CHAPTER 33

Regulated and Constitutive Secretion Studied In Vitro: Control by GTPases at Multiple Levels

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A. Introduction

All eukaryotic cells renew and expand their cell surface by a continuous supply of new plasma membrane components. These are provided by vesicles that bud from the Golgi complex and fuse with the plasma membrane. This process occurs continuously in the absence of any external signal and is thus named "constitutive secretion". In more differentiated cells, constitutive secretion alone is insufficient to meet other physiological needs. For example, in cells secreting chemical messengers as a means of communication, the special secretory products are often packaged in storage granules that undergo exocytosis only when the cells are appropriately stimulated by external signals. This process is termed "regulated secretion" (for reviews, see BURGESS and KELLY 1987; MOORE 1987; MILLER and MOORE 1990a). Many cells possess more than one type of constitutive or regulated secretory pathway. A well-known example is the polarized epithelium with two constitutive pathways for delivery of newly synthesized proteins to either the apical or the basolateral surface (for reviews, see SIMONS and FULLER 1985; RODRIGUEZ-BOULAN and NELSON 1989). The presence of multiple types of vesicles therefore offers the possibility of segregating secretion of different products both spatially and temporally.

In cells with more than one form of secretion, the pathways generally diverge at the level of the *trans* Golgi network (TGN; ORCI et al. 1987; TOOZE et al, 1987; TOOZE and HUTTNER 1990; SOSSIN et al. 1990), although sorting of constitutive proteins from the regulated secretory proteins may continue in immature secretory granules (CASTLE 1990; GRIMES and KELLY 1992). Proteins secreted via the constitutive and the regulated pathways traverse the same endoplasmic reticulum (ER) and Golgi compartments until they reach the TGN, where they are segregated into distinct sets of vesicles. It is now generally accepted that transfer between the early secretory compartments, and delivery from the TGN to the cell surface, is achieved by successive budding of transport vesicles carrying "cargos" and fusion of these vesicles with the next target compartment (reviewed in FARQUHAR 1985; ROTHMAN and ORCI 1990, 1992; MELLMAN and SIMONS 1992). At each step, a sorting event occurs in which cargo proteins are incorporated into transport vesicles whereas resident proteins are retained.

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Sorting at the TGN is considerably more complex, since it must also include sorting among different secretory vesicles. In general, the problem of vesicular transport can be divided into two parts: sorting and transport machinery.

Recent genetic and biochemical studies have greatly advanced our knowledge of the mechanisms for sorting and the components involved in vesicular transport. An important realization is that many of the underlying principles are similar from step to step, and several common components are repeatedly used throughout the secretory pathway. Perhaps the most surprising finding is that components that were once considered as modulators of regulated secretion, i.e., GTPases, are now known to control membrane traffic through the constitutive pathway. Despite these similarities, unique components must also operate in each step to maintain the membrane selectivity of fusion and to differentiate secretion spatially and temporally. In this chapter, we will focus on the transport steps between the TGN and the cell surface in mammalian cells. We shall emphasize in particular the similarities and differences between constitutive and regulated secretion.

B. The Regulated Secretory Pathway: A General Mechanism for the Control of Cell–Cell Communication and Plasma Membrane Activities

Most cells have the capacity to secrete via a constitutive pathway. Unicellular organisms usually secrete their protein products constitutively. The budding yeast *Saccharomyces cerevisiae*, for example, appears to secrete all proteins by a single pathway (NOVICK and SCHEKMAN 1983; SCHEKMAN 1985); the same set of mutations affect transport of plasma membrane proteins, invertase, and also the pheromone alpha factor which is proteolytically processed by the *KEX2* gene product at pairs of basic residues (FULLER et al. 1989). Maturation and secretion of hormones in higher cells, by contrast, often occur in the regulated secretory pathway, even though a set of processing enzymes similar to Kex2p are involved (SMEEKENS and STEINER 1990; SMEEKENS et al. 1991; THOMAS et al. 1991). The regulated secretory pathway in animal cells has long been recognized as a secondary pathway used exclusively for the discharge of specialized secretory products (TARTAKOFF et al. 1978) and is often associated only with "professional" secretory cells such as endocrine, exocrine, and neuronal cells. Recent studies, however, indicate that this type of secretory pathway is much more common than originally thought. The pathway appears to serve as a general mechanism for regulating cell–cell communication, plasma membrane transporter activities, and formation of specialized membrane domains.

Table 1 summarizes the types of molecules transported to the cell surface in a regulated fashion in different cells. In general, these pathways

Table 1. Examples of secretion regulated at the level of exocytosis in mammalian cells

Tissue type	Molecules transported and packaged
Regulated secretory pathway – biosynthetic type	
Posterior pituitary	Oxytocin, arginine vasopressin, neurophysins
Hypothalamus	Corticotropin-releasing hormone; thyrotropin-releasing hormone; growth-hormone-releasing hormone; somatostatin; luteinizing hormone-releasing hormone; prolactin-releasing hormone; prolactin-inhibiting hormone
Anterior pituitary	Growth hormone, prolactin; β-lipotropin; adrenocorticotropic hormone; thyroid-stimulating hormone; follicle-stimulating hormone; luteinizing hormone; endopeptidase PC1/3; carboxypeptidase; peptidylgycine alpha-amidating monooxygenase; secretogranins
Brain	Enkephalins; substance P; etc.
Thyroid	Thyroglobulin
Pancreas, endocrine	Insulin; glucagon; somatostatin; pancreatic polypeptide; carboxypeptidase
Adrenal medulla	Norepinephrine; epinephrine; secretogranins; dopamine β-hydroxlyase; phenylethanolamine-*N*-methyltransferase
Kidney, endocrine	Renin
Heart, atria	Atrial natriuretic factor
Respiratory mucosa	Mucin
Pancreas, exocrine	Trypsinogen; chymotrypsinogen; proelastase; procarboxypeptidase A; prophospholipase A; α-amylase; esterase; deoxyribonuclease; ribonuclease; lipase
Gastrointestinal system	Cholecystokinin; gastrin; secretin; enteroglucagon; vasoactive intestinal peptide; gastric inhibitory peptide; motilin; substance P; bombesin; somatostatin; neurotensin; neuropeptide Y; peptide YY
Salivary gland	α-amylase; mucin
Blood vessels, endothelia	Von Willebrand factor; CD62
Platelets	Clotting factors; serotonin; ADP; ATP
Neutrophil, granulocytes	Gelatinase; CD11; CD18; etc.
Kidney, epithelia	Apical membrane proteins
Mast cells	Histamine
Regulated secretory pathway – recycling type:	
Central and peripheral nerves	Acetylcholine; serotonin; glycine; gamma-aminobutyric acid; etc.
Stomach, parietal cells	H,K-ATPase
Adipose	Glucose transporters GLUT4, GLUT1
Kidney, collecting duct epithelia	Water channels
Kidney, distal renal tubules	Ca^{2+} channels or regulators
Lung alveoli	Surfactant

can be classified into two categories based on the origin of their secretory vesicles. The "biosynthetic" type of regulated secretory pathway utilizes vesicles that are made in the TGN, and these vesicles usually contain peptides synthesized on membrane-bound ribosomes. In the "recycling" type of regulated secretory pathway, newly synthesized vesicle membrane components are first transported to the cell surface via the constitutive pathway (CUTLER and CRAMER 1990; REGNIER-VIGOUROUX et al. 1991). Assembly of vesicles then occurs during endocytosis and recycling from the endosomes (LINSTEDT and KELLY 1991). Molecules transported by the biosynthetic, regulated pathway include classical peptide hormones and their processing enzymes (GUEST et al. 1991; MILGRAM et al. 1992) in endocrine glands, and digestive enzymes in exocrine glands. The recycling regulated pathway, on the other hand, is involved in release of non-peptide neurotransmitters at synapses and endocrine cells. Cells capable of regulated secretion are also found in a variety of tissues. For example, the gastrointestinal system contains cells that secrete a variety of peptides; epithelia contain cells that secrete mucin; the heart atria contain cells secreting atrial natriuretic factor; and platelets, mast cells, neutrophils, and endothelial cells are triggered to secrete a number of factors during defense mechanisms. Even cells such as T-helper lymphocytes are now suspected to have a regulated secretory pathway (TAPLITS and HODES 1989). Thus, regulated secretion is not restricted to a few organs, but occurs in a wide range of tissues.

The function of regulated secretion is not limited to secreting soluble products. There is increasing evidence suggesting that a major role of this pathway is to regulate plasma membrane ion transporter activities. Gastric HCl secretion is regulated by insertion and retrieval of H,K-ATPase stored in membrane vesicles (for review, see FORTE et al. 1989), and the water permeability of the kidney collecting duct epithelium is controlled by vasopressin-induced recycling of water channels between an intracellular compartment and the plasma membrane (LENCER et al. 1990). Likewise, activation of calcium channels in renal epithelium may also involve agonist-induced exocytosis (BACSKAI and FRIEDMAN 1992). Adipocytes respond to insulin by translocating the glucose transporters GLUT1 and GLUT4 to the cell surface in a similar manner (SLOT et al. 1991; SMITH et al. 1991). In addition, a novel form of regulated secretion has been described for the generation of the apical plasma membrane during differentiation of epithelial cells (VEGA-SALAS et al. 1987). In this case, structures carrying apical membrane proteins, known as the vacuolar apical compartment (VAC), undergo exocytosis and contribute to the rapid formation of the apical surface (RODRIGUEZ-BOULAN and NELSON 1989). It is possible that most, if not all, cells possess some types of storage vesicles for communication and/or regulation of their membrane activities.

C. Controlling Passage Through the Regulated Secretory Pathway – Distinctions Between Constitutive and Regulated Secretion

I. Exocytosis

The most obvious distinction between the constitutive pathway and the regulated pathway is control of exocytosis. Exocytosis from the regulated pathway is modulated by extracellular signals, whose exact nature depends on the particular physiological process involved (see Table 1). The intracellular responses, however, are often elicited by a similar set of intracellular mediators. Studies using permeabilized cells have shown that exocytosis from the regulated pathway differs from the constitutive pathway in two respects: calcium and GTP. Regulated secretion in most cases is stimulated by micromolar concentration of free calcium ions. By comparison, constitutive secretion must be operational at resting levels of free Ca^{2+} (50–200 n*M*) since it is ongoing even in the absence of external signals. Such differences in the Ca^{2+} sensitivity may arise in one of two ways. First, a unique Ca^{2+}-responsive component may be specifically sorted to regulated secretory granules, but not constitutive secretory vesicles; this would make the regulated granules uniquely sensitive to calcium. Alternatively, the same Ca^{2+}-responsive component may be present on both types of vesicles, but modified in such a way that they differ in their affinities for Ca^{2+} – constitutive vesicles requiring physiological Ca^{2+} and regulated granules requiring higher levels for optimal secretory activities. Using a semi-intact Chinese hamster ovary (CHO) cell system to reconstitute constitutive secretion, we found that transport from the *trans* Golgi to the plasma membrane does not require physiological Ca^{2+} (Miller and Moore 1991). Thus, constitutive and regulated secretion appear to differ fundamentally with regard to their requirements for calcium (see Fig. 1). Recent studies have shown that calcium-binding proteins of the annexin family, along with other factors (Morgan and Burgoyne 1992), may mediate the responses of calcium during regulated secretion (Ali et al. 1989; Blackwood and Ernst 1990; Sarafian et al. 1991), and also during endocytosis (Lin et al. 1992). The distinct requirements for calcium may be explained by the participation of these components in exocytosis of regulated, but not constitutive, vesicles.

Evidence for a second modulator of regulated secretion comes from studies using guanine nucleotides. Addition of nonhydrolyzable GTP analogs to semi-intact cells enhances regulated secretion from the biosynthetic pathway in a variety of cell types (reviewed in Gomperts 1990). These agents also induce regulated exocytosis from the recycling pathway, as has been shown for the translocation of GLUT4 to the plasma membrane

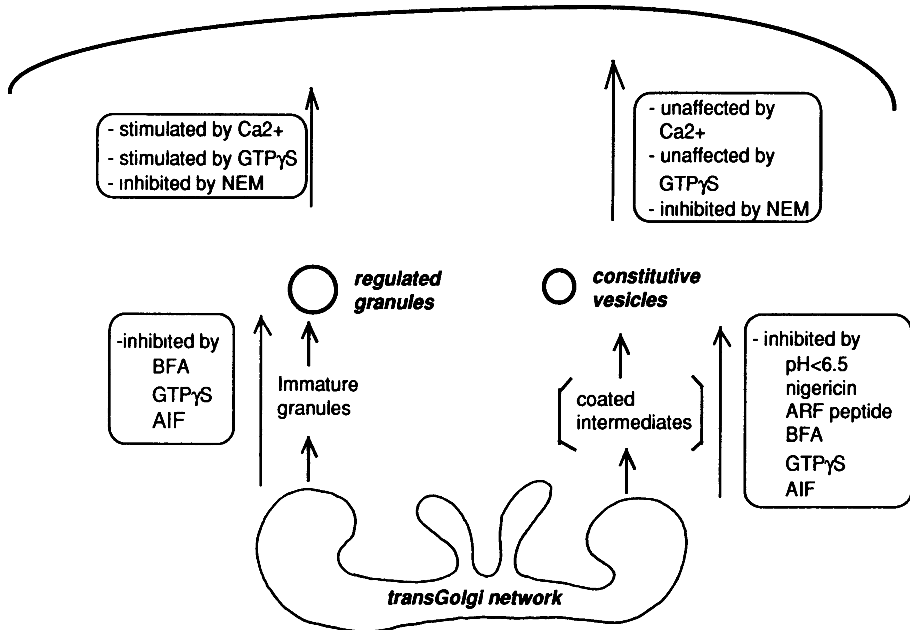

Fig. 1. Characteristics of transport between the *trans* Golgi network and the cell surface via *constitutive vesicles* and *regulated granules*. *BFA*, Brefeldin A; *ARF*, ADP-ribosylation factor; *NEM*, N-ethylmaleimide

in permeabilized adipocytes (BALDINI et al. 1991). These studies indicate that one or more GTPases may participate in late step(s) of regulated secretion. In contrast, GTPγS does not stimulate exocytosis of constitutive vesicles in semi-intact cells (see below), indicating that the exocytotic machinery for constitutive and regulated secretion must also differ in the GTPases involved.

As summarized in Table 1, many cell types possess a regulated secretory pathway. It remains to be determined whether all cells utilize a common set of calcium sensors and GTPases for regulated exocytosis, or each cell utilizes similar but distinct components.

II. Formation of Granules

Protein secretion by the regulated pathway is controlled at multiple points. In addition to granule exocytosis, the formation of secretory granules from the TGN is also likely to be regulated. Using sulfated glycosaminoglycan chains as a bulk-flow marker, we found that traffic out of the TGN via the constitutive and regulated pathways is differentially sensitive to the protein synthesis inhibitor cycloheximide. Constitutive secretion persists in the absence of new protein synthesis for several hours, whereas transport to the regulated secretory pathway is inhibited upon arrest of protein synthesis

(BRION et al. 1992). It is possible that the formation of storage granules is triggered by newly synthesized hormones and thus, in the absence of hormones in the Golgi complex, no new granules are formed. This would provide a simple way to regulate the number of secretory granules within the cell, i.e., by directly regulating the amount of hormone synthesized. If this model is indeed correct, then the question is how the hormones induce budding. The situation could be similar to budding of some enveloped viruses, in which binding of nucleocapsids to the membrane triggers budding of viral particles (SIMONS and FULLER 1987). Alternatively, binding of hormones to Golgi membrane could trigger a signal across the membrane to initiate vesicle coat assembly in a manner analogous to G-protein-mediated signal transduction across the plasma membrane. As will be discussed below, multiple GTPases are known to participate in intracellular transport steps. It will be of interest to determine if one of the trimeric G-proteins is involved in this signaling event.

III. Sorting of Contents

In addition to regulating formation of storage granules, cells also control the types of molecules entering this pathway. It is now generally accepted that sorting is accomplished by special structural features on proteins targeted to the regulated granules; proteins that are not actively segregated away are exported by a passive-flow process (for reviews, see MOORE et al. 1989; HUTTNER and TOOZE 1989; KELLY 1991). DNA transfection studies have shown that the sorting mechanism(s) in the different cell types mentioned in Table 1 appear to be quite similar; there are now numerous examples in which a given hormone can be recognized and targeted to the secretory granules of a different cell type upon transfection. Von Willebrand's factor provides an interesting case. This protein is normally targeted to a regulated secretory organelle, the Weibel-Palade bodies, in endothelial cells. When transfected into the pituitary cell line AtT-20, it induces the formation of a second regulated organelle resembling Weibel-Palade structures (WAGNER et al. 1991). This suggests that the assembly of regulated secretory granules may be triggered by the transported protein itself, as mentioned earlier. Is there, then, a single universal mechanism for sorting proteins into regulated secretory granules from the biosynthetic pathway? The answer probably is, no. Several cells are known to contain granules with distinct contents (FUMAGALLI et al. 1985; FISHER et al. 1988), and von Willebrand's factor is sorted into a different population of secretory granules from the endogenous hormone ACTH in transfected AtT-20 cells (WAGNER et al. 1991). These findings indicate that sorting of different molecules must be governed by different sets of biochemical interactions. This notion is supported by studies of the properties of the transported proteins in vitro. Several acidic proteins (including secretogranins, mast cell granule matrix, and sulfated proteoglycans) aggregate at low pH and/or high calcium in vitro, conditions

that mimic the Golgi milieu (GERDES et al. 1989; FERNANDEZ et al. 1991; H.-P.H. MOORE, unpublished). This may provide a mechanism for sorting and concentrating this class of proteins at the TGN. Hormones, however, do not precipitate under the same conditions (H.-P.H. MOORE, unpublished), and mutations affecting the oligomeric states of insulin also do not abolish its ability to be sorted into regulated granules (QUINN et al. 1991). A similar finding was obtained with a mutant *Tetrahymena* cell line in which a mucocyst content protein fails to condense but is still sorted into dense core granules (TURKEWITZ et al. 1991).These proteins therefore must employ different sorting mechanisms.

D. Regulation of Traffic Through the Constitutive Pathway

The above discussion points out the differences between the constitutive secretory pathway and the regulated secretory pathway. While membrane traffic through the regulated secretory pathway is controlled at the levels of granule formation, sorting, and exocytosis, traffic through the constitutive pathway is not subject to these same types of regulation. This, however, does not mean that constitutive secretion is not regulated at all. In fact, recent evidence suggests that constitutive secretion is not only regulated, but the factors involved appear to be similar to those regulating exocytosis from storage granules. Thus, the nomenclature should be clarified. Regulated and constitutive secretion should not be viewed as a regulated vs. a nonregulated process, but should be both considered as regulated processes. The distinction is that regulated secretion is controlled *additionally* at other levels by extracellular signals as outlined above.

What regulates constitutive secretion? The first clue that constitutive secretion may be regulated by GTPases came from studies of the yeast gene encoding Sec4p. Temperature-sensitive mutants of this gene accumulate post-Golgi secretory vesicles at the restrictive temperature (NOVICK et al. 1981). Sequence analysis showed that it bears striking sequence homology to a family of small GTPases, including the proto-oncogene product Ras (SALMINEN and NOVICK 1987). This was quite unexpected, for GTPases were known to be associated with signal-transducing receptors that are involved in regulated secretion. Further support for the involvement of GTP in exocytosis of constitutive vesicles came from in vitro studies using reconstituted systems. In the system we have developed, CHO cells were perforated with the bacterial toxin streptolysin-O (SL-O). Transport from the TGN to the cell surface is reconstituted by the addition of an adenosine triphosphate (ATP) regenerating system and cytosol and can be monitored by insertion of a viral glycoprotein into the plasma membrane or by secretion of a bulk-flow marker – sulfated glycosaminoglycan chains (MILLER and MOORE 1992). Addition of micromolar concentrations of nonhydrolyzable GTP analogs to this system effectively blocks secretion, indicating that

transport requires the hydrolysis of GTP. Nonhydrolyzable GTP analogs also block transport in earlier steps of the constitutive secretory pathway, indicating that this is a general phenomenon (MELANCON et al. 1987; BAKER et al. 1988; MAYORGA et al. 1988; RUOHOLA 1988; GRAVOTTA et al. 1990; MILLER and MOORE 1991).

Although constitutive secretion and regulated secretion are both regulated by GTP analogs, the effects are quite different. Whereas GTPγS effectively blocks constitutive secretion, it stimulates regulated exocytosis. There are two possible scenarios to explain this. One is that both effects are mediated by Sec4p-like small GTPases, but the two proteins involved have opposite states of activation by guanine nucleotides; for instance, exocytosis from the constitutive pathway may require the Sec4p-like protein in its GDP-bound state to allow exocytosis, whereas regulated exocytosis may require it to be in the GTP-bound state. An alternative hypothesis is that the different effects reflect regulation by distinct classes of GTPases. We prefer the latter hypothesis based on kinetic analysis of the GTPγS effects. Constitutive secretion is blocked only when GTPγS is added early in the transport reaction, during or soon after budding of vesicles from the TGN (see Fig. 1). Once the vesicles are formed, addition of GTPγS no longer exerts an inhibitory effect. This indicates that the exchange of GTP for GDP on Sec4p-like proteins must occur early during vesicle assembly, even though hydrolysis may take place later during vesicle fusion. If the Sec4p-like protein works in a similar fashion in regulated secretion, GTPγS should not affect Sec4p-like proteins when added to permeabilized systems measuring exocytosis of preformed granules. The stimulatory effects of GTPγS commonly observed in these systems are thus likely to be mediated by other types of GTPases such as G_e (see Chap. 42). This model also predicts that transport through the regulated secretory pathway should be inhibited by GTPγS if it is added during granule formation. Indeed, using a semi-intact system that reconstitutes transport via the regulated secretory pathway from the TGN to the cell surface, we found that GTPγS inhibits transport when added early, but stimulates secretion when added late after the transported molecules have entered mature granules. Thus, vesicular transport through the secretory pathway appears to involve more than one type of GTPase operating at several different levels.

E. GTPases and Intracellular Membrane Transport

During the last few years, several in vitro systems have been developed to examine the molecular components involved in Golgi-to-plasma-membrane transport (CURTIS and SIMONS 1988; TOOZE and HUTTNER 1990; SALAMERO et al. 1990; GRAVOTTA et al. 1990; MILLER and MOORE 1991). In this section, we shall summarize the findings from these systems. Some of the components discussed in this section are speculation based on analogy to other transport

steps, and their participation in Golgi-to-cell-surface transport needs to be confirmed experimentally.

As we have discussed earlier, GTPases play a major role in vesicular transport. For the sake of our discussion, we shall classify them into four groups: SAR1, ARF, rab, and heterotrimeric G-proteins. Not all of these have been shown to operate between the TGN and the cell surface, although it is likely that components similar to these are involved in ER-to-Golgi, intra-Golgi, and Golgi-to-surface transport.

I. SAR1

The SAR1 gene of *S. cerevisiae* was identified as a multicopy suppressor of the yeast ER-to-Golgi transport mutant *sec12* (NAKANO and MURAMATSU 1989). It shares sequence homology with ras and other small GTPases, with the strongest homology to adenosine diphosphate (ADP) ribosylation factors (ARFs). Sar1p is bound to the ER membrane via a transmembrane protein, Sec12p (D'ENFERT et al. 1991), which interacts with the products of a group of genes (such as SEC23, SEC13, SEC16) controlling the formation of ER-to-Golgi transport vesicles (KAISER and SCHEKMAN 1990). Thus it is likely to participate in an early step to initiate vesicle budding. Although there is as yet no evidence for a Sar1 homolog in mammalian cells, a mammalian Sec23 homolog does exist; antibodies directed against yeast Sec23p recognize an 85-kDa protein from a variety of mammalian tissues (ORCI et al. 1991). The mammalian Sec23p is very similar to the yeast protein. Using polymerase chain reaction (PCR) amplification, we have isolated a 110 base pair cDNA fragment from human hepG2 cells. The fragment shares 88% sequence identity with the yeast SEC23 gene and thus probably encodes a bona fide mammalian homolog. Like the yeast protein, the mammalian sec23p exists in both cytosolic and membrane-bound forms. The protein purified from rat liver cytosol is complexed with a 110-kDa protein similar to that found in yeast (HWANG et al. 1991; HICKE and SCHEKMAN 1989). Addition of this protein complex to an yeast ER-to-Golgi transport assay inhibits transport, indicating that the mammalian components can interact with the transport machinery in yeast cells.

How does Sec23p/Sar1p control vesicle budding? A clue to the solution comes from localization studies in mammalian cells. Immunoelectron microscopic analyses of rat pancreatic cells with affinity-purified antibodies against Sec23p showed that the immunoreactivity was confined to the transitional zone between the ER and the *cis* Golgi complex; little immunoreactivity was found elsewhere (ORCI et al. 1991). Moreover, when cells were treated with energy poison to deplete the formation of vesicles from this region, the immunoreactivity remained localized to the fibrillar aggregates in the ER transitional zone that were previously reported by PALADE and coworkers (MERISKO et al. 1986). This raises the possibility that the Sec23p complex is

normally anchored to this fibril network, and its function is to restrict vesicle budding spatially to transitional ER by activating Sar1p and subsequent coat assembly only in this region of the ER network. Consistent with this hypothesis, we found that 30% of the Sec23p in the particulate fraction is resistant to extraction by 1% octoxynol (Triton X-100). It will be of interest to analyze the molecular components of this cytoplasmic matrix.

At present, it is unknown whether budding of exocytic vesicles from the TGN is also controlled by similar complexes. Antibodies against Sec23p do not detect any immunoreactivity in the Golgi or the cytoplasm near TGN. This indicates that Sec23p participates specifically in ER-to-Golgi transport. This finding, of course, does not exclude the possibility that an immunologically distinct, Sec23p-like complex is involved in budding of exocytic vesicles. Future work will be necessary to determine if this is the case.

II. Trimeric G-proteins

Transport from the Golgi complex to the cell surface via both the constitutive pathway and the regulated pathway is inhibited by the addition of aluminum fluoride (see Fig. 1; L. Carnell and H.-P.H. Moore, unpublished; Barr et al. 1991). Since this agent is known to activate heterotrimeric G-proteins without affecting small GTPases (Kahn 1991), this result implies that trimeric G-proteins also control transport in some fashion. The heterotrimeric G-protein $G_{\alpha i3}$ is localized to the Golgi apparatus, and its overexpression in LLC-PK1 cells slows down secretion of a heparan sulfate proteoglycan (Stow et al. 1991). Pretreatment of cells with pertussis toxin reverses this effect. It remains to be established if this G-protein participates in TGN-to-cell-surface transport via the constitutive pathway. Whether the same G-protein regulates budding of regulated granules is also not known. Cells with regulated secretory function also contain other pertussis toxin-sensitive G-proteins, i.e., $G_{\alpha i1}$ and $G_{0\alpha}$ (reviewed in Kaziro et al. 1991). It will be important to determine which of these G-proteins is involved in constitutive and regulated transport from the TGN.

Although the determination of the exact role of trimeric G-proteins in TGN-to-surface transport requires further studies, it probably works by a similar mechanism as in intra-Golgi transport. Activation of trimeric G-proteins by GTPγS causes an increase in binding of coat proteins to the Golgi membrane, which is reversed by the addition of $\beta\gamma$ subunits of G-proteins (Donaldson et al. 1991) and inhibited by a peptide derived from ARF sequences (Donaldson et al. 1992). Likewise, aluminum fluoride causes an increase in the amount of ARF and coat proteins (see below) associated with coated vesicles generated during an in vitro Golgi transport reaction (Serafini et al. 1991). These studies are consistent with a model in which activation of a G-protein stimulates ARF and coat assembly, and persistent activation by AlF inhibits the uncoating step.

How does a trimeric G-protein activate coat assembly? Recent studies (Donaldson et al. 1992) showed that stimulation of coat binding to Golgi membrane by AlF requires GTP. Since activation of trimeric G-proteins by AlF does not require GTP, this result suggests that activation of a G-protein does not directly stimulate binding of coat proteins. Instead, the effect is probably indirectly mediated by other factors. For instance, a trimeric G-protein may activate other small GTPases such as ARF, which is essential for coat binding (Donaldson et al. 1992). It may also activate other cellular pathways required for ARF/coat binding. Recent studies (Zeuzem et al. 1992) have shown that the binding of ARF to Golgi membranes requires a low intravesicular pH that is established by a vacuolar H^+-ATPase. Thus, regulation of ion transport may be a possible downstream effect of activation of a trimeric G-protein. It is interesting to note that trimeric G-proteins have been implicated in the regulation of intravesicular acidity; acidification of endosomes by the vacuolar H^+-ATPase is stimulated by GTPγS, and the effect is blocked by pertussis toxin (Gurich et al. 1991). Activation of heterotrimeric G-proteins may therefore regulate the assembly of coat proteins via changes in acidification of intracellular organelles.

The above model is consistent with in vitro transport studies. Transport between the *trans* Golgi and the cell surface requires acidification of the lumen of the *trans* Golgi by a vacuolar-type H^+-ATPase (Miller and Moore 1990b; S. Miller and H.-P.H. Moore, manuscript in preparation); it is blocked by protonophores, inhibitors of the vacuolar proton pump, or removal of chloride ions which limits the activity of the electrogenic pump. Pulse-chase experiments using nigericin to abolish the pH gradient across the TGN have demonstrated that the block to secretion occurs kinetically early in the reaction. Secretion of [^{35}S]-labeled glycosaminoglycan chains rapidly becomes insensitive to the addition of nigericin. Interestingly, this nigericin-insensitive population is also insensitive to inhibition by GTPγS, suggesting that the GTPγS-sensitive step occurs at the same point as, or earlier than, the nigericin-sensitive step (see Fig. 1). Taken together, these results are consistent with the hypothesis that inhibition of the vacuolar H^+-ATPase or neutralization of the lumenal pH blocks the assembly of coat proteins onto Golgi membranes, and thereby blocks vesicular transport at a kinetically early step.

III. The ADP-Ribosylation Factor Family

ADP-ribosylation factors (ARF), initially identified as cofactors required for in vitro cholera toxin-catalyzed ADP-ribosylation of the α subunit of the trimeric GTPase $G_{s\alpha}$ (Kahn 1986), are members of a growing gene family related to the ras-like small GTPases (see Chaps. 34, 35). Several reports have shown that ARF is involved in intra-Golgi transport; it is present on coated vesicles generated in intra-Golgi transport assay (Serafini et al.

1991), and depletion of two ARF-related factors from cytosol abolishes GTPγS-dependent inhibition of intra-Golgi transport (Taylor et al. 1992). Preliminary studies from our laboratory indicate that ARF is also involved in *trans* Golgi-to-cell-surface transport by the constitutive pathway, since a peptide corresponding to the N-terminal domain of ARF1P inhibits secretion of sulfated glycosaminoglycan chains (L. Carnell, R. Kahn and H.-P.H. Moore, unpublished).

How does ARF control vesicular traffic? Recent studies using isolated Golgi membranes showed that ARF is required for binding of non-clathrin coatomer proteins to the membranes (Donaldson et al. 1992). While binding of ARF to the membrane is inhibited by Brefeldin A (BFA), subsequent binding of coat proteins to the membranes is not affected by BFA. This indicates that ARF participates in an early stage of coat assembly, and that BFA interferes with this step. Consistent with this model, we found that BFA blocks export from the TGN to the cell surface via the constitutive pathway; addition of BFA rapidly and reversibly inhibited cell surface transport of VSV G-protein that had been accumulated in the TGN by incubation of infected baby hamster kidney (BHK-21) cells at 20°C (Miller et al. 1992). The block to secretion was not due to collapse of TGN to the ER, since VSV G-protein blocked in treated cells resided in compartments that were distinct from the ER/Golgi system. In addition to transport of membrane proteins, constitutive secretion of a soluble marker – sulfated glycosaminoglycan chains – from the TGN is also blocked by BFA. A similar effect was observed for export via the regulated secretory pathway; BFA potently inhibited secretion of sulfated secretogranin-II induced by K^+-depolarization from PC-12 cells. Inhibition was at the level of granule formation, since BFA had no effect on regulated secretion from preformed granules. Thus, budding of constitutive vesicles and regulated granules from the TGN appears to be governed by a common mechanism for coat assembly that is sensitive to BFA.

Although budding of constitutive vesicles and regulated granules from the TGN are both inhibited by BFA, the exact coat proteins involved may not be identical. Indeed, BFA not only affects the assembly of the non-clathrin coatomer protein, β-COP, but also the clathrin-associated coat protein, γ-adpatin, from the Golgi (Wong and Brodsky 1992). The targets for BFA, however, appear to be different in these two cases, since in MDCK and PtK1 cells the assembly of γ-adaptin is sensitive to BFA even though the assembly of β-COP is BFA-resistant. Since β-COP is found on the same membrane as the vesicular stomatitis virus (VSV) G-protein blocked at 20°C, it is a likely candidate for the budding of constitutive vesicles from the TGN. Clathrin, on the other hand, is found on maturing regulated granule membranes (Orci et al. 1985; Tooze and Tooze 1986) and thus is a good candidate for the generation of regulated granules. Whether this is indeed the case requires future functional tests.

IV. The rab Family

The rab proteins have been implicated in the control of vesicle targeting. Vesicular transport requires accurate delivery of cargos to the correct target compartment. Unlike the budding and fusion events, components involved in vesicle targeting must be specific to the individual step to prevent inappropriate delivery of cargos to the wrong membranes. H. BOURNE proposed a model to explain the role of rab proteins in this process based on the function of the elongation factor, EF-Tu, in protein synthesis (BOURNE 1988). According to this model, the GTPases act as a molecular switch that couples the hydrolysis of GTP to membrane fusion. Only when the vesicles interact with the correct target membranes will hydrolysis and fusion occur. The role of these proteins is thus to ensure the specificity of vesicle targeting. The model thus predicts the presence of a family of these small GTPases, each mediating targeting of a specific set of transport vesicles. Data accumulated thus far are consistent with such a prediction. Molecular cloning of Sec4p-related proteins in mammalian cells, also known as the "rab" proteins, indicated that this class of proteins belongs to a large family – approximately 20 or so of these proteins have been cloned (ZAHRAOUI et al. 1989; ELFERINK et al. 1992; CHAVRIER et al. 1992; H. YU, D. LEAF and H.P. MOORE, manuscript in preparation), and many more remain to be discovered. Localization of some of these proteins indicates that they are specifically localized to different membranes – another prediction of the model.

In the budding yeast *S. cerevisiae*, consitutive secretion is mediated by the product of the *SEC4* gene (SALMINEN and NOVICK 1987; GOUD et al. 1988). Thus far, the only functional *SEC4* homolog that has been isolated is the *YPT2* gene from *Schizosaccharomyces pombe* (HAUBRUCK et al. 1990). Of the rabs known from mammalian and other sources, the two that share the closest homology with Sec4p and Ypt2p are rab8 and rab10 (CHAVRIER et al. 1990). Canine rab8 and rab10, also identified as the *ORA1* and *ORA2* genes from the electric fish *Omata* (NGSEE et al. 1991) and the MEL gene from human fibroblasts (NIMMO et al. 1991), show 48% and 49% amino acid sequence identity with the *S. cerevisiae* Sec4p, respectively. This degree of sequence homology is higher than for rab proteins involved in different steps of vesicular transport (for example, rat rab1 and rab2 are 35% identical). However, we found that neither rab8 nor rab10 could functionally complement a temperature-sensitive mutant of *sec4* (Y.T. CHEN and H.P. MOORE, unpublished). The lack of complementing activity means either rab8/10 performs a different function from Sec4p, or the post-Golgi pathways in mammalian cells are sufficiently different such that the rab protein or proteins involved in constitutive secretion are no longer conserved.

To analyze the intracellular location of rab8/10 and other members of this growing gene family, we have designed a mammalian expression vector

with an influenza hemagglutinin epitope tag engineered at its extreme N terminus (the C terminus was not used to avoid interference with isoprenylation). To test whether the tag interferes with proper protein folding and function, we epitope-tagged the yeast Sec4p and tested its ability to rescue a mutant temperature-sensitive allele of yeast *sec4* cells. Yeast cells transformed with this altered *SEC4* gene can still grow at a restrictive temperature, indicating that the modified protein is functional. Moreover, when expressed in mammalian cells the Sec4 protein was localized to the cell surface. Thus, its localization signal is recognized by the mammalian secretory machinery. In transfected cells, rab8 is found on the cell surface, with the highest concentration in the ruffling regions of the cell periphery (CHEN, HOLCOMB and MOORE, submitted). On the other hand, rab10 is localized to the Golgi region as judged by co-staining with the Golgi markers, NBD-ceramide and β-COP. The cellular location of rab8 is consistent with a possible role in a late step of the constitutive secretory pathway. The staining in membrane ruffles may indicate that these regions are particularly active in exocytotic activities. In this regard, it is interesting to note that new membrane insertion and recycling often occurs in a polarized fashion. For example, newly synthesized membrane proteins are preferentially inserted at the leading edge or margin of cultured cells (MARCUS 1962; BERGMAN et al. 1983). Recycling receptors such as transferrin receptor, LDL receptor, and fibronectin receptor are also inserted at the leading edge (BRETSCHER 1983, 1989). It will be of interest to determine if rab8 and/or rab10 indeed participate in constitutive transport between the *trans* Golgi and the cell surface in mammalian cells.

As discussed earlier, many cells contain pathways for regulated secretion, and they generally fall into two groups: the biosynthetic type and the recycling type. To date, only rab3A has been clearly localized to the synaptic vesicles in nerve terminals and neuroendocrine cells – a recycling type of regulated secretion (FISCHER VON MOLLARD et al. 1990). As shown in Table 1, the recycling type of regulated secretory vesicles is found in a wide variety of tissues.

rab3A, however, is restricted to a few tissues (i.e., neural and endocrine). This raises the possibility that regulated secretion in other tissues may be mediated by functionally similar, but distinct, rab proteins. Indeed, several rab proteins that are closely related to rab3A, i.e., rab3B, rab3C, and rab3D, have been identified; rab3D, for example, is enriched in adipocytes and may participate in the regulated insertion of the glucose transporter GLUT4 (BALDINI et al. 1992). Many other *rab* genes also have closely related isoforms (>85% identify). There are at least three rab5's (CHAVRIER et al. 1992), four rab3's (BALDINI et al. 1992), three rab1's (ZAHRAOUI et al. 1989; VIELH et al. 1988; H. YU, D. LEAF and H.-P.H. MOORE, unpublished), and two rab11's (R. Chavez and H.-P.H. MOORE, unpublished). Their functional significance is not yet clear.

Whether rab3 also participates in biosynthetic type of regulated secretion

is not as clear. A peptide derived from the effector domain stimulates amylase secretion from permeabilized pancreatic cells, suggesting that rab3-like proteins may be involved (Padfield et al. 1992). Since the same peptide also inhibits ER-to-Golgi transport (Plutner et al. 1990), it may interact with effectors of other rab proteins. The protein rab3A is predominantly localized to the recycling type of microvesicles in chromaffin cells (Fischer von Mollard et al. 1990), although some has been also found on chromaffin granules (Darchen et al. 1990). Multiple GTPases have been detected by GTP-blotting and other techniques on exocytotic granules from a variety of tissues (e.g., Rubins et al. 1992). It will be important to determine which of these GTPases functions in targeting of dense-core granules. We have cloned a number of rab proteins from the mouse anterior pituitary cell line, AtT-20 (Yu et al. 1993). Their possible role(s) in granule targeting and membrane recycling are being investigated.

The rab proteins are thought to ensure the specificity of vesicle targeting by interacting with specific cellular targets. A possible factor associated with the target compartment is the GTPase activating protein (GAP). At present, it is unclear whether each rab has its own GAP, or a given GAP can act on more than one rab protein. We have begun to address this question by isolating the GAP involved in constitutive secretion. The effector domain of yeast Sec4p has the sequence FITTIGIDF (residues 32–40 on Ras). This region of the Ras protein is known to interact with the GAP protein. Several mammalian rabs have effector domains very similar to this sequence; rab8 and rab10 have the sequence FISTIGIDF, and *RAM* – a gene isolated from rat megakaryocytes – has a sequence encoding FITTVGIDF in this region (Nagata et al. 1990). It is therefore possible that a mammalian GAP involved in exocytosis may interact with the yeast Sec4p. Using Sec4 protein purified from baculovirus-infected Sf9 cells, we have detected a GAP activity from crude rat liver extracts. Preliminary results show that the activity purifies as a 150-kDa and a 400-kDa complex. Interestingly, the GAP for rab3A also purifies as a large complex. It will be important to determine if rab proteins involved in constitutive and regulated secretion share the same GAP protein.

F. Conclusions

In summary, recent progress has uncovered a very large number of components involved in vesicular transport. Many of these turn out to be GTPases and their modulators. Collectively, they serve to control vesicular transport between cellular compartments. Figure 2 proposes a possible model in which sequential interactions of GTPases result in coat assembly, vesicle budding, uncoating, and targeting/fusion. It should be kept in mind that at the present time, many aspects of the model are hypothetical.

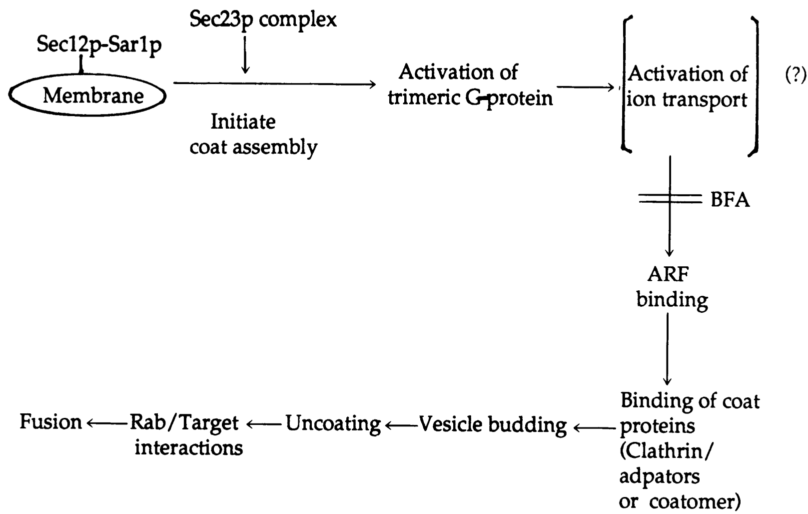

Fig. 2. A hypothetical model for the control of vesicular transport by GTPases. *ARF*, ADR-ribosylation factor; *BFA*, Brefeldin A

However, the model provides a useful conceptual framework for future experimentation. Several important questions remain. Does budding of exocytic vesicles from the TGN involve Sar1-like GTPases? If so, are they regulated by Sec23p-like proteins? Are coatomer and clathrin involved in the generation of constitutive and regulated granules from TGN, respectively, or are other coat proteins required? Do ARF proteins participate in the formation of regulated granules as well as constitutive vesicles, and if so, does each pathway utilize a different member of this family? What is the identity and downstream effector(s) of trimeric G-protein's regulatory vesicle traffic, and are they the same or different for constitutive and regulated vesicles? Finally, how is the rab protein in regulated secretion controlled differently such that fusion does not occur immediately upon correct targeting of vesicles to the membrane? Hopefully, answers to these questions will be forthcoming in the near future.

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