Pathways of Protein Secretion in Eukaryotes

Regis B. Kelly

Summary. Protein secretion from cells can take several forms. Secretion is constitutive if proteins are secreted as fast as they are synthesized. In regulated secretion newly synthesized proteins destined for secretion are stored at high concentration in secretory vesicles until the cell receives an appropriate stimulus. When both constitutive and regulated protein secretion can take place in the same cell a mechanism must exist for sorting the correct secretory protein into the correct secretory vesicle. The secretory vesicle must then be delivered to the appropriate region of plasma membrane. Transfection of DNA encoding foreign secretory proteins into regulated secretory cells has provided insight into the specificity of sorting into secretory vesicles.

The general pathway of protein secretion in eukaryotes has been clear for many years (*1*). It is possible to show by labeling experiments that secreted proteins pass from their site of synthesis in the rough endoplasmic reticulum (RER) to the Golgi apparatus, the stack of characteristic membrane cisternae where glycosylation takes place. From the Golgi, newly synthesized vesicles travel via transport vesicles to the cell surface. Protein secretion has been found in almost every cell type and is consistent with the universal presence of RER and Golgi in nucleated cells.

Until recently, it was believed that a cell secreted all its proteins by the same pathway. In exocrine cells and liver cells, all secretory products are found in all regions of the RER and Golgi, an indication that there is no regional specialization (*2, 3*). In yeast, mutants in secretion affect the externalization of all known secreted proteins (*4*). Furthermore, immunocytochemical studies indicated that the same pathway—RER to Golgi to surface—appeared to be taken by plasma membrane proteins (*3, 5*). In agreement with these observations, mutants in yeast that blocked protein secretion also blocked plasma membrane protein externalization (*6*), and kinetic and pharmacological analyses showed that the routes of externalization of membrane and secreted proteins were not significantly different (*7*).

Although the basic pathway of protein secretion is similar in many kinds of cells from yeast to mammalian cells, individual steps along the pathway show variability from cell type to cell type. As emphasized by Tartakoff and Vassalli (*8*), secretory cells fall into two classes, called here constitutive and regulated secretory cells (Fig. 1). Antibody-secreting lymphocytes are good examples of a constitutive secretory cell. They do not have a large intracellular pool of antibodies. The newly synthesized proteins reach the cell surface minutes after leaving the Golgi. Since their transport vesicles have such a short half-time, they are hard to find in electron micrographs of the cell's cytoplasm. When they are found, they do not have the electron-opaque "dense core" found in electron micrographs of conventional secretory vesicles (*9*). Transport vesicles fuse with the plasma membrane constitutively to release their contents by exocytosis. There is no evidence that an external stimulus has to trigger exocytosis by altering the level of a cytoplasmic second messenger such as calcium (*8*). Constitutive secretory cells are probably the most common class and include liver cells, fibroblasts, muscle cells, and perhaps yeasts. Alteration of the rate of protein secretion by constitutive secretory cells is effected by altering the rate of protein synthesis.

Regulated secretory cells as a class are specialized to release, for a brief period, large amounts of protein at a rate much higher than the synthetic rate. Rapid transient rates of release are achieved by storing newly synthesized proteins in secretory vesicles that have a half-time of days. Because of the long half-life, the cytoplasm becomes filled with secretory vesicles, which provide one of the characteristic morphological features of these cells. Unlike the transport vesicles of constitutive secretory cells, secretory vesicles are prevented from fusing with the plasma membrane until the level of a cytoplasmic messenger, usually calcium, is altered.

In most types of regulated secretory cell, the material inside the mature secretory vesicle is condensed to give the electron-opaque dense core in electron micrographs. The appearance of the dense core correlates with an increase in concentration of the secretory product. In endocrine cells the secreted proteins are concentrated as much as 200-fold (*10*) during their passage from the last cisterna of the Golgi to the mature secretory vesicle. In exocrine cells, concentration occurs in specialized condensing vacuoles, but may be more modest—only around ninefold relative to the RER level (*2*). The concept of a relation between concentration and the formation of the dense core is strengthened by experiments in which the secretory product remained bound to the dense core after removal of the secretory vesicle membrane by detergent (*11*). Although condensation occurs in the region of the Golgi, and exocytosis is regulated at the cell surface, it nonetheless appears a useful generalization that cells that regulate their secretion also condense their secretory products, whereas those that secrete constitutively do not.

Cells can regulate their sites of exocytosis as well as the rate. Cells such as fibroblasts do not regulate their site of secretion but may secrete components of the extracellular matrix anywhere on their cell surface. Mast cells or neutrophils secrete the contents of their secretory vesicles toward any part of the plasma membrane that is stimulated. Such cell types can be considered nonpolarized secretory cells. Epithelial cells, like liver, endocrine, or exocrine cells, secrete some of their products through a specialized domain of their plasma membrane. Neurosecretory cells, such as the hypothalamic neurons that release hormones only from nerve terminals in the posterior pituitary, are extreme examples of this type of directed or polarized secretion. Such cells can

The author is professor and vice-chairman in the Department of Biochemistry and Biophysics, University of California, San Francisco 94143.

be considered polarized secretory cells. A secretory cell, therefore, can be regulated or constitutive, polarized or nonpolarized (Table 1).

Table 1. A classification scheme for protein-secreting cells. While alternative classification schemes may exist, and cell classes may exist that do not readily fall into this classification, it is a convenient one for the viewpoint expressed in this article. Cells that have regulated secretion may also show constitutive secretion. Although there is no direct evidence yet available, polarized cells may also have nonpolarized secretion.

Regulated	Constitutive
Polarized	
Neurons	Liver parenchymal
Endocrine	
Exocrine	
Spermatocytes (acrosome reaction)	
Nonpolarized	
Neutrophils	Fibroblasts
Mast cells and basophils	Chondrocytes
Platelets	Macrophages
	B lymphocytes
Egg cells during fertilization	

Sorting in Secretory Cells

Both secreted proteins and lysosomal enzymes are made in the same RER, yet end up at different locations in the cell. The mechanisms by which newly synthesized lysosomal enzymes and secretory proteins are segregated from each other have been clarified in recent years. In one such mechanism the asparagine-linked high mannose sugars of lysosomal enzymes are selectively modified in the *cis* cisternae of the Golgi apparatus, the compartment first entered by proteins leaving the RER, to acquire a mannose phosphate group. Receptors for the mannose phosphate group in the Golgi cisternae bind the newly synthesized lysosomal enzymes (*12*). Although the molecular nature of the sorting signal now seems clear, the site at which secretory and lysosomal proteins first segregate remains in dispute. There must also be alternative routes of lysosomal enzyme sorting in mammalian cells, since cells that lack the 215-kilodalton mannose phosphate receptors (*13*), or the transferase that specifically phosphorylates lysosomal enzymes (*14*), retain the capacity to store lysosomal enzymes. Yeast also provides an intriguing alternate sorting mechanism. Carboxypeptidase, a vacuolar enzyme in yeast analogous to a lysosomal enzyme in mammals, is directed to the vacuole by information residing in the amino acid sequence of a carboxypeptidase precursor, not the oligosaccharides (*15*).

If newly synthesized lysosomal enzymes in the lumen of the Golgi are recognized and sorted into lysosomes, there is no need, a priori, for a sorting mechanism for secretory proteins. If there is bulk flow from Golgi to surface, any protein in the lumen of the Golgi that lacks a lysosomal sorting domain would be transported out of the Golgi to the plasma membrane.

In this article, the view is presented that the transport of secretory protein in constitutively secreting cells may indeed involve a passive, bulk flow mechanism in which no sorting is required. Regulated secretory cells, however, do not always lose the constitutive pathway when they acquire the regulated one. If two pathways exist to the cell surface and secreted proteins show a preference for one pathway over another, secretory protein sorting must occur. A plausible model is that the constitutive pathway remains bulk flow, while proteins destined for storage in regulated secretory cells carry targeting information. Finally, the coexistence of regulated and constitutive secretion in the same cell leads to speculation about the possible coexistence of polarized and nonpolarized secretion. For example, polarized secretion may coexist with nonpolarized secretion.

Evidence for More Than One Alternative Secretion Pathway

A cell with regulated secretion (Table 1) may also show constitutive secretion. On logical grounds alone it has been clear that there must be more than one pathway to the surface of cells. For example, polarized secretory cells, such as exocrine cells, secrete digestive enzymes only from the apical surface, secrete components of the extracellular matrix from their basolateral surface, and have different plasma membrane components in the two surfaces. Direct experimental evidence that all membrane proteins do not arrive at the plasma membrane in secretory vesicle membranes was obtained with the endocrine cell line AtT-20. Secretory vesicles containing newly synthesized adrenocorticotropin hormone (ACTH) did not contain any newly synthesized gp70 membrane protein, encoded by an endogenous murine leukemia virus (*16*). The AtT-20 cell line stored only mature ACTH in its secretory vesicles and released only mature ACTH on exposure to an external stimulus. In the absence of stimulation, however, it "leaked" newly synthesized precursor to ACTH, proopiomelanocortin (POMC). Since the kinetics of externalization of POMC were indistinguishable from the kinetics of externalization of the gp70 membrane protein, it was speculated that each cell of this cell line has at least two pathways of protein secretion. In the regulated pathway, POMC precursor is stored in secretory vesicles, processed proteolytically to mature ACTH, and released only on stimulation. In the constitutive pathway, POMC precursor could be externalized in the absence of stimulation by the same route that carries membrane protein.

Subsequent experiments have been consistent with this model. The initial comparison in AtT-20 cells was of a membrane protein and a secreted protein. All newly synthesized proteins secreted by AtT-20 cells, however, fall into either the rapidly released constitutive class or the regulated class in which they are stored in vesicles and released on stimulation (*17*). We have recently identified one of the proteins secreted by the constitutive pathway as laminin (*18*). The two pathways could also be distinguished pharmacologically since chloroquine, a weak base known to raise the *p*H of intracellular organelles, blocked

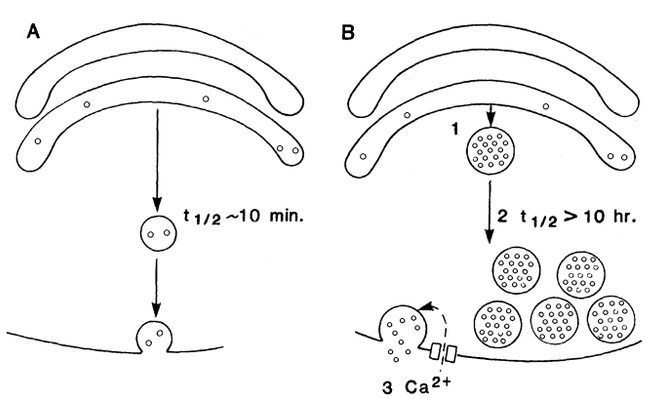

Fig. 1. Two pathways of protein secretion. (A) In constitutive secretion, newly synthesized molecules are not concentrated; there is no post-Golgi storage pool; and the transport vesicle has a short transient time from Golgi to surface. (B) In regulated secretion, the secretory products are condensed in electron-dense core forms (*1*); the dense core vesicles accumulate in the cytoplasm because they can have a half-life of many hours (*2*); and fusion requires altering the level of an intracellular messenger, such as calcium.

newly synthesized POMC from entering the regulated but not the constitutive pathway (*19*). That somatostatin inhibits growth hormone secretion but not membrane protein externalization can also be interpreted readily by assuming two pathways (*20*).

Evidence for Sorting Domains in Pituitary Cells

If two secretion pathways coexist in one cell type, a protein in the lumen of the Golgi apparatus may need to be assigned a route to the cell surface. Blobel (*21*) has suggested that the molecular basis on which such choices may be made is a "sorting domain" on the secreted protein and a "carrier" protein that transports the secreted protein to its correct secretory organelle (Fig. 2A). An alternative model (Fig. 2B) combines a carrier-mediated pathway with a passive, bulk-flow process, analogous to fluid-phase endocytosis of horseradish peroxidase, which accompanies receptor-mediated endocytosis (*22*). Since membrane has to flow to the cell surface to allow cell growth and membrane turnover, passive bulk phase flow would be mandatory for soluble proteins pinched off inside transport vesicles unless there is an exclusion mechanism. In the first two models, sorting is necessary. Another possibility is that soluble proteins in the lumen of the Golgi are not sorted but their probability of going by one pathway or the other is proportional only to the volume of the transport vesicles and the number that leave the Golgi per unit time (Fig. 2C). If the vesicles in different pathways have different surface-to-volume ratios, the ratio of membrane to secretory proteins in the two pathways can differ.

With the cloning of the genes for many secretory proteins and the availability of transfection procedures to introduce and express those genes in cultured cell lines, it has become possible to investigate models such as those in Fig. 2. DNA encoding rat or human proinsulin was first introduced into the COS cell line, in which transient expression is very high (*23*). The transformed cells synthesized proinsulin, and secreted it. According to the model we had presented, secretion of proinsulin by COS cells was constitutive and therefore analogous to the secretion of POMC and gp70 by AtT-20 cells. When stable lines of AtT-20 that expressed human proinsulin as well as the endogenous ACTH were generated, proinsulin was packaged into secretory vesicles just as well as the ACTH (*24*). Furthermore, unlike the results with COS cells, the proinsulin was proteolytically processed in the secretory vesicles to a form that migrated with authentic insulin during electrophoresis. This form of insulin was released along with mature ACTH when the cells were stimulated to secrete (*24*). Human growth hormone has also been expressed in AtT-20 cells (*25*) and parathyroid hormone in GH4 cells (*26*). In all cases the foreign peptide is packaged into secretory vesicles and released on stimulation. It is possible therefore that all peptide hormone precursors can be packaged into the secretory vesicles in any endocrine cells, independent of their species of origin.

The packaging of peptide hormones encoded by foreign DNA into AtT-20 secretory granules could be interpreted by any of the three models in Fig. 2. A bulk flow argument can be eliminated only by showing that some identified molecule in the lumen of the Golgi is partitioned differently between the pathways. If sulfated glycosaminoglycans are assumed to be synthesized in the lumen of the Golgi before sorting occurs, their preference for the constitutive pathway over the regulated one (*27*) is incompatible with an entirely bulk flow model (Fig. 2C). We have also shown that endogenous laminin is sorted differently from endogenous ACTH (*18*). To determine whether all secretory proteins encoded by foreign DNA are packaged into regulated secretory vesicles, we introduced into AtT-20 cells DNA encoding a fragment of a viral membrane protein that had lost its hydrophobic tail (truncated G) (*28*). It was not packaged into secretory vesicles (*25*). Sorting of proteins in cells of the AtT-20 type must therefore require some type of sorting domain. Although not definitive, the evidence favors bulk flow of nonsorted proteins (Fig. 2B) over carrier-mediated sorting into both pathways (Fig. 2A). The observation that newly synthesized ACTH precursor can take either route to the surface (*17*) is readily explained by inefficient sorting and escape of the unsorted molecules by bulk flow. The alternative is to have carrier proteins for ACTH in both pathways. The ability of chloroquine to divert newly synthesized ACTH precursor from the regulated pathway to the constitutive one (*19*) also makes it more likely that proteins are sorted into the regulated pathway and that no information is required to enter the constitutive one. Finally, an artificially generated secretory protein such as truncated G, which might not be expected to have sorting information, escapes constitutively (*25*). Firmer evidence may result from in vitro mutagenesis of proteins secreted by both pathways, or the formation of proteins that are hybrids of constitutively and regulated secretory proteins.

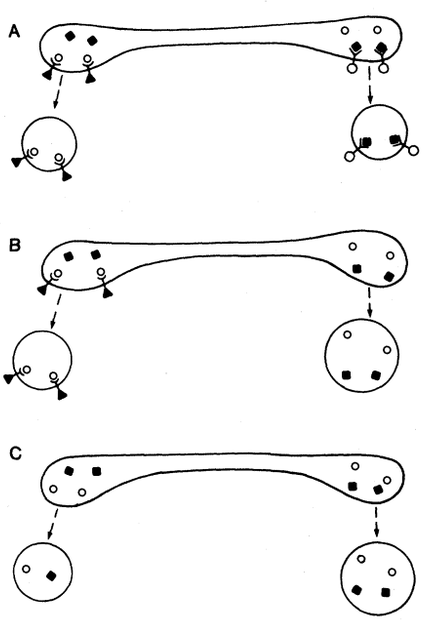

Fig. 2. Alternative sorting pathways when there is more than one pathway of protein secretion. (A) Both types of secreted protein have sorting domains that are recognized by an appropriate carrier molecule. (B) Only one type of protein has a sorting domain. Proteins that lack sorting domains or do not find the correct carrier escape to the surface by nonspecific, fluid phase bulk flow. (C) There is no sorting. In this model, if there are two types of secretory vesicle, the amount entering a pathway depends on the internal volume of the vesicle and the number that form per unit time. To distinguish between the models, consider two secretory proteins X and Y. If the ratio of X to Y is different in the two pathways, a bulk flow mechanism (C) is unlikely. If X takes exclusively the left pathway and Y the right, model A is favored. If X takes both pathways and Y only the left, a mixed model (B) is more likely, but whether the A or the B pathway is carrier-mediated is unresolved. In principle, the carrier-mediated pathway could be determined by constructing fusions of the A and B proteins. This model is for sorting events that occur at the same time. Neutrophils have two forms of regulated secretory vesicle, but these are made sequentially during development. Such "temporal" as opposed to "spatial" sorting might only be available to cells that do not regenerate their secretory vesicles after discharge.

Aberrant Secretion of Lysosomal Enzymes

Additional support for the idea that the constitutive pathway of secretion may not need specific domains comes from the aberrant secretion of incorrectly targeted lysosomal enzymes. Although the correct destination of newly synthesized lysosomal enzymes is the lysosome, a considerable amount can be secreted from the cell. This secretion can usually

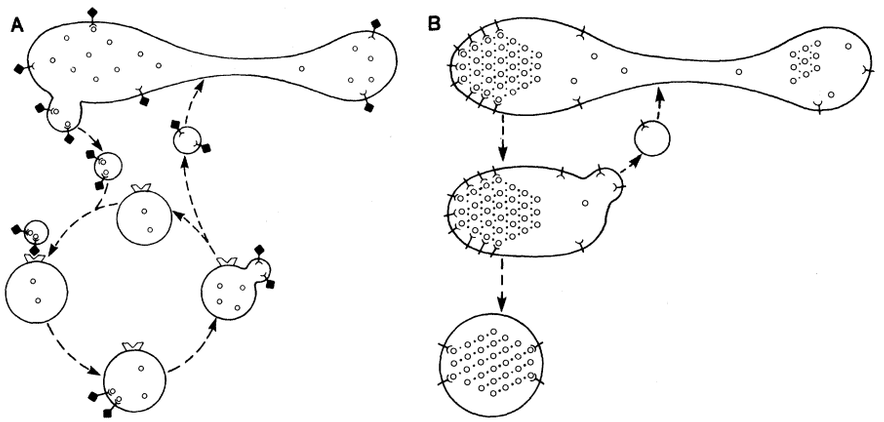

Fig. 3. Carrier-mediated sorting can come before (A) or after (B) the condensation of secreted proteins to give a dense core. (A) A receptor picks up a single secretory protein from the lumen of a *trans* Golgi region. The transport vesicle carrying the newly synthesized protein recognizes the surface of an immature secretory granule and fuses with it. The secreted protein must then dissociate from its receptor perhaps in a low *p*H compartment. The dissociated receptor returns to the Golgi for a second cycle. (B) In the lumen of the *trans* Golgi regions, secreted proteins begin to self-aggregate (for simplicity, only two are shown, represented by open circles and dots). Receptors recognize the aggregate, which then buds out of the Golgi, like a virus budding from a cell. Some association of membrane protein and content is suggested on morphological grounds (*33*) and is to be expected since secretory vesicles have unique membrane proteins. Concentration often requires decreasing the surface-to-volume ratio, and so recycling of excess membrane is proposed. Co-aggregation models (B) suggest that genetic deletion of some secretory granule products could effect the secretion of normal proteins.

be enhanced in mammalian cells by interfering with the ability of lysosomal enzymes to be recognized by the mannose phosphate receptor, either by removing the receptor, eliminating the mannose phosphate residues on the lysosomal enzymes, or by raising the *p*H of intracellular compartments (*29*). Since this secretion is aberrant, it is unlikely that lysosomal enzymes carry a sorting domain for carrier-mediated secretion as described in Fig. 2A. These cells do not have a regulated secretory pathway; hence it is likely that secretion is by a constitutive pathway and involves passive, fluid-phase flow to the surface (Fig. 2B).

Protein secretion by yeast cells also favors specific diversion away from a bulk phase constitutive pathway. Secretion of proteins in yeast probably parallels the constitutive secretion in mammalian cells since, by genetic arguments, membrane and secreted proteins take the same pathway and are externalized in the absence of any known stimulus (*4*). This pathway too does not seem to need a specific sorting domain since proteins diverted into it experimentally have no trouble being externalized. The vacuole in yeast, equivalent to the lysosome in other cells, contains the enzyme carboxypeptidase. If the sorting mechanism for procarboxypeptidase is saturated by overexpressing the protein genetically, procarboxypeptidase does not accumulate in the Golgi but is secreted from the yeast cell (*30*). At present, therefore, it may be appropriate to think of the constitutive pathway as an escape route for any soluble protein in the lumen of the Golgi that lacks the sorting domains characteristic of lysosomal enzymes and of proteins destined to be stored in regulated secretory vesicles. A similar combination of carrier-mediated and bulk flow transport has been suggested from the transport of secreted proteins from the RER to the Golgi (*31*).

Extension of the Model to Other Secretory Proteins

It is now possible to test directly the similarities between types of secretory cells by transfecting into one cell type the DNA encoding a secretory protein of a different cell type. Recently, a complementary DNA (cDNA) encoding the rat exocrine protein trypsinogen has been expressed in AtT-20 cells (*18*). Trypsinogen was packaged into the ACTH-containing secretory vesicles and released from the cells on stimulation. The implication is that at least one protein found in exocrine secretory vesicles can be sorted into endocrine secretory vesicles. Neurons appear also to have a capacity to recognize and sort exocrine proteins. When cDNA encoding the human growth hormone was expressed in the pheochromocytoma cell line PC12, growth hormone was found to be packaged into synaptic vesicles having physical properties similar to those that contain the neurotransmitter norepinephrine (*32*). On stimulation with carbachol, cells released both norepinephrine and human growth hormone rapidly and with the same kinetics.

An implication of these DNA transfection experiments is that the secreted proteins so far examined share a common sorting mechanism. To date, no obvious consensus amino acid sequence has emerged that is shared by these proteins or by all endocrine or exocrine proteins. Moreover, the signal peptide, which directs newly synthesized proteins to the RER, has no obvious amino acid consensus sequence. Identification of shared endocrine and exocrine sorting domains, if they exist, may be possible by in vitro mutagenic techniques.

Possible Carrier-Mediated Sorting Mechanisms for Secretory Proteins

If sorting of secretory proteins occurs, it is reasonable to expect it to resemble protein sorting in other systems. The two well-characterized mechanisms of protein sorting involve lysosomal enzymes [for review see (*33*)] and receptor-mediated endocytosis [for review see (*22*)]. Both processes share (i) membrane receptors, (ii) concentration of receptors in coated pits, (iii) dissociation of ligand from the receptor in a compartment that is sensitive to lysosomotropic drugs such as ammonia or chloroquine (*34*), (iv) return of the receptor, either to the Golgi membranes for the mannose phosphate receptor or the cell surface in receptor-mediated endocytosis, and, in many cases, (v) delivery of the ligand, but not the receptor, to the lysosome. If we assume that the proteins on the regulated pathway are sorted into secretory vesicles, it is reasonable to expect that a similar sorting mechanism might hold for secretory proteins (Fig. 3A). The expectation is fulfilled for at least one aspect of the model, the involvement of a step sensitive to lysosomotropic drugs. When ACTH-secreting cells were exposed to chloroquine before being labeled, the newly synthesized precursor was not delivered to the secretory granules but was secreted directly presumably by the constitutive pathway (*19*).

Such a carrier-mediated model implies that one ligand is carried per receptor per cycle (*35*). A characteristic of the regulated pathway, however, is the concentration of secreted proteins and their condensation to form a dense core, even in the *trans* cisternae of the Golgi on

occasion (*36, 37*). This leads to consideration of an alternative sorting model (Fig. 3B), in which proteins destined to be secreted together form aggregates in the Golgi cisternae. In a process that is analogous to virus budding from the plasma membrane the aggregate associates with secretory vesicle membrane proteins, some of which could be sorting proteins, and buds off. The aggregate must interact with a subpopulation of membrane proteins to permit specific vesicle membrane proteins to be in the same organelle as the vesicle contents. Excess membrane, and soluble material not included in the core, could be returned to the Golgi to effect condensation and perhaps remove inappropriate proteins from the immature secretory granule. Morphological evidence in favor of exactly such an association between core and Golgi membranes has recently appeared for insulin-secreting cells (*36*) (Fig. 4). If a vesicle containing a carrier protein takes an aggregate to an immature secretory vesicle and then recycles to the Golgi for another round, and if the recycling is blocked by chloroquine, then the two models in Fig. 3 become formally the same except that one molecule (Fig. 3A) or many (Fig. 3B) can be taken at a time per receptor. Obviously, carrying more than one protein per ligand per cycle is much more efficient.

Besides its efficiency, a budding model has the advantage that it readily explains de novo biogenesis of secretory vesicles. If, as postulated, the regulated secretory pathway is a nonessential one that appears during differentiation, then when, for example, a precursor cell becomes an endocrine cell, secretory vesicles must arise where none had existed before. The carrier model (Fig. 3A) suffers from the logical difficulty that the transit vesicle carrying the newly synthesized protein has to find a preexisting target organelle, the immature secretory vesicle. Models that invoke preexisting organelles are incompatible with de novo biogenesis. The generation of membrane-coated viruses by specific self-assembly may be our best model for the de novo biogenesis of an organelle.

A common feature of both models is that a receptor analogous to the mannose phosphate receptor resides in the Golgi, where it recognizes some common feature of proteins that enter secretory vesicles. Such a receptor has not been identified, but it may account for the binding of insulin to pancreatic membranes (*38*) and the association of insulin with the membranes of Golgi in immunoelectron microscopy (*36*).

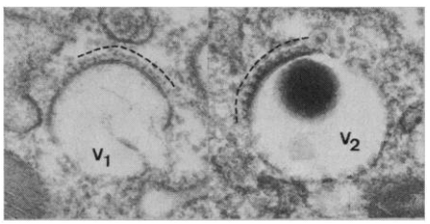

Fig. 4. Morphological evidence that condensation and sorting might occur simultaneously. The above electron micrographs show two sections through the Golgi area of a pancreatic B cell, illustrating the types of immature organelles that accumulate in monensin-treated cells. The vacuole V_1 has no dense core but has a clathrin-coated region indicated by the dashed line. The vacuole V_2 has a secretory granule core-like structure associated with the vacuolar membrane. [From (*36*), with the kind permission of L. Orci and the MIT Press]

If a budding model is correct (Fig. 3B), the specificity of sorting could belong in part to molecules in the lumen of the Golgi that can co-aggregate with secreted proteins. There are certainly proteins in the lumen of secretory vesicles that are candidates for aggregation-inducing molecules. These include, for example, the proteins common to chromaffin granules, prolactin-containing granules, and the vesicles that contain parathyroid hormone, or the protein neurophysin in the hypothalamus, or the four proteins present in large amounts in ACTH-containing granules (*17, 39*). Sulfated proteoglycans, which have an unusually high negative charge, are found in a wide range of secretory vesicles, including cholinergic synaptic vesicles, and mast cell, zymogen, and chromaffin granules (*40*). Proteoglycans have been for many years a popular candidate for a condensing molecule (*1*). Unfortunately, a recent attempt to establish directly that intravesicular proteoglycans are involved in sorting had a negative result. Reducing the amount of an intravesicular chondroitin sulfate proteoglycan, either pharmacologically or by using a cell line deficient in its biosynthesis, had no detectable effect on sorting of ACTH in AtT-20 cells (*41*).

If it turns out that the same receptor can recognize a molecule either individually (Fig. 3A) or as part of an aggregate (Fig. 3B), then whether a cell sorts and then condenses or condenses and then sorts could depend simply on the rate of protein synthesis relative to sorting. It may be noteworthy that the cases mentioned above, in which dense cores were seen with high frequency in Golgi cisternae, were from highly stimulated preparations or after block of intracellular movement with monensin.

Proteolytic Processing Is Not Involved in Sorting

In many endocrine and neuronal cells, a peptide hormone precursor undergoes proteolytic cleavage before being packaged in secretory vesicles (*42*). A similar conversion is found for some granulocyte enzymes before they are delivered to granules (*43*) and in lysosomal enzymes before they are delivered to the lysosomes (*44*). It is unlikely that proteolytic cleavage precedes sorting of proteins, since the proteolytic fragments of processed hormones are found in equimolar stoichiometry in secretory vesicles (*45*). If processing came first, every fragment would require a sorting domain. More direct support came from the observation that in the hypothalamus, cleavage of the oxytocin and vasopressin precursors came after the proteins had left the cell body and were being transported down the axon (*46*). Proteolytic cleavage is not required for sorting, since not all regulated secretory proteins undergo proteolysis. In transfected AtT-20 cells, human growth hormone is sorted normally even though it undergoes no known proteolytic processing in the cell. Inhibition of proinsulin conversion with amino acid analogs also had no effect on secretion (*47*).

Proteolysis at dibasic amino acids is not an exclusive feature of the regulated pathway. Yeasts, which by present knowledge have only a constitutive secretory pathway, also have a peptidase that cleaves the α-factor precursor at dibasic amino acids (*48*). Proalbumin is converted to albumin in liver cells, also thought to lack the regulated pathway (*42*). In DNA transfection studies, proparathyroid hormone expressed in L cells (*26*) and prosomatostatin in COS (*49*) cells are cleaved at their dibasic amino acids. Perhaps the peptidase is more readily detectable in regulated secretory vesicles because the vesicles turn over much more slowly. An unresolved puzzle of proteolytic processing is how proteolysis occurs in secretory vesicles of hypothalamic neurons (*46*) during axonal transport, while dense core granules form in the cell body.

Regulated and Nonregulated Exocytosis in the Same Cell

Secretory vesicles of regulated secretory cells fuse with the plasma membrane only when levels of intracellular calcium or an alternative second messenger are altered. Constitutive release apparently does not require a change in the

level of second messenger. The presence of both pathways in one cell raises the question of why one type of secretory vesicle fuses with the plasma membrane constitutively while another secretory vesicle in the same cell does not.

Constitutive membrane fusion must be occurring constantly within cells. Membrane recycling occurs during receptor-mediated endocytosis, whether or not receptors are occupied (*22*). Transitional vesicles from the RER fuse with the Golgi membranes, and transport vesicles from the Golgi fuse with the plasma membrane. Indeed it is possible that all subcellular organelles are in a constant process of vesicle budding and fusing except, perhaps, during cell division (*50*). Implicit in this picture of frantic membrane pinching-off and fusing is that each membrane must have the means of recognizing the classes of membranes with which it can fuse. In view of the specificity of the interaction, fusion must involve target recognition proteins and cannot be a simple phospholipid bilayer interaction. Transport vesicles from the Golgi then would have surface proteins that specifically recognize and induce fusion with the plasma membrane.

In this context, the surprising feature of mature secretory vesicles relative to constitutive transport vesicles is their remarkably low probability of fusing either with the plasma membrane or other mature vesicles (*10*) under resting conditions. Since the half-life of a secretory vesicle can be 20 to 100 times greater than that of a transport vesicle, the probability of fusion per vesicle must be commensurately smaller. Either the plasma membrane fusion proteins found in constitutive secretory vesicles are excluded from the membrane of the regulated secretory vesicle, or they are present but inhibited. If the latter, a constitutive secretory vesicle could be converted to a regulatory one by introducing regulatory elements into the fusion machinery in such a way that fusion then requires an alteration in cytoplasmic calcium or another cytoplasmic messenger molecule.

Attempts have been made to study the putative regulatory elements. The involvement of calmodulin in calcium-mediated exocytosis (*51*) has stimulated the search for calmodulin binding sites on the membranes of secretory vesicles. Calcium-dependent and calcium-independent calmodulin binding sites have been found and in some cases characterized (*52*). As yet, no evidence is available—either from studies in which antibodies to the calmodulin binding sites are microinjected or those in which fusion is reconstituted in vitro—that the calmodulin binding sites are directly involved in fusion.

Polarity of Exocytosis

Because much is known about the regulation of secretion, the focus in this article has been on how regulated and constitutive pathways of secretion can exist at the same time. A second, but more poorly understood, difference between secretory cell types is whether they are polarized or nonpolarized. Liver cells release albumin only at the sinusoidal surface; exocrine cells release at their apical surfaces (away from their basal lamina), and endocrine cells, such as pancreatic islet cells, adrenal medullary cells, and mammatrophs (*53*), release hormone at their basolateral surfaces (toward the basal lamina). Other cell types show no evidence of polarized secretion.

The nerve cell is the best understood example of polarized secretion. After leaving the Golgi apparatus, membrane vesicles targeted to the nerve terminal associate with microtubules in the axon. This causes movement at 2 to 5 μm/sec in the correct direction. The vesicle-microtubule interaction can now be reconstituted in vitro to give adenosine triphosphate–dependent movement at approximately the correct rate (*54*). The example of the neuron emphasizes that polarized secretion can involve two steps: first, selective association of the secretory vesicle with the cytoskeleton to move to the correct secretion zone; then, insertion by exocytosis into the appropriate domain of plasma membrane. Accumulation of secretory vesicles around zones of secretion, so dramatic in nerve cells, can also be seen in endocrine cells. For example, in the rare intermediate cells of the pancreas that have both zymogen granules and endocrine-like dense core granules, the latter are excluded from the apical region (*55*). A second example of targeting secretory vesicles to specific cytoplasmic domains occurs when adrenal medullary cells or the ACTH-secreting cell line, AtT-20, is grown under conditions that favor long process formation. The secretory vesicles accumulate at the growing tips (*56*) (Fig. 5).

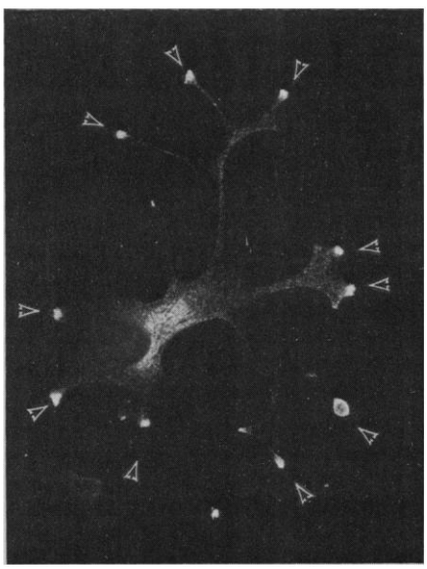

Fig. 5. Endocrine secretory vesicles can accumulate in specialized cytoplasmic regions. Giant AtT-20 cells were grown in culture medium containing 8-bromo-adenosine 3′,5′-monophosphate for 1 week, permeabilized, and stained for immunofluorescence with rabbit antibody to ACTH (courtesy of T. Schroer). Secretory vesicles detected by their ACTH content accumulate at the growing tips (arrows), a result confirmed by electron microscopy (*56*). ACTH is also observed in a juxtanuclear region, presumably Golgi-associated.

The evidence that microtubules are involved in secretory vesicle movement in secretory cell types other than neurons is strong but circumstantial. The most common approach has been to disrupt microtubules with colchicine and similar antimitotic agents. In regulated polarized cells (Table 1), including the endocrine and exocrine cells (*57*), and constitutive polarized cells, such as liver cells (*58*), exposure to antimitotic drugs leads to the accumulation of newly synthesized secretory vesicles in the region of the Golgi. The common view is that microtubules are required for intracellular transport of vesicles carrying newly synthesized membrane and secretory proteins but are not required for exocytosis of mature vesicles. There are some intriguing reports, however, of externalization that is insensitive to antimitotic drugs. Biochemical data and electron microscopy (*59*) suggest that while movement to the apical domain of epithelial cells is inhibited, movement to the basolateral domains is not. This is consistent with the tenfold higher concentration of microtubules in the apical region than in the basolateral one (*60*). Externalization of vesicular stomatitis virus G protein is not slowed down by colchicine, although polarity is lost in migrating cells (*61*). Perhaps microtubules are involved in polarized insertion of membrane vesicles, but not in nonpolarized insertion (*62*). If both polarized and nonpolarized secretion can take place in the

same cell then another exciting form of sorting must take place. Polarized and nonpolarized secretory vesicles must associate differently with the cytoskeleton and have different cytoskeleton recognition proteins on their surfaces.

Conclusion

Because there are two pathways of protein secretion in the same cell, there must be sorting. As with endocytosis it may be possible to distinguish two types of transport, one carrier-mediated and one passive. Carrier-mediated mechanisms could be difficult to unravel if aggregation precedes sorting. The continuous fusion of constitutive transport vesicles with the plasma membrane suggests that regulated secretory vesicle membranes may have placed their constitutive fusion apparatus under calcium regulation. Finally, the marked tendency of regulated secretory vesicles to accumulate in certain regions of the cytoplasm and fuse with specialized regions of plasma membrane has drawn our attention to the specific membrane-cytoskeleton interactions that must occur in these cells.

The presence of two pathways of secretion in the same cell has altered somewhat our ideas of how the pathways developed. Earlier it had been assumed that the regulated pathway of secretion was the normal pathway and that constitutively secreting cells, such as lymphocytes, represented "a special variant of the model in which the secretory products do not undergo concentration as a prerequisite for storage" (*63*). The position taken in this review is that constitutive secretion is the normal form present in most cells, and a regulated secretory cell derives from a precursor that does not lose the constitutive pathway on differentiation. Acquisition of regulated secretion is thus a developmental option for a precursor cell, analogous to the option to become a striated muscle cell. The DNA transfection experiments indicate that endocrine cells, exocrine cells, and neurons might make use of the same developmental option.

The knowledge that there are diverse pathways of protein secretion within a single cell leads to a number of biological questions. Is it possible to identify sorting domains on secretory proteins that determine pathway, and if so, what proteins recognize the domains? How, in turn, are the sorting proteins targeted? Are the rules for sorting vesicle membrane proteins different from those for their contents? If membrane vesicles move in an orderly fashion in the cytoplasm along different highways of cytoskeleton, how do the cytoskeletal highways differ, and how do membrane vesicles recognize the differences? The answers to these questions, now more accessible because of developments in molecular and cellular biology, will help us to outline the rules by which a cell generates its internal order.

References and Notes

1. G. Palade, *Science* **189**, 747 (1975); A. M. Tartakoff, *Int. Rev. Exp. Pathol.* **22**, 227 (1980); J. Rothman *Science* **213**, 1212 (1981); M. G. Farquhar and G. E. Palade, *J. Cell Biol.* **91**, 77s (1981); A. R. Hand and C. Oliver, *Methods Cell Biol.* **23**, 137 (1981).
2. J. W. Slot and H. J. Geuze, *J. Histochem. Cytochem.* **31**, 1049 (1983); M. Bendayan, *Histochem. J.* **16**, 85 (1984); ———, J. Roth, A. Perrelet, L. Orci, *J. Histochem. Cytochem.* **28**, 149 (1980).
3. G. J. A. M. Strous *et al., J. Cell Biol.* **97**, 1815 (1983).
4. P. Novick, S. Ferro, R. Schekman, *Cell* **25**, 461 (1981); P. Novick, C. Field, R. Schekman, *ibid.*, **21**, 205 (1980).
5. J. E. Bergmann and S. J. Singer, *J. Cell Biol.* **97**, 1777 (1983); J. Green, G. Griffiths, D. Louvard, P. Quinn, G. Warren, *J. Mol. Biol.* **152**, 663 (1981); J. J. M. Bergeron *et al., J. Cell Biol.* **94**, 36 (1982).
6. P. Novick and R. Schekman, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 1858 (1979); *J. Cell Biol.* **96**, 541 (1983).
7. R. L. Rotundo and D. M. Fambrough, *Cell* **22**, 595 (1980); G. J. A. M. Strous and H. F. Lodish, *ibid.*, p. 709.
8. A. M. Tartakoff and P. Vassalli, *J. Cell Biol.* **79**, 694 (1978).
9. ———, *J. Exp. Med.* **146**, 1332 (1977); H. J. Geuze and J. W. Slot, *Am. J. Anat.* **158**, 161 (1980).
10. M. M. Salpeter and M. G. Farquhar, *J. Cell Biol.* **91**, 240 (1981).
11. A. Zanini *et al., ibid.* **86**, 260 (1980).
12. H. J. Geuze, J. W. Slot, G. J. A. M. Strous, A. Hasilik, K. von Figura, *ibid.* **98**, 2047 (1984); H. J. Geuze *et al., Cell* **37**, 195 (1984); W. J. Brown and M. E. Farquhar, *ibid.* **36**, 295 (1984); W. J. Brown, E. Lonstatinescu, M. G. Farquhar, *J. Cell Biol.* **99**, 320 (1984).
13. A. Waheed *et al., Biochem. Biophys. Res. Commun.* **105**, 1052 (1982); M. Owada and E. F. Neufeld, *ibid.*, p. 814.
14. C. A. Gabel, D. E. Goldberg, S. Kornfeld, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 775 (1983); C. A. Gabel and S. Kornfeld, *J. Cell Biol.* **99**, 296 (1984).
15. S. Emr, personal communication; H. Schwaiger, A. Hasilik, K. von Figura, A. Wiemken, W. Tanner, *Biochem. Biophys. Res. Commun.* **104**, 950 (1982).
16. B. Gumbiner and R. B. Kelly, *Cell* **28**, 51 (1982).
17. H. P. Moore, B. Gumbiner, R. B. Kelly, *J. Cell Biol.* **97**, 810 (1983).
18. T. Burgess, C. Craik, R. B. Kelly, *J. Cell Biol.* **101**, 639 (1985).
19. H. P. Moore, B. Gumbiner, R. B. Kelly, *Nature (London)* **302**, 434 (1983).
20. R. Green and D. Shields, *J. Cell Biol.* **99**, 97 (1984).
21. G. Blobel, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 1496 (1980).
22. R. M. Steinman, I. S. Mellman, W. A. Muller, Z. A. Cohn, *J. Cell Biol.* **96**, 1 (1983).
23. O. Laub and W. J. Rutter, *J. Biol. Chem.* **258**, 6043 (1983); P. T. Lomedico, *Proc. Natl. Acad. Sci. U.S.A.* **79**, 5798 (1982).
24. H. P. H. Moore, M. D. Walker, F. Lee, R. B. Kelly, *Cell* **35**, 531 (1983).
25. H. P. H. Moore and R. B. Kelly, *J. Cell Biol.*, in press.
26. J. G. Hellerman *et al., Proc. Natl. Acad. Sci. U.S.A.* **81**, 5340 (1984).
27. T. Burgess and R. B. Kelly, *J. Cell Biol.* **99**, 2223 (1984).
28. J. K. Rose and J. E. Bergmann, *Cell* **30**, 753 (1982).
29. A. R. Robbins and R. Myerowitz, *J. Biol. Chem.* **256**, 10623 (1981); A. Hasilik and E. F. Neufeld, *ibid.* **255**, 4937 (1980); K. von Figura, V. Gieselmann, A. Hasilik, *EMBO J.* **3**, 1281 (1984); M. L. Reitman, A. Varki, S. Kornfeld, *J. Clin. Invest.* **67**, 1574 (1981); A. Hasilik, A. Waheed, K. von Figura, *Biochem. Biophys. Res. Commun.* **98**, 761 (1981); A. Gonzales-Noriega, J. H. Grubb, V. Talkad, W. S. Sly, *J. Cell Biol.* **85**, 839 (1980).
30. T. H. Stevens, G. Payne, R. Schekman, in preparation.
31. E. Fries, L. Gustafsson, P. A. Peterson, *EMBO J.* **3**, 147 (1984); H. F. Lodish, N. Kong, M. Snider, G. J. A. M. Strous, *Nature (London)* **304**, 80 (1983); C. A. Gabel and S. Kornfeld, *J. Cell Biol.* **99**, 296 (1984).
32. E. S. Schweitzer and R. B. Kelly, *J. Cell Biol.* **101**, 667 (1985).
33. W. S. Sly and H. D. Fischer, *J. Cell Biochem.* **18**, 67 (1982); A. Hasilik *et al.*, in *Protein Synthesis*, A. K. Abraham, T. S. Elkhorn, J. F. Pryne, Eds. (Humana, Clifton, N.Y., 1983), pp. 349–370.
34. It is usual to interpret a chloroquine block in terms of a block in the ability to dissociate a ligand from its carrier. Raising the *p*H of intracellular compartments may well have other effects, however. Unoccupied low-density lipoprotein receptors do not recycle in chloroquine [S. K. Basu *et al., Cell* **24**, 493 (1981)], and fluid phase uptake is blocked *(22)*.
35. It might be thought that transporting secreted molecules one at a time out of the Golgi would be too slow a process to fill the secretory vesicles in a reasonable time. This does not seem to be the case. The pancreatic acinar cell is estimated to synthesize 5×10^5 molecules of chymotrypsinogen per hour, and chymotrypsinogen makes up about 15 percent of the zymogen granule content [R. M. Case, *Biol. Rev.* **53**, 211 (1978)]. The Golgi therefore has to transport 3×10^6 molecules per hour. If we assume that one-third of the Golgi area of 1300 μm^2 [R. P. Bolender, *J. Cell Biol.* **61**, 269 (1974)] is the *trans* face, then this corresponds to 7×10^3 molecules per hour per square micrometer of Golgi membrane. The rate of uptake of mannosyl fucosyl glycoproteins per hour (2.2×10^6 per cell) for alveolar macrophages, of 900 μm^2 surface area (*22*) is 2.4×10^3 molecules per hour per square micrometer of plasma membrane. As judged by these admittedly imprecise calculations, a mechanism similar to that used for receptor-mediated endocytosis could be used for secretory protein sorting.
36. L. Orci *et al., Cell* **39**, 39 (1984).
37. R. D. Broadwell and C. Oliver, *J. Cell Biol.* **90**, 474 (1981); A. R. Hand and C. Oliver, *J. Histochem. Cytochem.* **32**, 403 (1984); L. Orci, *Diabetes* **31**, 538 (1982); ———, M. Ravazzola, A. Perrelet, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 6743 (1984); M. G. Farquhar, in *Subcellular Structure and Function in Endocrine Organs*, H. Heller and K. Lederis, Eds. (Cambridge Univ. Press, Cambridge, England, 1971), pp. 79–122.
38. B. D. Noe and M. W. Moran, *J. Cell Biol.* **99**, 418 (1984).
39. P. Rosa, G. Fumagalli, A. Zanini, W. B. Huttner, *J. Cell Biol.*, in press; D. V. Cohn *et al., Proc. Natl. Acad. Sci. U.S.A.* **79**, 6056 (1982); P. Rosa and A. Zanini, *Eur. J. Cell Biol.* **31**, 94 (1983).
40. F. Slaby and M. G. Farquhar, *Mol. Cell. Endocrinol.* **18**, 33 (1980); H. A. Reggio and G. E. Palade, *J. Cell Biol.* **77**, 288 (1978); W. L. Kiang, T. Krusius, J. Finne, R. U. Margolis, R. K. Margolis, *J. Biol. Chem.* **257**, 1651 (1982); S. S. Carlson and R. B. Kelly, *ibid.* **258**, 11082 (1983).
41. T. L. Burgess and R. B. Kelly, *J. Cell Biol.* **99**, 2223 (1984).
42. K. Docherty and D. F. Steiner, *Annu. Rev. Physiol.* **44**, 625 (1982); D. F. Steiner, K. Docherty, R. Carroll, *J. Cell Biochem.* **24**, 121 (1984).
43. A. Hasilik, R. Pohlmann, R. L. Olsen, K. von Figura, *EMBO J.* **3**, 2671 (1984).
44. V. Gieselman, A. Hasilik, K. von Figura, *J. Cell Biol.* **97**, 1 (1983).
45. R. E. Mains and B. A. Eipper, *ibid.* **89**, 21 (1981).
46. H. Gainer, Y. Sarne, M. J. Brownstein, *Science* **195**, 1354 (1977).
47. P. A. Halban, *J. Biol. Chem.* **257**, 13177 (1982).
48. D. Julius, R. Schekman, J. Thorner, *Cell* **36**, 309 (1984); D. Julius *et al., ibid.* **37**, 1075 (1984).
49. T. G. Warren and D. Shields, *ibid.* **39**, 547 (1984).
50. G. Warren, J. Davoust, A. Cockcroft, *EMBO J.* **3**, 2217 (1984); T. R. Hersketh, M. A. Beaven, J. Roger, B. Burke, G. B. Warren, *J. Cell Biol.* **98**, 2250 (1984); B. Burke, G. Griffiths, H. Reggio, D. Louvard, G. Warren, *EMBO J.* **1**, 1621 (1982); G. Warren, C. Featherstone, G. Griffiths, B. Burke, *J. Cell Biol.* **97**, 1623 (1983);

P. R. Sager, P. A. Brown, R. D. Berlin, *Cell* **39**, 275 (1984).
51. J. Trifaro and N. Kenigsberg, *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **42**, 961 (1983); R. A. Steinhardt and J. M. Alderton, *Nature (London)* **295**, 154 (1982).
52. J. E. Hooper and R. B. Kelly, *J. Biol. Chem.* **259**, 148 (1984); *ibid.*, p. 141; R. D. Burgoyne and M. J. Gelson, *FEBS Lett.* **131**, 127 (1981); S. Grinstein and W. Furaya, *ibid.* **140**, 49 (1982); S. F. Olsen, J. Slaninova, M. Trieman, T. Saemark, N. A. Thorn, *Acta Physiol. Scand.* **118**, 355 (1983); N. Moskowitz, W. Schook, K. Beckenstein, S. Puszkin, *Brain Res.* **263**, 243 (1983).
53. E. Vila-Porcile and L. Oliver, in *Synthesis and Release of Adenohypophyseal Hormones*, M. Jutisz and K. W. McKerns, Eds. (Plenum, New York, 1980), pp. 67–103; W. D. Matthews, L. Tsavaler, L. F. Reichardt, *J. Cell Biol.* **91**, 257 (1981); K. Kawai, I. Ipp, L. Orci, A. Perellet, R. H. Unger, *Science* **218**, 447 (1982).
54. S. P. Gilbert and R. D. Sloboda, *J. Cell Biol.* **99**, 445 (1984); S. T. Brady, R. J. Lasek, R. D. Allen, *Science* **218**, 1129 (1982); R. D. Vale, B. J. Schnapp, T. S. Reese, M. P. Sheetz, *Cell*, in press; T. Schroer, S. Brady, R. B. Kelly, *J. Cell Biol.* **101**, 568 (1985).
55. R. N. Melmed, C. Beniter, S. J. Holt, *J. Cell Sci.* **11**, 449 (1972).
56. R. B. Kelly *et al.*, *Cold Spring Harbor Symp. Quant. Biol.* **47**, 697 (1984); J. M. Trifaro and R. W. H. Lee, *Neuroscience* **5**, 1533 (1980).
57. F. Malaisse-Lagae, M. Amherdt, M. Ravazzola, A. Sener, J. C. Hutton, L. Orci, W. J. Malaisse, *J. Clin. Invest.* **63**, 1284 (1979); S. L. Howell, M. Tyhurst, *Cell Tissue Res.* **190**, 163 (1978); J. A. Williams, *Methods Cell Biol.* **23**, 247 (1981).
58. C. M. Redman, D. Banerjee, S. Yu, *Methods Cell Biol.* **23**, 231 (1981); G. Bennett, S. Parsons, E. Carlet, *Am. J. Anat.* **170**, 521 (1984).
59. G. Bennett, E. Carlet, G. Wild, S. Parsons, *Am. J. Anat.* **170**, 545 (1984); J. Blok, L. A. Ginsel, A. A. Mulder-Stapel, J. J. M. Onderwater, W. Th. Daems, *Cell Tissue Res.* **215**, 1 (1981); A. Quaroni, K. Kirsch, M. M. Weiser, *Biochem. J.* **182**, 213 (1979).
60. E. P. Reaven and G. M. Reaven, *J. Cell Biol.* **75**, 559 (1977).
61. A. A. Rogalski, J. E. Bergmann, S. J. Singer, *ibid.* **99**, 1101 (1984).
62. Although studies have been made on the effects of colchicine on bone marrow–derived secretory cells, the results cannot readily be compared to those on other regulated secretory cells. Since platelets and neutrophils and resting mast cells synthesize little, if any, of their secretory products, it is difficult to look at the fate of newly synthesized material. Secondly, in platelets and neutrophils, secretory vesicles do not fuse with plasma membrane at the periphery; they release their contents on stimulation into internal membranes. There is no need therefore for directed transport to the cell surface [J. G. White, in *Cell Biology of the Secretory Process*, M. Cantin, Ed. (Karger, Basel, 1984), pp. 546–569; J. E. Smolen, H. M. Korchak, G. Weissmann, in *ibid.*, pp. 517–545; T. W. Martin and D. Lagunoff, in *ibid.*, pp. 481–516].
63. M. G. Farquhar and G. E. Palade, *J. Cell Biol.* **91**, 77s (1981).
64. The author expresses his deepest gratitude to members of his laboratory in discussion with whom the ideas presented here have arisen, evolved and matured. The contributions and comments of Drs. Daniel Cutler, Barry Gumbiner, Suzanne Pfeffer, Ira Mellman, Stuart Kornfeld, Peter Walter, and Jack Rose are also gratefully acknowledged. And, once again, Leslie Spector outdid herself in patience and precision while putting together the many drafts of this article.

RESEARCH ARTICLE

Structure of the GDP Domain of EF-Tu and Location of the Amino Acids Homologous to *ras* Oncogene Proteins

Frances Jurnak

During protein biosynthesis in *Escherichia coli*, the elongation factor (EF-)Tu recognizes, transports, and positions the codon-specified aminoacyl–transfer RNA onto the A site of the ribosome (*1*). In this role, EF-Tu interacts with several cellular components, including guanosine diphosphate (GDP) and guanosine triphosphate (GTP), which act as allosteric effectors to control the protein conformation required during the elongation cycle.

In comparison to adenosine-binding proteins, there are relatively few proteins that bind to guanosine, with EF-Tu being the most widely characterized. It was, therefore, of considerable interest when a family of *ras* oncogene proteins, termed p21, were found to be GDP- and GTP-binding proteins (*2*). In subsequent comparisons of the amino acid sequence of EF-Tu with the human Harvey C-H *ras* 1 gene product (H-*ras*) as well as with other known GDP-binding proteins (*4, 5*), four principal regions of homology were identified if conserved amino acid substitutions are included. In addition, several invariant amino acids were identified in all GDP-binding proteins that have been sequenced (*4*). I now present my biochemical and preliminary high-resolution crystallographic results on a trypsin-modified form of EF-Tu-GDP. The locations of the amino acid sequence homology between EF-Tu and the H-*ras* p21 protein are described.

EF-Tu-GDP was isolated from *Escherichia coli* B cells and treated with TPCK-trypsin (TPCK, tosylamidophenylethyl chloromethyl ketone) (*6*). In all preparations, the protein was routinely passed over a G-100 Sephadex column after the trypsin digestion. A minor peak, trailing the major peak identified as trypsin-modified EF-Tu, was routinely detected by absorbance at 238 nm. In the original preparation, the minor peak was isolated and sequenced (*7*). The minor peak contained a 14-amino-acid peptide, now known to be Ala^{45} to Arg^{58} (*8*). Not only had the minor peak been detected in the trypsin-modified EF-Tu-GDP preparations used for the crystallographic study described below, but the sequences of the NH_2-terminal fragments were determined (*9*). Sequencing was carried out on a Beckman 890M with the use of a 0.1*M* Quadrol program and 2.0 mg of Polybrene as a carrier (*10*). The major NH_2-terminal fragment was identified as Gly-Ile-Thr-Ile-Asn and corresponds to residues 59 to 63 in the EF-Tu sequence. Less than 10 percent of the material contained a second NH_2-terminal fragment, Leu-Leu-Asp-Glu, which corresponds to residues 264 to 267. As was expected, the acetylated NH_2-terminus of the protein was not detected by this method. However, an NH_2-terminal fragment, previously shown to correspond to residues 1 to 44 was detected by a sodium dodecyl sulfate (SDS) gel electrophoretic analysis (*11*). The NH_2-terminus of the 14-amino-acid fragment, residues 45 to 58, produced by the trypsin digestion, was not detected by any method in the material that was used for crystallization. Therefore, the findings indicate that the trypsin-modified EF-Tu used in the x-ray diffraction analysis described below contained only two major fragments corresponding to residues 1 to 44 and 59 to 393. A small portion of the latter fragment also contained a cleavage site at Lys^{263}. Activity assays demonstrated that the trypsin-modified protein was fully active toward GDP and GTP exchange.

The trypsin-modified EF-Tu-GDP was crystallized by vapor diffusion techniques, with the parameters described previously (*6*). Two heavy atom derivatives were identified, the sites were located and refined by conventional methods, and the structure was solved at 5 Å resolution in the space group $P4_32_12$ as reported previously (*7*). As a result of a space group transformation promoted by contaminants in the polyethylene glycol

F. Jurnak is an assistant professor in the Department of Biochemistry, University of California, Riverside 92521.