Neuron, Vol. 23, 419–422, July, 1999, Copyright ©1999 by Cell Press

Tailoring Uniform Coats for Synaptic Vesicles during Endocytosis

Minireview

Bing Zhang,*§ Barry Ganetzky,† Hugo J. Bellen,* and Venkatesh N. Murthy‡§
*Department of Human and Molecular Genetics
Howard Hughes Medical Institute
Baylor College of Medicine
Houston, Texas 77030
†Laboratory of Genetics
The University of Wisconsin
Madison, Wisconsin 53706
‡Department of Molecular and Cellular Biology
Harvard University
Cambridge, Massachusetts 02138

One of the distinctive features of clear synaptic vesicles (SVs) in both central and peripheral nerve terminals is their uniform small size. It is striking that they maintain this uniformity despite undergoing numerous cycles of fusion and fission. This suggests that the reformation of SVs during endocytosis is a highly regulated process, which might be important to ensure faithful synaptic transmission by minimizing the variability of postsynaptic response at a synapse. Here, we will review several recent genetic studies addressing the link between endocytosis and uniformity of SV size. These genetic results will be further discussed in the context of biophysical mechanisms underlying clathrin cage formation.

Reformation of Synaptic Vesicles following Exocytosis

SVs are recycled and reused at the nerve terminal following exocytosis. There are currently two views of how SVs discharge neurotransmitter and are subsequently recycled at nerve terminals. One view, referred to as "kiss and run," holds that neurotransmitter is released from SVs through a transient pore formed during brief contact with the plasma membrane (reviewed by De Camilli and Takei, 1996). Following discharge of transmitter, SVs do not collapse into the plasma membrane but instead are rapidly recycled into the cytosolic vesicle pool by simple closure of the pore. In this review, we will not further discuss kiss and run, because SVs recycled through this pathway would never change their individual identity as defined by their size and protein composition.

An alternative, more favored view is that SVs in most nerve terminals collapse into the plasma membrane upon exocytosis and that their recycling is mediated by clathrin and its adaptor proteins (De Camilli and Takei, 1996). Clathrin-mediated endocytosis involves a number of highly coordinated sequential steps, including formation of clathrin-coated vesicles, pinching off from the plasma membrane, and, finally, removal of clathrin coats prior to exocytosis. In addition, SVs can be regenerated from the endocytic intermediates, called cisternae, which themselves are thought to be derived from membrane taken up in bulk from the plasma membrane (see Zhang et al., 1998, and references therein).

§To whom correspondence should be addressed (e-mail: bxz@bcm.tmc.edu [B. Z.] and vnmurthy@fas.harvard.edu [V. N. M.]).

Intermingling of SVs and the plasma membrane during exocytosis poses a serious challenge to the clathrin-dependent endocytic machinery because the composition of vesicular lipids and proteins is different from that of the plasma membrane. This prompts the question of how clathrin and its adaptor proteins correctly distinguish the vesicular components and efficiently reassemble them into functional SVs without altering vesicle identity. One possible solution is to regenerate a fully equipped vesicle through clathrin-mediated reassembly directly from the plasma membrane. This scenario satisfies the demand for rapid turnover of vesicles during exo- and endocytic cycles. Another possibility is to fuse freshly internalized vesicles with the endosome and sort out size and composition at a later time. In the latter case, regulation of vesicle size at the plasma membrane may not be necessary, because the final size of SVs will be determined by clathrin-independent rebudding from the endosome. Recent evidence, however, suggests that a significant fraction of SVs do not pass through the endosome before they are reused for exocytosis (Takei et al., 1996; Murthy and Stevens, 1998). Consistent with these findings, the genetic studies discussed here demonstrate that SV size and composition are regulated during endocytosis at the plasma membrane via a clathrin-mediated process.

Clathrin Coat and Adaptors

Ever since clathrin coats were discovered as major mediators of intracellular membrane trafficking, the subject of clathrin cage formation has captured the attention of cell biologists. Not surprisingly, clathrin coats are also transient homes for endocytic SVs. Clathrin is organized as a three-legged complex called a triskelion consisting of three pairs of heavy-chain (180 kDa) and light-chain proteins (25–30 kDa) (Pearse and Crowther, 1987) (Figures 1 and 2). Clathrin triskelia further polymerize into polyhedral lattices or cages composed of pentagonal and hexagonal faces (Figure 1) to capture SV membrane during coated vesicle formation.

However, clathrin triskelia do not directly interact with SV membrane. The membrane association is mediated by clathrin adaptor proteins, including AP1, AP2, AP3, and AP180, which are sandwiched between the clathrin cage and the captured cargo membrane (De Camilli and Takei, 1996). Whereas AP180 is a monomeric polypeptide, the other adaptors are heterotetrameric complexes consisting of four noncovalently bound subunits. Each of these complexes is composed of two large subunits ($\gamma/\beta1$, $\alpha/\beta2$, and $\delta/\beta3$, for AP1, AP2, and AP3, respectively), one medium subunit ($\mu1$, $\mu2$, and $\mu3$), and one small subunit ($\sigma1$, $\sigma2$, and $\sigma3$). The β class subunits are similar to each other, but the other subunits are unique to the adaptor and appear to play an important role in targeting each adaptor to the proper compartment within the cell. Because of their specific subcellular localization, these adaptor proteins participate in distinct pathways of protein sorting and vesicle transport. AP1 is involved primarily in protein sorting and transport from the *trans* Golgi network to the endosome, whereas AP2 and AP180 are involved in receptor and SV endocytosis

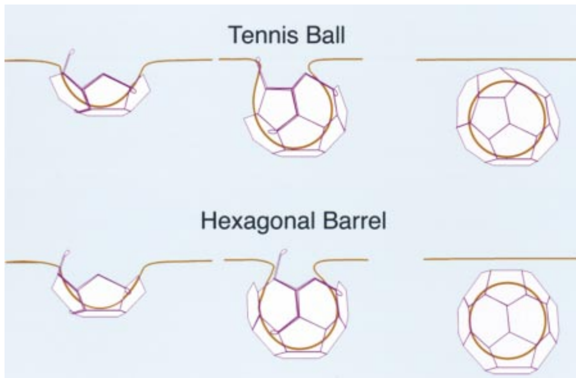

Figure 1. Formation of a Clathrin-Coated Vesicle

This figure illustrates how a slight difference in the initial clathrin lattice can lead to different coat structures. Two hypothetical sequences of steps in coat formation are presented. In the top series, the initial coated pit consists of a hexagon at the bottom, five pentagons (two of which are visible), and one hexagon on the sides. This is shown to lead to a structure resembling a tennis ball (the pentagons trace the continuous joint of a tennis ball). The vesicle membrane is shown by the light brown lines. Single clathrin triskelia are drawn in some panels to illustrate how they fit into the lattice. In the bottom series, the initial pit has one hexagon and six pentagons. This leads to a barrel structure, which is of nearly the same size as the tennis ball above. For the sake of clarity, adaptors and vesicular proteins are not indicated.

at the plasma membrane. AP3 acts between *trans* Golgi and lysosomes as well as in endosomes (Faundez et al., 1998).

Genetic Perturbations of Endocytic Proteins Alter Synaptic Vesicle Size

Clathrin triskelia are used to generate vesicles of different sizes in different systems. The homogeneity of SVs therefore cannot be simply due to the intrinsic properties of the clathrin triskelion; other factors must be involved. For example, it is known that in addition to serving as adaptors to sort cargo membrane and proteins, AP1, AP2, and AP180 also increase the efficiency of clathrin cage assembly. Furthermore, AP2 and AP180 have the ability to regulate the final size of the clathrin cage assembled in vitro. When clathrin cages (coats without vesicles inside them) are formed in vitro from pure clathrin triskelia, the diameters of the resulting cages are nonuniform and range from 40 to 120 nm (Zaremba and Keen, 1983). However, when such empty cages form in the presence of AP2 or AP180, they have a much more uniform size (78 $\pm$ 5 nm; Zaremba and Keen, 1983; Ye and Lafer, 1995). These studies suggest that these adaptors regulate clathrin cage assembly, providing a plausible mechanism for SV size regulation during endocytosis.

A series of genetic perturbation experiments strongly support a role for adaptor proteins and their binding partners in regulation of SV size during clathrin-dependent reassembly. Besides binding to clathrin, AP2 and AP180 also directly bind to each other and form a complex in vitro that efficiently stimulates clathrin assembly (Hao et al., 1999). Their roles in SV endocytosis in vivo have also been recently examined. The α subunit (α-adaptin) of AP2 is essential for SV endocytosis, as the nerve terminals of *Drosophila* mutant embryos are expanded in surface area and completely devoid of vesicles (Gonzalez-Gaitan and Jackle, 1997). In contrast, AP180 appears to be a regulatory protein required to maintain a homogeneous size of SVs in vivo. In *Drosophila* (*lap*; Zhang et al., 1998) and *C. elegans* (*unc-11*; Nonet et al., 1999) AP180 mutants, SV endocytosis is impaired and SVs remaining at nerve terminals are variably enlarged. The increased vesicle size results in an increase in quantal size recorded in muscle cells of *lap* mutants, indicating that these unusually large vesicles are properly refilled with neurotransmitter and are competent for exocytosis. This alteration in SV size occurs even when slow axonal transport of newly synthesized vesicles is abolished by severing the axon from its soma. In addition, when *lap* mutant synapses are examined after recovery from depletion of SVs, the quantal size remains as large and variable as it was prior to depletion. This demonstrates that altered SV size in the mutant reflects a specific impairment in synaptic endocytosis, rather than defects in vesicle biosynthesis in the soma. Along with alterations in SV size, the SV protein synaptobrevin is unexpectedly mislocalized to axonal membranes in *unc-11* mutants (Nonet et al., 1999). This suggests that AP180 also maintains the fidelity of vesicle reassembly

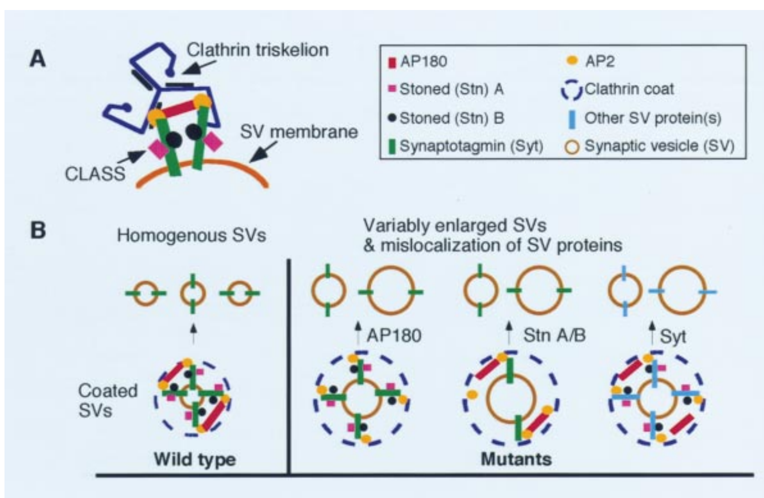

Figure 2. A Proposed Model for Regulation of SV Size and Protein Composition by CLASS during Endocytosis

(A) CLASS proteins may adjust the flexibility of the clathrin triskelion leg angles to determine the final size of clathrin-coated vesicles. For simplicity, only one triskelion is shown to coat a small patch of SV membrane.

(B) A schematic illustration showing how mutations in CLASS proteins might alter SV size and protein composition. Clathrin coat size is restricted by CLASS proteins during SV reassembly to generate uniform SVs. Mutations of CLASS proteins are thought to weaken this restriction on clathrin trikelia and lead to variable and enlarged vesicles.

by ensuring that synaptobrevin returns from the plasma membrane to SVs.

The observation that SVs of variable size can be generated by mutations in AP180 raises another issue: do mutations in protein partners that have been shown to directly or indirectly associate with these adaptors affect SV size as well? AP2 has been shown to bind to the SV membrane protein synaptotagmin (Zhang et al., 1994). This suggests that in addition to its putative calcium-sensing role, synaptotagmin may also function as an anchor for AP2 during endocytosis. Indeed, in vivo evidence indicates that synaptotagmin participates in SV endocytosis in *C. elegans* (Jorgensen et al., 1995), *Drosophila* (Reist et al., 1998), and squid (Fukuda et al., 1995). Recently, Reist et al. (1998) discovered that a subset of SVs in *synaptotagmin* (*syt*) mutant flies shows a significant increase in diameter. These observations lead us to hypothesize that a complex of interactive proteins regulates SV size during endocytosis.

This hypothesis is further supported by genetic and electrophysiological analyses of the stoned (*stn*) mutant in *Drosophila* (Stimson et al., 1998; see Fergestad et al., 1999, and references therein). The *stn* locus encodes a dicistronic message producing stoned A and stoned B proteins. Stoned A is a novel protein, whereas stoned B exhibits a partial homology with the μ2 subunit of AP2. Both proteins bind to the C2B domain of synaptotagmin in vitro (Stimson et al., 1998), suggesting that the stoned proteins may also play a role in SV endocytosis. Absence of stoned B or both proteins causes defects in SV recycling, increases the size of SVs and quanta, and accumulates multivesicular bodies at neuromuscular junction (NMJ) synapses (Fergestad et al., 1999). While the origin of multivesicular bodies remains elusive, the alteration in vesicle size in *stn* is reminiscent of those reported for AP180 and synaptotagmin mutants. Unlike AP180, the stoned proteins are required for proper localization and sorting of synaptotagmin, which in *stn* mutants is reduced in level and mislocalized to axonal plasma membrane where it is not normally present (Stimson et al., 1998; Fergestad et al., 1999). Taken together, studies of AP180, synaptotagmin, and stoned A and B proteins in vivo clearly demonstrate that these proteins play a modulatory role in endocytosis by recruiting vesicular proteins and regulating the final size of SVs.

The Mechanics of Coat Formation and Implications for Synaptic Vesicle Size Regulation

Small-diameter clathrin coats resemble fullerenes ("buckyballs") that are almost exclusively composed of hexagons and pentagons. Geometric considerations dictate that there be twelve pentagons in the cage; the number of hexagons varies and determines the size of the cage (see Shraiman, 1997). The smallest cage will contain no hexagons; the structures most commonly observed in coated vesicles prepared from brain appear to have eight hexagons (Zaremba and Keen, 1983; Pearse and Crowther, 1987). An emerging feature of SV recycling is that regulation of vesicle size and composition is tightly coupled during the initial formation of clathrin-coated vesicles. It appears reasonable to postulate that the size of an SV is directly determined by the size of the clathrin coat that captures it. The puzzle of the uniformity of vesicle size will then be solved if we can understand how uniform clathrin coats are assembled during endocytosis.

Two general questions can be framed regarding the mechanics of coat formation. First, are coats constructed by remodeling preformed flat hexagonal clathrin lattices through a precise series of dislocations and disassembly (Mashl and Bruinsma, 1998), or are they constructed de novo with pentagons and hexagons added appropriately as the coat vesicle grows, without any further editing (Shraiman, 1997)? Second, what determines the final curvature, and therefore the size, of the clathrin coat?

Regarding the first question, available evidence appears to favor de novo formation (at least at synapses) since flat lattices are normally not observed, and the insertion of pentagons into the interior of a hexagonal lattice may be energetically unfavorable (Kirchhausen, 1993). Figure 1 illustrates the addition of pentagons and hexagons as vesicles undergo endocytosis and shows further that similar sizes result from the same pentagon and hexagon composition despite different assembly details. In the top series, the coat starts with a hexagon at the bottom, five pentagons, and one hexagon on the sides. The final coat is a tennis ball shaped structure with eight hexagons and twelve pentagons. In the bottom series, the initial coat has one hexagon and six pentagons. The resulting coated vesicle is a hexagonal barrel—also with twelve pentagons and eight hexagons. These two structures are approximately the same size and are probably the smallest possible coats that can accommodate a vesicle inside.

The second question is how the correct curvature of the coat is achieved. Two biophysical mechanisms have been proposed to account for the assembly of uniform clathrin coats. In the first model, the intrinsic curvature of clathrin triskelia, in conjunction with specific interactions with adaptors, target membrane lipids, or proteins defines the size of membrane to be retrieved. After vesicles have undergone exocytosis, adaptors and the SV membrane recruit clathrin and regulate the spontaneous curvature adopted by the triskelion such that small and uniform vesicles with high curvature are formed.

In the second model, the size of the clathrin cage can be governed by the kinetics of assembly (Shraiman, 1997). Cage assembly at high concentrations of free clathrin triskelia occurs rapidly, and hexagonal faces are favored, leading to relatively flat and large hexagonal lattices. Formation of pentagons is energetically less favorable, because pentagons induce curvature, and will occur only when cage assembly is slow. The size of the cage will be determined by the number of pentagons inserted, and therefore the kinetics of assembly will determine the size of the cage. Numerical implementation of the model showed that at low concentrations of free clathrin, about 90% of cages assembled are with eight hexagons (Shraiman, 1997). More importantly, the variance of the cage size was small for a reasonable range of parameters used. The varying kinetics of assembly and the cytosolic concentration of clathrin triskelia may lead to different sized vesicles in different systems. In addition, the presence of specific adaptors might serve to regulate the availability of free clathrin or its assembly kinetics.

These two biophysical models (intrinsic curvature model and the kinetic model) can be tested directly by

biological manipulations that alter SV sizes. Similarly, the aforementioned genetic results can be interpreted in terms of the biophysical explanations for the uniformity of clathrin coats. Both in vitro and in vivo studies of AP180 appear to conflict with the assembly kinetics hypothesis. In vitro, AP180 potently stimulates clathrin cage assembly and significantly enhances its assembly rate even at low clathrin concentrations (Ye and Lafer, 1995, and references therein). Hence, the kinetic model would predict that clathrin cages assembled in the presence of AP180 would be more variable instead of homogenous in size. If the availability of clathrin could regulate the kinetics of clathrin cage assembly, one would expect the rate of coat assembly to become slower in *lap* due to its reduced levels of clathrin (Zhang et al., 1998), thus favoring the formation of smaller vesicles. However, SVs become more variable and larger in *lap* mutants than in wild type. Without the knowledge of whether clathrin concentration is also altered in *syt* and *stn* mutants, it would be difficult to conclude how the assembly kinetics model applies here. But it is clear that AP180 alone is insufficient to ensure uniform coat size, as demonstrated by *syt* and *stn* mutant phenotypes (Reist et al., 1998; Fergestad et al., 1999). Moreover, the *syt* study points to the possibility that cargo membrane protein or lipid participates in regulation of clathrin coat assembly as well.

If the pucker angle of clathrin triskelion can be regulated to achieve uniform clathrin-coated vesicles, the results from *lap*, *unc-11*, *syt*, and *stn* mutants can be reconciled with the intrinsic curvature model. The biochemical data on AP180 (Ye and Lafer, 1995) and the morphometric data on *lap* and *unc-11* NMJs strongly argue that this protein restricts the size of clathrin cages and thus defines the amount of membrane to be retrieved during assembly of clathrin-coated vesicles. This modulatory activity of AP180 can be further enhanced through its interactions with a complex of proteins, including AP2, synaptotagmin, and the stoned proteins (Figure 2A). When the complex of clathrin–APs–synaptotagmin–stoned proteins (CLASS) is intact, clathrin triskelia are able to form cages of uniform size, leading to a homogeneous population of SVs (Figure 2B). In the absence of any of these CLASS proteins, we propose that the complex is unable to induce the unique conformation of the triskelia. Consequently, coated vesicle formation is either blocked (as in α-*adaptin* mutants) or severely impaired, vesicle size becomes variable and larger, and vesicular proteins are inefficiently recovered. Likely, other proteins or lipids (both vesicular and cytosolic) may also help fine tune clathrin-mediated reassembly of SVs during endocytosis.

Conclusions

Recent genetic studies have significantly advanced our understanding of the role of endocytosis in SV recycling. It is increasingly apparent that the regulation of vesicle size and composition is coupled during clathrin-dependent reassembly at the plasma membrane. Further, SV proteins, such as synaptotagmin, can no longer be considered as exocytic proteins only, but also have an active role in the recycling process. These studies leave many important questions to be addressed in the future. An immediate goal will be to dissect the mechanistic details by which the CLASS proteins affect clathrin cage assembly. A systematic in vitro and in vivo structure–function analysis should afford a better understanding of the origin of the uniformity of coated vesicles, including those at synapses. Another interesting direction is to examine whether SV size can be regulated under physiological conditions and if such modulations contribute to synaptic plasticity.

Selected Reading

De Camilli, P., and Takei, K. (1996). Neuron *16*, 481–486.

Faundez, V., Horng, J.-T., and Kelly, R.B. (1998). Cell *93*, 423–432.

Fergestad, T., Davis, W.S., and Broadie, K. (1999). J. Neurosci., in press.

Fukuda, M., Moreira, J.E., Lewis, F.M.T., Sugimori, M., Ninobe, M., Mikoshiba, K., and Llinas, R. (1995). Proc. Natl. Acad. Sci. USA *92*, 10708–10712.

Gonzalez-Gaitan, M., and Jackle, H. (1997). Cell *88*, 767–776.

Hao, W., Luo, Z., Zheng, L., Prasad, K., and Lafer, E.M. (1999). J. Biol. Chem., in press.

Jorgensen, E.M., Hartwieg, E., Schuske, K., Nonet, M.L., Jin, Y., and Horvitz, H.R. (1995). Nature *378*, 196–199.

Kirchhausen, T. (1993). Curr. Opin. Struct. Biol. *3*, 182–188.

Mashl, R.J., and Bruinsma, R.F. (1998). Biophys. J. *74*, 2862–2875.

Murthy, V.N., and Stevens, C.F. (1998). Nature *392*, 497–501.

Nonet, M.L., Holgado, A.M., Brewer, F., Serpe, C.J., Norbeck, B.A., Holleran, J., Wei, L., Hartwieg, E., Jorgensen, E.M., and Alfonso, A. (1999). Mol. Cell Biol. *10*, 2343–2350.

Pearse, B.M.F., and Crowther, R.A. (1987). Annu. Rev. Biophys. Biophys. Chem. *16*, 49–68.

Reist, N.E., Buchanan, J., Li, J., DiAntonio, A., Buxton, E.M., and Schwarz, T.L. (1998). J. Neurosci. *18*, 7662–7673.

Shraiman, B.I. (1997). Biophys. J. *72*, 953–957.

Stimson, D.T., Estes, P.S., Smith, M., Kelly, L.E., and Ramaswami, M. (1998). J. Neurosci. *18*, 9638–9649.

Takei, K., Mundigl, O., Daniell, L., and De Camilli, P. (1996). J. Cell Biol. *133*, 1237–1250.

Ye, W., and Lafer, E.M. (1995). J. Neurosci. Res. *41*, 15–26.

Zaremba, S., and Keen, J.H. (1983). J. Cell Biol. *97*, 1339–1347.

Zhang, J.Z., Davletov, B.A., Südhof, T.C., and Anderson, R.G. (1994). Cell *78*, 751–760.

Zhang, B., Koh, Y.H., Beckstead, R.B., Budnik, V., Ganetzky, B., and Bellen, H.J. (1998). Neuron *21*, 1465–1475.