Spatial Segregation of the Regulated and Constitutive Secretory Pathways

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Abstract. Recent experiments using DNA transfection have shown that secretory proteins in AtT-20 cells are sorted into two biochemically distinct secretory pathways. These two pathways differ in the temporal regulation of exocytosis. Proteins secreted by the regulated pathway are stored in dense-core granules until release is stimulated by secretagogues. In contrast, proteins secreted by the constitutive pathway are exported continuously, without storage. It is not known whether there are mechanisms to segregate regulated and constitutive secretory vesicles spatially. In this study, we examined the site of insertion of constitutive vesicles and compared it with that of regulated secretory granules. Regulated granules accumulate at tips of processes in these cells. To determine whether constitutively externalized membrane proteins are inserted into plasma membrane at the cell body or at process tips, AtT-20 cells were infected with ts-O45, a temperature-sensitive mutant of vesicular stomatitis virus in which transport of the surface glycoprotein G is conditionally blocked in the ER. After switching to the permissive temperature, insertion of G protein was detected at the cell body, not at process tips. Targeting of constitutive and regulated secretory vesicles to distinct areas of the plasma membrane appears to be mediated by microtubules. We found that while disruption of microtubules by colchicine had no effect on constitutive secretion, it completely blocked the accumulation of regulated granules at special release sites. Colchicine also affected the proper packaging of regulated secretory proteins. We conclude that regulated and constitutive secretory vesicles are targeted to different areas of the plasma membrane, most probably by differential interactions with microtubules. These results imply that regulated secretory granules may have unique membrane receptors for selective attachment to microtubules.

Some eucaryotic cells have the capacity to target proteins to specific areas of the plasma membrane. This spatial segregation of proteins can lead to important functional specialization. For example, the polar organization of epithelial cells allows transport, absorptive, and secretory functions to be carried out vectorially (39). In these cells, newly synthesized membrane proteins are segregated from one another in the Golgi complex and are targeted to the apical or basolateral domains of the plasma membrane (23, 39). Cell types other than epithelial cells can also localize distinct sets of membrane proteins to separate cell surface domains. However, the mechanisms by which this spatial segregation is achieved are much less well-understood. Influenza virus and vesicular stomatitis virus (VSV) bud asymmetrically from the surface of cultured pancreatic islet cells, providing evidence for polarization of plasma membrane domains in endocrine cells (19). Electrophysiological, ultrastructural, and immunocytochemical studies (cf. references 17, 30–32, 36) have revealed regional differences in structure and function between the cell body and axons of neurons. Small and Pfenninger (41) observed differences in membrane composition between the cell body and axons of growing neurons using freeze-fracture techniques. Na^+ channels were found to be restricted to the nodal zone of the axolemma (9), and membrane-bound acetylcholinesterase was found to be confined to the fibers but not cell bodies of cultured sympathetic neurons (37). It is not known how the diversity in cell surface composition is established. It could arise from the selective delivery of proteins in distinct transport vesicles to specific regions of the plasma membrane (5, 12). Alternatively, proteins might be inserted into the plasma membrane randomly and subsequently become segregated by retention of the proteins at specific domains of the plasma membrane by elements of the cytoskeleton or extracellular matrix.

The AtT-20 mouse pituitary tumor cell line provides an excellent model for studying mechanisms of spatial segregation of proteins. This neuroendocrine cell line sorts proteins into regulated and constitutive secretory vesicles (for reviews, see references 5, 13, 26). It is not known if these different types of vesicles are targeted to different regions of the plasma membrane. In AtT-20 cells, dense-core secretory granules containing the endogenous regulated protein hormone, ACTH, accumulate at tips of processes extending from the cell (4, 6, 14, 24, 44). Microtubules have been implicated in the transport of regulated granules to process tips (24, 44).

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The site of exocytosis of constitutively secreted proteins and the role of microtubules in directing constitutive vesicles to the cell surface in AtT-20 cells remain uncertain.

In this study we asked two questions concerning the mechanism of transport and site of externalization of constitutive and regulated secretory proteins. (*a*) Does the site of secretion/membrane insertion of constitutively externalized membrane proteins differ from that of regulated secretory proteins? (*b*) Do microtubules play different roles in constitutive and regulated secretion? We found that a constitutively externalized membrane protein is first inserted into the plasma membrane of the cell body, not at the process tips where regulated granules accumulate. In addition, the constitutive and regulated secretory pathways show differential sensitivity to microtubule disruption. The results indicate that constitutive and regulated secretory vesicles have distinct mechanisms for targeting to their release sites.

Materials and Methods

Materials

Poly-D-lysine HBr (M_r = 130,000), 8-bromo-adenosine 3′-5′ cyclic monophosphate, and 1,4-diazobicyclo (2.2.2) octane (DABCO) were obtained from Sigma Chemical Co. (St. Louis, MO). [^{35}S]methionine, [^{35}S]cysteine, and the mouse monoclonal anti-β-tubulin antibody were from Amersham Corp. (Arlington Heights, IL). Glutaraldehyde and paraformaldehyde were from Polysciences, Inc. (Warrington, PA). Rhodamine- and fluorescein-conjugated antibodies were from Organ on Teknika-Cappel (Malvern, PA). Laminin was obtained from Bethesda Research Laboratories (Gaithersburg, MD).

Cell Culture and DNA Transfection

AtT-20 cells were cultured in DME-H21/10% FCS at 37°C in a humidified 15% CO_2 incubator. Stable clones expressing VSV G protein (DRG-7), human growth hormone (GH-9), or rat insulin (Ins-6B) were obtained by transfecting AtT-20 cells with an RSV-expression vector containing cDNA encoding VSV G (35), human growth hormone (22), or genomic DNA encoding rat insulin (20).

Cells were grown on substrates (plastic culture dishes or glass coverslips) that had been coated with poly-D-lysine (1 mg/ml in 0.1 M Na borate, pH 8.4) for 8–16 h at room temperature. Unless otherwise noted, polylysine-coated dishes or coverslips were subsequently coated with 10 μg/ml laminin (24) in water (coverslips) or Ca^{++} Mg^{++}-free PBS (dishes) for 16 h at 4°C to stimulate rapid process outgrowth. Cells were routinely grown for 48 h before experiments were begun. Extensive process outgrowth was evident several hours after plating on the lysine/laminin-coated dishes or coverslips.

Infection of AtT-20 Cells with ts-O45

Cells grown on lysine/laminin-coated coverslips were rinsed with DME-H21 and incubated with ts-O45 VSV (6.6×10^7 pfu/ml of DME-H21/18 mM Hepes, pH 7.4/9%, tryptose phosphate broth) for 40 min at 37°C. Infection medium containing virus was removed and the cells were rinsed with DME-H21/2% FCS/25 mM Hepes, pH 7.4, and incubated in a 40°C (nonpermissive temperature) water bath for 3 h, then transferred to a 32°C (permissive temperature) water bath to allow synchronous movement of VSV G protein out of the ER. Coverslips were maintained for various times at the permissive temperature before fixation and processing for immunofluorescence.

Immunofluorescence

Cells were examined by indirect immunofluorescence using modifications of the procedure of Rogalski et al. (34). Cells grown on lysine/laminin-coated coverslips were fixed in 3.7% paraformaldehyde in PBS for 30 min and (unless otherwise noted) were permeabilized for 5 min in PBS/0.1% Triton X-100. Antibody incubations were for 30 min, followed by three 10-min rinses with PBS/0.1 M glycine. VSV G protein was detected with a mouse monoclonal antibody (reference 16; antibody I1, 1:100 dilution of ascites fluid) kindly provided to us by Dr. Leo Lefrancois (Scripps Clinic and Research Foundation, La Jolla, CA). Insulin was detected with a mouse monoclonal antibody (1:100 dilution of ascites fluid) directed against amino acids 8–10 of the insulin A-chain (hybridoma HB 125; American Type Culture Collection, Rockville, MD). After incubations with mouse primary antibodies, the coverslips were successively incubated with affinity-purified fluoresceinated goat anti-mouse antiserum (1:75 dilution; Cappel Laboratories, Malvern, PA) and fluoresceinated rabbit anti-goat antiserum (1:50 dilution; Cappel Laboratories). Human growth hormone was detected with goat anti-hGH serum (Antibodies, Inc., Davis, CA; 1:100 dilution) followed by incubation with fluoresceinated rabbit anti-goat antiserum (1:100 dilution; Cappel Laboratories). ACTH was detected using affinity-purified rabbit anti-porcine ACTH serum (generated by the method described in reference 21; 1:30 dilution) followed by incubation with affinity-purified fluoresceinated goat anti-rabbit serum (1:20 dilution; Cappel Laboratories).

Microtubule staining patterns in control and colchicine-treated cells were examined using a modification of the protocol of Schulze and Kirschner (38). Cells grown on lysine/laminin-coated coverslips were rinsed in PBS, then permeabilized for 5 min in 80 mM Pipes (pH 7.0)/5 mM EGTA/1 mM $MgCl_2$/0.5% NP-40/20% glycerol. Cells were fixed for 10 min in 80 mM Pipes (pH 7.0)/5 mM EGTA/1 mM $MgCl_2$/0.5% glutaraldehyde. Glutaraldehyde autofluorescence was quenched by a 7-min incubation with sodium borohydride (1 mg/ml in PBS). The cells were then rinsed in antibody buffer (PBS with 1% Triton X-100) and incubated for 60 min with a 1:500 dilution of a mouse monoclonal antibody directed against β-tubulin (Amersham Corp.) followed by successive 30-min incubations with affinity-purified fluoresceinated goat anti-mouse antiserum (1:100 dilution; Cappel Laboratories) and fluoresceinated rabbit anti-goat antiserum (1:100 dilution; Cappel Laboratories). Each antibody incubation was followed by extensive washes in antibody buffer (30 min, five buffer changes). Coverslips were mounted on glass slides in 90% glycerol in PBS containing 2.5% DABCO and were sealed with nail polish. Microtubules were completely depolymerized by a 2-h treatment with 10 μM colchicine.

Visualization of the Golgi Complex Using Wheat Germ Agglutinin

The Golgi complex was visualized with rhodamine-labeled wheat germ agglutinin using a modification of the procedure of Lipsky and Pagano (18). Cells were rinsed with PBS, fixed for 30 min with 3.7% paraformaldehyde in PBS, rinsed again in PBS, and incubated for 15 min in a permeabilization buffer containing 1% BSA, 0.1% Triton X-100, 1 mM $MnCl_2$, 1 mM $MgCl_2$, and 1 mM $CaCl_2$ in PBS. Rhodamine-labeled wheat germ agglutinin (E.Y. Laboratories, San Mateo, CA) was diluted to 50 μg/ml in the permeabilization buffer and centrifuged for 15 min at 10,000 *g* to remove aggregates. The supernatant was incubated with the permeabilized cells for 30 min at room temperature. The cells were rinsed for 15 min in permeabilization buffer, and then mounted on slides and processed as described above.

Metabolic Labeling and Immunoprecipitation

Cells grown on lysine/laminin-coated dishes were pretreated with 10 μM colchicine in DME-H21/2% FCS/25 mM Hepes, pH 7.4, for 2.0 h to completely disrupt microtubules before pulse-labeling. After 1.5 h of pretreatment, the medium was gently aspirated and the cells were washed with methionine-free (for labeling of TG) or cysteine-free (for labeling of insulin) DME-H21. During the last 30 min of pretreatment the cells were incubated in DME-H21/25 mM Hepes lacking either methionine or cysteine. The cells were then labeled with 0.12 mCi [^{35}S]methionine (labeling of TG) or [^{35}S]cysteine (labeling of insulin) for 30 min in methionine or cysteine-free DME-H21/25 mM Hepes. The cells were washed and chased in complete medium (DME-H21/2% FCS/25 mM Hepes). Colchicine (10 μM) was maintained in the labeling and chase medium to prevent microtubule repolymerization.

Medium and cell extracts were harvested, subjected to double immunoprecipitation, and analyzed on SDS polyacrylamide gels as described (28). Insulin was immunoprecipitated with a guinea pig anti-porcine insulin serum (Linco Research, Inc., Eureka, MO). Truncated G protein was immunoprecipitated with a rabbit serum directed against VSV which was generated according to the method of Fan and Sefton (10). For quantification of secretion, autoradiograms were scanned and the area under the peaks was determined. ACTH RIA and solid phase insulin RIA were conducted as described previously (29). ^{125}I-ACTH and ^{125}I-insulin for radioimmunoassay were generously supplied by the Metabolic Research Unit, University of California, San Francisco.

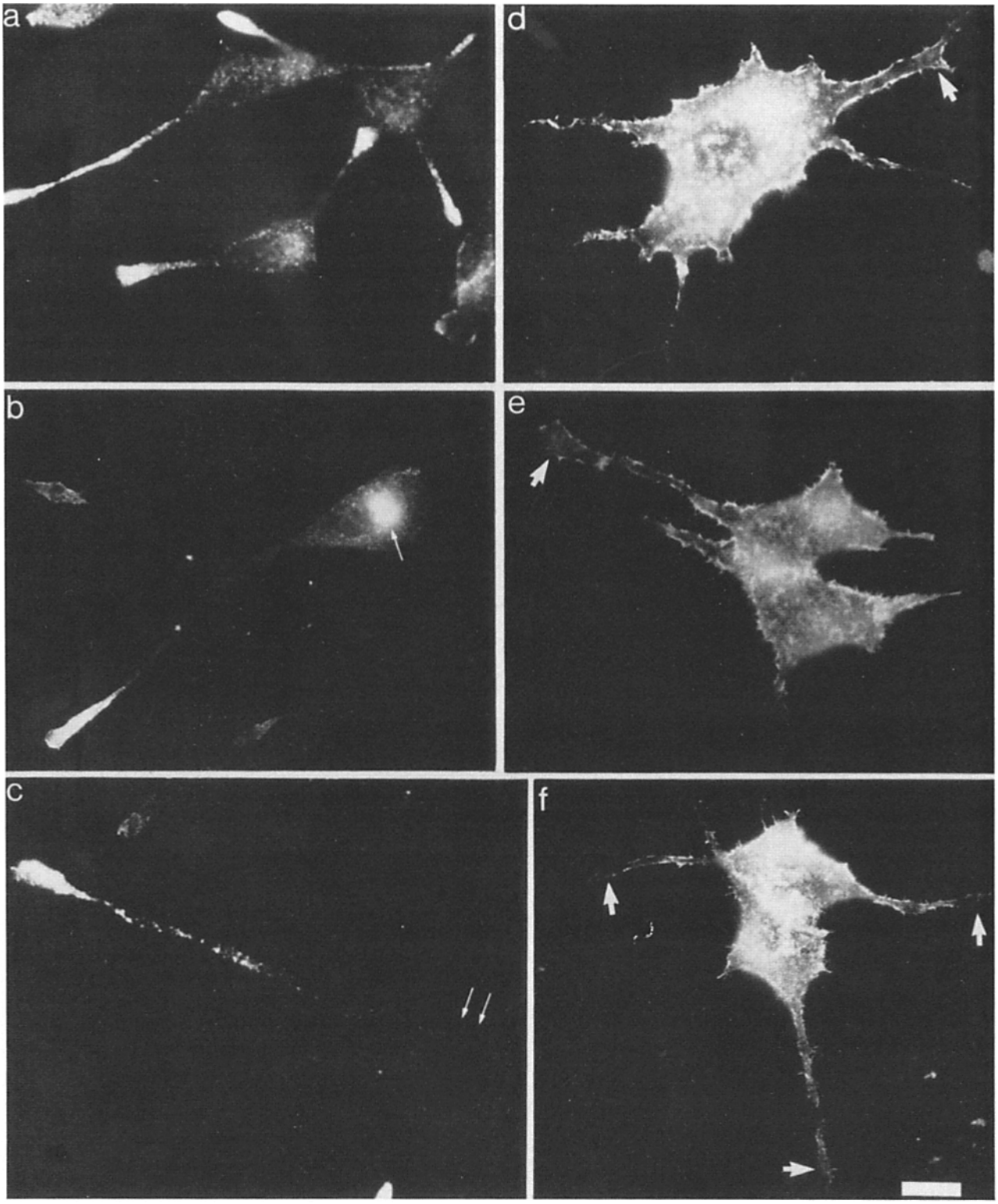


Figure 1. Indirect immunofluorescence localization of VSV G protein and regulated proteins in AtT-20 cells. (*a*) Insulin-secreting AtT-20/Ins 6B cells labeled with anti-insulin antibody. (*b*) Growth hormone-secreting AtT-20/GH9 cells labeled with anti-growth hormone antibody. The Golgi region is shown by the arrow. (*c*) AtT-20/DRG7 cells, which stably express VSV G protein, labeled with anti-ACTH antibody. The area of the cell body is shown by the arrows. In *a–c*, note accumulation of protein hormones at process tips. Golgi staining was more prominent in cells stained for growth hormone than in cells stained for ACTH or insulin; this is probably due to a greater affinity of the anti-ACTH and insulin antibodies for mature forms of these hormones found in secretory granules than for precursor forms in the Golgi. (*d–f*) AtT-20/DRG7 cells labeled with antibody against VSV G protein. G protein is evenly distributed in the plasma membrane of both cell body and processes; G protein does not accumulate at process tips (*arrowheads*). All secondary and tertiary antibodies in *a–f* were fluorescein conjugated. Bar, 20 μm.

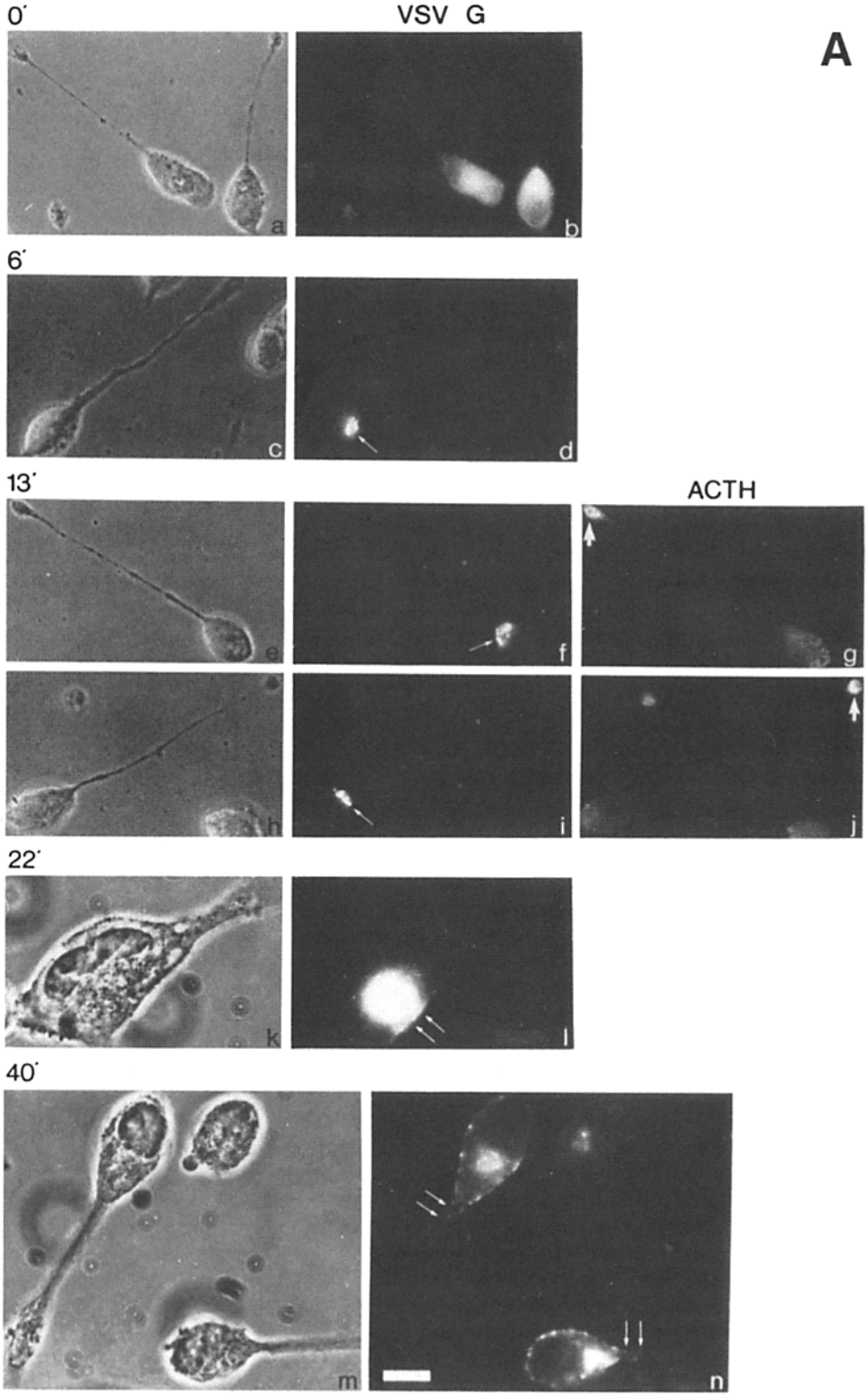
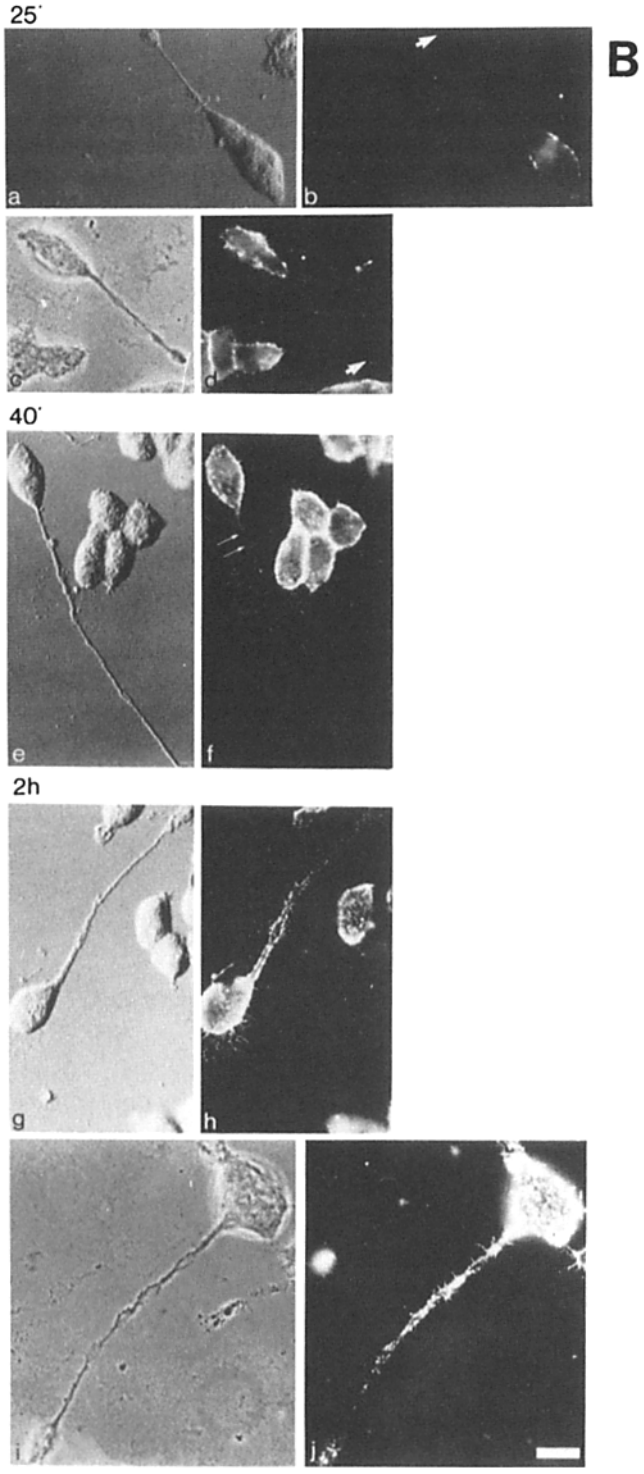


Figure 2. Transport of VSV G protein from ER to Golgi region to cell surface. Cells were infected with ts-045, incubated for 3 h at the nonpermissive temperature, shifted to the permissive temperature, and fixed after incubation for various times as indicated. VSV G proteins were localized with a mouse anti-G protein antibody followed by fluorescein-conjugated antibodies. Cells in *A*, *e–g* and *h–j* were two cells that have been double labeled for G protein (*A*, *f* and *i*) and ACTH (*A*, *g* and *j*). Phase-contrast or Nomarski images are shown immediately to the left of the corresponding fluorescence image. (*A*) Surface and intracellular staining of cells permeabilized with Triton X-100. (*a* and *b*) At 0 min before the temperature-shift; note diffuse G protein staining in the ER. (*c* and *d*) At 6 min after the temperature-shift; G protein staining concentrated in the Golgi region (*arrow*). (*e–j*) At 13 min; G protein staining remains concentrated in the Golgi region (*arrows in f* and *i*). ACTH accumulation at process tips is shown in the same cells (*arrowheads in g* and *j*). (*k* and *l*) At 22 min; insertion of G proteins into the plasma membrane close to the Golgi region (*arrows*). (*m* and *n*) At 40 min; G protein present in the plasma membrane of both the cell body and proximal shaft of the processes (*arrows*). (*B*) Surface staining of nonpermeabilized cells. (*a–d*) At 25 min; insertion of G protein into the plasma membrane of the cell body but not process tips. Process tips are shown by arrowheads. (*e* and *f*) At 40 min; G proteins present in the membrane of cell body and proximal shaft of processes (*arrows*). (*g–j*) At 2 h; G proteins present in the plasma membrane of both cell body and processes. Bars: (*A*) 15 μm; (*B*) 20 μm.

Results

VSV G Protein Stably Expressed in AtT-20 Cells Does Not Accumulate at Process Tips

Regulated secretory granules accumulate at process tips in AtT-20 cells. To determine if constitutively secreted proteins are also targeted predominantly to process tips or to different regions of the plasma membrane, we studied the constitutive externalization of the membrane glycoprotein G of VSV. VSV G protein has been used extensively in studies of assembly, intracellular transport, and site of plasma membrane insertion in constitutive secretory cells (cf. references 1, 2, 8, 40, 46). When expressed in AtT-20 cells by DNA transfection, G protein was found to exit the cell via the constitutive



secretory pathway; regulated granules isolated from transfected cells did not contain detectable amounts of G protein (Chung, K.-N., and H.-P Moore, unpublished observation).

To promote rapid and extensive process outgrowth, AtT-20 cells were routinely grown on polylysine/laminin-coated substrates. We examined the steady-state distribution of G protein by indirect immunofluorescence in an AtT-20 cell line stably transformed with VSV G protein, AtT-20/DRG7. G protein was found to be evenly distributed on the plasma membrane of both cell body and processes (Fig. 1, *d–f*). Although bright staining was observed in the juxtanuclear Golgi region, no intracellular accumulation of G protein was observed at process tips (Fig. 1, *d–f*). In contrast, the endogenous regulated hormone, ACTH, did accumulate at process tips in AtT-20/DRG7 cells (Fig. 1 *c*). Two exogenous regulated protein hormones, insulin (Fig. 1 *a*) and growth hormone (Fig. 1 *b*), were also found to accumulate at process tips in transfected AtT-20 cell lines expressing these proteins. Thus, in contrast to regulated proteins, G protein does not accumulate intracellularly at process tips.

Newly Synthesized VSV G Protein Is Inserted Predominantly Into the Plasma Membrane of the Cell Body, Not at Process Tips

The uniform cell surface distribution of VSV G protein at steady-state could arise from random insertion of constitutive vesicles into the plasma membrane. Alternatively, it could result from localized insertion of constitutive vesicles into the cell body or the process tips followed by diffusion within the plane of the membrane. Since vesicles carrying G protein do not accumulate to a large extent intracellularly, the site of membrane insertion cannot be easily determined in cells stably expressing G protein. To determine the site of insertion of newly synthesized G protein, AtT-20 cells were infected with the temperature-sensitive mutant VSV, ts-O45. In cells infected with this virus, G protein transport is conditionally blocked in the ER at the nonpermissive temperature (40°C); after switching to the permissive temperature (32°C), VSV G protein rapidly leaves the ER and is synchronously transported to the Golgi and then to the cell surface (cf. references 1, 3, 15, 40, 46). We used this virus to determine by indirect immunofluorescence the site where newly synthesized G protein is first inserted into the plasma membrane.

After a 3-h incubation of ts-O45–infected cells at the nonpermissive temperature, diffuse cytoplasmic staining was evident (Fig. 2 *A*, *a* and *b*), indicating that viral G protein was blocked in the ER. Within 6 min after the temperature shift to the permissive temperature, G protein was strikingly concentrated in the Golgi region (Fig. 2 *A*, *c* and *d*), which showed a reticular pattern of staining. A similar concentration of G protein was seen in the Golgi region at 13 min; as of 6 min, no cytoplasmic or plasma membrane staining was evident (Fig. 2 *A*, *e* and *f*, *h* and *i*). The location of ACTH at process tips in double-stained cells is included for comparison (Fig. 2 *A*, *g* and *j*).

VSV G protein insertion was first detected at 22 min after the temperature-shift at the plasma membrane of the cell body (Fig. 2 *A*, *k* and *l*). At 40 min, G protein was present in detectable amounts in the plasma membrane of the cell body only, not along the membrane of the processes (Fig. 2 *A*, *m* and *n*). To investigate the surface distribution of G proteins more clearly, we examined the staining patterns of G proteins in nonpermeabilized cells (Fig. 2 *B*). G proteins were restricted to the plasma membrane of the cell body at 25 min after temperature shift (Fig. 2 *B*, *a* and *b*, *c* and *d*). At 40 min, G protein was also detectable along the proximal membrane of the processes, but was either not detectable or was present in small amounts along the distal membrane of the process tips (Fig. 2 *B*, *e* and *f*). Only at later times (1 and 2 h) was G protein clearly detectable along the entire distal process membrane and at the tips of the process in most cells (Fig. 2 *B*, *g* and *h*, *i* and *j*).

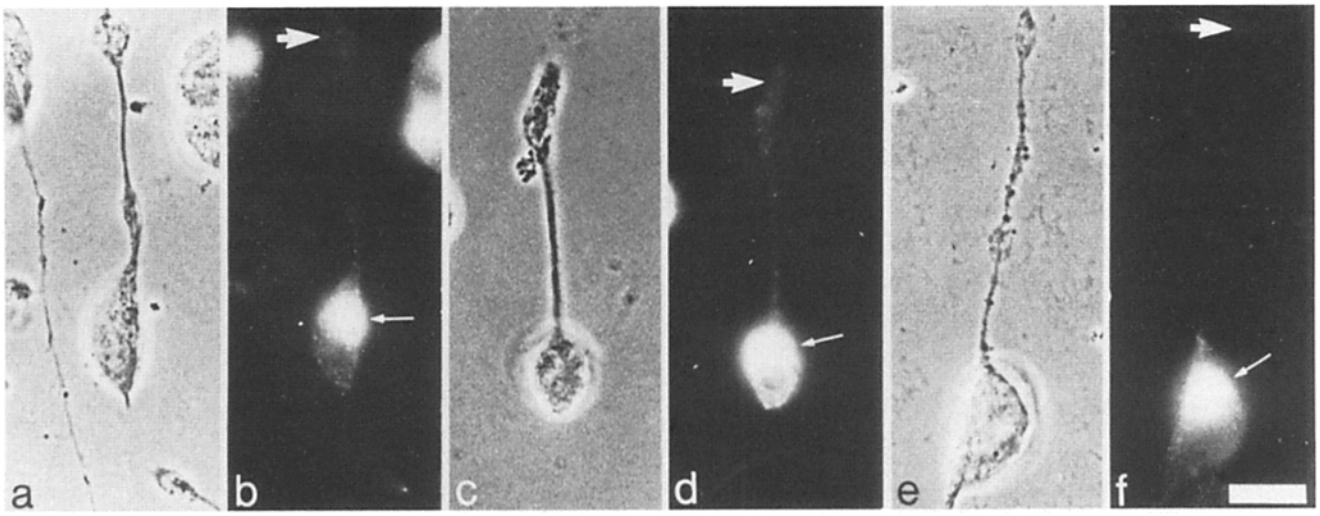


Figure 3. Intracellular localization of VSV G protein in ts-045–infected cells. After incubation at the nonpermissive temperature, cells were incubated at the permissive temperature for varying times before fixation. Fixed cells were preincubated with rabbit anti-VSV G protein antiserum to block cell surface G protein staining, then permeabilized and labeled with mouse anti-G protein antibody followed by fluoresceinated secondary and tertiary antibodies to localize intracellular G protein. Note bright staining (*small arrows*) in area of Golgi at 25 min (*b*), 40 min (*d*), and 2 h (*f*); no accumulation of VSV G protein was noted at process tips (*arrowheads*). Phase-contrast images corresponding to *b*, *d*, and *f* are shown in *a*, *c*, and *e*, respectively. Bar, 20 μm.

The uniform distribution of VSV G protein on the plasma membrane of the cell body and processes at later times after the temperature down-shift may be due to diffusion of G protein in the plane of the membrane from the cell body into the processes, or to movement of constitutive vesicles into the processes. To determine the location of intracellular vesicles carrying G protein at later times after the temperature down-shift, cell surface G protein staining was blocked by preincubation of fixed cells with a rabbit antiserum directed against VSV; the location of intracellular G protein was determined by permeabilizing the cells and processing for indirect immunofluorescence with the mouse monoclonal antibody against G protein as described above. At 25 min, 40 min, and 2 h after the temperature down-shift, the intracellular G protein staining was observed mostly in the cell body near the Golgi region, not in processes or at process tips (Fig. 3). These data suggest that the majority of VSV G protein is inserted at the membrane of the cell body and moves down the processes by diffusion in the plasma membrane.

In contrast to the staining of G protein vesicles in the cell body, ACTH accumulation was maintained at process tips of virus-infected cells (Fig. 2 *A*, *g* and *j*). To show that transport of organelles was occurring normally, infected cells were examined by video microscopy. We found that both rounded and well-processed cells exhibited a similar degree of non-Brownian movement of organelles.

These results show that constitutive and regulated secretory vesicles differ in their sites of insertion: newly synthesized VSV G protein is inserted into the plasma membrane first at the cell body close to the site of synthesis, whereas regulated granules accumulate at process tips where they are exocytosed in response to secretagogue stimulation. VSV G protein eventually becomes distributed in ts-O45–infected AtT-20 cells along the entire surface of the plasma membrane in a manner similar to the steady-state distribution of VSV G protein observed in AtT-20/DRG7 cells.

Differential Effects of Microtubule Disruption on Constitutive and Regulated Secretion

We are interested in the mechanism for the differential targeting of regulated and constitutive vesicles to different areas of the plasma membrane. Results obtained in mitotic AtT-20 cells suggest that microtubules may be involved in transporting regulated granules (24, 44). Constitutive vesicles carrying VSV G protein may lack targeting information for transport on microtubules, or they may recognize a different set of microtubules for targeting to their release sites. We therefore investigated the role of microtubules in constitutive and regulated secretion. We found that colchicine treatment of AtT-20 cells preferentially affected transport of regulated but not constitutive secretory proteins.

Effects on Regulated Secretion. Disruption of microtubules dramatically altered the spatial segregation of regulated granules. Microtubules in AtT-20 cells (Fig. 4 *a*) were completely depolymerized by a 2-h treatment with 10 μM colchicine (Fig. 4 *b*). Treatment with colchicine caused retraction of processes extending from the cell body, and the Golgi elements become dispersed throughout the cytoplasm (Fig. 4 *d*, and references 33, 43). In contrast to untreated cells (Fig. 4 *e*), ACTH granules became randomly dispersed in the cytoplasm of colchicine-treated cells (Fig. 4 *f*). Even in cells grown on polylysine, which do not extend long processes, colchicine exerts similar effects and disperses ACTH granules from their specialized release sites (Fig. 4, *g* and *h*). Thus, clustering of regulated granules at special release sites is maintained by microtubules.

In addition to preventing accumulation of granules at process tips, colchicine also affects proper storage of newly synthesized hormones. AtT-20 cells stably transfected with the rat insulin gene (AtT-20/Ins 6B) were pretreated with 10 μM colchicine for 2.0 h. The cells were pulse-labeled with [^{35}S]cysteine for 30 min to label newly synthesized proinsu-

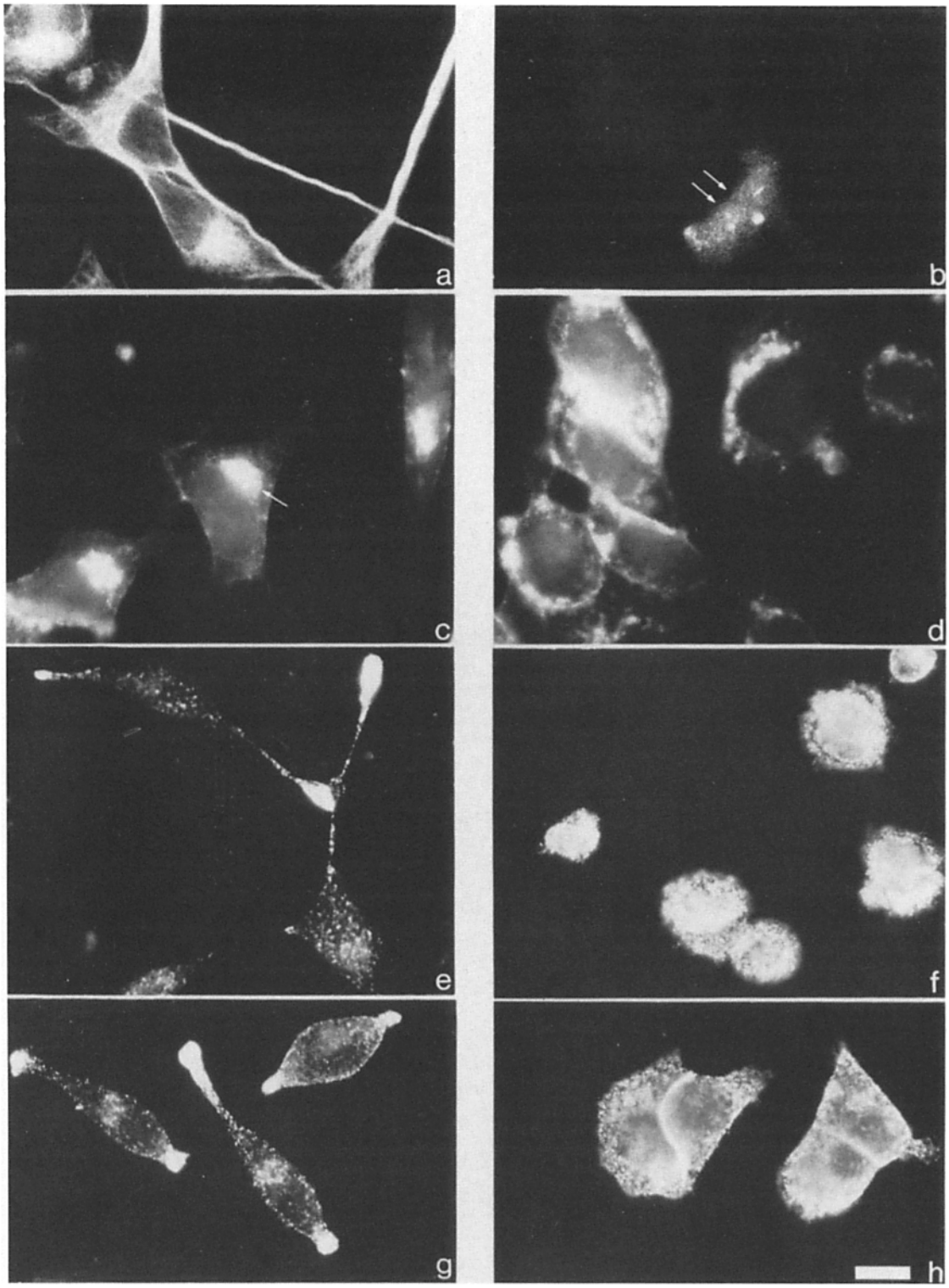


Figure 4. Effect of colchicine on microtubules, Golgi distribution, and ACTH accumulation in AtT-20 cells. (*a* and *b*) Cells stained with a mouse monoclonal anti-tubulin antibody and fluoresceinated secondary and tertiary antibodies. Treatment with 10 μM colchicine for 2 h (*b*) caused complete depolymerization of microtubules; arrows point to a clump of cells. (*c* and *d*) Cells stained with rhodamine-wheat germ agglutinin. In *c* note prominent staining of Golgi region (*arrow*). In colchicine-treated cells (*d*), Golgi elements are dispersed throughout the cytoplasm. (*e–h*) Cells immunostained for ACTH. (*e* and *f*) Cells grown on laminin substrate, (*g* and *h*) on polylysine-coated coverslips. While laminin promotes extension of long processes, cells grown on polylysine form bipolar protuberance. ACTH-containing granules accumulate at process tips in cells on laminin (*e*), and also in the protuberance in cells on polylysine (*g*). In colchicine-treated cells, ACTH granules are dispersed throughout the cytoplasm of cells grown on laminin (*f*) or polylysine (*h*). Bar: (*a–d*) 15 μm; (*e–h*) 20 μm.

Table I. Colchicine Reduces the Efficiency of Storage of Newly Synthesized (Pro) Insulin

	Percent secreted			Percent remaining in cell		
	P*	I‡	Total hormone	P	I	Total hormone
Control						
0–1 h	17.4 ± 0.4	1.9 ± 0.2	19.3 ± 0.4	16.1	65.1	81.2
1–2 h	3.5 ± 0.6	1.9 ± 0.3	5.4 ± 0.6	–	74.6 ± 0.4	74.6 ± 0.4
Colchicine-treated						
0–1 h	35.9 ± 0.2	6.6 ± 0.4	42.5 ± 0.4	23.9	33.7	57.6
1–2 h	6.4 ± 0.6	4.9 ± 0.9	11.3 ± 1.1	–	48.2 ± 1.5	48.2 ± 1.5

AtT-20/Ins 6B cells were treated with colchicine for a total of 4.5 h. At 2.0 h, the cells were labeled with [35S]cysteine for 30 min, then chased for two 1.0-h periods. Cell extract and medium samples were immunoprecipitated and run on SDS-PAGE gels. Release (mean ± SEM) was quantitated as a percentage of the sum of total hormone secreted plus total hormone in cell extracts; total hormone equals insulin plus proinsulin. The percent proinsulin or insulin remaining in the cell after the first-hour chase was calculated from single dishes extracted after that chase.

* *P*, proinsulin.

‡ *I*, mature insulin.

lin and then chased for two 1-h periods. We have shown previously that in AtT-20 cells a portion of newly synthesized hormone apparently escapes packaging and becomes secreted rapidly even in the absence of secretagogue stimulation (27, 29). After the second hour of chase, 74.6% of the total labeled proinsulin in control cells was processed to mature insulin and stored intracellularly (Table I). Cells treated with colchicine showed reduced storage of insulin. 42.5% of labeled hormone was secreted during the first hour chase (Table I). This was a 2.2-fold increase over the amount secreted by control cells during the same period (19.3%). At the end of the second hour of chase, only 48.2% of labeled hormone was stored in colchicine-treated cells. Thus, microtubules facilitate proper storage of regulated secretory proteins.

Effects on Constitutive Secretion. We chose a truncated form of the VSV G protein (TG) to study the effect of colchicine on constitutive secretion. TG consists of the extracellular domain of VSV G protein but lacks the entire membrane-spanning and cytoplasmic domains. When stably transfected into AtT-20 cells, TG is secreted solely by the constitutive pathway (27). AtT-20/TG-17 cells, an AtT-20 cell line stably expressing TG, were treated with 10 μM colchicine for 5.5 h. At 2.0 h the cells were pulse-labeled with [35S]methionine for 30 min to label newly synthesized TG and then chased for two 1.5-h periods. Cell extract and medium samples were immunoprecipitated and analyzed as described for newly synthesized (pro) insulin. The release of TG was not inhibited during either time period in colchicine-treated cells (Table II).

We also examined the insertion of VSV G protein into the plasma membrane by indirect immunofluorescence under conditions of microtubule disruption. AtT-20 cells infected with ts-O45 were incubated during the 3-h incubation at the nonpermissive temperature with 10 μM colchicine, or with 10 μM lumicolchicine, a photo-inactivated analogue of colchicine which does not disrupt microtubules. In colchicine-treated cells at 13 min after the temperature-shift, G protein staining appeared in small fluorescent elements which were dispersed throughout the cytoplasm (Fig. 5 *b*). This dispersal of Golgi elements containing G protein was similar to the disruption of the Golgi seen in colchicine-treated cells stained with rhodamine-labeled wheat germ agglutinin (Fig. 4 *d*). In contrast, at 13 min in lumicolchicine-treated cells, the Golgi complex retained its juxtanuclear, reticular structure (Fig. 5 *a*). At 25 min, VSV G protein was inserted into the plasma membrane of colchicine-treated cells (Fig. 5 *C*). Thus, in the absence of microtubules, insertion into the plasma membrane of constitutive vesicles carrying VSV G protein or truncated G protein is not blocked.

Discussion

Determining the site of insertion of newly synthesized membrane proteins is important for understanding how eucaryotic cells maintain polarized cellular functions. Here we present evidence that in the neuroendocrine cell line, AtT-20, insertion of two types of secretory vesicles takes place at different sites in the plasma membrane. A constitutively exported membrane protein, VSV G, is inserted into the plasma membrane at the cell body. In contrast, regulated secretory granules accumulate at process tips, which are presumably the predominant site at which these granules are inserted into the plasma membrane. Such spatial segregation of vesicles may also occur in other regulated secretory cells. In neurons, the predominant site of membrane insertion during neurite elongation is thought to be at or near the growth cone (see reference 11). However, insertion of membrane proteins at the cell body has also been reported. For example, in growing neurons, freeze-fracture analyses suggest that some membrane proteins insert at the cell body and then diffuse into the plasma membrane of the growing axon (30–31, 41). Biochemical studies have also shown that some membrane pro-

Table II. Secretion of Newly Synthesized Truncated G Protein from AtT-20/TG-17 Cells Treated with Colchicine

Chase	Control	Colchicine-treated
First	61.6 ± 5.6	56.4 ± 12.1
Second	30.7 ± 3.5	25.8 ± 1.0

AtT-20/TG-17 cells were treated with colchicine for a total of 5.5 h. At 2.0 h, the cells were labeled with [35S]methionine for 30 min, then chased for two 1.5-h periods. Cell extract and medium samples were immunoprecipitated and run on SDS-PAGE gels. Release (mean ± SEM) was quantitated as the percentage of the total TG present (cell extract plus medium) which was released into the medium.

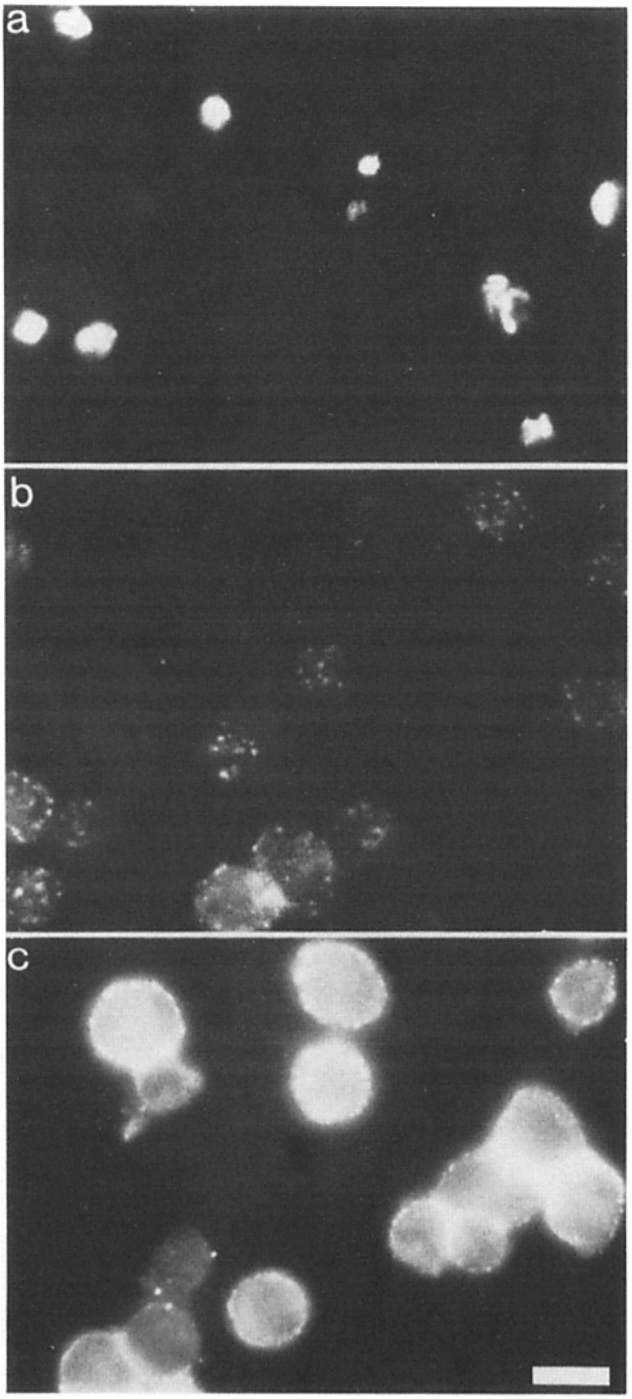


Figure 5. ts-O45 VSV-infected cells treated with lumicolchicine or colchicine. Cells were treated with lumicolchicine or colchicine during incubation at the nonpermissive temperature and stained with monoclonal anti-G protein antibody after the switch to the permissive temperature. (*a*) Lumicolchicine-treated cells, fixed, and permeabilized at 13 min after switching to the permissive temperature. Note staining of VSV G protein in the Golgi region. (*b*) Colchicine-treated cells, fixed, and permeabilized at 13 min; G protein staining is present in small fluorescent elements which are dispersed throughout the cytoplasm. (*c*) Nonpermeabilized colchicine-treated cells, fixed at 25 min; G protein is present in the plasma membrane of the cell body. Bar, 20 μm.

teins insert at the cell body (7, 42). The apparent discrepancy of insertion of membrane components at both the growth cone and the cell body could be explained by insertion of different classes of vesicles at different sites in the plasma membrane in a manner similar to the segregation of regulated and constitutive vesicles in AtT-20 cells. It is interesting to note that vesicles which are relatively poor in membrane proteins have been proposed as the source of membrane used for neurite outgrowth (cf. references 7, 41, 45). Since regulated granules are relatively poor in membrane proteins (25), the secretory vesicles providing membrane for neurite outgrowth at the growth cone might be derived from regulated granules.

We found that microtubule disruption did not inhibit constitutive secretion but did affect formation and accumulation of regulated granules. The differential effect of microtubule disruption suggests that regulated and constitutive vesicles differ in their ability to associate with microtubules. One intriguing possibility is that regulated granules possess unique membrane receptors for a microtubule-based motor such as kinesin, but constitutive vesicles lack such receptors and thus do not associate with the motor.

Our results show that microtubules are not necessary for constitutive secretion in AtT-20 cells. Similarly, Rogalski et al. (34) found that in NRK cells (which do not have a regulated secretory pathway), complete disassembly of microtubules did not affect the rate or extent of G protein sialylation or the transport of G protein to the cell surface. However, Amheiter et al. (1) reported that VSV G protein–containing vesicles in BHK cells move in a saltatory manner through the cytoplasm, suggesting possible cytoskeletal involvement in the transport of these vesicles. It remains to be determined whether cytoskeletal elements other than microtubules are involved in transporting constitutive secretory vesicles.

No accumulation of VSV G protein at process tips was observed in AtT-20 cells. These results are in agreement with the biochemical characteristics of the constitutive pathway (13): newly synthesized constitutively secreted proteins are not concentrated in a post-Golgi storage pool; transport vesicles carrying these proteins have a short transit time from Golgi to cell surface. In contrast to our results with VSV G protein, Matsuuchi et al. (24) found that a soluble marker of the constitutive pathway, kappa light chain, does accumulate at tips of processes in AtT-20 and PC12 cells. The reason for this difference is unclear. Since kappa light chain and G protein have not been shown to exist in the same vesicles, it remains possible that these two proteins are exported in distinct classes of constitutive secretory vesicles.

In conclusion, microtubules in AtT-20 cells appear to be important for formation and accumulation of regulated granules, but are not necessary for constitutive secretion. In addition to the sorting events that segregate proteins into the regulated and constitutive pathways in the Golgi, there appear to be post-Golgi mechanisms that result in differences in intracellular storage and targeting to the plasma membrane between the two secretory pathways.

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