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The SNAREs vti1a and vti1b have distinct localization and SNARE complex partners

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Two mammalian proteins, vti1a and vti1b, are homologous to the yeast Q-SNARE Vti1p which is part of several SNARE complexes in different transport steps. In vitro experiments suggest distinct functions for vti1a and vti1b. Here we compared the subcellular localization of endogenous vti1a and vti1b by immunofluorescence and immuno-electron microscopy. Both proteins had a distinct but overlapping localization. vti1a was found predominantly on the Golgi and the TGN, vti1b mostly on tubules and vesicles in the TGN area and on endosomes. vti1a coimmunoprecipitated with VAMP-4, syntaxin 6, and syntaxin 16. These four SNAREs could assemble into a SNARE complex of conserved structure because one SNARE motif of each subgroup is present. vti1a-β, VAMP-4, syntaxin 6, and syntaxin 16 are coenriched with small synaptic vesicles and with clathrin-coated vesicles isolated from rat brain synaptosomes. Therefore, this SNARE complex may have a role in synaptic vesicle biogenesis or recycling.

Introduction

SNARE proteins on transport vesicles and target membranes have an important role in vesicle targeting and fusion (Jahn and Südhof, 1999). They form SNARE complexes thereby bridging the opposing membranes and promoting membrane fusion. SNAREs are evolutionary conserved proteins with a common domain structure. They interact via a highly conserved SNARE motif of about 60 amino acid residues next to the C-terminal membrane anchor. The neuronal exocytic SNARE complex consists of four different SNARE motifs forming an extended parallel four helix bundle (Sutton et al., 1998). Amino acid side chains from all helices point into the center of the bundle and interact in 16 layers. Most layers are hydrophobic but the central 0 layer consists of one arginine (R) and three glutamine (Q) residues. They are extremly conserved and led to a reclassification into R-SNAREs and Q-SNAREs (Fasshauer et al., 1998). According to sequence alignments three different groups of Q-SNAREs, Qa, Qb and Qc, can be distinguished (Bock et al., 2001). Therefore, SNARE complexes should have a uniform structure and each SNARE should occupy one specific position in the four helix bundle. This is supported by a structural analysis of the late endosomal SNARE complex (Antonin et al., 2002).

The yeast Qb-SNARE Vti1p is part of four different SNARE complexes and is required for retrograde traffic to the cis Golgi, for traffic from the TGN to the late endosome, and for several pathways to the vacuole (Lupashin et al., 1997; Fischer von Mollard et al., 1997; Fischer von Mollard and Stevens, 1999). The mammalian homologs vti1a and vti1b are only distantly related sharing about 30% amino acid identity with each other as well as with yeast Vti1p (Lupashin et al., 1997; Fischer von Mollard and Stevens, 1998; Advani et al., 1998). Did vti1a and vti1b evolve into proteins of more specialized function, distinct localization and different SNARE partners? Distinct functions for vti1a and vti1b are observed in in vitro fusion assays. Antibodies specific for vti1a block fusion of early endosomes while antibodies against vti1b block fusion of late endosomes but not vice versa (Antonin et al., 2000a). In addition, microinjection of antibodies specific for vti1a (also referred to as vti1-rp2) into Vero cells prevents transport of VSV G-protein to the cell surface and results in its accumulation in the Golgi area (Xu et al., 1998). However, despite these functional data a detailed analysis of the intracellular distribution of vti1a and vti1b is still awaiting. vti1a is localized to the Golgi by immunofluorescence microscopy (Xu et al., 1998) and the brain-specific splice variant vti1a-β, but not vti1b, enriches with small synaptic vesicles (Antonin et al., 2000c). So far, localization data are only available for myc-tagged vti1b which overlaps with markers for Golgi and TGN (Advani et al., 1998). Recently, SNARE partners for vti1b have been identified. The SNARE

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complex consisting of vti1b, syntaxin 7, syntaxin 8 and endobrevin/VAMP-8 has similar structural properties as the neuronal SNARE complex (Antonin et al., 2002). By contrast, vti1a coimmunoprecipitates with syntaxin 5 (cis-Golgi) and syntaxin 6 (TGN), but syntaxin 5 and syntaxin 6 are not part of the same SNARE complex (Xu et al., 1998).

In this study we compared the localization of endogenous vti1a and vti1b using immunofluorescence and immuno-electron microscopy. We identified a novel SNARE complex containing the neuronal vti1a-β, syntaxin 6, syntaxin 16, and VAMP-4. Furthermore, all members of this SNARE complex were found on small synaptic vesicles and clathrin-coated vesicles isolated from nerve terminals.

Material and methods

Materials

Reagents were used from the following sources: enzymes for DNA manipulation from New England Biolabs (Beverly, MA), secondary antibodies for immunofluorescence from Dianova (Hamburg, Germany), brefeldin A from Calbiochem (San Diego, CA), nitrocellulose and PVDF membranes from Schleicher & Schüll (Dassel, Germany), protein A- and protein G-sepharose from Amersham Pharmacia Biotech (Uppsala, Sweden) and Ni-NTA agarose from Qiagen (Hilden, Germany). All other reagents were purchased from Sigma (St. Louis, MO). Plasmid manipulations and protein expression were performed in the *E. coli* strains XL1Blue or BL21(DE3) using standard media.

Cloning of rat VAMP-4 and syntaxin 16B

Sequence data from rat expressed sequence tags (ESTs, GeneBank AW144777 and D86817) were used to assemble the sequence of rat VAMP-4. The coding sequence for the soluble domain of VAMP-4 (amino acids 1 – 117) was PCR amplified from a rat cerebellum cDNA λ ZAPII library (Stratagene, La Jolla, CA) using the primers CGGGATCCATGCCTCCCAAGTTCAAGC and GGAATTCATTTTATTTTGCATCCACGCC and cloned into pGexKG.

Primer construction for the amplification of rat syntaxin 16B was based on the sequences of human syntaxin 16B, a rat EST and two mouse ESTs (GeneBank AF008936, AW524540, AI426461, and AI956297). Two primer pairs were used for nested PCR amplification from a rat cerebellum cDNA λ ZAPII library, first GGGGTCRCAAAGGGTGAGAC and CTAGCGAGACTTTACGGCGACGAG, second CGGGATCCATGGCCACCAGGCGTTTAAC and GGAATTCACTTCCGRTTCTTCTTYTGRATG. The PCR product was cloned into pGEM-Teasy (Promega, Madison, WI) and used to PCR amplify the coding sequence for the N-terminal domain (amino acids 1 – 239) with the primers GCATATGGCCACCAGGCGTTTAAC and GGAATTCACTCTCGCTCCCTCTCCTC. The PCR fragment was cloned into pET-28a (Novagen, Madison, WI).

Protein purification and antibody generation

Recombinant glutathione S-transferase GST-VAMP-4 (amino acids 1 – 117) was expressed in XL1Blue cells and purified from the supernatant using glutathione-agarose according to the manufacturer's instructions. 6 × His-syntaxin 16 (amino acids 1 – 239) was expressed in BL21(DE3) and purified from the pellet fraction. After solubilization with 7.2 M urea in PBS 6 × His-syntaxin 16 was bound to Ni-NTA agarose and eluted with 6.4 M urea in 0.1 M sodium phosphate, pH 4.5. The eluate was dialyzed against 3 M and 1 M urea in PBS. Antisera were raised in rabbits. The antiserum against syntaxin 16 was affinity purified using antigen immobilized on nitrocellulose after SDS-PAGE.

Immunofluorescence microscopy

NRK cells were grown on coverslips. Where indicated, cells were treated with 10 μM brefeldin A (BFA) for 15 min at 37 °C. Cells were fixed with 3% paraformaldehyde in PBS and permeabilized with 0.05% Triton X-100 in PBS. An affinity-purified rabbit antiserum against vti1a and vti1b (Antonin et al., 2000c) and monoclonal antibodies against giantin (Linstedt and Hauri, 1993) were used. Secondary antibodies were conjugated with Cy2 or Cy3. The staining was analyzed by confocal microscopy (ZEISS LSM410).

Immuno-electron microscopy of ultrathin cryosections

Ultrathin cryosections were prepared as described (Tokuyasu, 1973). Human fibroblasts (König cells) were incubated with 5 nm BSA-gold for 10 min as indicated and fixed with 2% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M sodium phosphate, pH 7.4 for 30 min at room temperature directly on cell culture dishes. Cells were postfixed with 4% paraformaldehyde and 0.1% glutaraldehyde for 2 h on ice and removed from the dishes with a cell scraper. After washing twice with PBS, cells were embedded in 10% gelatin, cooled on ice and cut into small blocks. Adult rat brain was isolated after transcardial perfusion with 4% paraformaldehyde, cut into 200-μm slices using a vibratome, and blocks containing the mossy fiber area were cut out. Blocks were infused with 2.3 M sucrose overnight, mounted on small metal pins, frozen and stored in liquid nitrogen. Ultrathin sections were cut at −110 °C using a diamond knife (Diatome) in an ultracryomicrotome (Leica), collected using a 1:1 mixture of 1.8% methyl-cellulose and 2.3 M sucrose, and deposited on formvar- and carbon-coated nickel grids. For immunolabeling, sections were incubated with affinity-purified antibodies against vti1a (1:20), vti1b (1:20) or transferrin receptor (1:30) for 1 h followed by a 20-min incubation with protein A-gold (10 nm). For double labeling experiments, sections were fixed for 5 min with 1% glutaraldehyde after incubations with the first antibody and protein A-gold (5 nm). After quenching with glycine and blocking with BSA sections were incubated with the second antibody and protein A-gold (10 nm). Results were not dependent on the order of antibody incubations in double labeling experiments for vti1a and vti1b. Sections were contrasted with uranyl acetate/methyl-cellulose for 10 min on ice, embedded in the same solution and examined with a Philips CM120 electron microscope.

Immunoprecipitation

Polyclonal antisera against vti1a and syntaxin 16 were bound to protein A-sepharose, and monoclonal antibodies against syntaxin 6 (BD Transduction Laboratories) to protein G-sepharose and crosslinked with dimethylpimelimidate. A rat brain was homogenized in TBS, debris were removed by a centrifugation with $300g_{av}$, and crude synaptosomes were pelleted at $9000g_{av}$. Synaptosomes were solubilized with 1% Triton X-100 in TBS with 1 mM EDTA at a protein concentration of 1 mg/ml. The extracts were cleared at $200\,000g_{av}$ and incubated with the coupled antibodies or with protein A-sepharose for 2 h at 4 °C. Beads were washed five times in TBS containing 1% Triton X-100 and eluted with 0.1 M glycine-HCl, pH 2.5. Starting material, unbound material and eluates were analyzed by SDS-PAGE and Western blotting.

Isolation of organelles

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

Results

Localization of endogenous vti1a and vti1b

Our first goal was to compare the subcellular localization of endogenous vti1a and vti1b using specific rabbit polyclonal antisera. vti1a was found on perinuclear structures of NRK cells by immunofluorescence microscopy (Fig. 1C). Many of these structures were also positive for the Golgi marker giantin (B, overlay A). vti1b localized also to the perinuclear area but vti1b – stained structures extended further into the periphery of the cell (G). Little overlap (E) was observed between vti1b and

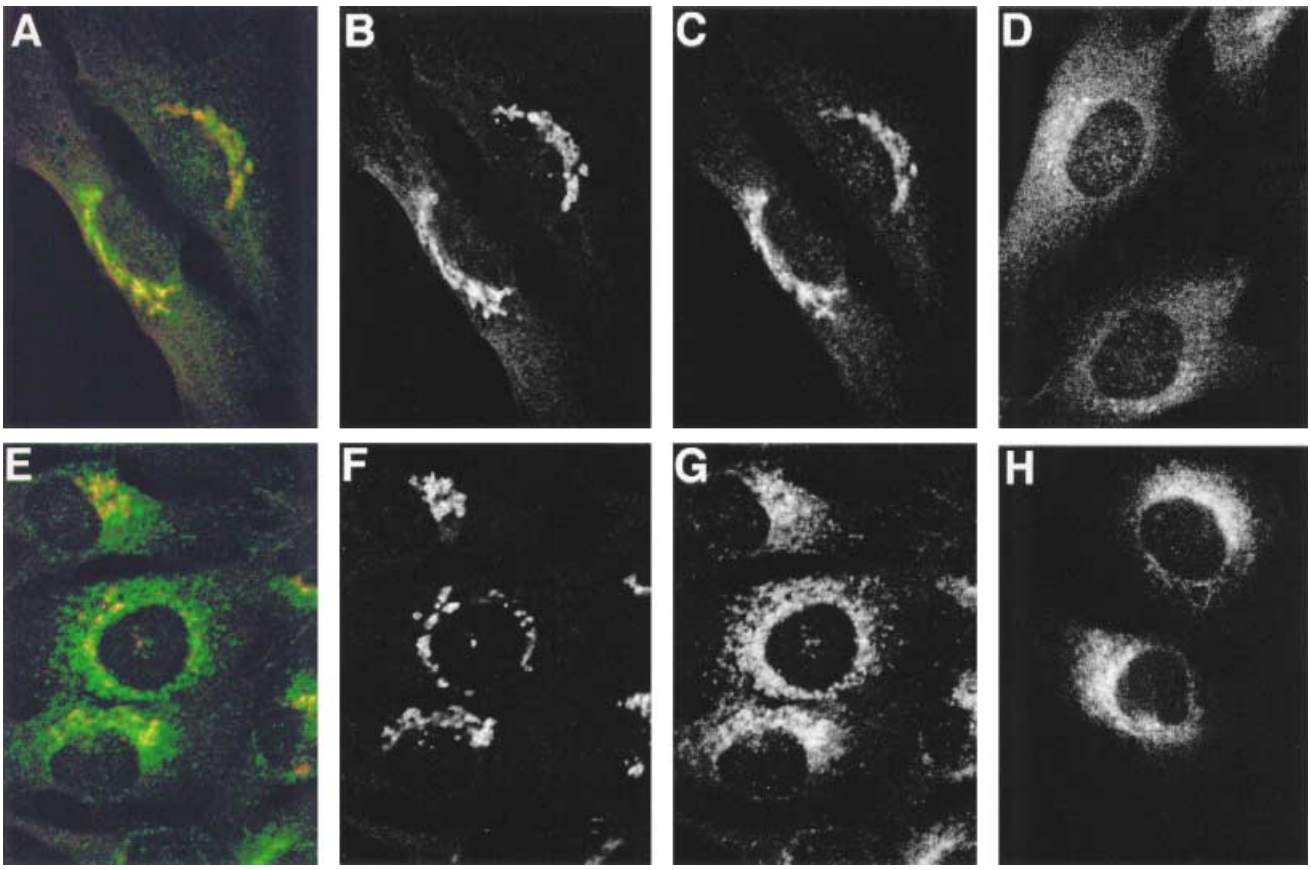


Fig. 1. vti1a and vti1b have a distinct localization by immunofluorescence microscopy. NRK cells were stained for the Golgi marker giantin (**A, B, E, F;** *red*) and for vti1a (**A, C;** *green*) or vti1b (**E, G;** *green*) and analyzed by confocal microscopy. Giantin and vti1a were found on the same structures while vti1b did not overlap with giantin. NRK cells were treated with 10 μM BFA for 15 min at 37 °C and stained for vti1a (**D**) or vti1b (**H**). Most perinuclear localization of vti1a in untreated cells (**C**) was lost upon BFA treatment (**D**). Most perinuclear staining of vti1b (**G**) remained after BFA treatment (**H**).

giantin (F). As Golgi, TGN and recycling endosomes are found in the perinuclear area we used brefeldin A (BFA) to distinguish Golgi from TGN and endosomes. Golgi membranes redistribute into more peripheral ER structures while the TGN fuses with endosomal membranes (Lippincott-Schwartz et al., 1991). Most perinuclear staining of vti1a (C) was lost and a scattered staining emerged upon BFA treatment (D). These data indicate that vti1a was localized to the Golgi. vti1b localization (G) was less affected by BFA (H) suggesting a localization outside the Golgi.

We performed immuno-electron microscopy to obtain a detailed view of the distribution of vti1a and vti1b. Human fibroblasts were incubated with 5 nm BSA gold for 10 min to label early endosomes, and ultrathin cryosections were stained with affinity-purified antiserum specific for vti1a and 10 nm protein A gold (Fig. 2A – C). vti1a was localized to Golgi stacks and to the TGN. Lower levels of vti1a labeling were found in early endosomes containing BSA gold. Sections of human fibroblasts were double labeled for vti1b (10 nm gold) and transferrin receptor (5 nm gold, Fig. 2D – F). Most labeling for vti1b was seen in the TGN region, often in vesicles or clathrin-coated vesicles (Fig. 2D) and on endosomal structures. Considerable labeling for vti1b was detected on early endosomes containing transferrin receptor. Low levels of vti1b were observed on Golgi stacks, and on lysosomes.

Cryosections were double labeled for vti1a and vti1b to be able to compare the localization of vti1a and vti1b directly (Fig. 2G – I). The fraction of gold particles associated with different organelles was determined for vti1a and vti1b in a quantitative analysis (Table I). 15% of the labeling for vti1a was found within Golgi stacks and 48% on the TGN. Labeling was also detected on tubules and vesicles not assigned to the TGN or plasma membrane (13%), tubulovesicular structures near

Tab. I. Quantification of vti1a and vti1b in fibroblasts.

Compartment	Percent gold particles vti1a	vti1b
Golgi	15	3
TGN	48	19
Tubules, vesicles not assigned to TGN or PM	13	27
Tubulovesicular structures near PM	7	15
PM	2	1
MVB/endosomes	7	23
Lysosomes	0.7	4
unclassified	7	7

Cells were fixed and double labeled for vti1a and vti1b as described in Materials and methods. For quantification, a total of 448 gold particles for vti1a and 272 gold particles for vti1b were counted. PM, plasma membrane; MVB, multivesicular bodies.

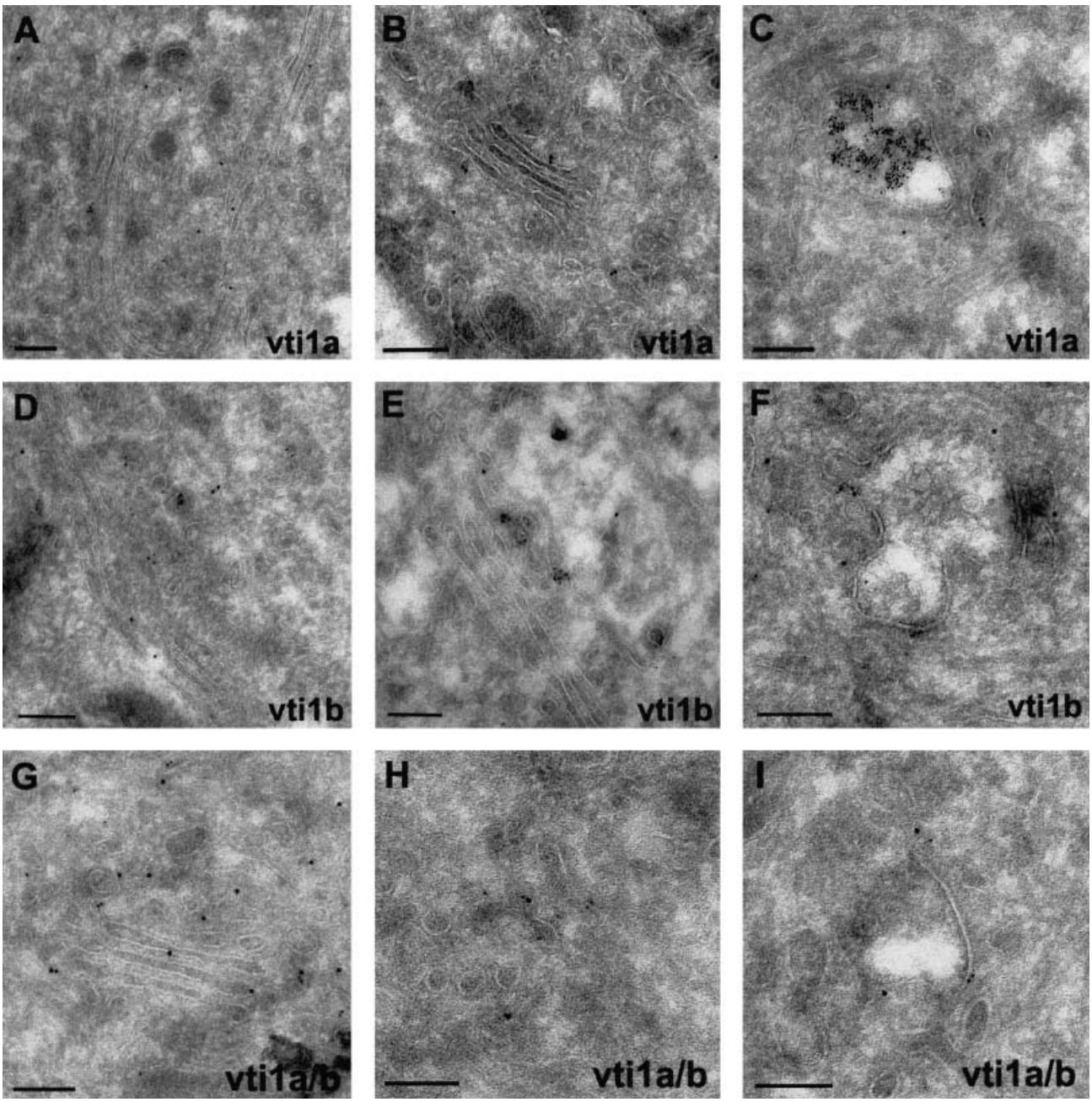


Fig. 2. vti1a is found predominantly in the Golgi and TGN, vti1b in tubules, vesicles and endosomes by immuno-electron microscopy. vti1a (10 nm gold) is localized to Golgi stacks and to the TGN (**A, B**). Low levels of vti1a can be found on early endosomes identified by BSA-gold (5 nm) taken up during a 10-min incubation period (**C**). (**D – F**) vti1b (10 nm gold) was seen mostly in the TGN, on tubules, vesicles and clathrin-coated vesicles in the TGN area and on endosomes. Colocalization with transferrin receptor (5 nm gold) was seen in early or recycling endosomes. (**G-I**) Double labeling of vti1a (10 nm gold) and vti1b (5 nm gold). The overall labeling intensity is higher for vti1a than for vti1b. vti1a is most prominent in the Golgi and TGN (**G**), vti1b is more abundant in tubules and vesicles (**H**) and endosomes (**I**). Bars: 200 nm.

the plasma membrane (7%) and endosomes and multivesicular bodies (7%). By contrast, most vti1b was localized on tubules and vesicles which were not assigned to the TGN or plasma membrane (27%) and on endosomes and multivesicular bodies (23%). vti1b was also detected on the TGN (19%), on tubulovesicular structures near the plasma membrane (15%), and on lysosomes (4%). These data confirmed that vti1a and vti1b have a distinct localization. However, both proteins have a broad and partially overlapping distribution. The subcellular localization of vti1a and vti1b is in agreement with earlier functional data.

Using subcellular fractionation we have previously shown that a brain-specific splice variant of vti1a, vti1a-β is enriched on small synaptic vesicles. To substantiate these findings we studied vti1a localization in brain at the ultrastructural level. Ultrathin cryosections were obtained from mossy fiber synap-

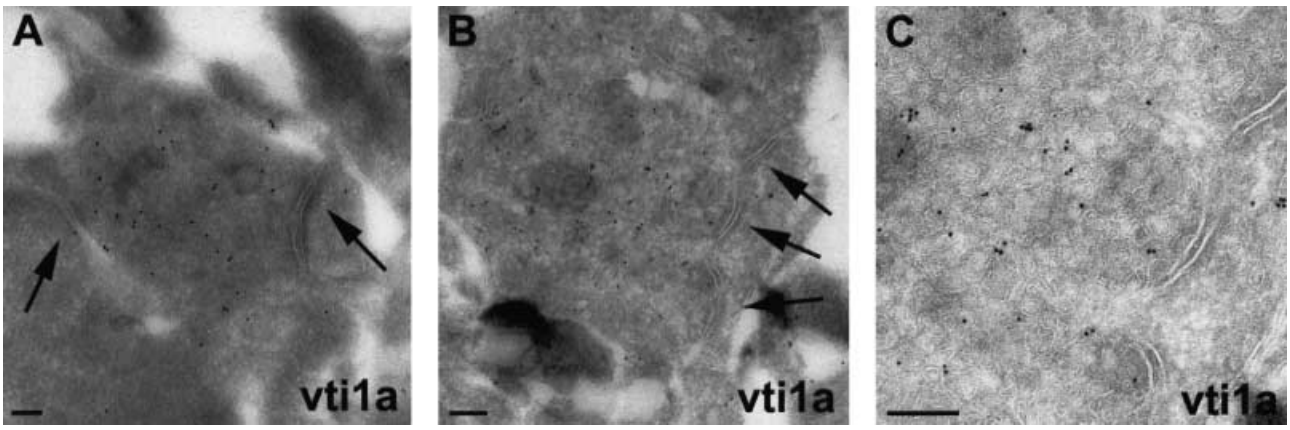


Fig. 3. vti1a is localized to small synaptic vesicles in the mossy fiber nerve terminals by immuno-electron microscopy. Pre- and post-synaptic membranes are seen at synaptic clefts (*arrows*). Some sections were collected in sucrose-methylcellulose to see the overall structure (**A**), others in sucrose to optimize visibility of small vesicles (**B** and **C**) and stained for vti1a (10 nm gold). Bars: 200 nm.

ses and stained with an antiserum which recognizes both vti1a and vti1a-β (Fig. 3). Nerve terminals were strongly decorated with antibodies against vti1a and protein A gold (Fig. 3A). Small synaptic vesicles labeled with anti-vti1a antibodies were visible in presynaptic nerve terminals at higher resolution (Fig. 3B – C).

SNARE partners of vti1a

Considering the distinct but overlapping distribution of vti1a and vti1b we were interested in the SNARE partners of both proteins. vti1b forms a SNARE complex with endobrevin, syntaxin 7 and syntaxin 8 (Antonin et al., 2000a). vti1a coimmunoprecipitates with the TGN-localized syntaxin 6 and with the Golgi-localized syntaxin 5 (Xu et al., 1998). However, syntaxin 5 and syntaxin 6 were not part of the same complex indicating that vti1a participates in at least two SNARE complexes. In addition, syntaxin 6 and the TGN protein VAMP-4 coimmunoprecipitate (Steegmaier et al., 1999). We asked whether VAMP-4 and vti1a are present in one complex. It is an emerging concept that SNARE complexes have a common structure with four distinct SNARE motifs. One helix is contributed by an R-SNARE, such as VAMP-4. The three Q-SNARE helices can be distinguished by their amino acid sequence. vti1a corresponds to the N-terminal (Qb), syntaxin 6 to the C-terminal helix of SNAP-25 (Qc). Therefore, a syntaxin (Qa) was missing from this complex. As best candidates we tested syntaxin 12/13 which is localized in early and recycling endosomes (Tang et al., 1998; Prekeris et al., 1998) and syntaxin 16. Epitope-tagged syntaxin 16 is found on the Golgi (Simonsen et al., 1998).

We generated an antiserum specific for syntaxin 16. This antiserum detected a single band of about 41 kDa in accordance with the predicted molecular mass of 37 kDa (Fig. 4). Syntaxin 16 was most abundant in adrenal, brain, heart and kidney. Lower levels were found in liver, pancreas and thymus. All potential SNARE partners for vti1a are abundant in brain. The brain-specific splice variant of vti1a, vti1a-β, is enriched in synaptic vesicles and clathrin-coated vesicles isolated from nerve terminals. vti1a-β is part of a SNARE complex of unknown composition (Antonin et al., 2000c). To characterize this SNARE complex and to remove Golgi membranes we isolated crude synaptosomes from rat brain and generated Triton X-100 extracts as starting material. vti1a-β, syntaxin 16,

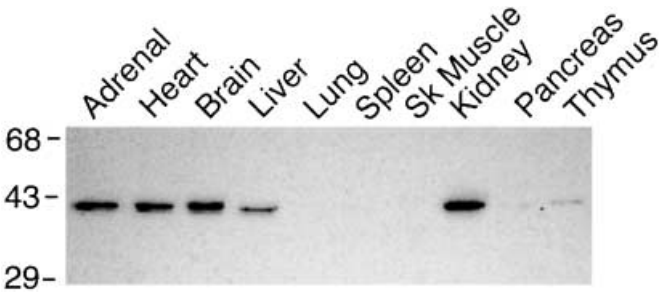


Fig. 4. Syntaxin 16 protein levels in different tissues. Homogenates were prepared from the indicated mouse tissues, separated by SDS-PAGE, and Western blots were stained with affinity-purified antiserum against syntaxin 16. A specific band of 41 kDa was recognized. sk skeletal.

and syntaxin 6 were immunoprecipitated and the fractions analyzed by immunoblotting for the presence of different SNAREs (Fig. 5). VAMP-4, syntaxin 16, and syntaxin 6 were identified in vti1a immunoprecipitates. By contrast, syntaxin 13 was not detected. Both, vti1a-β and syntaxin 6 were found after immunoprecipitation with anti-syntaxin 16 antibodies. VAMP-4 levels in immunoprecipitates using anti-syntaxin 16 and anti-syntaxin 6 antisera and syntaxin 16 levels in immunoprecipitates using anti-syntaxin 6 antisera were probably below the detection limit. Both, anti-VAMP-4 and anti-syntaxin 16 antisera gave only weak signals with the starting material. Antibodies specific for VAMP-4 coimmunoprecipitated little vti1a indicating that the antiserum may recognize SNARE complexes with low efficiency (data not shown). These experiments identified a SNARE complex composed of VAMP-4, syntaxin 16, vti1a, and syntaxin 6. As other well characterized SNARE complexes it consisted of one R-, Qa-, Qb-, and Qc-SNARE.

VAMP-4, syntaxin 6 and syntaxin 16 are coenriched in synaptic vesicles

As the next step we investigated whether the other members of the newly identified SNARE complex were enriched on synaptic vesicles and clathrin-coated vesicles as observed for vti1a-β. Synaptic vesicles (CPG3) were isolated from rat brain homogenate via synaptosomes (P2) and the high speed pellet of

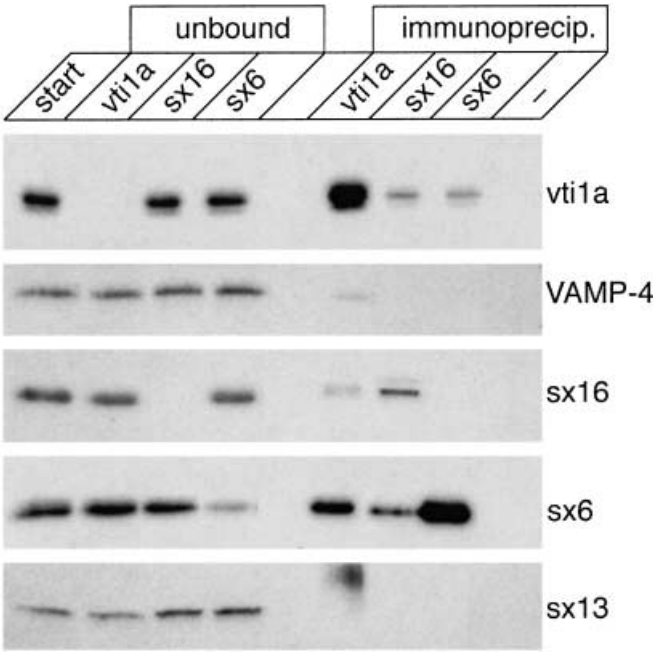
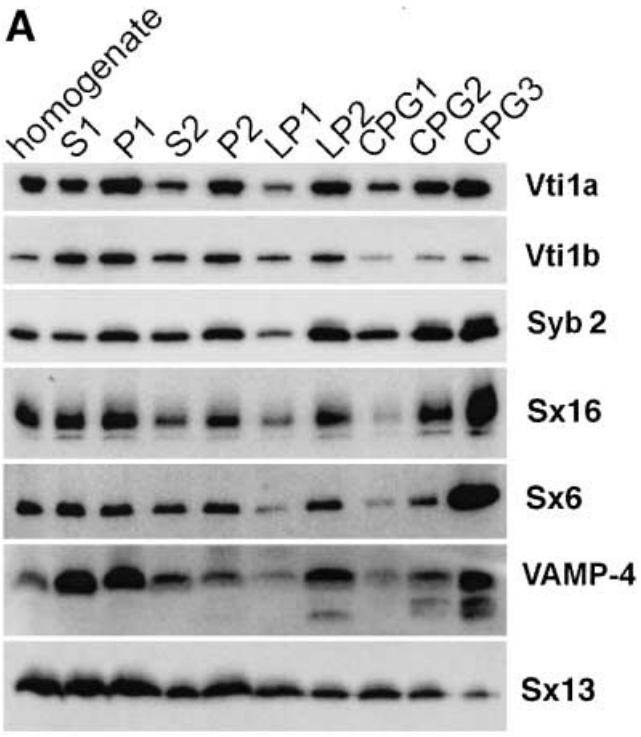


Fig. 5. vti1a forms a SNARE complex with syntaxin 6, syntaxin 16 and VAMP-4. Antibodies against vti1a, syntaxin 16 or syntaxin 6 coupled to protein A- or protein G-sepharose were used for immunoprecipitations from Triton X-100 extracts prepared from rat brain synaptosomes. Protein A-sepharose carrying no antibodies was used as negative control (–). Starting material, unbound fractions, and eluates from the immunoprecipitations were separated by SDS-PAGE. vti1a, VAMP-4, syntaxin 16 (sx16), syntaxin 6 (sx6) and syntaxin 13 (sx13) were detected by immunoblotting.

lysed synaptosomes (LP2, Fig. 6A). vti1a-β as well as syntaxin 16, syntaxin 6, and VAMP-4 copurified together with the synaptic vesicle protein synaptobrevin 2 in P2, LP2, and CPG3. These five SNAREs were more abundant in the CPG3 than in CPG1 and CPG2 fractions (contaminating membranes). Therefore, they are specifically enriched on synaptic vesicles. By contrast, syntaxin 13 did not copurify with synaptic vesicles and was much more abundant in brain homogenate, P2 and LP2. During exocytosis the synaptic vesicle membrane is incorporated into the plasma membrane of the nerve terminal. This membrane is endocytosed via clathrin-coated vesicles, and synaptic vesicles are reformed (Hannah et al., 1999). Clathrin-coated vesicles (CCVs) from nerve terminals were enriched from rat brain homogenate via the fractions crude synaptosomes, Ficoll load, supernatant after Ficoll and D_2O load (Fig. 6B). vti1a-β, syntaxin 16, syntaxin 6, and VAMP-4 were



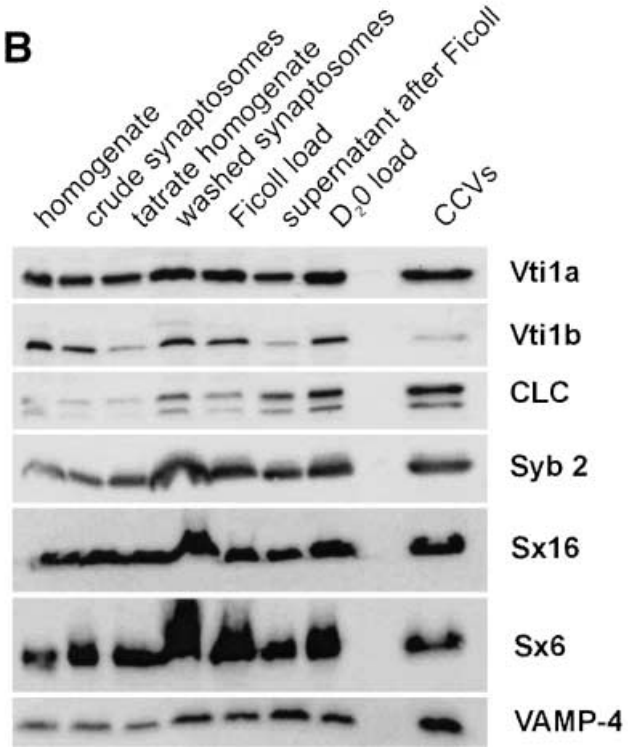


Fig. 6. SNARE partners of vti1a-β are enriched on small synaptic vesicles and clathrin-coated vesicles isolated from nerve terminals. (**A**) Synaptosomes (P2) were isolated from rat brain homogenate, osmotically lysed and separated into a low-speed membrane fraction (LP1) containing synaptic plasma 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 as seen by the enrichment of the synaptic vesicle protein synaptobrevin 2 (Syb 2). vti1a-β, syntaxin 16 (Sx16), syntaxin 6 (Sx6) as well as VAMP-4 were enriched in the synaptic vesicle fraction CPG3 compared to the homogenate and fractions P2 and LP2. By contrast, syntaxin 13 (Sx13) is not a synaptic vesicle protein as it is much more abundant in the homogenate, P2 and LP2 than in CPG3. (**B**) Clathrin-coated vesicles (CCVs) were isolated from synaptosomes with rat brain homogenate as starting material. Clathrin-coated vesicles were enriched via a high-speed pellet of lysed synaptosomes (Ficoll load), supernatant after Ficoll and D_2O load. vti1a-β, syntaxin 16, syntaxin 6, and VAMP-4 copurified in parallel with the synaptic vesicle marker synaptobrevin 2 in clathrin-coated vesicles. Clathrin light chain (CLC) was highly enriched.

present in highly purified clathrin-coated vesicles and displayed a similar distribution as the synaptic vesicle marker synaptobrevin 2. These data indicate that vti1a-β, syntaxin 16, syntaxin 6, and VAMP-4 were present on synaptic vesicle membranes throughout their life cycle. This supports the view that they form a complex that might be functional in the life cycle of synaptic vesicles.

Discussion

Both, immunofluorescence and immuno-electron microscopy revealed that vti1a and vti1b had distinct but overlapping subcellular distributions. Redistribution of vti1a into more peripheral structures upon BFA treatment indicated that a substantial amount of vti1a was localized on Golgi membranes. Quantification of the immuno-electron microscopical labeling indicated that most vti1a was found in the TGN (48%) and in the Golgi (15%).

vti1b was localized to early endosomes, multivesicular late endosomes, and tubules and vesicles not connected with the TGN. Lower levels of vti1b were seen on the TGN and lysosomes. A broad distribution among different organelles with a concentration in one or two organelles is also observed for SNARE partners of vti1a and vti1b and may be the result of recycling or biosynthetic transport of newly synthesized proteins. SNARE partners of vti1b are localized as following: endobrevin/VAMP-8 to early endosomes (Wong et al., 1998) or to early and late endosomes (Antonin et al., 2000b), syntaxin 7 to early endosomes (Prekeris et al., 1999) or to late endosomes (Mullock et al., 2000) and syntaxin 8 to the TGN (Prekeris et al., 1999) or to early endosomes (Subramaniam et al., 2000). The SNARE partners of vti1a, VAMP-4 and syntaxin 6, were found on the TGN (Steegmaier et al., 1999; Bock et al., 1997). Therefore, the steady-state localization does not allow for the prediction of SNARE complexes or functions. However, these localizations are compatible with a role of endobrevin, syntaxin 7, vti1b, and syntaxin 8 in late endosome fusion and a possible role for the VAMP-4, syntaxin 16, vti1a, syntaxin 6 complex in early endosomal/TGN trafficking.

The newly identified SNARE complex consisting of vti1a-β, VAMP-4, syntaxin 6 and syntaxin 16 provides more evidence for a conserved structure of SNARE complexes. Structural studies of the neuronal (Sutton et al., 1998) and the late endosomal SNARE complex (Antonin et al., 2002) as well as sequence analysis (Fasshauer et al., 1998; Bock et al., 2001) indicate that all SNARE complexes consist of a parallel bundle of four distinct α-helices, one R-SNARE and three different Q-SNAREs. VAMP-4 is the R-SNARE of this newly identified complex. Syntaxin 16 possesses a phenylalanine residue in the -3 layer and an arginine in the -8 layer which are characteristic for syntaxins (Qa). Syntaxin 6 is most similar to syntaxin 8 and belongs to the SNAREs which are related to the C-terminal domain of SNAP-25 (SN2, Qc) characterized by a small amino acid residue, a serine, in the -3 layer and a bulky amino acid residue, a glutamine, in the -8 layer. vti1a is most similar to the N-terminal domain of SNAP-25 (SN1, Qb) and has small amino acid residues in both the -3 and -8 layers (glycine and serine, respectively).

Related complexes are present in yeast and *Arabidopsis*. *Arabidopsis* SYP41 and SYP42 (syntaxin 16 homolog) form a complex with VTI12 and SYP61 (syntaxin 6) on the TGN (Sanderfoot et al., 2001). Yeast Tlg1p (syntaxin 6) and Tlg2p (syntaxin 16) form a complex with Vti1p and function in endocytosis and trafficking between early endosomes and the TGN (Pelham, 1999). The related mammalian SNARE complex may function in similar transport steps. Syntaxin 6 is required for the homotypic fusion of immature secretory granules (Wendler et al., 2001) which are derived from the TGN. In vitro fusion of early endosomes is inhibited by antibodies specific for vti1a (Antonin et al., 2000a) and syntaxin 6 (Mills et al., 2001). However, antibodies directed against syntaxin 6 had no effect in a different study (McBride et al., 1999). Fusion of early endosomes isolated from fibroblasts is also inhibited by antibodies against syntaxin 13 (McBride et al., 1999; Mills et al., 2001). Syntaxin 13 is localized to recycling endosomes and is required for transferrin receptor recycling (Prekeris et al., 1998). Syntaxin 13 is a syntaxin (Qa) but its SNARE partners have not yet been identified. We were not able to detect a coimmunoprecipitation of syntaxin 13 with vti1a (Fig. 5) or syntaxin 6 (Antonin et al., 2000a) in rat brain and rat liver, respectively. However, low levels of coimmunoprecipitation of syntaxin 13 may have escaped detection.

vti1a-β is part of a SNARE complex in nerve terminals which is distinct from the exocytic SNARE complex (Antonin et al., 2000c). We have now isolated the vti1a-β SNARE complex from synaptosomes and demonstrated that the SNARE partners vti1a-β, VAMP-4, syntaxin 6 and syntaxin 16 were found on small synaptic vesicles and on clathrin-coated vesicles isolated from nerve terminals. We propose that this complex acts in an early endosomal fusion step within the nerve terminal. Early endosomes have been identified by the specific marker rab5 in nerve terminals (Fischer von Mollard et al., 1994) and probably serve a dual function. On the one hand they play a role in the biogenesis of small synaptic vesicles (Hannah et al., 1999). Synaptic vesicle proteins leave the TGN in vesicles destined for the constitutive secretory pathway. After transport through the axon they undergo constitutive exocytosis and recycling through early endosomes to mature into synaptic vesicles. Secondly, early endosomes play a role in recycling of a subpopulation of synaptic vesicles (Blumstein et al., 2001).

Recently, it has been suggested that fusion may not require SNAREs because Ca^{2+}-stimulated exocytosis of synaptic vesicles is reduced more than 100-fold while spontaneous fusion is only decreased 10-fold in synaptobrevin 2 knockout mice (Schoch et al., 2001). The second SNARE complex on synaptic vesicles identified in this study may be responsible for the residual exocytosis. Alternatively, VAMP-4 may substitute for synaptobrevin by interacting with syntaxin 1 and SNAP-25 because this promiscuous complex forms in vitro (Yang et al., 1999). Therefore, it cannot be ruled out that the residual fusion is SNARE dependent.

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