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Biogenesis of Neurosecretory Vesicles

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1 Introduction

Neurosecretory vesicles are defined as the vesicles which mediate the usually calcium-dependent, regulated release of signaling molecules in the nervous system. At least two types of neurosecretory vesicles can be distinguished by their structure and content. The first type are the synaptic vesicles of neurons, which mediate the storage and release of classical neurotransmitters (e.g., acetylcholine, glutamate, GABA, and glycine) but lack secretory proteins. Synaptic vesicles have a counterpart in certain endocrine cells, the synaptic-like microvesicles (SLMVs). The second type are the large dense core vesicles of neurons, which mediate the storage and release of neuropeptides. These vesicles are the neuronal equivalent of the secretory granules found in cells capable of regulated protein secretion, notably endocrine cells. (Since large dense core vesicles are essentially similar, if not identical, to endocrine secretory granules, we shall also use the term "secretory granules" when referring to large dense core vesicles of neurons.) This chapter summarizes recent data obtained in our laboratory concerning the biogenesis of secretory granules from the *trans*-Golgi network (TGN) and of SLMVs from early endosomes in the neuroendocrine cell line PC12.

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2 Biogenesis of Secretory Granules

2.1 Regulation by Heterotrimeric G Proteins

Using a cell-free system derived from PC12 cells, which reconstitutes the formation of secretory granules from the TGN (formation being defined as the physical detachment of newly budded secretory granules from the TGN) (Tooze and Huttner 1990), we had previously observed that this process is inhibited by nonhydrolyzable analogues of GTP, suggesting an involvement of GTP-binding proteins (Tooze et al. 1990). Work carried out during the past 2 years has shown that one class of GTP-binding protein involved in secretory granule formation are the heterotrimeric G proteins. The first indication for this came from experiments which examined the effects of aluminum fluoride, an activator of heterotrimeric but not small ras-like GTP-binding proteins, and of purified heterotrimeric G protein $\beta\gamma$ subunits in the cell-free system. Aluminum fluoride was found to inhibit secretory granule formation to the same extent as the nonhydrolyzable GTP analogue GTP γ S (Barr et al. 1991). Conversely, addition of purified $\beta\gamma$ subunits stimulated the formation of secretory granules (Barr et al. 1991). Further work showed that multiple heterotrimeric G proteins of the Gi/Go and Gs class are associated with the TGN (Leyte et al. 1992). Activation of Gi/Go by mastoparan, a peptide which mimicks the effect of an activated receptor on Gi/Go by promoting guanine nucleotide exchange on these G proteins, results in the inhibition of secretory granule formation from the TGN in the cell-free system. Pretreatment of cells with pertussis toxin, which renders Gi/Go unable to interact with receptors, not only blocks this effect of mastoparan but by itself results in an increase in cell-free secretory vesicle formation (Leyte et al. 1992). These findings suggest that guanine nucleotide exchange factors operate on Gi/Go in the cell-free system, hinting at the existence of receptors which act in such a manner. Activation of Gs by cholera toxin, on the other hand, stimulates cell-free secretory vesicle formation. Thus, Gi/Go and Gs exert opposing regulatory effects on secretory vesicle formation from the TGN (Leyte et al. 1992).

2.2 Coat Proteins

The effector systems through which the various heterotrimeric G proteins exert their stimulatory and inhibitory effects on secretory granule formation are unknown. However, two lines of evidence suggest that coat proteins, implicated in vesicle formation at other sites in the endomembrane system, are also involved in the formation of secretory granules from the TGN and may be targets for regulation by heterotrimeric G proteins. The first line of evidence comes from a study on the effect of brefeldin A on secretory granule formation. Brefeldin A, a fungal metabolite that inhibits vesicular transport along the secretory pathway (Klausner et al. 1992), prevents the membrane binding of components of the nonclathrin and clathrin coat, which are thought to be required for the budding of vesicles that mediate biosynthetic protein traffic (Rothman and Orci 1992). Brefeldin A has been found to inhibit the formation of secretory granules in vivo (Miller et al. 1992; Rosa et al. 1992) and in the cell-free sys-

tem (Rosa et al. 1992). If we assume a common mechanism of action for brefeldin A irrespective of the donor compartment affected by the drug, the inhibition of secretory granule formation from the TGN by brefeldin A suggests that coat protein binding to the TGN is prevented by the drug, and that this is the underlying cause for the inhibition of secretory granule formation. It will be important to determine whether the clathrin coat of immature secretory granules or other coat proteins are the targets for brefeldin A that are relevant in this context.

The second line of evidence comes from a study on the effect of ARF (ADP-ribosylation factor), a small GTP-binding protein, on secretory granule formation. We have found that a synthetic, N-terminally myristylated peptide corresponding to the N-terminal domain of ARF-1 stimulates the formation of secretory granules in the cell-free system, whereas the corresponding nonmyristylated peptide, or peptides corresponding to the N-terminal domain of ARF-4, do not (Barr and Huttner in prep.). Thus, ARF-1 or an ARF-1-like protein may be involved in secretory granule formation.

If one extrapolates from the results obtained by other investigators using Golgi membranes, it may be that the effects of brefeldin A and of the ARF-1 peptide on cell-free secretory granule formation from the TGN are connected. Brefeldin A inhibits an activity associated with Golgi membranes that catalyzes the guanine nucleotide exchange on ARF (Donaldson et al. 1992b; Helms and Rothman 1992), which is a prerequisite for its binding to membranes (Donaldson et al. 1992a). This, in turn, is required for the membrane binding of other coat components (Donaldson et al. 1992a). Thus, by analogy with the formation of vesicles mediating transport through the Golgi complex, the sequence of events in secretory granule formation from the TGN may be: TGN-associated catalysis of guanine nucleotide exchange on ARF → binding of ARF to TGN membranes → binding of coat proteins to the TGN → budding of immature secretory granules, with brefeldin A inhibiting the first event.

2.3 Sorting Signals

We have previously identified a highly conserved domain common to two widespread regulated secretory proteins of neurons and endocrine cells, chromogranin A and chromogranin B (secretogranin I; Huttner et al. 1991), which consists of a disulfide-bonded 20-amino-acid-long loop structure encoded by a separate exon (Benedum et al. 1987; Pohl et al. 1990). Exploiting the observations by other investigators that disulfide bond formation can be inhibited in living cells by the addition of thiol reducing agents (Alberini et al. 1990; Braakman et al. 1992), the role of this intramolecular disulfide bond in the sorting of chromogranin B to secretory granules of PC12 cells has been studied (Chanat et al. 1993). Reduction of the disulfide bond resulted in the selective missorting of chromogranin B to constitutive secretory vesicles, whereas the sorting of the related regulated secretory protein secretogranin II, which lacks disulfide bonds, was unaffected. The reduced chromogranin B still exhibited the low pH/calcium-induced aggregation implicated in its sorting (Chanat et al. 1993; Chanat and Huttner 1991). These data therefore suggest that aggregation, although in principle capable of causing the segregation of regulated from constitutive secretory pro-

teins within the lumen of the TGN (Chanat et al. 1991; Tooze et al. 1993), is not alone sufficient to allow the sorting process to go to completion, i.e., to generate secretory granules from the TGN. Rather, in the case of chromogranin B, the sorting process can only be completed if the structural information associated with the disulfide bond is preserved. It is tempting to speculate that this structural information may be required for the interaction of soluble chromogranin B with receptors in the TGN membrane involved in sorting.

2.4 Secretory Granule Maturation

Immature secretory granules have previously been shown to be a short-lived intermediate in the biogenesis of mature secretory granules (Tooze et al. 1991; Tooze et al. 1993). The characterization of the fate of immature secretory granules in PC12 cells has revealed that there are at least two maturation routes. Immature secretory granules may mature (1) into larger secretory granules by self-fusion (Tooze et al. 1991), or (2) into smaller secretory granules by condensation of the contents (Bauerfeind et al. 1993), accompanied in both cases by the removal of the excess membrane. It will be important to determine the fate of the excess membrane removed from immature secretory granules during their maturation. This membrane may be used (1) for the recycling of sorting receptors to the TGN, (2) for the segregation of lysosomal proteins and residual constitutive secretory proteins from regulated secretory proteins, or (3) for the removal of certain products of proteolytic processing of regulated secretory proteins (Bauerfeind and Huttner 1993). Whatever the fate of the membrane may be, the immature secretory granule may well turn out to be a post-TGN sorting compartment on the biosynthetic secretory pathway.

3 Biogenesis of SLMVs

3.1 SLMVs Originate from Early Endosomes

We have previously shown that in PC12 cells, newly synthesized synaptophysin, a major synaptic vesicle membrane protein, travels in constitutive secretory vesicles from the TGN to the plasma membrane, cycles several times between the plasma membrane and early endosomes, and is then packaged into SLMVs (Régnier-Vigouroux et al. 1991). This suggested that the *de novo* formation of SLMVs occurs from early endosomes, although their formation directly from the plasma membrane was not strictly excluded. Recent experiments extending this work show that the fluid phase marker horseradish peroxidase, pre-internalized into early endosomes, is in part chased to SLMVs, thus providing evidence for the formation of SLMVs directly from early endosomes (Bauerfeind et al. 1993).

3.2 SLMVs and Early Endosomes Take Up and Store Classical Neurotransmitters

It has been suggested, but not unequivocally shown, that SLMVs store classical neurotransmitters (Reetz 1991). Using metabolic labeling of PC12 cells with radioactive choline followed by subcellular fractionation and analysis of acetylcholine, we have recently shown that SLMVs do indeed contain biosynthetic acetylcholine (Bauerfeind et al. 1993). Interestingly, a substantial portion of the biosynthetic acetylcholine is found in early endosomes, and both early endosomes and SLMVs contain an ATP-dependent acetylcholine uptake system (Bauerfeind et al. 1993). These findings are consistent with the early endosomal origin of SLMVs.

3.3 SLMVs and the Storage of Biogenic Amines

Because PC12 cells are capable of neurotransmitter uptake and storage in SLMVs, and also express the uptake system for biogenic amines such as catecholamines, one can use these cells to address the question whether or not SLMVs also contain catecholamines. Our experiments indicate that, at least for undifferentiated PC12 cells, this is not the case (Bauerfeind et al. 1993). In these cells, catecholamine storage is confined to mature secretory granules, with no detectable storage in SLMVs. Consistent with this, the vesicular amine transporter is present in the membrane of mature secretory granules. Interestingly, the vesicular amine transporter is also detected in early endosomes, but not in SLMVs (Bauerfeind et al. 1993). If our interpretation that this reflects the recycling of the amine transporter after exocytosis of secretory granules to the TGN is correct, then this implies that the early endosomes of these PC12 cells differentially sort two neurotransmitter uptake systems, the acetylcholine transporter to SLMVs and the amine transporter to vesicles destined to the TGN.

The finding that SLMVs of undifferentiated PC12 cells lack catecholamines has implications about the nature of the so-called small dense core vesicles (SDCVs) of sympathetic neurons, which lack secretory proteins, contain catecholamines, and because of their small size have been considered to be related to the synaptic vesicles. The latter notion is supported by our observation (Bauerfeind and Huttner in prep.) that SDCVs contain synaptophysin. If SDCVs should indeed prove to be catecholamine-storing synaptic vesicles, this would imply that the vesicular amine transporter expressed in neurons is sorted differently than the one expressed in undifferentiated PC12 cells. This possibility offers an interesting perspective to identify sorting signals underlying the traffic of membrane proteins to synaptic vesicles.

4 Conclusions

(1) The formation of secretory granules from the TGN is regulated by multiple heterotrimeric G proteins in a stimulatory (G_s) and inhibitory (G_i/o) fashion. (2) Effector systems of the TGN-associated heterotrimeric G proteins may include ARF and other coat proteins. (3) Receptor system stimulating guanine nucleotide exchange on the

TGN-associated heterotrimeric G proteins may recognize specific structures in regulated secretory proteins such as the conserved disulfide-bonded loop of chromogranin B which appears to contain sorting information. (4) The membrane retrieval from immature secretory granules occurring during secretory granule maturation may provide a mechanism for recycling of sorting receptors to the TGN. (5) Membrane proteins destined to SLMVs travel from the TGN via the constitutive secretory pathway to the plasma membrane and from there to early endosomes, the compartment from which the de novo formation of SLMVs occurs. (6) SLMVs contain classical neurotransmitters (acetylcholine) but, at least in undifferentiated PC12 cells, lack catecholamines, despite the presence of both the acetylcholine transporter and vesicular monoamine transporter in early endosomes, implying differential sorting of two distinct neurotransmitter uptake systems in this compartment.

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