalized with Golgi markers and Vti1b with Golgi and the *trans*-Golgi network or endosomal markers in fibroblast cell lines. Here we study the distribution of Vti1a and Vti1b in brain. Vti1b was found in synaptic vesicles but was not enriched in this organelle. A brain-specific splice variant of Vti1a was identified that had an insertion of seven amino acid residues next to the putative SNARE-interacting helix. This Vti1a-β was enriched in small synaptic vesicles and clathrin-coated vesicles isolated from nerve terminals. Vti1a-β also copurified with the synaptic vesicle R-SNARE synaptobrevin during immunoisolation of synaptic vesicles and endosomes. Therefore, both synaptobrevin and Vti1a-β are integral parts of synaptic vesicles throughout their life cycle. Vti1a-β was part of a SNARE complex in nerve terminals, which bound *N*-ethylmaleimide-sensitive factor and α-SNAP. This SNARE complex was different from the exocytic SNARE complex because Vti1a-β was not coimmunoprecipitated with syntaxin 1 or SNAP-25. These data suggest that Vti1a-β does not function in exocytosis but in a separate SNARE complex in a membrane fusion step during recycling or biogenesis of synaptic vesicles.

Key words: SNARE; synaptic vesicle; clathrin-coated vesicle; endosome; Vti1; nerve terminal; membrane traffic

Transport between different organelles requires complex formation between specific members of the *N*-ethylmaleimide-sensitive factor attachment protein (SNAP) receptor (SNARE) protein family on both transport vesicles (v-SNARE) and target membranes (t-SNARE) (Rothman, 1994). SNARE proteins have common structural features. Most of them contain a C-terminal membrane anchor. They interact with each other via predicted coiled coil domains close to the membrane. These SNARE proteins are conserved in evolution, numbering 21 family members in yeast and so far over 35 in mammals (Jahn and Sudhof, 1999) The SNARE complex required for exocytosis of synaptic vesicles has been studied extensively. It consists of the synaptic vesicle v-SNARE synaptobrevin and the plasma membrane t-SNAREs syntaxin 1 and SNAP-25. Synaptobrevin and syntaxin contribute one helix and SNAP-25 two helices to a parallel four-helix bundle (Sutton et al., 1998). A conserved arginine in synaptobrevin interacts with the three glutamines from the t-SNAREs in a central layer of the SNARE complex. The SNARE complex required for exocytosis in yeast also consists of a four-helix bundle with interactions between one arginine and three glutamine containing helices (Rossi et al., 1997), suggesting that this is a common feature of SNARE complexes (Fasshauer et al., 1998b). Therefore, SNARE proteins have been reclassified as R-SNAREs (arginine) or Q-SNAREs (glutamine). All t-SNAREs are Q-SNAREs, whereas v-SNAREs are either R-SNAREs or Q-SNAREs.

The composition of SNARE complexes required for intracellular traffic is less clear. A single SNARE can be part of different complexes. Yeast Vti1p interacts with the *cis*-Golgi t-SNARE Sed5p in retrograde traffic to the *cis*-Golgi (Lupashin et al., 1997), with the endosomal t-SNARE Pep12p in traffic from the Golgi to the endosome (Fischer von Mollard et al., 1997) and with the vacuolar t-SNARE Vam3p in biosynthetic transport pathways to the vacuole/lysosome (Fischer von Mollard and Stevens, 1999) and in homotypic vacuolar fusion (Ungermann et al., 1999). Vti1p binds to the t-SNAREs Tlg1p (early endosome) and Tlg2p [*trans*-Golgi network (TGN)] (Holthuis et al., 1998). Two proteins related to yeast Vti1p were identified in mammals (Lupashin et al., 1997; Fischer von Mollard and Stevens, 1998). Mouse Vti1a and Vti1b share only 30% amino acid identity and have a similar degree of homology with the yeast protein (33 and 27%). Tagged Vti1b/Vti1-rp1 overlapped with Golgi and TGN proteins (Advani et al., 1998). Vti1-rp1/Vti1b was found in endosomes according to unpublished results mentioned by Xu et al. (1998). Vti1-rp2/Vti1a was localized to the Golgi apparatus. Vti1a could be coimmunoprecipitated with the *cis*-Golgi t-SNARE syntaxin 5 and the TGN syntaxin 6. Antibodies against Vti1a block intra-Golgi traffic (Xu et al., 1998).

Here we describe a brain-specific splice variant of Vti1a that is localized to synaptic vesicles. This Vti1a-β is not part of the exocytic SNARE complex but may function in a novel SNARE complex required for synaptic vesicle recycling or biogenesis.

MATERIALS AND METHODS

Materials. Reagents were used from the following sources: enzymes for DNA manipulations from New England Biolabs (Beverly, MA); secondary antibodies from Jackson ImmunoResearch (West Grove, PA); Eupergit C1Z methacrylate microbeads from Röhm Pharma (Darmstadt, Germany); glutathione (GSH)-Sepharose 6B and cyanogen bromide (CNBr)–Sepharose 4B (Amer-sham Pharmacia Biotech, Uppsala, Sweden); and Ni-NTA agarose from Qiagen (Hilden, Germany). All other reagents were purchased from Sigma (Deisenhofen, Germany). Plasmid manipulations were performed in the *Echerichia coli* strain XL1Blue.

N-ethylmaleimide-sensitive factor (NSF) and α-SNAP in pQE-9 plasmids encoding for His6-tagged fusion proteins were kindly provided by S. Whiteheart and J. E. Rothman (Sloan-Kettering Center, New York, NY).

Cloning of rat Vti1a and Vti1a-β. Sequence data from rat expressed sequence tags (ESTs) (GenBank accession numbers AI010508, AI227646, and AI555622) were used to assemble the sequence of rat Vti1a. Oligonucleotide primers annealing a few base pairs upstream of the start codon

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Correspondence should be addressed to Gabriele Fischer von Mollard, Zentrum Biochemie und Molekulare Zellbiologie, Abteilung Biochemie II, Universität Göttingen, Heinrich-Düker Weg 12, 37073 Göttingen, Germany. E-mail: mollard@uni-bc2.gwdg.de.

and downstream of the stop codon of rat Vti1a (GGG GTA CCG GAG CTG CCA TGT CAG and CGG GAT CCG CCT CAG TGT CCT CTG AC) were used to PCR amplify DNA from rat lung and rat cerebellum cDNA λ ZAPII libraries (Stratagene, La Jolla, CA). The PCR products derived from cerebellum cDNA were reamplified using the same primers and cloned into pGEM-Teasy (Promega, Madison, WI). The nucleotide sequences of inserts of three clones with slightly smaller and three clones with slightly larger inserts were determined. The smaller clones encoded Vti1a and the larger clones Vti1a-β with an insertion of 21 base pairs.

Reverse transcription-PCR. Total RNA was isolated from different rat tissues (cerebellum, cortex, hippocampus, lung, liver, kidney, and spleen; 200 mg each) using TRIZOL reagent (Life Technologies, Rockville, MD) following the instructions of the manufacturer. RNA (5 μg) was used for an RT-PCR reaction using the Superscript II kit (Life Technologies) following the instructions of the manufacturer in a total volume of 20 μl. Ten percent of the products of the RT-PCR were used as a template for the specific PCRs. The three oligonucleotides gagaaccagagggcacatc (Vti1a, annealing with codons 110–116 and therefore only with Vti1a), ttgataaaattacgtgaggag (Vti1a-β, annealing with the vti1-β specific codons 115–121), and gagaagaagcaaatggttg (MB7, annealing with codons 33–38) were used as forward primers each in combination with the oligonucleotide gggatcctagcggttttggatgattcttc (rVbsol, annealing with codons 187–180) as a reverse primer.

Protein purification. Recombinant NSF and α-SNAP were purified as described previously (Hanson et al., 1995). Recombinant glutathione *S*-transferase (GST)-rat Vti1a [amino acids (aa) 1–114] and GST-rat Vti1a (aa 115–192) were purified using GSH-Sepharose 6B following the instructions of the manufacturer.

Antibodies. Antisera were raised in rabbits against a fusion protein containing GST and the amino acids 1–207 of mouse Vti1b (pBK9) or amino acids 1–187 of mouse Vti1a (pBK10) purified from *E. coli*. The antisera were affinity purified with Affigel 10 columns with covalently bound 6His-mVti1b (amino acids 1–207, pBK38) or 6His-mVti1a (amino acids 1–187, pBK39), respectively, for immunofluorescence. Vti1a antiserum specific for the N-terminal or C-terminal region of the protein was affinity-purified using recombinant GST-rat Vti1a (aa 1–114) or GST-rat Vti1a (aa 115–192), respectively, coupled to CNBr–Sepharose 4B. The affinity-purified antisera were specific for their targets [the C-terminal antiserum did not recognize GST-Vti1a (aa 1–114) and the N-terminal antiserum did not detect GST-Vti1a (aa 115–192)].

The following antibodies were described previously: synaptophysin (monoclonal antibody, Cl7.2) (Jahn et al., 1985); rab5 (monoclonal antibody, Cl 621.3) (Fischer von Mollard et al., 1994); rab5 (rabbit antiserum R6) and rab3a (monoclonal antibody, Cl42.2) (Matteoli et al., 1991); SNAP-25 (Cl71.2) (Bruns et al., 1997); and synaptobrevin (monoclonal antibody, Cl69.1). The following antibodies were kind gifts: syntaxin 1 (monoclonal antibody HPC-1; provided by Dr. C. Barnstable, New Haven, CT) (Barnstable et al., 1985); and Sec61α (rabbit serum; provided by Dr. E. Hartmann, Göttingen, Germany). Commercial sources were used for the following antibodies: secretory carrier membrane protein (SCAMP) (rabbit serum; Synaptic Systems, Göttingen, Germany); and syntaxin 6 (monoclonal antibody; Transduction Laboratories, Lexington, KY).

Immunofluorescence. Adult female Sprague Dawley rats were anesthetized, perfused, and post-fixed as described previously (Mugnaini and Dahl, 1983), with modifications. Briefly, a rat was perfused transcardially with ice-cold 0.9% NaCl, followed by fixative (4% formaline, 0.9% NaCl, and 0.5% $ZnCl_2$). The brain was dissected and immersed in the same fixative overnight at 4°C. After rinse in 0.1 M Tris-HCl, pH 7.2, the tissue was incubated overnight in 20% sucrose containing 0.1 M Tris-HCl, pH 7.2, and then sectioned on a cryostat at 8 μm. The sections were mounted on poly-L-lysine-coated glass slides and incubated in PBS containing 3% goat serum and 0.3% Triton X-100 (GSDB) for 30 min. The sections were incubated overnight with the respective antibodies, washed with PBS, and incubated for 1 hr at room temperature with secondary antibodies (Cy2-conjugated goat anti-mouse antibody and Cy3-conjugated goat anti-rabbit antibody (Jackson ImmunoResearch) in GSDB. After washing with PBS, the sections were coverslipped with mounting solution (Dako, Glostrup, Denmark) and analyzed with a confocal microscope (LSM-410-invert; Zeiss, Göttingen, Germany).

Culturing of neurons from the hippocampi of neonatal rats (Sprague Dawley) was done as described previously (Rosenmund et al., 1995). After 3 weeks in culture, the cells were processed for immunofluorescence as described previously (Hannah et al., 1998) using Triton X-100 as detergent. The staining was analyzed with a confocal microscope (LSM-410-invert; Zeiss).

Electron microscopy. For immunogold labeling, purified synaptic vesicles (as described below) were adsorbed to glow discharged nickel grids. Thereafter, labeling with diluted respective antibodies [synaptophsin antiserum (G 95), 1:100, Vti1a affinity-purified serum, 1:50)] and 10 nm goat anti-rabbit IgG gold conjugates diluted at 1:100 in 1% BSA in phosphate buffer were performed. The samples were post-fixed for 10 min with 2% glutharaldehyde in phosphate buffer, washed with H_2O, rinsed with 3 drops of 1% uranyl acetate, and immediately dried with filter paper.

Isolation of organelles. Small synaptic vesicles were purified as described previously (Huttner et al., 1983). Clathrin-coated vesicles were purified from rat brain synaptosomes as described previously (Maycox et al., 1992).

For immunoisolation of organelles, monoclonal antibodies Cl 69.1 (anti-synaptobrevin), Cl 42.2 (anti-rab3a) and Cl 621.3 (anti-rab5) were covalently coupled to Eupergit C1Z methacrylate microbeads as described previously (Burger et al., 1989). Rat brain was homogenized in 25 ml of homogenization buffer [320 mM sucrose, 5 mM HEPES, pH 7.4, 1 mM EDTA, 0.1 mM GTPγS, and protease inhibitors (10 μg/ml soybean trypsin inhibitor, 1 μg/ml pepstatin, 11 μg/ml benzamidine, 1 μg/ml antipain, 1 μg/ml leupeptin, and 0.1 mM phenylmethylsulfonyl fluoride)] using a glass Teflon homogenizer (10 strokes, 1000 rpm). Postnuclear supernatant (PNS) was generated by centrifugation at 1000 × g_{av} for 10 min. PNS was further centrifuged for 30 min at 50.000 × g_{av} to remove myelin increasing the unspecific binding to the beads. The resulting supernatant (800 μg of protein) was incubated in 800 μl of homogenization buffer with 20 μl of the appropriate beads for 1 hr at 4°C. The incubation mixture was layered on top of a sucrose cushion (0.5 ml, 0.8 M) and centrifuged for 5 min at 4600 × g_{av}. The supernatants were centrifuged for 30 min at 200,000 × g_{av} at 4°C using a Beckman TLA120.2 rotor to sediment nonbound membranes. The bead pellets were washed five times with PBS. Aliquots of each sample as well as the starting PNS were analyzed by SDS-PAGE and immunoblotting.

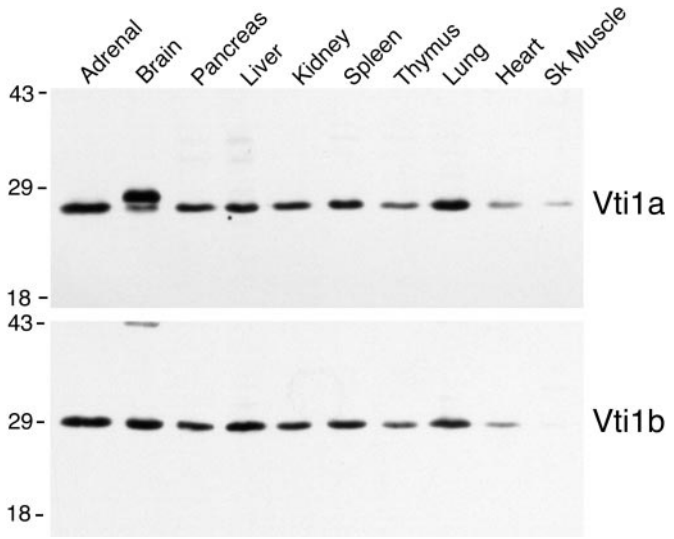

Figure 1. Vti1a and Vti1b were expressed in all tissues. Homogenates were prepared from the indicated mouse tissues, and 20 μg separated by SDS-PAGE Western blots were stained with antisera against Vti1a or Vti1b. The Vti1a antiserum recognized a ubiquitous protein of 27 kDa and an additional slightly larger brain-specific band. Vti1b antiserum bound to a single band of 29 kDa.

Immunoprecipitation. A synaptosomal fraction (P2) was solubilized in extraction buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, and 1% Triton X-100) at a final protein concentration of 0.5 mg/ml for 1 hr at 4°C. Lysates were clarified by centrifugation at 200,000 × *g* for 10 min. After transfer of the supernatant to a fresh tube, immunoprecipitations were conducted for 2 hr at 4°C with monoclonal antibodies against syntaxin 6, syntaxin 1 (HPC-1), SNAP-25 (71.2), synaptobrevin (69.1), or affinity-purified antibody specific for Vti1a. Antibodies were bound to Protein A-Sepharose beads (Amersham Pharmacia Biotech) for 60 min, sedimented, and washed eight times with extraction buffer. The supernatants were precipitated (Wessel and Flügge, 1984). The immunoprecipitates and 30% of the precipitated supernatants were analyzed by SDS-PAGE and immunoblotting using the above antibodies. In the case of the detection of SNAP-25, an anti-mouse Fc antibody was used as a secondary antibody to exclude a cross-reactivity with the light chain of the antibodies used for immunoprecipitation.

Binding of NSF and α-SNAP to SNARE complexes. Detection of a 20 S complex containing Vti1a-β was done as described previously (Söllner et al., 1993a), using the LP2 fraction from rat (high-speed membrane fraction of lysed synaptosomes) as starting material instead of bovine brain membranes. In brief, the LP2 fraction (0.5 mg/ml final protein concentration) was solubilized in 20 mM HEPES, pH 7.4, 100 mM KCl, 1 mM EDTA, and 0.5% Triton X-100 at 4°C for 1 hr. Insoluble material was removed by centrifugation at 200,000 × g_{av} for 10 min. The supernatant (1 ml) was incubated with recombinant NSF (0.3 μM) and α-SNAP (0.9 μM) for 1 hr at 4°C and then layered on top of a 10–35% (w/v) glycerol gradient containing the same buffer as above and subjected to centrifugation for 19 hr in an SW41 rotor (Beckman) at 40,000 rpm at 4°C. Fractions (1 ml) were collected, and the proteins were precipitated by trichloroacetic acids, separated by SDS-PAGE, and electrotransferred to nitrocellulose. The blots were immunodecorated with the indicated antibodies. In a control reaction, NSF and α-SNAP were omitted.

SNARE complex disassembly reaction. LP2 fractions (high-speed membrane fraction of lysed synaptosomes, 0.5 mg/ml final concentration of protein) were preincubated with 3 μM NSF, 9 μM α-SNAP, 3 mM $MgCl_2$,

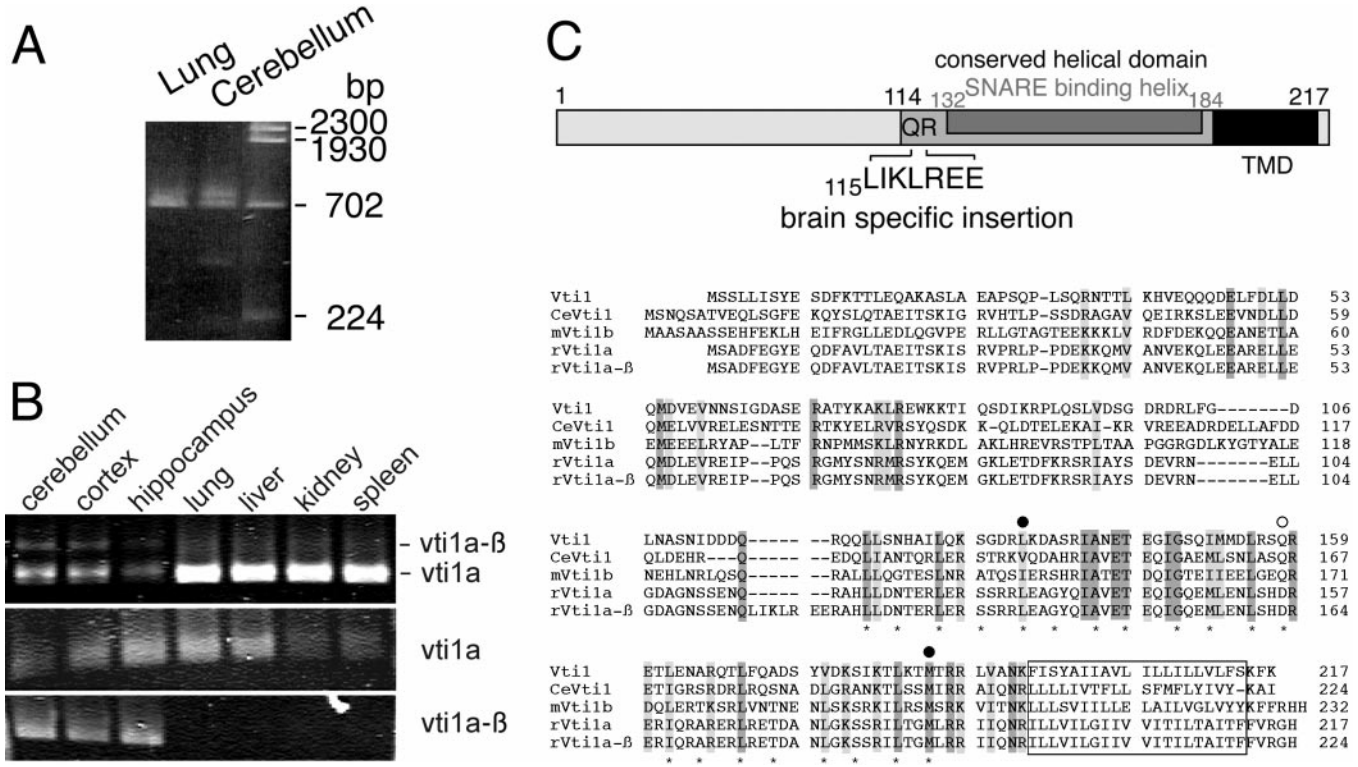

Figure 2. The brain-specific splice variant Vti1a-β had an insertion of seven amino acid residues. *A*, A single band was PCR-amplified from a lung cDNA library, a double band from a cerebellum cDNA library using primers specific for Vti1a. The slightly larger band encoded Vti1a-β with a insertion of seven amino acids after Q114. *B*, Expression pattern of Vti1a and Vti1a-β in different tissues. RT-PCRs were performed using primer pairs amplifying both Vti1a and Vti1a-β (expected sizes, 511 and 490 bp; *top*), Vti1a only (forward primer annealing with codons 110–116, 265 bp; *middle*), or Vti1a-β only (forward primer annealing with Vti1a-β-specific codons 115–121, 253 bp; *bottom*). Vti1a was amplified from all tissues examined. Vti1a-β was amplified from the neuronal tissues cerebellum, cortex, and hippocampus, but not from lung, liver, kidney, or spleen. *C*, Alignment of *Saccharomyces cerevisiae* Vti1p, the C-terminal part of a predicted *C. elegans* protein (GenBank accession number CAB16506), mouse Vti1b, rat Vti1a (GenBank accession number AF262221), and rat Vti1a-β (GenBank accession number AF262222). *Filled circles* indicate the beginning and end of the predicted SNARE-interacting helix (SNARE motif). The *open circle* marks the position of the conserved glutamine or aspartate residue in layer 0, and *asterisks* indicate hydrophobic positions in the heptade repeats.

and 3 mM ATP in 50 mM HEPES, pH 7.4, for 10 min at 30°C. Then, trypsin was added to a final concentration of 0.05 mg/ml, and the samples were incubated for another 15 min at 30°C. Reaction was stopped by adding soybean trypsin inhibitor and phenylmethylsulfonyl fluoride to a final concentration of 100 μg/ml and 1 mM, respectively. SDS sample buffer was added, and the samples were heated immediately for 5 min at 100°C. As a control, the reaction was either performed in the absence of NSF and α-SNAP or the ATPase activity of NSF was abolished by replacing ATP with 3 mM ATPγS or $MgCl_2$ with 10 mM EDTA, respectively.

RESULTS

Identification of a brain-specific Vti1a splice variant

For the characterization of Vti1a and Vti1b proteins, we generated polyclonal antibodies in rabbits using purified recombinant fusion proteins as antigens (see Materials and Methods for details). Both antisera reacted with single bands of 27 (Vti1a) and 29 (Vti1b) kDa, respectively, close to the predicted molecular weight of the proteins. A tissue survey by immunoblotting of homogenate extracts revealed that both Vti1a and Vti1b are widely distributed in all tissues examined (Fig. 1). In brain extracts, the Vti1a-specific antiserum recognized an additional band with a slightly lower mobility that was not observed in other tissues. To determine whether this higher molecular weight band is produced from a splice variant of the Vti1a mRNA, the coding sequences of Vti1a were amplified from lung and cerebellum rat cDNA libraries (Fig. 2*A*). A single band was amplified from the lung cDNA library. In contrast, two bands, one band of slightly lower mobility than the PCR product from lung, were amplified from the cerebellum

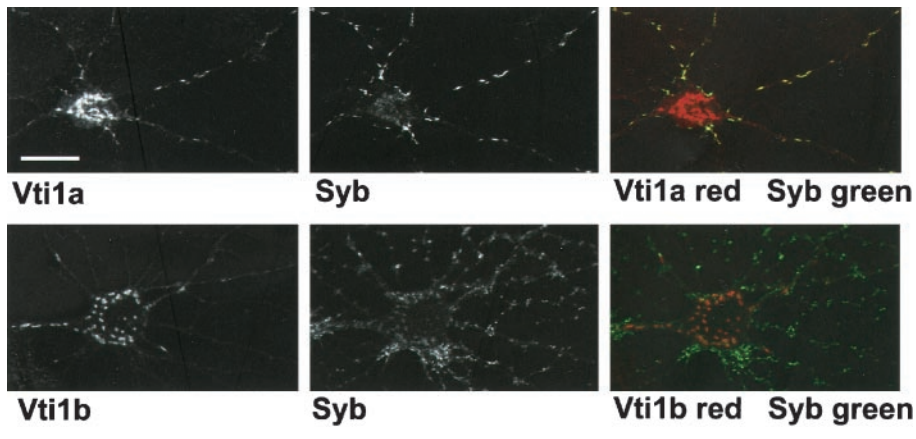

Figure 3. Vti1a localized to the cell body as well as to nerve terminals of hippocampal neurons. Cultured hippocampal neurons were double stained for Vti1a or Vti1b and synaptobrevin. Vti1a and Vti1b were found in the cell body. In addition, Vti1a colocalized with synaptobrevin in nerve terminals. Scale bar, 20 μM.

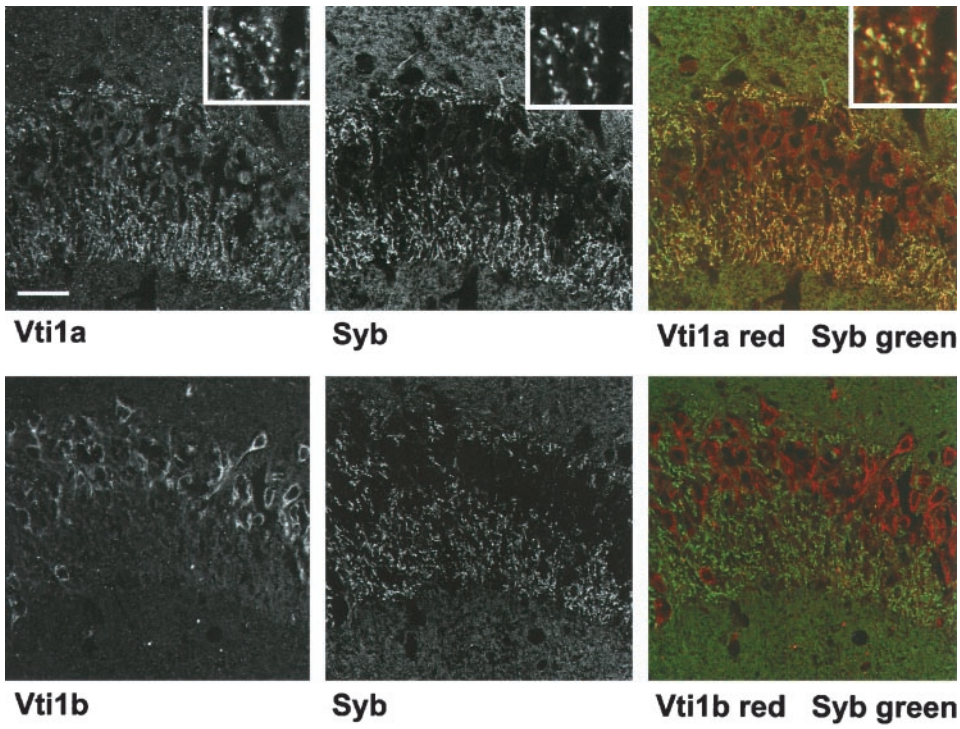

Figure 4. Vti1a localized to the cell body as well as to mossy fiber terminals in hippocampal sections. Rat hippocampal sections were double stained for Vti1a or Vti1b and synaptobrevin. Vti1a and Vti1b were found in the cell bodies of pyramidal cells. Vti1a also colocalized with synaptobrevin in mossy fiber nerve terminals. The *insets* in the *top panels* show a threefold magnification of an area from the mossy fiber nerve terminals. Scale bar, 30 μM.

cDNA. Both bands were cloned into plasmids, and the inserts were sequenced. The *top band* corresponded to rat Vti1a, except for an insertion of 21 nucleotides. This sequence encoded the brain-specific splice variant Vti1a-β with the insertion of the amino acid sequence LIKLREE C-terminal of residue 114. Residue 114 is located at the beginning of a domain, which is conserved among the Vti1 proteins from different species and is predicted to form an α-helix (Fig. 2*C*). Alignment with the different SNAREs predicts that the SNARE-interacting helix (SNARE motif) of Vti1a starts at residue 132. The *bottom band* encoded the rat Vti1a.

To further analyze the expression patterns of both splice variants, RT-PCRs were performed with RNAs isolated from different rat tissues. Primer pairs were designed to amplify both splice variants (Fig. 2*B*, *top*), Vti1a only (*middle*), or Vtia-β only (*bottom*). Vti1a was amplified from all tissues examined. Vti1a-β was expressed in the neuronal tissues cerebellum, cortex, and hippocampus, but not in lung, liver, kidney, or spleen. These data confirm that Vti1a-β is a brain-specific splice variant.

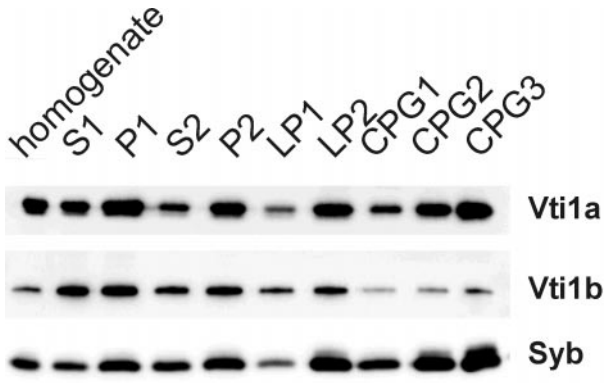

Figure 5. Vti1a-β copurified with synaptic vesicles. Synaptosomes (*P2*) were isolated from rat brain homogenate (*H*). The synaptosomes were osmotically lysed and separated into a low-speed membrane fraction (*LP1*) containing synaptic plasma membranes and a high-speed pellet (*LP2*) with synaptic vesicles. The synaptic vesicles were further purified by sucrose density gradient centrifugation and chromatography on a CPG column yielding the highly enriched fraction CPG3. Vti1a-β copurified with the synaptic vesicle marker synaptobrevin (*Syb*). Vti1b was present in synaptic vesicles but was not enriched compared with homogenate.

Mouse and rat Vti1a, as well as a human Vti1a assembled from the EST database, have an aspartate residue at position 156 instead of the conserved glutamine found in the center of the SNARE helix in other Q-SNAREs and all other Vti1 proteins. Still, an aspartate residue would be able to form a strong ionic interaction with an arginine residue in the center of the SNARE motif. Rat and mouse Vti1a were 96% identical in their amino acid sequence. Conserved exchanges were found in nine amino acid residues (D/E, R/K, A/S, and A/T). The C-terminal part of a predicted *Caenorhabditis elegans* protein (GenBank accession number CAB16506) had a high degree of homology with mouse Vti1a (41% amino acid identity) and a lower degree of homology with mouse Vti1b (25% amino acid identity).

Vti1a localized to cell bodies and nerve terminals in neurons

In previous studies, Vti1a was localized to the Golgi apparatus of fibroblasts (Xu et al., 1998). The localization of Vti1b is less clear because it was found to colocalize with either Golgi and TGN (Advani et al., 1998) or with endosomal markers (Xu et al., 1998). To investigate the localization of both proteins in neurons, we performed indirect immunofluorescence on both cultured hippocampal neurons and hippocampal tissue sections. In cultured neurons, Vti1b was localized to large cisternae in the cell body and to a few structures in processes close to the cell bodies (Fig. 3). No overlap was found between the staining of Vti1b and of synaptobrevin 2, an R-SNARE specific for synaptic vesicles. In contrast, the staining pattern for Vti1a was different. Vti1a was also found in the perinuclear region of the cell body, but Vti1a and Vti1b stained different structures. An additional pool of Vti1a was observed in the processes and overlapped there with synaptobrevin in the nerve terminals. To further analyze the subcellular distribution, hippocampal sections were stained (Fig. 4). Again, Vti1b antiserum stained the cell bodies of pyramidal cells but not the nerve terminals of the mossy fibers. The staining for Vti1b and synaptobrevin did not overlap. Vti1a and synaptobrevin clearly colocalized in the large nerve terminals of the mossy fibers. Vti1a was also found in the cell bodies of pyramidal cells. These data indicate that a subfraction of Vti1a was localized to nerve terminals.

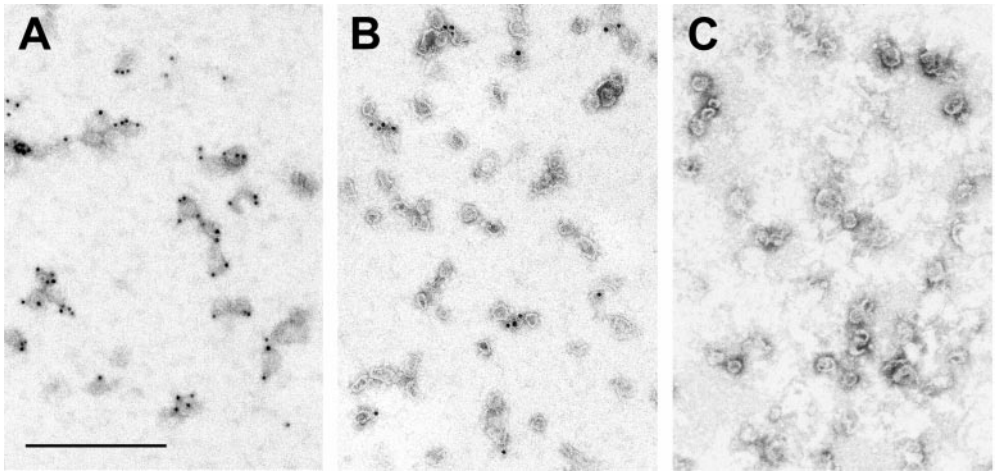

Figure 6. Vti1a was localized to synaptic vesicles using immunogold electron microscopy. The CPG3 fraction was adsorbed to coated grids and incubated with antisera against the synaptic vesicle marker synaptophysin (*A*), against Vti1a (*B*), or without antiserum (*C*) followed by 10 nm goat anti-rabbit IgG gold conjugates and negative staining. Scale bar, 500 nm.

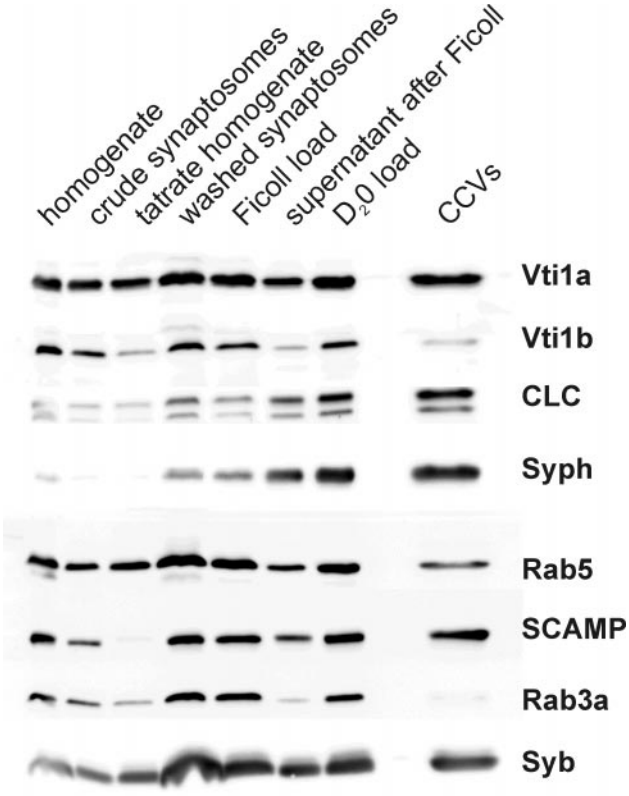

Figure 7. Vti1a-β copurified with clathrin-coated vesicles isolated from nerve terminals. Clathrin-coated vesicles (*CCVs*) were isolated from synaptosomes with brain homogenate as the starting material. Highly enriched clathrin-coated vesicles were isolated from the high-speed pellet of lysed synaptosomes (Ficoll load) via the fractions Ficoll SN and D_2O load. Vti1a-β copurified in parallel with the synaptic vesicle markers synaptobrevin (*Syb*), synaptophysin (*Syp*), and SCAMP in clathrin-coated vesicles. Clathrin light chain (*CLC*) was highly enriched. Very low amounts of Vti1b were found in clathrin-coated vesicles.

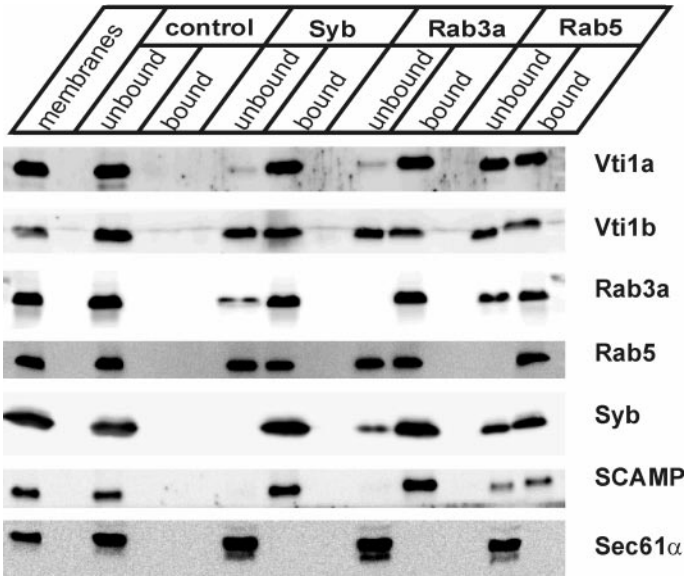

Figure 8. Vti1a-β was found on immunoisolated synaptic vesicles and endosomes. A 50,000 $\times$ *g* supernatant of rat brain homogenate was incubated with antibodies against synaptobrevin (*Syb*), rab3a, or rab5 coupled to Eupergit beads. Glycine was coupled to control beads. The bead fractions were washed and unbound, and bound fractions were separated by SDS-PAGE and analyzed by immunoblotting for different markers. Most Vti1a-β-containing organelles could be isolated with synaptobrevin and rab3a beads. A large fraction of Vti1a-β was found on rab5-containing organelles. Vti1b could also be immunoisolated with synaptobrevin, rab3a, and rab5 beads but to a smaller extend than Vti1a-β. Control beads did not bind any proteins, and the endoplasmic reticulum protein Sec61α was not bound to the immunobeads, indicating that the immunoisolations of organelles were specific.

Vti1a-β was enriched in synaptic vesicles

Next, we wanted to distinguish between Vti1a and the splice variant Vti1a-β and localize them to different compartments within the nerve terminal by subcellular fractionation and immunoblotting. First, synaptic vesicles were purified according to Huttner et al. (1983). Synaptic vesicles (CPG3) were isolated from brain homogenate (H) via the fractions synaptosomes (P2) and high-speed pellet of lysed synaptosomes (LP2) (Fig. 5). Vti1a-β copurified together with synaptic vesicles as indicated by the marker synaptobrevin in the fractions P2, LP2, and CPG3. The lower molecular weight ubiquitous Vti1a and the slightly larger brain-specific Vti1a-β were both present in early fractions of the purification, such as H and P1. In contrast, only Vti1a-β was found in the purified fractions LP2 and CPG3. These data indicate that Vti1a-β was enriched in synaptic vesicles.

Vti1b was present throughout the purification but was not enriched in the CPG3 fraction. By comparing immunoblots of purified synaptic vesicles and known amounts of recombinant Vti1b and Vti1a, it was estimated that synaptic vesicles contain at least 10 times less Vti1b than Vti1a-β (data not shown).

To confirm that Vti1a-β localized to synaptic vesicles, the CPG3 fraction was analyzed by immunogold electron microscopy. Vti1a antiserum specifically stained synaptic vesicles (Fig. 6*B*). The labeling intensities were low because the antisera were used at high dilutions to avoid background staining (Fig. 6*C*). The antiserum against the abundant synaptic vesicle protein synaptophysin gave a stronger staining (Fig. 6*A*). Only small structures representing

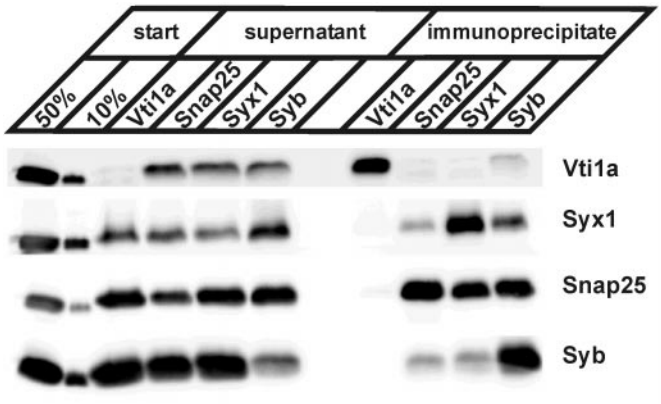

Figure 9. Vti1a-β was not found in the exocytic SNARE complex. SNARE complexes were isolated from Triton X-100 extracts of synaptosomes using antisera against Vti1a, SNAP-25, syntaxin 1 (*Syx1*), or synaptobrevin (*Syb*). Vti1a antiserum did not coimmunoprecipitate syntaxin 1, SNAP-25, or synaptobrevin. SNAP-25, syntaxin 1, and synaptobrevin coimmunoprecipitated as a SNARE complex.

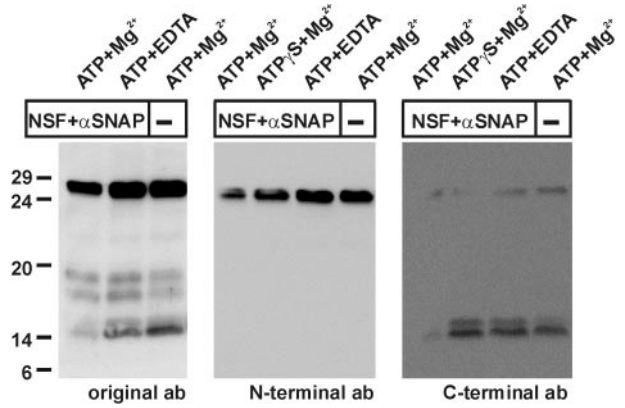

Figure 11. A C-terminal 14 kDa fragment of Vti1a-β with the SNARE motif was protease-protected under conditions of SNARE complex assembly but not disassembly. Detergent extracts from lysed synaptosomes (high-speed membrane fraction LP2) were incubated with NSF and α-SNAP plus ATP + Mg^{2+} (disassembly of SNARE complexes), ATPγS + Mg^{2+}, or ATP + EDTA (inhibition of disassembly). SNARE complexes remained assembled without addition of NSF and α-SNAP. Fractions were incubated with trypsin and separated by SDS-PAGE. Immunoblots were developed with antisera against Vti1a (*left*), against the N-terminal half of Vti1a (*middle*), or against the C-terminal half of Vti1a (*right*).

synaptic vesicles were decorated by Vti1a antiserum in the crude LP2 fraction; larger membranes were not labeled (data not shown). These data confirm that Vti1a was specifically localized to synaptic vesicles.

Vti1a-β was enriched in clathrin-coated vesicles

Next, we wanted to examine whether Vti1a-β is present on the synaptic vesicle membrane throughout the synaptic vesicle recycling pathway. The synaptic vesicle membrane is incorporated into the plasma membrane upon exocytosis and endocytosed via clathrin-coated vesicles. Synaptic vesicles are reformed either by uncoating of these clathrin-coated vesicles or via an endosomal intermediate (Hannah et al., 1999). Clathrin-coated vesicles were isolated from synaptosomes (Maycox et al., 1992). Clathrin light chain and the synaptic vesicle proteins synaptophysin and synaptobrevin were used as marker proteins for the enrichment of clathrin-coated vesicles (Fig. 7). Vti1a-β coenriched in parallel with synaptophysin and synaptobrevin, indicating that Vti1a-β was localized to clathrin-coated vesicles. Synaptophysin was less enriched than clathrin light chain because the major pool of synaptophysin is associated with synaptic vesicles. In contrast, less Vti1b was present on purified clathrin-coated vesicles compared with the starting material. Therefore, Vti1b was only a minor component of clathrin-coated vesicles in the nerve terminal. As shown previously (Maycox et al., 1992; Fischer von Mollard et al., 1994), rab3a and rab5 were deriched in clathrin-coated vesicles compared with the starting material of the last purification step (D_2O load).

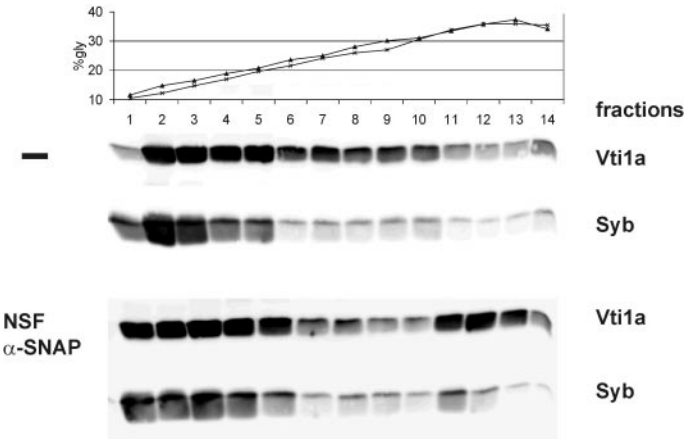

Figure 10. Vti1a-β was part of a SNARE complex that bound NSF and α-SNAP. Detergent extracts from lysed synaptosomes (high-speed membrane fraction LP2) were incubated without additions (−) or with NSF and α-SNAP and separated on a glycerol gradient. Fractions were analyzed by SDS-PAGE and immunoblotting. A subfraction of Vti1a-β and synaptobrevin (*Syb*) were shifted to denser fractions 11–13 in the presence of NSF and α-SNAP.

Vti1a-β was found on immunoisolated synaptic vesicles and endosomes

By subcellular fractionation, we have shown that Vti1a-β was enriched in synaptic vesicles. To verify this finding, we used an immunoisolation procedure as an independent method to purify organelles. Antibodies against synaptobrevin, rab3a, or rab5 were coupled to Eupergit beads and used for isolation of organelles from a 50,000 × *g* supernatant of brain homogenate (Burger et al., 1989). Synaptic vesicles are the predominant organelle in this starting material because each neuron forms ~1000 synapses containing numerous synaptic vesicles. Golgi membranes, clathrin-coated vesicles, endocytic intermediates, and endosomes are much less abundant. Synaptobrevin as a membrane protein is present on synaptic vesicle membranes throughout their life cycle, i.e., is present on mature synaptic vesicles as well as on membranes during the biogenesis and in the process of recycling, such as clathrin-coated vesicles, endocytic intermediates, and endosomes. Rab3a and rab5 have overlapping but not identical distributions. Rab3a is enriched in synaptic vesicles but absent from clathrin-coated vesicles and Golgi membranes. Rab5 is found predominantly on endosomes but also on a subpopulation of synaptic vesicles. Almost all Vti1a-β-containing organelles present in the starting material were bound to synaptobrevin beads, as well as to rab3a-beads (Fig. 8). Organelles containing synaptobrevin were quantitatively isolated by synaptobrevin beads and rab3a beads. These data confirm that Vti1a-β was localized to synaptic vesicles. Less Vti1a-β-containing organelles were isolated with rab5 beads. Some Vti1a-β, as well as some synaptobrevin, was not bound to rab5 beads because some synaptic vesicles do not contain rab5. These data indicate that Vti1a-β together with synaptobrevin was present on synaptic vesicle membranes throughout their life cycle.

A fraction of Vti1b-containing organelles were immunoisolated with synaptobrevin, rab3a, and rab5 beads. More than 50% of Vti1b remained unbound in these immunoisolations. Because synaptic vesicles are so abundant in the starting material, these data indicate that Vti1b was present on synaptic vesicles at lower concentrations, as well as on other membranes not connected to the synaptic vesicle pathway at higher concentrations.

Vti1a-β did not coimmunoprecipitate with the exocytic SNARE complex

Because we identified Vti1a-β as a synaptic vesicle protein, we wanted to determine whether Vti1a-β was in a complex with the SNAREs required for fusion of synaptic vesicles with the plasma membrane. SNARE complexes were immunoprecipitated from Triton X-100 extracts of synaptosomes using antibodies against Vti1a, SNAP-25, syntaxin 1, and synaptobrevin, respectively. SNAREs in these immunoprecipitates were detected by immunoblotting (Fig. 9). Syntaxin 1, SNAP-25, and synaptobrevin were not present in the Vti1a immunoprecipitates, although Vti1a-β was quantitatively removed from the extract. Vti1a antiserum coimmunoprecipitated syntaxin 6 as described by Xu et al. (1998), indicating that the Vti1a antiserum was able to immunoprecipitate a SNARE complex (data not shown). The three SNAREs of the exocytic SNARE complex were coimmunoprecipitated with antibodies against synaptobrevin, syntaxin 1, or SNAP-25, indicating that SNARE complexes were present under our experimental conditions. Vti1a-β was not coimmunoprecipitated by antibodies against syntaxin 1 and SNAP-25. These data indicate that Vti1a-β was not part of the exocytic SNARE complex. A small amount of Vti1a-β was present in the synaptobrevin immunoprecipitates. It is likely that this interaction is weak or nonspecific because synaptobrevin was absent from Vti1a immunoprecipitates.

Vti1a-β was part of an NSF- and α-SNAP-containing SNARE complex in nerve terminals

After establishing that Vti1a-β was not part of the exocytic SNARE complex, we wanted to determine whether Vti1a-β was part of a novel SNARE complex in nerve terminals. SNARE complexes have the characteristic ability to bind NSF and α-SNAP and form a 20 S complex. NSF disassembles these complexes in the presence of ATP and Mg^{2+} (Söllner et al., 1993b). Two different approaches were taken to study whether Vti1a-β in nerve terminals exists in a SNARE complex, which can bind NSF and α-SNAP and is disassembled by NSF. First, detergent extracts from the high-speed pellet of lysed synaptosomes (LP2) containing mostly synaptic vesicles were incubated with NSF and α-SNAP under conditions that favor formation of a 20 S complex or without addition of NSF and α-SNAP. These extracts were separated on a glycerol density gradient, and Vti1a-β and synaptobrevin were identified by immunoblotting (Fig. 10). Significantly more Vti1a-β and synaptobrevin were found in the higher density fractions 11–14 in the presence of NSF and α-SNAP compared with the extract without additions. These data indicate that a subfraction of Vti1a-β was present in a SNARE complex that bound NSF and α-SNAP.

In the second approach, we used the observation that the four-helix bundle in the assembled SNARE complex (core complex) is much more protease-resistant than the rest of the molecules or the disassembled SNAREs (Fasshauer et al., 1998a; Poirier et al., 1998). Therefore, we looked for a protease-resistant Vti1a-β fragment under conditions in which a SNARE complex is stable. Detergent extracts from the high-speed pellet of lysed synaptosomes (LP2) were treated with trypsin under different conditions: SNARE complex disassembly by addition of NSF, α-SNAP, ATP, and Mg^{2+} (Fig. 11*A*, *first lane*); inhibition of disassembly in the presence of NSF and α-SNAP by EDTA (*second lane*); or without additions, maintaining SNARE complexes (*third lane*). Vti1a-β was detected in these fractions by immunoblotting. A protease-resistant Vti1a-β fragment of 14 kDa was identified in fractions favoring SNARE complex formation but not under conditions of SNARE complex disassembly. Next, we wanted to determine whether the 14 kDa fragment was part of the N-terminal or C-terminal half of Vti1a-β with the SNARE motif. The antiserum against Vti1a was affinity-purified with an immobilized GST fusion with either the amino acid residues 1–114 of Vti1a or residues 115–192. The resulting antibodies were specific for the N-terminal or C-terminal part of Vti1a, respectively (data not shown). SNARE complex disassembly was also inhibited by addition of ATPγS to exclude effects of EDTA. The 14 kDa fragment was only recognized by the antiserum specific for the C-terminal half (Fig. 11*C*) but not by the antiserum against the N-terminal part (Fig. 11*B*). These data indicate that the C-terminal part of Vti1a-β with the SNARE motif was protected from proteases only under conditions in which SNARE complexes are stable. Therefore, Vti1a-β was part of a novel SNARE complex in nerve terminals.

DISCUSSION

We identified Vti1a-β as a brain-specific splice variant of Vti1a. Our results show that the brain-specific Vti1a-β but not the ubiquitous Vti1a is enriched in synaptic vesicles and nerve terminal-derived clathrin-coated vesicles. Vti1a-β and synaptobrevin were isolated to a similar degree by rab5 beads used to immunoisolate endosomes and rab5-containing synaptic vesicles. Our data indicate that both synaptobrevin and Vti1a-β are integral components of the synaptic vesicle membrane throughout the vesicle life cycle. Vti1b was found at low concentrations on synaptic vesicles and nerve terminal-derived clathrin-coated vesicles. These data indicate that Vti1b may only be a minor component or only present on a subpopulation of synaptic vesicles.

The brain-specific Vti1a-β had an insertion of seven amino acid residues at position 114 of the ubiquitous Vti1a. This insertion is located at the beginning of an evolutionary conserved predicted α-helix. The domain that forms the SNARE-interacting helix according to sequence alignments (Fasshauer et al., 1998b) is close by and starts at amino acid residue 132. The close proximity of the brain-specific insert to the SNARE motif raises the possibility that binding of SNARE partners is influenced by these additional amino acid residues. The insertion may be important for sorting of Vti1a-β to synaptic vesicles. Consensus signals for targeting of proteins to synaptic vesicles are still unknown. However, it was found that the SNARE motif of synaptobrevin is important for localization to synaptic-like microvesicles (SLMV) in the neuroendocrine cell line PC12 (Grote et al., 1995).

Two lines of evidence demonstrated that Vti1a-β was present in a SNARE complex, which was disassembled by the addition of NSF and α-SNAP in nerve terminals. First, Vti1a-β moved to denser fractions in a glycerol gradient upon the addition of NSF and α-SNAP, suggesting the presence of Vti1a-β in a SNARE complex that bound NSF and α-SNAP. In addition, a C-terminal fragment of Vti1a-β with the SNARE motif was protected from protease digestion only under conditions in which SNARE complexes are stable but not when they are disassembled. Although enriched on synaptic vesicles Vti1a-β coimmunoprecipitated with neither syntaxin 1 nor with SNAP-25. These data indicate that Vti1a-β does not function in the SNARE complex required for regulated exocytosis. These results provide further evidence for the specific formation of SNARE complexes *in vivo*. Although it was observed that noncognate SNARE complexes can form *in vitro* and have similar properties as cognate SNARE complexes (Fasshauer et al., 1999; Yang et al., 1999), these noncognate SNARE complexes could not be isolated by coimmunoprecipitation from cell extracts (Fasshauer et al., 1999). Because we identified a novel, Vti1a-β-containing SNARE complex within the nerve terminal, our data also indicate that a second membrane fusion step is required at some point in the life cycle of synaptic vesicles and that Vti1a-β functions in this step.

Vti1a-β may be required for a fusion step during synaptic vesicle recycling. Synaptic vesicle membranes can undergo several hundred rounds of exocytosis and reloading with neurotransmitter. Therefore, it is critical that the protein composition of synaptic vesicles is maintained or restored during recycling. Several models have been suggested for synaptic vesicle recycling (Cremona and De Camilli, 1997). Synaptic vesicles may transiently fuse with the plasma membrane, release their content via the fusion pore, and reseal. This kiss-and-run mechanism may be favored under conditions of strong stimulation and avoids mixing of synaptic vesicle proteins with plasma membrane proteins (Alés et al., 1999). Clathrin-coated vesicles are intermediates in synaptic vesicle recycling, as was demonstrated using several different approaches (Mill-

er and Heuser, 1984; van der Bliek and Meyerowitz, 1991). However, it is still under debate whether uncoating of clathrin-coated vesicles results directly in synaptic vesicles without any further intermediates and without fusion steps (Takei et al., 1996; Murthy and Stevens, 1998) or whether an endosomal intermediate is involved. Nerve terminals contain early endosomes, as indicated by the presence of the early endosome marker protein rab5 (Fischer von Mollard et al., 1994). According to a model with a sorting compartment, uncoated clathrin-coated vesicles would fuse with endosomes requiring a SNARE complex. Vti1a-β may be involved in this fusion step. Synaptic vesicles are generated by budding from these endosomes. Both pathways could exist in parallel. Synaptic vesicles may reform from clathrin-coated vesicles as long as they have the correct protein composition. A passage through the endosome may be required as a sorting step to restore the protein composition of synaptic vesicles. Budding of SLMV has been reconstituted *in vitro* with PC12 cells from both an endosomal compartment and a subplasmalemmal compartment still connected with the plasma membrane. Synaptic vesicle proteins accumulate in a subplasmalemmal tubulocisternal compartment that is devoid of the endosomal marker transferrin receptor. SLMV budding from this compartment is dependent on clathrin, AP-2, and other proteins required for formation of clathrin-coated vesicles (Schmidt and Huttner, 1998; Shi et al., 1998). On the other hand, SLMV are formed from endosomes isolated from PC12 cells (Clift-O'Grady et al., 1998). The resulting SLMV are devoid of transferrin receptor present in the donor compartment. This budding step is dependent on the adaptor complex AP-3. The transport of endocytosed synaptobrevin to early endosomes and the budding of SLMV from tubular extensions of early endosomes has been observed in PC12 cells using immunoelectron microscopy (de Wit et al., 1999)

It is possible that Vti1a-β or Vti1b have a role in the biogenesis of synaptic vesicles. The biogenesis of synaptic vesicles is still unclear. Most data indicate that synaptic vesicle proteins leave the TGN in vesicles destined for the constitutive secretory pathway (Hannah et al., 1999). These vesicles are transported through the axon by fast axonal transport. Mature synaptic vesicles are formed after several rounds of constitutive exocytosis and recycling via an endosome. In yeast, Vti1p is not involved in constitutive secretion and does not bind the plasma membrane t-SNAREs Sso1p/Sso2p. In contrast, yeast Vti1p binds all endosomal, vacuolar–lysosomal, and Golgi t-SNAREs (Fischer von Mollard et al., 1997; Holthuis et al., 1998). Therefore, a role in constitutive exocytosis seems unlikely.

So far we do not know the SNARE partners of Vti1a-β and Vti1b in the nerve terminal. Candidates would be endosomal SNARE proteins. Three R-SNAREs have been localized to the endosomal system: endobrevin/ vesicle-associated membrane protein –8 (VAMP-8), which is expressed at low levels in brain (Advani et al., 1998; Wong et al., 1998), VAMP-7/TI-VAMP (Advani et al., 1999), and VAMP-4 (Steegmaier et al., 1999). Recently, endogenous VAMP-7/TI-VAMP has been localized to somatodendritic tubules and vesicles in cultured neurons but not to nerve terminals (Coco et al., 1999). Therefore, VAMP-7 and Vti1a-β do not colocalize in synapses. Q-SNAREs identified in TGN or endosomal membranes are syntaxin 6 (Simonsen et al., 1999), syntaxin 12/13 (Prekeris et al., 1998; Tang et al., 1998b), syntaxin 7, syntaxin 8 (Wang et al., 1997; Prekeris et al., 1999), syntaxin 11, which is expressed at low levels in brain (Valdez et al., 1999), and syntaxin 10 (Tang et al., 1998a).

The localization of Vti1a-β to synaptic vesicles suggests that this SNARE is more specialized for certain trafficking steps and may only bind to a subset of SNARE proteins required for these trafficking steps *in vivo* than the promiscuous yeast Vti1p.

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