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Secretory Granule Formation

The Morphologist's View

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INTRODUCTION

All eukaryotic cells have a constitutive secretory pathway that transports secretory proteins from the RER via the Golgi apparatus and postGolgi secretory vesicles to the plasma membrane. Fusion of the secretory vesicles with the plasma membrane, a constitutive event, then liberates the content of the vesicles to the exterior of the cell and integrates the secretory vesicle membrane, lipid, and protein into the plasma membrane.

REGULATED SECRETORY PATHWAYS

A comparatively small number of cell types have, in addition to a constitutive exocytic pathway, a second, regulated secretory pathway *(1)*. Both endocrine and exocrine cells have regulated secretory pathways. The hallmarks of the regulated secretory pathway are first that the postGolgi secretory granules fuse with the plasma membrane only after the cell has received a specific, external signal that triggers secretion. The signal involves the binding of a secretagog to the plasma membrane followed by signal transduction to the interior of the cell. The second characteristic of the regulated pathway is that only a subset of the cell's total complement of secretory proteins enters it. In other words, the regulated secretory proteins must be sorted from the constitutively secreted proteins in the

same cell. Secretagog-stimulated exocytosis and sorting of regulated secretory proteins appear to be the two fundamental characteristics of regulated secretory pathways.

Dense Core Secretory Granules

Regulated secretory proteins are made in very large amounts in endocrine and exocrine cells. For example, in exocrine, well over 90% of total proteins synthesized are pancreatic digestive enzymes and proenzymes. Because of this, regulated secretory proteins reach such high concentrations in regulated secretory vesicles that they form osmotically inert aggregates of precipitated protein—in effect coprecipitates of protein. These aggregates of precipitated protein form dense cores in the secretory vesicles, which, therefore, are referred to as dense core secretory granules. In short, the storage of regulated secretory proteins in these cored secretory granules are secondary characteristics of the regulated exocytic pathway.

Polarized Epithelia with Regulated Secretory Pathways

In endocrine cells, including those in the pancreas, neither the constitutive nor the regulated exocytic pathways are polarized. The constitutive secretory vesicles and the regulated secretory granules can fuse with any part of the plasma membrane that is not divided into apical and basolateral domains. Exocrine pancreatic cells, by contrast, are epithelial. Their plasma membrane is differentiated by tight junctions into an apical domain and a basolateral domain. The regulated pathway in these cells is polarized such that zymogen granules move to the apical region of the cell and fuse exclusively with the apical plasma membrane after a secretagog signal is received at the basolateral membrane.

In summary, all cells have a constitutive secretory pathway. A small number of cell types have, in addition to the ubiquitous constitutive secretory pathway, a regulated secretory pathway. The regulated pathway involves sorting of secretory proteins. In an even smaller subset of cells (polarized epithelial cells with a regulated exocytic pathway), the latter is polarized. Since the constitutive secretory pathway is ubiquitous, it is no doubt the ancestral pathway from which the regulated exocytic pathway evolved in some specialized cells.

Ultrastructure of Secretory Granule Formation

Electron microscopic evidence shows that secretory granules of the regulated exocytic pathway form at the *trans*-Golgi network (GN), and the immature granules bud from the TGN, which is the site of divergence of the constitutive and regulated exocytic pathways (*2*). In AtT20 cells, an anterior pituitary tumor cell line, for example, condensation of the

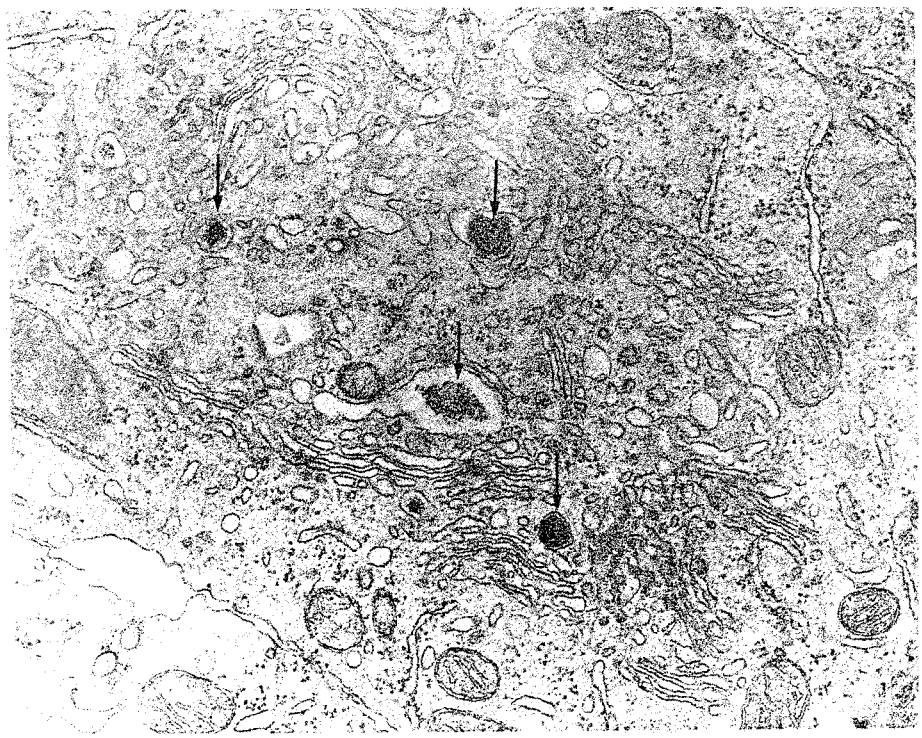

Fig. 1. The Golgi region of an AtT20 cell. Secretory protein (arrows) is seen condensing into secretory granule cores at the *trans*-side of Golgi stacks in an AtT20 cell. Note that the secretory protein becomes progressively more compact and spherical as the cores of the granules develop.

regulated secretory protein, proopiomelanocorticotropic (POMC) can be seen in the electron microscope (*3*). The condensing secretory protein compacts into a spherical dense core that buds from the TGN to form an immature secretory granule as Figs. 1 and 2 show. The same events take place in exocrine cells.

We can conclude from electron microscopic observations that secretory proteins destined for the cores of regulated secretory granules come out of solution in the lumen of the TGN at a very early stage in granule formation. This not only concentrates these proteins, but also renders them osmotically inert.

Involvement of Clathrin-Coated Vesicular Transport

The surfaces of the TGN and of immature secretory granules in AtT20 cells are sites of clathrin binding (Fig. 3), whereas mature secretory granules do not have clathrin coats. This difference is equally true of condensing vacuoles in exocrine pancreas and mature zygomen granules.

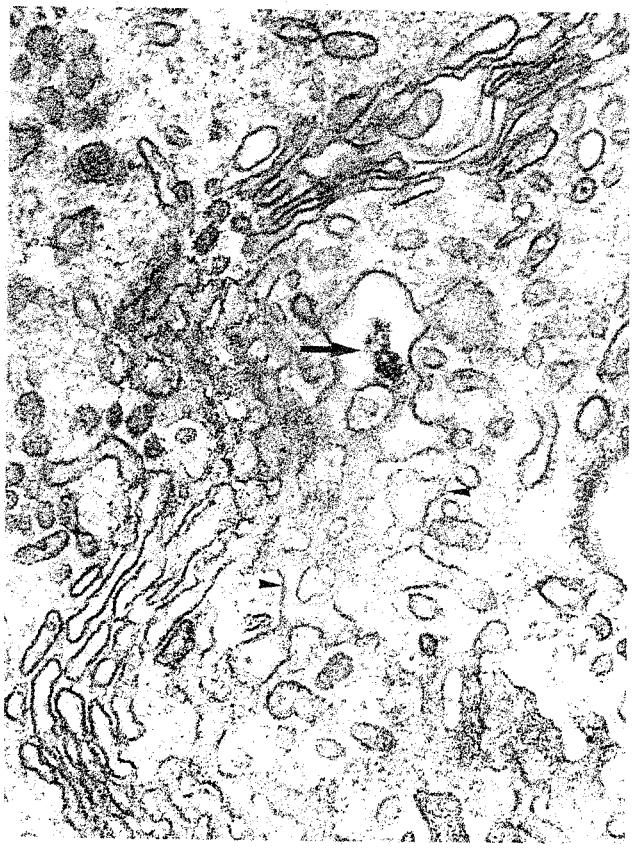

Fig. 2. The *trans*-Golgi network (TGN) is an AtT20 cell. This micrograph shows the reticular structure of the TGN (arrowheads) and condensing secretory protein in a region of the TGN (arrow).

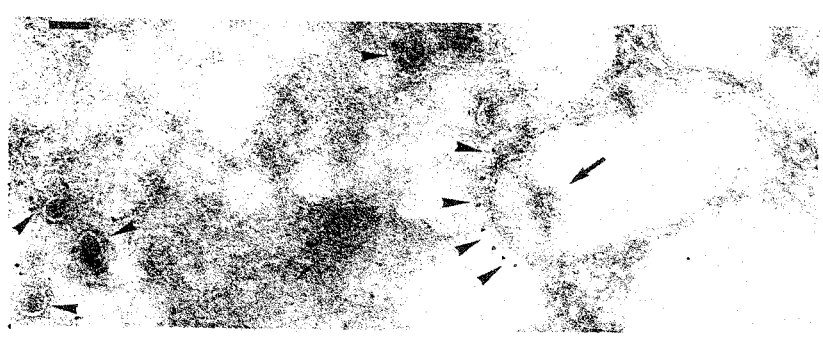

Fig. 3. A cryosection of an AtT20 cell labeled with anticlathrin antibody. Immunogold labeling reveals clathrin coats on vesicles and the surface of the TGN in AtT20 cells (arrowheads). Condensing secretory protein is indicated by the arrow.

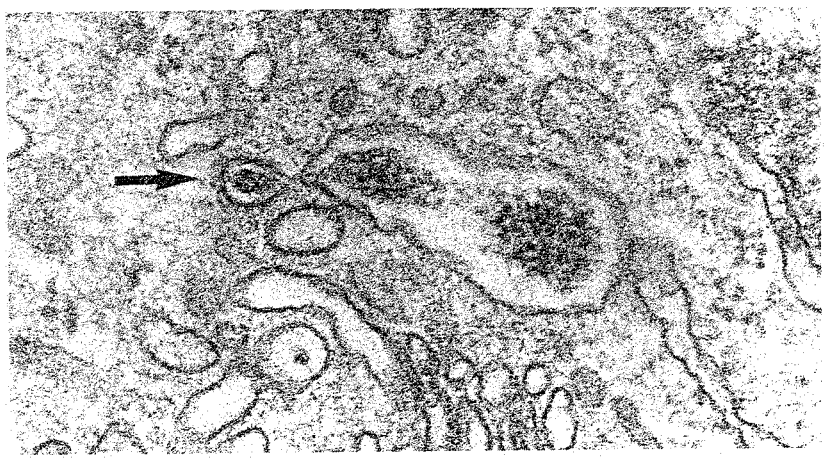

Fig. 4. Vesicular transport of condensed secretory protein in an AtT20 cell. This micrograph shows secretory protein in an AtT20 cell and secretory protein condensing in the TGN. A clathrin-coated vesicle (arrow) that contains condensed secretory protein appears to be budding from the TGN or possibly fusing with it. This micrograph and others establish that condensed secretory protein is transported in clathrin-coated vesicles.

Figure 4 shows a clathrin-coated vesicle that contains condensed secretory protein associated with an element of the TGN in an AtT20 cell. Irrespective of whether this vesicle is budding from, or fusing with, the TGN we can conclude from electron micrographs such as this that there is clathrin-coated vesicular transport of already condensed secretory protein in these cells. The presence of secretory protein is revealed by immunolabeling (Fig. 5).

Packaging Into Secretory Granule Before Cleavage

The regulated secretory proteins of exocrine pancreatic cells do not undergo activation by proteolytic cleavages until after secretion, whereas endocrine regulated secretory proteins undergo proteolytic cleavage to yield mature products within the cells in which they are made. In endocrine cells, where does the primary translation product undergo specific proteolytic cleavage(s) to produce the mature hormones? In particular, does cleavage precede the sorting and packaging into dense core secretory granules, or does it occur afterwards? Obviously, if cleavage precedes sorting and packaging, the complexity of the sorting event is increased, since the multiple products, instead of a single precursor, have to be sorted. We approached this question using AtT20 cells in which POMC is cleaved to yield ACTH and other hormones. We *(4)* raised an antibody against a short

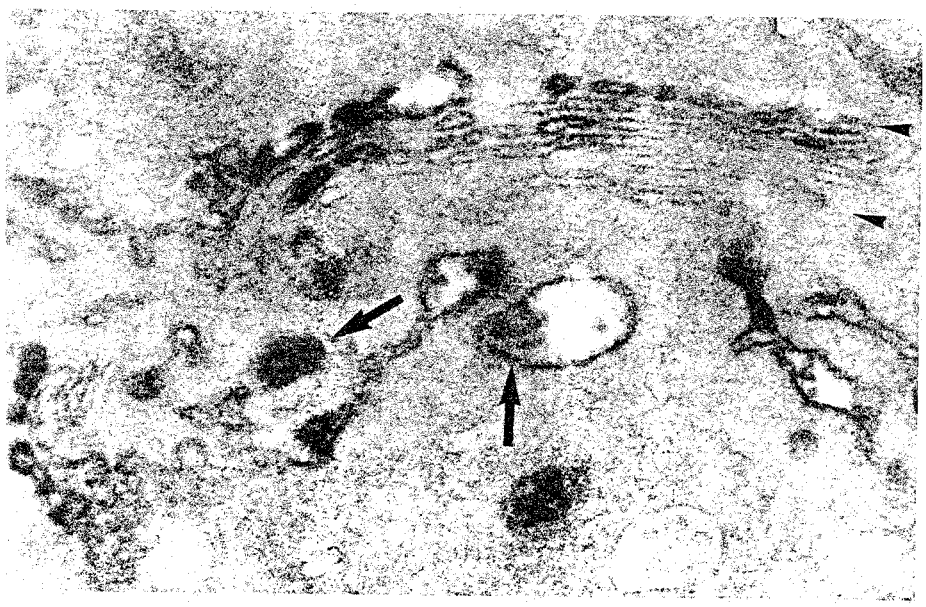

Fig. 5. Immunoperoxidase labeling of ACTH and POMC in AtT20 cells. This micrograph shows a Golgi stack with the cisternae containing peroxidase reaction product (arrowheads), indicating the presence of ACTH and POMC. Note the heavy deposits of reaction product in the TGN (arrows).

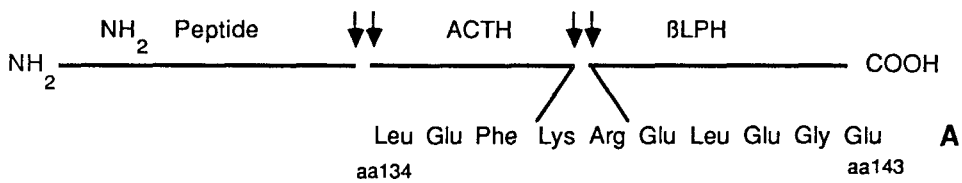

Fig. 6. Diagram of POMC and the cleavage sites on either side of the ACTH sequence. The paired arrows are cleavage sites that on cleavage liberate ACTH from POMC. The sequence A corresponds to that of cleavage site at COOH terminus of ACTH. An antibody was raised against a synthetic peptide with the sequence shown in A.

synthetic peptide with a sequence corresponding to the sequence around one of the POMC cleavage sites (Fig. 6). After cleavage the two basic amino acids in the middle of this sequence are removed, which means the epitope must be destroyed. This antibody, as expected, recognized POMC, but not ACTH (Fig. 7). When we immunolabeled AtT20 cells (Fig. 8) and cryosections (Fig. 9), some of the dense core secretory granules were specifically labeled and, therefore, must contain POMC. We concluded that at least some POMC is sorted to the secretory granules prior to its matura-

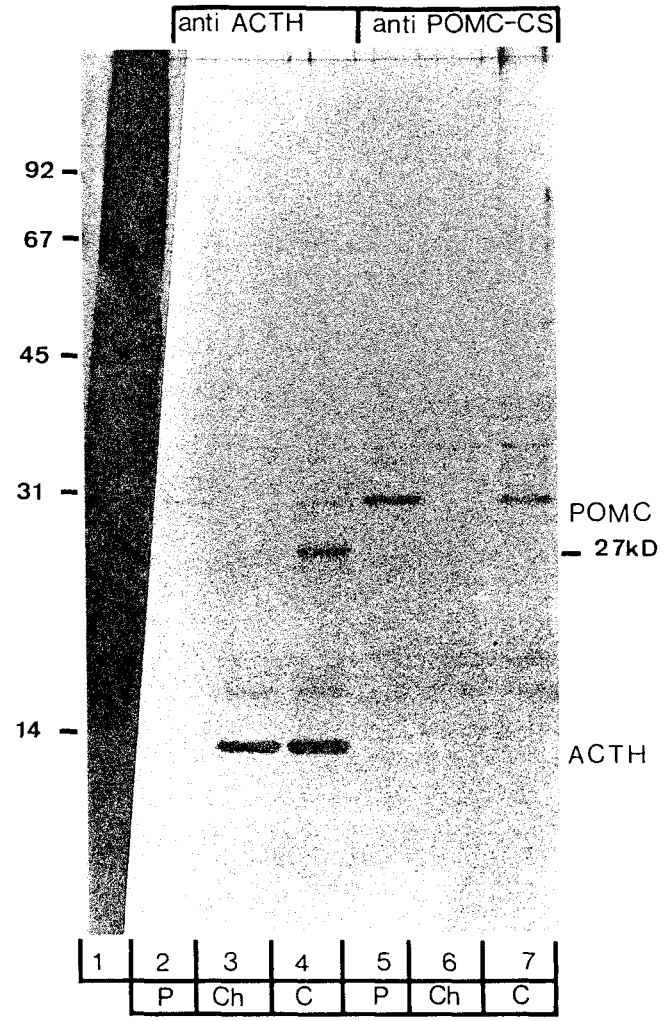

Fig. 7. An immunoblot of labeled ACTH cell extracts with the antibody raised against sequence A in Fig. 6. AtT20 cells were pulsed and then chased with ^{35}S methionine (P, Ch) or continuously labeled (C). Western blots were done with antibody against ACTH and against the cleavage site sequence shown in A, Fig. 6. This antibody was designated antiPOMC-cleavage site (POMC-CS). As this Western blot shows, antibody POMC-CS was specific for POMC and did not recognize ACTH. Anti ACTH recognized POMC and ACTH.

tion by proteolytic cleavage. We suspect that, in fact, all of the POMC is sorted to the granules before cleavage, but cannot with these experiments prove that the cleavage never precedes sorting. In other neuroendocrine cell types, evidence both for sorting before cleavage, and cleavage before sorting has been presented. In the endocrine pancreas, proinsulin appears to be sorted prior to cleavage, like the POMC in AtT20 cells, as the work of Orci's group has shown *(5)*. On the other hand, in neuroendocrine bag

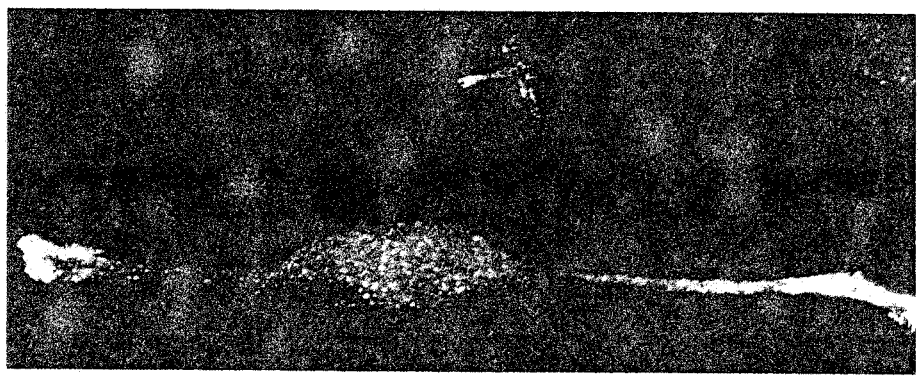

Fig. 8. Immunofluorescence labeling of an AtT20 cell with antiPOMC-CS. The antibody POMC-CS, specific for POMC-labeled secretory granules in the cell body and at the tips of processes in AtT20 cells.

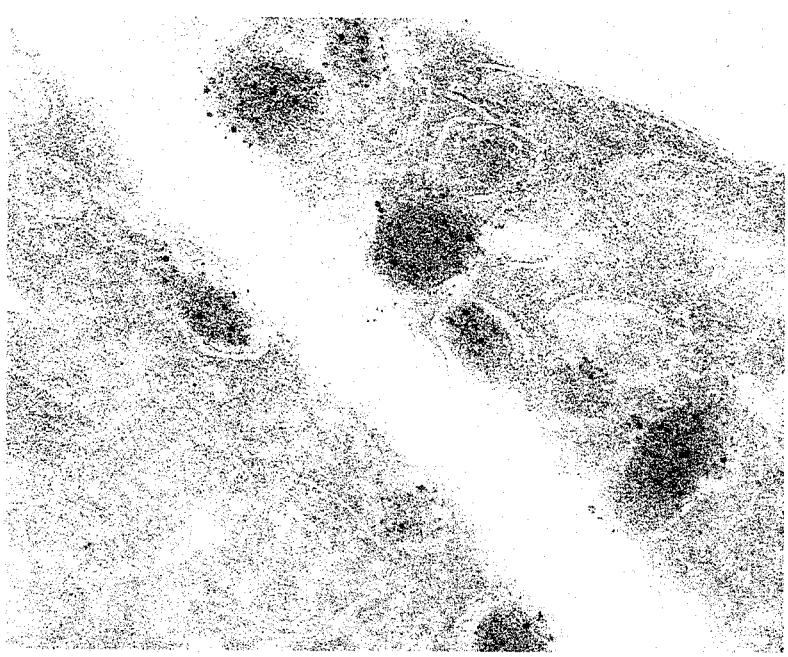

Fig. 9. Secretory granules double-labeled for POMC and ACTH. In this cryo section, secretory granules at the periphery of an AtT20 cell were labeled with rabbit antibody POMC-CS and large gold to reveal POMC, and then guinea pig antibody against ACTH and small gold to reveal ACTH. The secretory granules contain both POMC and its product ACTH.

cells in *Aplysia*, there is evidence for cleavage of regulated secretory proteins before sorting and packaging (*6*). It seems, therefore, that the timing of cleavage varies from cell type to cell type. One inescapable consequence of cleavage after sorting to the granules is that on secretion the cleavage products are released together.

How Are Regulated Secretory Proteins Sorted?

One of the fundamental questions posed by the regulated exocytic pathway in endocrine and exocrine cells is that of protein sorting. First, how are soluble regulated secretory proteins, the cargo of secretory granules, sorted into the dense cores of secretory granules to the exclusion of soluble constitutively secreted proteins? Second, how are specific membrane proteins sorted to the membrane of secretory granules? We know where sorting takes place, the TGN, but we do not know how it is achieved.

Model of Sorting Regulated Cargo Proteins: Receptor-Mediated Sorting

Sorting of soluble lysosomal enzymes from TGN to lysosomes involves membrane-bound mannose-6-phosphate receptors. Endocytosis of many species of ligands at the plasma membrane involves specific receptors, which may or may not recycle. By analogy with these events, it has been suggested that membrane-bound receptors in the TGN, and immature secretory granules budded from it, might be responsible for sorting regulated secretory proteins from constitutively secreted proteins and delivering the former to regulated secretory granules.

Basically, this model envisages binding of regulated secretory protein in the TGN, clustering of receptor–ligand complexes, budding of the immature secretory granule, and recycling of the receptor from the detached secretory granule back to the TGN. Since in endocrine cells, at least, the pH of the granule is lower than that of the TGN, by analogy with the mannose-6-phosphate receptor, one can postulate that the affinity of the putative sorting receptor for the ligand is high at the pH of the TGN and low at the pH of the granule. In exocrine pancreas, the zymogen granules have a higher pH than condensing vacuoles, if DAMP labeling is a reliable indicator of intracellular pH. In the case of the exocrine pancreas, one would have to argue that the receptor had lower affinity at higher pH.

The vehicle for receptor recycling could be clathrin-coated vesicles, because in exocrine and endocrine cells, clathrin occurs in patches at the surface of the TGN and on the surface of immature secretory granules near the Golgi apparatus. Moreover, as shown here, there is clathrin-coated vesicular transport of secretory protein. Clathrin is not, however, seen on

the surface of mature endocrine or exocrine secretory granules. There can be no doubt that clathrin-coated vesicles play some role in secretory granule formation, but there is still no clear indication of what that role really is. The presence of clathrin on the TGN and secretory granule membrane does not address the question of whether or not there are membrane-bound receptors for anything other than clathrin. To my mind, the receptor sorting hypothesis for the sorting events involved in the formation of secretory granules is, in its simplest form, wrong. The fundamental problem with this model is that it does not take account of the very large amounts of cargo protein to be sorted. Receptors in membranes are two-dimensional; they are a surface. The core of a secretory granule is a large volume of highly concentrated protein, so concentrated that it is osmotically inert out of solution. I do not believe a membrane-bound receptor, a two-dimensional sheet, could have the capacity needed to fill the volume of a secretory granule core. Membrane-bound receptor-mediated sorting is able to sort proteins at low concentrations, e.g., lysosomal enzymes in the TGN or growth factor in the medium, but not the bulk sorting or regulated secretory proteins. Imagine filling your car's petrol tank with 20 gallons of petrol, spread as a monolayer. To make a volume from a sheet, you laminate, as in a book, and secretory granules are not filled with laminated membrane; quite the opposite, they are spheres and use the minimum possible amount of membrane to envelope a given volume of precipitated protein.

Condensation as a Sorting Mechanism

The condensation sorting model of secretory granule core formation *(1)* envisages that regulated secretory proteins have evolved such that they come out of solution as coprecipitates when they reach a particular concentration in compartments of the secretory pathway. In all probability, the ionic milieu and the pH may play roles in determining at what concentration coprecipitation is possible. Clearly under normal circumstances the coprecipitation is restricted to the TGN.

If regulated secretory proteins coprecipitate in the TGN whereas constitutively secreted proteins do not, the precipitation event brings about both sorting and concentration of the former; it also renders them osmotically inert. I do not wish to imply that constitutively secreted proteins can never precipitate or crystallize from solution; they can, but not in vivo where they are not made in amounts comparable to the regulated secretory proteins of exocrine and endocrine cells.

Is there any evidence in favor of the condensation sorting model? Condensation of regulated secretory proteins can be seen in the EM normally in the TGN. However, as the work of Farquhar *(7,8)*, Hopkins *(9)*, Broadwell and Oliver *(10)*, and others has shown, when endocrine cells

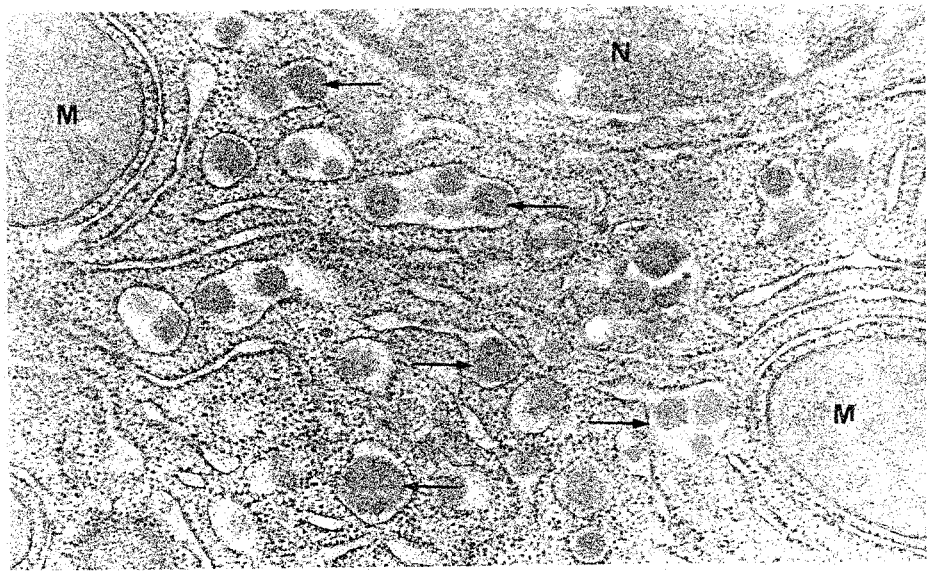

Fig. 10. Intracisternal granules in guinea pig exocrine pancreas. Several cisternae of the rough endoplasmic reticulum in this section of a guinea pig exocrine pancreatic cell contain intracisternal granules (arrows) that are aggregates of the set of pancreatic secretory proenzymes and enzymes that normally form the cores of zymogen granules.

are hyperstimulated, condensation occurs in earlier compartments of the pathway, in the *cis* and medial Golgi, and even in the RER. As long ago as 1956, Palade *(11)* reported intracisternal granules, interpreted as spheres of condensed secretory protein, in the RER of guinea pig exocrine pancreas. What all these observations tell us is that condensation of secretory proteins is not dependent on the environment of the TGN. It cannot, therefore, depend on receptors found only in effective concentration in the TGN and secretory granule membranes.

Recently Horst Kern, Kathryn Howell, Steve Fuller, and I *(12)* have shown that intracisternal granules in the RER of guinea pig pancreas (Fig. 10) contain the complete set of secreted proenzymes and enzymes that are found in zymogen granules. However, they did not include two abundant soluble resident RER proteins, heavy chain binding protein (BiP) and protein disulfide isomerase (PDI). Therefore, the formation of ICGs is a sorting event. Of course, ICGs are not zymogen granule cores. First, most of the proteins in the ICGs are crosslinked by intermolecular disulfide bonds, rendering them completely insoluble in the absence of reducing agents, which is certainly not the case for zymogen granule cores. Second, the ICGs never progress down the exocytic pathway. When they

leave RER, they go to autophagic vacuoles. They are not secreted. Nevertheless, their formation is a sorting event, and it can occur in the environment of the RER. Likewise, in the RER of rat pancreas, amylase can crystallize *in situ*, and the crystals contain other zymogens, but not PDI and BiP. Crystallization is, of course, a condensation event in which the condensed molecules are packed in a regular array. In short, cocondensation of regulated secretory proteins can sort them from other soluble proteins in the same compartment, and there is ample evidence to show that cocondensation of these proteins is not restricted to the TGN even though that is its normal site.

The Granule Membrane—An Unsolved Problem

Cocondensation of soluble secretory proteins can generate cores; however, it does not in any way explain how the cores become enveloped in secretory granule membrane with a specific protein composition. The granule membrane is clearly derived from regions of the membrane of TGN. As yet, we do not know how the membrane associates with the cargo proteins condensed in the cores, nor do we know how and when the specific protein composition of the granule membrane is achieved. There might well be a place for membrane-bound receptors in the TGN and immature granule membranes that recognize precipitated aggregates of secretory proteins. This in effect would involve the recognition of one surface, that of the spherical core, by a second surface, the TGN/immature granule membrane. Such receptors, if they exist, should not recognize monomeric secretory proteins. One can also postulate that the clathrin-coated vesicular transport from immature granules serves to remove inappropriate membrane proteins from the granule membrane. Finally, the secretory granule must include proteins able to cause fusion of the granule with the plasma membrane only after a signal is relayed following the binding of a fusogen to the plasma membrane. The molecules that allow constitutive fusion of constitutive secretory vesicles with the plasma membrane must be excluded from the membrane of regulated secretory granules or inactivated if they are present.

CONCLUSION

A deeper understanding of the regulated exocytic pathway, and for that matter the constitutive exocytic pathway, will depend on our ability to characterize the proteins in the vesicle membranes. Characterizing the protein composition of secretory granule membrane has proven to be a formidable task, and as far as I know, the work done to date has

not told us a great deal about the mechanisms involved in sorting the contents of regulated secretory granules, or bringing about constitutive or regulated fusion with the plasma membrane. Without knowing a great deal more about the membranes, there seems to be little prospect of real further progress in understanding the key properties of the regulated exocytic pathway.

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