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Lipid Rafts Association of Synaptotagmin I on Synaptic Vesicles

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Abstract—We confirmed the raft association of synaptotagmin I (syt I) in synaptic vesicles by sucrose density gradient centrifugation, cholesterol depletion, and temperature dependence, and Ca^{2+} was found to positively regulate this association. Furthermore, using syt I mutants we found that the transmembrane domain (TMD) of syt I plays an important role in localizing syt I into the lipid rafts of synaptic vesicles, and the raft association of the TMD can be regulated by its phosphorylation status.

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Lipid rafts are a class of special lipid microdomains. They exist in the membranes of almost all eukaryotic cells [1]. They play important roles in many biological processes [1, 2]. Recent investigations also showed that lipid rafts are likely to perform a critical function in exocytosis [3-5]. The core-fusion machinery SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptors) proteins were found to be enriched in rafts [4], implying that rafts may regulate fusion events by organizing SNARE proteins.

Synaptotagmin I (syt I) is a constituent of synaptic vesicles in neurons and neuroendocrine cells. It has one transmembrane domain. Synaptotagmin I is believed to play a critical function as a calcium sensor in regulated neurotransmission [6]. But whether syt I is associated with raft is disputed [3, 5, 7]. And noticeably, all previously reported results were based on the lyses of synaptosomes or whole cells [4, 7]. The samples used in those studies were mixtures of synaptic vesicles, other organelles, and cell membranes. Thus, whether syt I has a lipid raft association on synaptic vesicles needs to be further clarified.

The goal of this study was to investigate the raft association of syt I on purified synaptic vesicles.

Abbreviations: MbCD) methyl-β-cyclodextrin; SNAP-25) synaptosomal-associated protein of 25 kD; SNARES) soluble N-ethylmaleimide-sensitive factor attachment protein receptors; syt I) synaptotagmin I; TMD) transmembrane domain of syt I; VAMP) vesicle-associated membrane protein.

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MATERIALS AND METHODS

Reagents. Antibodies against syt I and rabphilin were from BD Biosciences (USA). Antibody against Xpress was from Invitrogen (USA). Antibodies against VAMP2 and HRP-conjugated second antibody and also antibodies against rab6, calnexin, and synaptophysin were from Santa Cruz Corp. (USA). The Amplex Red cholesterol assay kit was from Molecular Probes (USA). The Na^+/K^+-ATPase assay kit was provided by Nanjing Jiancheng Bioengineering Institute (China). All other reagents were from Sigma (USA).

Plasmid constructs. The cDNA encoding rat syt I was kindly provided by T. C. Sudhof (Dallas, USA) [8]. The deletion mutant of syt I, ΔC2AB, was constructed by PCR using the following sets of primers with appropriate restriction enzyme sites (underlined): 5′-GGATCCATGGTGAGTGCCAGT-3′ (sense primer), 5′-GCGAATTCCTTGGGCTCGTCCTTTTCTT-3′ (antisense primer).

Three site-directed mutants of ΔC2AB, ΔC5A (Cys74, Cys75, Cys77, Cys79, and Cys82 to Ala), ΔT3A (Thr112, Thr125, and Thr128 to Ala), and ΔT3E (Thr112, Thr125, and Thr128 to Glu) were constructed by PCR-mediated overlapping using two subsequent PCR amplifications as described previously [9, 10] using the following oligonucleotides: 5′-CGCCGCCTTCGCTGTCGTAAGAAAGCTTTGTTCAAAAAGAAAAACAA-3′ (ΔC5A, sense), 5′-CAAAGCTTTCTTAGCGACAGCGAAGGCGGCGGTCACGACTAGAAGGAC-3′ (ΔC5A, antisense), 5′-TTAAGGATGACGATGCTGAAGCCGGACTGGCTGAT-3′ (ΔT3A, sense), 5′-GCATCGTCATC-

CTTAAGGGCCTGATCCTTCATGGCCTT-3′ (ΔT3A, antisense), 5′-GGCCCTTAAGGATGACGATGCTGA-AGAAGGACTGGAAGATGGAG-3′ (ΔT3E, sense), 5′-TTCAGCATCGTCATCCTTAAGGGCCTGATC-CTTCATTTCCTTCCC-3′ (ΔT3E, antisense).

All mutants were subcloned into the *BamH*I and *EcoR*I sites of the Xpress vector pcDNA4/HisMax C (Invitrogen) and confirmed by DNA sequencing.

Cell culture and transfection. The 293 cells were cultured in DMEM medium supplemented with 10% FCS, 100 units/ml of penicillin, and 100 μg/ml of streptomycin. Cells were incubated in a humidified atmosphere of 5% CO_2. Cells were transiently transfected using the calcium phosphate coprecipitation method. Then cells were harvested and analyzed two days post-transfection.

Preparation of synaptic vesicles from rat brain. Synaptic vesicles were purified using an established procedure [11]. Two 4-week-old Sprague–Dawley rat brains were used for each experiment. All procedures were performed at 4°C using pre-cooled reagents. For methyl-β-cyclodextrin (MbCD) or saponin treatment, the sample was solubilized for 30 min either in Mes with 10 mM MbCD or in 0.5% Triton with 0.5% saponin (v/v).

Detergent solubilization and sucrose gradient fractionation. Lipid rafts were isolated from synaptic vesicles as follows. The purified synaptic vesicles (2 mg protein) were solubilized with 1 ml ice-cold Mes-buffered saline (MBS) (25 mM Mes, 150 mM NaCl, pH 6.5) containing 1% Triton X-100 (v/v) in the presence of protease and phosphatase inhibitors and incubated at 4°C for 20 min. The solubilized synaptic vesicles were then homogenized using a Dounce glass–Teflon homogenizer. The homogenate was adjusted to 40% (w/v) sucrose by adding 80% sucrose in MBS buffer and overlayed successively with 30 and 5% sucrose with a ratio of 3 : 6 : 4 (5/30/40% v/v). After centrifugation at 240,000*g* for 18 h, thirteen fractions were collected from the top of the gradient with the addition of a deposit partition. Cytosolic proteins and solubilized membrane proteins remain in the 40% sucrose layer (fractions 11-13). Insoluble lipid rafts, on the other hand, have a lower buoyant density and float to the interface between the 30 and 5% sucrose layers (fractions 4-6). The influence of temperature was investigated using the same procedure but at 37°C instead of 4°C. The synaptic vesicles were also extracted by 4% Chaps or 1% Brij98 in Mes following the same procedure as described above, except that Brij98 treatment was carried out at 37°C. Lipid rafts were isolated using flotation of cell lysates on discontinuous sucrose density gradients as described [4], similar to the procedure of synaptic vesicle raft isolation. Cells ($5 \cdot 10^7$) were used for each experiment. Each experiment was repeated 3 times ($n = 3$).

Western blotting [12, 13]. Equal volumes of each fraction separated by the sucrose gradient fractionation were analyzed by SDS-PAGE (12% w/v), and then the gel was transferred to nitrocellulose membrane for western blotting analysis. The membranes were blocked for 1 h with Tris-buffered saline (TBS) (25 mM Tris, 100 mM NaCl, pH 7.4) supplemented with 0.1% Tween 20 (TBST) and 5% (w/v) defatted powdered milk at room temperature. Then the membranes were incubated with the corresponding primary antibody diluted in blocking buffer at 4°C overnight. After three washes with TBST, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody diluted in blocking buffer for 1 h. Blots were developed using ECL detection reagents (Santa Cruz Corp.) and were visualized using Kodak X-Omat K Film (Kodak, USA). Then blots were scanned using a scanner (Nikon LS-9000 ED FILM SCANNER) (Nikon, USA).

For the quantitative estimation of the raft-associated portion of syt I and mutants, syt I of PC12 cells or rat brain synaptic vesicles was immunoblotted with mouse monoclonal antibody against syt I (BD Biosciences) and mutants were detected with the mouse monoclonal antibody against the Xpress epitope tag. Fractions 4-6 were combined and defined as the detergent-resistant raft fraction (*R*); fractions 11-13 were combined and defined as the solubilized fraction (*S*). Equal volumes of the two fractions were analyzed by SDS-PAGE (12% w/v), and the gel was transferred to nitrocellulose membrane for western blotting analysis. Blots were quantified by computerizing the gray scale values with the ImageJ program (NIH Image software). Total immunoreactivity was calculated as the sum of the intensities of *R* and *S* (as these fractions contained the vast majority of syt I and mutants). The raft-associated portion was estimated from the following equation: $[R/(R + S)] \cdot 100\%$.

Statistical analysis [14]. The data are expressed as mean ± standard deviation. Statistical significance was calculated by Student's *t*-test. A probability value of $p < 0.05$ indicated a statistically significant difference.

RESULTS AND DISCUSSION

Synaptotagmin I has raft distribution on synaptic vesicles, with Ca^{2+} identified as a positive regulator. We studied the raft association of syt I on synaptic vesicles, because syt I is a synaptic vesicle protein and the biogenesis and the property of synaptic vesicles are different from other organelles. First, the purified synaptic vesicles were assessed by morphological criteria (electron microscopy analysis). Figure 1a shows uniform vesicles with a radius of around 50-100 nm, which is consistent with previous reports [11].

Second, Na^+/K^+-ATPase, the general plasma membrane marker, is absent from purified synaptic vesicles. We tested the plasma membrane contamination by measuring the enzyme activities of the Na^+/K^+-ATPase. Almost no activity of the Na^+/K^+-ATPase can be observed in the purified synaptic vesicles (data not shown). Therefore,

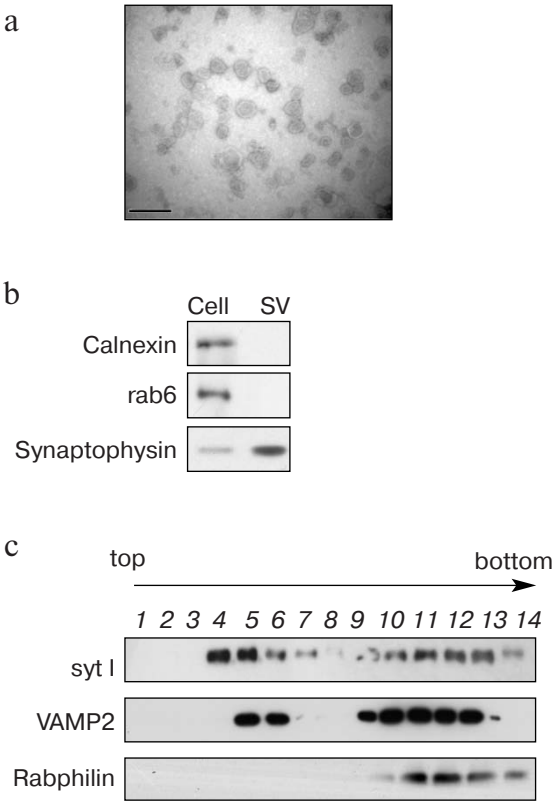

Fig. 1. Synaptotagmin I association with lipid rafts prepared from synaptic vesicles using Triton X-100. a) Electron micrograph of the purified synaptic vesicle fraction, with a bar of 200 nm. b) Integrated PC12 cells and the purified synaptic vesicles (SV) from rat brain were analyzed for calnexin, rab6, and synaptophysin by SDS-PAGE and immunoblotting. c) Syt I was associated with lipid rafts on synaptic vesicles. Equal volumes of the recovered fractions were separated by SDS-PAGE and blotted.

the contamination of plasma membrane in the synaptic vesicle portion is quite low. Furthermore, neither calnexin nor rab6, the marker for endoplasmic reticulum or Golgi apparatus, was significantly detected in the synaptic vesicle fractions (Fig. 1b). This method yields a fraction of synaptic vesicles that is virtually free of contamination by other organelles.

It is well known that the typical lipid rafts are detergent-insoluble membrane domains. So, they are also named detergent-resistant membranes. The detergent-resistant membranes on synaptic vesicles were analyzed by treatment with the nonionic detergent Triton X-100 at 4°C [15].

Upon solubilization in Triton X-100, a considerable amount of syt I (about 40%, $n = 3$) was found to be present in the rafts of purified synaptic vesicles (see Fig. 1c). Some other synaptic proteins were tested at the same time. Vesicle-associated membrane protein (VAMP2) was present in lipid rafts, which is in agreement with previous reports [4]. In contrast, rabphilin was not in the rafts (Fig. 1c). We have already tested the protein and cholesterol distribution after preparation of rafts (data not shown). The result showed a typical raft protein distribution and cholesterol enrichment like previous reports [4, 7].

When samples were lysed in Triton X-100 at 37°C instead of 4°C, the detergent-resistant membranes should be destroyed [16]. Figure 2a shows that syt I became completely soluble at the higher temperature (37°C), confirming its raft-associated property.

Lipid rafts are also defined as cholesterol-rich domains in membranes. Cholesterol is essential for the retention of lipid raft structures, so cholesterol depletion can disrupt rafts [15]. The cholesterol dependence of the raft distribution of syt I was then tested using MbCD and saponin, which deplete cholesterol via different mechanisms [4]. Figure 2b shows that the syt I distribution in the lipid rafts could be effectively eliminated by cholesterol depletion. Thus, syt I had the property expected of a raft protein.

Some researchers argued that Triton X-100 might promote the aggregation of membrane components [17]. To minimize the potential risk associated with the use of Triton X-100, we explored other detergents, Chaps and Brij98 [15], because Chaps and Brij98 are common detergents used for isolation of detergent-insoluble lipid rafts and are distinct from Triton X-100 in character. Chaps was selected because it is a zwitterionic detergent [18], while Triton X-100 is a nonionic detergent. Brij98 was

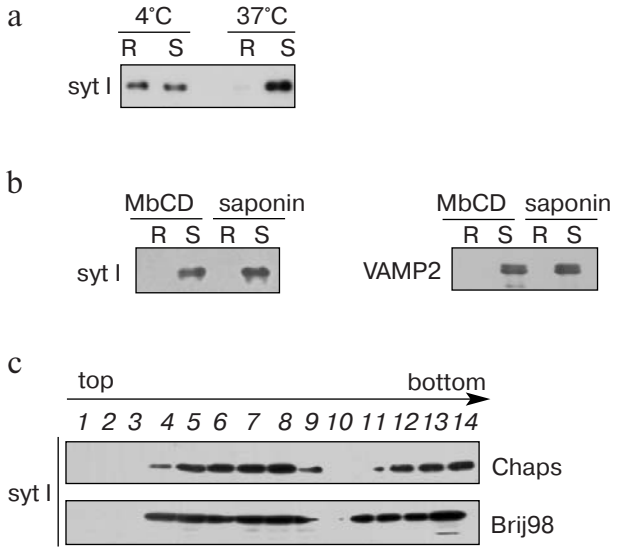

Fig. 2. Synaptotagmin I association with lipid rafts prepared from synaptic vesicles using different detergents. a) The influence of temperature on the raft association of syt I. Proteins in detergent-resistant raft (*R*) fractions (fractions 4-6) and solubilized (*S*) fractions (fractions 11-13) were detected by western blotting (37°C was applied with a control of 4°C). b) Cholesterol dependence of raft association of syt I and VAMP2. Cholesterol was depleted in 1% Triton X-100 with 10 mM MbCD (*MbCD*) or 0.5% Triton X-100 with 0.5% saponin (*saponin*). c) Syt I was associated with lipid rafts, which were prepared from synaptic vesicles using different detergents.

selected because it isolates lipid raft domains at 37°C and avoids the formation of nonspecific membrane aggregates at low temperature [19]. The raft isolation by Brij98 or Chaps showed that syt I was localized to the lipid raft microdomains of synaptic vesicles (Fig. 2c), which gave information consistent with that of Triton X-100 treatment. The similar phenomenon obtained with different types of detergents led to the conclusion that there are typical lipid rafts on synaptic vesicles and indicated that syt I associates with the synaptic vesicle lipid rafts.

Next we analyzed whether Ca^{2+} could modulate the raft association of syt I, since the function of syt I is highly related to Ca^{2+} influx [6, 20]. We found that the association of syt I with synaptic vesicle rafts exhibited a high degree of Ca^{2+} sensitivity. As shown in Fig. 3, the raft-associated portion of syt I was around 47 ± 2% (n = 3) when treated with Ca^{2+}, while the amount was only 27 ± 2% (n = 3) when treated with EDTA ($p < 0.05$). This Ca^{2+}-induced enrichment of syt I in rafts could be eliminated by cholesterol depletion with MbCD (data not shown), indicating that this association was still cholesterol dependent. Other synaptic proteins were tested at the same time. The results showed that neither the raft protein VAMP nor the non-raft protein rabphilin changed their distribution in the presence of Ca^{2+} (Fig. 3). So the increased association is likely caused by the specific interaction between syt I and Ca^{2+}.

Recent reports indicated that rafts might be fusion "hot spots". SNAREs and the Ca^{2+}-channel were concentrated in rafts [3, 4]. Munc-18 was localized outside of rafts to set free the t-SNARE for fusion [21]. SNARE proteins are proposed to form the structure of heteromeric complexes that are required for membrane fusion. Syt I is considered as the primary Ca^{2+} sensor for transmitter release. Therefore, the Ca^{2+}-triggered enrichment of syt I in rafts may up-regulate its availability at fusion sites. This may facilitate the fusion process. The detailed mechanism needs to be further explored.

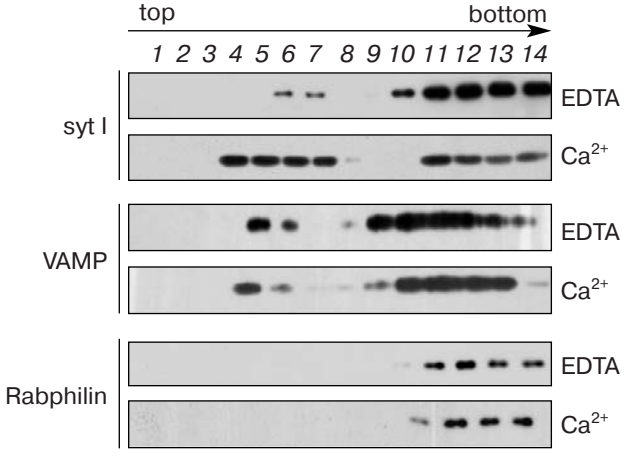

Fig. 3. Raft association of syt I was significantly enhanced by Ca^{2+}. Rafts were prepared from synaptic vesicles using 1% Triton X-100 in the presence of 4 mM EDTA or 1 mM Ca^{2+} in the buffer. The raft protein VAMP2 and the non-raft protein rabphilin were detected in the same sample.

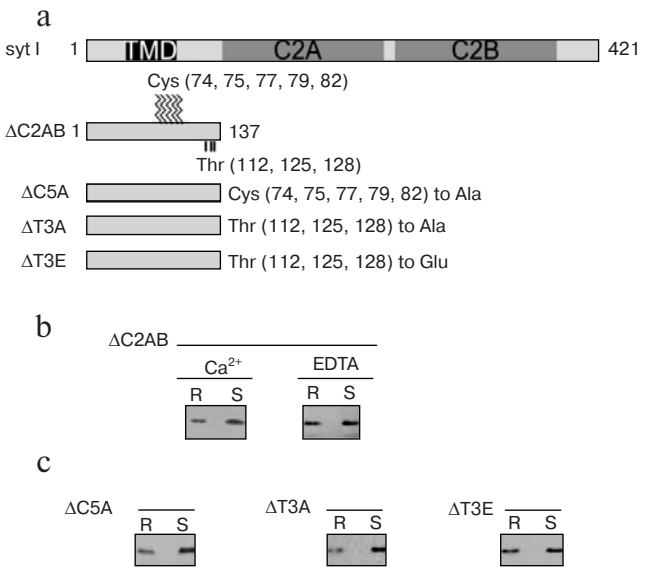

Fig. 4. Synaptotagmin I TMD association with rafts. a) Schematic representation of deletion mutants of syt I including the position of the point mutations. ΔC2AB encodes amino acids 1-137 of syt I. Site-directed mutants of the TMD of ΔC2AB including ΔC5A (Cys74, Cys75, Cys77, Cys79, and Cys82 to Ala), ΔT3A (Thr112, Thr125, and Thr128 to Ala), and ΔT3E (Thr112, Thr125, and Thr128 to Glu). b) Association of TMD with rafts. ΔC2AB was transiently transfected into 293 cells. Rafts were prepared using 1% Triton X-100 in the presence of 4 mM EDTA or 1 mM Ca^{2+}. c) The plasmids with ΔC5A, ΔT3A, and ΔT3E were transfected into 293 cells separately. Proteins in detergent-resistant raft (*R*) fractions (fractions 4-6) and solubilized (*S*) fractions (fractions 11-13) were detected by western blotting.

The transmembrane domain (TMD) of syt I is localized into rafts, which can be regulated by phosphorylation status. Most raft proteins acquire their raft association through their TMD and/or nearby modifications. There are many well-studied examples in this aspect, such as Src, Fyn, and Ras [22, 23]. The mutants of raft proteins with different affinities for rafts provided approaches to understand the mechanisms in regulating the raft association [9, 10, 24].

We first constructed a truncated mutant of syt I without its cytoplasmic C2AB domain (Fig. 4a). This mutant, named ΔC2AB, contains the intravascular N-terminus, the TMD, and a flexible linker region. This fragment was transiently transfected into 293 cells using the calcium phosphate method. Transient transfection of 293 cells is a convenient method for protein expression and analysis [25]. Then rafts were prepared and isolated as described [4]. Briefly, cells were lysed using 1% Triton X-100 and fractionated on sucrose density. The raft fractions (fractions 4-6) and non-raft fractions (fractions 11-13) were

collected and subjected to immunoblotting. As shown in Fig. 4b, the raft association levels of ΔC2AB in the presence of 4 mM EDTA or 1 mM Ca^{2+} were nearly invariable, both around 36-39%. These results indicate that the raft association of TMD of syt I is not sensitive to Ca^{2+}. The findings reported here suggest that the TMD is important for syt I–raft association. The lack of response of the raft related TMD to Ca^{2+} could be explained by the lack of the C2 domain, the Ca^{2+} binding motif [26-28] in the TMD. Since Ca^{2+} could enhance the raft association of syt I, the results here strongly suggested that syt I may obtain the Ca^{2+}-induced enrichment in rafts through the cooperation of its TMD and C2 domains.

Next we tested whether the palmitoylation of syt I is involved in the raft association, since syt I is palmitoylated in/near its TMD (Fig. 4a) [9]. The mutant ΔC5A at the palmitoylation sites was constructed. The analysis of raft affinity showed that 35 ± 2% (n = 3) of ΔC5A was associated with rafts (Fig. 4c), which was almost equal to that of the wild type. Thus, it is unlikely that the raft association of its TMD is significantly affected by palmitoylation.

This result together with that of Salaun et al. [24] reemphasizes that the relationship between protein palmitoylation and raft targeting is not simple and certainly not easy to predict. For example, Salaun et al. found that the increased palmitoylation of SNAP-23 compared to SNAP-25 results in the enhanced association of SNAP-23 to lipid rafts. They also showed an opposite example, the cysteine string protein, which is palmitoylated on at least 11 cysteine residues but is completely excluded from raft domains [29]. Therefore, it will be fascinating to identify regions outside of the palmitoylation domain of syt I that regulate the level of its raft association.

Phosphorylation of some proteins, such as CD3/TCR [30], showed to be a necessary modification for raft association. Next we tested the influence of phosphorylation on the raft association of syt I. We employed the mutant ΔT3A, whose phosphorylation sites were mutated to non-phosphorylatable alanines (Fig. 4a) [10]. The result showed that about 23 ± 3% (n = 3) of ΔT3A was recovered in raft fractions, which was much less than that of the wild type ΔC2AB ($p < 0.05$). Then, a phosphorylation mimic, ΔT3E, was constructed to test if the phosphorylated syt I had a high affinity for rafts. Expectedly, this mutant showed a remarkably increased raft association (48 ± 2%, n = 3) ($p < 0.05$) (Fig. 4c), suggesting that phosphorylation is likely to play a regulatory role in the association of syt I with synaptic vesicle rafts.

Synaptotagmin I was reported to undergo dynamic phosphorylation during the process of exocytosis [20]. Previous studies supported the hypothesis that exocytosis and the "kiss-and-run" mechanism use the same core molecular machinery and the ratio between exocytosis and "kiss-and-run" is controlled by protein phosphorylation [31, 32]. So the regulation of raft association of syt I by the level of phosphorylation may be significant in the control of exocytosis.

Altogether, our results clearly show the raft association of syt I on synaptic vesicles, highlighting a new possible role of syt I in the fusion process.

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