Prohormone Processing in the *trans*-Golgi Network: Endoproteolytic Cleavage of Prosomatostatin and Formation of Nascent Secretory Vesicles in Permeabilized Cells

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Abstract. Many peptide hormones are synthesized as larger precursors which undergo endoproteolytic cleavage at paired basic residues to generate a bioactive molecule. Morphological evidence from several laboratories has implicated either the TGN or immature secretory granules as the site of prohormone cleavage. To identify the site where prohormone cleavage is initiated, we have used retrovirally infected rat anterior pituitary GH_3 cells which express high levels of prosomatostatin (proSRIF) (Stoller, T. J., and D. Shields. *J. Cell Biol.* 1988. 107:2087–2095). By incubating these cells at 20°C, a temperature that prevents exit from the Golgi apparatus, proSRIF accumulated quantitatively in the TGN and no proteolytic processing was evident; processing resumed upon shifting the cells back to 37°C. After the 20°C block, the cells were mechanically permeabilized and proSRIF processing determined. Cleavage of proSRIF to the mature hormone was ∼35–50% efficient, required incubation at 37°C and ATP hydrolysis, but was independent of GTP or cytosol. The in vitro ATP-dependent proSRIF processing was inhibited by inclusion of chloroquine, a weak base, CCCP, a protonophore, or by preincubating the permeabilized cells with low concentrations of *N*-ethylmaleimide, an inhibitor of vacuolar-type ATP-dependent proton pumps. These data suggest that: (*a*) proSRIF cleavage is initiated in the TGN, and (*b*) this reaction requires an acidic pH which is facilitated by a Golgi-associated vacuolar-type ATPase. A characteristic feature of polypeptide hormone-producing cells is their ability to store the mature hormone in dense core secretory granules. To investigate the mechanism of protein sorting to secretory granules, the budding of nascent secretory vesicles from the TGN was determined. No vesicle formation occured at 20°C; in contrast, at 37°C, the budding of secretory vesicles was ∼40% efficient and was dependent on ATP, GTP, and cytosolic factors. Vesicle formation was inhibited by GTPγS suggesting a role for GTP-binding proteins in this process. Vesicle budding was dependent on cytosolic factors that were tightly membrane associated and could be removed only by treating the permeabilized cells with high salt. After high salt treatment, vesicle formation was dependent on added cytosol or the dialyzed salt extract. The formation of nascent secretory vesicles contrasts with prosomatostatin processing which required only ATP for efficient cleavage. Our results demonstrate that prohormone cleavage which is initiated in the TGN, precedes vesicle formation and that processing can be uncoupled from the generation of nascent secretory vesicles.

PEPTIDE hormones of less than ∼50 amino acids are initially synthesized as large inactive polyprotein precursors or prohormones. Many prohormones have complex structures comprising precursors encoding multiple repeating units of identical or unrelated peptides flanked by pairs of basic amino acids or, less frequently, single basic residues (20, 29). Endoproteolytic cleavage at specific sets of basic residues results in excision of the peptide which may then undergo additional biochemical modifications such as acetylation, amidation, sulfation, etc., to generate a biologically active hormone. Morphological and biochemical evidence suggests that these modifications occur sequentially in the distal secretory pathway, including the TGN, immature and mature secretory granules (20, 45, 49, 65). Consequently, the biosynthesis of peptide hormones represents a good model to understand protein processing and membrane trafficking. In the past few years several prohormone cleavage enzymes have been identified by using PCR techniques and oligonucleotides corresponding to the active site of the

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yeast, *S. cerevisiae*, pro-α-factor processing enzyme Kex-2p (for recent reviews see 51, 57). This approach has identified a family of proteases including prohormone convertases or "PC's", that cleave prohormones, and a related set of enzymes, designated "furins", which process constitutively secreted and membrane glycoproteins (22, 39, 58). The coordinated expression of a subset of PC's, along with that of a particular prohormone substrate, may in part explain cell and tissue-specific processing of peptide hormone precursors (9, 63, 75). Several recent reports have demonstrated that the PC's themselves undergo posttranslational processing (2, 26, 68). At present, relatively little is known about the intracellular sorting and processing of the PC's or how they are copackaged with their respective prohormone substrates to effect proteolytic processing.

A characteristic feature of polypeptide hormone-producing cells is their ability to concentrate and store the mature hormone in dense core secretory granules. Upon stimulation, these granules fuse with the plasma membrane in a calcium-dependent process, and release their contents into the external milieu. This has been designated "regulated" secretion (13). In contrast, fibroblasts, hepatocytes, and plasma cells manifest "constitutive" secretion whereby secretory and plasma membrane proteins are transported in vesicles that continuously fuse with the plasma membrane in a calcium-independent process (13). Since endocrine cells possess both constitutive and regulated secretory pathways, a mechanism must exist whereby the cell is able to sort different classes of proteins to their appropriate secretory vesicles. Morphological evidence from several laboratories has implicated the TGN as the site of this protein sorting event (24, 31, 44, 49, 56, 65). Orci and colleagues (3, 42–45) using immunoelectron microscopy techniques demonstrated that proinsulin cleavage is initiated in acidic, clathrin-coated immature secretory vesicles that bud from the TGN, whereas mature insulin is stored in secretory granules devoid of clathrin. Subsequent morphological data from a number of laboratories (31, 49, 56, 65) have provided evidence that prohormone endoproteolytic processing and sorting may occur in the TGN. Tooze et al. (65) and Schnabel et al. (49) demonstrated that the TGN is the site of pro-opiomelanocortin (POMC)[1] processing and packaging into nascent secretory granules. Recent morphological studies on somatostatin biosynthesis have also suggested that its precursor is processed in the TGN (31). During and after sorting and packaging into regulated pathway vesicles, peptide hormone precursors undergo posttranslational processing to generate bioactive molecules. However, the temporal and spatial relationship between prohormone processing, selective packaging of mature hormones, and secretory granule formation is poorly understood.

To identify the intracellular site of prohormone processing and packaging of polypeptide hormones into nascent secretory vesicles, we have established a permeabilized cell system derived from rat anterior pituitary GH_3 cells infected with a recombinant retrovirus encoding preprosomatostatin (preproSRIF). Cell-free and permeabilized cells have been used to successfully reconstitute intraGolgi transport (47) and ER to Golgi vesicular transport (5) as well as other vesicular transport pathways (27, 48). By using permeabilized cells or in vitro systems, several groups (23, 25, 38, 64) have recently investigated polypeptide sorting in the TGN and the budding of secretory vesicles. Tooze and Huttner (64) characterized the formation of two populations of immature secretory granules in rat pheochromocytoma PC12 cells in which secretogranin II was packaged into regulated secretory pathway vesicles and heparin sulfate proteoglycan was sorted to constitutive vesicles; the formation of both classes of vesicle required ATP and GTP. More recently, Grimes and Kelly (25) observed the ATP-dependent formation of both constitutive and regulated immature secretory vesicles in mechanically permeabilized PC12 cells. The constitutive vesicular transport of plasma membrane glycoproteins from the TGN has also been investigated using permeabilized cells and shown to be dependent on ATP hydrolysis (18). Likewise, the sorting of influenza virus hemagglutinin (HA) to the apical surface of permeabilized MDCK cells (23) required ATP and was inhibited by GTPγS, a nonhydrolyzable analogue of GTP. Similarly, transport of vesicular stomatitis virus G protein (VSV-G) from the Golgi apparatus to the plasma membrane could be reconstituted in CHO cells and was dependent on ATP, GTP, and cytosolic factors (38). Although earlier studies have investigated proinsulin processing in isolated secretory granules (46), to our knowledge permeabilized cells have not been exploited previously to investigate a possible role for the Golgi apparatus/TGN in prohormone processing.

Somatostatin (SRIF) is a 14-amino acid peptide hormone that is synthesized as a part of a larger ~120 residue precursor, preproSRIF (28). Cleavage of the precursor at a single Arg-Lys pair, located near the carboxyl terminus of the molecule, results in excision of the mature tetradecapeptide (41). Using GH_3 cells which secrete endogenous growth hormone (GH), we have previously demonstrated (59) that proSRIF was accurately and efficiently processed and the mature hormone packaged into regulated secretory vesicles. Data from other investigators have shown that GH_3 cells express only the PC2 member of the prohormone processing enzyme family (52). We have now used a 20°C temperature block to accumulate polypeptides in the TGN (18) and prepared permeabilized GH_3 cells. This system was able to support both prohormone cleavage and the formation of nascent secretory vesicles. Here we demonstrate that: (*a*) proSRIF cleavage was initiated in the TGN; (*b*) cleavage required ATP hydrolysis to generate an intralumenal acidic pH; and that (*c*) prohormone cleavage preceded the formation of nascent secretory vesicles. In contrast to prohormone cleavage, secretory vesicle formation required both GTP and cytosolic factors as well as ATP. The cytosolic factors necessary for vesicle budding were tightly membrane associated and could be removed only by treatment with high salt. Our data demonstrate that prohormone cleavage in the TGN precedes vesicle budding and that processing can be uncoupled from the formation of immature secretory granules.

Materials and Methods

Materials

[^{35}S]Cysteine was purchased at the highest available specific activity from

1. *Abbreviations used in this paper:* BFA, brefeldin A; CCCP, carbonylcyanide m-chlorophenylhydrozone; GH, growth hormone; HA, hemagglutinin; NEM, N-ethylmaleimide; POMC, pro-opiomelanocortin; SRIF, somatostatin.

DuPont New England Nuclear (Boston, MA). Brefeldin A (BFA) was purchased from Epicentre (Madison, WI). Carbonyl-cyanide *m*-chlorophenylhydrozone (CCCP) was purchased from CalBiochem (La Jolla, CA). *N*-ethylmaleimide (NEM) was purchased from Pierce (Rockford, IL). Chloroquine was purchased from Sigma Chem. Co. (St. Louis, MO). GTPγS and ATPγS were purchased from Boehringer Mannheim, Germany. Reverse phase HPLC columns were purchased from Vydac (Hesperia, CA) or Dynamax Corporation, Rainin Instruments (Woburn, MA). Centricon S-10 concentrators were obtained from Amicon® (Beverly, MA).

Antibodies. A monoclonal antibody to ribophorin I was a gift from Dr. Gert Kriebich, New York University Medical Center; rabbit anti TGN-38 was from Dr. Paul Luzio, University of Cambridge, England. A rabbit antipeptide antiserum to the carboxy terminal 10 residues of rat growth hormone (RRFAESSCAF) was prepared exactly as described (19); initially this serum was generously provided by Dr. Richard Mains, Johns Hopkins Medical School.

Methods

Cell Culture. Cells were grown at 37°C in an atmosphere of 7.5% CO_2. GH_3 cells were grown in Ham's F10 medium (3 g/ml $NaHCO_3$) supplemented with 15% equine serum, 2.5% FBS, 2 mM glutamine, 25 U/ml penicillin, and 25 mg/ml streptomycin.

Pulse-Labeling and Immunoprecipitation. GH_3.S18.9 cells were pulse-labeled with 500 μCi [^{35}S]cysteine/ml as previously described (59) and chased for various times as indicated at 20 or 37°C in the presence of 5 mM nonradioactive cysteine. At the end of the incubation, the cells and medium were treated with either anti-GH or anti-SRIF antibodies as previously described, except that SDS was omitted before antibody addition (59). The samples were then treated with protein A-Sepharose and the immunoprecipitable material analyzed by SDS-PAGE or reverse phase HPLC (16) (see below).

HPLC Analysis of SRIF-Immunoreactive Polypeptides. The antibody-antigen complexes bound to protein A-Sepharose were incubated in 500 mM Tris-HCl, pH 8.8, 20 mM EDTA, 8 M urea ("TEU") containing 100 mM DTT at 65°C for 15 min followed by carboxymethylation (59). The protein A-Sepharose beads were centrifuged at 15,000 *g* for 5 min and the eluted peptides applied to either of two different reverse phase HPLC columns: Gradient A elution conditions: Vydac C_{18} (25 cm × 4.6 mm) column eluted by 0–5 min 5% CH_3CN; 5–6 min 5–20% CH_3CN; 6–36 min 20–35% CH_3CN; 36–51 min 35–50% CH_3CN; 51–52 min 50–80% CH_3CN. Gradient B: Dynamax C_4 (5 cm × 4.6 mm) column (Rainin Instruments) eluted with 0–4 min 5% CH_3CN; 4–6 min 5–25% CH_3CN; 6–21 min 25–32% CH_3CN; 21–33 min 32–46% CH_3CN; 33–37 min 46% CH_3CN; 37–38 min 47–80% CH_3CN. Each gradient was eluted at a flow rate of 1.5 ml/min and all solutions contained 0.1% trifluoroacetic acid. The columns were eluted using a Waters-Millipore HPLC system, fractions of 1 min were collected and the radioactivity determined by liquid scintillation counting. Prosomatostatin processing efficiency was determined by comparing the radioactivity in only the peak fraction of mature SRIF divided by that present in the peak fraction of proSRIF plus mature SRIF.

Preparation of Permeabilized GH_3.S18.9 Cells. Each experiment was repeated at least twice. Approximately 2 × 10^6 cells were pulse-labeled with [^{35}S]cysteine (500 μCi/ml) for 10 min at 37°C, washed with PBS (prewarmed to 20°C), and chased for 2 h at 20°C in medium containing 5 mM Cys. To ensure maintenance of a neutral pH during the chase period, the incubation media were supplemented with 25 mM Hepes KOH, pH 7.2. At the termination of incubation, cells were permeabilized exactly as described by Beckers et al. (8). Briefly, cells were incubated at 4°C in swelling buffer for 5 min, the buffer aspirated and replaced with 1 ml "breaking buffer" (90 mM KCl, 10 mM Hepes, pH 7.2) after which the cells were broken by scraping with a rubber policeman. The cells were centrifuged at 800 *g* for 5 min, washed in 3–5 ml of breaking buffer, and resuspended in 5 vol of breaking buffer. This procedure resulted in >95% of cell breakage, evaluated by staining with Trypan blue. Incubations were in a final volume of 300 μl and contained: 120 μl permeabilized cells (equivalent to 5–8 × 10^5 cells), 120 μl cytosolic extract (∼180–240 μg protein), 2.5 mM $MgCl_2$, 0.5 mM $CaCl_2$, 110 mM KCl, 1 mM ATP, 0.02 mM GTP, 10 mM creatine phosphate, 80 μg/ml creatine phosphate kinase and protease inhibitors (16). Incubations were at 20 or 37°C as indicated, at the end of which aliquots were treated with cell lysis buffer (above) followed by incubation with anti-GH and/or anti-SRIF antibodies.

Preparation of Cytosol. GH_3.S18.9 cells were pelleted at 800 *g* for 5 min, and washed once in homogenization buffer containing 125 mM KCl, 25 mM Hepes KOH, pH 7.4 (7). The cells were resuspended in homogenization buffer (1 vol of cell pellet to 5 vol of buffer) followed by homogenization using a stainless steel ball bearing homogenizer. The homogenate was centrifuged for 1 h at 39,000 rpm in a Beckman SW 50.1 rotor and the supernatant applied to a Sephadex G-25 column equilibrated with homogenization buffer (Beckman Instr., Inc., Fullerton, CA). Desalted cytosol was concentrated using Centricon-3 concentrator and frozen in aliquots in liquid nitrogen.

Formation of Nascent Secretory Vesicles in Permeabilized Cells. The permeabilized cells were incubated for 90 or 120 min at either 37 or 20°C in the presence of 1 mM ATP, 0.02 mM GTP and 1 mg cytosolic protein/ml. At the end of the incubation, aliquots were digested with 100 μg proteinase K/ml at 4°C for 30 min; when present Triton X-100 was used at a final concentration of 1%. Proteolysis was terminated by addition of 2 mM PMSF. After proteinase K digestion, the permeabilized cells were centrifuged in a Brinkman microfuge (15,000 *g*) for 2 min at 4°C. The pellets were treated with cell lysis buffer (59) and the lysates from permeabilized cells and the in vitro formed vesicles were treated with buffer A (2% Triton X-100, 150 mM NaCl, 0.2 mM EDTA, 1 mM PMSF, 50 mM Tris HCl, pH 8.3) followed by 5 μl of rabbit anti-GH or anti-SRIF serum.

High Salt Extraction of Permeabilized Cells. Approximately 2–5 × 10^5 GH_3.S18.9 cells were pulse-labeled for 10 min at 37°C, chased 2 h at 20°C, and permeabilized (8). The permeabilized cells were washed three times with 3 ml of a physiological salt solution (100 mM KCl and 20 mM Hepes-KOH, pH 7.4) and resuspended in a volume corresponding to three times the packed cell volume. The salt concentration was adjusted to 400 mM KCl and the permeabilized cells incubated for 10 min at 4°C. After high salt treatment, the permeabilized cells were centrifuged for 5 min at 800 *g* and the pellet resuspended in a volume of incubation buffer equivalent to that of the starting material. The salt extractable material in the supernatant was dialyzed extensively against several changes of 1× incubation buffer (100 mM KCl, 2 mM $MgCl_2$, 10 mM Hepes KOH, pH 7.2) at 4°C and concentrated in a Centricon-10 concentrator to a final concentration of 0.5 mg protein/ml.

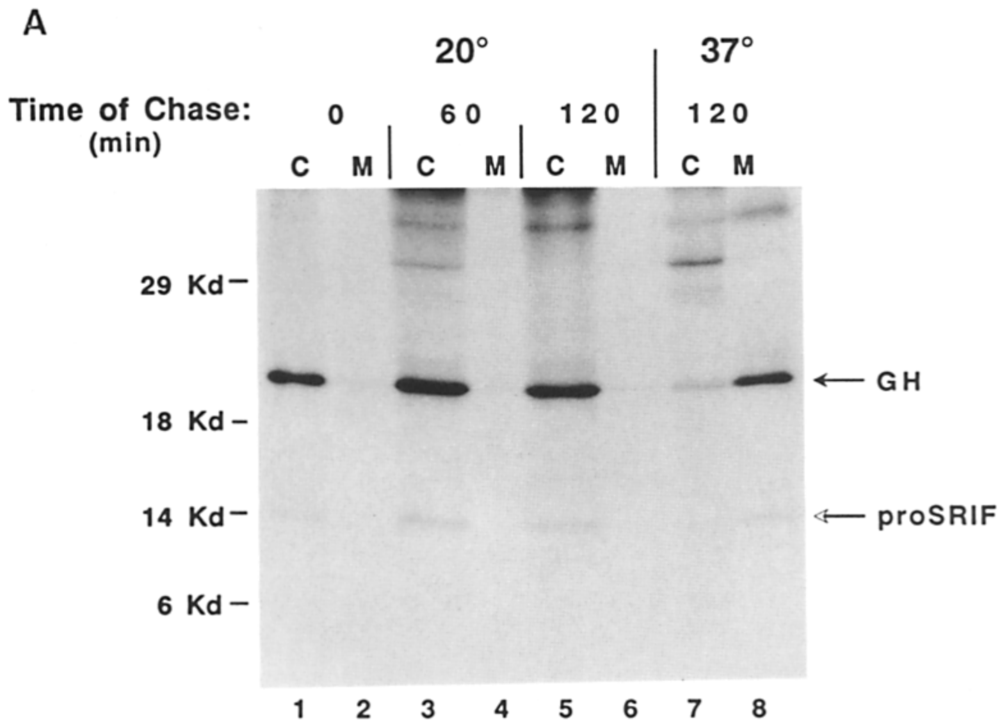
Equilibrium Density Sucrose Gradients. To isolate an enriched Golgi membrane fraction from GH_3 cells, cells were homogenized using four strokes of a stainless steel ball-bearing homogenizer in 0.25 M sucrose, 10 mM Tris-HCl, pH 7.4, and 1 mM $MgAc_2$ (1 vol of cell pellet per 5 vol of homogenizing medium). The homogenate was adjusted to 1.4 M sucrose and loaded onto a step gradient comprising: 2 ml of a 2.0 M sucrose cushion overlaid with ∼2 ml of adjusted homogenate (loading material), 7 ml of 1.2 M sucrose, and 2.2 ml of 0.8 M sucrose; all solutions contained 10 mM Tris-HCl, pH 7.4, and 1 mM $MgAc_2$. The gradients were centrifuged for 4.5 h at 39,000 rpm in a Beckman SW41Ti rotor. 1-ml fractions were collected from the top of each gradient and assayed for total protein by the method of Bradford (12), galactosyl transferase activity (14), sialyl transferase activity (11), TGN-38 (33) and ribophorin I (73), the latter two by Western blot. Each fraction was also assayed for GH and SRIF by sequential immunoprecipitation using appropriate antibodies followed by protein A-Sepharose (as above).

Galactosyl Transferase and Sialyl Transferase Assays. Galactosyl transferase and sialyl transferase activities were determined as described by Chaney et al. (14) and Bergeron et al. (11), respectively.

Densitometry. The band intensity in each fluorograph was quantitated using a Molecular Dynamics Model 300A computing densitometer and the data analyzed using the "Image Quant" 3.2 program (Molecular Dynamics, Inc., Sunnyvale, CA).

Results

We have previously established and analyzed a cell line designated GH_3.S18.9 which is derived from rat anterior pituitary GH_3 cells infected with a recombinant retrovirus encoding angler fish preproSRIF-I (59). These cells process proSRIF to the mature hormone with ∼75% efficiency and target ∼55% of the mature peptide to the regulated secretory pathway, however the endogenous GH is poorly stored (59). Our initial experiments were aimed at establishing conditions whereby GH and proSRIF would accumulate intracellularly in the TGN. To this end, we incubated GH_3.S18.9 cells at 20°C, a temperature which has previously been shown to inhibit transport of membrane glycoproteins from the TGN (36). Cells were pulse-labeled for 10 min at 37°C with [^{35}S]Cys, followed by a 0–120-min chase at 20°C. The



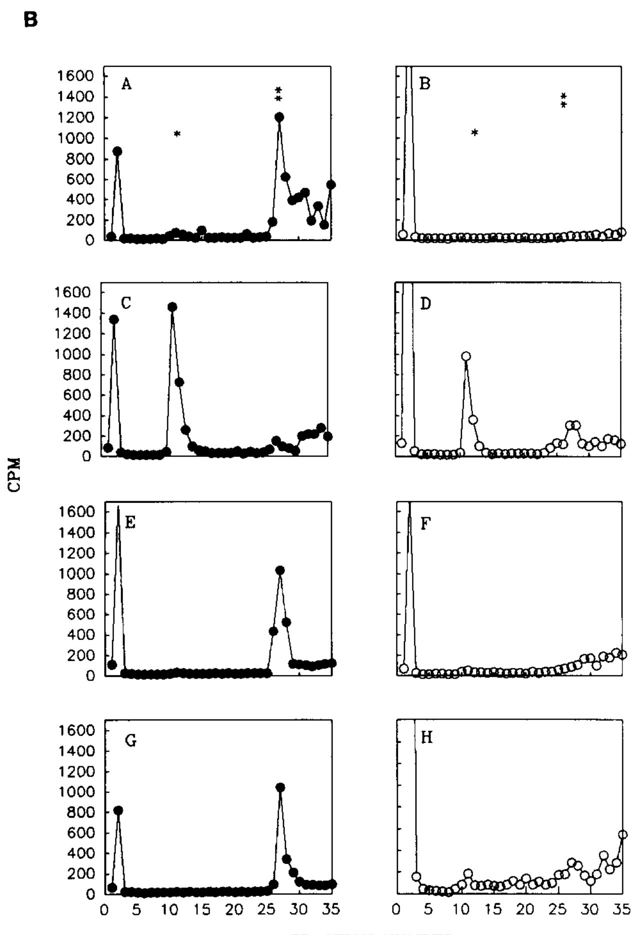


Figure 1. (*A*) Incubation at 20°C inhibits GH secretion. GH_3.S18.9 cells were pulse-labeled with 500 μCi/ml [^{35}S]cysteine for 10 min and chased for the indicated times at 20°C (lanes *1–6*) or at 37°C (lanes *7* and *8*). Samples of the culture medium (lanes *2*, *4*, *6*, and *8*) and cell lysate (lanes *1*, *3*, *5*, and *7*) were treated sequentially first with anti-SRIF antisera followed by protein A–Sepharose, and then with rabbit anti-GH antibodies and protein A–Sepharose (59). The GH-immunoprecipitable material was analyzed on a 15% polyacrylamide gel and subjected to fluorography. Arrows indicate the position of GH and residual proSRIF. (*B*) Incubation at 20°C inhibits proSRIF processing and secretion. The SRIF-immunoreactive material was eluted from the protein A–Sepharose beads (Methods) and the peptides resolved on a C_4 reverse-phase HPLC column using a gradient of CH_3CN in 0.1% TFA/water (Gradient B, Methods). Intracellular material: *A*, *C*, *E*, and *G*: ●—●. Secreted material: *B*, *D*, *F*, and *H*: ○—○. *A* and *B*) SRIF immunoreactive material after 10-min pulse with [^{35}S]Cys at 37°C. (*C* and *D*) SRIF immunoreactive polypeptides after 120-min chase at 37°C. (*E* and *F*) SRIF-immunoreactive polypeptides chased at 20°C for 60 min. (*G* and *H*) As *C* and *D* but chased at 20°C for 120 min. Mature SRIF and proSRIF eluted at fractions 11 and 27, indicated by single and double asterisks, respectively.

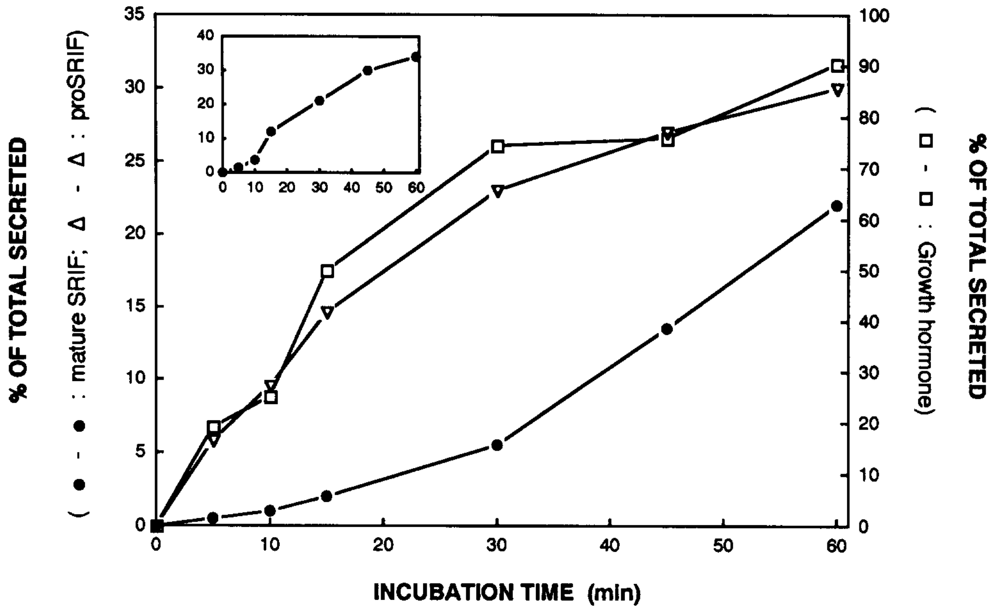


Figure 2. Kinetics of GH secretion and proSRIF processing after shift from 20 to 37°C. GH_3.S18.9 cells were pulse labeled with [^{35}S]Cys for 10 min at 37°C, and chased for 2 h at 20°C after which they were shifted back to 37°C and incubated for the indicated times. Secreted and intracellular GH-immunoreactive material was determined by SDS-PAGE followed by fluorography and densitometry. Somatostatin polypeptides were analyzed from the same samples by sequential immunoprecipitation with anti-SRIF antibodies and the peptides resolved by reverse phase HPLC. Secretion of mature SRIF: ●—●; proSRIF secretion: △—△; GH secretion: □—□. Inset: intracellular mature SRIF: ●—●. Percent of SRIF-immunoreactive secreted material was calculated as:

$$\frac{\text{secreted SRIF material}}{\text{total (intracellular + secreted SRIF)}} \times 100\%.$$

Note differences in left and right ordinates. Data are the average of two separate experiments.

intracellular and secreted material was then analyzed for GH and SRIF-immunoreactive material (Fig. 1). In marked contrast to incubation at 37°C where 85–90% of pulse-labeled GH was secreted by 120 min of chase (Fig. 1 *a*, lanes *7* and *8*; Ref. 59), at 20°C ~95% of the GH accumulated intracellularly (lanes *3–6*). Similarly, there was virtually no secretion of unprocessed proSRIF at this temperature (Fig. 1 *b*). In agreement with our previous observations (59), at 37°C proSRIF was cleaved with ~80% efficiency by 120 min of chase (*C* and *D*) and ~60% of the mature hormone (fraction 11, 12) was stored intracellularly, although there was some constitutive secretion of unprocessed precursor and mature SRIF (*D*). Most significantly, even after 120 min incubation at 20°C, there was minimal proSRIF cleavage to the mature hormone (~6%) and like GH, greater than 95% of the precursor accumulated within the cells.

It was possible that after incubation at 20°C, GH, and proSRIF, might be irreversibly trapped within the Golgi apparatus and would not be packaged into secretory vesicles when the cells were returned to 37°C. To investigate this possibility, GH_3 cells were pulse-labeled with [^{35}S]Cys, chased for 60 min at 20°C, and then shifted to 37°C for various times and the secretion of GH and proSRIF determined (Fig. 2). Within 10 min of incubation at 37°C, ~25% of GH was secreted and greater than 70% was recovered in the medium by 30 min of chase. Thus the kinetics of growth hormone secretion were virtually identical to those of control cells incubated at 37°C alone (Fig. 1; Ref. 59). Similarly, the fraction of unprocessed proSRIF secreted constitutively was also rapidly released upon shift from 20 to 37°C (Fig. 2). In contrast to GH secretion, there was a lag in proSRIF processing after the shift from 20 to 37°C and in the appearance of the fraction of mature SRIF that is constitutively released. Most importantly, these data show that in the GH_3 cells the 20°C block is completely reversible for both secretion and prohormone processing. These kinetics demonstrating differential GH and mature SRIF secretion are consistent with our previous observations that SRIF is preferentially targeted to the regulated secretory pathway (59). We interpret the lag in SRIF appearance to result from the requirement of proSRIF to undergo a series of reactions before secretion, namely endoproteolytic cleavage, aggregation, and packaging into nascent secretory vesicles.

The 20°C Block Results in Prohormone Accumulation in the TGN

The foregoing experiments suggested that GH and proSRIF accumulated intracellularly at 20°C. Based on data from previous investigators (31, 49, 65), we hypothesized that these molecules would be in the TGN. To identify the site of accumulation, we first used cell fractionation techniques (Fig. 3). Cells were pulse-labeled for 10 min at 37°C, chased for 2 h at 20°C, homogenized, and the post-nuclear supernatant fractionated on an equilibrium sucrose gradient designed to separate the ER and Golgi apparatus (8). An aliquot of each gradient fraction was transferred to nitrocellulose membranes and probed with antibodies to the TGN marker antigen TGN-38 (Fig. 3, panel *A*) and the ER marker ribophorin I (*B*). Each gradient fraction was also analyzed by sequential immunoprecipitation for SRIF- and GH-immunoreactive material (*D* and *E*, respectively), and the Golgi marker enzymes, sialyl and galactosyl transferases (*C*). Most of the proSRIF- and GH-immunoprecipitable material was recovered at the top of the gradient (fractions 2, 3, and 4) in the same fractions as sialyl and galactosyl transferase activities (fractions 3 and 4) and TGN-38 (fraction 3). Some residual proSRIF and most of the total protein remained in the load zone of the gradient (fractions 10 and 11)

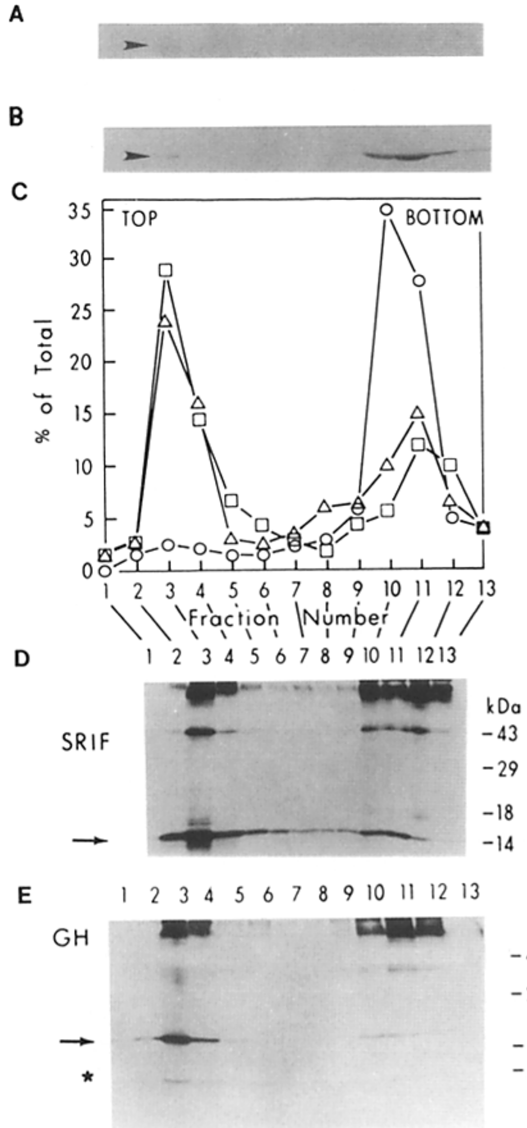


Figure 3. Intracellular accumulated GH and proSRIF cofractionate with Golgi markers. Cells were pulse-labeled with [^{35}S]cysteine for 10 min at 37°C, chased at 20°C for 120 min, and homogenized. A post-nuclear supernatant was layered over a 2 M sucrose cushion and overlaid with 1.2 M and 0.8 M sucrose and centrifuged for 4.5 h at 150,000 *g* in a Beckman SW41Ti rotor (Methods). 1-ml gradient fractions were collected from the top and assayed for: (*A*) TGN-38 detected by Western blotting; (*B*) Ribophorin I, detected by Western blot; (*C*) galactosyl transferase (□—□), sialyl transferase (△—△) and total protein (○—○); (*D*) SRIF-immunoreactive material; (*E*) immunoprecipitable GH material. Immunoprecipitates in *D* and *E* were analyzed by SDS-PAGE followed by fluorography. The arrows (*D* and *E*) indicate the position of proSRIF and GH, respectively. The asterisk (*E*) indicates residual proSRIF present in the GH immunoprecipitate.

as did greater than 95% of the ER marker ribophorin I (*B*). These results suggest that both proSRIF and GH accumulate in the TGN after incubation at 20°C.

Additional evidence that GH and proSRIF accumulated in the TGN at 20°C, was provided by use of the fungal metabolite brefeldin A (BFA). This drug inhibits secretion by preventing vesicle exit from the ER and leads to dissolution and redistribution of the Golgi apparatus (30). However, at

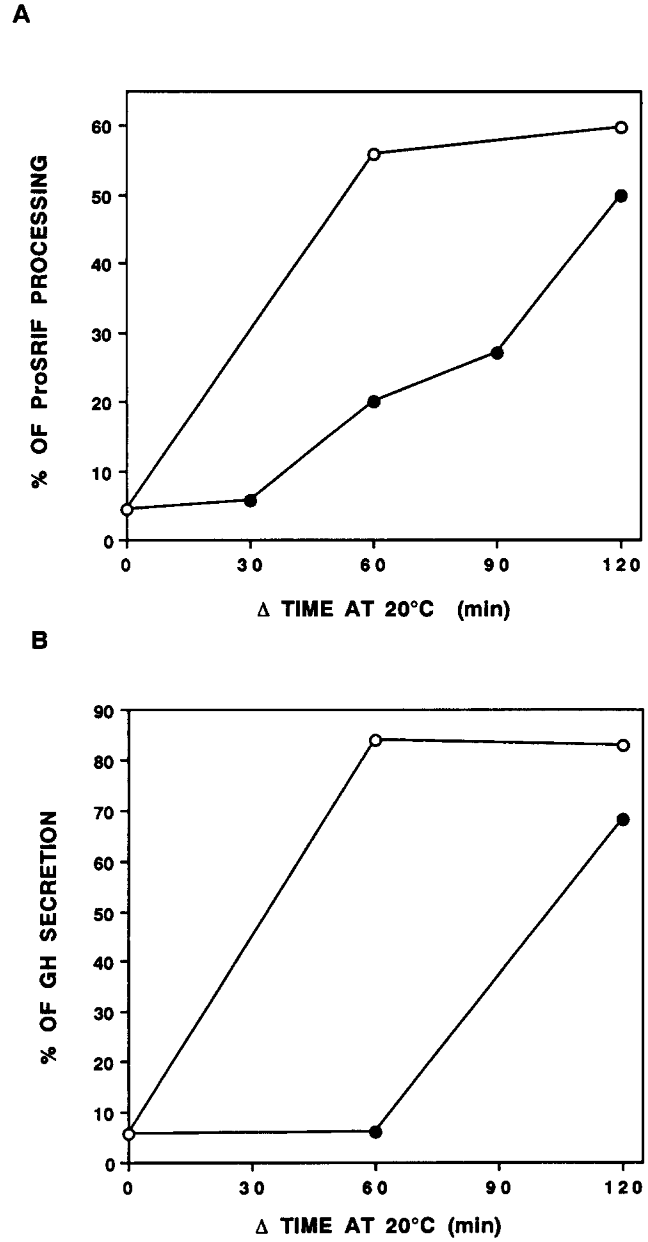


Figure 4. Kinetics of proSRIF and GH transport to a BFA-resistant compartment (TGN) at 20°C. (*A*) ProSRIF processing occurs in a BFA-resistant compartment: Cells were pulse-labeled for 10 min at 37°C with [^{35}S]cysteine and chased at 20°C for 30, 60, 90, or 120 min. At each time point, cells were incubated for an additional 60 min at 37°C in the absence (○—○) or presence (●—●) of 10 μg BFA/ml. The media and cell lysate was then treated with anti-SRIF antibodies and the immunoreactive material analyzed by HPLC. Percent processing is expressed as:

$$\frac{\text{total mature SRIF}}{\text{mature SRIF + proSRIF}} \times 100\%.$$

(*B*) GH transport from the late Golgi/TGN is not affected by BFA. Aliquots of the intracellular and secreted material from the 60- and 120-min samples (*A*) were treated with anti-GH antibodies and the GH-immunoprecipitable polypeptides analyzed by SDS-PAGE. The fluorograph was quantitated by densitometric scanning. GH secretion in the absence of BFA, ○—○; GH secretion in the presence of 10 μg BFA/ml, ●—●.

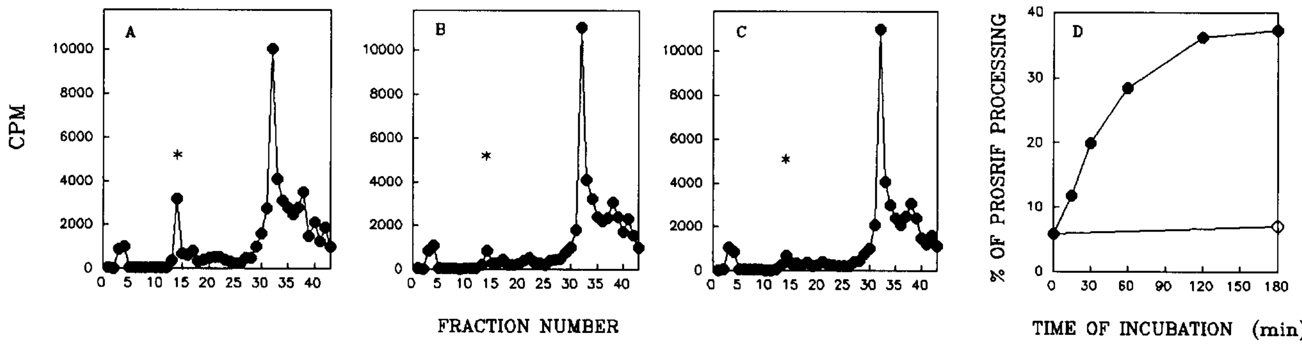


Figure 5. ProSRIF processing in permeabilized GH_3.S18.9 cells. Cells were pulse-labeled for 10 min at 37°C, chased for 120 min at 20°C and permeabilized as described (Ref. 8 and Methods). The permeabilized cells were incubated for 90 min with cytosol and an energy generating system ("EGS" ATP, GTP, CP, and creatine phosphate kinase; Methods) either at 37°C (*A*), 4°C (*B*) or at 37°C in the absence of "EGS" (*C*). At the end of the incubation, samples were treated with anti-SRIF antibodies and the immunoprecipitable material analyzed using a C_{18} HPLC column (gradient A; Methods). Asterisks indicate the elution position of mature SRIF; proSRIF eluted at fractions 32 and 33. (*D*) Kinetics of proSRIF cleavage in permeabilized cells. Samples were incubated exactly as in *A* for up to 180 min at 37°C (●—●) or at 20°C (○—○) and proSRIF processing quantitated after resolution of precursor and mature immunoreactive forms by HPLC.

early times after treatment, BFA has relatively little effect on the integrity of the TGN (15). Since the TGN has been implicated as the site of prohormone endoproteolytic cleavage, we used BFA to determine (*a*) whether prohormone processing was sensitive to BFA and (*b*) the transit time at 20°C for proSRIF to reach a BFA-insensitive compartment, presumably the TGN. Cells were pulse-labeled for 15 min at 37°C, chased for up to 120 min at 20°C, and subsequently incubated for 1 h at 37°C in the presence or absence of 10 μg BFA/ml (Fig. 4). At early chase times (up to 60 min), proSRIF processing was inhibited by BFA consistent with the precursor residing in an early BFA-sensitive Golgi compartment. By 60–90 min of incubation at 20°C, a low but significant level of mature SRIF was apparent (~25%). At 120 min of chase, proSRIF had traversed the site of the BFA block and the level of processing was significant (~50–55%) approaching that of control non-BFA-treated cells preincubated at 20°C. After 60 min of chase at 20°C, GH secretion was also BFA sensitive. However, when cells preincubated for 120 min at 20°C were then incubated at 37°C in the presence of BFA, GH secretion was similar to control levels (Fig. 4 *B*). These data show that in GH_3 cells, BFA has little or no effect on late stages of secretion, i.e., TGN to plasma membrane, but significantly inhibits prohormone processing before the precursor reaches the TGN compartment. These results also suggest that proSRIF takes ~90 min at 20°C to traverse the site of the BFA block and that processing occurs in a post-BFA sensitive compartment.

A GH_3 Permeabilized Cell System Supports Prohormone Cleavage

Having established that it was possible to accumulate significant levels of proSRIF in the TGN, permeabilized GH_3.9 cells were prepared by the method of Beckers et al., (8). To investigate proSRIF processing in vitro, cells were pulse-labeled with [^{35}S]cysteine for 10 min at 37°C, chased at 20°C for 120 min, transferred to 4°C, and permeabilized cells prepared. The permeabilized cells were incubated for 90 min at 37°C in the presence of cytosol, GTP, an ATP-generating system (complete system), and analyzed for SRIF-immunoreactive material by HPLC (Fig. 5). After incubation at 37°C (*A*), ~28% of proSRIF (fraction 32) was cleaved to the mature fourteen amino acid peptide (fraction 14) and eluted with precisely the same retention time as SRIF or SRIF synthesized in intact cells. In contrast, incubation at 37°C in the absence of ATP and GTP (*B*) or with the complete system at 4°C (*C*) resulted in minimal background processing of ~5%. Proteolytic processing was linear up to ~90 min incubation at 37°C whereas only background cleavage was observed at 20°C (*D*). Efficient processing (~25–35%) to mature SRIF-14 was evident in the absence of cytosol and addition of up to 2 mg cytosolic protein/ml had little effect on processing efficiency (data not shown).

These data suggested that only ATP and/or GTP might be necessary to effect prohormone cleavage in the TGN. To assess their respective requirement, permeabilized cells were incubated in the absence of either ATP, GTP, or both (Fig. 6). Omission of ATP from the incubation system drastically inhibited proSRIF processing. In contrast, incubation in the absence of 0.02 mM GTP had relatively little effect on prohormone processing, resulting in ~21% processing in the absence of GTP compared to ~25% in the complete system (Fig. 6). Similarly, incubation in the presence of GTPγS, a non-hydrolyzable GTP analogue which inhibits vesicular transport in vitro (67) or with GDP-CP, had no significant effect on processing efficiency. This result suggested that neither GTP hydrolysis nor GTP-binding proteins are required for prohormone processing. As expected, omission of both ATP and GTP from the incubation system resulted in no proSRIF processing. The data demonstrate that cleavage of proSRIF to the mature tetradecapeptide hormone requires only ATP.

ATP Hydrolysis Is Required to Generate an Acidic pH in the TGN

Numerous reports (3, 40, 43, 60) have demonstrated that an acid environment is required for prohormone processing, sorting to the regulated secretory pathway and that the TGN and immature secretory granules have an acidic pH (3). Furthermore, several reports have demonstrated the presence of a vacuolar type H^+-ATPase in the Golgi apparatus and secretory granule membranes (1, 21, 72, 76). Therefore, we hypothesized that the ATP requirement for proSRIF processing was to provide energy to establish a proton gradient in

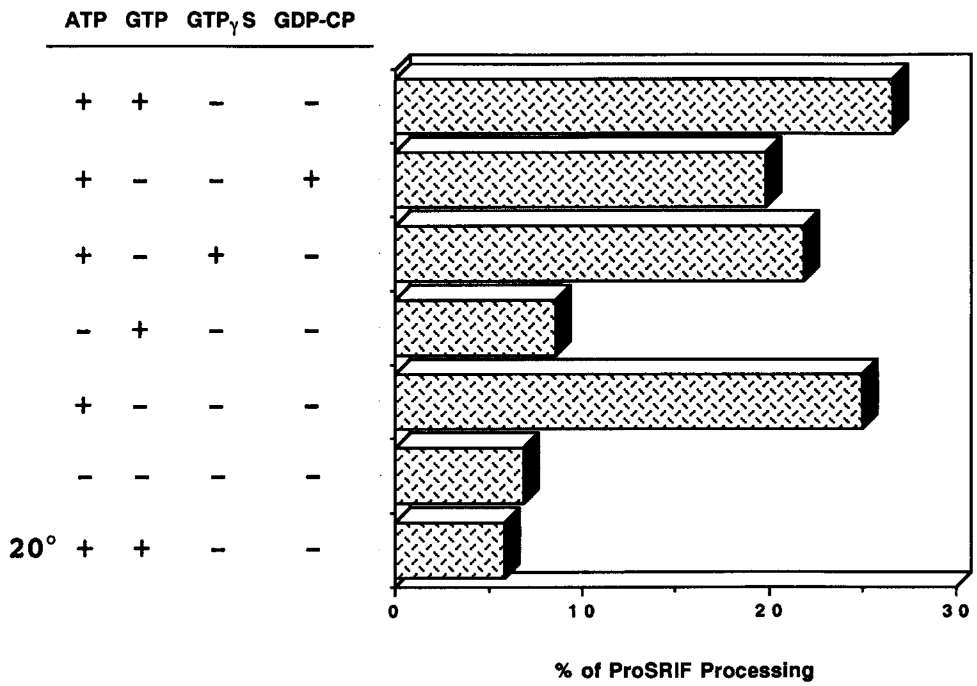


Figure 6. Requirements for proSRIF processing in permeabilized cells. Pulse-labeled cells, chased for 120 min at 20°C, were permeabilized and then incubated at 37°C with 1 mg/ml cytosol, under the indicated conditions for 90 min followed by treatment with anti-SRIF antibodies. Immunoreactive proSRIF and mature SRIF were resolved by reverse-phase HPLC and the efficiency of processing quantitated. When present, GTPγS and GDP-CP were at a final concentration of 20 μM. The data are the average from two separate experiments.

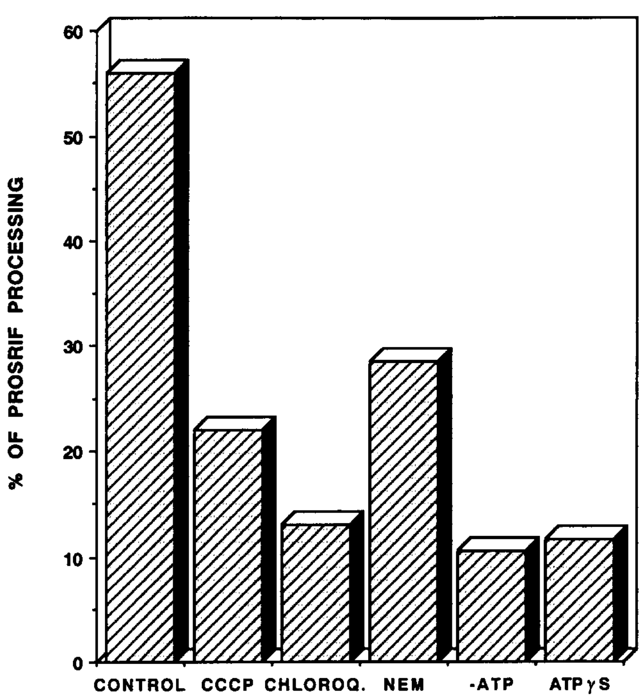


Figure 7. ATP hydrolysis is required to generate a proton gradient in the TGN. Permeabilized cells were preincubated with 15 μM CCCP, 25 μM chloroquine (CHLOROQ.), 10 μM NEM for 15 min at 4°C; control permeabilized cells were preincubated with buffer alone. The samples were then incubated for 120 min at 37°C with 2 mM ATP and the various inhibitors. Samples: "-ATP," (incubation in the absence of ATP), and ATPγS (incubation in the presence of 2 mM ATPγS). SRIF-immunnoreactive polypeptides were analyzed by HPLC.

$$\text{Percent processing} = \frac{\text{mature SRIF}}{\text{mature SRIF} + \text{proSRIF}} \times 100\%.$$

Data were averaged from two separate experiments.

the TGN and that this acidic pH would facilitate processing by the PC2 enzyme. To test this idea, permeabilized cells were incubated with ATP or the non-hydrolyzable analogue ATPγS (Fig. 7). ProSRIF cleavage was dependent on ATP hydrolysis since in the presence of 2 mM ATPγS there was minimal processing above background levels. That ATP hydrolysis was required to generate an acidic milieu in the TGN was demonstrated by incubating the system in the absence or presence of either CCCP (a protonophore which would collapse a proton gradient), chloroquine, a weak base that neutralizes acidic organelles or with *N*-ethylmaleimide, which inactivates vacuolar ATPases (1) (Fig. 7). Consistent with the

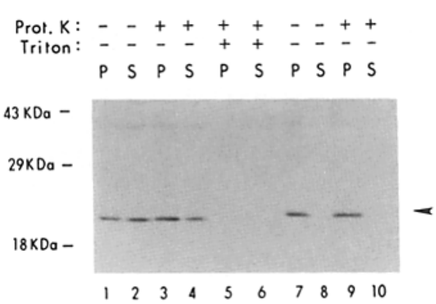


Figure 8. Budding of nascent secretory vesicles from the TGN of permeabilized GH_3.S18.9 cells. Cells were pulse-labeled with [^{35}S]cysteine for 10 min at 37°C, chased at 20°C for 2 h, permeabilized and incubated for an additional 2 h at 37°C (lanes *1–6*) or at 20°C (lanes *7–10*) in the presence of cytosol, ATP, and GTP. At the end of the incubation, aliquots were either untreated (lanes *1*, *2*, *7*, and *8*) or treated with 100 μg proteinase K/ml (lanes *3–6*, *9* and *10*) at 4°C for 30 min in the absence (lanes *3*, *4*, *9*, and *10*) or presence (lanes *5* and *6*) of 1% Triton X-100. Control and proteinase K-digested samples were then centrifuged for 2 min at 15,000 *g* at 4°C, and the pellet and supernatant treated with anti-GH serum. The arrowhead indicates the migration of rat GH.

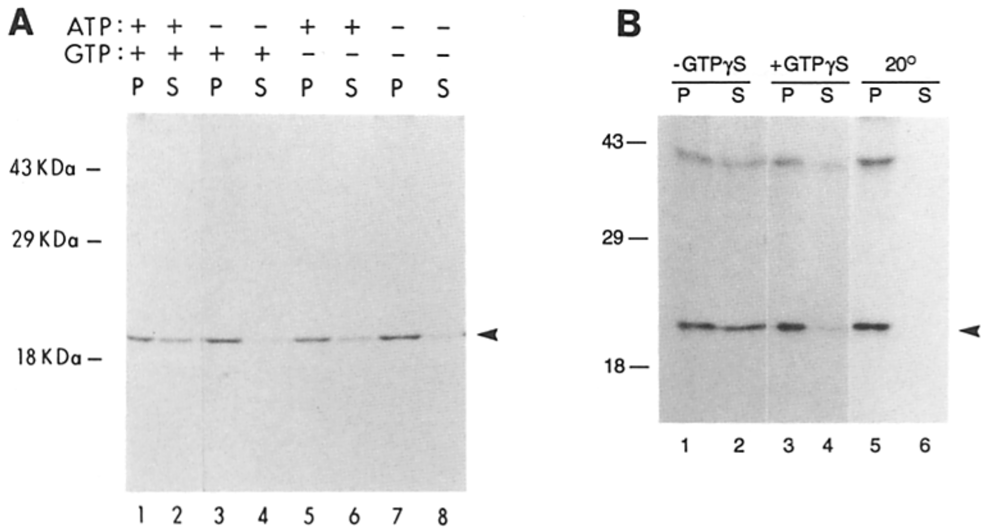


Figure 9. Requirements for vesicle budding from the TGN. (*A*) Energy requirements: Pulse-labeled cells, chased for 2 h at 20°C, were permeabilized and then incubated with cytosol at 37°C for 2 h in the absence and presence of ATP and GTP as indicated. At the end of the incubation, samples were centrifuged for 2 min at 15,000 *g*, the supernatants and pellets were treated with anti-GH serum. (*B*) Vesicle budding is inhibited by GTPγS: Pulse-labeled permeabilized cells, prepared as in *A* (Methods), were incubated for 2 h at 37°C with cytosol, ATP, and GTP (lanes *1* and *2*) or at 20°C (lanes *6* and *7*) or with cytosol, ATP, GTP, and 20 μM GTPγS (lanes *3* and *4*). The samples were then centrifuged, and the supernatant and pellets treated with anti-GH serum and the immunoreactive material analyzed by SDS-PAGE, followed by fluorography. The arrowheads (*A* and *B*) indicate the position of rat GH.

above hypothesis, incubation with either CCCP or chloroquine decreased proSRIF cleavage by ∼65–80%, respectively, and pretreatment with low concentrations of N-ethylmaleimide inhibited processing by ∼50%. These results suggest that at least part of the requirement for ATP hydrolysis is to generate an acidic milieu in the lumen of the TGN.

Formation of Nascent Secretory Vesicles

To determine if this system could support vesicle budding from the TGN and formation of immature secretory granules, cells were pulse labeled with [^{35}S]cysteine, chased for 2 h at 20°C, permeabilized, and incubated for 2 h at 37°C. At the end of the incubation, aliquots were digested with proteinase K in the absence and presence of 1% Triton X-100 followed by brief centrifugation in a microfuge at 15,000 *g*. Control samples were incubated in the absence of proteinase K followed by brief centrifugation.

Aliquots of each pellet and supernatnant were then treated with either anti-GH (Fig. 8) or anti-SRIF antibodies (Fig. 10). Our rationale was that small nascent secretory vesicles would not sediment after short periods of centrifugation (Methods) and the polypeptides contained within the lumen would be protease resistant if the membrane vesicles formed in vitro were sealed. Approximately 40–60% of the GH-immunoreactive material was found in the supernatant (lanes *1* and *2*) and ∼70% of this latter material was resistant to proteinase K digestion (lanes *3* and *4*). All the protease-resistant GH was present in membrane bound vesicles since it was digested quantitatively when proteolysis was performed in the presence of Triton X-100 (lanes *5* and *6*). Consistent with our data from whole cells, there was no vesicle budding when the permeabilized cells were incubated at 20°C (lanes *7* and *8*). These data demonstrated that GH is packaged into nascent secretory vesicles in vitro and that vesicle release is temperature-dependent.

In contrast to prohormone cleavage which required only ATP, vesicle budding from the TGN was dependent on both ATP and GTP (Fig. 9 *A*; lanes *1* and *2*). Omission of ATP (lanes *3* and *4*), GTP (lanes *5* and *6*), or both (lanes *7* and *8*) from the incubation resulted in only minimal (∼8–12%) or no vesicle formation. Based on observations using PC-12 cells, we expected that budding of nascent secretory vesicles from the TGN would be inhibited by non-hydrolyzable analogues of GTP such as GTPγS (6, 25, 64). The permeabilized GH_3 cells were incubated with GTPγS in the presence of GTP and the formation of immature vesicles monitored (Fig. 9 *B*). Vesicle budding was inhibited significantly by addition of GTPγS (compare lanes *2* and *4*) suggesting that GTP-binding proteins are involved in this process. Quantitation of the data in Figs. 8 and 9, by densitometry (Fig. 10 *A*), demonstrated an absolute requirement for both ATP and GTP in the formation of nascent secretory vesicles. To determine if mature SRIF and the uncleaved prohormone were also packaged into immature secretory vesicles, the pellets and supernatant fractions from the above incubations were also analyzed for SRIF-immunoreactive material using HPLC methods and the data quantitated (Fig. 10, *B* and *C*). As with GH-containing vesicles, the formation of proSRIF or mature SRIF-containing vesicles required ATP and GTP. Vesicle release was inhibited by at least 50% when the reaction contained 20 μM GTPγS (Fig. 10 *B*); however, neither GTPγS nor GDP-CP (non-hydrolyzable GTP analogues) affected proSRIF processing (Fig. 6). As expected SRIF-vesicle formation was inhibited upon incubation at 20°C (Fig. 10 *C*).

When intact cells were preincubated at 20°C and shifted back to 37°C, ∼15% of GH was secreted by 15 min (Fig. 2). In contrast, the kinetics of vesicle budding in permeabilized cells demonstrated that there was a lag of 15–20 min before the appearance of GH-containing vesicles upon incubation at 37°C (Fig. 11). This suggests that the membranes have to be "primed" perhaps by recruitment of cytoplasmic factors (see below) before vesicles can bud from the TGN. After the lag, vesicle budding was linear for up to 120 min at which time there was an abrupt plateau suggesting that the putative factor or factors may have become rate-limiting.

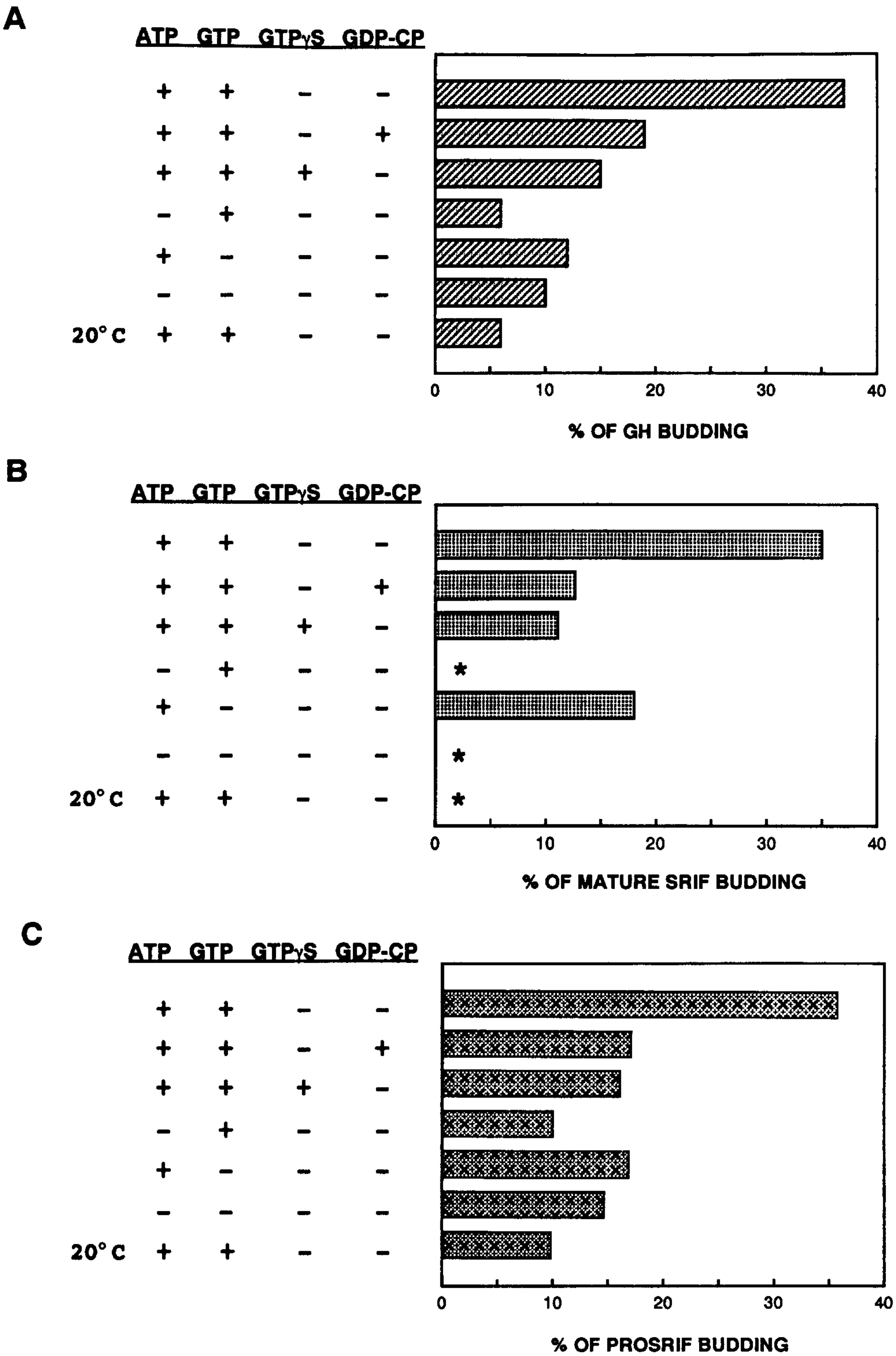


Figure 10. Budding efficiency of nascent secretory vesicles containing GH- and SRIF-polypeptides. Pulse-labeled cells were incubated at 20°C for 2 h after which they were permeabilized and further incubated at 37 or 20°C for an additional 120 min under the indicated conditions. Samples were then separated into pellet and supernatant fractions by centrifugation at 15,000 *g* for 2 min, treated with anti-GH or anti-SRIF serum. The immunoprecipitates were resolved by SDS-PAGE (GH) or HPLC (mature and pro-SRIF). (*A*) The intensity of each GH band in the pellet and supernatant fractions, in fluorographs identical to those shown in Figs. 8 and 9, was determined by densitometry. Percent budding is defined as:

$$\frac{\text{GH intensity in supernatant}}{\text{GH intensity in pellet+supernatant}} \times 100\%.$$

(*B*) Budding of vesicles containing mature SRIF.

$$\%\ \text{budding} = \frac{\text{mature SRIF in supernatant}}{\text{mature SRIF in pellet+supernatant}} \times 100\%.$$

The asterisks indicate that mature SRIF was undetectable since proSRIF cleavage does not occur under these conditions. (*C*) Budding of pro-SRIF containing vesicles.

$$\%\ \text{budding} = \frac{\text{proSRIF in supernatant}}{\text{proSRIF in pellet+supernatant}} \times 100\%.$$

In each case the data are the average of two separate experiments.

Role of Cytosolic Components in Vesicle Budding

The preceding budding assays were performed in reactions supplemented with cytosol (a post-ribosomal supernatant). Based on other studies in which intracellular vesicular transport (8, 35, 47, 69) had an absolute requirement for cytosol, we hypothesized that cytosolic components would be necessary to facilitate budding from the TGN. Surprisingly, when the permeabilized cell system was washed sequentially three times with buffer containing 100 mM KCl and the washed preparation incubated in the absence of cytosol, vesicle formation still occurred (Fig. 12; lanes *7–12*). Indeed under these conditions there was an ∼50% increase in vesicle budding efficiency compared to control levels (Table I). Furthermore, there was no stimulation of vesicle formation above control levels when the washed permeabilized cells were incubated in the presence of cytosol. This rather unexpected result suggested that if cytosolic factors were necessary for vesicle formation, they would be bound tightly to the TGN

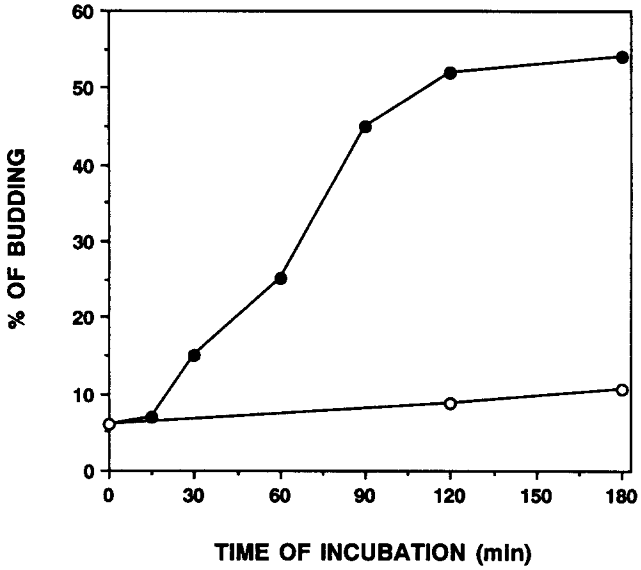


Figure 11. Kinetics of nascent vesicle formation. Pulse-labeled permeabilized cells were incubated in the presence of cytosol, ATP, and GTP at 37°C (●—●) or at 20°C (○—○). At the indicated times, aliquots were removed and separated into supernatant (vesicle) and pellet fractions by centrifugation at 15,000 *g* for 2 min. Each fraction was treated with anti-GH serum and the immunoprecipitable material resolved by SDS-PAGE followed by fluorography. The fluorogram was then analyzed by densitometry.

membranes. To test this hypothesis, permeabilized cells were treated with high salt buffer (400 mM KCl) (Fig. 12, lanes *1–6*) and the high salt-treated permeabilized cells assayed for vesicle budding in the absence or presence of control cytosol or the dialyzed salt extract. Consistent with our hypothesis, the high salt-treated preparation was unable to support vesicle budding in the absence of added cytosol (lanes *3* and *4*). Furthermore, vesicle budding could be restored by addition of cytosol (compare lanes *1* and *2* and *3* and *4*). Most significantly, when the dialyzed salt extract was added to the salt-washed preparation, vesicle budding was restored to control levels (lanes *5* and *6* and Table I). As expected, high salt treatment did not inhibit proSRIF processing to the mature peptide (Table I) and there was no stimulation of prohormone processing by incubation in the presence of the salt extract. These data are in agreement with our hypothesis that cytosolic components required for vesicle budding are tightly bound to the surface of the TGN membrane.

Discussion

Recent evidence using EM immunogold techniques suggests that prohormone processing occurs in or is initiated in the TGN (31, 49, 65), although earlier morphological studies (43, 45) had implicated immature secretory granules as the site of proinsulin processing. Despite the recent identification of several key proteases involved in prohormone processing (51, 57), relatively little is known about the interaction of these enzymes with their prohormone substrates. For example, it is unclear how prohormones and the processing enzymes are sorted in the TGN and the mechanism of selective packaging of mature hormone into nascent secretory granules is poorly understood. To understand the temporal and spatial relationship between prohormone processing and formation of nascent secretory vesicles, we have employed a permeabilized cell system similar to that used by other investigators to investigate ER to Golgi transport, and exocytotic pathways (8, 35). By using a permeabilized cell system, our goal is to identify the intracellular site of and factors necessary for prohormone processing. Furthermore, such an approach should also facilitate identification of vesicular intermediates that mediate selective sorting and packaging of peptide hormones into immature secretory granules. During protein sorting into nascent secretory granules, peptide hormone precursors undergo one or several posttranslational modifications to generate a biologically active molecule from the relatively inert precursor. Consequently, the identification of the site and mechanism

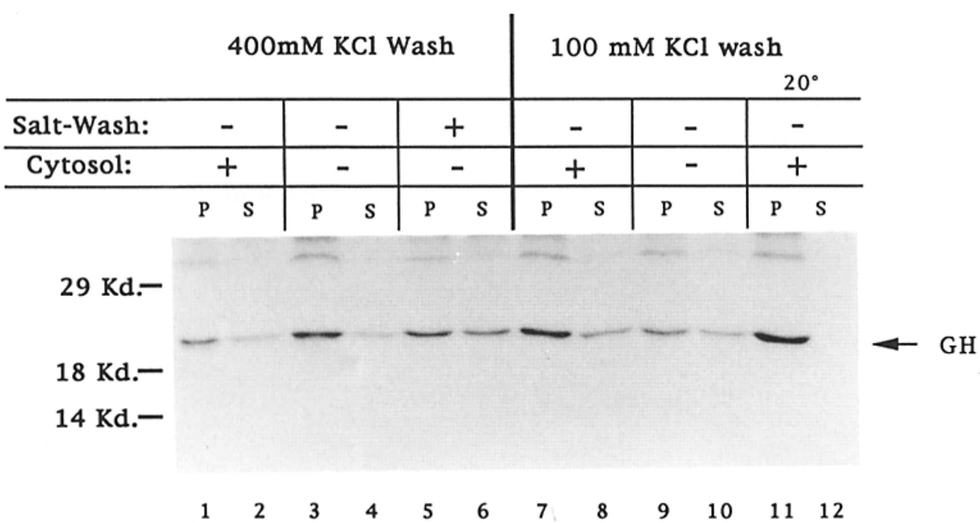


Figure 12. Cytosolic factors are required for nascent secretory vesicle formation. Pulse-labeled cells were incubated at 20°C for 2 h, permeabilized, and washed three times with physiological salt (100 mM KCl, lanes *7–12*) or once with high salt (400 mM KCl, lanes *1–6*). The 100 mM KCl treated permeabilized cells were incubated for 2 h at 37 or 20°C (lanes *11* and *12*) in the presence (lanes *7, 8, 11,* and *12*) and absence (lanes *9* and *10*) of 1 mg cytosolic protein/ml. High salt-treated permeabilized cells were incubated at 37°C with 1 mg/ml control cytosol (lanes *1* and *2*), no cytosol (lanes *3* and *4*) or with 0.25 mg protein/ml of dialyzed high salt extract (lanes *5* and *6*). All samples contained ATP and GTP. After incubation, samples were separated into pellet and supernatant fractions which were treated with anti-GH serum. The immunoprecipitable GH material was resolved by SDS-PAGE followed by fluorography. The arrow indicates the position of rat GH.

Table 1. Effect of High Salt Treatment on proSRIF Processing and Vesicle Budding from the TGN

Treatment	% proSRIF‡ Processing		% Vesicle‖ Budding	
		% Control		*% Control*
Control*	34.8	100	24.2	100
Control, no cytosol	32.7	94	37.0	152
High salt + control cytosol§	27.8	80	27.6	114
High salt wash, no cytosol	31.1	89	9.4	39
High salt wash + high salt extract	29.6	85	30.0	124
20°C	4.6	13	3.1	13

* Control system: Permeabilized cells, washed three times with physiological salt and incubated with 1 mg cytosolic protein/ml, 1 mM ATP, 0.02 mM GTP, for 120 min at 37°C.
‡ Determined from a single experiment.
§ High salt wash: ∼2-5 × 10^5 permeabilized cells were treated with 400 mM KCl, resuspended in reaction buffer (see Methods), and incubated as for control samples. The 400 mM KCl extracted material was dialyzed extensively against incubation buffer (100 mM KCl, 2 mM $MgAc_2$, 0.5 mM $CaCl_2$, 10 mM Hepes, pH 7.4) before adding to the salt-treated permeabilized cells.
‖ Average of two experiments.

of prohormone processing and formation of nascent secretory granules represents a critical step in understanding hormone action.

The 20°C Block Prevents Prohormone Processing

To address the first question, namely the site of prohormone cleavage, we exploited an experimental protocol that has been used to trap viral envelope glycoproteins in the TGN, i.e., a 20°C block (18). By incubating cells at 20°C, we were able to accumulate quantitatively both GH and proSRIF intracellularly. Under these conditions there was no prohormone processing or secretion. Several lines of evidence are consistent with the transport and processing block occurring in the TGN. First, proSRIF and GH cofractionated on equilibrium sucrose gradients with the distal Golgi/TGN marker enzymes galactosyl and sialyl transferases as well as the marker protein TGN-38. Second, processing occurred in a "late compartment" that was resistant to the drug BFA. At early chase times proSRIF cleavage was inhibited by BFA whereas at 90 min, processing was BFA-resistant. Third, the 20°C block itself has been demonstrated to be selective for inhibiting vesicular transport from the TGN (18, 36). Finally, the most compelling evidence that prohormone cleavage is initiated in the TGN comes from experiments in which efficient processing occurred in the presence of ATP alone (Fig. 6) or after high salt treatment of the permeabilzed cells (Table I). Under either of these conditions, there was no vesicle budding from the TGN, yet proSRIF cleavage was ∼40–50% efficient. Taken together, our results are consistent with the hypothesis that cleavage is initiated in the TGN and are in agreement with recent EM immunogold studies (31) demonstrating that proSRIF cleavage occurred in this organelle in rat hypothalamic neurons. However, our data do not exclude the possibility that processing continues in immature and mature secretory granules (17, 43, 46).

The 20°C temperature block was particularly stringent in GH_3 cells and only minimal levels of processing and secretion (∼6%) were seen even after 120 min incubation at this temperature. Our data therefore demonstrate that the 20°C block precedes the prohormone processing step in the secretory pathway. These results contrast with earlier reports that employed mouse pituitary AtT-20 cells (70) or primary cultures of rat cortical or hypothalamic cells (31). In AtT-20 cells, POMC processing was reduced at 20°C because it was retained in the ER; however, if POMC progressed to the Golgi apparatus, although its cleavage kinetics were slowed, processing was not inhibited (70). Surprisingly, incubation of rat brain cortical fragments at 19°C resulted in the intracellular accumulation of mature SRIF-immunoreactive polypeptides. In particular the content of SRIF-28, an NH_2-terminally extended form of the tetradecapeptide, was increased significantly at reduced temperatures (31). The reason for the differences in temperature sensitivity between various cell lines is unclear at present but might reflect differences in the respective lipid composition of their Golgi membranes. Most importantly, the use of the 20°C block to accumulate proSRIF in the TGN enabled us to develop a permeabilized cell system for investigating prohormone cleavage and formation of nascent secretory vesicles.

ProSRIF Cleavage in Permeabilized Cells

GH_3 cells express the PC2 prohormone processing enzyme exclusively (52) and proSRIF cleavage occurred with ∼35–50% efficiency in the permeabilized cells (Figs. 5 and 7). In contrast to vesicular transport which requires cytosolic factors, ATP, GTP, and GTP-binding proteins (5, 47), proSRIF processing needed only ATP hydrolysis (Fig. 7). We propose that the requirement for ATP hydrolysis is, in part, to provide energy for a vacuolar type H^+-ATPase present in the Golgi membrane (72). Since it is likely that the PC2 processing enzyme and proSRIF are in close proximity within the TGN, we postulate that efficient cleavage requires only conditions that are necessary for maximal enzyme activity. Presumably this can be achieved by the H^+-ATPase which pumps protons into the lumen of the TGN thereby generating an acidic pH to activate the PC2 enzyme. Our data are consistent with this hypothesis, since inclusion of either the weak base chloroquine, the protonophore CCCP, or *N*-ethylmaleimide (an inhibitor of vacuolar ATPases) in the incubation resulted in substantial (60–80%) inhibition of proSRIF processing. We speculate that the acidic pH may also induce conformational changes within proSRIF that expose paired basic residues to the processing enzyme. In addition, the acidic pH may function in facilitating vesicular transport per se. In support of this hypothesis, Zeuzem et al. (76) showed that ADP-ribosylation factor, a small GTP-binding protein that functions in Golgi vesicle transport (30), required a low pH in the *trans*-Golgi to promote its membrane binding.

Our present data agree with previous reports from our laboratory and others (3, 40, 60) using whole cells which demonstrate that an acidic milieu is required for prohormone processing and packaging to the regulated secretory pathway. In contrast, Mains and May (34) have provided evidence that POMC processing and storage of mature peptides did not require a low pH compartment. However, our observation that an acidic pH in the TGN is required for processing is also in agreement with earlier reports that proinsulin processing in isolated islet β-cell secretory granules required ATP to activate an H^+-ATPase which generated an acidic pH necessary for enzyme activity (46). Davidson et al. (17) showed that β-cell granules possess two proinsulin processing enzymes, designated type I and II, which were subsequently shown to correspond to PC1/PC3 and PC2, respectively (4, 10). It is noteworthy that the islet type II activity (PC2) was optimal at ~pH 6.0–6.2 and at a Ca^{2+} concentration compatible with conditions prevailing in the late Golgi apparatus (17). Our demonstration that permeabilized GH_3 cells, which express only the PC2 enzyme, require an acidic pH in the TGN for proSRIF processing are quite consistent with these earlier reports and more recent data showing that purified PC1/PC3 and PC2 exhibit acidic pH optima (4, 55, 74). Surprisingly, there was little or no proSRIF processing at 20°C either in intact GH_3.S18.9 cells or in the permeabilized cell system, even after prolonged incubation. This suggests that the 20°C block may arrest proSRIF and/or PC2 in the TGN before the site of processing and that further transport or packaging, which requires incubation at 37°C, is necessary to bring the enzyme and substrate together to effect cleavage. Given the slight lag in proSRIF processing after the shift from 20 to 37°C (Fig. 2), this explanation seems reasonable. Alternatively, the PC2 enzyme itself may be temperature sensitive; further experiments are in progress to address these points.

Formation of Nascent Secretory Vesicles

In polypeptide hormone secreting cells two classes of secretory vesicles have been characterized on the basis of their kinetics of exocytosis, namely constitutive- and regulated-pathway vesicles. Mature regulated secretory vesicles can be readily identified morphologically by their dense core semicrystalline protein content. These vesicles are stored intracellularly and only released upon stimulation. In contrast, constitutive vesicles are less dense than regulated vesicles, are not stored, and continuously deliver their contents to the plasma membrane (13). Although these two pathways have been studied in some detail for the past several years, little is known about the molecular mechanism by which the cell discriminates between proteins destined for different vesicle populations. Morphological evidence has demonstrated that differential sorting and packaging of proteins occurs in the TGN (24, 44, 56) and the expression of chimeric proteins in heterologous cells suggests that the propeptide of some precursors may play a role in targeting to the regulated pathway (50, 54, 61).

We also used the permeabilized GH_3 cells to investigate the relationship between prohormone processing and formation of secretory granules. Unlike PC12 cells where secretory vesicle formation could be achieved using a cell homogenate (64), we were unable to obtain vesicle budding if a post-nuclear supernatant was used rather than permeabilized cells. Similar observations have been made for ER to Golgi transport (5); thus in GH_3 cells it may be necessary to maintain the structural integrity of the Golgi apparatus to facilitate vesicle budding (5, 35). Vesicle budding and release of nascent secretory granules required GTP and cytosolic factors in addition to ATP (Figs. 10 and 12). These results show that prohormone processing in the TGN precedes vesicle budding and that these two processes are separate biochemical reactions. Consequently, it should now be possible to separate immature secretory granules from constitutive vesicles as well as isolate vesicular intermediates in the formation of nascent secretory vesicles. Such studies are currently in progress.

Several lines of evidence suggest that vesicle budding did not result from leakage of content from the TGN or from non-specific fragmentation of the Golgi apparatus: (*a*) the released GH, proSRIF, and mature SRIF were resistant to protease digestion (Fig. 8) and were only protease sensitive in the presence of Triton X-100, confirming that these polypeptides are enclosed in membrane-bounded vesicles; (*b*) we observed no budding at 20°C even in the presence of ATP, GTP, and cytosol showing that budding requires physiological temperatures; (*c*) there was little or no budding in the absence of ATP or GTP; and (*d*) high salt wash of the permeabilized cells inhibited vesicle formation significantly at 37°C, demonstrating cytosolic factors are necessary to promote vesicle budding. Finally, the nascent secretory vesicles contained <20% of total sialyl transferase activity (data not shown) suggesting that most of the Golgi vesicles remained associated with the permeabilized cells.

Inhibition of Vesicle Formation by GTPγS

In recent years, several laboratories have used in vitro systems to investigate both regulated and constitutive secretory vesicle budding from the TGN (23, 25, 38, 64) and more recently fusion with the plasma membrane (27, 69). Using PC12 cell homogenates, elegant studies from Tooze and Huttner (64) demonstrated the formation of both classes of vesicle which could be separated by appropriate sucrose gradients. Vesicle budding required ATP and was inhibited by GTPγS implying that GTP-binding proteins are required for this process (66). The immature secretory vesicles derived from the TGN were also demonstrated to be intermediates in the formation of mature secretory granules (67). Grimes and Kelly (25) have also obtained evidence for release of constitutive and immature regulated secretory granules derived from the TGN in vitro; in this case vesicle formation also required both ATP and GTP. Recently, heterotrimeric G-proteins have been implicated in regulating the formation of both constitutive and regulated pathway secretory vesicles (6, 25, 32, 62). In agreement with the observations of Tooze et al. (66), inclusion of GTPγS in the incubation, inhibited GH- and SRIF-containing vesicle formation by ~50%, suggesting the involvement of either small GTP-binding proteins or trimeric G-proteins in this process. Our GTPγS results and those of Tooze et al. (66) suggest that there may be a fundamental difference between the formation of post-TGN vesicles and those which mediate intraGolgi transport. In the latter case, Melançon et al. (37) showed that GTPγS did not inhibit vesicle budding from Golgi membranes in vitro, but rather lead to accumulation of coated vesicles which were unable to fuse with acceptor Golgi membranes. Furthermore, the difference in these two vesicular processes is not related to formation of regulated pathway vesicles per se, since Gravotta et al. (23) and Miller and Moore (38) showed that post TGN constitutive vesicle formation is also inhibited by GTPγS. We are currently analyzing high salt-treated Golgi membranes (see below) to identify putative GTP-binding proteins which may be specific for budding from the TGN.

Role of Cytosol

Rothman and colleagues have identified a number of cytosolic factors which have been characterized in detail and are required for vesicle budding and fusion during intraGolgi transport (47). In addition, in all permeabilized cell systems described to date, cytosolic extracts or factors have been found necessary to support vesicle budding (53), exocytosis (48, 69), and endocytosis (47). Consequently, it was surprising that upon washing the permeabilized cells sequentially with physiological strength buffers, the system maintained its capacity for vesicle budding in the absence of added cytosol (Fig. 12). Indeed, in several experiments, vesicle formation in the absence of cytosol was ∼50% higher than in the complete system (Table I). One interpretation of this data is that inhibitory factors may be loosely associated with the TGN membrane and prevent vesicle release. Such putative factors would be expected to cycle between the cytoplasm and Golgi membrane and function to prevent vesicle budding in the absence of "cargo molecules," i.e., mature hormones. In this context, it is noteworthy that Barr et al. (6) showed heterotrimeric G proteins can inhibit secretory vesicle formation in vitro in PC-12 cells. More recently, Leyte et al. (32) demonstrated that the Gαi subunit of trimeric G proteins can negatively regulate budding of immature secretory granules from the TGN of PC12 cells. In agreement with the hypothesis that vesicle formation could be regulated by GTP-binding proteins, we also observed that vesicle budding was inhibited by GTPγS. Furthermore, in the presence of GTP alone, budding was actually lower than when only ATP was present in the reaction (Figs. 3 and 5 *B*). This inhibition is consistent with the existence of a Gαi protein which could be activated by excess GTP; analysis of our Golgi preparations will determine if such a hypothesis is correct.

To explain the lack of a cytosol requirement for budding, we hypothesized that cytosolic factors may be tightly bound to the TGN and would require more stringent conditions for release. This hypothesis was correct since pretreatment of the permeabilized cells with high salt (400 mM KCl) resulted in no vesicle budding, unless the system was supplemented with either fresh cytosol or the dialyzed salt extract. In either case, vesicle formation was restored to control levels. Prohormone processing was unaffected by high salt treatment of the permeabilized cells and the efficiency of proSRIF cleavage was identical to that of controls (Table I). By analogy to intraGolgi transport (47, 71), we speculate that high salt treatment may remove coat proteins necessary for generating regulated pathway vesicles. These might include coat components common to all vesicular transport systems, as well as those unique to the regulated pathway such as specific clathrin-adaptor molecules and GTP-binding proteins. Currently, we are analyzing the salt extract to identify components that specifically mediate budding of regulated pathway secretory vesicles.

Finally, this work demonstrates that prohormone cleavage, which requires an acidic milieu, is initiated in and precedes the release of nascent secretory vesicles from the TGN. We have shown that prohormone processing and vesicle formation are separate biochemical reactions that occur during sorting to the regulated secretory pathway. Having demonstrated that both prohormone cleavage and budding of immature secretory vesicles can occur in vitro, we can now exploit this system to identify components that facilitate the packaging of peptide hormones into specific regions of the TGN; these experiments are currently in progress.

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Note Added in Proof. Kuliawat and Arvan (*J. Cell Biol.* 1992. 118:521–529) have previously demonstrated that proinsulin processing is also inhibited when isolated rat islets are incubated at 20°C and is resumed upon warming to 37°C.

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