11

Analysis of SNARE-Mediated Exocytosis Using a Cell Fusion Assay

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1. Introduction

Exocytosis is the fusion of transport vesicles with the plasma membrane. By exocytosis, eukaryotic cells secrete soluble proteins and endogenous chemicals to the extracellular space, and deliver new membrane proteins and lipids to the plasma membrane. A large body of work has demonstrated that the interactions of SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) proteins on vesicles (v-SNAREs) and on target membranes (t-SNAREs) catalyze intracellular vesicle fusion events, including exocytosis (Bonifacino and Glick, 2004; Jahn et al., 2003; Rothman, 1994) (Fig. 1). The vesicle-associated membrane proteins (VAMPs), *i.e.*, VAMPs 1, 2, 3, 4, 5, 7 and 8, are v-SNAREs that reside in various post-Golgi vesicular compartments, and have been implicated in exocytosis. In this chapter, we review recent progress of using a novel cell fusion assay to analyze the specificity and membrane fusion activities of VAMPs (Hasan et al., 2010).

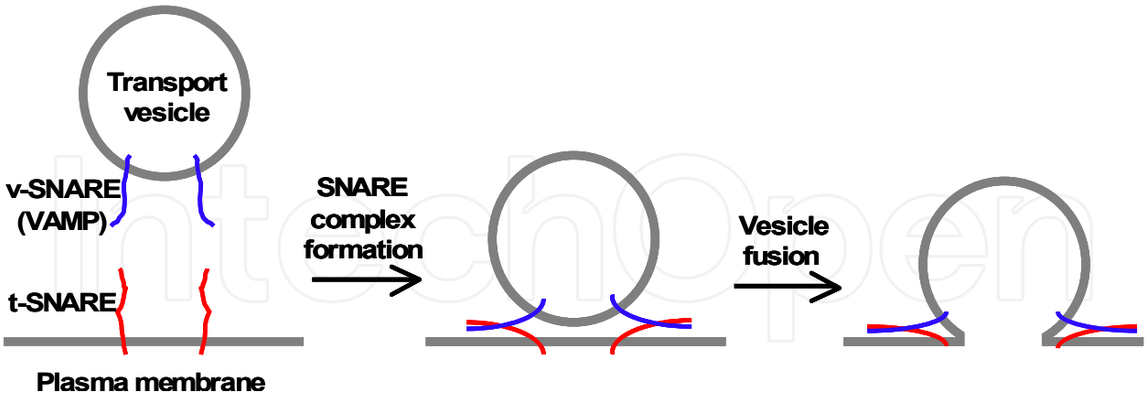

Fig. 1. Interactions of VAMPs and plasma membrane t-SNAREs drive exocytosis.

2. SNAREs – Core machinery of vesicle fusion

SNAREs are cytoplasmic oriented type I membrane proteins. SNAREs share one homologous domain, the 'SNARE motif,' which contains eight heptad repeats ready for coiled-coil formation. The SNARE proteins that mediate the fusion of synaptic vesicles with the presynaptic plasma membrane are well studied (Sollner et al., 1993b). In synapses, the v-

SNARE VAMP2 resides in synaptic vesicles, whereas t-SNAREs syntaxin1 and synaptosomal-associated protein of 25 kD (SNAP-25) are located in the plasma membrane. Syntaxin1 and SNAP-25 constitute an acceptor complex for VAMP2 (Fasshauer and Margittai, 2004). The cytoplasmic domains of VAMP2, syntaxin1 and SNAP-25 form an extremely stable complex that is resistant to sodium dodecyl sulfate (SDS) (Hayashi et al., 1994) and heat stable up to ~90°C (Yang et al., 1999), indicating that SNARE complex formation is thermodynamically favorable. One α-helix from VAMP2, one α-helix from syntaxin1 and two α-helices from SNAP-25 intertwine to form a four-helix bundle (Sutton et al., 1998). Assembly of SNARE complexes is initiated at the N-termini and proceeds to the transmembrane domains at the C-termini in a zipper-like fashion (Stein et al., 2009). When v- and t-SNARE proteins are incorporated into liposomes, they spontaneously drive liposome fusion (McNew et al., 2000b; Weber et al., 1998), demonstrating that SNAREs form the minimal machinery for membrane fusion. Using a cell fusion assay, we showed that v- and t-SNARE proteins ectopically expressed on the cell surface spontaneously drive cell-cell fusion (Hu et al., 2003; Hu et al., 2007), providing further proof that SNAREs form the core machinery for intracellular membrane fusion. After membrane fusion, the adapter protein SNAP (soluble NSF attachment protein) and the ATPase NSF (N-ethylmaleimide-sensitive factor) dissociate v-/t-SNARE complexes at the expense of ATP (Mayer et al., 1996; Sollner et al., 1993a) to free SNAREs for the next round of fusion.

Genomic analysis indicates that there are 36 SNAREs in humans (Bock et al., 2001). Individual members of the SNARE family localize to distinct subcellular organelles (Chen and Scheller, 2001), suggesting that each SNARE has a selective role in vesicle trafficking. Using yeast SNARE proteins as models, a series of experiments showed that to a remarkable degree the specificity of intracellular membrane fusion can be predicted from the pattern of liposome fusion mediated by isolated v- and t-SNARE proteins (McNew et al., 2000a; Parlati et al., 2002). However, membrane fusion by SNAREs in mammalian cells is more promiscuous (Brandhorst et al., 2006; Shen et al., 2007). Here we show that with the exception of VAMP5, VAMPs are essentially redundant in mediating membrane fusion with plasma membrane t-SNAREs (Hasan et al., 2010).

3. Roles of VAMPs in exocytosis

VAMPs have been implicated in vesicle fusion with the plasma membrane, the *trans*-Golgi network (TGN) and endosomes. VAMP1 (synaptobrevin 1) and VAMP2 (synaptobrevin 2) mediate regulated exocytosis in neurons and endocrine cells (Hanson et al., 1997; Kesavan et al., 2007; Morgenthaler et al., 2003). In addition, VAMP2 is involved in the exocytosis of the water channel aquaporin 2 (Procino et al., 2008) and α5β1 integrin (Hasan and Hu, 2010), as well as insulin-stimulated translocation of the glucose transporter GLUT4 (Randhawa et al., 2000). Enriched in recycling endosomes and endosome-derived vesicles (Galli et al., 1994; McMahon et al., 1993), VAMP3 (cellubrevin) mediates the recycling of transferrin receptors to the cell surface (Galli et al., 1994), integrin trafficking (Luftman et al., 2009; Proux-Gillardeaux et al., 2005; Skalski and Coppolino, 2005), and the secretion of α-granules in platelets (Feng et al., 2002; Polgar et al., 2002). Present primarily in the TGN, VAMP4 participates in the transport between the TGN and endosomes (Mallard et al., 2002; Steegmaier et al., 1999), as well as in homotypic fusion of early endosomes (Brandhorst et al., 2006). Expressed in muscle cells, VAMP5 (myobrevin) is associated with the plasma

membrane and intracellular vesicles (Zeng et al., 1998). In addition to vesicular transport from endosomes to lysosomes (Advani et al., 1999), the tetanus neurotoxin-insensitive VAMP (VAMP7) is involved in apical exocytosis in polarized epithelial cells (Galli et al., 1998; Pocard et al., 2007). Associated with early endosomes (Advani et al., 1998; Wong et al., 1998), VAMP8 (endobrevin) is required in regulated exocytosis in pancreatic acinar cells (Wang et al., 2004).

VAMPs have high sequence homology in their SNARE motifs (Fig. 2). All VAMPs possess a conserved arginine residue at the center of SNARE motifs (Fig. 2), and have been classified as R-SNAREs based on crystal structures (Fasshauer et al., 1998). The N-terminal 51 residues of VAMP4 contain a dominant signal for targeting to the TGN, while the SNARE motif of VAMP5 is responsible for its targeting to the plasma membrane (Zeng et al., 2003). In VAMP7, the N-terminal 'longin domain' regulates subcellular targeting (Pryor et al., 2008).

VAMPs 3, 4, 7 and 8 have broad tissue distribution (Advani et al., 1998; McMahon et al., 1993). Originally identified in nervous tissues, VAMPs 1 and 2 are also detected in skeletal muscle, fat and other tissues (Jagadish et al., 1996; Martin et al., 1998; Procino et al., 2008;

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VAMP1           ------------------------------------------------------------
VAMP2           ------------------------------------------------------------
VAMP3           ------------------------------------------------------------
VAMP4           ------------------------------------------------------------
VAMP5           ------------------------------------------------------------
VAMP7           MAILFAVVARGTTILAKHAWCGGNFLEVTEQILAKIPSENNKLTYSHGNYLFHYICQDRI 60
VAMP8           ------------------------------------------------------------

VAMP1           ------------------------------MSAPAQPPAEGTEGTAPGGGPPGPPPNMTS 30
VAMP2           ------------------------------MSATAATAPP--AAPAGEGGPPAPPPNLTS 28
VAMP3           ------------------------------MST--------------------GPTAATGS 11
VAMP4           ------------MPPKFKRHLNDDDVTGSVKSERRNLLEDDSDEEEDFFLRGPSGPRFG 47
VAMP5           ------------------------------------------------------MAG 3
VAMP7           VYLCITDDDFERSRAFNFLNEIKKRFQTTYGSRAQTALPYAMNSEFSSVLAAQLKHHSEN 120
VAMP8           ------------------------------------------------MEEASEG 7
                                  SNARE motifs
VAMP1           --NRRLQQTQAQVEEVVDIIRVNVDKVLERDQKLSELDDRADALQAGASQFESSAAKLKR 88
VAMP2           --NRRLQQTQAQVDEVVDIMRVNVDKVLERDQKLSELDDRADALQAGASQFETSAAKLKR 86
VAMP3           --NRRLQQTQNQVDEVVDIMRVNVDKVLERDQKLSELDDRADALQAGASQFETSAAKLKR 69
VAMP4           PRNDKIKHVQNQVDEVIDVMQENITKVIERGERLDELQDKSESLSDNATAFSNRSKQLRR 107
VAMP5           ---IELERCQQQANEVTEIMRNNFGKVLERGVKLAELQQRSDQLLDMSSTFNKTTQNLAQ 60
VAMP7           KGLDKVMETQAQVDELKGIMVRNIDLVAQRGERLELLIDKTENLVDSSVTFKTTSRNLAR 180
VAMP8           GGNDRVRNLQSEVEGVKNIMTQNVERILARGENLEHLRNKTEDLEATSEHFKTTSQKVAR 67

VAMP1           KYWWKNCKMMIMLGAICAIIVVVIVRRD---------------------------- 116
VAMP2           KYWWKNLKMMIILGVICAIILIIIIVYFST-------------------------- 116
VAMP3           KYWWKNCKMWAIGITVLVIFIIIIIVWVVSS------------------------- 100
VAMP4           QMWWRGCKIKAIMALVAAILLLVIIILIVMKYRT---------------------- 141
VAMP5           KKCWENIRYRICVGLVVVGVLLIILIVLLVVFLPQSSDSSSAPRTQDAGIASGPGN 116
VAMP7           AMCMKNLKLTIIIIIVSIVFIYIIVSPLCGGFTWPSCVKK---------------- 220
VAMP8           KFWWKNVKMIVLICVIVFIIILFIVLFATGAFS----------------------- 100
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Fig. 2. Sequence alignment of human VAMP proteins. The conserved arginine residues in the center of SNARE motifs are labeled red.

Randhawa et al., 2000; Veale et al., 2010). Therefore, multiple VAMPs are co-expressed in mammalian cells. However, it is not clear if the seven VAMPs have differential membrane fusion activities. Using a cell fusion assay, we compare the membrane fusion activities of VAMPs.

3. Cell fusion assays

3.1 Expression of flipped SNAREs at the cell surface

By fusing the pre-prolactin signal sequence, which specifies translocation across the endoplasmic reticulum, to N-termini of the neuronal SNAREs VAMP2, syntaxin1 and SNAP-25, the cell fusion assay was originally developed in Dr. James Rothman's lab (Hu et al., 2003). The engineered SNAREs are called 'flipped' SNAREs because the orientation of their SNARE motifs against cellular membranes is flipped. A Myc tag is inserted between the signal sequence and N-termini of SNAREs to detect flipped SNARE proteins (Fig. 3A). When COS-7 cells are transfected with flipped SNARE constructs, flipped SNARE proteins are expressed at the cell surface (Fig. 3B). To express plasma membrane t-SNAREs, flipped syntaxins 1 or 4 are cotransfected with flipped SNAP-25. SNAP-25 does not contain a transmembrane domain. After expression, flipped SNAP-25 proteins are anchored to the cell surface by assembling with flipped syntaxin proteins.

Flow cytometry is used to measure the expression levels of SNARE proteins at the cell surface (Figs. 3C and D). When flipped VAMPs 1, 3, 4, 5, 7 and 8, and syntaxins 1 and 4 plasmids are transfected at the same concentration, cell surface expression of VAMPs 5 and 8 is higher than VAMPs 1, 3, 4 and 7, and cell surface expression of syntaxin4 is higher than syntaxin1 (Hasan et al., 2010). To express the v- and t-SNAREs at the same level, we optimized the concentration of each flipped SNARE plasmid used in transfection (concentrations see Section 4.2), so that VAMPs 1, 3, 4, 5, 7 and 8, and syntaxins 1 and 4 are expressed at the same level at the cell surface, respectively (Fig. 3D).

3.2 Microscopic cell fusion assay

Multiple readout systems have been developed to detect fusion of the cells that express flipped v-SNAREs (v-cells) and the cells that express flipped t-SNARE proteins (t-cells) (Hasan et al., 2010; Hu et al., 2003; Hu et al., 2007). In the microscopic assay shown in Fig. 4A, flipped v-SNARE constructs are cotransfected with a plasmid that encodes the green fluorescent protein EGFP. In t-cells, the flipped t-SNARE constructs are cotransfected with a plasmid that encodes the red fluorescent protein DsRed2. Fusion of the v- and t-cells results in fused cells that contain both EGFP and DsRed2. In the merged channel, the cytoplasm of the fused cells is yellow (arrows, Fig. 4A).

3.3 Enzymatic cell fusion assay

The microscopic cell fusion assay becomes less efficient when used to analyze multiple v-/t-SNARE combinations quantitatively. To develop a quantitative cell fusion assay, we take advantage of the strong transcriptional activation by binding of the tetracycline-controlled transactivator (tTA) to the tetracycline-response element (TRE) (Gossen and Bujard, 1992). Two plasmids in CLONTECH's Tet-Off gene expression system are used (Hasan et al., 2010). The first plasmid pTet-Off encodes the transcriptional activator tTA, and the second

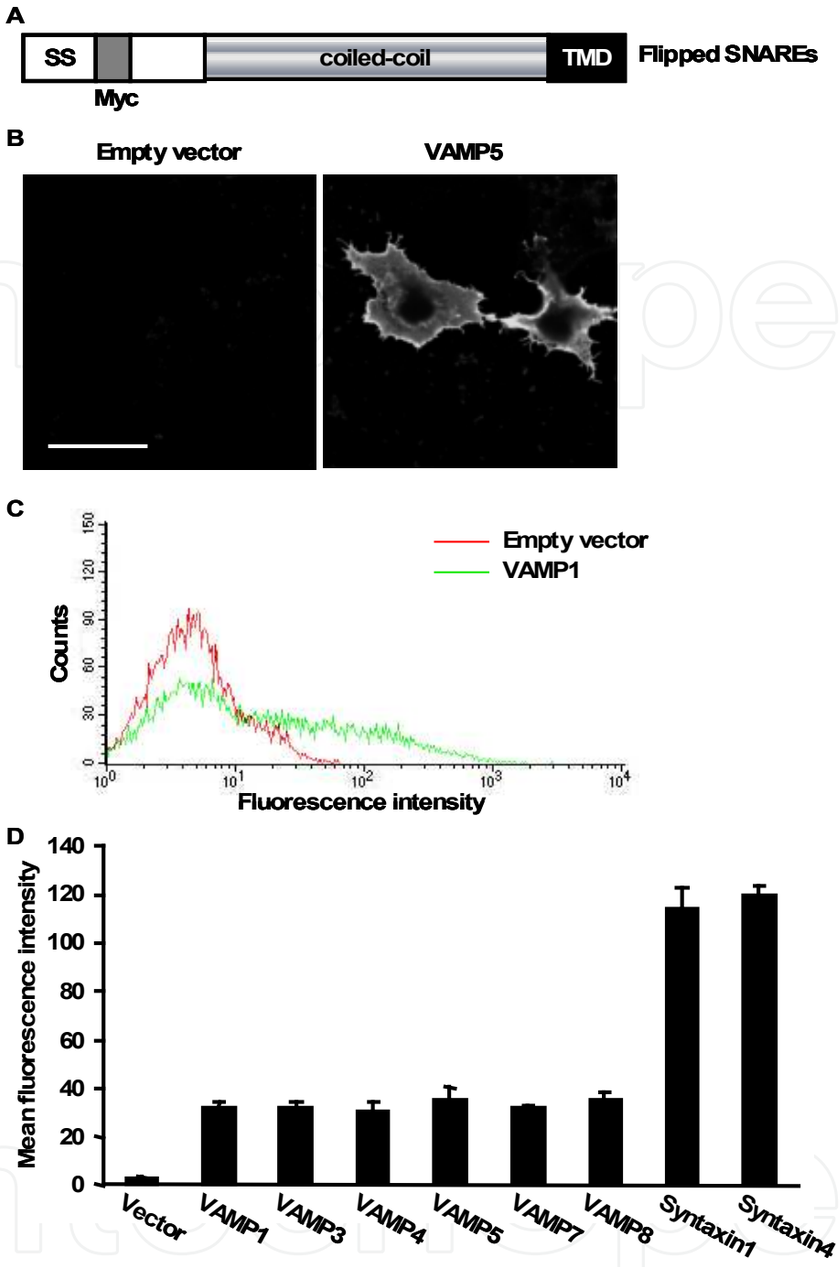

Fig. 3. Expression of flipped SNAREs at the cell surface. (A) Domain structure of flipped SNAREs. (B) Twenty-four hours after transfection with empty vector pcDNA3.1(+) or flipped VAMP5 plasmid, unpermeabilized COS-7 cells are stained with an anti-Myc antibody. Representative confocal images are shown. Scale bar, 50 μm. (C and D) Twenty-four hours after transfection with empty vector or flipped SNARE plasmids, unpermeabilized cells are stained with the anti-Myc antibody and analyzed by flow cytometry. (C) Representative FACS profiles of the cells transfected with empty vector or flipped VAMP1. (D) To express VAMPs and syntaxins at the same level at the cell surface, flipped SNARE plasmids are transfected at titrated concentrations. The mean fluorescence intensity of staining is obtained using CellQuest Pro software.

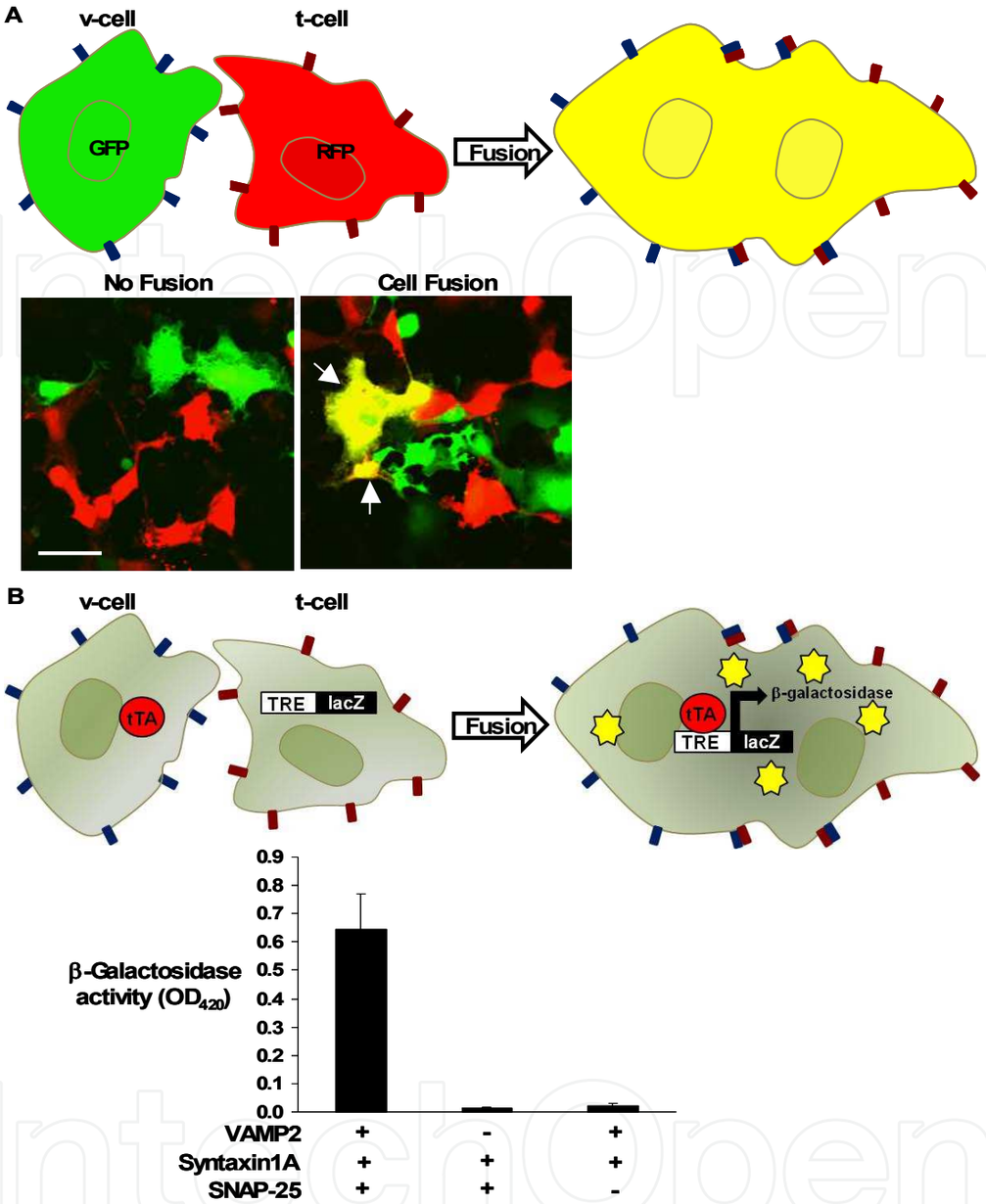

Fig. 4. Cell fusion assays. (A) Microscopic cell fusion assay. Cells that express flipped v-SNAREs (v-cells) are labeled by the green fluorescent protein EGFP, whereas cells that express flipped t-SNAREs (t-cells) are labeled by the red fluorescent protein DsRed2. Fusion of v- and t-cells results in fused cells (arrows) whose cytoplasm is yellow under fluorescence microscope. Scale bar, 50 μm. (B) Enzymatic cell fusion assay. The tetracycline-controlled transactivator (tTA) is expressed in v-cells, and a reporter plasmid that encodes β-galactosidase under control of the tetracycline-response element (TRE-LacZ) is transfected into t-cells. Fusion of the v- and t-cells leads to the binding of tTA to TRE and the expression of β-galactosidase, which is measured using a colorimetric method by absorbance at 420 nm. Only baseline β-galactosidase activity is detected when either flipped VAMP2 or SNAP-25 is not expressed.

plasmid pBI-G encodes the *LacZ* gene under control of the tetracycline-response element (TRE-*LacZ*). In the absence of tTA, transcription of the *LacZ* gene in TRE-*LacZ* is silent. When tTA is present, it binds to the TRE and activates the transcription of *LacZ*, resulting in the expression of β-galactosidase. We hypothesize that if tTA is located in v-cells and TRE-*LacZ* is located in t-cells, β-galactosidase will not be expressed. Fusion of the v- and t-cells would result in the binding of tTA to TRE and transcriptional activation of *LacZ* (Fig. 4B).

The neuronal SNAREs are used to test feasibility of the assay. VAMP2 is coexpressed with tTA in v-cells, and syntaxin1 and SNAP-25 are coexpressed with TRE-*LacZ* in t-cells. When the v- and t-cells are combined, robust β-galactosidase expression is indeed detected (Fig. 4B). However, when either VAMP2 or SNAP-25 is not expressed, only baseline β-galactosidase activity is detected, indicating that cell fusion and expression of β-galactosidase rely on interactions of v- and t-SNAREs.

3.4 Experimental procedures

3.4.1 Cell culture and reagents

COS-7 cells were obtained from the American Type Culture Collection, and cultured in Dulbecco Modified Eagle's Medium (DMEM) supplemented with 4.5 g/l glucose and 10% fetal bovine serum (FBS). The anti-Myc monoclonal antibody 9E10, developed by Dr. Bishop, was obtained from the Developmental Studies Hybridoma Bank maintained by the University of Iowa. pEGFP-N3, pDsRed2-N1, pTet-Off and pBI-G were obtained from CLONTECH. Plasmid transfection is done with Lipofectamine according to the manufacturer's instructions (Invitrogen).

3.4.2 FACS analysis

Expression levels of SNAREs at the cell surface are measured using immunostaining and flow cytometry as we previously reported (Hasan and Hu, 2010). Briefly, COS-7 cells are seeded in 6-well plates. Twenty-four hours after transfection with the flipped SNARE, pTet-Off and pBI-G plasmids, cells are fixed with 1% paraformaldehyde in PBS++ (PBS supplemented with 0.1 g/l $CaCl_2$ and 0.1 g/l $MgCl_2$). After labeling with the anti-Myc monoclonal antibody 9E10 and FITC-conjugated secondary antibodies, the cells are scraped off the plates with a cell scraper. 15,000 cells are analyzed using a FACSCalibur flow cytometer (BD Biosciences). The mean fluorescence intensity of each sample is obtained using CellQuest Pro software.

3.4.3 Microscopic cell fusion assay

The day before transfection, 1.2×10^6 COS-7 cells are seeded in each 100-mm cell culture dish, and 5×10^4 COS-7 cells are seeded on sterile 12-mm glass coverslips contained in 24-well plates. For v-cells, 5 μg of flipped v-SNARE and pEGFP-N3 are cotransfected into the cells grown in each 100-mm culture dish. For t-cells, 0.25 μg each of flipped syntaxin, SNAP-25 and pDsRed2-N1 are cotransfected into the cells seeded in the 24-well plates. Twenty-four hours after transfection, the v-cells are detached from culture dishes with EDTA (Enzyme-free Cell Dissociation Buffer (Invitrogen)). Detached cells are counted with a hemocytometer and resuspended in HEPES-buffered DMEM supplemented with 10% FBS,

6.7 μg/ml tunicamycin and 0.67 mM DTT. v-Cells (1.2×10^5) are added to each coverslip already containing the t-cells. After 24 hours at 37°C in 5% CO_2, the cells are gently washed once with PBS++, then fixed with 4% paraformaldehyde. Confocal images are collected on an Olympus laser scanning confocal microscope. The 488 nm argon laser line is used to excite EGFP and the 543 nm HeNe laser line is used to excite DsRed2. To prevent cross-contamination between EGFP and DsRed2, each channel is imaged sequentially before merging the images.

3.4.4 Enzymatic cell fusion assay

The day before transfection, 1.2×10^6 COS-7 cells are seeded in each 100-mm cell culture dish, and 2×10^5 COS-7 cells are seeded in each well of 6-well plates. For v-cells, 5 μg each of flipped v-SNARE plasmid are cotransfected with 5 μg of pTet-Off into the cells in each 100-mm culture dish. Control cells are cotransfected with empty vector pcDNA3.1(+) and pTet-Off. For t-cells, 1 μg each of flipped syntaxins 1 or 4, SNAP-25 and pBI-G are cotransfected into the cells in each well of the 6-well plates. There are putative N-glycosylation motifs (Asn-X-Ser/Thr) in VAMPs. Our previous studies (Hu et al., 2003; Hu et al., 2007) showed that N-glycosylation disrupts the function of flipped SNAREs, and that treatment of tunicamycin, an antibiotic that inhibits N-glycosylation (Elbein, 1984), restores the fusion activities of flipped SNAREs. To prevent N-glycosylation of VAMPs 1, 4, 5, 7 and 8, v-cells expressing these VAMP proteins and control cells are incubated in cell culture medium containing 10 μg/ml of tunicamycin during transfection. Since we have mutated the putative N-glycosylation sites in flipped VAMP2 (Hu et al., 2003) and VAMP3 proteins (Hu et al., 2007), v-cells that express VAMPs 2 or 3 are not treated with tunicamycin. Since flipped syntaxins 1 or 4, SNAP-25 proteins are not N-glycosylated (Hu et al., 2003; Hu et al., 2007), the t-cells are not treated with tunicamycin.

Twenty-four hours after transfection, the v-cells are detached from the culture dishes with Enzyme-free Cell Dissociation Buffer, and added (4.8×10^5 cells) to each well already containing the t-cells. After 6, 12 or 24 hours at 37°C in 5% CO_2, the expression of β-galactosidase is measured using the β-Galactosidase Enzyme Assay System with Reporter Lysis Buffer according to the manufacturer's instructions (Promega). The cells are washed twice with PBS, and then lysed in Reporter Lysis Buffer. Cell lysates are mixed with equal volume of Assay 2× Buffer. As a blank control, Reporter Lysis Buffer is mixed with Assay 2× Buffer. After 90 minutes, the colorimetric reaction is stopped by adding 1 M sodium carbonate, and absorbance at 420 nm is measured using a HITACHI 100-40 spectrophotometer.

4. Membrane fusion by VAMPs and plasma membrane t-SNAREs

4.1 Fusogenic interactions of VAMPs and plasma membrane t-SNAREs

Using the enzymatic fusion assay (Fig. 4B), we examine the fusogenic pairings between the seven VAMPs and two plasma membrane t-SNARE complexes, syntaxin1/SNAP-25 (Fasshauer and Margittai, 2004) and syntaxin4/SNAP-25 (Reed et al., 1999). Robust β-galactosidase expression is detected when VAMPs 1, 2, 3, 4, 7 or 8 are combined with syntaxin1/SNAP-25 (Fig. 5A) or syntaxin4/SNAP-25 (Fig. 5B), indicating that these VAMPs mediate membrane fusion with plasma membrane t-SNAREs. With syntaxin1/SNAP-25, the

six VAMPs drive fusion to a similar degree. With syntaxin4/SNAP-25, VAMP8 fuses less efficiently than VAMPs 1, 2, 3 and 4 (31% lower fusion activity and $P = 0.046$ vs. VAMP1, Fig. 5B). In contrast, when VAMP5 is combined with the t-SNAREs, only baseline β-galactosidase activity is detected (Figs. 5A and B), suggesting that VAMP5 does not drive membrane fusion with the t-SNAREs. The stronger fusion activities of syntaxin4/SNAP-25 than syntaxin1/SNAP-25 (compare Figs. 5A and B) are likely caused by higher cell surface expression of syntaxin4/SNAP-25 than syntaxin1/SNAP-25 and higher fusion activity of syntaxin4 than syntaxin1 (Hasan et al., 2010). Together, the data shown in Fig. 5 indicate that VAMPs 1, 2, 3, 4, 7 and 8, but not VAMP5, drive membrane fusion when partnering with plasma membrane t-SNAREs.

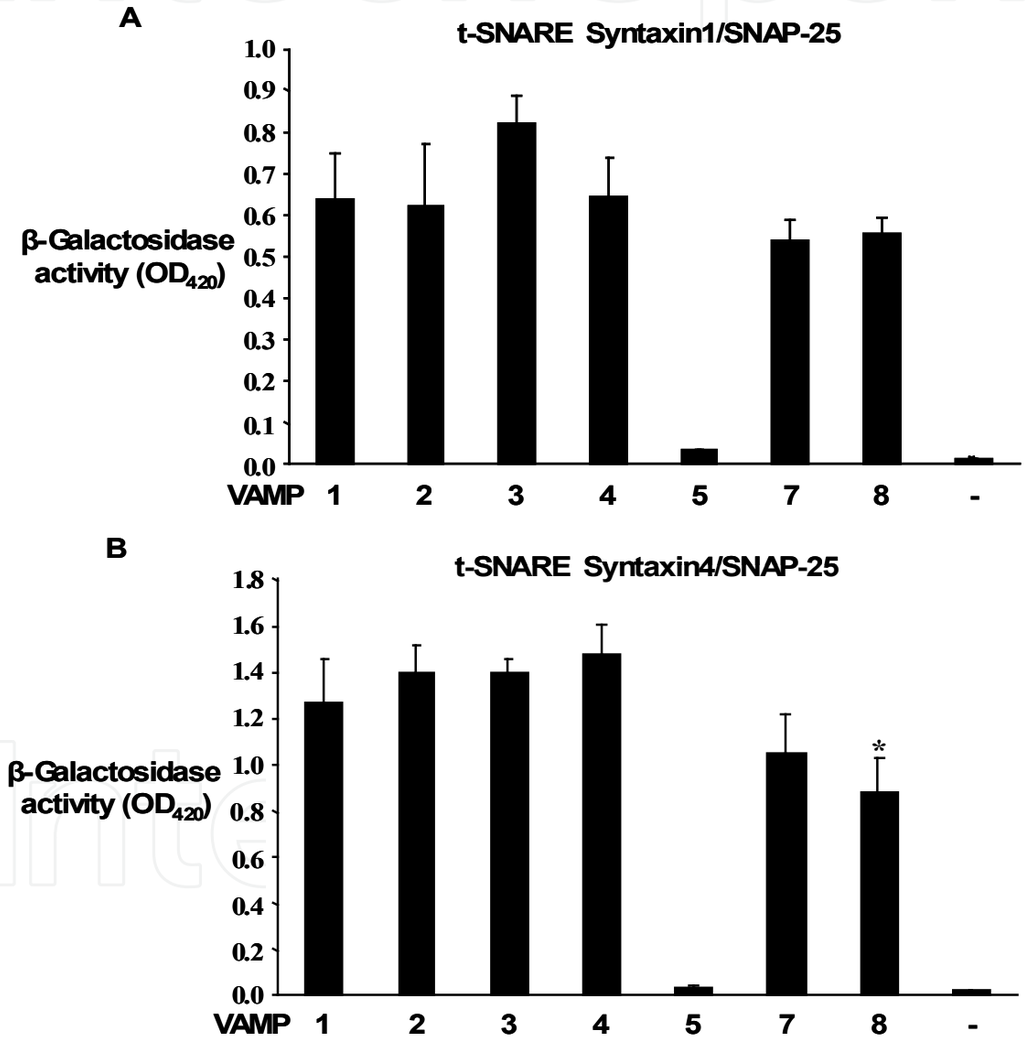

Fig. 5. Cell fusion by VAMPs and plasma membrane t-SNAREs. Twenty-four hours after combining v-cells that express VAMPs 1, 2, 3, 4, 5, 7 or 8 and t-cells that express (A) syntaxin1/SNAP-25 or (B) syntaxin4/SNAP-25, cell fusion is quantified using the enzymatic cell fusion assay. Control cells (-VAMP) are transfected with the empty vector. The flipped SNARE plasmids are transfected at the same concentration. Error bars represent standard deviation of three independent experiments. * $P < 0.05$ vs. VAMP1.

VAMPs 1, 2, 3, 7 and 8 are known to drive vesicle fusion with the plasma membrane. The above data provide additional evidence that vesicles that carry either one of these five VAMPs are capable of fusing with the plasma membrane. Since these VAMPs are functionally redundant, they may compensate each other in loss of function studies (Borisovska et al., 2005).

To rule out the possibility that the baseline β-galactosidase activity detected in the VAMP5 combinations (Fig. 5) is caused by residual membrane fusion activity of VAMP5, we perform the microscopic cell fusion assay, which analyzes individual cell fusion events and can detect rare fusion events. In multiple experiments, no cell fusion is observed in the microscopic assay when VAMP5 is combined with syntaxin1/SNAP-25 or syntaxin4/SNAP-25, whereas VAMP4 drives fusion efficiently with both t-SNARE complexes (Hasan et al., 2010) (data not shown). Together, the results using enzymatic and microscopic cell fusion assays suggest that VAMP5 is unable to mediate membrane fusion with the plasma membrane t-SNAREs.

VAMP5 is expressed in muscle cells, in which it is mainly associated with the plasma membrane as well as intracellular vesicles (Zeng et al., 1998; Zeng et al., 2003). It is still possible that VAMP5 forms fusogenic interactions with other plasma membrane t-SNAREs to mediate exocytosis in muscle cells. Indeed, SNAP-23, a ubiquitously expressed SNAP-25 homolog, is expressed in skeletal muscle (Bostrom et al., 2010). The role of VAMP5 in exocytosis needs further investigation.

4.2 Differential membrane fusion activities of VAMPs

Do VAMPs have differential membrane fusion capacities? An ideal experimental system to answer this question will require equal expression of VAMP proteins and a quantitative membrane fusion assay. Using anti-Myc staining and flow cytometry as measurement (Figs. 3C and D), we express VAMP proteins at the same level at the cell surface. Furthermore, using the quantitative enzymatic cell fusion assay, we compare their membrane fusion activities.

When COS-7 cells are transfected with the flipped SNARE plasmids at the same concentration, cell surface expression of VAMPs 5 and 8 is more than 2 fold higher than VAMPs 1, 3, 4 and 7, and cell surface expression of syntaxin4 is 1.8 fold higher than syntaxin1 (Hasan et al., 2010) (data not shown). To express the v- and t-SNAREs at the same level, we titrate and optimize the concentration of each flipped SNARE plasmid used in transfection. Flipped SNARE plasmids are transfected at the following concentrations (per 10 cm^2 growth area, *i.e.*, per well in 6-well plates): VAMP1, 0.2 μg; VAMP3, 0.5 μg; VAMP4, 0.5 μg; VAMP5, 0.05 μg; VAMP7, 1.0 μg; VAMP8, 0.1 μg; syntaxin1, 0.5 μg; syntaxin4, 0.05 μg. tTA, TRE-LacZ and flipped SNAP-25 are cotransfected at 1 μg per 10 cm^2 growth area. Under such conditions, VAMPs 1, 3, 4, 5, 7 and 8 are expressed at same level at the cell surface, while syntaxins 1 and 4 are expressed at the same level (Fig. 3D). Because the flipped VAMP2 protein does not contain a Myc tag (Hu et al., 2003), we are not able to compare its expression level with the other VAMPs.

After expressing VAMPs 1, 3, 4, 5, 7 and 8 at the same level, we compare their membrane fusion activities using the enzymatic cell fusion assay. With syntaxin1/SNAP-25, VAMPs 1, 3, and 8 have comparable and the highest fusion activities, whereas VAMPs 4 and 7 have 50% and 30% lower fusion activities, respectively (Fig. 6A). With syntaxin4/SNAP-25,

VAMPs 1 and 3 have comparable and the highest fusion activities, whereas VAMPs 4, 7 and 8 have 36%, 26% and 54% lower fusion activities, respectively (Fig. 6B). As expected, only baseline β-galactosidase activity is detected when VAMP5 is paired with the t-SNAREs. These data indicate that VAMPs have differential membrane fusion activities with plasma membrane t-SNAREs. However, when expressed at higher levels, VAMP4 drives membrane fusion as efficiently as VAMPs 1 and 3 (Fig. 5). Since the differences of fusion activities among the fusogenic VAMPs are within a factor of 2, these results imply that with the exception of VAMP5, VAMPs are essentially redundant in mediating membrane fusion with plasma membrane t-SNAREs.

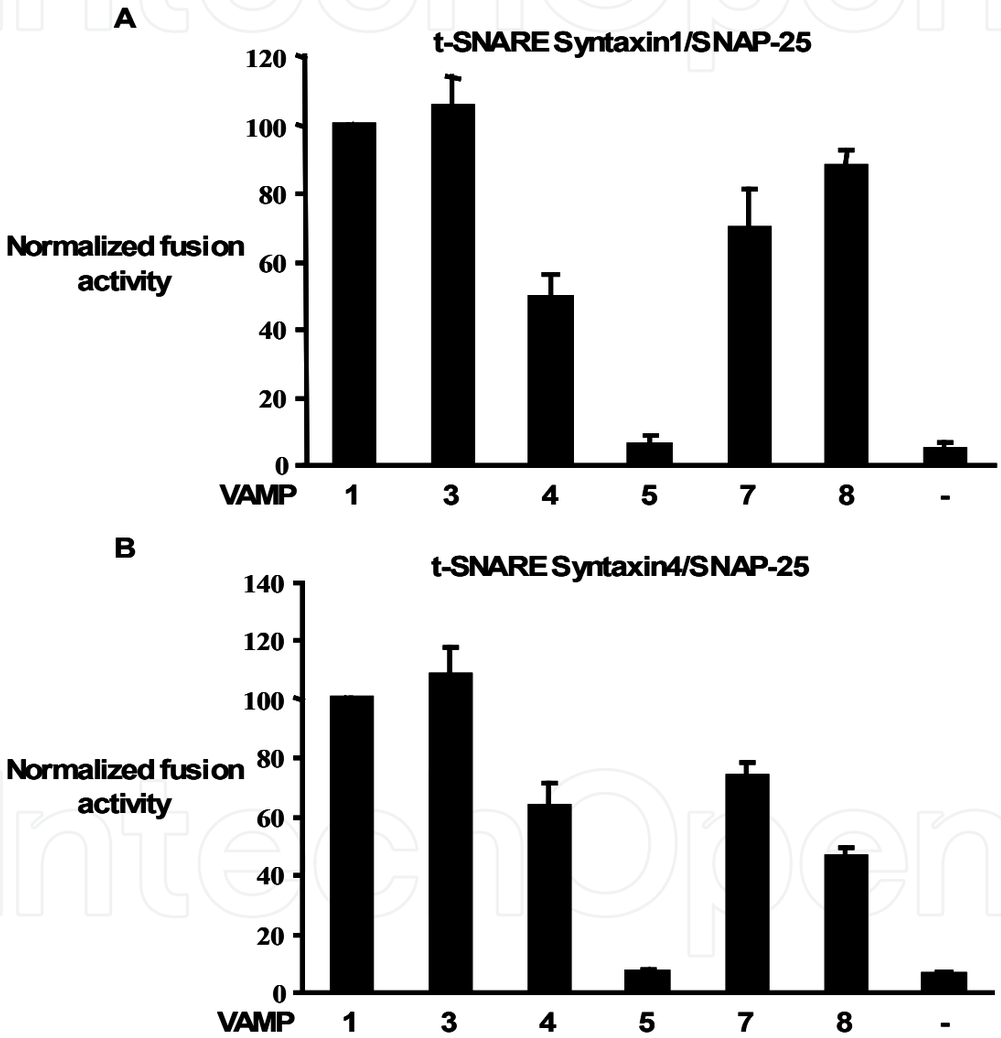

Fig. 6. Comparison of fusion activities of VAMPs. With optimized transfection conditions, VAMPs 1, 3, 4, 5, 7 and 8 are expressed at the same level at the cell surface, while syntaxin1/SNAP-25 (A) and syntaxin4/SNAP-25 (B) are expressed at the same level. Cell fusion is quantified using the enzymatic fusion assay. The fusion activities (OD_{420}) of control cells (-VAMP) and the v-cells expressing VAMPs 3, 4, 5, 7 or 8 are normalized to the fusion activity of the v-cells expressing VAMP1. Error bars represent standard deviation of four independent experiments.

4.3 Cooperativity of VAMP proteins in the cell fusion reaction

The number of SNARE complexes that cooperate to mediate vesicle fusion is under active investigation. To investigate the cooperativity of VAMPs in membrane fusion, we determine the dependence of cell fusion activity on cell surface expression level of VAMP1. We choose VAMP1 in this experiment because it has high membrane fusion activity (Fig. 6). In t-cells, the cell surface expression levels of syntaxin1 and SNAP-25 are kept constant. v-Cells are transfected with increasing concentrations of the flipped VAMP1 plasmid. At each concentration, we measure the cell surface expression level of VAMP1 proteins using flow cytometry, and determine cell fusion activity of VAMP1 with syntaxin1/SNAP-25 using the enzymatic fusion assay. Cell fusion activity is then plotted as a function of the mean fluorescence intensity of VAMP1 staining (Fig. 7A). The correlation is best fit with a polynomial regression. The hyperbolic instead of sigmoidal correlation (Fig. 7A) suggests that there is no cooperativity of VAMP1 proteins in driving cell fusion.

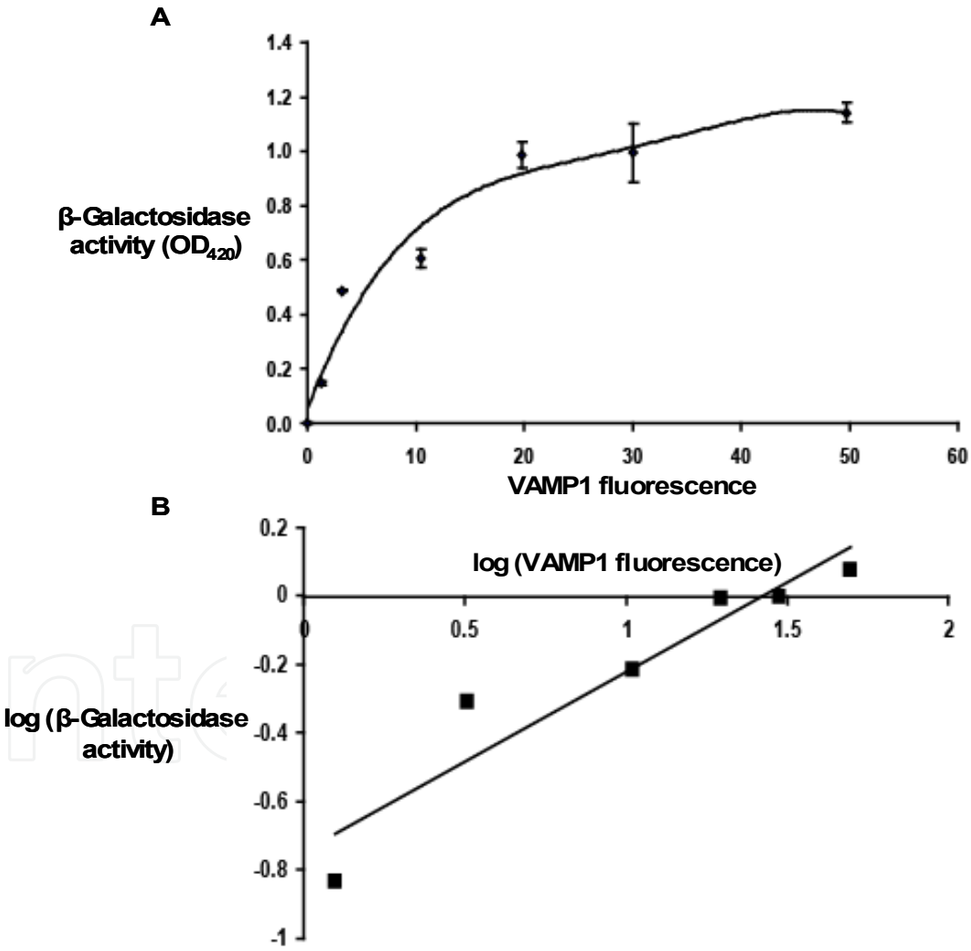

Fig. 7. Dependence of cell fusion activity on cell surface density of VAMP1. (A) v-cells expressing increasing amount of VAMP1 at the cell surface are combined with t-cells expressing syntaxin1/SNAP-25. Cell fusion activities are quantified and correlated with the mean fluorescence intensity of VAMP1 staining. (B) Log-log plot of cell fusion activity vs. mean fluorescence intensity of VAMP1 staining.

The log-log plot has been used to determine the cooperativity of viral fusion proteins in membrane fusion (Danieli et al., 1996). Using log-log plot, we further analyze the cooperativity of VAMP1 proteins. If two VAMP1 (V) proteins take part in the cell fusion reaction, *i.e.*, V + V → Fusion, the rate of fusion (F) = k $[V]^2$. Therefore, log (F) = log (k) + 2 log [V], and the slope of the resulting log-log plot will be 2. A log-log analysis of the dependence of cell fusion activity on VAMP1 cell surface density is shown in Fig. 7B. Linear regression is performed to model the log-log correlation (Fig. 7B), and the resulting slope is 0.52. These results further suggest that the cell fusion reaction does not involve concerted action of VAMP1 proteins.

Previous studies have estimated that 1 to 11 SNARE complexes are needed for membrane fusion (Domanska et al., 2009; Hua and Scheller, 2001; Karatekin et al., 2010; van den Bogaart et al., 2010). In the cell fusion reaction, we do not observe cooperativity of VAMP1 proteins, suggesting that concerted action of multiple SNARE complexes is not required to fuse cellular membranes. However, to achieve fast exocytosis in intact cells, concerted action of multiple SNARE complexes is clearly needed (Mohrmann et al., 2010). Such cooperativity of SNARE complexes may be organized by the binding of regulatory proteins such as synaptotagmins and Munc18 (Chicka et al., 2008; Shen et al., 2007).

5. Conclusion

To examine v-/t-SNARE interactions quantitatively, we developed an enzymatic cell fusion assay that utilizes activated expression of β-galactosidase and spectrometric measurement. Using this assay, we show that VAMPs 1, 2, 3, 4, 7 and 8 mediate membrane fusion efficiently with plasma membrane t-SNAREs syntaxin1/SNAP-25 and syntaxin4/SNAP-25, whereas VAMP5 does not drive fusion with the t-SNAREs. By expressing VAMPs 1, 3, 4, 7 and 8 at the same level, we further compare their membrane fusion activities. VAMPs 1 and 3 exhibit comparable and the highest fusion activities, whereas VAMPs 4, 7 and 8 have 30 - 50% lower fusion activities. Collectively, these data indicate that VAMPs have differential membrane fusion activities, and imply that with the exception of VAMP5, VAMPs are essentially redundant in mediating membrane fusion with plasma membrane t-SNAREs. Furthermore, no cooperativity of VAMP1 proteins is observed in the cell fusion reaction, suggesting that concerted action of multiple SNARE complexes is not required to fuse cellular membranes.

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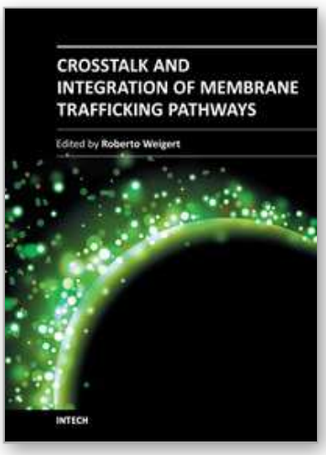

Crosstalk and Integration of Membrane Trafficking Pathways
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Membrane traffic is a broad field that studies the complex exchange of membranes that occurs inside the cell. Protein, lipids and other molecules traffic among intracellular organelles, and are delivered to, or transported from the cell surface by virtue of membranous carriers generally referred as "transport intermediates". These carriers have different shapes and sizes, and their biogenesis, modality of transport, and delivery to the final destination are regulated by a multitude of very complex molecular machineries. A concept that has clearly emerged in the last decade is that each membrane pathway does not represent a close system, but is fully integrated with all the other trafficking pathways. The aim of this book is to provide a general overview of the extent of this crosstalk.

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