Indication for differential sorting of the rat v-SNARE splice isoforms VAMP-1a and -1b

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Indication for differential sorting of the rat v-SNARE splice isoforms

VAMP-1a and -1b

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Abstract: SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) proteins are essential constituents of the intracellular trafficking machinery. The variable C-terminus in the two rat VAMP-1 splice isoforms VAMP-1a and -1b potentially acts as a sorting signal since similar changes at the C-terminal end of a human VAMP-1 splice isoform resulted in its sorting to mitochondria. To evaluate differences in the subcellular localization of these two v-SNARE proteins, GFP and RFP tagged VAMP-1a and -1b proteins were expressed in HeLa, COS-7 and MDCK cells and evaluated by conventional confocal as well as total internal reflection fluorescence (TIRF) microscopy. Regions consistent with the endoplasmic reticulum (ER) and Golgi apparatus demonstrated a major overlap of both signals. In the periphery, vesicular structures were observed that expressed mainly one of both isoforms. Within our experimental settings, we could not observe sorting of any of the two isoforms to mitochondria or peroxisomes, whereas both isoforms were found expressed in a minor subset of singular vesicles, which sporadically appear to colocalize with the exocyst marker EXOC3/Sec6. Since vesicular structures were seen that expressed only one of the two splice variants, it is possible that VAMP-1a and VAMP-1b are sorted to distinct cellular compartments which require further characterization.

Keywords: VAMP, SNARE, sorting, intracellular trafficking, subcellular compartment

Introduction

Intracellular transport of vesicles and their regulated fusion with target membranes such as the plasma membrane or intracellular compartments is a fundamental process in cell biology (Bennett and Scheller 1993; Rothman and Warren 1994). So-called SNAREs (soluble N-ethylmaleimide-sensitive factor attachment protein receptors) are proteins that participate in constitutive or regulated vesicle docking and fusion events (Jahn et al. 2003; Sollner et al. 1993). VAMPs (vesicle associated membrane proteins) as members of this apparatus have initially been characterized as components of synaptic vesicles in *Torpedo californica* (Trimble et al. 1988) and were independently identified in rat where they were termed synaptobrevins (Baumert et al. 1989). They belong to a small family of membrane proteins (Kutay et al. 1995) that are highly conserved from yeast to mammals (Bennett and Scheller 1993; Gerst et al. 1992) and have been characterized in many species such as yeast, drosophila, rat and human (Archer et al. 1990; Chin et al. 1993; DiAntonio et al. 1993; Elferink et al. 1989; Protopopov et al. 1993; Sudhof et al. 1989). Their involvement and importance for vesicle fusion is confirmed by the observation that specific cleavage of VAMPs by zinc-metalloproteases such as clostridial neurotoxins inhibits regulated membrane fusion (Link et al. 1993; Schiavo et al. 1992; Schiavo et al. 1994; Schiavo et al. 1993; Yamasaki et al. 1994). In a 20S complex of SNAP25, NSF and VAMP-2, the VAMP protein serves as a receptor that is located on the vesicle membrane and links the vesicle to attachment factors on the target membrane (Sollner et al. 1993). Furthermore, yeast studies involving VAMP homologues revealed a role of VAMP not only in vesicle docking (Broadie et al. 1995; Hunt et al. 1994) but also in targeted vesicle transport (Dascher et al. 1991; Ossig et al. 1991). In these studies, mutational changes in the supposed sorting domain lead to an alternative subcellular localization (Ferro-Novick and Jahn 1994; Gerst 1997;

Grote et al. 1995). Even earlier it has been proposed that transport pathways between distinct compartments require their own subset of SNARE components (Calakos et al. 1994; Grote et al. 1995; Parlati et al. 2000; Sollner et al. 1993) and that compartmental specificity is encoded in SNARE proteins (McNew et al. 2000). While the highly conserved hydrophilic domain of VAMP located in the cytosol serves as a binding site for syntaxin thereby acting as a fusion mediator (Archer et al. 1990; Elferink et al. 1989), the function of its C-terminus is not completely understood. Since the C-terminal end that is predicted to extend into the vesicle lumen comprises only a few amino acids, no isoform specific antibodies could be generated so far that could distinguish the VAMP-1 isoforms (Elferink et al. 1989; Sudhof et al. 1989). A VAMP-1 isoform, referred to as VAMP-1b, which differs from the original VAMP-1a in its C-terminal sequence was previously characterized in mammals (Mandic et al. 1997). It has been demonstrated that it is generated by alternative RNA splicing, a process that earlier has been described for VAMP homologues in *Drosophila melanogaster* (Chin et al. 1993). VAMP-1a and -1b show tissue-specific expression levels thereby pointing to a tissue specific function of these isoforms (Mandic et al. 1997). The first evidence that the C-terminus of different VAMP-1 isoforms may encode distinct sorting signals was provided by Isenmann and co-workers (Isenmann et al. 1998). Here the C-terminus was shown to function as a sorting signal in a human VAMP-1 isoform (VAMP-1B), which is different from rat VAMP-1b, since this VAMP-1 isoform was targeted to mitochondria instead of endosomal-like structures and the plasma membrane as observed by the same group. In the present study, we evaluated potential differences between the subcellular localization of the rat VAMP-1 isoforms VAMP-1a and -1b by using fluorescence microscopy techniques on GFP- and RFP-tagged VAMP-1 proteins in conjunction with immunocytochemistry.

Furthermore we used total internal reflection fluorescence (TIRF) microscopy to study the localization of VAMP-1a and -1b in living cells.

Materials and methods

Generation of VAMP-1 expression vectors

A vector, containing the entire coding sequence of VAMP-1 was kindly provided by W. Trimble (University of Toronto and Division of Cell Biology, Toronto, Ontario, Canada) and was used as a template for subsequent PCR-based cloning procedures. HindIII and EcoRI restriction sites were introduced into the respective 5' and 3' primers. Since the first 371 nucleotides in both isoforms are identical, the same 5'-primer, containing a HindIII restriction site (5'-TTACAAGCTTCGATGTCTGCTCCAGCTCAGCCACCTGC-3'), was used for amplification of both isoforms. The VAMP-1a and -1b specific C-terminal sequence was included in the respective primers at the 3'-end together with an EcoRI restriction site (VAMP-1a - reverse complement: 5'-AGAATTCGATCAAGTAAAAATGTAGATTACAATTA-3'; VAMP-1b - reverse complement: 5'-AGAATTCGATCAGCGATACTTACTTACAATTACTACCACGATGATGG-3'). All primers were obtained from Invitrogen GmbH (Karlsruhe, Germany). PCR was carried out with a thermocycler from Biometra (Model T Professional, Göttingen, Germany), with the following settings: 96°C x 1min, 60°C x 2min, 72°C x 3min (40 cycles) and 72°C x 15min. PCR products were run on a 1.2% agarose gel followed by gel-purification (QIAGEN® Gel Extraction Kit, QIAGEN, Hilden, Germany) according to the manufacturers' instructions. Fluorescence protein encoding vectors (pEGFP-C1, pDsRed-Monomer-C1) were from Clontech Laboratories, Inc. (Mountain View, CA, USA). The purified PCR products and both vectors were double-digested

with HindIII and EcoRI (New England Biolabs, Ipswich, MA, USA) and gel purified. Subsequently vectors and products were treated with T4 DNA ligase (New England Biolabs) to generate GFP-VAMP-1a, GFP-VAMP-1b, RFP-VAMP-1a and RFP-VAMP-1b encoding expression vectors. Selection was performed after transformation of DH5 α competent cells (Life Technologies GmbH, Darmstadt, Germany). Plasmids were purified with the QIAGEN Plasmid Midi kit. All constructs were sequenced by a commercial provider (4BaseLab, Reutlingen, Germany).

Cell culture and transfection

HeLa, COS-7 and MDCK cells (Gluzman 1981; Scherer et al. 1953; Gaush et al. 1966) were cultured in DMEM (Dulbecco's Modified Eagle Medium) in the presence of 10% FCS, 1% L-glutamine, 100 U/l penicillin and 100 μ g/ml streptomycin. Cell culture medium was replaced every three days. Cells were grown on cover slips, placed in 6-well-plates until reaching 90% confluence followed by transfection with 4 μ g of the VAMP-1 encoding GFP/RFP plasmids using Lipofectamine® 2000 (Life Technologies GmbH).

Antibodies

Anti-VAMP-1 rabbit polyclonal antibody (ab3346) and GFP mouse monoclonal antibody (ab1218) were purchased from Abcam (Cambridge, UK). Mouse monoclonal antibodies directed against beta-actin (A5316) were purchased from Sigma-Aldrich (Munich, Germany), and DsRed rabbit polyclonal antibody (632496) from Clontech Laboratories, Inc.. The anti-Rab4 (610888) and anti-Rab11 (610657) antibodies were from BD Biosciences (Heidelberg, Germany) and the anti-EXOC3/Sec6 antibody (ADI-VAM-SV021, Stressgen®) from Enzo Life Sciences, Inc. (Farmingdale, NY, USA). The anti-mouse Alexa Fluor 647 antibody (A-21235,

Invitrogen™) was purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA). All other antibodies were obtained from Santa Cruz Biotechnologies Inc. (Santa Cruz, CA, USA): Anti-TOM20 (sc-17764, mouse monoclonal), donkey anti-mouse IgG-Rhodamine (sc-2300), goat anti-mouse IgG-horseradish peroxidase (sc-2005), goat anti-rabbit IgG-horseradish-peroxidase (sc-2054).

Western Blot analysis

Cells were trypsinized and collected in 4 ml DMEM, followed by pelleting at 350 g for 5 min. After washing the pellet in PBS and centrifugation, the supernatant was discharged. For sample preparation, the pellet was solved in two (pellet) volumes of lysis buffer (137mM NaCl, 20mM TrisHCl/pH 7.5, 10% glycerol, 1% Nonidet P-40, containing protease and phosphatase inhibitors), followed by incubation for 30 min at 4°C. After centrifugation for 15 min (13,000 rpm, 4°C), the supernatant was collected followed by measuring the protein concentration (Bradford 1976). 5-40 µg of protein per lane was separated on a 15% SDS-Gel (Laemmli 1970). Proteins were subsequently transferred to a nitrocellulose membrane (Towbin et al. 1979). After transfer, membranes were incubated in blocking buffer (TBS, 0.1% Tween-20, 5% non-fat dry milk) for 30 min. Primary antibodies (anti-VAMP-1, anti-GFP, anti-DsRed, anti-beta-actin) were incubated over night at 4°C in blocking buffer. Subsequently, membranes were washed thrice for 5 min in blocking buffer, followed by addition of the secondary HRP (horse radish peroxidase)-coupled antibody (1:2000, 1h at RT). Membranes were washed thrice as described above followed by addition of ECL (enhanced chemiluminescent) solution (Thermo Fisher Scientific) to each membrane and subsequent exposure to x-ray film (Optimax Typ TR, PROTEC, Obererstenfeld, Germany).

Fluorescence Microscopy

For fluorescence microscopy, involving GFP or RFP expressing plasmids only, transfected cells were washed twice for 5 min in phosphate buffered saline (PBS) 48h after of transfection followed by fixation for 5 min in ice-cold (-20°C) methanol. Subsequently, cells were washed twice in PBS and once in distilled water followed by covering cells with Mountain Dew Fluorescence Medium (DakoCytomation, Hamburg, Germany). If immunocytochemistry was used in addition, cells were washed thrice in PBS, fixed in precooled (-20°C) methanol for 5 min, followed by 3x PBS washing. After 15 min incubation in staining buffer (PBS, 3%BSA, 0.3% NP40), primary antibodies (anti-rab4, anti-rab11, anti-EXOC3/Sec6, anti-TOM20) were applied in the same buffer at a dilution of 1:250. After 3x washing, secondary antibodies (anti-mouse, anti-rabbit or anti-goat antibodies, conjugated to Rhodamine (1:500) or Alexa Fluor 647 (1:250) were added for 1h, followed by a washing step in staining buffer, PBS, 1µg/ml DAPI (in H₂O) for 10min and finally in distilled water. Subcellular localization was evaluated by confocal fluorescence microscopy (Axio Imager M2, Carl Zeiss GmbH, Jena, Germany) using the AxioVision Rel. 4.8 software. 48 hours after transfection, cells were evaluated by TIRF microscopy (LEICA DMI 6000 B microscope, LEICA Microsystems GmbH, Wetzlar, Germany). Conventional confocal microscopy: GFP was excited at 488 nm (AR⁺-laser) and emission was monitored at 500-520 nm. RFP and Rhodamine were excited with a HeNe-laser at 543 nm and emission was detected at 560-620 nm. To visualize Alexa 647, the specimens were exposed to 633 nm (excitation) light (HeNe-laser) and emission was detected at 645-675 nm. TIRF microscopy: 488 nm (300 mW) and 532 nm (500 mW) solid-state lasers were used for excitation of GFP and RFP, respectively. The corresponding filter cube settings are shown in supplementary Fig. 1. The penetration depth of the evanescent light was 110 nm. The total length of the

series shown in Figures 3 and 4 was 5min and 17.141s. The total number of exposures in this series was 182 corresponding to 91 frames, each frame comprising of images from 2 channels which were recorded successively (GFP: exposure time=277ms, gain=2.7 and RFP: exposure time=932ms, gain=5.0). Fluorescent beads were used for technical validation of the TIRF microscopical settings (see supplementary Fig. 1). Subsequent image analysis was performed with the LAS AF Version 2.6.0 software (LEICA Microsystems GmbH).

Results

Expression of GFP- and RFP-tagged VAMP-1a and -1b proteins in COS-7 and HeLa cells was evaluated by Western blot analysis and immunocytochemistry (Fig. 1). All constructs expressed proteins of predicted size exhibiting immunoreactivity to antibodies directed against GFP, RFP as well as VAMP-1 (Fig. 1A). Transfection of HeLa cells with the same constructs revealed a strong and distinct intracellular-vesicular and plasma membrane fluorescence for all VAMP-1 fusion proteins (Fig. 1B). To evaluate the level of colocalization between the different VAMP-1 isoforms, GFP-VAMP-1a and RFP-VAMP-1b or GFP-VAMP-1b and RFP-VAMP-1a encoding plasmids were cotransfected into HeLa cells with subsequent analysis by fluorescence microscopy (Fig. 2A). As expected for VAMP-1 proteins, a major overlap of green and red fluorescence was seen in regions consistent with the endoplasmic reticulum (ER) and Golgi apparatus. In peripheral regions of the cell, vesicular structures could be seen that carried only one of the two tested isoforms, being visible in only one of the channels (GFP or RFP) (Fig. 2A, arrows). After cotransfecting same but differently tagged VAMP-1 isoforms (GFP-VAMP-1a and RFP-VAMP-1a or GFP-VAMP-1b and RFP-VAMP-1b), an extensive overlap of the fluorescence signals could be seen as expected (Fig. 2B). Similarly as in Fig. 2A,

vesicular structures were observed that appeared to carry only one of the fusion proteins, either GFP- or RFP-VAMP-1. However, when looking at the single fluorescence channels (GFP and RFP) in Fig. 2B, the differently tagged identical VAMP-1 proteins are found to reside in the same vesicular structure (arrow), which was not obvious when looking only at the merged image. Therefore, different N-terminal GFP- and RFP-tags do not appear to have a major influence on VAMP-1 sorting. However, due to the limitations of studies using fixed cells no final conclusion about the extent of colocalization of both VAMP-1 isoforms was possible. To overcome these limitations, total internal reflection fluorescence (TIRF) microscopy was deployed to investigate vesicle movement in living COS-7 cells, cotransfected with both VAMP-1 isoforms. Next to regions of colocalization, distinct red vesicles could be observed carrying mainly the VAMP-1b isoform, thereby pointing to the presence of a vesicle population to which VAMP-1b preferentially sorts (Fig. 3A, see also supplemental file S1_movie). Tracking several of the vesicles (1-6) over time reveals major differences in the extent of vesicle motility (Fig. 3B). In addition, vesicles could be observed coming into close vicinity to each other without obvious fusion (Fig. 4A, see supplemental file S2_movie), whereas other vesicles contact each other followed by subsequent fusion (Fig. 4B, see supplemental file S3_movie). Interestingly, not only vesicular but also tubular structures, positive for both isoforms, could be seen during TIRF microscopy (Fig. 3A and 4) possibly representing near-membrane endosomal structures.

VAMP-1a and VAMP-1b do not colocalize with mitochondria.

No colocalization of VAMP-1a and -1b was seen with the mitochondrial marker TOM20 (Fig. 5A and B), largely excluding sorting of any of the two isoforms to mitochondria. This observation is in line with observations made for the human

isoform of rat VAMP-1a as reported by Isenmann and colleagues (Isenmann et al, 1998).

Resemblance of the C-terminus of VAMP-1b with peroxisomal targeting signal 1 (PTS-1)

Since the C-terminal sequence -SKYR as found in VAMP-1b shows a striking similarity to peroxisomal targeting signal-1 (PTS-1) -SKL, we were interested in the question if -SKYR could also act as a PTS. Addition of the known PTS1 to the C-terminus of GFP, results in sorting of the GFP-fusion protein to peroxisomes (Fig. 6A, upper image). However, addition of the similar C-terminal sequence present in VAMP-1b (-SKYR) to GFP did not result in peroxisomal targeting of the protein but rather exhibited a diffuse cyto- and nucleoplasmic distribution typically seen for wild type GFP (Fig. 6A, lower image). Since membrane association of the VAMP-1 protein could be a prerequisite for proper sorting, RFP plasmids encoding full length VAMP-1 proteins were cotransfected with the GFP-SKL encoding plasmid. No colocalization of any of the two VAMP-1 splice isoforms could be seen with the peroxisomal marker protein GFP-SKL, making it unlikely that the C-terminal sequence -SKYR in VAMP-1b serves as a peroxisomal sorting signal (Fig. 6B).

Co-staining of VAMP-1a and -1b with markers of the secretory pathway.

To evaluate, if VAMP-1a and -1b show a compartment-specific preference for sorting, we performed co-staining of cells transfected with one of the VAMP-1 isoforms and markers of the endocytic (rab4 and rab11) and exocyst (EXOC3/Sec6) compartments. No obvious major co-localization of the VAMP-1 isoforms with rab4 (supplementary Fig. 2) or rab11 (supplementary Fig. 3) was noted in our experimental settings. When co-staining with EXOC3/Sec6 (supplementary Fig. 4),

there mainly was no overlap (white arrows) except for single minor regions pointing to possible co-localization (supplementary Fig. 4, asterisks). Similarly, preliminary studies with markers of the lysosomal (LAMP-3), early endosomal (EEA1) and autophagosomal (LC3 β) compartments could not exclude a minor co-localization of both VAMP-1 isoforms with these cellular regions (data not shown).

Furthermore, to evaluate if inhibition of membrane transport and subsequent release (Matlin and Simons 1983) differentially affected VAMP-1a and -1b, we incubated MDCK cells, co-expressing GFP-VAMP-1a and RFP-VAMP-1b, for 5h at 20°C in the presence of 100 μ M cycloheximide to inhibit *de novo* protein synthesis. Since both VAMP-1 isoforms are expected to accumulate in the Golgi, one could expect that after releasing the block (incubation at 37°C) VAMP-1a and -1b would possibly more clearly show sorting into different compartments than when observed during regular culture at steady state conditions. One hour after raising the incubation temperature to 37°C, which is known to be sufficient for releasing the membrane block, we could not see a dramatic change in localization that would point to a major effect on VAMP-1 sorting (supplementary Fig. 5). However, since these are only preliminary observations, less pronounced subtle effects on sorting cannot be judged within the scope of these experimental settings.

Discussion

The potential role of the new C-terminal sequence -SKYR found in the rat splice isoform VAMP-1b could not be unraveled yet. Since the variable C-terminus in VAMP-1 was implicated in protein sorting (Isenmann et al. 1998), the present study used fluorescence microscopy-based techniques to evaluate differences in the subcellular distribution of the rat VAMP-1b isoform in comparison to the initially described isoform VAMP-1a.

The C-terminus of VAMP-1 as a possible sorting signal

Isenmann and colleagues described a human VAMP-1 splice variant (Isenmann et al. 1998) which similarly to the reported rat VAMP-1 splice variant (Mandic et al. 1997) differs from the initially reported VAMP-1 protein (Trimble et al. 1988) at its short C-terminal end that is predicted to extend into the vesicle lumen. The mechanism of alternative subcellular targeting by generating isoforms with a differing C-terminus was reported earlier for Syntaxin 2 (Quinones et al. 1999). Interestingly, Isenmann and colleagues could demonstrate that the very C-terminal sequence -RRD of their newly discovered isoform VAMP-1B serves as a sorting signal for VAMP-1B to mitochondria. Similarly, mitochondrial sorting of cytochrome b5 is mediated by a C-terminal hydrophobic motif flanked by basic amino acids (De Silvestris et al. 1995). Using fluorescence microscopy, we could rule out a significant targeting of any of the two rat VAMP-1 isoforms to mitochondria.

Similarity of the C-terminal sequence in VAMP-1b with the sorting signal of peroxisomal proteins

Peroxisomal proteins carry signals that ensure their proper targeting to peroxisomes. One of these signals -SKL (peroxisomal targeting signal 1, PTS1), is located at the C-terminus of peroxisomal proteins (Subramani 1998) and exhibits a striking similarity with the C-terminal sequence -SKYR found in VAMP-1b. Addition of the C-terminal sequence -SKL to a protein like GFP is sufficient for peroxisomal targeting (Monosov et al. 1996). One of the differences between these two sequences however is, that peroxisomal matrix proteins carrying the PTS1 do not need to be membrane attached to be targeted to peroxisomes. In contrast, VAMP-1b proteins are membrane associated and carry the C-terminal sequence -SKYR, which

is expected to be directed to the vesicle lumen (Elferink et al. 1989; Sudhof et al. 1989). In our studies, neither GFP-SKYR nor full length VAMP-1b could sort to peroxisomes as visible after cotransfection with the peroxisomal marker GFP-SKL. In a similar manner, peroxisomal proteins which are membrane bound are primarily inserted into membranes of the ER before reaching their target organelle, as shown for Pex15p (Elgersma et al. 1997) or peroxisomal APX (Mullen and Trelease 2000). Although sorting of VAMP-1b to peroxisomes appears unlikely, the similarity of both sequences supports the notion that -SKYR could represent a protein sorting signal. Furthermore, -SKYR may yet turn out to be a PTS but under conditions that have yet to be identified.

Other compartments and vesicle fusion.

According to the SNARE-hypothesis, for membrane fusion to proceed, each membrane requires a transmembrane domain of a SNARE protein, defining the v-SNARE containing membrane as the donor and the t-SNARE containing membrane as the acceptor compartment (Bock et al. 2001; Fasshauer et al. 1998). In early and late endosomes, homotypic fusion events are a common observation (Antonin et al. 2000). Vesicle contact can result in complete fusion but can also proceed without final fusion exhibiting so-called “kissing” or “kiss and run” phenotypes characterized by temporary fusion events and exchange of vesicle content with subsequent separation of the vesicles lacking a final complete fusion step (Luzio et al. 2009). An example for the regulation of an intracellular fusion event by exchange of a certain VAMP isoform for another was given by Pryor and colleagues (Pryor et al. 2004), who demonstrated how the exchange of VAMP-8 for VAMP-7 in a complex together with Vti1b/Syntaxin-8 could mediate a switch from heterotypic fusion of late endosomal and lysosomal vesicles to homotypic fusion between late endosomes. In

our studies, we observed vesicles containing both VAMP-1 isoforms on same vesicles that were capable of fusing with each other. At the same time, there were single vesicles carrying only one of both VAMP-1 isoforms. Although a function of VAMP-1 in this context has not been described to date, such a role appears possible since other isoforms as VAMP-7 and VAMP-8 were allocated to lysosomal membranes and even to the phagosomal compartment (Advani et al. 1999; Fasshauer et al. 1999; Furuta et al. 2010). It seems conceivable that *in vivo*, the subcellular localization of proteins could be crucial for the formation of SNARE complexes, mediated by regulatory components as tethering factors for example (Parlati et al. 2000). Since only one of the two VAMP-1 splice isoforms dominates in a single tissue (Mandic, et al., 1997), it is possible that next to cellular functions which both isoforms have in common, thereby substituting for each other, there also is an isoform specific function that could be required in a specific tissue, e.g. VAMP-1a in neural and VAMP-1b in non-neural tissues (Mandic, et al., 1997).

Implications of VAMP-1 and its splice isoforms in disease

In a 2007 study, the authors could relate a nonsense mutation in the VAMP-1 gene to a so-called lethal wasting mutant in the mouse, which is characterized by progressive immobilization and death approximately 2 weeks after birth without showing histopathological cerebral changes (Nystuen et al. 2007). The functional role of VAMP-1 is assumed to be related to calcium dependent transmission within the neuromuscular junction (Liu et al. 2011). Furthermore, expression levels of VAMP-1 were found to be associated with diverse neurological disorders and reactive pathological conditions such as hypoxia. For instance, generalized hypoxia in the brain tissue results in elevated VAMP-1 expression levels whereas another SNARE protein, Syntaxin 1, is suppressed under these conditions (Fei et al. 2007). In a study

on Alzheimer patients, VAMP-1 was the only tested SNARE protein that was found to be down regulated in all of the investigated brain regions, whereas the t-SNAREs SNAP-25 and syntaxin exhibited normal expression levels (Sze et al. 2000). VAMP-1 appears reduced in the superior temporal gyrus of schizophrenia patients, which is required for speech recognition (Sokolov et al. 2000). Next to motoneurons, VAMP-1 is also expressed in sensory neurons where it is involved in CGRP (*calcitonin-gene-related-peptide*) secretion that plays a role in migraine related vasodilation and can be inhibited by botulinum toxin (Meng et al. 2007). All of these reports refer to the role of the initially discovered VAMP-1 isoform (murine VAMP-1a or human VAMP-1A). Interestingly, in a 2012 study by Bourassa et al., defective mRNA splicing of the VAMP-1 gene was implicated as an underlying cause for hereditary spastic ataxia (HSA) 1 (Bourassa et al. 2012). In this study, the authors identified a mutation at the splicing donor site of exon 4 in the VAMP-1 gene to be responsible for the lack of VAMP-1a transcripts in the brain, which is the dominant VAMP-1 isoform in neural tissues. Instead, transcripts corresponding to VAMP-1b (-1D in humans) were generated, which the authors interpreted as a *loss of-function mutation* of the VAMP-1 gene. Lack of VAMP-1A in the brain stem and cerebellum (Elferink et al. 1989; Trimble et al. 1990) could therefore explain the classic symptoms of the disease, which not only include movement disorders of the lower extremities but also such of the eyes and tongue. However, alternative expression of a VAMP-1b (-1D) like isoform in the central nervous system of patients with spastic ataxia could not be excluded. In this case, one could hypothesize that VAMP-1b (-1D) proteins do not fully substitute for VAMP-1a (-1A), which supports a tissue specific function of both isoforms and could also be associated with the altered cellular function because of differential subcellular sorting of the isoforms.

In the present study we could further narrow down differences in the subcellular localization of both VAMP-1 isoforms. However, more studies are required to further look into the functional role of the very C-terminus in VAMP-1 and its possible implication in VAMP-1 protein sorting.

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Fig. 1. Expression of GFP- and RFP tagged VAMP-1a and -1b proteins in COS-7 and HeLa cells. GFP- and RFP-VAMP-1 constructs were generated as described in the material and methods section. (A) Expression of the different constructs was evaluated by Western blot analysis of lysates derived from COS-7 cells transfected with the indicated constructs. Single bands could be observed after deploying antibodies directed against GFP or RFP (upper and middle panel in A). Specific bands, corresponding to the size of the GFP and RFP fusion proteins, were observed when using a VAMP-1 specific antibody (lower panel in A). Here an additional smaller sized band (*) was observed that is not seen on the GFP and RFP blots and likely represents a cleavage product. (B) Confocal microscopy images of methanol-fixed HeLa cells transfected with the indicated constructs reveals a vesicular cytoplasmic fluorescence pattern as well as plasma membrane localization of all four fusion

proteins. Abbreviations: U=untransfected, V1=VAMP-1, G=GFP, Ga=GFP-VAMP-1a, Gb=GFP-VAMP1b, Ra=RFP-VAMP-1a, Rb=RFP-VAMP-1b.

Fig. 2. Indications for differential sorting of VAMP-1a and -1b in HeLa cells. HeLa cells were transfected with the indicated constructs and fixed in methanol as described in the materials and methods section. (A) Coexpression of GFP- and RFP-tagged VAMP-1a and -1b isoforms reveals major regions of overlap, consistent with the expected colocalization of both isoforms in the ER and Golgi. However, in more peripheral regions of the cell, vesicular structures are seen that appear to carry only one of the isoforms (Merge), which becomes more obvious, when comparing the single green (GFP) and red (RFP) channels showing a lack of the respective structure seen in the merged image in one of the single channels (arrows in GFP-V1a/RFP-V1b and GFP-V1b/RFP-V1a). (B) After control cotransfections of same VAMP-1 isoforms (GFP-V1a/RFP-V1a and GFP-V1b/RFP-V1b), vesicular structures showing only red or green fluorescence in the merged images can be observed too. However, when comparing the single channels, the same structure can also be found positive for the differentially tagged same VAMP-1 isoform with a weaker signal (arrows), implying that same VAMP-1 isoforms are sorted to the same compartment independently of their N-terminal (GFP or RFP) tag. Confocal microscopy images of methanol-fixed HeLa cells.

Fig. 3. Evidence for single vesicle populations carrying predominantly one VAMP-1 isoform. (A) Deployment of total internal reflection fluorescence (TIRF) microscopy on vital COS-7 cells cotransfected with GFP-VAMP-1a (GFP-V1a) and RFP-VAMP-1b (RFP-V1b) reveals vesicles that mainly contain one VAMP-1 isoform (red vesicles 1-6). The intense signal seen in the center of the cell (asterisk) could represent a near

plasma membrane accumulation of GFP/RFP-rich structures or regions of epifluorescence rather than TIRF. (B) The extent of vesicle movement varies between different vesicles within the observed time frame. Some of the vesicles (1 and 5) remain rather stationary whereas others exhibit a marked movement appearing to meet in distinct areas (2-4 and 6).

Fig. 4. Vesicle interaction depicted by TIRF microscopy. (A) Vesicle contact without fusion. Shown is a detail from the same experiment as in Fig. 3 (orange rectangle labeled A in Fig. 3A). Depicted are two vesicles (1 and 2), each carrying mainly one of the two VAMP-1 isoforms, that come into close vicinity and appear to contact each other. No vesicle fusion can be observed. (B) Evidence for homotypic fusion events of vesicles carrying both VAMP-1 isoforms (monitored region corresponds to white rectangle labeled B in Fig. 3A). TIRF microscopy reveals vesicles carrying both VAMP-1 isoforms (1 and 2) that fuse with each other after coming into close vicinity, resulting in the generation of a larger-sized single vesicle (1+2). RFP-V1b=RFP-VAMP-1b, GFP-V1a=GFP-VAMP-1a.

Fig. 5. VAMP-1a and -1b are not localizing to mitochondria. (A) No colocalization is seen between GFP-VAMP-1a or -1b (GFP-V1a or -V1b) and the mitochondrial marker protein TOM20. (B) Detail, showing the lack of colocalization of both VAMP-1 splice isoforms and mitochondria. Confocal microscopy images of methanol-fixed HeLa cells. Arrows in (A) and (B) point to mitochondria (red) and VAMP-1 (green)

Fig. 6. VAMP-1a and -1b do not sort to peroxisomes. (A) Attaching the C-terminal peroxisome targeting signal 1 (PTS1) -SKL to GFP results in sorting of the GFP-SKL protein to peroxisomes in CHO cells (Monosov et al. 1996) (upper image, typical

punctate fluorescence pattern), whereas attachment of the similar C-terminal sequence -SKYR that is found in VAMP-1b did not result in peroxisomal targeting (lower image, diffuse cyto- and nucleoplasmic fluorescence pattern). (B) Similarly, no colocalization was seen between RFP-VAMP-1a (RFP-V1a) or RFP-VAMP-1b (RFP-V1b) respectively and the peroxisome marker GFP-SKL in HeLa cells. Confocal microscopy images of methanol-fixed CHO and HeLa cells respectively.

Supplementary Figure 1

Comparison of TIRF and epifluorescence using fluorescent beads (Flow Check Pro Fluorospheres, 10µm, Beckman Coulter). Shown are the wavelengths (nm) used for excitation (Ex), emission (Em) and by the dichroic (DC) filter. Note the differences between evanescent light induced fluorescence (TIRF) and epifluorescence.

Supplementary Figure 2

No major colocalisation (white arrows) of the early and sorting endosome marker rab4 with the VAMP-1 splice isoforms VAMP-1a and -1b is seen in methanol-fixed MDCK cells. Confocal microscopy images.

Supplementary Figure 3

No major colocalisation (white arrows) of the recycling endosome marker rab11 with the VAMP-1 splice isoforms VAMP-1a and -1b is seen in methanol-fixed MDCK cells. Confocal microscopy images.

Supplementary Figure 4

Possible colocalization of the VAMP-1 splice isoforms with EXOC3/Sec6 positive vesicles. Most of the EXOC3/Sec6 positive vesicles do not show any colocalization

with the two VAMP-1 isoforms (white arrows). However, sporadically one can observe regions of potential overlap (yellow signal, asterisks). Confocal microscopy images of methanol-fixed MDCK cells.

Supplementary Figure 5

Inhibition and subsequent release of membrane transport does not seem to differently affect VAMP-1a and VAMP-1b distribution in MDCK cells. VAMP-1 transfected MDCK cells were grown under standard culture conditions (DMEM, 10% FCS, 37°C, 5% CO₂) and were then shifted to 20°C (blocking membrane transport) in the presence of 100µM cycloheximide (CHX) (blocking new protein synthesis=pulse) for 5h. Subsequently cells were shifted to 37°C to allow for membrane transport to proceed (Matlin and Simons 1983). No major differences can be seen when comparing the colocalization of untreated cells (37°C, no CHX) with cells 1h after release of the membrane transport block (t=60min @ 37°C + 100µg/ml CHX). Confocal microscopy of methanol-fixed MDCK cells.

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Draft

Fig. 1

A

B

GFP-V1a/RFP-V1b

GFP-V1b/RFP-V1a

Biochemistry and Cell Biology

GFP-V1a/RFP-V1a

GFP-V1b/RFP-V1b

A

GFP

RFP

Dapi

Merge

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B

Fig. 2

A

B

Fig. 3

A

B

A

GFP-SKL

GFP-SKYR

B

GFP

RFP

Dapi

Merge

Fig. 6