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Protein Hormone Storage in Secretory Granules: Mechanisms for Concentration and Sorting*

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I. Introduction: Concentrating Protein Hormones in Secretory Granules

ALL CELLS have secretory pathways for transport of proteins to the plasma membrane or to lysosomes. Proteins in this pathway are synthesized on polysomes attached to the endoplasmic reticulum and pass through its membranes into its lumen. Vesicular or tubular structures transport proteins from there to the *cis*-Golgi region, and the proteins travel through the stacks of the Golgi complex to the *trans*-Golgi side. At this point, several kinds of sorting into different vesicles occur, such as separation of lysosomal proteins from those bound to the cell surface.

The secretory pathway in neuroendocrine cells, exocrine cells, peptidergic neurons, mast cells, and other secretory cells that store proteins has an additional process that concentrates specific proteins into secretory granules. Protein hormones, made in large amounts in neuroendocrine cells, are stored in large dense-core vesicles packed with protein, commonly known as secretory granules. Cells retain the granules until stimulated, when they release the contents through exocytosis, so that large amounts of hormones may be rapidly available when needed. Concentration of hormones in granules is extensive; PRL, for example, is 200 times more concentrated in the dense cores of secretory granules than in the lumen of the endoplasmic reticulum (1). How cells concentrate hormones is a major unanswered question in endocrinology.

Although the question is unanswered, models have been proposed based on the possibility of a sorting signal to direct or retain proteins in secretory granules and on the possibility that aggregation of hormones serves both a concentration and a sorting function. Mechanisms are beginning to be understood for sorting of some transmembrane and soluble proteins in the secretory pathway, but in the case of proteins that are concentrated, it is not even clear whether there is any sorting process other than aggregation. Many reviews have been written about sorting of hormones into granules (2–20). This review focuses on mechanisms by which soluble protein hormones are concentrated into secretory granules. It differs from previous reviews in that two points are emphasized: 1) new findings and new interpretations of transport through the secretory pathway affect conclusions about sorting into secretory granules; and 2) there is unexpected specificity in handling of secretory granule cargo proteins.

II. A Well Characterized Sorting Mechanism: Lysosomal Hydrolases

A. Sorting is linked to transport

Probably the best characterized system of sorting of soluble proteins is that of lysosomal hydrolases, which are separated from other proteins after transport through the Golgi complex (Fig. 1) (21, 22). The Golgi complex is a broad, multilayered ribbon of enclosed membrane stacks (23). Proteins in the secretory pathway proceed from the *cis* to the *trans* side of this complex, and, in the outermost layer called the *trans*-Golgi network, proteins are sorted and carried to different locations in the cell (15).

Lysosomal hydrolases have mannose-6-phosphate groups attached to their carbohydrate moieties, and these groups are recognized by a transmembrane protein in the *trans*-Golgi network called the mannose-6-phosphate receptor. Lysosomal hydrolases bind to this transmembrane protein in the lumen of the Golgi cisternae, and a protein called AP-1 (adaptor protein 1) binds to this protein on the cytosolic side (21, 22, 24). Clathrin, a protein identified years ago as a component of a caged structure around certain small membrane vesicles, binds to AP-1, and the membrane in this area begins to invaginate (22). The invagination buds off into a separate vesicle through an active process that involves a protein, dynamin (25), and the vesicle carries the enclosed lysosomal hydrolyses to the endosomes, the next membrane compartment en route to the lysosomes. The two processes, sorting and transport, are linked, because the mannose-6-phosphate receptor that binds the vesicular cargo of lysosomal enzymes on the lumen side also binds the proteins necessary to form the vesicle on the cytosolic side. AP-1 is an adaptor protein that links clathrin to transmembrane proteins, and the mannose-6-phosphate receptor, although traditionally called a receptor, is also an adaptor protein linking specific soluble cargo proteins to part of the machinery necessary for forming vesicles.

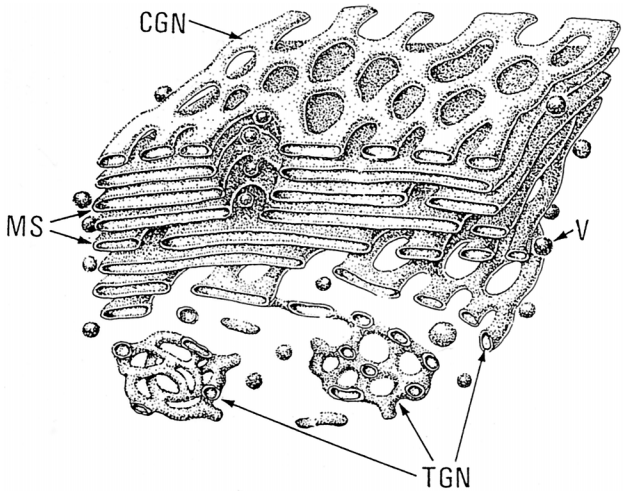

FIG. 1. A portion of the Golgi apparatus of an epithelial nonciliated cell of the ductuli efferentes. Fusing vesicles originally from the endoplasmic reticulum form the *cis*-Golgi network (CGN). Proteins progress through the stack and are eventually sorted at the *trans*-Golgi network (TGN). V, Vesicle; MS, midsaccules, also called medial cisternae. [Reproduced with permission from Y. Clermont *et al.*: *Anat Rec* 242:289–301, 1995 (23). © Wiley-Liss, Inc., a subsidiary of John Wiley and Sons, Inc.]

B. Specific transport is associated with specific vesicles

This example of sorting is also an example of the specificity of vesicles involved in transport through the secretory pathway, because clathrin-coated vesicles are only involved in certain phases of transport, and additional vesicle classes have been characterized. Clathrin-coated/AP-1 vesicles transport lysosomal hydrolases from the *trans*-Golgi network to the endosomes; using a similar protein, adaptor protein-2 (AP-2), clathrin-coated/AP-2 vesicles transport proteins from plasma membrane to endosomes (22). Vesicles with completely different coats designated COPII (coat protein complex II) transport proteins from the endoplasmic reticulum to the region where the *cis*-Golgi layer forms, and vesicles with coats designated COPI transport vesicles back from the Golgi complex to the endoplasmic reticulum as well as through the Golgi cisternae (26–30). In addition to those identified above, other kinds of vesicles have been described emerging from the *trans*-Golgi network (15), and sorting occurs for which coat proteins have not been identified; therefore, this area continues to develop.

In neuroendocrine cells, clathrin-coated patches occur on parts of the *trans*-Golgi membrane where dense cores of secretory granules form and on parts of the membrane of immature secretory granules (31, 32). Immature granules contain lysosomal enzymes that are removed as granules mature (33, 34), and it seemed likely, therefore, that clathrin-coated vesicles remove lysosomal enzymes from immature secretory granules as well as from the *trans*-Golgi network. Arvan and co-workers have recently demonstrated that sorting of these enzymes from the immature granules uses the same system involving the mannose-6-phosphate receptor as elsewhere in the *trans*-Golgi network (34, 35).

The elucidation of the role of mannose-6-phosphate in the sorting of soluble proteins in the secretory pathway occurred early on (22) and was one of the factors that led to models in which sorting signals were predicted to be present on other proteins, including those destined for secretory granules. Sorting of proteins clearly occurs in the *trans*-Golgi network, which led to the assumption that all important sorting occurs there. Recent work has shown, however, that sorting linked to transport also occurs in the endoplasmic reticulum.

III. Sorting of Soluble Transported Proteins in the Endoplasmic Reticulum

Soluble proteins in the endoplasmic reticulum that assist in protein folding are retained there or are returned to that location if they do leave (36–38). Investigations have shown retention usually occurs through a KDEL sequence on the retained proteins, and the initial view was that soluble proteins are passively carried from the endoplasmic reticulum unless they are actively retained by this sequence. A more recent finding is that sorting occurs not only by retention, but also that proteins that are not meant to stay in the endoplasmic reticulum may be selected and actively transported from there.

The first evidence that sorting occurs came from Mizuno and Singer (39), who showed that the soluble secretory protein serum albumin is concentrated in discrete areas of the endoplasmic reticulum before transport to the *cis* face of the Golgi complex. Vesicular stomatitis virus glycoprotein, a membrane protein, is also concentrated in the course of transport from the endoplasmic reticulum (40).

A. Sorting in yeast

Further evidence that sorting occurs to select proteins for transport out of the endoplasmic reticulum has been found in yeast, and the mechanisms are beginning to be identified (28). So far, the principle that emerged from sorting of lysosomal enzymes, that sorting of soluble cargo proteins is linked to proteins involved in vesicle formation, is emerging in this sorting as well, shown by biochemical and genetic investigations. COPII vesicles carry proteins from the endoplasmic reticulum, and Sar1, Sec23, and Sec24, three components of yeast COPII, form a complex with proteins that are transported from the endoplasmic reticulum, including amino acid permeases, which are integral membrane proteins, and glycosylated pro-α-factor, which is a soluble secreted protein (41). Proteins that remain in the endoplasmic reticulum are not included in the complex, demonstrating specificity. Binding of the soluble pro-α-factor inside the lumen to the coat proteins on the cytoplasmic side of the membrane must be mediated by a transmembrane adaptor protein not yet identified. There is likely to be more than one adaptor component, since a consensus sequence among transported proteins has not been found, and therefore more than one sorting signal for leaving the endoplasmic reticulum is predicted.

Genetic evidence for more than one sorting system to carry proteins out of the endoplasmic reticulum comes from yeast with temperature-sensitive mutants of a COPI component, Sec21. Mutations in Sec21 allow normal secretion of two proteins, invertase and HSP159, but two others, carboxypeptidase Y and pro-α-factor, remain in the endoplasmic reticulum. Carboxypeptidase Y-invertase hybrids, however, leave the endoplasmic reticulum, suggesting there is a signal for forward transport in the invertase molecule (42). COPI vesicles transport proteins from the Golgi complex back to the endoplasmic reticulum. The simplest explanation for why mutations in a retrograde pathway affect transport forward through the secretory pathway is that mutations in COPI components trap adaptors in the Golgi cisternae that are unable to return to fulfill their function of causing forward transport, because COPI vesicles recycle adaptor proteins from the Golgi complex back to the endoplasmic reticulum. A candidate for these adaptor proteins in yeast is the p24 family, because deletion of one member of this family, Emp24, affects the transport of invertase and not carboxypeptidase Y (43).

B. Sorting in mammalian cells

These investigations in yeast have shown there is active sorting by more than one system for transport from the endoplasmic reticulum. Recent evidence indicates such sorting also occurs in mammalian cells, at least for some proteins. Transfer from the endoplasmic reticulum occurs through irregularly shaped vesicles that emerge from the endoplasmic reticulum and fuse in the *cis*-Golgi region (44). This area has been called vesicular-tubular clusters or the endoplasmic reticulum-Golgi intermediate compartment, usually abbreviated ERGIC (45). Recent genetic studies indicate that ERGIC-53, a protein found primarily in this location, is involved in selective transport of certain proteins in people. Combined deficiency of coagulation factors V and VII is an autosomal recessive bleeding disorder resulting from alterations to a single gene, ERGIC-53 (46). In addition to its location in the cell, ERGIC-53 has other characteristics that might be expected of a protein involved in sorting: it binds mannose and associates with the COPII component, Sec23 (47). Since COPII vesicles carry proteins forward from the endoplasmic reticulum, the association of COPII components with ERGIC-53 is further evidence for a link between vesicle formation and sorting.

Although ERGIC-53 is necessary for normal secretion of coagulation factors V and VII, what is perhaps surprising is that there are no obvious deficiencies in other secreted proteins in people in whom ERGIC-53 is mutated, suggesting that ERGIC-53 is necessary for a limited set of proteins. The deficiency of the two coagulation factors, however, is not complete, for they are present in the serum at reduced levels (46). Nichols and co-workers (46) suggest that partial deficiencies in the transport of other proteins may exist but may not be as clinically apparent.

C. Implications for sorting into granules

What difference does it make to investigations of sorting into secretory granules if there is some sorting that occurs in the endoplasmic reticulum? One immediate difference is that interpretation of experiments to detect sorting signals becomes more complex. It has been known for some time that proteins must be folded correctly to leave the endoplasmic reticulum and proceed further along the secretory pathway (36, 48), and it has seemed reasonable that mutations in secreted proteins that resulted in their retention in the endoplasmic reticulum did so by disrupting folding. There is now an alternate interpretation: that a sorting signal necessary for transport out of the endoplasmic reticulum may have been affected. Prosequences of secretory proteins have been shown to facilitate transport from the endoplasmic reticulum (49). Such facilitation could be caused because the sequence increases the rate of correct folding, allows the formation of oligomers, or reacts with adaptors to increase active transport from the endoplasmic reticulum.

If all proteins that are transported from the endoplasmic reticulum remain mixed in common vesicles as they proceed forward through the Golgi complex, even if more than one set of adaptor proteins is involved, then what happens before the Golgi complex may not be important in so far as secretory granule sorting is concerned. Much sorting occurs on the *trans* side of the Golgi complex in the vesiculating region known as the *trans*-Golgi network; if all proteins are in common vesicles, they will still be mixed when they reach this point. There is, however, one piece of evidence that suggests

some sorting may occur earlier and that all proteins are not in the same *trans*-Golgi network. Ladinsky and co-workers (50) developed three-dimensional reconstructions of *trans*-Golgi regions of rat kidney NRK cells, using high-voltage electron microscopy. They found that individual regions of the *trans*-Golgi network with no tubular connections to each other each produced vesicles of only one type, either clathrin-coated or a type not previously described with a lacey coat (50). From what has been learned so far about vesicular transport, different coats imply different cargos. The authors predicted from this observation that some sorting of proteins occurs before the *trans*-Golgi network. What gets sorted, and whether there is any relation to the sorting that occurs in the endoplasmic reticulum is unknown at present.

IV. Two Models for the Functioning of the Golgi Complex

A. Vesicular transport of proteins vs. maturation of stacks

The model of Golgi function usually described and presented in textbooks is one in which proteins in the secretory pathway, including soluble secreted proteins, are carried forward from one layer of the Golgi complex to the next by COPI-coated vesicles (26, 51, 52) (Fig. 2). Each layer of the Golgi complex is a fixed compartment containing different sets of resident enzymes; secretory proteins pass through each distinct layer. A second model, less frequently described, has been called the directed maturation model (45). In this model, the Golgi stacks are made of progressively maturing, discontinuous compartments with no forward transport by vesicles (Fig. 2). Instead, a layer of the Golgi complex forms at the *cis* side by fusion of vesicles from the endoplasmic reticulum, and enzymes that process the proteins are brought by COPI vesicles to the most recently formed layer back from layers established earlier. The layer itself progresses from the *cis* to the *trans* side of the complex as new layers form behind it, and as the ones ahead vesiculate and disappear in turn in the final sorting process. Implicit in the model is the consumption of each layer in the final sorting. Bannykh and Balch (45) discuss how this model fits in well with current experimental findings. Among the evidence for this model are the results of investigators who examined the Golgi complex by three-dimensional electron microscopy and concluded that the *trans*-Golgi layer is completely consumed by the final budding of vesicles or by fusing to the plasma membrane in certain cell types (53, 54).

B. Implications for sorting into granules

The directed maturation model for protein traffic through the Golgi complex may have implications for sorting. If this model is correct, it is easier to envision mechanisms by which separate sorting systems for transport from the endoplasmic reticulum might result in the kind of segregation in the *trans*-Golgi region found in NRK cells. In addition, if the first model of vesicular traffic forward through the Golgi complex occurs, then aggregation of proteins that will be concentrated in secretory granules must be restricted to a size that will fit in the small COPI-coated vesicles until the last layer is reached. In the directed maturation model, no such restriction applies, and formation of large aggregates may begin earlier.

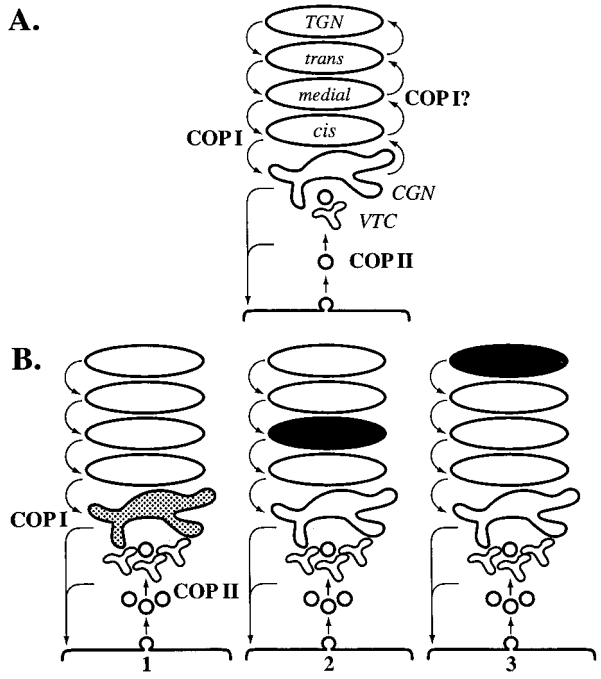

FIG. 2. Two models for transport through the Golgi complex. A, Vesicular transport model. COPII vesicles transport proteins from the endoplasmic reticulum to vesicular-tubular clusters (VTC) and the *cis*-Golgi network (CGN). COPI vesicles retrieve proteins and membrane back from each layer of the Golgi complex, *cis*, medial, *trans*, and the *trans*-Golgi network (TGN) to the endoplasmic reticulum. Transport of secretory proteins forward through the stack of Golgi cisternae are mediated by small vesicles at each step. Each compartment is distinct. B, Directed maturation model. Assembly of COPII vesicles forms vesicular-tubular clusters that merge to ultimately form the first layer, the *cis*-Golgi cisternae. Secretory proteins do not exit the first layer; instead, COPI vesicles carry processing enzymes to it from more mature layers. As new layers form behind, the layer moves up through the stack until it is consumed by sorting in the *trans*-Golgi network. [Reproduced with permission from S. I. Bannykh and W. E. Balch: *J Cell Biol* 138:1–4, 1997 (45). © The Rockefeller University Press.]

V. Models for Formation of Secretory Granules

A. Active budding off of secretory granules vs. active budding off of everything else

The usual version of secretory granule formation is that secretory granule proteins are brought to specific areas of the *trans*-Golgi network, sometimes called condensing vacuoles, and that secretory granules bud off from the *trans*-Golgi network by an active process that may resemble a virus budding (2, 14, 52). Formation of secretory granules in this model does not differ in principle from the formation of other vesicles except for the size. Immature secretory granules have detached from the *trans*-Golgi network, although they may still have some characteristics of that area, including lysosomal enzymes (33, 35) and clathrin coats attached in patches on the surface (31, 55–57). Immature granules may

fuse with each other before the final mature granules form (33, 58).

A different vision of the formation of secretory granules comes from the work of Rambourg and co-workers (53), who examined PRL-producing cells of lactating rats by three-dimensional electron microscopy. PRL is found in aggregates in the lumen of the *trans*-Golgi cisternae; these lumps of PRL are in the plane of the layer of *trans*-Golgi complex, scattered throughout (Fig. 3) and not in a sequestered region projecting above the layer. The morphology is consistent with this *trans*-layer beginning to peel away from the Golgi stack as small vesicles form from it, some clathrin-coated and some not, removing membrane that is not directly around the aggregated PRL. As these vesicles are removed, the remaining membrane of the layer, no longer an uninterrupted surface, takes on the tubular-vesicular appearance of the *trans*-Golgi network. What has been termed immature granules are, in this view, those aggregates in which removal of membrane by clathrin-coated and other vesicles is not complete (Fig. 3). What has been interpreted as immature granules that have fused to each other may be two or more protein aggregates that were drawn near each other as the *trans*-Golgi layer is consumed (Fig. 3), although this does not rule out fusion of separate vesicles as well. Further removal of membrane eventually leads to mature granules. Membrane removal has been known to occur from secretory granules for some time (59); the difference in models is whether secretory granules have budded first. In the more traditional view, secretory granules bud from the *trans*-Golgi network; in this view, everything else buds off, leaving the secretory granules, and the difference between the *trans*-Golgi network and immature secretory granules is a quantitative one, depending on the amount of membrane removal, rather than a qualitative one.

Such a mechanism clearly occurs in mammary gland cells, where very little buds from the *trans*-Golgi stacks, which are filled with milk proteins; these stacks each fuse in turn in their entirety with the plasma membrane (23, 54). The only endocrine cell type to be examined so far by three-dimensional electron microscopy is the lactotroph; it will be interesting to see whether the morphology of other neuroendocrine cells is similar.

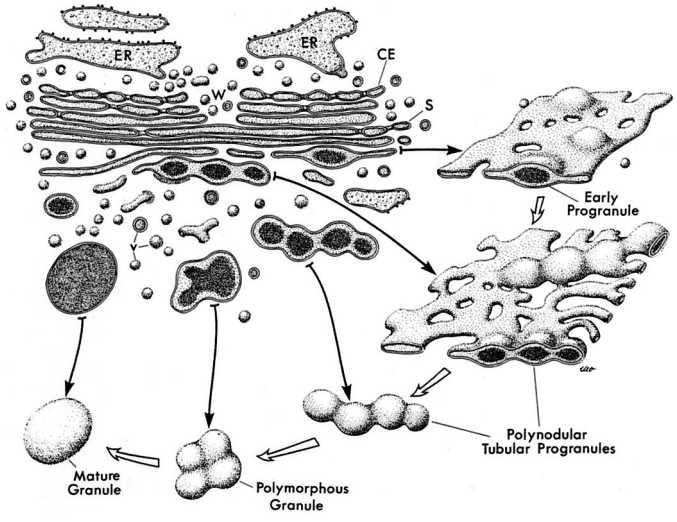

FIG. 3. A portion of the Golgi apparatus of a rat lactotroph. PRL is made in the endoplasmic reticulum (ER) and transported to the Golgi complex. W, Well, a gap between the *cis*-elements (CE) of the Golgi apparatus. S, Saccule, also called a Golgi cisternae. PRL aggregates are detected in the *trans*-Golgi cisternae, shown in two and three dimensions. What appear in two dimensions to be fused immature granules are neighboring aggregates of PRL in the lumen. The *trans*-Golgi layer progressively vesiculates through vesicles (V) budding off, until the mature secretory granules remain. [Reproduced with permission from A. Rambourg *et al.*: *Anat Rec* 232:169–179, 1992 (53). © Wiley-Liss, Inc., a subsidiary of John Wiley and Sons, Inc.]

B. Implications for sorting into granules

If everything buds off, leaving membrane containing aggregated protein inside, what are the consequences for models of secretory granule formation and sorting into secretory granules? One consequence is that there is no need for a separate budding mechanism for secretory granules from the *trans*-Golgi network. Coat proteins play a role in formation of vesicles, but not in delivery of vesicles to their destinations; other proteins in and associated with the membranes of vesicles direct fusion to the correct sites, and vesicles must lose their coats to fuse (26, 60). Secretory granules will still need to obtain the proteins necessary for regulated delivery to the plasma membrane, but will not need to obtain separate proteins for budding. The same mechanisms that other cells use for constitutive transport to lysosomes and plasma membrane and for recovery of proteins that belong in the Golgi complex will be all that is needed as long as the components of these pathways exist in sufficient quantity to remove excess membrane from secretory granules. Several groups have attempted to determine the requirements necessary for secretory granule formation in cell-free systems (61–65). What they may actually be detecting in these reconstituted systems are requirements for budding of other vesicles, and what remains is the granules.

A second consequence of a model in which aggregation before vesicle budding occurs is the potential for aggregation to be an excellent mechanism for sorting in and of itself. Some vesicle types, such as those coated with clathrin, are small and, when forming, could enclose soluble proteins or aggregates of small size, but not the large aggregates of PRL that have formed in the *trans*-Golgi lumen before the vesiculation of that Golgi layer begins (Fig. 3). The effect of preexisting large aggregates may be that proteins that are not aggregated will be carried off by other vesicles as they form, leaving the proteins that have aggregated in the resulting secretory granules. If the directed maturation model of Golgi transport is correct, the aggregate need not form all at once in the *trans*-Golgi layer, but may begin to form as the protein hormone enters the Golgi complex, developing in size until it reaches the large size detected in the *trans*-Golgi lumen.

Finally, it is possible that aggregation is not just a sorting mechanism, but may be, in addition, an important factor driving formation of secretory granules. Such a function will exist if the membrane proteins that do not aggregate, but that must localize in granules for correct function, recognize some aspect of the aggregate to remain in the membrane that becomes part of the granules. This possibility is discussed further in later sections. An important point is that aggre-

gates alone are not sufficient to form secretory granules. Fibronectin, which aggregates easily, forms membrane-enclosed aggregates in AtT20 cells, but these vesicles do not behave as secretory granules, and regulated release of fibronectin can not be induced (66). There must be some form of recognition between the contents of the granule and the membrane proteins necessary for functioning of the granule.

VI. Hormone Aggregates in Cells

A. Evidence for their existence

The appearance, detected with electron microscopy, of the dense material in the *trans*-Golgi lumen and secretory granules of rat lactotrophs certainly looks like an insoluble mass of protein (53), but the cells have been fixed and subjected to other procedures in the processes necessary to obtain these pictures. Does an insoluble aggregate of hormone exist before cells are subjected to these procedures? For rat PRL, the answer is yes. Rat PRL granules are large and dense and may be easily separated from other organelles, after which their membranes are removed by the addition of nonionic detergent (67). This procedure leaves the dense cores of the granules intact, in a form that sediments rapidly and that appears the same by electron microscopy as the dense cores in secretory granules in cells (67). PRL in granules is therefore in a form so aggregated that it behaves as large particles rather than soluble protein, and aggregation is not an artifact of electron microscopy. Clumps of rat PRL in the lumen of the *trans*-Golgi cisternae have the same appearance as the dense cores in granules, so it is most likely that the same insoluble aggregates have already formed in this area of the secretory pathway.

The location where the aggregates first start to form is important, because it is the location of PRL aggregates relatively early in the sorting process in the *trans*-Golgi lumen that raises the possibilities that aggregation is an effective sorting mechanism and is a factor in the formation of granules. Rat PRL is not a complex molecule; the signal sequence that is necessary for its import into the endoplasmic reticulum is rapidly removed, and after that, no further processing or addition is necessary for storage (68).

Other hormones, however, do require processing. Insulin traverses the secretory pathway as proinsulin until it is cleaved into insulin and C-peptide, a process that occurs in immature granules (31, 55). Rat insulin does become insoluble in granules in a crystalline form that sediments rapidly when the contents of the granules are isolated, and, in these conditions, C-peptide is soluble (69). The crystalline form, however, does not occur in all species [*e.g.*, guinea pigs package insulin into secretory granules whose contents remain homogeneous, rather than separating into a compact insulin core with a lucent halo of C-peptide, as rat and mouse insulins do (70, 71)]. Mice that have deficient or mutated processing enzymes do not process proinsulin to insulin well, but package proinsulin into granules that resemble morphologically those of the guinea pig (72, 73). Therefore, the crystalline form of insulin is not necessary for concentrated storage in secretory granules.

The crystalline form of insulin occurs relatively late in the secretory pathway in immature granules and is not analogous to the aggregates of PRL found before extensive formation of vesicles occurs from the *trans*-Golgi cisternae. Concentrated masses of proinsulin are detected by electron microscopy in the *trans*-Golgi network in forms that look similar to aggregates of PRL (31, 55). It has not been shown by means independent of electron microscopy that these masses of proinsulin are some kind of aggregate. If aggregation of proinsulin functions as a sorting mechanism, the aggregate must be porous enough or reverse easily enough to allow processing enzymes to function rapidly. Although proteases eventually chew up crystals of proteins, the processing of insulin occurs relatively rapidly, and, if there is an aggregate, it is simplest to imagine cleavage to insulin occurring if the aggregate is loose, as a kind of colloid or gel.

ACTH is produced by processing the prohormone, POMC. Dense masses of POMC are also detected in the *trans*-Golgi network by electron microscopy (57, 74), but, again, there is no separate evidence that an aggregate of POMC forms. Because processing of POMC, proinsulin, and other precursors is relatively rapid once it begins, it will be difficult to try to isolate aggregates of prohormones from intact cells. Use of mice or cell lines deficient in processing enzymes may allow a better determination of what the physical states of prohormones are in cells.

B. Properties of hormone aggregates in cells

Apart from the demonstrated existence of some hormone aggregates, the properties of hormone aggregates in the secretory pathway are largely unknown. One property that may be deduced from electron microscopy is that aggregation is a specific process. Somatomammotrophs of the bovine pituitary gland produce both PRL and GH, two proteins of similar size with structural homology to each other. In these bovine cells, the two proteins are frequently found in separate granules or in separate aggregates in the same granules (75, 76). The separation occurs because these proteins form distinct aggregates even when they are present in the same *trans*-Golgi lumen. Another example of separate aggregation occurs in bag cells of *Aplysia californica*. The precursor form of egg-laying hormone is cleaved in the *trans*-Golgi lumen into a C-terminal portion and an N-terminal portion. These are packaged into separate granules, and the separation appears to occur by aggregation, since separate dense masses of the N-terminal part and C-terminal part are found in the same *trans*-Golgi lumen (77).

In addition to specificity, a second property may be deduced: hormone in aggregates in granules must remain in a conformation so that it can dissociate reversibly and relatively rapidly after exocytosis. Changes in conformations of proteins can trigger aggregation (78). In an extreme example, denaturing proteins by boiling exposes hydrophobic surfaces that encourage essentially irreversible intermolecular binding in an aqueous environment. Changes in conformation in proteins *in vivo* are also responsible for some types of aggregation; for example, the normal cellular form of the prion protein is mostly α-helix with no β-sheets, but in infected cells the protein has more than 30% β-sheet structure and forms aggregates (79). Formation of β-sheets is involved

in aggregation of other proteins as well (78, 80), but these aggregates do not dissociate easily and are unlikely to be models for concentrating proteins into secretory granules. It remains possible that a less drastic change in conformation of the tertiary structure of the hormone occurs during aggregation and the change may facilitate the process.

There have been suggestions that intermolecular disulfide bonds are involved in storage of PRL in secretory granules (81, 82), although such bonds would not allow rapid dissociation of PRL. Intermolecular disulfide bond exchange may occur between proteins in secretory granules retained in cells for extended periods and may occur during preparation of granules, especially if stored frozen, but are not essential for storage in granules because there are few intermolecular disulfide bonds in rat PRL from freshly prepared granules (83, 84).

Clearly, the environmental conditions in the part of the secretory pathway in which aggregates form will influence that process. In all cells examined, acidification of the lumen occurs in the *trans*-Golgi network, so that the pH decreases to about 6 there (85, 86). Ca^{2+} concentrations in the endoplasmic reticulum are high in all cells, since intracellular stores of Ca^{2+} for signaling are kept there (87). Other than these generalizations, there are only specific pieces of information about a few cell types. In PC12 cells, total Ca^{2+} in the endoplasmic reticulum, in most of the Golgi complex except for the *trans*-Golgi network, and in the granule dense cores is at least 10 mM (88). Insulin-containing granules have been isolated and have very high cation concentrations: in addition to 42 mM insulin, they contain 23 mM Zn^{2+}, 120 mM Ca^{2+}, and 42 mM Mg^{2+} (89). Other granules have not been characterized for divalent cations as quantitatively. Qualitatively, secretory granules and the Golgi complex in the rat anterior pituitary gland stain for Zn^{2+}, especially in somatotrophs, corticotrophs, and thyrotrophs (90). All granules in any one cell do not stain; whether this heterogeneity is an artifact or whether granules are heterogeneous is not resolved. More complete knowledge of divalent cation concentrations and other conditions all along the secretory pathway will be useful in understanding how and when aggregation occurs.

VII. Hormone Aggregates in Solution

The aggregation properties of secretory granule proteins in solution under a variety of conditions have been investigated, intensively in some cases, using several different techniques. Because the environment in the cell in which aggregation occurs has not been completely characterized, it is not possible to confirm that conditions in solution exactly mimic conditions in the cell. Some techniques, such as measurements of turbidity or of precipitated protein, measure the formation of relatively large aggregates, as found in the dense cores of granules, but these techniques do not allow any characterization of the type of aggregation that occurs; therefore, it cannot be determined whether the aggregate resembles what occurs in intact cells. There is usually more than one way that a protein may aggregate (78), and some are unlikely to have relevance in intact cells. Other more sophisticated techniques include osmometry, dynamic light scattering, and equilibrium or velocity sedimentation analysis. These techniques measure the presence of soluble forms of proteins and can be used in distinguishing monomers from oligomers, and in characterizing the oligomers, but are not useful when the proteins come out of solution. Assays using these techniques cannot usually be performed at high cation concentrations or at the pH found in insulin-containing secretory granules. Since insolubility is likely to be a key characteristic of protein aggregates in cells, working under conditions in which proteins remain soluble may also give results that do not resemble what occurs in cells.

The investigations using turbidity or precipitation as measures of aggregation have demonstrated that secretory granule proteins aggregate in the presence of Ca^{2+}, mildly acidic pH, or both, conditions that are likely to exist in the *trans*-Golgi cisternae and in immature secretory granules (91–96). There is some specificity, in that proteins not found in secretory granules do not aggregate under these conditions (92–94). The limitations of these techniques mean that there could be no characterization of the aggregates formed, and so the physical basis for the aggregation has not been elucidated.

Investigations of aggregation of secretory granule proteins under conditions in which they remain in solution have demonstrated these proteins form dimers or oligomers under certain conditions. Proinsulin and insulin form dimers and hexamers that include Zn^{2+} and Ca^{2+} (97); human GH will form dimers (98) and human PRL will form oligomers (99) in the presence of Zn^{2+}; and chromogranins A and B will form dimers and, at lower pH, tetramers, in the absence of divalent cations (100, 101). Formation of these oligomers appears to be building blocks for higher molecular weight forms in cells, but the demonstration that they exist in solution does not prove the same forms exist in cells.

One clear-cut finding relative to sorting has emerged from investigations of aggregation in solution, *i.e.*, a single specific sequence in proteins may cause formation of large aggregates. Bovine GH will aggregate so much that it precipitates under conditions in which human GH will not, and a discrete, transferable sequence causes this aggregation, because interchanging 8 amino acids in a 24-amino acid stretch transfers the ability to aggregate to the human hormone (102–104). Although this aggregation occurs under conditions unlikely to have physiological relevance, the investigations demonstrate that a protein sequence that causes large aggregates may be as small and specific as sequences associated with sorting signals. Therefore, the modification of sequences that affect sorting do not in themselves discriminate between a sorting signal and a sequence that affects aggregation.

VIII. Does Aggregation Cause Sorting in Cells?

A. Ways to measure sorting

The experiments to address the role of aggregation or the existence of a sorting signal are conducted by causing cells to express mutant proteins and determining whether they are present in secretory granules. The most direct way to assess the presence in secretory granules is by immunostain-

ing at the level of electron microscopy, which is used sometimes. An alternate, more rapid, approach that is amenable to multiple samples is to compare basal hormone release to that in the presence of an agent that stimulates exocytosis. The ability to stimulate hormone secretion indicates regulated exocytosis. In addition to secretory granules, there are other sources of regulated exocytosis (19), and at least one source exists in neuroendocrine cells, the small synaptic-like microvesicles (105), but it is usually a safe assumption that any such source that might exist in endocrine cells will contain little, if any, hormone relative to that in granules. Measurements of stimulated hormone release indicate the hormone is in secretory granules, but are not a measure of whether it is concentrated there, an important distinction, if, as discussed further below, secretory granules are a default pathway for proteins not sorted elsewhere. The only way to demonstrate concentration, and not just presence, in granules is by electron microscopy using immunogold techniques.

Constitutive secretion is caused by release from vesicles not subject to regulated exocytosis and includes 1) release of soluble proteins carried in vesicles from the Golgi complex directly to the plasma membrane, and 2) release of soluble proteins carried first to the endosomes and then to the plasma membrane (106). Hormone secretion through the pathway through the endosomes, described for proinsulin, is called constitutive-like secretion (107).

Basal release of hormone in the absence of stimulation has sometimes been assumed to be caused only by constitutive secretion from vesicles that are not secretory granules, but it need not be. Spontaneous PRL release from lactotrophs, for example, can be almost completely inhibited by addition of dopamine (108), indicating that this basal release is primarily through the regulated pathway of secretory granules, not through a constitutive one, in these cells. There is also little hormone release from insulin-producing cells except through granules. Insulin is present only in granules, so insulin release is a marker for release from granules (69). Perifused pancreatic rat islets release about 5–6% of the total insulin immunoreactivity as proinsulin (109, 110). The amount of proinsulin in rat secretory granules is 2% of the total in the crystalline cores and 8% in the soluble fraction of the granule (69). Proinsulin in the crystalline cores is a minimal estimate of the amount present in granules, because proinsulin is more soluble than insulin; therefore, more may be in the soluble fraction of the granules. Proinsulin in the soluble fractions is an upper estimate because immature granules may have contaminated the granule preparation. At most, however, only a few percent of release of insulin immunoreactivity occurs through nongranular pathways, because most of the proinsulin release is accounted for by what is in the granules. Unlike tumor cells, the primary source of basal release from normal cells or secretory tissue is release of secretory granules.

Cell lines that grow continuously in culture produce less hormone than primary tissue, usually by at least 1 order of magnitude. They seem likely to have more membrane traffic and therefore more chance to remove hormone to constitutive pathways. For these reasons, perhaps, it has been suggested that pituitary cell lines may not be appropriate for measuring concentration into secretory granules (111). Basal secretion from a PRL and GH-producing cell line, GH_3, may be inhibited up to 80% by somatostatin (112), indicating most secretion comes from a regulated pathway. The ratio of stimulated PRL release to basal PRL release and the patterns of stimulated release are quite similar when PRL release from primary cultures of lactotrophs and from GH_4C_1 cells, a subclone of GH_3 cells, were compared, indicating that the cell line behaves as normal cells in culture (113). Pituitary, adrenal, and some insulin-producing cell lines used as models store hormones concentrated in dense core granules (57, 58, 114, 115). Therefore, although cell lines do not usually make as much hormone as primary cultures do, they treat the hormone they do make in the same way normal cells do, indicating that cell lines are appropriate models for investigating how proteins are concentrated into secretory granules.

B. Does sorting in cells correlate with aggregation in solution?

Experiments to address whether aggregation is important for sorting are based on expression of mutant proteins in cells. To make conclusions about the importance of aggregation, a comparison of results in cells aggregation in solution should be made. Since at present there is not definitive evidence that aggregates in solution reflect what occurs in cells, it is not surprising results from these investigations are somewhat mixed.

1. Insulin. One type of familial hyperproinsulinemia is associated with the mutation, HB10D (histidine at the 10th position in the B chain of insulin mutated to aspartic acid) (116, 117). The histidine that is mutated is a ligand for Zn^{2+} in insulin and proinsulin hexamers, and the mutation reduces the ability to form hexamers (116). The presence of high levels of proinsulin in the blood is consistent with enhanced secretion through constitutive pathways, since processing to insulin occurs in immature secretory granules (85). This mutant was investigated further in transgenic mice expressing the human HB10D mutant and in AtT20 cells transfected with proinsulin. After islets from the mice received a pulse of radioactive amino acids, newly synthesized human proinsulin was released more rapidly than mouse insulin and proinsulin; the human hormone that remained in the cells was processed to insulin (116). These data are consistent with a mutant proinsulin that aggregates less well being sorted into secretory granules less well. In AtT20 cells, the mutant proinsulin was also rapidly released, and its release could not be stimulated (117).

Quinn *et al.* (118) also examined packaging of proinsulin mutants into AtT20 cells by immunogold electron microscopy, selecting two mutants, HB10D and SB9D, that both affect aggregation in solution. HB10D allows formation of dimers, but not hexamers, and SB9D prevents formation of either (119). Each mutant was concentrated into granules, although HB10D was less effectively concentrated than SB9D or wild-type insulin, which is consistent with the rapid release found in the studies mentioned above. All forms, however, were still effectively concentrated compared with the

density of staining in the lumen of the Golgi cisternae (118). One interpretation is that aggregation is not a sorting mechanism or necessary for concentration, and the authors proposed that concentration occurs because proinsulin is brought to granules by a sorting signal. An alternate interpretation is that the conditions used to measure self-association of insulin in solution do not mimic the conditions that cause aggregation in cells.

Support for the second interpretation comes from the work of Grodsky and colleagues (120). They used a double mutant of insulin, SB9D and TB27E, which is monomeric in the assay conditions usually used, but, as they pointed out, does aggregate in high Ca^{2+} concentrations (120). These workers made transgenic mice expressing either human wild-type insulin or SB9D,TB27E-insulin; the mice made about half human insulin and half endogenous mouse insulin. They examined basal and stimulated release of insulin immunoreactivity from perfused pancreas preparations of these mice. Release of insulin immunoreactivity was normal in preparations from mice expressing wild-type human insulin. They found no basal release in the absence of Ca^{2+}, and glucose stimulated release and somatostatin inhibited release in the presence of Ca^{2+}, characteristics of insulin release from secretory granules. Release of insulin immunoreactivity showed similar patterns in preparations from mice expressing the mutant hormone in the presence of Ca^{2+}, but differences appeared in the absence of Ca^{2+}. Basal release of insulin immunoreactivity was elevated in preparations with the mutant, and there was no further stimulation of release by glucose and no inhibition of release with somatostatin. These experiments did not distinguish whether it is aggregation of insulin or proinsulin that may be important. The mutations will be expected to interfere with self-association of both forms, and the authors did not measure whether the insulin immunoreactivity was in the form of proinsulin or insulin. The simplest interpretation of these results is that lack of aggregation in the absence of Ca^{2+} causes insulin immunoreactivity to be diverted to and released from a constitutive pathway, rather than being packaged into secretory granules. The implication of this conclusion is that aggregation properties in certain, but not all, conditions in solution may be relevant to what occurs in the cell, and the studies of insulin in high Ca^{2+} concentrations are the ones that support aggregation as having a role in cells.

The above experiments showed that Ca^{2+} had to be removed to see SB9D,TB27E-insulin immunoreactivity diverted to a constitutive pathway but did not have to be removed to see a diversion of HB10D-proinsulin. The explanation of Carroll and co-workers (116) for why HB10D-proinsulin is diverted to a constitutive pathway is an interesting explanation for the different behavior of the mutants. HB10D-insulin binds with higher affinity to the insulin receptor than wild-type insulin, and the authors propose it is actively diverted to other pathways from secretory granules by binding to the insulin receptor. Further evidence for this proposal was obtained by using an additional mutation to make VA3L,HB10D-insulin, which has reduced affinity for the insulin receptor by about 2 orders of magnitude (121). This mutant was no longer diverted into alternate pathways. These results by themselves suggest that the default pathway for protein hormones may be secretory granules (121). Combined with the evidence for a role of aggregation in the results of Ma *et al.* (120), they raise the possibility that a loose aggregate of proinsulin forms at high Ca^{2+} concentrations to get proper localization in secretory granules, but the interactions are sufficiently weak so that higher affinity interactions such as binding to the receptor may divert some proinsulin to constitutive pathways.

2. Chromogranins. Chromogranins are made and stored in secretory granules of many neuroendocrine cell types (122–124). Chromogranin B and secretogranin II are synthesized by the adrenal cell line PC12, and this line has been used to investigate sorting and storage of these proteins. Chromogranin B, but not secretogranin II, has an intermolecular disulfide bond. Adding dithiothreitol to PC12 cells reduces the disulfide bond in chromogranin B and results in rapid secretion of chromogranin B from the cells (125). Heparin sulfate proteoglycan, a protein of the extracellular matrix, is constitutively secreted by the cells from small vesicles, and the kinetics of secretion of the reduced form of chromogranin B as well as its distribution in subcellular fractions from sucrose gradients resemble that of heparin sulfate proteoglycan, rather than that of proteins in dense core granules, all consistent with the diversion of the reduced form of chromogranin B to a constitutive pathway. The effect of dithiothreitol is not a nonspecific effect on the formation of secretory granules, because the ability of secretogranin II, which does not have a disulfide bond, to be stored in dense granule fractions of cells does not change when cells are treated with dithiothreitol (125).

More recent results have used PC12 cells transfected with a virus expressing chromogranin B, or an N-terminal deletion form of secretogranin B that lacks the sequence with the disulfide bond, or both. The N-terminal deletion mutant is not stored in these cells, when expressed alone, but is secreted via a constitutive pathway. When coexpressed with the full-length protein, the N-terminal deletion form is stored. The authors suggest that the full-length form aggregates and the N-terminal deletion form does not, and when the two are coexpressed, the full-length form causes the deletion form to aggregate (126). An alternate explanation is that secretory granules only form properly when there is a good aggregate around which to form, but when they do form, they become the default pathway.

The aggregation properties of chromogranins have been extensively examined to determine whether there is correlation with what occurs in cells. Chromogranin B forms precipitable aggregates in cells with permeabilized membranes in buffer with high Ca^{2+} concentrations and acidic pH (95). This aggregation property of chromogranin B does not change when it is reduced with dithiothreitol, an action that drastically affects its ability to localize in secretory granules (127). There is better correspondence with properties in the cell and in solution when formation of oligomers is measured by gel filtration; reduction of the disulfide bond in chromogranin B reduces the ability to make soluble dimers and tetramers (101). These gel filtration assays were performed in the presence of EDTA with no divalent cations, conditions unlikely to resemble what occurs in the *trans*-Golgi lumen;

thus far, it is only investigations in solution in the absence of Ca^{2+} that support a role for aggregation for chromogranins. The authors suggest the rate of aggregation may be a crucial factor to explain this discrepancy (101).

3. PRL. A series of experiments designed to investigate the role of Zn^{2+} in human PRL storage also investigated the role of aggregation. Human PRL and human GH bind Zn^{2+} with similar affinity (98, 99). Zn^{2+} binding causes human GH to form dimers, which were predicted to be a storage form for GH (98). Human H27A-PRL, a mutant PRL with greatly reduced Zn^{2+} binding, was not stable and was secreted inefficiently when expressed in clones of transfected GH_4C_1 cells, unlike wild-type human PRL, suggesting that Zn^{2+} binding at the site involving H27 does play a role in PRL packaging and storage. Unexpectedly, the aggregation properties in solution of wild-type and H27A-PRL were similar: both aggregated in the presence of Zn^{2+}, assessed by dynamic light scatter and sedimentation equilibrium analysis (99), which indicated that lower affinity Zn^{2+} binding sites than the one involving the H27 site cause aggregation in solution. It is possible that there are differences in the kinds of aggregates of wild-type and H27A-PRL formed in the presence of Zn^{2+} that were not resolved by these assays for self-association, or that the conditions in which aggregation was assessed were too far removed from conditions in cells to reflect differences that occur there. As in the first two examples, however, this work also shows a discrepancy between some assays of aggregation in solution and biological effects in cells.

The discrepancies do not rule out a role for aggregation because, as indicated above, most methods of self-association analysis require that proteins be in solution, and conditions to keep proteins soluble may avoid the conditions that cause aggregation in cells. Although it is not satisfying that there is incomplete correlation between biological effects on storage and self-association of proteins in solution, these technical difficulties may preclude obtaining a complete correlation even if aggregation is the chief sorting method.

IX. What Keeps Secretory Proteins from Aggregating?

The finding that many proteins that are concentrated in secretory granules have tendencies to aggregate in solution raises the questions of why aggregation doesn't occur earlier in the secretory pathway and why it occurs only in specific cells. For example, at neutral pH, PRL self-associates at high concentrations, especially with divalent cations present (96, 98, 99). There are high concentrations of at least one divalent cation, Ca^{2+}, in the endoplasmic reticulum and Golgi cisternae (88). Proteins that act as a buffer and bind free Ca^{2+} will reduce Ca^{2+} levels, but free Ca^{2+} concentrations are still as high as 0.1 to 0.4 mM in the endoplasmic reticulum (128). Intense buffering of Ca^{2+} may be part of the way in which PRL is prevented from aggregating; in addition, interactions with chaperones in the endoplasmic reticulum may be crucial in preventing aggregation from occurring too early in the secretory pathway.

PRL will precipitate at low pH, even in the absence of cations (94). The pH is lower in the *trans*-Golgi region than in the earlier part of the secretory pathway in neuroendocrine cells, but a drop in pH in this region is not unique to cells that form secretory granules; the pH decreases in other cell types as well (86, 129, 130). PRL is made by the human decidua, but the cells do not make secretory granules, nor is there any evidence that PRL forms insoluble aggregates in these cells (131, 132). Decidual tissue contains 20 to 100 times less PRL per mg protein than pituitary tissue; the intracellular amounts reflect both synthesis and storage. Estimates of synthesis based on the amount secreted compared with intracellular amounts suggest decidual tissue probably synthesizes 10- to 100-fold less PRL per mg tissue than pituitary tissue (131). The GH_4C_1 pituitary cell line synthesizes about 100-fold less PRL than normal lactotrophs in culture (113) but still concentrates PRL in aggregates in dense-core secretory granules (114). At this time, we do not know why PRL aggregates only in specific locations in lactotrophs and not in decidual cells, but it seems likely the answer is more than a question of the difference in amounts of PRL made or differences in pH in the *trans*-Golgi network.

X. Sorting in the *trans*-Golgi Network

A. Transmembrane proteins

Lysosomal proteins, as described above, are sorted into clathrin-coated vesicles for transport to endosomes. Proteins that reside in the Golgi complex are removed for transport back to the earlier stages of the Golgi complex. The need for retrograde transport to retrieve enzymes that reside in the Golgi complex is obligatory in the directed maturation model of protein transport, but retrograde transport occurs regardless of which model is correct. Transport of proteins has been demonstrated to occur from the plasma membrane all the way back to the endoplasmic reticulum (133). There are also two known pathways for sorting to plasma membranes from the *trans*-Golgi network, apical and basolateral (15). These two pathways were first distinguished in polarized cells, but they also exist in other cell types, including neuroendocrine cells (15, 134, 135).

The principles for sorting transmembrane proteins are being elucidated. One mechanism involves the length of the transmembrane segment (136). Cholesterol concentrations are low in the endoplasmic reticulum and increase across the Golgi complex, resulting in a thicker membrane in the *trans*-Golgi region. Proteins destined for the plasma membrane have longer transmembrane segments than those that remain in the Golgi complex, suggesting that plasma membrane proteins can segregate into thicker patches of membrane when Golgi proteins can not. Since cholesterol-rich and cholesterol-poor domains exist in the same membrane bilayer (136), these domains provide an efficient way of sorting some proteins.

A second mechanism depends on amino acid motifs in cytoplasmic sequences of the transmembrane proteins (15, 111). For example, a tyrosine or double leucine motif in this region may allow transport of the protein that contains it to

the basolateral membrane (15, 111). Analyzing signals of membrane proteins is complex because the effect of different sorting signals for apical and basolateral membranes may vary with different cell types (137). In addition, many proteins have more than one sorting motif on the cytoplasmic side; for example, a plasma membrane protein with a motif to allow endocytosis to endosomes may also have a motif for basolateral sorting to allow it to return to the plasma membrane. To add to the complexity, there is a third mechanism: recognition of luminal signals. Destroying the cytoplasmic basolateral sorting motifs on transmembrane proteins causes them to be transported to the apical surface if they have N-linked carbohydrates; those without carbohydrates remain in the Golgi complex (137). O-linked glycosylation sites may also be a signal for apical sorting (138).

In addition to all these sorting mechanisms, there must be some sorting of transmembrane proteins to secretory granule membranes in cells with secretory granules. Some membrane proteins are necessary for regulated exocytosis, such as synaptotagmin and synaptobrevin, which are found both in secretory granule membranes and in the membranes of small vesicles found in neuroendocrine cells, termed synaptic-like microvesicles (105). More interesting, perhaps, are those that localize only in secretory granules. A class of transmembrane proteins that resemble protein tyrosine phosphatase receptors, ICA512 (or IA2) and phogrin, have a location primarily on secretory granules and not in the synaptic-like microvesicles (139–143). GH_4C_1 cells, rat pituitary cells, can be induced to contain more secretory granules; such induction stabilizes ICA512 (144), indicating that the stability of this protein is linked to the kind of membrane traffic that occurs.

Other proteins that must go to secretory granules are the processing enzymes that cleave precursors into hormones. Most of these are not transmembrane and could be imagined to end up in secretory granules because of binding to their substrates. Peptidylglycine α-amidating monooxygenase (PAM) amidates the C-terminal end of processed peptides and can be found in transmembrane and soluble forms, resulting from either cleavage of the transmembrane product or from alternate mRNA splicing (145, 146). AtT20 cells, pituitary tumor cells, normally have very low levels of PAM. Stable clones expressing more of the soluble form store it in a regulated pathway; clones expressing the transmembrane form accumulate PAM in the *trans*-Golgi region and store less POMC and ACTH (147–149). These results suggest that PAM plays more of a role in the cells than merely processing peptides. PAM appears to have a strong signal on the cytoplasmic side for localization to the *trans*-Golgi area, and expressing PAM at what is a relatively high level for AtT20 cells must interfere with normal maturation of secretory granules to reduce ACTH storage, perhaps by binding factors that would normally be used for such a function. Other proteins to which the cytoplasmic side of PAM binds are currently being identified (150, 151). Ultimately, understanding how transmembrane proteins localize to secretory granules and, more unexpectedly, affect hormone storage should give important information about the formation and subsequent transport of these granules.

B. Soluble proteins

Soluble proteins are subject to at least some of the same sorting that affects transmembrane proteins in the lumen. GH is secreted from both sides of Madin-Darby canine kidney cells unless it is glycosylated, in which case it is only secreted from the apical side (152), consistent with apical localization of transmembrane-glycosylated proteins. Sorting of soluble proteins will also be affected by proteins to which they bind, as work with the insulin mutants and the insulin receptor suggests (121). Where will soluble proteins go if they have no sorting signal?

Investigations of nonglycosylated GH secretion in the kidney cells suggest that soluble proteins may be secreted by more than one pathway, and so the default pathways for soluble proteins with no signals may be any pathway that can carry soluble proteins. Free diffusion of soluble proteins into all available pathways does not appear consistent with the investigations of soluble proteins out of the endoplasmic reticulum in yeast, where active transport was required for at least some soluble proteins to be carried forward. A difference may be that the endoplasmic reticulum is full of chaperones and chaperone-like proteins that bind and may retain secretory proteins, so more help may be necessary for certain proteins to leave this compartment than others.

In cells that make secretory granules, the only barrier to the localization of soluble proteins in the granules may be the aggregates, which by their sheer bulk may reduce the space available into which soluble proteins may diffuse, and, in addition, facilitate removal of soluble proteins from forming granules by exclusion from the aggregate. On the other hand, soluble proteins that bind to or are incorporated in aggregates may be retained in granules. The amount and quality of the aggregate may determine how much of the secretory pathway may be used as a default pathway. For proteins that do aggregate properly, secretory granules may be the default pathway (Fig. 3).

The need to postulate a sorting signal to carry proteins into secretory granules came from investigations showing all proteins were not detected in secretory granules (2). The presence of sorting signals to other pathways and the exclusion of proteins that are not concentrated by the aggregates that form early in the process may be what caused the appearance of active sorting.

XI. Sorting Signals and Sorting Signal Receptors for Secretory Granules

There are a number of reports that changing sequences of proteins that are concentrated into secretory granules will affect their behavior; therefore, it is possible to identify sequences that affect sorting into secretory granules or a regulated pathway (153–156). The difficulty is in interpretation of these results and in determining whether a true sorting signal that functions by binding to a sorting signal receptor has been identified. Without a definitive way of knowing which aggregates in solution are comparable to that which occurs in cells, it is not possible to rule out effects on aggregation. It is also clear that, because more sorting occurs than originally envisioned, changing amino acid sequences may

affect sorting in unanticipated ways. For example, glycosylated proteins that have been modified so that they have less tendency to aggregate may be carried actively into an apical pathway, which would reflect a sorting signal, but not a signal for secretory granules.

The sorting signal, as originally envisioned, was thought to recognize proteins and carry them to where secretory granules form to concentrate them (2). If there is not a special signal to bring proteins to where granules form, and instead, sorting proceeds by removal of proteins from this region, then it may seem that all proteins in the *trans*-Golgi network should behave as lysosomal proteins and appear in immature secretory granules before sorting is complete. The appearance of proteins other than secretory granule proteins in immature secretory granules, however, will depend on the amount and affinity of adaptor proteins that help with their removal, as well as the speed with which different transport vesicles form. Proteins not found in immature vesicles may not be there because they lack sorting signals to get them there, or, alternatively, may not be there because they are more rapidly removed from the *trans*-Golgi membranes than lysosomal enzymes.

The most conclusive way to demonstrate the existence of a sorting signal in proteins may be to identify receptors for the sorting signal. If a sorting signal exists for directing soluble proteins into secretory granules or for retaining them in granules once they are there, the sorting signal must bind to a protein in the membrane or to a specialized portion of the membrane, the receptor for the sorting signal. If the primary purpose of the sorting signal is to carry proteins to a location to be concentrated, then the receptor should detect soluble forms of proteins, monomers or oligomers, and bind these proteins better at neutral pH than under the more acidic conditions that exist in immature secretory granules. If the primary purpose of the sorting receptor is to retain proteins that have already passively self-associated because of pH and divalent cation concentrations or other factors, then such a receptor should have a higher affinity for large aggregates of the protein; an ability to bind oligomers or monomers well would be expected to interfere with retention of aggregates. In addition, a retention sorting signal receptor should bind the proteins best at lower pH, conditions found in regions of forming granules (85). Many secretory granule proteins aggregate in solution at lower pH, and determining the binding component for large insoluble aggregates clearly presents technical challenges not found in measuring binding of oligomers and monomers.

Two proteins in the secretory pathway have been proposed to be sorting signal receptors; they have the ability to bind soluble secretory granule proteins and have one characteristic of a sorting signal receptor that functions in retention in that they bind monomers or oligomers best at low pH. One is an intraluminal loop of the transmembrane inositol 1,4,5-trisphosphate receptor, which binds chromogranin A (157, 158). This receptor, however, is present in very low concentrations or not at all in secretory granules of neuroendocrine tissue and therefore cannot be a general sorting signal for chromogranins (159). The ability to bind proteins is, in itself, not sufficient to prove proteins are involved in sorting; chymotrypsinogen binds secretory granule proteins in a manner consistent with a sorting signal receptor (160) but is unlikely to play such a role.

A second protein identified as having the ability to bind secretory granule proteins is carboxypeptidase E, and in this case it has been reported that mice in which a mutation in this protein keeps it inactive in the endoplasmic reticulum (cpe^{fat} mice) do not sort POMC, proinsulin, and GH to regulated pathways (161–163). The same investigators find that the N-terminal end of POMC binds to carboxypeptidase E, and this sequence in POMC is necessary for proper sorting (156, 164). The authors propose that carboxypeptidase E binds to secretory granule membranes and serves as a sorting signal receptor. These results are controversial, for other laboratories have performed some of the same investigations with different results. Others have found that the N-terminal end of POMC is not necessary for packaging into secretory granules (165), and that removal of the C-terminal region of carboxypeptidase E that causes binding to membranes does not influence sorting of the enzyme (155). Most importantly, others have found that although mice with mutant carboxypeptidase E don't process proinsulin to insulin well, the unprocessed proinsulin is concentrated into secretory granules (72, 166). These discrepancies in results indicate that the question of whether carboxypeptidase E is a sorting receptor has not been resolved.

In the simplest model, aggregation is the sole means of sorting, but even with this most simple model, phogrin and ICA512 must have a way of recognizing dense-core granules, as they preferentially localize in granule membranes. One mechanism would be for phogrin and ICA512 to recognize some aspect of the cargo or to recognize adaptor molecules that recognize the cargo. It seems most logical that whatever recognizes the cargo recognizes the cargo proteins in their aggregated state, since recognition of monomers or small oligomers may lead to missorting into small vesicles. If the aggregate has the properties of a colloid, it may have specific structural attributes that could serve as properties to be recognized (167–169). An alternate to recognition of protein cargo may be recognition of differences in membrane lipid composition. If the membrane lipid composition changes around large aggregates because they induce membrane curvature or change other properties, then certain transmembrane proteins may preferentially localize in this region. At present there is no evidence one way or the other for differences in membrane composition around developing secretory granules compared with the rest of the *trans*-Golgi membrane. There is, however, evidence that membrane lipids are important in other types of sorting (15, 170). For example, sphingo-cholesterol microdomains in the membrane serve as sorting domains for some apical proteins (171, 172), and lysobisphosphatidic acid may play a role in sorting late endosomes (173, 174). A role for membrane lipids in sorting of secretory granule transmembrane proteins is possible.

XII. All Secretory Granule Proteins Are Not Treated Equally

Most experiments that investigated sorting into secretory granules have examined the effects of mutating proteins,

comparing the behavior of mutant proteins to that of wild type. A different approach is to compare behavior of endogenous secretory granule proteins to each other and to transfected exogenous secretory granule proteins. Since these proteins have not been modified by mutation, interpretation of results is not complicated by the possible unanticipated effects that mutations may cause, *e.g.*, creating or exposing sorting signals for other pathways. This type of experiment has demonstrated that cells do not treat all proteins that are cargo proteins for secretory granules in the same way.

In one set of experiments, Castle *et al.* (175) examined two salivary proteins, salivary amylase and proline-rich protein, expressed in transfected clones of AtT20 cells. They showed by electron microscopy with immunogold staining that proline-rich protein was in secretory granules, colocalized with ACTH. The colocalization of proline-rich protein was heterogeneous: some granules stained mainly for proline-rich protein, others mainly for ACTH, and some were mixed. The pattern of amylase staining by light microscopy indicated that amylase was also in secretory granules. Castle *et al.* (175) followed the ability to stimulate release of salivary proteins and ACTH as a function of time after synthesis of the proteins and found that, although the salivary proteins were concentrated into secretory granules, these proteins were not retained in cells as well as ACTH.

The authors interpret their data to mean that salivary proteins are removed from granules after they have formed and are released by a constitutive pathway (175). The implication of this model is that selective removal of granule contents occurs for long periods after granules have formed. One mechanism for selective removal of granule content is that a sorting signal retains ACTH; such a retention sorting signal would only be effective if it were able to recognize and retain large aggregates rather than oligomeric or monomeric forms. A second mechanism is that ACTH forms aggregates so large that they cannot be removed, and salivary proteins do not, but remain soluble, so only salivary proteins are removed in the small vesicles that bud off the granules. The presence of large concentrates of proline-rich protein, detected in secretory granules with immunogold staining, do not appear consistent with this second mechanism. A third mechanism is that large aggregates of salivary proteins are selectively removed.

An alternate model that does not exclude the above model is that granules with different cargos are treated differently, and granules with different cargos may be preferentially retained or released. Secretory vesicles, like other transport vesicles, need specialized proteins in their membranes to allow proper transport to the plasma membrane (176) and to ensure that exocytosis occurs in response to appropriate stimuli. If accumulation of these membrane proteins in secretory granules requires that the cargo around which they are assembling have certain properties, then their assembly may be more or less effective, depending on the cargo. If AtT20 cells form secretory granules best around the cargo that they normally make, then those formed around salivary proteins may be incomplete. Secretory granules containing mainly salivary proteins may lack components for proper retention, and, as a result, be more susceptible to basal release without stimulation; therefore, salivary proteins would not be retained in cells as well as ACTH.

The ability of cells to treat granules filled with different cargo proteins independently is difficult to test experimentally in most systems, but there is evidence that it occurs in two naturally occurring systems. One is bag cells of *Aplysia californica*. As described above, pro-egg-laying hormone forms two separate aggregates after cleavage, one of the N-terminal end and one of the C-terminal end, which are packaged into separate vesicles. Those of the C-terminal end go to neuronal processes for regulated release. The N-terminal part ends up either in large vesicles that are transported to lysosomes, or in smaller vesicles, separate from those containing the C-terminal part (77). The smaller N-terminal vesicles are transported to different locations in the cell than C-terminal vesicles, where they also undergo regulated release (177).

The second system is gonadotrophs. In rat gonadotrophs, LH and FSH are packaged into separate granules and all gonadotrophs have both types of granules. LH and FSH, however, do not have the same patterns of stimulated release, nor is the pattern of mobilization in the cell of the two kinds of granules the same after stimulation (178, 179). More FSH than LH is released basally, and stimulation releases more LH than FSH. An explanation for these differences is that secretory granules containing FSH are more likely to undergo basal release without stimulation. A possible explanation of how the differences may occur is that some of the membrane components in secretory granules that prevent exocytosis in the absence of large increases in Ca^{2+} are less effectively packaged into the membranes of FSH-containing granules.

A second set of experiments comparing behavior of secretory granule cargo proteins examined hormone storage in GH_4C_1 cells, rat pituitary tumor cells that make PRL, GH, and secretogranins. PRL storage in these cells is a specific, inducible process (114, 180–182). In the absence of estradiol, the cells store little PRL and contain few secretory granules, but in the presence of estradiol, especially with insulin and epidermal growth factor, they store more PRL and have 50-fold more secretory granules (114). Although the cells have many more granules, only the storage of PRL, not that of GH or secretogranin, increases (114, 182).

It was unexpected that the storage of only one cargo protein increased when the cells contained more granules. Storage of exogenous secretory granule proteins does not increase with treatment, since clones transfected with proinsulin stored more PRL, but not more proinsulin, after hormone treatment (183). The most interesting demonstration of the specificity of hormone storage was found in clones transfected with human PRL; in these clones hormone treatment induced neither rat nor human PRL storage (184). Two single amino acid mutations in human PRL, N31T and S34A, each ablated the ability of human PRL to block rat PRL storage, genetic proof that the effect on rat PRL is caused by human PRL (184). Expression of human PRL prevents the induction of rat PRL storage at a ratio as low as 1 human to 40 rat PRL molecules. If passive aggregation of rat PRL were the only factor that accounted for rat PRL storage, it should not be blocked by such a low ratio of human PRL. This kind

of specific antagonism resembles that found with receptors, but there is no evidence for a PRL receptor in GH_4C_1 cells (185), so the cause of specificity is currently unknown. The specific antagonism does, however, make a model of sorting based just on passive aggregation unlikely.

The ability to induce storage of one cargo protein in a cell that makes several may occur by inducing the retention of specific granules, a variation of the model suggested above. Hormone treatment may increase retention of granules containing mostly rat PRL. For the cells to make secretory granules that behave differently around different cargo, however, there must be a means to recognize the cargo, which gets us to the problem of how an aggregate can be specifically recognized, the same problem that exists in envisioning a more traditional sorting signal.

An alternate cause of the specificity found in inducing storage of only one secretory granule protein may be in the ability to cause PRL to aggregate; if aggregation is actively controlled, and does not just occur passively, it may be controlled in ways that will have specificity. Aggregation of proteins in the endoplasmic reticulum is controlled by chaperones and chaperone-like proteins, proteins that assist in the folding of other proteins (186). Chaperones are usually thought of as preventing or reversing the aggregation of proteins, but under certain conditions they may facilitate aggregation (187–189). Several roles for chaperones may be envisioned in sorting of secretory granule proteins. They may keep proteins destined for secretory granules from aggregating prematurely, they may keep such proteins in a conformation that is likely to aggregate in the proper conditions, they may induce aggregation in the appropriate place, or they may play a combination of these roles.

The first assumptions about chaperones were that they recognized hydrophobic sequences and were not very specific, but in at least some cases, more specificity does exist. GRP-78 (glucose-regulated protein-78) also known as BiP, recognizes linear sequences of 7 amino acids with some stringency in what the residues are, so that there are only 12 sequences to which BiP binds in the heavy chain of immunoglobulin (190). A member of the cyclophilin family, NinaA, in *Drosophila*, binds and transports rhodopsin I to rhabdomeres, but does not bind all rhodopsins (191, 192). Hsp47 binds with high affinity to the N-terminal end of the amino peptide of the procollagen A Iα1(I)chain, but not the procollagen Iα2(I) chain, to facilitate folding (193). Hsp47 and cyclophilin B form a complex with procollagen that remains with the proteins until they reach the intermediate compartment between the endoplasmic reticulum and *cis*-Golgi region, and have been proposed to act first as chaperones to keep the proteins from aggregating and then as antichaperones to concentrate collagen (194). It seems likely that chaperones will play an extensive role in transport through the secretory pathway because many secretory granule proteins have tendencies to aggregate in high Ca^{2+} concentrations, the conditions that exist in the environment through which they are being transported.

It is not necessary to imagine separate and distinct chaperones for each protein; different combinations of known chaperones may be effective. In addition, there are proteins in the secretory pathway of neuroendocrine cells, such as RESP18 and 7B2, that, because of their restricted location, must be related to endocrine cell function, and may play specialized chaperone-like roles for protein hormones (195–198). Secretogranins have also been proposed to play such a role for some proteins (124, 199, 200).

Chaperones and chaperone-like molecules will not be the only factors that control aggregation. Proton and divalent cation pumps that regulate pH and Ca^{2+}, Zn^{2+}, and possibly other cations obviously will play important roles, although the ways in which each secretory granule protein may be kept from improperly aggregating and the factors that best induce its aggregation are likely to be different for each protein. Each cell type may therefore be set up to create optimal conditions for the aggregation of proteins that the cell normally makes, so that cells would be designed for controlled aggregation of only certain proteins.

Controlled aggregation allows an explanation for the specific induction of PRL storage in GH_4C_1 cells; hormone treatment could induce the conditions necessary to cause aggregation of rat PRL, but not GH or secretogranins. Human PRL may reduce rat PRL storage by binding to a site that is necessary for rat PRL aggregation, but not responding there as rat PRL, and so reducing aggregation, and therefore storage, of both. If aggregation is controlled in a cell-specific fashion, it explains why PRL aggregates in pituitary cells, and not in decidual cells.

Finally, if factors that actively control aggregation do so with some specificity, they offer a means for secretory granule membrane proteins to localize in certain granules without specifically recognizing the cargo; instead, the membrane proteins may localize to where the factors are that control aggregation.

XIII. Summary

Recent findings in cell biology have demonstrated there are several kinds of active sorting from the *trans*-Golgi network in all cells. The presence of several sorting pathways, using more than one sorting signal, in neuroendocrine cells means that mutations that direct a hormone to a constitutive pathway instead of a regulated one may not simply be interpreted as a signal for sorting to a regulated pathway. The use of three-dimensional electron microscopy of lactotrophs and the possibility that the *trans*-Golgi network is consumed during sorting has suggested a major role for hormone aggregation, not only as a sorting mechanism, but also as a mechanism for granule formation, in that other transport vesicles may bud from the *trans*-Golgi network, leaving the aggregated protein as the dense core granule. If aggregation plays such a role, it is unclear how it works in cases where the prohormone must be processed one or more times; does a porous aggregate or colloid form? Obtaining information about the kinds of aggregates that occur in cells is difficult, because at this time there is not a definitive way of knowing whether an aggregate that occurs in solution also occurs in cells. Although secretory granule proteins tend to aggregate relatively easily in solution, the separate treatment of different secretory granule proteins in the same cell make it unlikely that aggregation is a purely passive process, but

suggests that the process of aggregation of each hormone is actively controlled in cells. Even if the ability to aggregate accounts for most of the sorting of cargo-secretory granule proteins into granules, other sorting must still occur to get correct membrane proteins necessary for transport and exocytosis into secretory granule membranes. Possible recognition sites for these secretory granule membrane proteins include the cargo itself in an aggregated form, membrane lipids in some unrecognized way, or the proteins and factors that specifically control aggregation of the cargo.

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