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Designing the lipid raft marker protein for synaptic vesicles①

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Abstract

Lipid rafts are cholesterol-enriched microdomains and implicated in many essential physiological activities such as the neurotransmitter release. Many studies have been carried out on the function of rafts in the plasma membranes, whereas little is known about the information of such microdomains in subcellular compartments especially synaptic vesicles (SVs). In the well-studied plasma membranes, several proteins have been recognized as raft markers, which are used to label or trace rafts. But the raft marker protein on SVs has not been identified yet. Although some SV proteins, including VAMP and CPE, have been found in raft fractions, they cannot be used as markers due to their low abundance in rafts. In this work, we designed several chimera proteins and tested their characteristics for using as SV raft makers. First, we detected whether they located in SVs, and then the chimeras exhibiting the better localization in SVs were further examined for their enrichment in raft using detergent treatment and gradient density floatation analysis. Our results indicate that one of the chimeric proteins is primarily located in SVs and distributed in raft microdomains, which strongly suggests that it could be served as a raft marker for SVs.

Key words: lipid rafts, synaptic vesicles, marker protein

0 Introduction

Lipid rafts are specialized membrane domains. They are characterized by comprising large amount of cholesterol and ordered phospholipids[1]. Rafts have been implicated in diverse processes including signal transduction, endocytosis and cholesterol trafficing[1]. Recent reports demonstrate that lipid rafts participate in regulating neurotransmitter release. Synaptic vesicles (SVs) mediate fast-regulated secretion of classical neurotransmitters such as acetylcholine (Ach), monoamines, and amino acid transmitters[2]. However, the existence of lipid rafts on synaptic vesicles (SVs) has not been fully investigated. Our previous study systematically analyzed the existence and properties of lipid rafts on purified SVs by methods of sucrose density gradient centrifugation, cholesterol depletion, and temperature variation[3].

Recent studies provided an overview of the functional importance of rafts, with a particular focus on their role in exocytosis[4-5]. A powerful and widely used method for studying lipid rafts is to isolate rafts through their insolubility in cold non-ionic detergents[6]. Several SV proteins were found to be associated with rafts by this method[4-5, 7]. Nevertheless, we would like to emphasize one important aspect: there was no particular marker for the raft of SVs *in vivo*. As shown in previous studies, only the result of isolating lipid rafts is used to draw conclusions.

The absence of SV raft marker makes it difficult to directly observe and study the behavior and function of SV rafts *in vivo*. The SV raft marker will be a convenient and favorable sign to detect the raft and raft-associated proteins in SVs. Therefore, it is necessary to design a raft marker of SVs. In this work, we combined different domains of several SV proteins that were present in raft fractions in order to construct specific SV raft marker proteins. Western blot analysis of subcellular fractions revealed that several combined proteins existed in the fractions containing synaptophysin, a marker of synaptic vesicles[8]. Fluorescence microscopy further confirmed the spatial distribution of those chimeras on SV of PC12 cells. Then their raft association was studied using detergent treatment and gradient density floatation. The results revealed that one chimera was preferentially targeted to rafts in SVs. This chimera will be used as a SV raft marker and a useful tool in SV rafts research.

1 Materials and methods

1.1 Materials

The fetal bovine serum and equine serum were from

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Hyclone. The lipofectamine were from Invitrogen. RPMI 1640, poly-L-lysine, Methyl-beta-cyclodextrin (MbCD), TritonX-100 and other reagents were purchased from Sigma. The antibody against synaptophysin and FITC conjugated second antibody were from Santa Cruz Corp. The polyclonal anti-GFP antibody was from Clontech.

1.2 Generation of the fragments

The eukaryotic expression plasmid pCMV5-p38 carrying the cDNA of rat synaptophysin I (p38), was kindly provided by Tomoyuki Mashimo. The cDNA fragments comprising the Loop1 or Loop3 of synaptophysin were generated by PCR. The sense primer introduced a PstI site, while the antisense primer introduced an XhoI site.

The cDNAs of the TMD of CPE and the Helix1 and TMD of rat VAMP, with corresponding restriction endonucleases sites at two terminals, were synthesized by annealing and extension of two oligonucleotide primers. The sequences with introduced restriction endonuclease sites of all fragments were listed in Table 1.

Table 1 The fragments used in the design of the SV raft marker

Fragments	Origin	Restriction endonucleases sites at 5' and 3' end	Sequence of fragments
1	Helix1 of VAMP with mutations	EcoRI/SphI	GAATTCTGATGCTGCAGCAGACCCAGGCCCAGGTGGATGAGGT GGTGGCCATCATGAGGGTGGCTGTGGACAAGGTCCTGGAGCGG GACCAGAAGCTAGCATGC
2(2')	TMD of VAMP	SphI/PstI (SphI/BamHI)	GCATGCGCAGCCAAGCTCAAGCGCAAATACTGGTGGAAAAAC CTCAAGATGATGATCATCTTGGGAGTGATTTGCGCCATCATCC TCATCATCATCATCGTTTACTTCAGCACTCGGGATCC
3	Loop1 of synaptophysin	PstI/XhoI	CTGCAGTTCGCCTTTGCTACGTGTGGCAGCTACACCGGGGAGC TTCGGCTGAGCGTGGAGTGTGCCAACAAGACGGAGAGTGCCCT CAACATCGAAGTTGAATTCGAGTACCCCTTCAGGCTGCACCAA GTGTACTTTGATGCACCCTCCTGCGTCAAAGGGGGCACTACCA AGATCTTCCTGGTTGGGCTCGAG
4	Loop3 of synaptophysin	PstI/XhoI	CTGCAGAAAGGCCTGTCCGATGTGAAGATGGCCACGGACCCAG AGAACATTATCAAGGAGATGCCCATGTGCCGCCAGACAGGGAA CACATGCAAGGAACTGAGGGACCCTGTGACTCTCGAG
5	TMD of CPE	XhoI/BamHI	CTCGAGTCTGAAAGGAAAGAAGAGGAGAAGGAAGAATTGATG GAATGGTGGAAAATGATGTCAGAAACTTTAAATTTTCGG GATCC

1.3 Chimeric constructions

The obtained cDNA fragments were digested using the corresponding restriction endonucleases, purified and then used in ligation reactions according to Table 2. The ligated products were inserted into the pECFP-N1 mammalian expression vector (Clotech) to generate the pChimerias-ECFP plasmids.

Table 2 Construction of chimeric fluorescent proteins

Models	One TMD	Two TMD	
Chimeras	1	2	3
Combinations of the fragments	1 + 2'	1 + 2 + 3 + 5	1 + 2 + 4 + 5

1.4 Cell culture and transfection of PC12 cells

PC12 cells were cultured in RPMI 1640, 10% heat inactivated horse serum, 5% fetal bovine serum at 37℃ in a humidified 5% CO_2 incubator as described in Ref. [9]. Transfection of PC12 cells was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Then cells were harvested and analyzed on the 2nd day post-transfection. Transfected PC12 cells were cultured and plated on poly-L-lysine (50μg/mL)-coated dishes or on glass coverslips for immunofluorescent staining.

1.5 Immunoblot and immunofluorescence analyses

Gel electrophoresis and immunoblotting were carried out as described previously[10] with polyclonal anti-GFP antibody. Whole-cell extracts from PC12 cells were prepared by suspending the cell pellets in cell lysis buffer. Total protein (50 to 200μg) was resolved by SDS-PAGE, transferred to a nitrocellulose membrane, and subjected to immunoblot analysis. The horseradish peroxidase-conjugated secondary antibodies and the ECL Western blotting detection system were from Santa Cruz Corp.

Immunofluorescence was performed as described previously[10] using the primary antibody against VAMP and FITC-conjugated second antibody. For immunofluorescence analysis, cells were fixed in 4% formaldehyde, washed twice using PBS buffer and then incubated for 30min in PBTA (0.1% TritonX-100/0.3% BSA in PBS). Subsequently, the cells were incubated for 60min with the antibody and washed three times with PBTA. The

cells were then incubated with FITC-conjugated second antibodyfor 60min and washed three times in PBTA again.

1.6 Cell Fractionation

Cells were harvested and immediately suspended in the PBS containing protease inhibitors (1mM phenylmethylsulfonyl fluoride, 2μg/mL Leupeptin, 1μg/mL pepstatin A). Cells were then homogenized using a Dounce glass-Teflon homogenizer and centrifuged for 10min at 500g at 4℃ to remove nucleus. The samples were divided into three aliquots for different treatments.

To prepare highly purified plasma membranes, the supernatant (*L*1) was layered over 35% (w/v) sucrose in 50mM Tris-HCl, and centrifuged for 1h at 100,000g at 4℃. The interface was resuspended in 25mM sucrose, 50mM Tris-HCl (pH7.5) and centrifuged for 30min at 100,000g at 4℃. The membrane pellet was then washed with PBS, and centrifuged for 20min at 125,000g at 4℃ to obtain the purified plasma membrane fraction (M)[11].

The standard procedure for vesicles purification was as follows[12]. Briefly, the supernatant (*L*1) was centrifuged at 10,000g for 10min, and the resulting supernatant was loaded onto a 5% ~ 25% glycerol gradient (10mM HEPES, 150mM NaCl). After a centrifugation at 287,000g for 45min, fractions were collected from the top to the bottom. The presence of synaptic vesicles was confirmed by immunoblot analysis of each fractions using anti-synaptophysin antibody. Where indicated, the pooled synaptic vesicle peak included the fractions 6-8.

1.7 Detergent treatment and gradient density floatation

Lipid rafts were isolated by flotation of cell lysates on discontinuous sucrose density gradients as described in Ref.[4]. Cells (5×10^7) were used for each experiment. Solubilized cells were homogenized in 1mL MBS containing 1% TritonX-100 and protease inhibitors (1mM phenylmethylsulfonyl fluoride, 2μg/mL Leupeptin, 1μg/mL pepstatin A) using a Dounce glass-Teflon homogenizer. After incubation at 4℃ for 20min, the homogenate was adjusted to 40% sucrose (w/v) by addition 80% sucrose solution in MES and overlaid successively with 30% and 5% sucrose with a ratio of 3:6:4 (5%/30%/40%, vol/vol). After centrifugation at 240,000g for 18h, thirteen fractions were collected from the top of the gradient. The procedure used for isolation of rafts from purified SVs was similar to that used for whole cells. The fractions 4-6 (the raft fractions, *R*) or the fractions 10-13 (the non-raft fractions, *N*) were combined and concentrated by 3h centrifugation at 485,000 g. The pellets were resuspended and subjected to SDS-PAGE. When indicated, SVs was pretreated with MbCD (10mM) or filipin (5μg/mL) for 30min in order to disrupt lipid raft[13,14].

2 Results and discussion

In the nervous system, communication normally depends on the regulated secretion of chemical messengers. These neurotransmitters are first stored in distinct types of secretory vesicles that will fuse with the plasma membrane and release their content upon appropriate stimulation[15]. Synaptic vesicles contain a unique collection of membrane proteins required for the storage of neurotransmitters and regulated exocytosis[12].

Lipid raft is now in hot discussion and intensive research[1]. Most studies of raft are related to the plasma membranes[16]. Little is known about the lipid rafts of the inner subcellular compartments especially synaptic vesicles. Several proteins of SV have been suggested to be localized in the raft and lipid raft has been thought to participate in regulating neurotransmitter release[3-5, 7]. The basic idea of the raft hypothesis envisions the lipid raft as the platform within which membrane bound proteins assemble to sense cellular signals and trigger exocytosis. However, there is a lack of fundamental understanding about the raft domains in SVs. How long does a SV raft protein exist in raft? Which physiological signals influence the SV raft dynamics? To answer these questions, it is urgent to investigate the function of lipid rafts in the synaptic system.

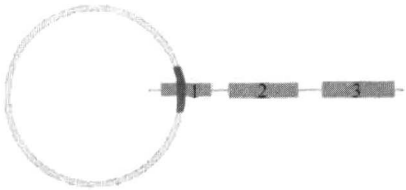
At present, only the method of detergent insolubility is used in the study of the SV raft proteins. As is often the case, scientific research requires harmonization of data from a variety of sources. In the well-studied plasma membrane (PM) raft, some proteins, such as flotillin and caveolin[16], have served as the PM raft markers that are very useful for the observation and study of the PM raft. The absence of SV raft marker makes it difficult to directly observe and study the behavior of SV rafts *in vivo*. Therefore it is necessary to design a SV raft marker that can be used to study the role of rafts in SVs during the exocytosis process *in vivo*.

By deletion, point mutagenesis, and chimeric protein construction, we tried to combine segments of membrane proteins domains that are responsible for site-directed transport and specific membrane localization. The constructed chimeric proteins were used as the candidates of SV raft markers.

2.1 Components of the marker

The raft marker of SVs will be used to detect the raft of SVs *in vivo*, and to study other protein's distribution in the raft of SVs. Therefore the constructed SV raft marker should contain at least three major parts: raft lo-

cating domain for raft association, SVs targeting signal for SVs localization, and the fluorescent fragment for detection and observation (Fig.1).



The fragments represent raft-locating sequence (1), SV targeting signal (2), and the fluorescent fragment (3) respectively.

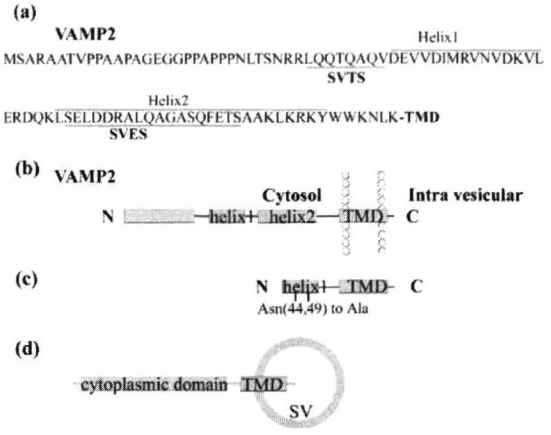
Fig.1 Schematic representation of the recombinant proteins

Almost all raft proteins are transmembrane proteins, such as the well-known raft markers of plasma membrane, namely caveolin and flotillin. Previous data suggested that the structure of a protein 's transmembrane domain had a great impact on the raft localization[17]. Hence, we designed the raft markers containing one or two transmembrane domains that were derived from the raft proteins to ensure their raft localization.

2.2 Construction of the chimeras

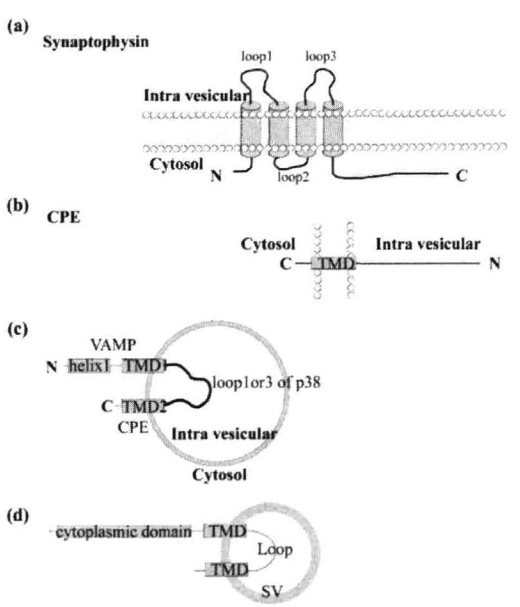
Several domains for efficient localization of SV have been identified in the cytoplasmic domains of SV proteins[15]. We focused on the SV constituent protein, VAMP2, which is enriched in rafts[4]. VAMP2 is a SV membrane protein required for membrane fusion. It is attached to the cytoplasmic face of synaptic vesicles by a C-terminal transmembrane anchor[18]. Its cytoplasmic domain, which is adjacent to the transmembrane anchor, contains two amphipathic α helices (helix 1, from amino acids 39 to 53; helix 2, from amino acids 60 to 88)[2,18]. Helix 1 is involved in the synaptic vesicle-targeting, and the extent of synaptic vesicle targeting correlates with the hydrophobicity of helix 1, while helix 2 may represent a synaptic vesicle exclusion signal[12, 19]. To facilitate synaptic vesicle targeting, we deleted the helix 2 of VAMP2 and substituted some amino acids to decrease the hydrophilicity of helix 1 (substitution of Ala for Asn-49 or Asp-44). The obtained chimera 1 with one transmembrane domain belonged to the Model 1 of the designed SV raft marker (Fig.2).

Carboxypeptidase E (CPE) is a lipid-raft associated protein resident in the trans Golgi network[7]. Its C-terminal transmembrane-spanning region is required for the raft association and sorting[7]. Synaptophysin[20] is an integral membrane protein of SVs containing four transmembrane domains and a C-terminal cytoplasmic domain[21]. Two plasmids were designed to express fusion proteins that appended the transmembrane domain of CPE to the C-terminal of the deleted VAMP2 via the loop1 (chimera 2) or loop3 (chimera 3) of synaptophysin. The obtained chimeras contained two transmembrane domains as illustrated in the Model 2 (Fig.3).



(a) SV targeting signal presents at the cytoplasmic tail of VAMP. The distinct motifs shown to be involved in targeting events or endocytosis have been marked. Abbreviations are SVTS: synaptic vesicle targeting domain; SVED: synaptic vesicle exclusion domain[15]. (b) Schematic representation of VAMP2. (c) Schematic representation of the Model 1 recombinant protein—chimera 1. To increase synaptic vesicle targeting, we deleted the helix 2 of VAMP2, moved helix 1 closer to the transmembrane domain, and substituted some amino acids to decrease the hydrophilicity of helix1 (Substitution of Ala for Asn-49 or Asp-44). (d) Model 1: chimera with one transmembrane domain.

Fig.2 Schematic representation of the Model 1 recombinant protein—chimera 1



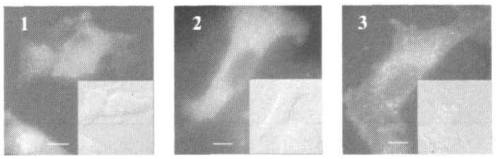
(a) Schematic representation of synaptophysin with three loops, two of which are hydrophilic intra-vesicular loops[20]. (b) Schematic representation of CPE with the raft locating sequence at the C-terminal. (c) Schematic representation of the Model 2 recombinant proteins ($VAMP_{helix1\ mutant}$-$VAMP_{TMD}$-$synaptophysin_{loop}$-CPE_{TMD}). To create chimeras with two transmembrane domains, we appended the transmembrane domain of CPE, via the loops of synaptophysin, to the C-terminal of the transmembrane anchor of VAMP. (d) Model 2: chimera with two transmembrane domains

Fig.3 Schematic representation of the Model 2 recombinant proteins—chimera 2 and 3

2.3 Screen out the chimeras that locate in SVs

The rat pheochromocytoma-derived cell line PC12 is a model system for the study of synaptic vesicle membrane protein. PC12 cells have a population of small vesicles with sedimentation and density properties very similar to those of rat brain synaptic vesicles[12, 22]. In addition, PC12 synaptic vesicles contain all of the known synaptic vesicle membrane proteins including VAMP and synaptophysin.

The constructed chimeras were fused to the NH_2-terminal of ECFP to directly observe their subcellular location by fluorescence microscopy. When expressed in PC12 cells, they were congregated in the intracellular compartments like synaptic vesicles (Fig.4), while the ECFP alone showed a diffuse distribution pattern (data not shown).

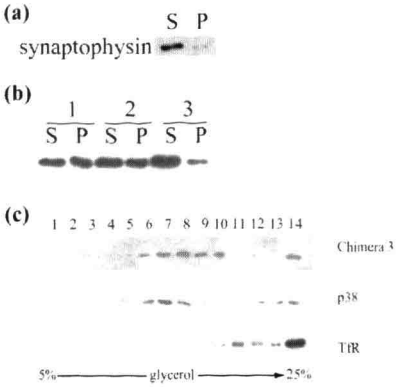


From left to right, there were PC12 cells expressing chimera 1, 2 and 3 respectively. Inset: differential interference contrast (DIC) image. Scale bar: 10μm.

Fig.4 Subcellular localization of chimeric polypeptides——ECFP fusion proteins by fluorescence microscopy

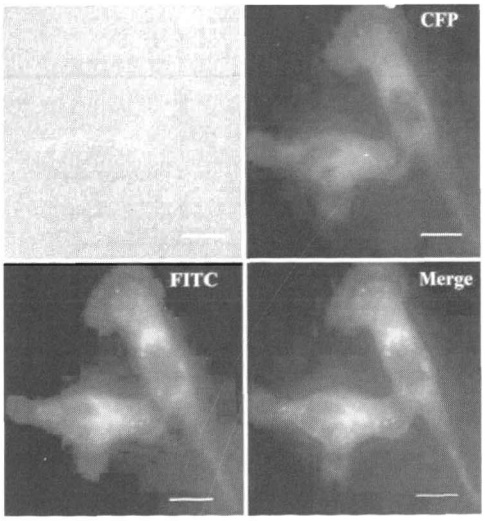
To further examine the localization of the chimeras, plasma membranes and SVs were isolated from the PC12 cells transfected with these chimeras. As shown in Fig.5b, all chimeras were present in both of these two fractions, while chimera 3 was mainly localized in SVs instead of in plasma membranes. Further velocity gradient analysis showed that chimera 3 sedimented in the fractions that contained synaptophysin, the representative vesicle protein, but excluded transferring receptor (TfR), the non-vesicle protein (Fig.5c), which indicated the SV localization of chimera 3. The mechanisms responsible for specific targeting SV proteins to the SV membrane were poorly understood[15]. Similarly, little was known about the intracellular routes taken by these proteins to reach the SVs[15]. However, it was reported that SV proteins were first sorted to the plasma membrane and then to the synaptic vesicles after several rounds of exo-endocytosis[8]. Therefore these chimeras were not totally localized in the SVs but partially distributed in the PM.

To confirm the subcellular localization of the protein, we performed immunofluorescence experiments. The result showed that chimera 3 was colocalized with SV protein synaptophysin (Fig.6).



(a) SVs (S) and plasma membrane (P) were isolated using established procedures and were tested by immunoblot staining for the unique vesicle protein synaptophysin. (b) The subcellular localization of the chimeras was measured (chimeras 1, 2, 3 were shown from left to right). (c) Velocity gradient purification of synaptic vesicles. Fractionation of synaptic vesicle enriched supernatants from PC12 cells expressing chimera 3 through 5% – 25% glycerol. The unique vesicle protein——synaptophysin was tested as the control. The distribution of the TfR was also shown.

Fig.5 The subcellular localization of the chimeras



DIC was the differential interference contrast image of the cells. The chimera 3 was visualized in cyan CFP fluorescence (CFP); synaptophysin was visualized by green FITC fluorescence (FITC); the colocalization of chimera 3 and synaptophysin was shown simultaneously in the merged image (Merge). Scale bar: 10μm.

Fig.6 The chimera 3 is colocalized with the SV protein synaptophysin

Taken together, these observations indicated that combination of TM domains of VAMP and CPE with the hydrophilic intravesicular loop3 of synaptophysin resulted in a good SV retention.

The fact that this series of chimeras were mostly targeted to SVs strongly suggested that the sorting steps were driven by molecular signals, which might mainly locate in the TM regions and the near cytoplasmic regions[23]. Actually, we constructed many other chimeras with the segments from several other SV proteins including synaptotag-

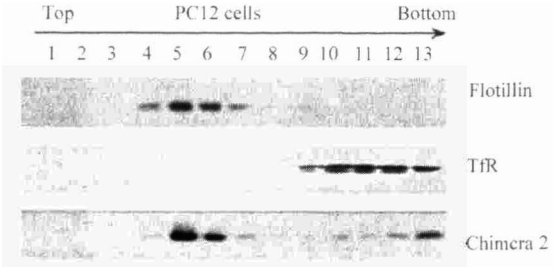
min, the vesicular acetylcholine transporter (VAChT) and the vesicular monoamine transporter (VMAT)[24]. We observed that some of the cells transfected with these chimeras did not exhibit the multipunctate immunofluorescence pattern but contained larger-appearing fluorescent structures possibly corresponding to cytoplasmic aggregates or, more likely, cytoplasmic dispersion (data not shown). We found that the chimeras made of the fragments from VAMP, synaptophysin, and CPE showed better subcellular location and raft resident. So, in this paper, we described this series only. Although one report suggested that VAMP required co-transfection with synaptophysin for its correct SV locating[25], our results and some other studies indicated that the SV-targeting signal in VAMP could direct the chimeras carrying this signal to SVs in PC12 cells accurately[12].

2.4 The chimera 3 is enriched in rafts by detergent treatment and gradient density floatation

It is well known that the typical lipid rafts are cholesterol-rich, liquid-ordered, and detergent-insoluble membrane domains with low buoyant density. It is therefore very convenient to study raft membranes by detergent treatment and gradient density floatation.

PC12 cells expressing the chimera 3 were extracted at 4℃ with 1% TritonX-100 and homogenized. The lysate was adjusted to 40% sucrose, and a 5% ~ 30% linear gradient was formed over the lysates. After centrifugation to equilibrium, fractions were harvested from the bottom of the tube and analyzed by immunoblotting.

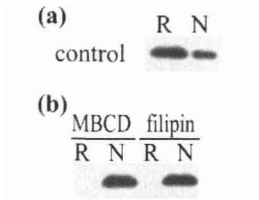
Upon solubilization in TritonX-100, most of the chimera 3 was detergent-insoluble and floated to the low-density fractions (fractions 4, 5, 6) (see Fig.7). At the same time, the large majority of flotillin, a representative raft-associated protein, was also recovered in these fractions, confirming that these fractions were enriched in lipid rafts. In contrast, the transferring receptor (TfR), which did not associate with lipid rafts, was absent from these fractions.



The chimera 3 was resistant to the extraction of TritonX-100 and floated to the low-density fractions of the sucrose gradients. PC12 cells were transfected with the chimera 3. Rafts were prepared as described in Section 1. Flotillin and TfR were used to mark the raft and non-raft fractions respectively.

Fig.7 Presence of the chimera 3 in raft fractions in PC12 cells

We further purified the rafts of SVs. Because the amount of proteins in each fractions was too less to be detected, pools were made with fractions 4-6 and with fractions 10-13, named samples R (raft fractions) and N (non-raft fractions), respectively. The same amount of total protein from samples R and N was subsequently analyzed by immunoblotting. As shown in Fig.8a, the chimera 3 was detected in the raft fractions.



(a) The chimera 3 is enriched in SV rafts. (b) Two agents were used to disrupt raft integrity: MbCD and filipin. Rafts were prepared as described in Section 1. The raft (R) and non-raft (N) fractions were tested at the same time respectively.

Fig.8 The chimera 3 is enriched in SV rafts

Cholesterol is critical to the structural integrity of raft domains[26]. Without cholesterol to maintain order, membrane rafts tend to dissolve or become dispersed. Thus, cholesterol depletion has been a well-documented method to disrupt raft domain. To confirm that the detergent insolubility of the chimera 3 was dependent on their localization to rafts, SVs were pretreated with agents that disrupted the raft integrity. Two agents have been widely used for this purpose: MbCD and filipin[26-30]. MbCD selectively depletes cholesterol from the membrane and is perhaps the most widely used agent for the disruption of raft structure[25-28], whereas filipin is a non-depleting agent that sequesters cholesterol in the membrane[29]. Thus these agents are chemically unrelated and act through very distinct mechanisms, yet have in common the ability to disrupt raft structures[30].

As shown in Fig.8b, the chimera 3 was totally solubilized by the TritonX-100 after MbCD treatment. Another sterol-binding agent, filipin, gave similar result (Fig.8b). These observations showed that the chimera 3 possessed the property of a raft protein.

The results here indicated that the chimera 3 was enriched in rafts. We chose this chimera as the SV raft marker candidate because it had all characters of known raft marker proteins and was specially enriched in SVs. Although the chimera 3 was not totally SV resident, it gave us a useful groundwork for the construction of the SV raft marker. We will further improve this chimera to be more accurate SV raft resident. Then researchers can use this constructed marker in the study of the SV raft and its resident proteins *in vivo*.

3 Conclusion

In this paper, we combined several synaptic vesicle-targating signals and raft-associating sequence, screened the chimera protein enriched in the lipid raft of synaptic vesicles to constructe the marker protein for SV raft. The result showed that one of the chimera proteins was enriched in SV raft and could be the candidate for SV raft marker. This work lay the foundation for screening out the well-chosen SV raft marker.

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