The role of lipid binding for the targeting of synaptic proteins into synaptic vesicles

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Synaptic vesicles (SVs) are key structures for synaptic transmission in neurons. Numerous membrane-associated proteins are sorted from the Golgi complex to the axon and the presynaptic terminal. Protein-protein and protein-lipid interactions are involved with SV targeting in neurons. Interestingly, many SV proteins have lipid binding capability, primarily with either cholesterol or phosphoinositides (PIs). As examples, the major SV protein synaptophysin can bind to cholesterol, a major lipid component in SVs, while several other SV proteins, including synaptotagmin, can bind to PIs. Thus, lipid-protein binding plays a key role for the SV targeting of synaptic proteins. In addition, numerous SV proteins can be palmitoylated. Palmitoylation is thought to be another synaptic targeting signal. Here, we briefly describe the relationship between lipid binding and SV targeting. [BMB reports 2009; 42(1): 1-5]

Biogenesis of SVs

There are several hypotheses regarding the formation of SVs (5). Some studies have indicated that SVs may be formed directly from the TGN, and then transported to the presynaptic terminal. Other studies suggested that mature synaptic vesicles are formed in the presynaptic terminal after the recycling of membrane carriers from the plasma membrane or from early endosomes.

Neurons are highly polarized cells. Neuronal dendrites receive synaptic input, and the presynaptic terminal of the axon releases neurotransmitters. Therefore, protein sorting to specific targets, either to the axon or to the dendrite, must be well orchestrated. Comparative EM studies have shown that the endosome structures between dendrites and the axon in neurons are quite different (6). There are extensive networks of tubular endosomes in dendrites and cell bodies, while in axons, early endosomes are found mainly in the presynaptic terminal and with significant varicosity. Moreover, the compositions of the endosomes in nerve terminals and dendrites are different. For example, endosomes in dendrites are transferrin receptor-positive, EEA1-positive and brefeldin A-sensitive. In contrast, endosomes in the nerve terminal are transferrin-negative, EEA1-negative and brefeldin A-insensitive. Thus, it is plausible that different sorting mechanisms are involved during the biogenesis of specific endosomes in the Golgi complex that allows discrimination for different target localizations of membrane-associated proteins.

Interestingly, EEA1, a well-known early endosome marker, has double zinc finger FYVE domains that can specifically bind to PI3P (7). Therefore, PI3P binding may mediate EEA1-positive early endosome targeting. However, synaptophysin can bind to cholesterol-rich domains in the Golgi complex, which promotes sorting to EEA1-negative endosomes. Thus, different lipid binding properties of proteins in Golgi may play crucial roles for sorting to either dendrites or the axon (Fig. 1B).

Roles of lipid binding in SV targeting

It was previously thought that lipids were simply structural components of cell membranes. However, more recent evi-
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Fig. 1. Lipid binding-dependent protein sorting in neurons. (A) Synaptic localization of apPDE4 long-form in an Aplysia sensory neuron. FLAG-tagged apPDE4 long-form was microinjected along with pNEX-synaptophysin-EGFP. Immunostaining used mouse anti-FLAG antibody and Cy3-conjugated secondary antibody. Scale bar = 20 μm. (B) Schematic diagram of protein sorting based on the major lipid components of each organelle in neurons. In Golgi complexes, PI3P binding of the proteins sorts to early endosomes for targeting to dendrite or soma. Cholesterol, or other lipids binding to proteins, sorts to endosomes of the axon and the presynaptic terminal in the Golgi complex.

dence suggests that several lipids are involved in numerous functions, including signaling and localization. Among these, the roles of cholesterol and Ps related to SV functions have been well studied (Fig. 1B) (8, 9).

Roles of cholesterol binding in SV targeting
It was previously thought that cholesterol might play a key role in SV biogenesis because of the relatively high ratio of phospholipid to cholesterol (1 : 0.8-1). Recently, SV protein and lipid components were extensively examined using mass spectrophotometry analysis (2). Interestingly, consistent with previous reports, the cholesterol content was relatively high (40% (w/w)), whereas the amount of phosphoinositol was quite low (less than 2% (w/w)). Moreover, lipid components can be asymmetrically localized in the bilayers. For example, Ps are primarily localized to the cytosolic sides of plasma membranes and the inner membrane layer contains higher contents of cholesterol than its outer counterpart (10). Given this, it is possible that the cholesterol contents in the outer layers of SVs may be much higher than their inner counterparts. If this is so, then cholesterol in the outer SV layers could be a marker for recruiting SV proteins. Interestingly, major synaptic proteins, such as synaptophysin, synaptotagmin and V-ATPase c, have been identified as cholesterol binding proteins (Table 1) (11). Synaptophysin, which has a four-spanning transmembrane domain, comprises 10% of total SV proteins. Recently, it was reported that synaptophysin can bind to cholesterol and is required for SV biogenesis (11). Using photo-activated cholesterol, cholesterol-binding pro-
PI4P is the most abundant PI, followed by PI4,5P2. PI4P, than 15% of the total phospholipids found in eukaryotic cells. Lipid components of eukaryotic membranes-they represent less species has a unique distribution in cells. Indeed, PIs are minor However, PIs are not distributed randomly in the cell, as each 6 is fused to GFP, which specifically binds to PI4,5P2, it is localized primarily in the plasma membrane. Therefore, using an electron microscope, Watt et al. (19) reported that PI4,5P2 was expressed to a minor extent in the Golgi, ER and endosomes, as well as in electron-dense structures within the nucleus. In neurons, several SV proteins, such as synaptotagmin and dynamin, can bind to PI4,5P2. Synaptotagmin is a synaptic protein that has a single transmembrane domain and two C2 regulatory domains for protein kinase C. These C2 domains can interact with PIs, including PI4,5P2. However, in the presence of Ca2+, the binding specificity changes from PI3,4,5P3 to PI4,5P2 (20). Thus, PI4,5P2 plays a key role in the endocytosis of SVs in many cases, including coated SV endocytosis (Table 1).

Regarding other PIs, PI3P and PI3,5P2 are localized mainly in early and late endosomes, respectively. Many early endosome-targeted proteins, such as EEA1 and Hrs, are mediated by PI3P binding to the specific target. Thus, PIs can be used as markers for different organelles. PI-modifying enzymes also play crucial roles for SV cycling.

### Table 1. Profiles of lipid-associated proteins in SV

<table>
<thead>
<tr>
<th>Lipid binding protein in SV</th>
<th>% in total SV proteins</th>
<th>Lipid binding</th>
<th>Function in Synapse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Synaptophysin</td>
<td>10.2</td>
<td>Cholesterol</td>
<td>SV biogenesis and protein recruitment</td>
</tr>
<tr>
<td>Synaptobrevin 2</td>
<td>8.6</td>
<td>PI4,5P2/cholesterol</td>
<td>SNARE complex</td>
</tr>
<tr>
<td>Synaptotagmin</td>
<td>7</td>
<td>PI4,5P2/cholesterol</td>
<td>SV exocytosis</td>
</tr>
<tr>
<td>Synapsin</td>
<td>6</td>
<td>Phospholipids</td>
<td>Trafficking</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Lipid modifying protein</th>
<th>Substrate/Product</th>
<th>Localization in synapse</th>
</tr>
</thead>
<tbody>
<tr>
<td>PI4KII</td>
<td>PI/PI4P</td>
<td>Synaptic terminal</td>
</tr>
<tr>
<td>PIPKII</td>
<td>PI4P,PI5P/PI4,5P,</td>
<td>Synaptic terminal</td>
</tr>
<tr>
<td>Synaptopojalin 1</td>
<td>PI4,5P/PI4P</td>
<td>SV</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Palmitoylation proteins in SV</th>
<th>% in total SV proteins</th>
<th>Palmitoylation motif</th>
</tr>
</thead>
<tbody>
<tr>
<td>Synaptobrevin 2</td>
<td>8.6</td>
<td>KNKWMIIILGV/AIIIUII</td>
</tr>
<tr>
<td>Synaptotagmin</td>
<td>7</td>
<td>VVTCFCFVCKKCL</td>
</tr>
<tr>
<td>SNAP25</td>
<td>–</td>
<td>LGKFCGLCVPCNKRSSDA</td>
</tr>
<tr>
<td>GAD65</td>
<td>–</td>
<td>ARAWCVNQKFGCGNNKCA</td>
</tr>
</tbody>
</table>

Putative palmitoylated cysteines are underlined. GAD65, glutamic acid decarboxylase (65 kDa); SNAP25, synaptosomal-associated protein (25 kDa). Based on the references: El-Husseini and Bredt, 2002; Rohrbough and Broadie, 2005; Takamori et al., 2006; Thiele et al., 2000.

Proteins were labeled in vivo, which allowed the identification of synaptophysin as a cholesterol binding protein. This work also showed that depletion of cellular cholesterol by MJCD blocked synaptic-like microvesicle (SLMV) biogenesis, but not the total endocytic activity. It was proposed that cholesterol-membrane protein interactions might increase the formation of highly curved structures, such as SVs. In addition, increases in cellular cholesterol content induced the augmentation of SVs, which eventually led to enhancing evoked and spontaneous activities in monitored synapses (autapse) in glia conditioned medium (12). Recently, it was shown that depletion of cellular cholesterol in hippocampal cultures severely impaired evoked synaptic activity (13). Thus, cholesterol is necessary for the formation and exocytosis of SV. In addition, synaptojanin can bind to other synaptic proteins, including VAMP2 and dynamin 1, which leads to the formation of functional synapses (14, 15). Therefore, it is plausible that cholesterol binding by synaptojanin initiates vesicle formation at the membrane, which then recruits other SV proteins through synaptophysin-dependent binding.

### Role of PI binding in SV targeting

PIs play a key role in protein targeting in cells (9). PIs are localized mainly at the cytosolic surface of lipid membranes. Normally, PIs are primarily synthesized in the endoplasmic reticulum (ER) and then transferred to other membranes. However, PIs are not distributed randomly in the cell, as each species has a unique distribution in cells. Indeed, PIs are minor lipid components of eukaryotic membranes-they represent less than 15% of the total phospholipids found in eukaryotic cells.

PI4P is the most abundant PI, followed by PI4,5P2. PI4P, which is generally used as a precursor for other PIs, is localized mainly to the Golgi, as assessed by fluorescence experiments. For example, the PH domain of CERT, which specifically binds to PI4P, is mainly concentrated at the TGN (16). In neurons, it is thought that PI4P is localized to the Golgi and SV, as PI4PKII is expressed in the Golgi complex and dense synaptic vesicles (17). Moreover, dephosphorylation of PI4,5P2 to PI4P by synaptojanin 1 promotes the uncoating of vesicles during synaptic recycling (18). Although PIs are not abundant in SVs, PI4P may be enriched in SVs and play a key role in SV cycling.

In contrast, PI4,5P2 is expressed mainly in the plasma membrane. For example, if the PH domain of PLC-δ1 is fused to GFP, which specifically binds to PI4,5P2, it is localized primarily in the plasma membrane. However, using an electron microscope, Watt et al. (19) reported that PI4,5P2 was expressed to a minor extent in the Golgi, ER and endosomes, as well as in electron-dense structures within the nucleus. In neurons, several SV proteins, such as synaptotagmin and dynamin, can bind to PI4,5P2. Synaptotagmin is a synaptic protein that has a single transmembrane domain and two C2 regulatory domains for protein kinase C. These C2 domains can interact with PIs, including PI4,5P2. However, in the presence of Ca2+, the binding specificity changes from PI3,4,5P3 to PI4,5P2 (20). Thus, PI4,5P2 plays a key role in the endocytosis of SVs in many cases, including coated SV endocytosis (Table 1).

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(46). PI4PKs are kinases that can phosphorylate PI to PI4P. In mammals, type I (PI4KIIa and PI4KIIb) and type III (PI4KIIIa and PI4KIIIb) are expressed. Of these, PI4KIIa is expressed in the Golgi and SV (17). Palmitoylation of the conserved, cysteine-rich domain in PI4KIIa induces its association with membranes (21). PI4P is a precursor of PI4,5P2, which is critical for the endocytosis of fused SV. However, it may play other roles, such as recruitment of SV proteins. PIP kinase is an enzyme that modifies PI4P to PI4,5P2. This kinase is expressed in SV and is involved with PI4,5P2 formation in the synaptic terminal (22). PIPKι/λ knock-out mice showed synaptic defects, including inhibition of miniature currents, enhanced synaptic depression, a smaller pool of readily releasable vesicles, delayed endocytosis and slower recycling kinetics (23). Thus, although the PI content of SVs is quite low, PIs may play important roles for SV function in neurons.

Roles of palmitoylation in SV targeting
Palmitoylation plays a key role for the membrane targeting of proteins that are otherwise located in the cytosol. Interestingly, many SV proteins are palmitoylated, including SNAP-25, synaptobrevin, GAD65 and synaptotagmin (Table 1) (24). It is thought that palmitoylation of proteins enhances the incorporation of the targeted protein into a lipid raft area within the membrane, which then accelerates targeting to the axon, and finally to the SV. For example, the palmitoylation motifs of GAP43, which contain 2 adjacent cysteines and nearby basic residues, mediates targeting to DIGs and sorting to axons (25).

Another palmitoylated protein, GAD65, can be localized to synaptic sites via palmitoylation (26). Amino acids 1-60 contain a potential localization sequence for SV. Palmitoylation of cysteines 30 and 45 is critical for the post-Golgi trafficking of GAD65 to presynaptic sites and for its relative exclusion from dendrites. Amino acids 1-23 contain a Golgi localization signal that is necessary for synaptic targeting, and amino acids 24-31 are required for membrane targeting. However, not all palmitoylated proteins are sorted to axons. For example, palmitoylation of PSD95 is necessary for sorting to dendrites and participates in the postsynaptic clustering of PSD95 in the postsynaptic destination.

Thus, other mechanisms, such as the lipid or protein binding specificities of palmitoylated proteins, are involved in synaptic targeting. Also, amino acids 1-11 of a GAP43-GFP fusion protein can localize in the axon and the presynaptic terminal. However, this fusion protein does not localize to lipid rafts (27). Consistent with this, synaptophysin, a major SV protein of PC12 cell SLMVs, is soluble in Triton-X 100 (28). Therefore, rather than lipid raft targeting of palmitoylated proteins, some other mechanisms, which include different lipid binding, is involved with SV targeting.

CONCLUSION
Specific lipid binding and protein targeting are significantly interrelated. Palmitoylation of proteins is also indirectly involved in this process, including lipid raft localization. Although PIs and cholesterol play critical roles for proper SV targeting, they appear to have different roles for targeting. Proteins that interact with phospholipids are involved with housekeeping functions, whereas proteins that interact with cholesterol are associated with specialized post-Golgi membrane function and trafficking. It will be interesting to examine this further in the future.

Acknowledgements
This work was supported by National Creative Research Initiative Program of the Korean Ministry of Science and Technology. D.-J. J. is supported by BK21 fellowship.

REFERENCES


