Prolactin and Growth Hormone Aggregates in Secretory Granules: The Need to Understand the Structure of the Aggregate

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Prolactin and GH form reversible aggregates in the trans-Golgi lumen that become the dense cores of secretory granules. Aggregation is an economical means of sorting, because self-association removes the hormones from other possible pathways. Secretory granules containing different aggregates show different behavior, such as the reduction in stimulated release of granules containing R183H-GH compared with release of those containing wild-type hormone. Aggregates may facilitate localization of membrane proteins necessary for transport and exocytosis of secretory granules, and therefore understanding their properties is important. Three types of self-association have been characterized: dimers of human GH that form with Zn^{2+}, low-affinity self-association of human prolactin caused by acidic pH and Zn^{2+} with macromolecular crowding, and amyloid fibers of prolactin. The best candidate for the form in most granules may be low-affinity self-association because it occurs rapidly at Zn^{2+} concentrations that are likely to be in granules and reverses rapidly in neutral pH. Amyloid may form in older granules. Determining differences between aggregates of wild type and those of R183H-GH should help to understand why granules containing the mutant behave differently from those containing wild-type hormone. If reversible aggregation of other hormones, including those that are proteolytically processed, is the crucial act in forming granules, rather than use of a sorting signal, then prohormones should form reversible aggregates in solution in conditions that resemble those of the trans-Golgi lumen, including macromolecular crowding. **(*Endocrine Reviews* 33: 254–270, 2012)**

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I. Introduction

Protein hormones are stored in concentrated forms in endocrine cells in membrane-bound vesicles with dense cores, viewed by electron microscopy. When the cells are stimulated, these vesicles fuse with the plasma membrane, so the dense cores are released and dissolve, causing a burst of hormone in the bloodstream on a time scale much faster than could result from increasing synthesis. Since these dense-core vesicles, or secretory granules, were first identified, investigators have asked how the hormones are concentrated there (1). In 1987, Burgess and Kelly (2) proposed two mechanisms for concentration: recognition of a receptor by a sorting signal sequence or self-association into a reversible protein aggregate. Over two decades of research followed,

Abbreviations: COPII vesicles, Vesicles with coat proteins II bound on the cytosolic side; EGFP, enhanced green fluorescent protein; ICA512, islet cell antigen 512; PEG, polyethylene glycol; proANP, proatrial natriuretic peptide.

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Figure 1.

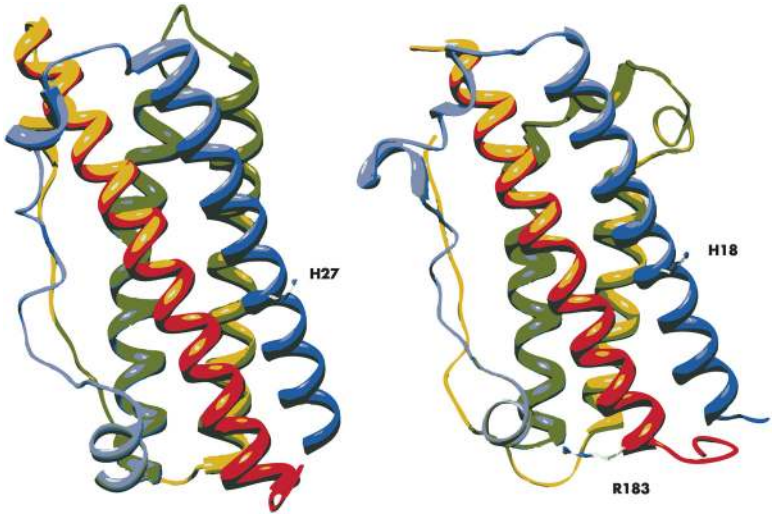

Figure 1. Structures of human GH (*right*) and human prolactin (*left*). Three of the amino acids discussed in the text are labeled.

attempting to identify sorting signal sequences and, to a lesser extent, to characterize hormone aggregates. Forming aggregates that reversibly dissociate to give native protein is an economical way to sort proteins in the secretory pathway, for it removes them in an energy-efficient manner from other sorting for soluble proteins. Isolation from other sorting appears increasingly important as our understanding of the complexity of the secretory pathway increases. Reversible hormone aggregates may also play a part in inducing proper location of membrane proteins necessary for function of secretory granules, although how such results are achieved is not understood.

This review covers what is known about the formation of reversible aggregates of prolactin and GH, two hormones related in structure. The process of concentrating these hormones into secretory granules should be straightforward, because all that happens to these hormones is transport from the endoplasmic reticulum and aggregation in the trans-Golgi layer. The only proteolytic processing of prolactin and GH, the removal of the signal peptide, occurs in the endoplasmic reticulum. These proteins are each monomeric four-α-helix bundles similar in structure and sequence (Fig. 1) (3, 4). The dense cores of prolactin secretory granules have been isolated and characterized (5–7). Although covalent modifications of prolactin have been reported, these do not always occur and are not necessary for packaging into granules (8). Understanding the aggregation process for prolactin and GH may provide a basis for understanding the principles that apply to storage of other hormones. This review summarizes what is known about aggregation of prolactin and GH, both in the secretory pathway of cells and in solution, and discusses whether conclusions about sorting of these hormones may be applied to other hormones, including those that are proteolytically processed.

II. Complexity of the Secretory Pathway

A. Transport between the endoplasmic reticulum and the Golgi complex

Transport of secretory proteins through the cell occurs through transport in membrane-enclosed vesicles. Full accounts of the specific membrane and cytosolic proteins and the mechanisms involved in forming and fusing vesicles at membrane-enclosed compartments in the cell are the subject of many reviews (9–13). Distinct vesicles are involved both in transporting newly made proteins to their destinations in the cells and in retrieving proteins to the compartments in which they function.

Transport of secretory proteins begins in the endoplasmic reticulum with the formation of vesicles with coat proteins II bound on the cytosolic side (COPII vesicles). These vesicles enclose soluble proteins from the lumen of the endoplasmic reticulum, often referred to as cargo, and bud from specific exit sites to carry the cargo proteins to a region near the cis-layer of the Golgi apparatus, referred to as the endoplasmic reticulum Golgi intermediate compartment or, more simply, as the intermediate compartment. According to a generally accepted model for transport of most cargo through the Golgi complex, that of cisternal maturation (14–18), vesicles from the endoplasmic reticulum arriving at the intermediate compartment fuse to form a new Golgi cisterna, and the proteins necessary for transport from the endoplasmic reticulum are cycled back by COPI vesicles. The newly formed Golgi cisterna progresses through the Golgi stacks from cis to medial to trans, as newer cisternae form behind it and older cisternae ahead mature, peel off, and are disassembled (19, 20). As the cisterna matures, proteins in the lumen may undergo an orderly series of enzymatic modifications, including phosphorylation, sulfation, and modification of sugars. The enzymes that cause these reactions and other resident proteins of the cis and medial Golgi layers are carried back by COPI vesicles to the newer cisternae that have formed behind the more mature ones. COPI vesicles form on all but the last two or three layers of the Golgi cisternae (21).

B. Transport from the trans-Golgi cisternae to other destinations

These last two or three trans-Golgi layers show progressively less cisternal volume than earlier layers because

they are disassembled by the formation of many tubular carriers (20–22). These layers and the emerging and newly formed tubules are called the trans-Golgi network. In the trans-Golgi layers, there are discrete regions for exporting cargo to different destinations (23–26), distinguished by differences in proteins associated with the cytoplasmic sides of the Golgi membranes, especially by proteins known as golgins (25). These distinct cargo export domains form tubular extensions that pull away and eventually separate, part of the disassembly of the cisterna. The tubular carriers are distinguishable by the cargo proteins in their membranes and lumens and the proteins associated with the cytoplasmic sides. Each cargo export carrier has a specific destination, such as the plasma membrane or early, late, or recycling endosomes (24). Other destinations are cell specific, and there may be more than one carrier for one destination (24). As many as six separate carriers may leave the trans-Golgi network, depending on the cell type (27).

The mechanisms that create different export domains in the trans-Golgi layer and cause the membranes to curve and form tubules are being investigated and are the subject of recent reviews (10, 24–26). Proposed mechanisms include the removal of proteins that keep the cis and medial Golgi layers flat and rigid, the recruitment of proteins to the cytoplasmic face of the domains to induce curvature, the activation of enzymes to modify the lipid composition of the membranes, and the formation of specific lipid domains called rafts. In addition, the arrival of tubular carriers with distinct protein and lipid compositions, which recycle components to the trans-Golgi network back from the plasma membranes and endosomes, influence the composition and curvature of the area where they fuse. There are multiple carriers arriving at, as well as departing from, the trans-Golgi network (27, 28).

The separation from the trans-Golgi layer and transport of these tubular carriers, once they have formed, are mediated through the attachment to microtubule proteins and actin filaments by protein connections (25, 26). Golgins, the cytoplasmic proteins that are specific for the exit domains, are good candidates for the proteins that make these connections (25). The pull of the membrane carriers along microtubules and actin filaments, in addition to enzyme-mediated changes in lipid composition and actions of proteins, such as dynamin, mediate fission of the tubular carriers from the trans-Golgi layers (26).

The amount and appearance of the trans layers of the Golgi complex vary greatly depending on the cell type (22). Cells that produce large amounts of proteins stored in granules, such as lactotrophs, have fewer tubular extensions of membranes from the trans layers than do many other cells (22, 29). Some of the Golgi membrane is used instead to surround the aggregates of hormone, which are too large to fit into the tubular carriers. The size of the trans-Golgi layers and the amount of tubule formation there also varies in a single cell type and increases with more secretory cargo (26). The trans-Golgi network is in a dynamic state with transport vesicles containing new cargo leaving for the plasma membrane, endosomes, and other destinations and transport vesicles arriving back from these regions to recycle enzymes and carrier proteins.

C. Alternate routes

Transport through the entire Golgi complex may not be the only way secretory proteins reach the cell surface. The drug sensitivity and kinetics of transport of proteins under some circumstances are consistent with more rapid pathways for secretory proteins through the cell than completely traversing the Golgi complex (30). At least one possible pathway for such transport in protein hormone secretion was demonstrated using three-dimensional electron tomography with β-cells of mouse islets that had been incubated in high glucose levels for 1 h (31). High glucose concentrations are a strong signal for increasing insulin production, and in these stimulated β-cells, there are tubular connections between Golgi cisternae at different levels of the stack; such connections have not been found in unstimulated β-cells (31). These connections could speed transport, although it is not yet known whether there is proinsulin in these tubules (31). Other proposed transport pathways include bypassing the Golgi stacks to go from the intermediate compartment or early Golgi cisternae to the plasma membrane (30). Such connections may be induced by the production needs of the cell, but when and how these alternate pathways are induced and used is not well defined. Induction of such connections, however, reinforces other findings that the secretory pathway is not static but changes in response to needs and signals and the proteins that are being produced (26).

D. Sorting proteins in the secretory pathway

With all these export carriers and multiple pathways, there must be much sorting to get proteins to their proper destination, much more than just sorting to a regulated pathway. The transport of certain transmembrane proteins is becoming increasingly well understood. Sorting begins in the endoplasmic reticulum and is linked to COPII coat formation on the cytosolic side of the transmembrane proteins (9, 32, 33). Specific cytosolic proteins recognize and bind to amino acid motifs on the transmembrane proteins, subsequently recruiting other proteins, resulting in multimer formations that cause membrane invagination, release of vesicles from the membranes of the endoplasmic reticulum, and transport to the intermediate compartment

(9). Some of these transmembrane proteins are receptors for soluble proteins and carry the soluble proteins forward, but there is also bulk flow transport for soluble proteins. The extent of receptor-mediated transport of soluble proteins *vs.* bulk flow from the endoplasmic reticulum has not been fully elucidated (32), but protein hormones produced in large amounts are most likely to be transported by bulk flow without receptor mediation.

Clathrin forms cytosolic coats linked to transmembrane proteins in a similar fashion in the trans-Golgi network. Adaptor proteins bind to motifs on the cytosolic side of specific transmembrane proteins and then bind clathrin, leading to clathrin-coated vesicle formation and transport. The composition of the coat proteins determines the destination of the carrier vesicles. Coat proteins are not apparent on some of the carrier export domains in the trans-Golgi layers, but recognition of transmembrane proteins in these areas seems likely to occur even if coats are not readily apparent (24, 26).

Sorting of soluble proteins in the lumen of the Golgi complex is less well understood. Some luminal soluble proteins are modified covalently, such as by phosphorylation or glycosylation, so that they will bind to transmembrane proteins and be carried to specific locations. The addition of glycosylphosphatidyl inositol causes proteins to associate preferentially with lipid rafts that go to the apical plasma membrane (10, 24). All soluble luminal proteins, however, are not covalently modified, and their sorting mechanisms are unknown. If soluble secretory proteins do not have a sorting signal, they may be carried passively to endosomes and then lysosomes, to early and recycling endosomes, and then to the plasma membrane, or even back to other layers of the Golgi complex, in addition to being included in vesicles headed directly for the plasma membrane. Such undirected transport is not efficient, but efficient transport requires an energy investment it may not be necessary to make for all proteins. Bard and Malhotra (10) point out that it will be difficult to address whether all soluble secretory proteins have specific receptors or mechanisms for sorting. Mutating these proteins in hopes of removing a sorting signal will influence binding to other components of the secretory pathway, and even low-affinity interactions may be important. Such an approach has been used to argue that there is a sorting signal on luminal secretory granule proteins, but modifications may generate new interactions with proteins in the various export domains, and change the destination, without involving a specific receptor.

Aggregation of protein hormones alone provides an efficient method of protein sorting, because the size of the aggregates alone is enough to exclude them from other pathways that have tubular export carriers with induced curvature, and it is a passive process, not requiring energy. In lactotrophs, the aggregates of prolactin appear as lumps in the trans-Golgi layer that are too large to fit in spherical or tubular vesicles (19). Clathrin-coated vesicles do bud off from the membrane around the aggregates, removing proteins destined for endosomes and lysosomes. Immature and finally mature secretory granules are formed from the trans-Golgi layer by removing these proteins and excess membrane.

Sorting proteins for secretory granules must also include sorting transmembrane proteins, but this process is also not well understood. There are membrane proteins specific for granules, such as islet cell antigen 512 [ICA512 (or IA2)] and phogrin, two tyrosine phosphotase homologues, and proteins necessary for transport and exocytosis, such as vesicle-associated membrane protein 2 (34). The means by which these proteins are brought to or retained in membrane surrounding secretory granule content is not known. The difference in behavior of granules with different cargo aggregates has led to the suggestion that recognition of specific properties of the aggregates influences the localization of appropriate membrane proteins, and therefore the properties of the aggregate are important (35).

III. Prolactin and GH Aggregation

A. Transport and aggregation in cells

Storage of rat prolactin was initially detected by electron microscopy as dense cores of material in secretory granules of rat lactotrophs (36). The process of forming the dense cores begins in the Golgi complex (37). Pulse-chase experiments using quantitative autoradiography and electron microscopy were used to follow the path of newly synthesized prolactin through the cell, and newly synthesized prolactin in dense cores is first detected in the Golgi complex of cultured lactotrophs 30 min after a 5-min pulse of [^{3}H]leucine (38). The dense cores were also found outside the cell in invaginations of the plasma membrane (36), which was recognized as evidence for exocytosis, the release of the granule contents by fusion of the secretory granule membrane with the plasma membrane. Although these dense cores retain their characteristic shape immediately after fusion and therefore dissolve slowly relative to the process of exocytosis, they begin to dissolve before the membrane invaginations disappear and therefore do not remain in an aggregated state for long (36), a characteristic of other hormone dense cores as well (39).

Evidence that formation of these dense cores is a specific process was found early in these investigations. Elec-

tron micrographs of bovine somatomammotrophs, bovine pituitary cells that make both prolactin and GH, showed separate cores, composed either of prolactin or GH, in the same granules and in the same trans-Golgi lumen (40).

Dense cores in secretory granules of lactotrophs seen by electron microscopy were shown to be actual aggregates of rat or bovine prolactin by isolating the cores and characterizing them (5–7). The dense cores are stable for over an hour at pH 6 after the membranes are dissolved by the detergent Lubrol, and more than 60% of the prolactin in the cores becomes soluble in 15 min at pH 7.4, the normal extracellular pH (5). Small amounts of sulfated proteins and glycosylaminoglycans are present in the prolactin granules and associate with the membraneless cores. These become soluble under different conditions than prolactin does (7) and appear at the edges of the dense cores rather than mixed with the prolactin aggregates (41).

Prolactin from freshly isolated granules with intact membranes is monomeric (42, 43). The redox potential of the secretory pathway is more oxidizing than that of the cytoplasm (44), which allows formation of disulfide bonds in prolactin as it folds in the endoplasmic reticulum. Chaperones such as disulfide isomerase facilitate the correct formation of these bonds before transport out of the endoplasmic reticulum (45, 46). The environment of secretory granules is acidic (47); an acidic pH stabilizes disulfide bonds and reduces disulfide bond interchange. Slow exchange of disulfide bonds in prolactin may occur in granule cores over time, because prolactin is so concentrated there, resulting in formation of disulfide-linked oligomers. Such exchange may occur to a small extent in older granules. Intermolecular disulfide bonds may also be likely to form if prolactin from granules is not assayed immediately after isolation in isotonic solution. Use of granules whose membranes are not intact after isolation or after storage has led to reports of a substantial amount of intermolecular disulfide bond formation, but these oligomers are not found in freshly isolated granules.

Overall, the early studies of prolactin and GH showed the storage form is aggregates of monomeric hormones that associate rapidly in the trans-Golgi lumen and rapidly dissociate in conditions similar to the extracellular environment.

B. Assay for aggregation in cells: protein and cell specificity

Two cell lines have been used for many investigations of storage of prolactin and GH and mutants of these proteins: GH_4C_1 and AtT-20 cells. GH_4C_1 cells are clonal strains of rat pituitary tumor cells that make GH and prolactin. The pattern of hormone release from these cells is similar to that from cultures of normal pituitary cells; there is rapid release within minutes of stored hormone, using depolarization or TRH (48). It was difficult to obtain good transfection efficiency initially with these cells, but the efficiency has improved somewhat over the years as new transfection agents have been developed. A complication that is possible with these cells is that there may be interactions between endogenously produced rat prolactin and GH and the transfected hormones. In addition, in pulse-chase experiments, it is necessary to distinguish endogenous rat hormones from transfected hormones such as the human ones. AtT-20 cells are a clonal strain of mouse pituitary cells that produce ACTH and β-endorphin; corticotropin-releasing factor and barium stimulate release of these peptides (49). They have a flatter morphology than GH_4C_1 cells, which makes identification of secretory granules by immunofluorescence staining of contents more dramatic and distinguishing secretory granules more easy. They also transfect more efficiently. Using these cells as models for human GH and prolactin storage avoids possible interactions with rat GH and rat prolactin but makes the assumption that these cells will package GH and prolactin in exactly the same way that cells that normally make the two hormones would, an assumption that may not be correct.

Cells may be transfected transiently and the results tested within 24 h, which reduces possible changes in the cell population that could occur over time as cells divide and multiply. An alternative, used especially before there were good transfection agents for GH_4C_1 cells, was to obtain stably transfected cells by cotransfecting the hormone sequences with a gene that allows for selection. The selection process and expansion of surviving clones is a lengthy process that may result in selection of cells with characteristics that differ from the majority of cells that express hormone in unselected populations.

We used the finding that prolactin granule cores were stable without membranes to investigate the process of hormone aggregation in cells. Newly synthesized prolactin in GH_4C_1 cells is soluble immediately after a 10-min pulse of ^{35}S-labeled amino acids when cells are lysed in 1.5% Lubrol. Thirty minutes later, 50% of the [^{35}S]prolactin is insoluble, and the insoluble form will sediment through 1.2 M sucrose, as dense cores of granules from lactotrophs do (50). The conversion to an insoluble form is inhibited when transport from the endoplasmic reticulum is blocked by brefeldin A or incubation at 15 C (50). These findings indicate aggregation measured this way behaves as expected from the earlier experiments using autoradiography (38), because aggregation occurs within 30 min and there must be transport from the endoplasmic

reticulum within that time. We found similar results with rat GH in GH_4C_1 cells.

Prolactin and GH also aggregate when expressed by transfection in AtT-20 cells. Aggregation of human GH and human prolactin in AtT-20 cells shows the same kinetics as that of the rat hormones in GH_4C_1 cells. There is protein specificity to the process, for all secreted proteins do not aggregate in pituitary cells. Human serum albumin, a protein secreted from the liver without aggregation, is secreted from AtT-20 cells with little aggregation, and the little that there is, 10% or less, does not increase with time (50).

There is also cell specificity to the process, for appreciable aggregation of human prolactin and GH does not occur as efficiently in all cell types. Over 40% of human GH and human prolactin labeled with a 10-min pulse of radioactive amino acids form aggregates in AtT-20 cells 30 min after synthesis, but only 10% of each hormone does so in COS cells, fibroblast cells that do not make secretory granules (50). Beuret *et al.* (51) concluded that other transfected secretory proteins, provasopressin, prooxytocin, proopiomelanocortin, secretogranin II, and chromogranin B, form aggregates in COS cells, characterizing the process primarily by light and electron microscopy. Meldolesi and co-workers (34) have specified molecular and functional properties that should be met for structures to be classified as secretory granules, including the presence of the vesicle-associated membrane protein 2 membrane protein. These criteria have not all been met for the aggregates in COS cells, and the aggregates are not in secretory granules, but the finding of Beuret *et al.* (51) indicates that the COS cell secretory pathway does have the ability to cause some secretory granule proteins to aggregate. The efficiency of aggregation was not measured directly, but the authors measured retention of chromogranin B and secretogranin II in COS cells. After a 90-min labeling period, up to 50% of [^{35}S]secretogranin II remains in the cells; chromogranin B is not as well retained. It is reasonable to assume retention is a measure of aggregation, and so secretogranins may aggregate more efficiently than human prolactin and GH in COS cells. A 90-min labeling period, however, must overestimate the amount retained. The longer the labeling period, the more the labeled protein will consist of stable components (in this case, protein remaining in the cell) and the more difficult it will be to detect labile components (in this case, secreted protein) (52). The apparent efficiency of aggregation will be less with a shorter labeling period, so the two results with GH and secretogranin in COS cells may not be as different as they appear. In any case, COS cells do not cause human prolactin or GH to aggregate as efficiently as pituitary cells do, so there must be specific factors or characteristics in the secretory pathway of pituitary cells that enhance aggregation of at least these two hormones.

C. Specificity in retention of aggregates

Retention of rat prolactin aggregates can be modified without modifying that of GH aggregates. Treating GH_4C_1 cells with hormones estradiol, insulin, and epidermal growth factor increases the storage of rat prolactin but not rat GH or other secretory granule proteins (53). Aggregation of labeled prolactin does not increase with hormone treatment, but retention of the labeled prolactin aggregates does. Untreated GH_4C_1 cells retain less than half of aggregates of prolactin and GH 120 min after synthesis, presumably through basal release of secretory granules (50). Treated cells retain about 80% of the prolactin aggregates 120 min after synthesis; retention of GH aggregates is unchanged. Prolactin storage increases because cells retain more granules containing prolactin.

The retention of secretory granules and the rate at which they are released is controlled by transmembrane secretory granule proteins and cytosolic proteins associated with these transmembrane proteins. Retention of secretory granules can be manipulated by changing the amounts of these membrane and associated proteins (54). Hormone treatment of GH_4C_1 cells increases accumulation of secretory granule membrane proteins, including ICA512, synaptosomal-associated protein of 25 kDa, which is necessary for secretory granule fusion to the plasma membrane, and synaptotagmin III (55, 56). The increase in ICA512 and synaptosomal-associated protein of 25 kDa is caused by stabilization of these two proteins, rather than by increases in synthesis, consistent with the longer retention of prolactin secretory granules, resulting in decreased turnover of their membrane proteins (55, 56). It is not known which proteins are responsible for the prolonged retention of prolactin granules after hormone treatment or why only prolactin-containing granules should be affected, but these results indicate that the behavior of granules may be regulated specifically and that the regulation distinguishes granules with different contents.

Human R183H-GH is a mutant that causes familial isolated autosomal dominant GH deficiency (type II) (57–59). Deladoëy *et al.* (58) have demonstrated that an individual with this mutation has releasable stores of GH, but GH release is severely impaired. In AtT-20 cells transiently transfected with either human wild-type or R183H-GH, we found that wild type and mutant are synthesized in the same amounts. Both form Lubrol-insoluble aggregates 40 min after synthesis and both are stored in granules, but retention of the aggregates differ (60). Fifty percent more

of the aggregated mutant is retained 120 min after synthesis than wild type, although the mutant protein folds correctly, is stable and not degraded for 2 h after synthesis, and has full biological activity. When hormone release is stimulated, less R183H-GH is released than wild-type hormone, and R183H-GH has a dominant effect partially suppressing hormone release when wild type and mutant are cotransfected (60). Clones of AtT-20 cells stably transfected with R183H-GH secrete less hormone into the medium after 4 h of incubation with forskolin than clones stably transfected with wild-type hormone, although accumulation in the medium is stimulated to the same extent after 8 h treatment with forskolin (61). Treatment for hours may affect factors in addition to release, but the difference at 4 h is consistent with our results of reduced release from transiently transfected cells. These results may be at least a partial explanation for what occurs in human subjects with this mutant, although there are obviously other considerations, such as long-term effects on pituitary gland development (60, 61). The striking finding in culture is that two proteins that differ by only a single amino acid will each form aggregates and be packaged into secretory granules, but the granules will behave differently. Differences between the aggregates of hormones that form the dense cores of secretory granules must influence the localization of proteins that cause granule transport and release. That content may affect behavior was noted early on (35); these experiments add to that finding by showing that a single amino acid change may have an effect. (Such a conclusion means that tags added to secretory granule proteins may have effects on the behavior of wild-type proteins.)

A second single amino acid mutant GH that is dominant negative, P89L-GH, has more marked effects than R183H-GH in stably transfected clones of AtT-20 cells, in that very little P89L-GH is secreted over 12 h compared with secretion of wild-type hormone, and secretion of ACTH is also reduced (61), an effect that may be either a direct disturbance of the secretory pathway or a bias that occurred during selection for stable clones, which could occur if clones producing P89L-GH that produce little ACTH grow better than those that produce normal amounts of ACTH. Because this selection doesn't happen with clones expressing wild-type hormone, there must be a disadvantage in expressing P89L-GH. We found P89L-GH aggregates in transiently transfected AtT-20 cells and is packaged into secretory granules (50), but we did not investigate granule retention and behavior in detail. The granules with this mutant would be predicted to behave differently from those containing wild-type hormone, based on the results in stably transfected clones (61). P89L-GH may be another example of a single amino acid change in the dense cores of granules that affects granule behavior.

D. Specificity in aggregation requirements

The environment of the trans-Golgi lumen has been thought to trigger aggregation by factors such as pH and divalent cations. The trans-Golgi compartment is more acidic than earlier parts of the secretory pathway; the lumen of the endoplasmic reticulum has a pH of 7.2, slightly lower than the extracellular pH of 7.4, and that of the lumen of the trans-Golgi, pH 6 (62). The pH of many secretory granules is even more acidic, eventually reaching as low as pH 5.2 in some cases (62). Acidic regions in cells may be neutralized by the membrane-permeant base chloroquin or an inhibitor of the proton pump, bafilomycin. These two agents slow aggregation of rat prolactin in GH_4C_1 cells and human GH in AtT-20 cells (50). Chloroquin, however, completely prevents aggregation of human prolactin in AtT-20 cells (63), so the aggregation of human prolactin is more dependent on an acidic pH than that of rat prolactin or human GH, and conditions necessary for efficient aggregation are not the same for hormones as similar in structure as these three.

E. Aggregation in solution

To understand how contents of granules influence their behavior, we need to know more about the aggregate itself. One approach is to study the reversible aggregation of protein hormones in solution. Such aggregates in solution must form within 30 min in conditions that exist in the pituitary Golgi complex to mimic what happens in cells. The aggregates must dissociate rapidly to native hormone when exposed to conditions found outside cells. Three different forms of reversible aggregation or self-association have been proposed for prolactin or GH or both: dimerization with Zn^{2+} through a high-affinity Zn^{2+}-binding site; the low-affinity self-association found in conditions of macromolecular crowding, which may be facilitated by low-affinity Zn^{2+} binding; and formation of aggregates of amyloid.

1. Formation of dimers with Zn^{2+} binding

Cunningham and co-workers (64) found that human GH binds Zn^{2+} with an apparent K_d of about 1 μM and that in the presence of Zn^{2+}, human GH forms dimers with a 1:1 ratio of GH to Zn^{2+}. The amino acids necessary for Zn^{2+} binding are H18, H21, and E74 (64). They proposed this Zn^{2+}:GH dimer may be the main storage form in the secretory granule for three reasons: the Zn^{2+}:GH complex is more stable than GH alone, most zinc in the pituitary gland is in the somatotroph secretory granules, and amino acids in the topologically equivalent positions

to those that serve as Zn^{2+} ligands in GH are conserved in placental lactogen, prolactin, and other GH (64). The experiment that they used to demonstrate increased stability was to measure GH denaturation by guanidine hydrochloride at pH 8 in the presence and absence of Zn^{2+}. It would be interesting to see the same experiment performed at an acidic pH, such as pH 5.5, more likely to resemble conditions in secretory granules.

Cunningham *et al.* (64) calculated that there is enough zinc in the pituitary gland to form dimers with human GH; the calculations were based on histochemical staining of the rat pituitary gland (65). Thorlacius-Ussing (65) found staining for Zn^{2+} over the secretory pathway in pituitary cells, including some of the secretory granules in somatotrophs, coricotrophs, thyrotrophs, and, in lactating rats, lactotrophs. Most of the Zn^{2+} staining is over somatotrophs. The staining measures chelatable Zn^{2+}, that is, Zn^{2+} that is not in a complex already. Chelatable Zn^{2+} is 5% of the total Zn^{2+} in the pituitary gland (65), so this study did not indicate where most of the Zn^{2+} is. Cunningham *et al.* (64) assume in their calculations that all Zn^{2+} is in the somatotroph secretory granules, and, with that assumption, there is enough Zn^{2+} in the pituitary gland to form dimers with GH. Zn^{2+} is required for many reactions and functions in all cells, so this assumption is not likely to be valid. It could be tested for rat GH secretory granules by isolating the granules and measuring the total amounts of Zn^{2+}. Even if Zn^{2+} is not present in high enough quantities for a 1:1 complex with GH, it may play a role in hormone storage. The presence of Zn^{2+} in the secretory pathway of pituitary cells might be one reason why prolactin and GH aggregate in pituitary cell lines and not well in fibroblasts.

Prolactin, as predicted by Cunningham *et al.* (64), also binds Zn^{2+} (66, 67), and histidine 27 in human prolactin (topologically equivalent to histidine 18 in GH) is necessary for the binding. Analysis was limited by the solubility of prolactin, but at least 0.7 nmol Zn^{2+} are bound per nanomole human prolactin (66). Zn^{2+} causes human prolactin to self-associate at pH 7.5, but not to form dimers, an unexpected result, based on the dimerization of human GH. H27A-prolactin, without the high-affinity Zn^{2+}-binding site, also self-associates in the presence of Zn^{2+} with parameters similar to self-association of wild-type hormone. Therefore, human prolactin does not form a dimer under conditions that human GH does, and the dimer does not appear to be a likely storage form of prolactin.

Although human prolactin does not behave as human GH does in the presence of Zn^{2+}, there is evidence for both hormones that the amino acids that bind Zn^{2+} with high affinity are necessary for normal production of both prolactin and GH. Much of newly synthesized human H27A-prolactin is degraded, and the newly synthesized mutant is secreted more slowly than wild-type hormone in clones of stably transfected GH_4C_1 cells (68). The GH_4C_1 clones that express human H27A-prolactin produce an average of 200-fold less rat prolactin than the clones that express human wild-type prolactin, and rat prolactin mRNA is also reduced, suggesting that cells that produced both rat prolactin and human H27A-prolactin did not grow well enough to be selected as clones. In contrast, in transiently transfected AtT-20 cells, newly synthesized human H27A-prolactin aggregates is not degraded and is secreted from cells with the same time course and to the same extent as wild-type human prolactin (63).

Iliev and co-workers (69) compared the production of wild-type human GH in transiently transfected GH_4C_1 cells with the production of mutants of GH in which the amino acids that bind Zn^{2+} with high affinity were changed to alanine in various combinations. Cells transfected with each of these mutants produce about 50% less human mutant GH than cells transfected with wild-type human GH (69). Cotransfection with wild-type human GH and the mutants does not reduce the production of wild-type hormone (69).

These results together suggest that the amino acid residues required for high-affinity Zn^{2+} binding in solution are required for the stability of both GH and prolactin in GH_4C_1 cells but not AtT-20 cells. These amino acids may be important for reasons unrelated to high-affinity Zn^{2+} binding, or high-affinity Zn^{2+} binding to human prolactin in cells is important even though dimer formation has not been detected and the amounts of Zn^{2+} are unlikely to be enough in the granules for the hormones to be stored as dimers with Zn^{2+}. At present, the reasons why these mutants of human GH and prolactin are not produced in the same amounts as wild-type hormone in GH_4C_1 cells remains to be clarified, but the reasons, when understood, may explain why these amino acids are so conserved.

2. Low-affinity self-association with macromolecular crowding

Macromolecular crowding in cells is an important factor affecting protein associations that is not always considered (70–72). A major difference between conditions usually used to investigate hormone association and aggregation in solution and conditions inside the secretory pathway is the available volume. In the cytoplasm of cells, 30% of the volume is occupied by large molecules, greatly reducing the volume in which large molecules may diffuse (Fig. 2) (72). The protein concentration in the cytoplasm is 200–300 mg/ml (70), and the lumen of the secretory pathway is likely to be as crowded. The concentration of insulin in secretory granules is 42 mM (over 200 mg/ml)

Figure 2.

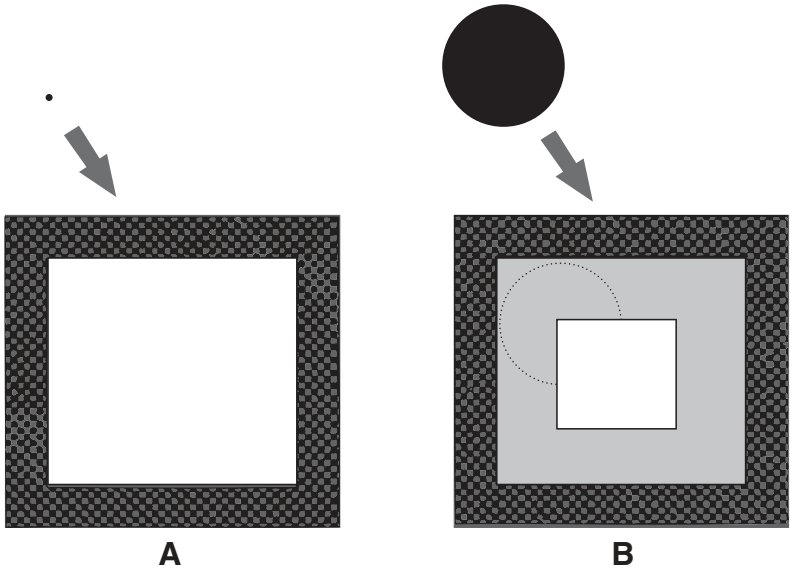

Figure 2. Influence of crowded conditions on available volume. A, A small object in a restricted space (*black square*) has the entire space available (*white area*) to its center of mass. B, A larger molecule has a smaller space available to its center of mass, which is excluded from the *gray area*. [Modified from A. P. Minton: The influence of macromolecular crowding and macromolecular confinement on biochemical reactions in physiological media. *J Biol Chem* 276:10577, 2001 (102), with permission. © American Society for Biochemistry and Molecular Biology.]

(73). In contrast, the maximum protein concentration used by investigators in solution is usually 1–5 mg/ml. The thermodynamic activity of proteins for reactions such as self-association increases dramatically at very high protein concentrations because the available volume is reduced. Crowding enhances the tendencies of macromolecules to bind to one another, but it does not create the tendencies. The reactions that predominate, however, may change (72). Investigations in relatively dilute solutions, such as 1–5 mg/ml protein, may not mimic what happens in crowded conditions in cells. Doing experiments at 200 mg/ml requires such large amounts of protein that many experiments are not usually feasible. It is possible, however, to mimic crowded conditions and affect the thermodynamic properties of proteins by adding large inert molecules, such as polyethylene glycol (PEG) or dextran, to dilute protein solutions to reduce the available volume, which mimics the reduction of available volume in cells. The ability of human prolactin to aggregate and the properties of the aggregate differ in several ways in the presence and absence of macromolecular crowding.

Macromolecular crowding reduces the solubility of human prolactin at acidic pH. A dilute solution of human prolactin, 5 μM, is soluble at both pH 7.4 and pH 6. In the presence of the crowding agent PEG at concentrations greater than 100 mg/ml, 5 μM prolactin is soluble at pH 7.4 but aggregates at pH 6. This pH dependency is consistent with its behavior in cells, where it is soluble in the endoplasmic reticulum but aggregates in the Golgi complex in a process that depends on an acidic pH (63).

Macromolecular crowding changes the interactions of human prolactin with Zn^{2+} at acidic pH. In the absence of PEG, 40 μM Zn^{2+} causes 90% of 5 μM human prolactin to aggregate at pH 7.4, but less than 25% at pH 6. This reduction in aggregation is consistent with the involvement of histidine as a ligand for Zn^{2+} binding, because protonation of histidine prevents Zn^{2+} binding (74). The high-affinity Zn^{2+}-binding site, however, is not required for this aggregation, because H27A-prolactin also aggregates with the same pH dependency (63). In the presence of 90 mg/ml PEG, a concentration that by itself does not cause aggregation, 40 μM Zn^{2+} causes 90% of 5 μM prolactin to aggregate both at pH 7.4 and pH 6. With crowding, interactions of Zn^{2+} with aspartate and glutamate may predominate over those with histidines, changing the pH dependence of aggregation so that it occurs more readily with Zn^{2+} at acidic pH.

Macromolecular crowding reduces the ratio of Zn^{2+} to human prolactin in the aggregate. Aggregates of human prolactin formed with Zn^{2+} in the absence of PEG have two or more Zn^{2+} per prolactin. Aggregates formed with Zn^{2+} in the presence of 90 mg/ml PEG have a ratio of 0.5 Zn^{2+} per prolactin (63). In unpublished studies using isolated rat prolactin secretory granules, we found less than one Zn^{2+} per prolactin, and it seems unlikely that there is enough Zn^{2+} in the human pituitary gland to have a greater ratio.

These aggregates form within minutes (63), appropriately for what must occur in cells, where prolactin is detected in aggregates 30 min after synthesis (38). Because the cores of granules begin to disintegrate quickly after exocytosis (36), aggregates in solution that mimic what occurs in cells should dissolve quickly. We found that over 80% of the prolactin that aggregated in the presence of 180 mg/ml PEG becomes soluble within 10 min at pH 7.4 (63).

We found no aggregation of human prolactin in the presence of Ca^{2+} in concentrations up to 10 mM in the presence or absence of macromolecular crowding and pH ranging from 7.5–5.5 (63). These results were unexpected, because Ca^{2+} is found in the secretory pathway, including secretory granules, in relatively high concentrations (75). Many secretory granule proteins aggregate in the presence

of Ca^{2+}, and this cation is likely to be important in their storage (76, 77). Although Ca^{2+} may play a role in secretory granules containing human prolactin, there is no evidence so far that it causes aggregation of this protein.

Macromolecular crowding does not make all secretory proteins aggregate. In conditions that cause human prolactin to aggregate, BSA, which is not stored in secretory granules, does not aggregate (63).

3. Amyloid fibers

Amyloids are fibrous protein structures with ordered β-sheets arranged so that the sheets are perpendicular to the axis of the fibril (78). Amyloid structures are unusually stable compared with other protein structures and have distinctive morphology and dye-binding characteristics (78). Amyloid proteins are found in some disease states, including Alzheimer's and Parkinson's diseases, and some have been proposed to have biological functions in bacteria and fungi (78).

Maji and co-workers (79) have proposed that amyloids function as the natural storage form of peptide hormones in secretory granules. They examined the structures of aggregates that formed in solution, using human prolactin and 41 peptide hormones whose length ranged from 10–41 amino acids. Prolactin and the peptides were incubated at 2 mg/ml at pH 5.5 to approximate the pH of secretory granules for extended periods from 14–30 d. Human prolactin does not form fibrils alone or in the presence of heparin, but does form fibrils in the presence of a greater than 4-fold excess of chondroitin sulfate. The morphology of these prolactin fibrils seen by electron microscopy is consistent with an amyloid structure, and prolactin fibrils bind Congo red and thioflavin T, two dyes that bind amyloid. The x-ray diffraction pattern of the prolactin fibrils shows a radial reflection at 10 Å, characteristic of a cross β-sheet with fibrils that are not aligned. The circular dichroism spectrum of prolactin fibrils shows a decrease in the characteristic pattern that results from α-helices, consistent with loss of α-helices from the normal conformation of the protein (79).

If these fibrils are the natural storage form of human prolactin, they must dissolve relatively rapidly. They are clearly stable, however, because the procedure for staining with Congo red involves two 20-min incubations and several rinses at alkaline pH (79). The solubilization of prolactin fibrils was measured at pH 7.4; half becomes soluble within 2 h (79). In contrast, more than half of the membraneless prolactin dense cores become soluble within 15 min at pH 7.4 (5), and more than half of the prolactin aggregates formed by crowding at pH 6 become soluble within 10 min at pH 7.4 (63).

The time that it takes for the aggregates to form is just as important as the time it takes for them to dissolve. Maji and co-workers (79) assessed fibril formation after 14 or 30 d of incubation and did not address the time course or the extent to which the conversion occurred. Newly synthesized prolactin forms aggregates in cells within 30 min after synthesis (38, 63), and aggregates of human prolactin form within minutes in solution at pH 6 under conditions of macromolecular crowding (63). These aggregates are unlikely to be amyloid, because they dissolve so quickly. If prolactin does form fibrils before secretory granules form, as proposed by Maji and co-workers (79), conditions must exist in the Golgi lumen that allow the formation of fibrils within minutes. Macromolecular crowding may enhance or inhibit amyloid formation (80–82), and its effects on prolactin amyloid formation have not been assessed.

Maji *et al.* (79) used fixed sections of a male mouse pituitary gland to demonstrate that thioflavin S, a fluorescent dye that stains amyloid, colocalizes with pituitary hormones. There is not much staining for prolactin in these sections because the mouse was male, but what there is does appear mostly to colocalize with prolactin. There are two reasons, however, to question whether thioflavin S is actually staining fibrils of prolactin in secretory granules. The first is that thioflavin S may stain more than hormones in the pituitary gland. Maji and co-workers (79) also stained for GH, and there is some colocalization of GH immunofluorescence and thioflavin S staining, but there are also structures in somatotrophs that stain brightly for thioflavin S and not GH. The second reason is that amyloid fibrils of prolactin that have previously been reported do not react with available antiserum for prolactin. Amyloid deposits are found in extracellular locations in pituitary glands of aging individuals and in some pituitary adenomas. These amyloid deposits bind Congo red (83). They were suspected to be of hormonal origin but do not react with available antibodies to prolactin. Westermark and co-workers (83) were able to isolate this amyloid material from the pituitary gland of an 86-yr-old male and showed the material has the amino acid sequence of prolactin. Antiserum made to the peptide that corresponds to amino acids 43–57 of human prolactin does stain the amyloid fibril in the gland, visualized by immunogold techniques (83). Because small amyloid deposits of prolactin accumulate in the pituitary gland with age, the formation of fibrils in solution by Maji *et al.* (79) may reflect this process, rather than what occurs in the normal process of storing hormones.

Meldolesi and co-workers (5, 6) found twisted filaments emanating from some membraneless rat prolactin dense cores that remain after most of the cores are dissolved by incubating at pH 8.5 for 1 h and found filaments

of the same appearance in some granules of bovine prolactin after the membranes were removed. The filaments are shaggy and roughened and more bent and twisted than the fibrils identified as prolactin amyloid (83). Their structure has not been investigated any further, and their stability is not known. The extent to which fixation in glutaraldehyde influences the appearance of the dissolving cores is unknown. Such filamentous structures have not been reported for dissolving granule cores after exocytosis; these are in a different environment than was used to investigate dissolving granule cores in solution and have not been exposed to isolation procedures, including centrifugation.

Maji and co-workers (79) examined the dissociation time of fibrils they had made from five peptides in addition to prolactin, and all of them take even longer than human prolactin to dissociate by a matter of hours. Rapid dissociation is expected for storage forms of peptide hormones in granules; without rapid dissociation, there would not be sharp peaks that are found in the pulsatile pattern of release of most hormones (84). Such rapidity has not been shown for any amyloid fibril form at this time.

Maji and co-workers (79) propose a model in which secretory granules form around hormone amyloid fibrils in the trans-Golgi lumen, but a rapid efficient association into fibrils has not yet been demonstrated for any hormone. In addition to prolactin, they have examined the ability of fully processed peptide hormones to make fibrils. It is, however, the precursors of peptide hormones and not the proteolytic products that are the majority of what is present in the trans-Golgi lumen. Maji and co-workers (79) found that ACTH and β-endorphin are in the same secretory granules in AtT-20 cells, which confirms earlier results (85). This colocalization occurs because most of the processing of proopiomelanocortin occurs after it is in secretory granules (85). The ability of prohormones to make fibrils, which is important if amyloid formation is a preliminary step in granule formation, was not addressed.

F. Current model for prolactin and GH secretory granule formation

Prolactin is soluble in the endoplasmic reticulum at pH 7.2. The pH drops in the trans-Golgi region to 6, and prolactin forms aggregates that will rapidly dissociate when exposed to the extracellular pH of 7.4. Protonation of histidines may be the event that increases the tendency of prolactin to self-associate at pH 6. There are nine histidines in human prolactin; two have a pK_a above 6. The pK_a of H27 is 6.64, that of H46 is 6.65, and that of H30 is 6.0 (86). Chelatable Zn^{2+} is present in secretory granules containing prolactin and may facilitate aggregation by binding to glutamates and aspartates and cross-linking small aggregates of prolactin into larger ones, so there is less than one Zn^{2+} per molecule in the aggregate. Secretory granule membrane proteins necessary for appropriate transport and exocytosis recognize an aspect of the aggregate and become localized in the membrane around the aggregate. Membrane and proteins not found in secretory granules are removed by clathrin vesicles and, to a lesser extent, other tubular export processes that occur in the trans-Golgi network until the mature secretory granules are left. These vesicles also remove soluble proteins, whether because the soluble proteins have a sorting signal, such as mannose-6-phosphate or simply because they are soluble and are part of the fluid of the lumen removed by the export carriers (Fig. 3).

We expect that GH will form granules in the same way but that specific details will vary. Two predictions are that the total amount of Zn^{2+} will be less than the amount of GH in secretory granules and that human GH will aggregate reversibly in crowded conditions with low-affinity interactions. Neutralizing the pH of the trans-Golgi lumen in cells slowed aggregation of human GH but did not reduce it to the extent that neutralization did to that of human prolactin (50, 63), so aggregation of GH under crowded conditions in solution would be predicted not to show as marked a dependency on pH as that of human prolactin, which would provide an explanation for some of the specificity of aggregation. Low-affinity interactions with Zn^{2+}, Ca^{2+}, or other factors may contribute, because there is something in pituitary secretory pathways not present in all cell types that makes prolactin and GH aggregate efficiently.

A predication is that the aggregates formed by R183H-GH differ in some as yet undetermined way from those formed by wild-type hormone. This single amino acid change is not a change in a putative sorting signal because the mutant protein is stable after synthesis, forms aggregates, and is packaged into secretory granules (60). Such a difference in aggregate properties may also contribute to differences from wild-type behavior described in cells transfected with P89L-GH, H18A-GH, H21A-GH, E174A-GH, and H27A-prolactin (61, 68, 69).

There is no sorting signal. Although others have proposed aggregation synergizes or complements a sorting signal, a sorting signal is not necessary in this model. Sorting occurs through the specificity of aggregation, caused by low-affinity interactions in crowded conditions.

IV. Distinguishing Aggregation from a Sorting Signal as a Means of Sorting

A. What would it take to prove sorting for prolactin and GH occurs through aggregation?

As indicated above, we expect that GH will also rapidly form aggregates in solution with macromolecular crowding and that

Figure 3.

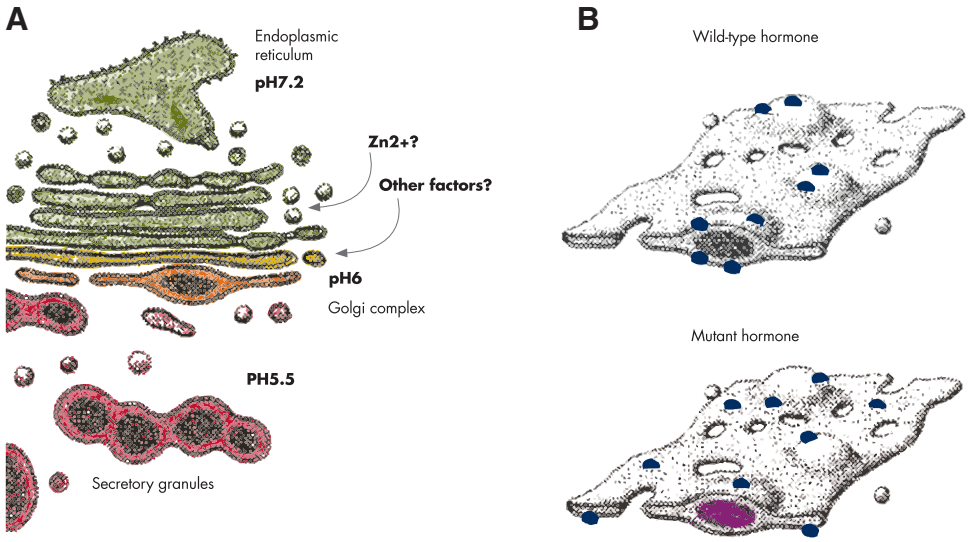

Figure 3. Current model for formation of secretory granules containing prolactin and GH. A, Cross-section of secretory pathway. Hormones are soluble in the endoplasmic reticulum and are carried to the Golgi complex where they form aggregates visible by electron microscopy in the trans-Golgi region. Factors enhancing the tendency to aggregate include the decrease in pH and must also include other factors or conditions that enhance aggregation in pituitary cells compared with fibroblasts. The presence of Zn^{2+} and Ca^{2+} is one possibility; there may be other factors as well, as yet unidentified. Excess membrane and unwanted proteins are removed by small vesicles. B, A section of the trans-layer of the Golgi complex. Membrane proteins necessary for granule function must correctly localize around the aggregate. Aggregates of mutant hormones such as R183-GH may not accumulate these proteins as efficiently as those of wild-type hormone. These figures are modifications of the representation of the secretory pathway of lactotrophs by Rambourg and co-workers (19), and a similar figure has been previously published (103). [Modified from Y. Rambourg *et al.*: Formation of secretory granules in the Golgi apparatus of prolactin cells in the rat pituitary gland: a stereoscopic study. Anat Rec 232:169, 1992 (19), with permission. © Wiley Periodicals.]

these aggregates will rapidly dissociate to yield native GH in conditions similar to the extracellular environment. Aggregation of GH will likely vary from that of prolactin in details such as the influences of pH and cations. A prediction from the model is that there are crucial differences between aggregates of wild-type and R183H-GH, which is crucial to explaining why granules that contain aggregates of the mutant do not behave the same as those containing the wild-type hormone. Finding physical differences in aggregates of the two proteins would be a useful step. Finding quantitative differences in the membrane proteins in the secretory granules with cores of wild-type or R183H-GH would provide support for the model. The most convincing support for the model would be to elucidate how the aggregate could influence the retention of membrane proteins around it and how such a relatively conservative change, arginine to histidine in R183H-GH, could affect this process.

What factors determine the proper localization of the membrane proteins around hormone aggregates of prolactin and GH in the trans-Golgi lumen? There may be direct interactions with the aggregates, but intermediates are also possible. Polyanionic molecules are candidates for intermediates in secretory granules containing prolactin. As indicated above, heparin sulfate, hyaluronic acid, and chondroitin sulfate coat the dense cores of prolactin aggregates in the secretory granules of lactotrophs (7). Both prolactin and GH bind heparin in solution (87, 88). Such glycosaminoglycans have polyanionic arrays that have diverse functions in cells (89). The glycosaminoglycans are present in granules in concentrations much lower than prolactin and are not an integral part of the aggregate, but because they coat the cores, they are in a position to be recognized by granule membrane proteins. Changing location and strength of positive charges on the surface of the aggregate may change the polyanion coating in a critical way. At this time, the role of these polyanions in prolactin secretory granules and the specificity of their binding to aggregates are unknown.

Rat GH secretory granules do not have the glycosaminoglycans that rat prolactin granules do (42), and so if the surface charge of the aggregate is important in facilitating location of membrane proteins, it cannot be formed in the same way for GH aggregates as is proposed for prolactin. The kinds of specificity reported above indicate that there will be differences in forming granules, and this difference may reflect one of them.

B. Is aggregation necessary and sufficient for storage of other hormones?

Formation of aggregates of prolactin and GH in cells can be assayed because they are stable when trans-Golgi

and granule membranes are dissolved in Lubrol. If other hormones use aggregation in the trans-Golgi lumen as a mechanism for sorting and formation of secretory granules, but the aggregates are not stable when the membranes are disrupted, then aggregation in cells cannot be detected even though it occurs and an important piece of evidence cannot be obtained. There is, however, some evidence that the ability to aggregate in solution correlates with the ability to be stored in granules for proteins other than prolactin and GH.

Proatrial natriuretic peptide (proANP) is stored in secretory granules with dense cores in atrial cardiocytes. In solution, proANP binds Ca^{2+} and aggregates; changing glutamates 23 and 24 to glutamines reduces Ca^{2+}-induced aggregation (90). Forskolin stimulates release of proANP in transfected AtT-20 cells, but not release of E23Q, E24Q-proANP (90). Perhaps surprisingly, mutating one more negatively charged amino acid restores both properties: Ca^{2+} causes E23Q, E24Q, D31N-proANP to aggregate in solution and forskolin stimulates its release from transfected AtT-20 cells (90). The means by which Ca^{2+} causes aggregation of wild type and the triple mutant, but not the double mutant, are not known, but these experiments demonstrate a good correlation between aggregation and storage.

Baertschi and co-workers (91) provided evidence that the contents of secretory granules affect the behavior of the granules by transfecting cultured atrial cardiomyocytes with constructs of proANP linked to enhanced green fluorescent protein (EGFP), so that the secretory granules could be observed in living cells (91). E23Q, E24Q-proANP-EGFP-containing vesicles with the mutant did not dock at the plasma membrane, although those containing the wild-type EGFP construct did (91). As indicated above, aggregation of E23Q, E24Q-proANP by Ca^{2+} is reduced in solution, but these experiments show the mutant does end up in granules when attached to EGFP, albeit in malfunctioning granules. EGFP itself, however, ends up in secretory granules (92, 93). It forms disulfide-bonded oligomers in cells, which are not necessary for some EGFP to be found in granules, but it is the ability to form aggregates that are not covalently bonded in solution in the conditions found in the Golgi lumen that should be tested and may be the reason why this protein ends up in granules.

The ability of Ca^{2+} to cause some secretory granule proteins to aggregate in relatively dilute solution may be enhanced. Jain and co-workers (94) found that proteins tagged with six histidines enhanced the calcium-induced aggregation of chromogranin A in dilute solution without being part of the aggregate (94). GH_4C_1 cells transfected with secreted alkaline phosphatase with the histidine tag have more stimulated secretion of chromogranin A compared with those transfected with untagged secreted alkaline phosphatase, indicating a correlation with increased aggregation in solution and increased storage (94). The authors suggest proteins in the secretory pathway may function as aggregation chaperones, facilitating aggregation without being part of it.

These observations indicate that aggregation correlates with storage in secretory granules. There are also predictions that may be tested. If secretory granule membrane proteins accumulate around aggregates in the trans-Golgi lumen, the aggregates that are important are the aggregates of prohormones, because most processing of prohormones occurs in the secretory granules. Testing aggregation of prohormones in conditions that resemble those of the trans-Golgi lumen, including macromolecular crowding, acidic pH, and cations such as Zn^{2+} and Ca^{2+}, is important. Because macromolecular crowding enhances the tendencies of proteins to bind to one another, but does not create these tendencies (72), detecting aggregation of prohormones under these conditions is evidence such a process is very likely to occur in cells.

Arvan and Halban (95) have proposed that the formation of dense cores of crystalline-like insulin retains insulin in granules and is responsible for sorting of insulin. [Such a mechanism does not occur in guinea pigs, because guinea pig insulin does not crystallize (95).] The evidence for insulin crystallization as a sorting mechanism is that some proinsulin is removed from secretory granules by the small vesicles that bud off from the granules as they form, removing membrane and proteins for other destinations. In pancreatic β-cells, the amount of proinsulin removed is very small (95). One explanation for the ability to remove proinsulin is that aggregation of proinsulin does not occur, so it is available for removal by the small vesicles; another is that proinsulin aggregation occurs but is not completely efficient and is easily reversible, so that there is some soluble proinsulin that may be removed. Proinsulin is more soluble than insulin in many conditions, but whether proinsulin aggregates under crowded conditions as the pH changes from neutral to mildly acidic should be investigated. Proinsulin is likely to be assembled into at least dimers before it leaves the endoplasmic reticulum (96), so aggregation of proinsulin must be a multistep process, first involving assembly into hexamers, and may not be as easy to elucidate in solution in crowded conditions as that of prolactin.

If aggregation of prohormones occurs in solution in conditions resembling those of the trans-Golgi lumen, then a second prediction that could be tested is to determine whether sequences that affect sorting in transfected cells affect aggregation in solution. An example is pro-

renin; mutating the cleavage sites for the processing enzymes reduces the ability to stimulate release of the mutant compared with wild type (97). These mutations changed basic amino acids to alanines, which could affect the ability to aggregate. Finding correlations in the ability to aggregate in crowded conditions and the ability of the prohormones to be stored in secretory granules would be support for aggregation as a primary sorting mechanism.

C. Is there good evidence for a sorting signal?

The main evidence for a sorting signal is that mentioned above: mutations in proteins that are made in large amounts and stored in secretory granules change the ability to simulate release of the proteins in transfected cells. This evidence as it is usually presented is not complete proof of a sorting signal for the following reasons.

1) The view that there are two pathways, regulated and constitutive, and that the constitutive pathway is the default pathway may be too simple. When a sorting signal for secretory granule proteins was first proposed, a constitutive pathway to the cell surface and storage in secretory granules, the regulated pathway, were thought to be the two choices available from the trans-Golgi layers for proteins that did not have signals such as mannose-6-phosphate (2). Current knowledge indicates several anterograde transport pathways as well as retrograde transport exist as the Golgi layers progress from the formation in the intermediate compartment to the disassembly in the trans-Golgi network. What determines which pathway soluble proteins take isn't known (10). Without a better understanding of this process, interpretation of mutations may be oversimplified, especially if the transfected protein is made in amounts much smaller than the amounts made by the cells that usually produce it, and the cells in which it is expressed have more active pathways than the cells that usually produce it.

2) Stability of transfected proteins is rarely measured. Degradation may occur to proteins at several stages in the secretory pathway (98). Measurements of stability and the amount of degradation require pulse-chase techniques, which are laborious. Short pulses of radioactive amino acids are necessary to detect the full amount of degradation if it occurs; longer labeling times preferentially label stable components (52). Radioactive amino acid incorporation doesn't stop when the pulse is ended because there are still internal pools of radioactive amino acids in the cells (52, 99, 100), so several time points are required during the chase to really determine whether the protein is stable. Steps intended to reduce background may remove the protein of interest as well (101). If the majority of a newly synthesized secretory protein is degraded before secretion, but a small proportion is detected in secretory granules because degradation does not occur there, it may be a stretch to say the protein has a sorting signal for granules.

3) The effects of mutations that reduce the ability to store the protein, such as changing basic amino acids necessary to cleave prohormones, on aggregation of the protein or peptide in solution in conditions found in the trans-Golgi lumen are not usually investigated, although sometimes effects on aggregation are dismissed without the investigation. If aggregates of wild-type hormone form in solution under conditions resembling those in the lumen of the trans-Golgi, including macromolecular crowding, and the ability is reduced in mutants that are not as well stored, it provides support for aggregation as the sorting means for secretory granules. If there are no conditions in which aggregation occurs, the need to postulate a sorting signal is strengthened.

V. Conclusions

Prolactin and GH provide simple models for secretory granule storage of protein hormones that are produced in large quantities. These hormones are stored as aggregates that are the dense cores of the secretory granules; the aggregates form rapidly and rapidly dissolve. The requirements for aggregation of prolactin and GH in cells differ, so the process is not identical for all hormones. The behavior of granules containing different hormones also is not identical. Because the behavior of granules is determined by proteins in the membrane that are necessary for granule transport and exocytosis, differences in their behavior must reflect differences in retention of these proteins in the membrane around the aggregate. The surfaces of aggregates of prolactin and GH are unlikely to be identical but may have features in common that are recognized with varying efficiency by the membrane proteins. It is therefore important to understand how aggregates of these hormones differ, especially how aggregates of wild-type GH differ from those of mutants such as R183H-GH, and to determine at what points the aggregates and the proteins in the membrane surrounding them interact.

If the prohormone forms of proteins that are processed form aggregates in solution under conditions of molecular crowding, and mutations that reduce storage in granules without affecting stability in cells reduce formation of these aggregates in solution, then these prohormones also may use the mechanisms of aggregation to form secretory granules. In such cases, investigating the properties of these prohormone aggregates will also be important.

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