Proteolytic Processing of Pro-ACTH/ Endorphin Begins in the Golgi Complex of Pituitary Corticotropes and AtT-20 Cells

Eva Schnabel*, Richard E. Mains, and Marilyn Gist Farquhar

Department of Cell Biology (E.S., M.G.F.)
Yale University School of Medicine
New Haven, Connecticut 06510

The Johns Hopkins University School of Medicine (R.E.M.)
Baltimore, Maryland 21205

The intracellular sites where proteolytic processing of pro-ACTH/endorphin or POMC take place have not yet been reliably identified. We have used affinity-purified antisera that recognize only the products of POMC processing and immunoelectron microscopy to identify the compartments of rat pituitary corticotropes and mouse AtT-20 cells in which cleavage occurs. Immunoperoxidase labeling of cryostat sections and immunogold labeling of ultrathin frozen sections were used for localization of the processing sites. By both procedures we detected processed peptides in Golgi cisternae and secretion granules. Within the Golgi, labeling was limited to the last or transmost cisterna and was most concentrated in its dilated rims which contain condensing secretory protein. No labeling of other Golgi cisternae was seen. All Golgi cisternae were labeled, however, when antisera that recognize unprocessed POMC were used for immunolabeling. We conclude that in AtT-20 and rat pituitary cells: 1) processing of POMC through at least two endo- and exoproteolytic cleavage steps and α-amidation of joining peptide begin in the trans Golgi subcompartment; 2) no detectable processing takes place before POMC reaches the trans Golgi cisterna; and 3) this Golgi cisterna as well as secretion granules contain the active enzymes necessary for proteolytic processing and α-amidation. (Molecular Endocrinology 3: 1223–1235, 1989)

INTRODUCTION

Many peptide hormones are synthesized as prohormone or polyprotein precursors that are proteolytically cleaved to yield active products during or before their release by exocytosis. The identification of the compartment(s) in which cleavage occurs has been a subject of considerable interest since the initial discovery of proinsulin. Although originally thought to be in part a Golgi function (1), recent biochemical, cell fractionation, and immunocytochemical data have led to the conclusion that proteolytic processing of several peptide hormones, including proinsulin (2, 3), propressophysin (4), and POMC or proACTH/endorphin (5) occurs largely or exclusively in secretion granules. The involvement of other compartments, especially the Golgi complex, in proteolytic processing has sometimes been inferred from analysis of kinetic data but has never been directly demonstrated due to the inherent limitations of these approaches. A recent immunocytochemical study (3) provided data supporting the conclusion that proteolytic processing of proinsulin occurs exclusively in secretion granules and pinpointed immature, clathrin-coated secretory vesicles as the main intragranular cleavage site. No evidence was obtained for processing within the Golgi complex.

In this paper, we have asked the question, in which compartment along the secretory pathway does processing of POMC begin? Specifically, we wondered if POMC cleavage products, *e.g.* ACTH and joining peptide (JP), can be detected in the Golgi complex? We have examined these questions by carrying out immunoelectron microscopy on rat anterior pituitary corticotropes and mouse AtT-20 cells using affinity-purified antisera to specific regions of the POMC molecule. We here report our findings based on both immunoperoxidase and immunogold localizations demonstrating that POMC processing does indeed begin in the Golgi complex, as processed POMC products are detectable in the last or transmost cisterna of this organelle.

RESULTS

Processing of POMC by Anterior Pituitary cells

The events in the proteolytic processing of POMC follow a distinct order (see Fig. 1). POMC is first cleaved

0888-8809/89/1223-1235$02.00/0
Molecular Endocrinology

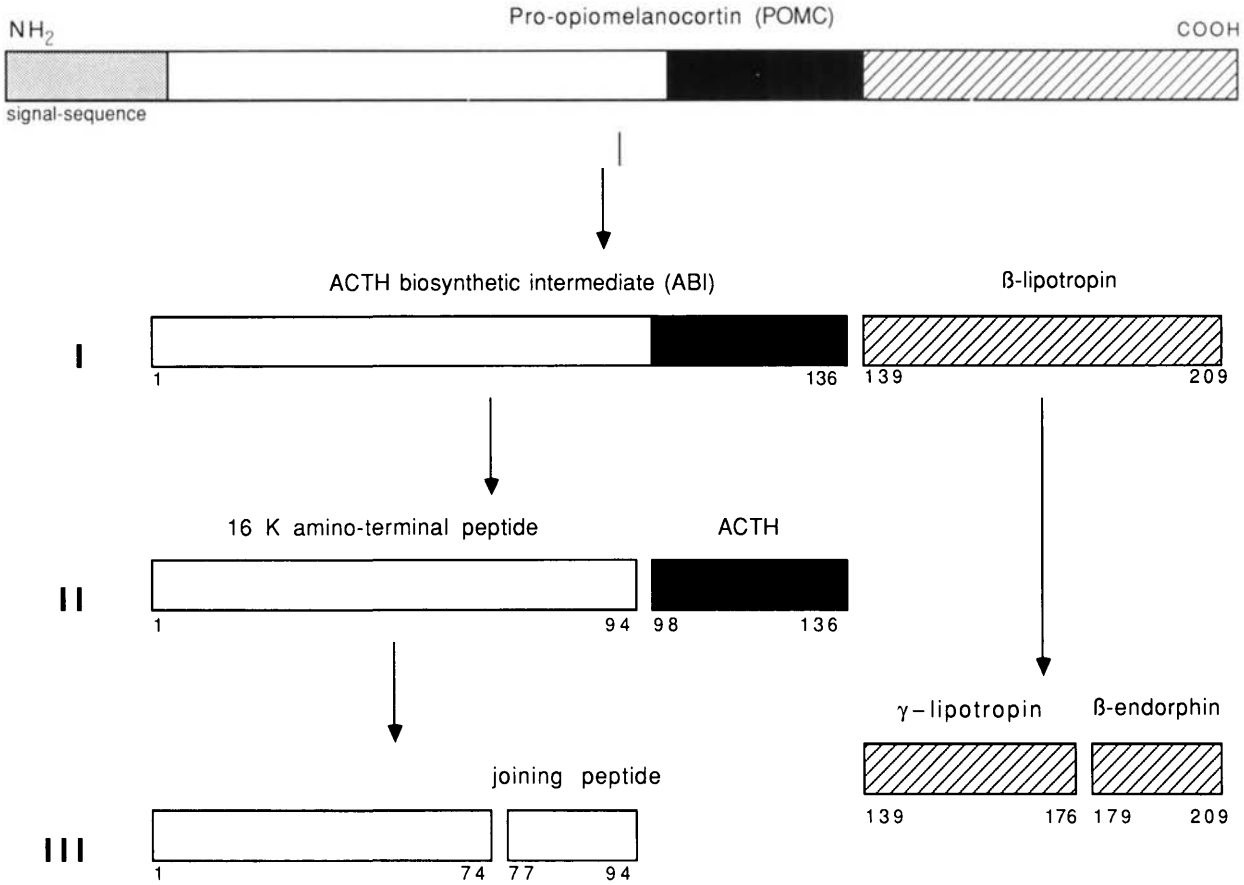

Fig. 1. Proteolytic Processing of POMC in AtT-20 Cells (Simplified) (6, 11, 26)

The amino-terminal signal sequence is cleaved during or shortly after translocation across the membranes of the rough ER. The remaining cleavages are carried out in orderly steps: 1, β-LPH and ACTH-biosynthetic intermediate (ABI) are generated. II, ACTH is cleaved from 16 K amino-terminal peptide. III, With a similar time course, the carboxy terminal end of JP is α-amidated, and cleavage at Arg^{75}-Arg^{76} occurs. Some of the β-LPH is processed further to β-endorphin in a rather slow time course.

in the region Lys^{137}-Arg^{138} to produce ACTH biosynthetic intermediate [ABI, POMC(1-136)] and β-lipotropin (β-LPH). Next, cleavage of ABI occurs in the region Lys^{96}-Arg^{97} to produce ACTH(1-39) and a 16 K amino-terminal peptide [POMC(1-95)]. Immunoprecipitation and tryptic peptide analysis have shown that more than 95% of the ABI is cleaved to produce POMC(1-95) and ACTH, rather than POMC(1-74) and a molecule containing JP and ACTH (6). In the final proteolytic processing event, 16 K amino-terminal peptide [POMC(1-95) and POMC(1-94)NH_2] is cleaved in the region Arg^{75}-Arg^{76} to produce a mixture of JP-(1-18)NH_2 and JP-(1-19). About 50% of the JP is α-amidated before cleavage of 16 K amino-terminal peptide [POMC(1-94)NH_2], and the remainder is cleaved before being α-amidated (6). JP is the major amidated peptide produced by cleavage of POMC in anterior pituitary corticotropes. In the anterior pituitary only a fraction of the β-LPH is processed further to β-endorphin with a rather slow time course. Cleavage of joining peptide from ACTH(1-39) is essentially complete before significant cleavage of β-LPH to β-endorphin occurs. This late and partial cleavage of β-LPH produces a mixture of β-LPH, β-endorphin, and γ-LPH. In the corticotropes of the anterior pituitary the cleavage of POMC ends here.

Antibody Specificity

The specificity of the antisera for processed products is critical to the interpretation of the staining patterns observed (see Table 1). Therefore specificity was assessed by immunoprecipitation as well as by immu-

Table 1. Specificities of Pro-ACTH/Endorphin Antisera[a]

	POMC	β-LPH	ABI	ACTH	16K	αJP	β-Endorphin	Ref.
Danielle	+	+	−	−	−	−	+	7
Rebecca	+	+	−	−	−	−	+	43
Georgie	+	−	+		+	+	−	8
Kathy	−	−	+	+	−	−	−	10
Jamie	−	−	−	−	+[b]	+	−	9

[a] Positive binding (+) has been defined by immunoprecipitation (as in Fig. 2), by cross-reaction in RIA at antibody concentrations comparable to those used for immunocytochemistry, by graded abolition of immunostaining in the presence of 10–200 nM peptide (6-10, 43) and failure of irrelevant peptides to block staining. Lack of binding (−) indicates lack of immunoprecipitation at high antibody concentration, and failure to show any signal in RIA.

[b] Jamie detects POMC(1-94)NH_2 but not POMC(1-95), both of which are in the 16K pool originally defined by use of antibody Georgie (6, 8, 9).

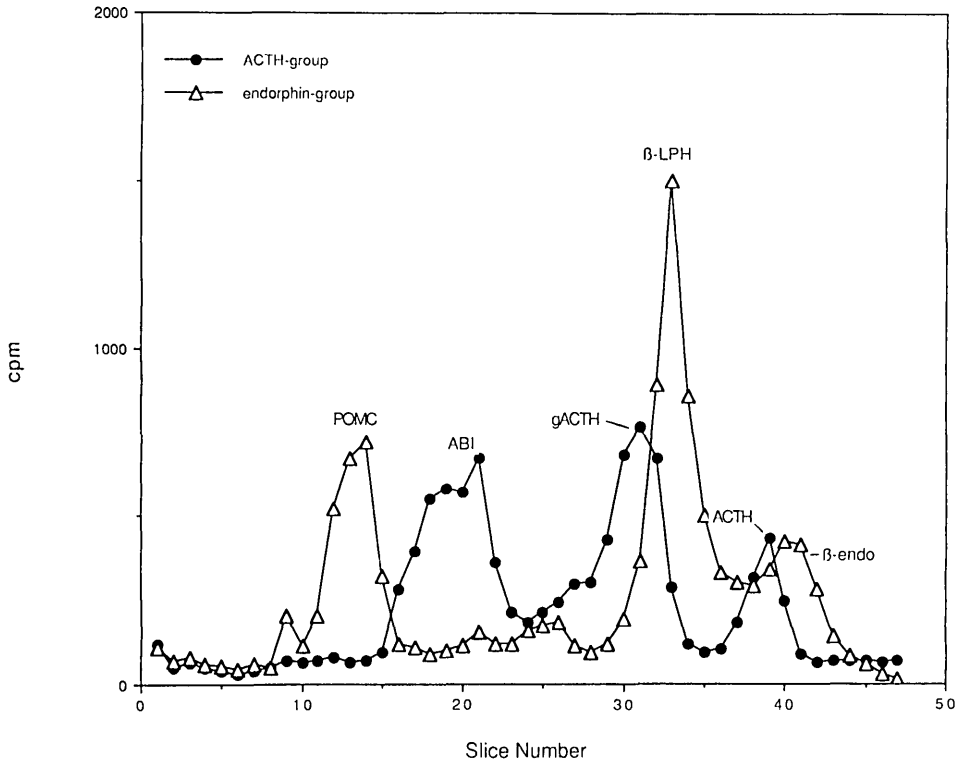

Fig. 2. Immunoprecipitation of Secretion Products from AtT-20 Cells

AtT-20 cells were incubated for 20 h in DMEM lacking phenylalanine and containing 60 μM [^{3}H]Phe (25 Ci/mmol) plus 10% NuSerum. Equal aliquots (20 μl of the 200 μl incubation medium) were immunoprecipitated with affinity-purified β-endorphin antiserum or affinity-purified C-terminal ACTH antiserum (~2 μg/ml). ACTH-group antibody Kathy (●) recognizes mature ACTH as well as ABI (products of the first cleavage step), but not intact POMC; β-endorphin group antibody Rebecca (△) recognizes POMC as well as β-LPH and β-endorphin. Samples were analyzed by sodium dodecyl sulfate gel electrophoresis, followed by elution and liquid scintillation counting. The peaks of radioactivity have been identified previously by peptide mapping and microsequencing (11). ACTH and gACTH, nonglycosylated and glycosylated ACTH(1-39); β-endo, β-endorphin(1-31).

noassay (Kathy, Jamie). AtT-20 cells incubated in medium containing [^{3}H]phenylalanine secreted labeled POMC as well as processed products into the medium (Fig. 2). Even at the high antibody concentrations used, the antiserum specific for the COOH-terminus of ACTH detected ABI, glycosylated ACTH and ACTH and failed to precipitate POMC (Fig. 2). Similarly stringent specificity was previously demonstrated for the α-amidated JP antibody (6).

AtT-20 Cells as a Model System for the Cleavage of POMC

In the anterior pituitary the corticotropes account for only 2–10% of the total cells. AtT-20 cells, a mouse anterior pituitary cell line, are known to synthesize POMC and proteolytically cleave it to mature end products in the same way as the corticotropes in the normal anterior pituitary. The pattern of immunoactive ACTH-related molecules found in AtT-20 cells is very similar to that shown for rat and mouse anterior pituitary extracts (7–11). In addition, the cells are known to package these products into morphologically recognizable secretion granules (12) which are discharged by regulated exocytosis (13, 14). Thus these cells represent a valid and convenient model system for corticotropes of the anterior pituitary, and they are frequently used for this purpose (11, 14, 15).

AtT-20 cells grown as a sparse monolayer form clusters connected by elongated cell processes with dilated tips (Fig. 3A). Hormone staining is most concentrated in the dilated tips of the cells, but staining specific for cleaved products from POMC is also present in perinuclear regions (16). By electron microscopy the cells are seen to have abundant rough ER and an elaborate Golgi complex which is organized into a stack of 4-6 cisternae (Fig. 3B). As in the case of other anterior pituitary cell types (17–19), condensing, immature secretory granules can often be found budding from the last or transmost Golgi cisterna (Fig. 3B). This cisterna is sometimes but not always set off from the stack, and it is often more reticular and less platelike than the stacked cisternae. It has been variously referred to as the trans Golgi reticulum (20), trans tubular network (21, 22), or trans Golgi network (23). Sometimes the membrane of the budding granules has a cage-like coat composed of clathrin and clathrin-associated proteins (12). A few secretion granules in different stages of

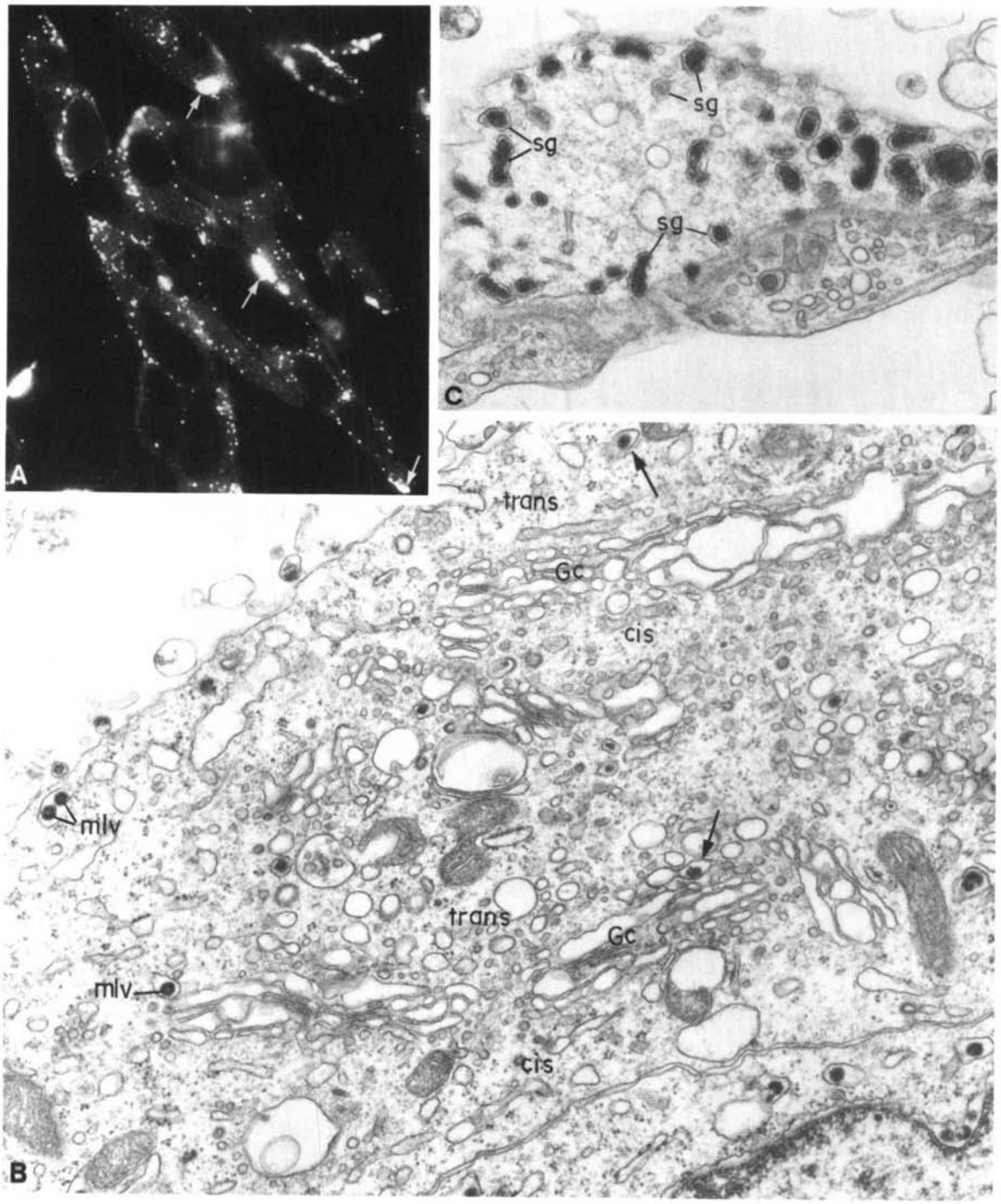

Fig. 3. AtT-20 Cells as Seen by Immunofluorescence and Electron Microscopy

A, Immunofluorescence localization of ACTH/ABI on a semithin (0.5 μm) frozen section demonstrating the distribution of secretion granules which are most concentrated at the tips of the cell processes (*arrows*), but are also present elsewhere in the cell. Primary antibody: Kathy. B, Golgi region of an AtT-20 cell showing two sets of stacked Golgi cisternae (Gc). Secretory granules can be seen on one side of each of the stacks (*arrows*) which can be identified as the trans (trans) side based on the presence of the granules. The cells also contain an endogenous murine leukemia virus (mlv) which can be recognized by its donut-shaped appearance. cis, cis or entry side of the Golgi complex. C, Electron micrograph of a dilated tip from a cell process of an AtT-20 cell, showing abundant secretion granules (sg) which are the source of the bright fluorescence signal seen in A. Ultrathin section from a culture fixed and embedded in the culture dish. A, ×217; B, ×30,000; C, ×32,500.

maturation typically are found scattered throughout the cytoplasm, and mature granules are concentrated in the dilated tips of the cell processes (Fig. 3C).

AtT-20 cells also produce an endogenous C-type virus immunologically very similar to Rauscher and Moloney murine leukemia virus (13, 24). The virus particles are recognizable in electron micrographs and distinguishable from secretion granules by their donut-shaped appearance (Fig. 3B).

Immunofluorescence Localization of POMC and its Products

All cleavage products of POMC studied in this investigation could be localized by immunofluorescence on semithin (0.5-1 μm) frozen sections of rat anterior pituitary and AtT-20 cells. In AtT-20 cells, immunofluorescence staining was typically distributed throughout the cytoplasm in a bright, punctate fluorescence pattern but was most concentrated in the dilated tips of the cell processes where the granules are located (Fig. 3A). In the corticotropes of the anterior pituitary the brightest fluorescence was seen along the cell border where the granules are normally located.

POMC-Related Peptides are Found throughout the Golgi Complex

In order to identify the intracellular compartments involved in cleavage of POMC we employed immunoperoxidase and immunogold labeling techniques to follow the distribution of POMC and several of its cleavage products in the rat anterior pituitary and mouse AtT-20 cell line.

When antibodies (Georgie or Danielle) which detect both the precursor POMC molecule and its processed products were used in immunoperoxidase experiments on AtT-20 cells, reaction product was seen throughout the Golgi stacks as well as over mature and condensing secretion granules (Fig. 4A). Within the Golgi complex, reaction product appeared to be most concentrated in the last cisterna on the trans or exit side of the stack which is typically associated with forming secretion granules. The same results were obtained by immunogold labeling of ultrathin frozen sections of both AtT-20 cells and corticotropes of the rat anterior pituitary (Fig. 4B): Golgi cisternae and all secretion granules independent of their stage of maturation were labeled with colloidal gold. Also, counts of gold particles over mature granules or over secretory protein condensing in the trans Golgi cisternae indicated that the labeling density was two to three times that in the cis and middle cisternae. This is in keeping with the fact that considerable (*i.e.* up to 200X) concentration takes place during granule packaging in anterior pituitary cells (25). These findings demonstrate that 1) POMC-related molecules are found throughout the stacked Golgi cisternae, and 2) concentration of POMC and/or its products occurs in the trans cisternae.

Processed ABI and ACTH Can Be Detected in the Trans Golgi Cisternae

When antibodies (Kathy) which recognize only processed products (ABI and ACTH) but not POMC were applied in immunoperoxidase experiments on AtT-20 cells (Fig. 5), reaction product was also regularly found over the Golgi cisternae as well as over secretion granules. In this case, however, staining of the Golgi was limited to the last or transmost cisterna where the reaction product was most concentrated over the secretory protein condensing in their dilated rims (Fig. 5, A and C). No labeling was seen in the remaining cisternae of the Golgi stack. Since the antibody used (Kathy) is specific for products of the first POMC cleavage step (ABI and ACTH), at least a fraction of the precursor must have already undergone cleavage in the transmost Golgi cisterna to create enough ABI and ACTH to be immunodetectable. Both endoproteolytic and exoproteolytic (carboxypeptidase B-like) activity is required to generate the antigenic determinant recognized by this antibody.

These findings were confirmed by the immunogold labeling technique carried out on ultrathin frozen sections of AtT-20 cells (Fig. 6A). Not only were all secretion granules covered with gold particles, but also the transmost Golgi cisterna was labeled, especially over its dilated rims where condensing secretory protein was visible.

The same findings pertain to corticotropes *in situ* in the rat anterior pituitary: processed forms of POMC could be detected in the dilated rims of the trans Golgi cisterna of pituitary corticotropes (Fig. 6B). This indicates that the findings are general and not the result of aberrations in the secretory pathway in the tumor-derived AtT-20 cells.

α-Amidated JP Can Also Be Detected in the Trans Golgi Cisterna of Corticotropes

We took advantage of the availability of an antibody (Jamie) which is highly specific for the α-amidated C-terminal of JP to localize a product of POMC processing whose formation requires endoproteolysis, exoproteolysis, and α-amidation (6, 9, 26). In immunogold labeling experiments on rat anterior pituitary corticotropes, we found, once again, that labeling with gold particles could also be detected in the dilated rims of the transmost Golgi cisterna and was not restricted to the secretion granules (Fig. 7). The distribution observed with the α-amidated JP-specific antibody was very similar to that seen with ACTH-specific antibodies. Since this antibody only recognizes the α-amidated C-terminal of JP and does not recognize POMC or nonamidated cleavage products (6, 9) these findings indicate that three enzymatic steps; endoproteolysis, exoproteolysis, and α-amidation, all begin in the transmost Golgi cisterna. It follows that the transmost Golgi cisterna must contain active forms of the enzyme(s) necessary for two sequential endoproteolytic cleavages of POMC and for

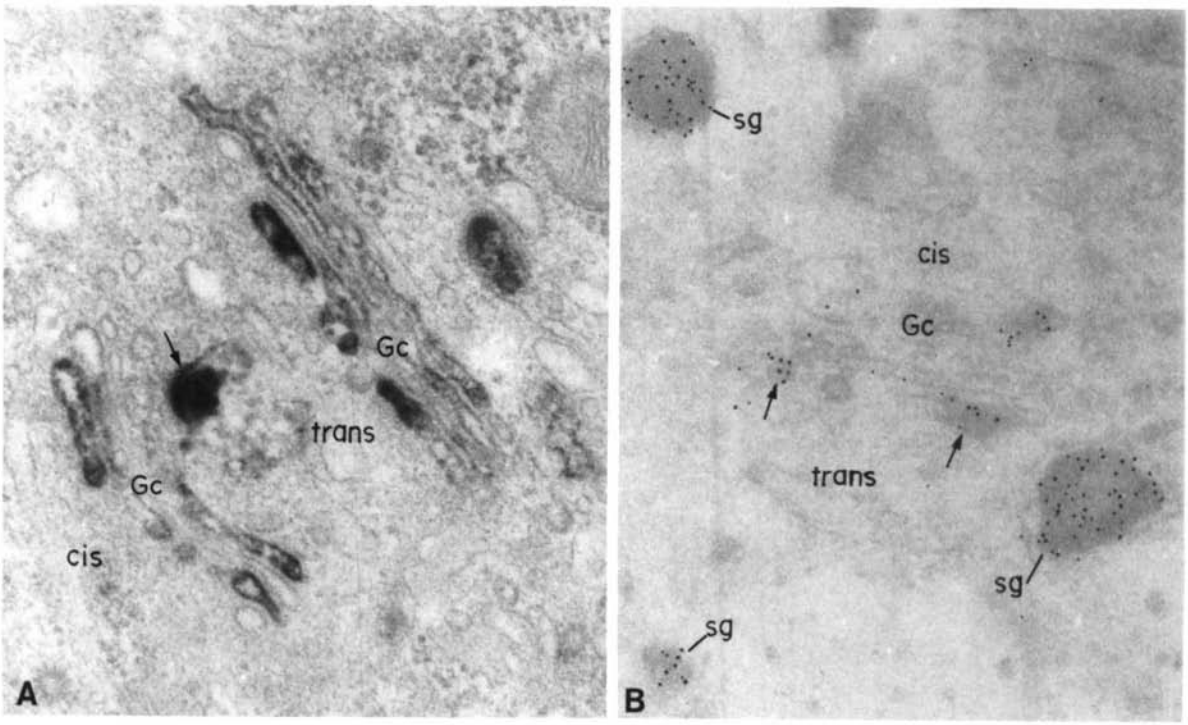

Fig. 4. POMC-Derived Peptides can be Detected throughout the Golgi Complex

A, Immunoperoxidase labeling showing the Golgi region of an AtT-20 cell stained with Georgie (detects both POMC and 16 K amino-terminal peptide). Two sets of stacked cisternae are present, and DAB reaction product is seen in all the Golgi cisternae (Gc) (cis, middle and trans) and over a condensing secretory granule (*arrow*). The densest deposits occur in the transmost cisternae and in the secretion granule. B, Immunogold labeling of an ultrathin frozen section of a rat pituitary corticotrope with Danielle (recognizes POMC as well as β-LPH and β-endorphin). Gold particles (5 nm) are seen over several Golgi cisternae (Gc) as well as over secretion granules (sg). Labeling is the heaviest over the secretion granules and over secretory protein condensing in the trans Golgi cisterna (*arrows*). Only a few gold particles can be found over the Golgi cisternae (Gc), probably because this technique is less sensitive than immunoperoxidase. A, ×81,250; B, ×70,000.

exoproteolytic trimming of basic residues from the COOH terminus of peptide as well as the enzyme responsible for α-amidation.

POMC Cleavage Products Are Packaged Together in the Same Secretion Granules

It has been concluded, based on sequential staining of serial sections (27, 28) and on their coordinate equimolar secretion (29), that all cleavage products of POMC are packaged into the same secretion granules. We used double immunogold labeling with two different sizes of gold particles and antibodies directed against different POMC cleavage products to determine whether or not two different products could be detected in the same granule. When β-LPH/β-endorphin (detected by Danielle) and either ACTH/ABI (recognized by Kathy) or the α-amidated C-terminal of JP (detected by Jamie) were localized on the same ultrathin frozen section of rat anterior pituitary, gold particles of both sizes were found over all secretion granules in all experiments (Fig. 8, A and B). We did not see secretion granules labeled with only one size of gold particle, and no gold particles were seen over secretion granules of other pituitary cell types, *e.g.* mammotropes (Fig. 8), somatotropes, or gonadotropes. These results directly demonstrate the simultaneous presence of all of these POMC products in the same secretion granule.

DISCUSSION

Taking advantage of antibodies that specifically recognize POMC cleavage products but not the unprocessed precursor, we have demonstrated the presence of processed ACTH, ABI, and α-amidated JP in the Golgi complex as well as in secretion granules of AtT-20 cells and rat pituitary corticotropes. We have further shown that the processed products are restricted to a single Golgi subcompartment; namely, the last or transmost cisterna on the exit side of the stack. The processed products were not seen in other Golgi elements or in any other location. Our findings unambiguously demonstrate that the transmost Golgi cisterna represents the first station along the exocytic pathway where endoproteolytic processing of POMC normally occurs. Moreover, at least two endoproteolytic cleavage steps as well as exoproteolysis and α-amidation can take place in this compartment as indicated by the presence of staining for the α-amidated C-terminal of JP in this

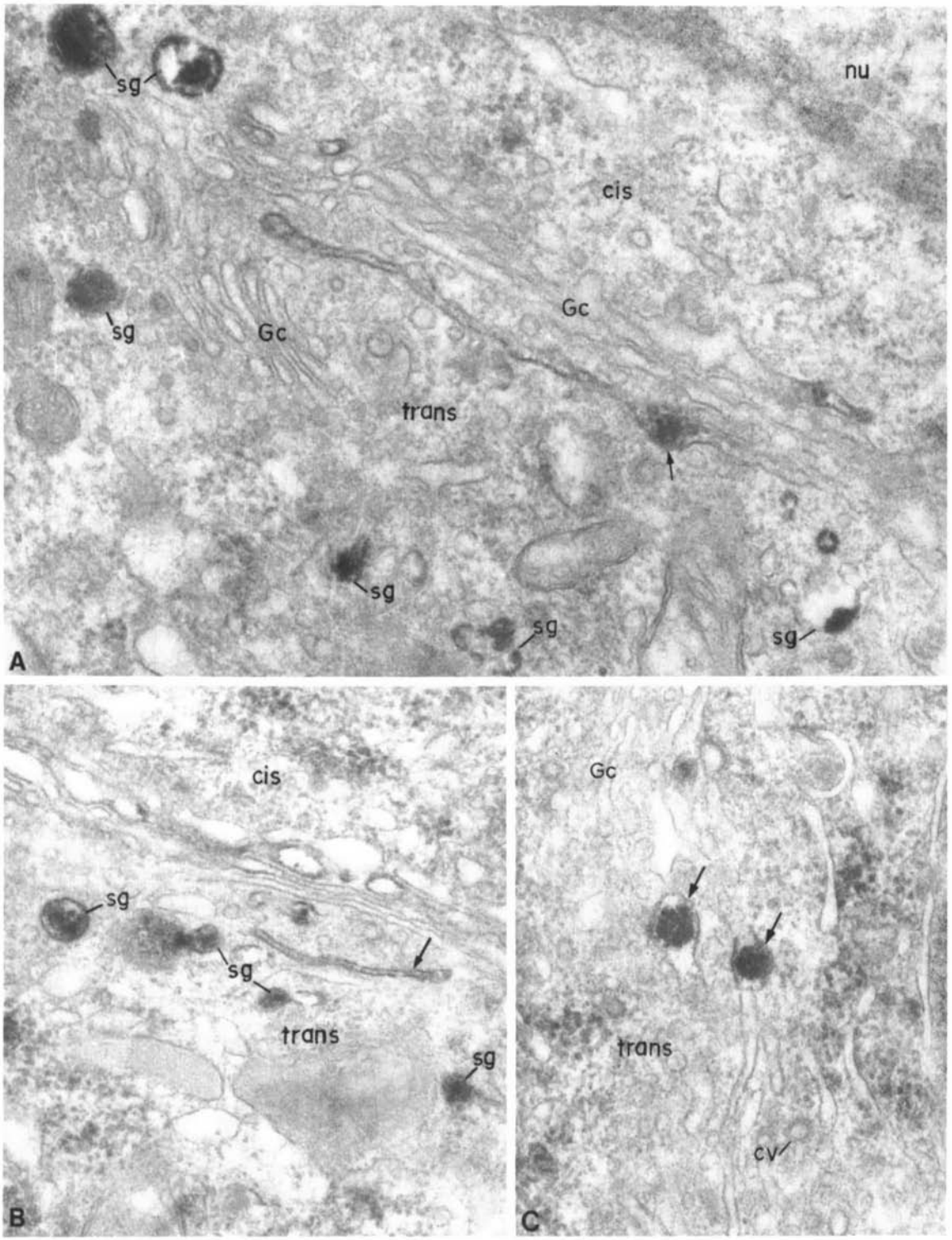

Fig. 5. Immunoperoxidase Localization of POMC Cleavage Products (ACTH and ABI) in the Transmost Golgi Cisterna of AtT-20 Cells

A, The last cisternae on the trans side of the Golgi stack (Gc) contains DAB reaction product and condensing secretory protein. Labeling is most dense over the forming granule (*arrow*). Several secretion granules (sg) are also heavily labeled. B, Another trans Golgi cisterna containing detectable POMC cleavage products (*arrow*). It is surrounded by maturing secretion granules (sg) which are also labeled. In this case the labeled trans cisterna is slightly set off from the stack and is less dilated than the remaining cisternae. C, Several budding or condensing secretion granules (sg) present in dilations of trans Golgi cisternae are densely labeled. All three specimens were reacted with Kathy. nu, Nucleus. A, ×75,000; B, ×50,000; C, ×37,500.

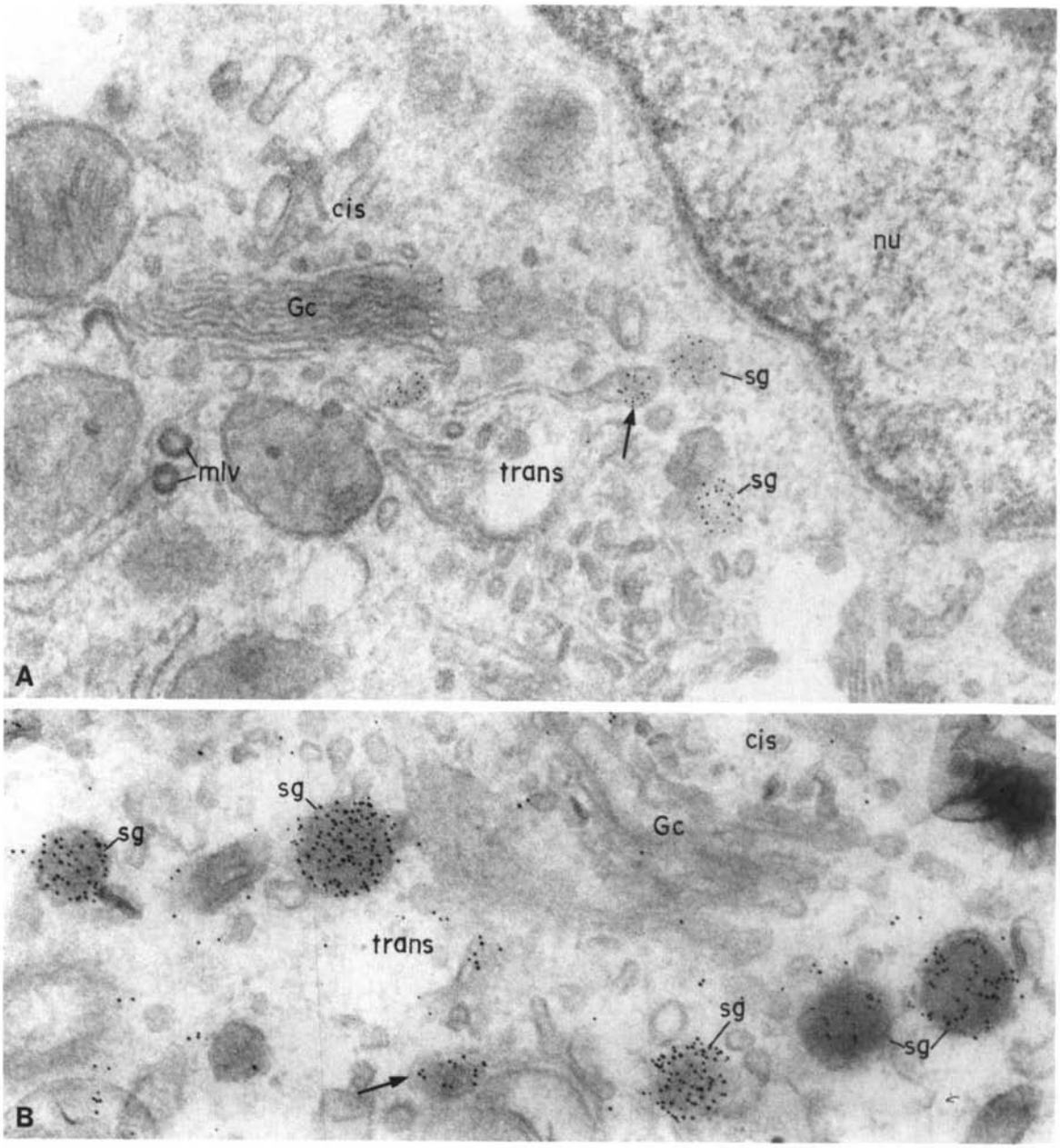

Fig. 6. Immunogold Labeling of Ultrathin Frozen Sections Demonstrating Products of POMC Cleavage in the Trans Golgi Cisterna
A, Golgi region of an AtT-20 cell. Colloidal gold is found over the dilated rim of a trans Golgi cisterna (*arrow*) containing condensing secretory protein. Two maturing secretion granules (sg) are also heavily labeled with colloidal gold. The labeled Golgi cisterna is set off from the Golgi stack (Gc) B, Golgi region of corticotrope from the rat pituitary. Secretory protein condensing within the dilated rim of a trans Golgi cisterna is heavily labeled with colloidal gold particles (*arrows*). The surrounding secretion granules (sg) are also heavily labeled. Sections incubated with Kathy. nu, Nucleus; mlv, murine leukemia virus; A, ×43,700; B, ×68,750.

location. This implies that the transmost Golgi cisterna contains all the proteolytic enzymes necessary to accomplish these cleavages as well as the α-amidating enzyme (9) and that these enzymes are active on the peptide substrate.

The question of where proteolytic processing of prohormones takes place is a long-standing one. Answers have varied with cell type studied and method used (1–5, 15, 30–32) but are often based on comparative kinetic data. However, the intracellular transit time of individual proteins varies considerably. Recent data indicate that most of the pregranule delay is created by the variable time newly-synthesized proteins spend maturing in the ER ($t_{1/2}$ = 10 min to 1.5 h) (33), as transit across the Golgi stack is rapid and similar from one protein to another ($t_{1/2}$ = 10–15 min). Thus the question of where in the cell proteolytic processing begins cannot be solved by kinetic or cell fractionation data due to the inherent limitations of these approaches. Like many questions involving analysis of the Golgi complex (22, 34), this question can best be investigated by immu-

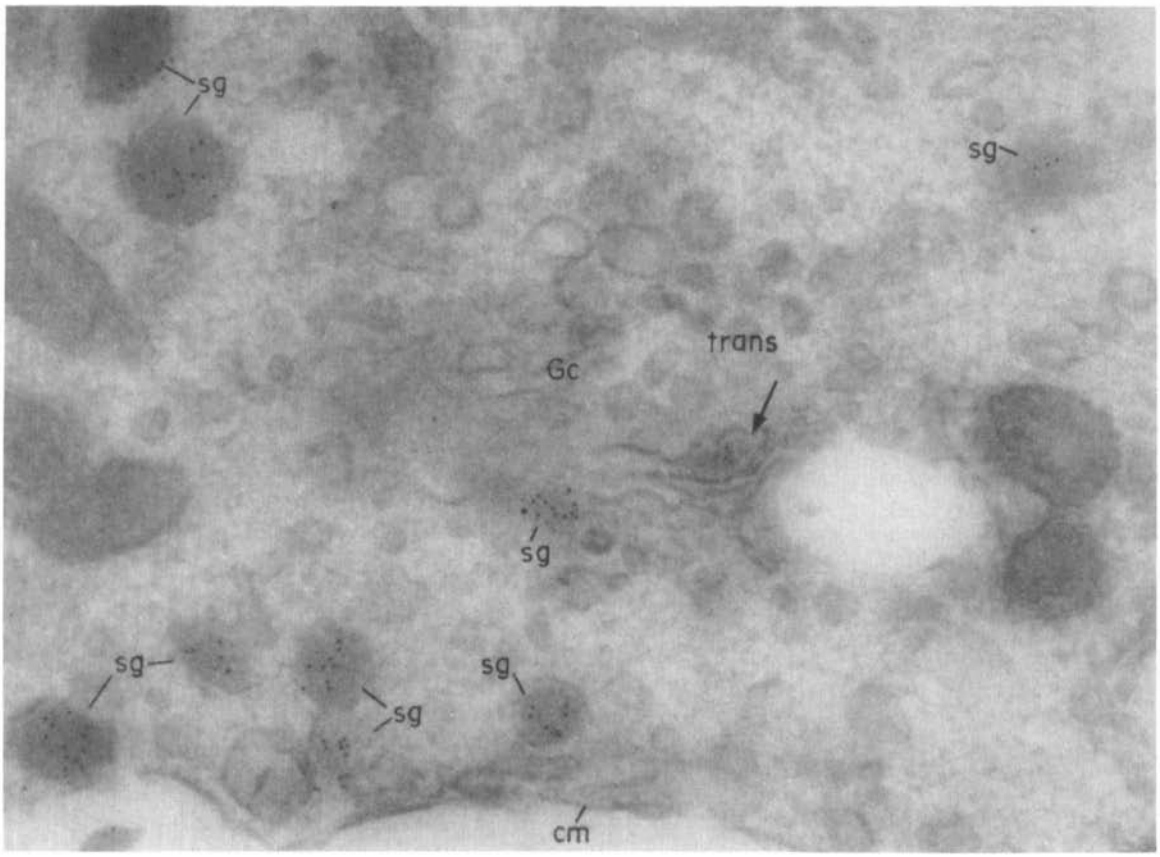

Fig. 7. Demonstration of the Presence of α-Amidated JP in the Trans Golgi Cisterna of Rat Pituitary Corticotropes
The dilated rim of a trans Golgi cisterna is labeled with colloidal gold particles (*arrow*). Secretion granules (sg) are also densely labeled. Immunogold preparation of an ultrathin frozen section reacted with Jamie (specific for α-amidated JP). ×62,500.

nolocalization of either the processing enzymes or of the products *in situ*.

Toward this end we localized POMC products using two different and complementary, preembedding immunocytochemical approaches carried out on aldehyde-fixed tissues, an immunoperoxidase method in which the antibodies are allowed to diffuse into cryostat sections which are subsequently embedded and sectioned, and an immunogold labeling technique carried out on ultrathin cryosections cut at liquid nitrogen temperatures. The former has the advantage of higher sensitivity due to the generation of DAB reaction product, and the latter has the advantage that antigens are uniformly exposed on the surface of the section. Our results were identical with both techniques: processed POMC cleavage products were regularly detected in the transmost Golgi cisterna as well as in immature and mature secretion granules. The only difference in the results with the two methods was that more consistent labeling of the trans cisterna was obtained by the immunoperoxidase procedure due to its superior sensitivity.

There have been only two other recent previous studies in which the products of proteolytic processing have been localized by immunoelectron microscopy. Using an antibody specific for a final product peptide of ELH, Fisher *et al.* (35) obtained evidence similar to ours indicating that the ELH prohormone of Aplysia neurons is processed in the transmost Golgi cisterna. On the other hand, using an antibody specific for the C-terminal of the B-chain of insulin, Orci *et al.* (3) obtained results indicating that B-chain/C-peptide cleavage occurs in clathrin-coated, immature secretion granules. Processed insulin was not detected in the stacked Golgi cisternae. However, Davidson *et al.* (36) recently showed that the initial cleavage of proinsulin is at the C-peptide/A-chain junction, and the B-chain/C-peptide cleavage occurs about 1 h after the initial cleavage of proinsulin. The site of the initial C-peptide/A-chain cleavage remains open.

Characteristics of the Transmost Golgi Cisterna

The transmost cisterna on the exit side of the stack of Golgi cisternae has been known for some time (19–22, 37) to have a variable and unusual morphology in that it often but not always has a reticular, rather than a plate-like organization. This region has been referred to under a variety of names, including GERL (37), trans Golgi reticulum (20), trans tubular network (21, 22), or trans Golgi network (23). It was shown some time ago that concentration of secretory proteins and granule

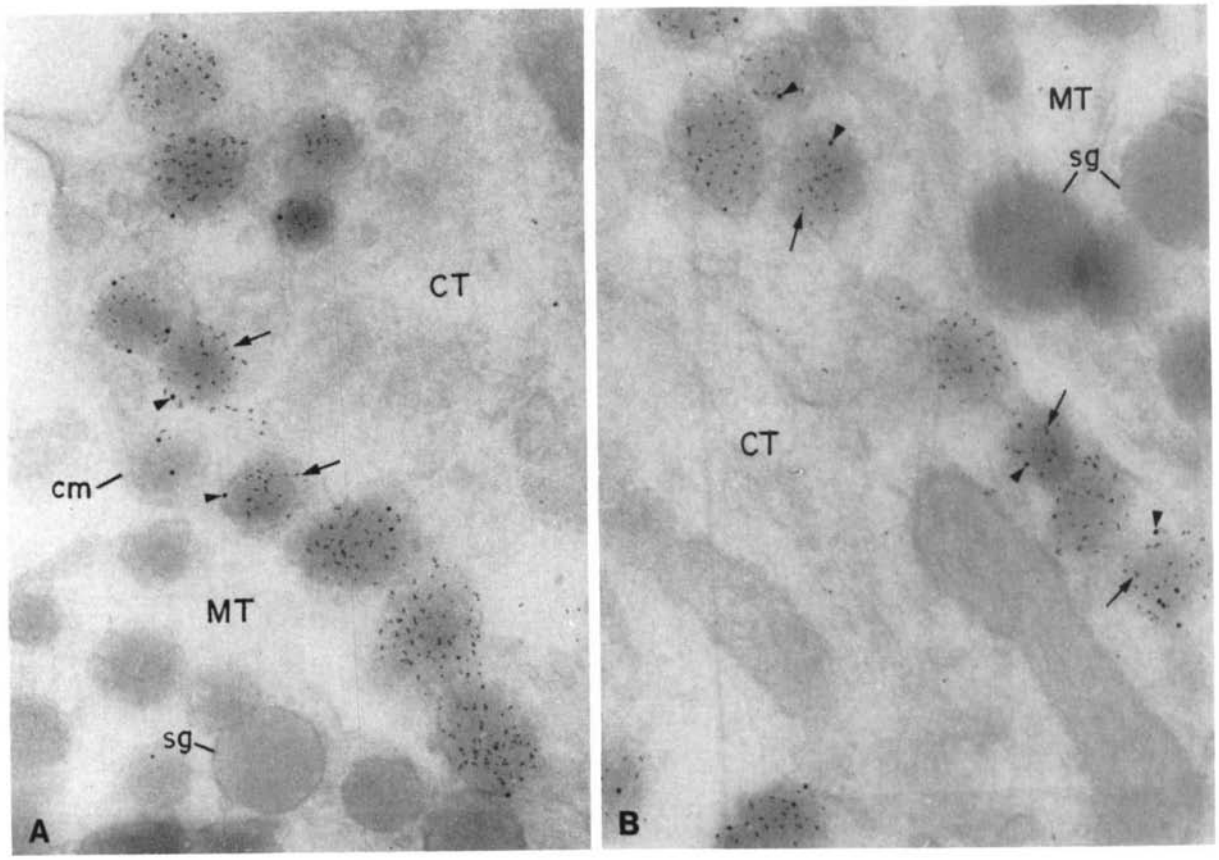

Fig. 8. Double Immunogold Labeling Demonstrating the Presence of Several POMC Cleavage Products in the Same Secretion Granules

A, Mature ACTH, detected with a 10 nm protein A-gold conjugate (*arrowheads*), and β-LPH/β-endorphin, detected with 5 nm protein A gold (*arrows*), are found over the same secretion granules. The latter are lined up along the cell membrane (cm) of the corticotrope (CT). No labeling is seen over secretion granules (sg) of the adjoining mammotrope (MT). B, α-amidated JP, detected with 10 nm protein A gold (*arrowheads*), and β-LPH/β-endorphin, detected with 5 nm protein A gold (*arrows*), are found within the same granules. No gold is seen over the secretory granules of the mammotrope (MT). ×62,500.

formation begins in this cisterna in anterior pituitary (17, 18) and in other cells (19). Recent evidence suggests that this last Golgi cisterna also represents the main site for the sorting of proteins secreted along the regulated pathway from membrane proteins which are delivered constitutively via a separate population of vesicles (38) and that sorting occurs concomitantly with formation of secretion granules (35, 38). The characteristics often ascribed to this Golgi subcompartment are that it is acid phosphatase-positive (17, 37), contains sialyltransferase activity (22), and has an acidic internal pH, at least in some cell types (39). This compartment is also known to be very labile and to enlarge at the expense of the stack under certain conditions, *i.e.* in cells incubated under N_2 (40) or in those infected with viruses and incubated at low temperature (20 C) (41). In spite of its variable and special morphology and of claims to the contrary (37), there is no doubt that this cisterna is a bona fide part of the Golgi complex, as it is the site of granule packaging (17–19) which is a traditional Golgi function, and it contains sialyltransferase activity (22) which is a classical Golgi marker enzyme (34, 42). The present results indicate that, in addition to granule packaging and terminal glycosylation, initiation of proteolytic processing and α-amidation can be added to the list of functions ascribed to this late Golgi subcompartment.

Intracellular Sites of Postranslational Modifications of POMC

Secretory proteins are known to move vectorially through the cell (ER→Golgi→granules) and to undergo modifications in transit. Based on the known intracellular location of processing enzymes for N-linked glycoproteins (34, 42), it can be assumed that POMC is synthesized and core glycosylated in the rough ER and transported to the cis or entry side of the Golgi complex where the oligosaccharides undergo trimming in transit through the cis and middle Golgi cisternae, and terminal glycosylation in transit through the trans Golgi cisternae. Based on the present observations, this is also where proteolytic cleavage and α-amidation begin. In the trans Golgi cisternae POMC and/or its products are

sorted from membrane proteins and other secretory proteins (38) and are concentrated and packaged into secretory granules which are stored for variable periods and eventually fuse with the plasma membrane and discharge their contents by regulated exocytosis. Our observations indicate that processing of at least some of the POMC occurs in the trans Golgi cisterna; however, since uncleaved POMC is found in mature granules at the cell periphery (32), processing must continue in the granules as suggested by pulse-chase labeling studies (43). Thus cleavage cannot be required for packaging or sorting.

Based on analogies to other sorting operations, sorting of ACTH and other regulated secretory proteins is believed to be receptor mediated and to occur by virtue of the interaction between a putative recognition signal present in proteins directed along the regulated pathway and a putative receptor present in the Golgi complex (14, 15, 44). Some credence has been given to this assumption by the recent isolation (44) of proteins from Golgi fractions prepared from canine pancreas that were shown to bind to several secretory proteins (PRL, insulin) and to be present in the Golgi region of AtT-20 cells by immunofluorescence. These molecules are thus candidates for Golgi binding proteins or sorters responsible for directing ACTH and other proteins into secretion granules at the level of the trans Golgi cisterna. Further characterization of this region of the Golgi is needed to understand the molecular mechanisms involved in the multiple functions of this cisterna. This remains a challenging problem for the future.

MATERIALS AND METHODS

Polyvinyl alcohol (mol wt = 10,000) and polyvinylpyrrolidone (M_r = 10,000) were obtained from Sigma Chemical Co. (St. Louis, MO). Protein A was purchased from Pharmacia (Piscataway, NJ), gold chloride from Fisher (Fair Lawn, NJ), and tannic acid from Mallinckrodt (St. Louis, MO). Glutaraldehyde was from EM Sciences (Fort Washington, PA), and Epox 812 from Fullam (Latham, NY).

Fluorescein isothiocyanate-conjugated sheep anti-rabbit $F(ab')_2$ was obtained from Zymed (South San Francisco, CA), and Fab fragments of sheep anti-rabbit immunoglobulin G (IgG) conjugated to horseradish peroxidase (HRP) were purchased from Biosys (Compiègne, France). Goat anti-rabbit IgG coupled to 5 nm colloidal gold (GAR 5) was from Janssen Life Science Products (Piscataway, NJ). Protein A-colloidal gold conjugates (5 and 10 nm) were prepared as described by Slot and Geuze (45).

Antibodies

The following affinity purified rabbit antibodies, which recognize different portions of the POMC molecule (see Table I) were used: Danielle (7) and Rebecca (43), which were raised against β-endorphin(1-31) and recognize POMC and β-LPH on an equimolar basis with β-endorphin by immunoprecipitation (Fig. 1); Georgie, which was raised against the 16 K amino-terminal region of POMC and detects POMC as well as the 16 K amino-terminal peptide, and both the α-amidated and glycine-extended forms of joining peptide (8, 9); Kathy, which was raised against ACTH(25-39) (10) and recognizes mature ACTH as well as ACTH biosynthetic intermediate (ABI), but not intact POMC; Jamie, which is specific for the α-amidated, C-terminal of JP (6, 9). Antisera were affinity purified using peptide resins as described (6).

Preparation of Pituitary Tissue for Immunocytochemistry

Adult male Sprague-Dawley rats (150–200 g) obtained from Charles River (Wilmington, MA), were anesthetized with ether, and their thorax was opened by right and left paramedian incisions. A 20-gauge needle connected to a syringe containing the perfusate was inserted into the left ventricle, the right auricle was opened, and the aorta was clamped just below the diaphragm. The upper half of the rat was first flushed with Dulbecco's Modified Eagle's Medium (DMEM) and then perfused with either 3% paraformaldehyde, 0.05% glutaraldehyde in 0.1 M sodium phosphate buffer, or McLean and Nakane's fixative (2% paraformaldehyde, 0.75 M lysine, 0.01 M sodium periodate in phosphate buffer) (46). The pituitary was removed and immersed in the same fixative for approximately 15 min, after which it was cut into 1-mm cubes. The latter were further fixed for a total of 1 h (in the case of the paraformaldehyde-glutaraldehyde fixative) or for a total of 4 h (in the case of McLean and Nakane's fixative). Tissue to be processed for immunoperoxidase was then cyoprotected by incubation in 10% dimethyl sulfoxide in PBS (1 h), frozen in isopentane at −70 C (47) and stored in liquid nitrogen. Tissue to be processed for ultrathin or semithin cryosectioning was cryoprotected by infiltration (1 h) with 2.3 M sucrose in phosphate buffer containing 50% polyvinylpyrrolidone, after which the pieces were mounted on aluminum nails and frozen in liquid nitrogen (48).

Preparation and Culture of AtT-20 Cells

AtT-20/D-16v cells were grown in DMEM supplemented with 10% horse serum in an atmosphere of 95% air, 5% CO_2 and were replated at 5-day intervals when they reached a density of approximately 5×10^6 per flask. For individual experiments they were plated onto 35-mm plastic tissue culture dishes and cultured for 4–5 days.

For morphological studies, AtT-20 cells were fixed for 1 h at room temperature with 2% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4. They were then postfixed at 4 C with 1% osmium tetroxide in acetate-veronal buffer (1 h), stained in block with 0.5% uranyl acetate in the same buffer (1–2 h), dehydrated in ethanol, and embedded in Epox. All fixation and embedding steps were carried out in the 35-mm culture dish. After polymerization overnight (60 C), pieces of the embedded cell layers were broken out of the culture dish, mounted on Epox blocks, and ultrathin sections (60–80 nm) were cut on a Reichert Ultracut E ultramicrotome. Sections were stained with 2% uranyl acetate and lead citrate, and micrographs were taken on a Philips 301 electron microscope operated at 80 kV.

Cells to be processed for immunofluorescence labeling on semithin (0.5–1 μm) frozen sections and immunogold labeling on ultrathin frozen sections were harvested by scraping into aldehyde fixative (3% paraformaldehyde, 0.05% glutaraldehyde in 0.1 M phosphate buffer). They were then pelleted in a Beckman microfuge for 10 min, and fixation of the pellet was continued for a total of 1 h. The pellets were cryoprotected in sucrose and frozen in liquid nitrogen as described above for pituitary tissue.

Immunoperoxidase Labeling of Pituitary Tissue

Immunoperoxidase labeling was carried out on cryostat sections (10–20 μm) as described in detail elsewhere (47). In brief, sections were incubated overnight at room temperature in one of the primary antibodies described above (diluted in 0.1 M PBS, 1% ovalbumin) followed by incubation for 2 h in Fab

fragments of sheep anti-rabbit IgG conjugated to HRP (1:54). They were then fixed in 1.5% glutaraldehyde containing 5% sucrose (1 h), and reacted with diaminobenzidine. After completion of the peroxidase reaction the sections were postfixed in 1% osmium tetroxide reduced with 1% ferrocyanide, dehydrated, and embedded in Epox. Ultrathin sections were stained for 1 min in lead citrate and examined at 60 kV in a Philips 301 electron microscope.

Immunoperoxidase Labeling of AtT-20 Cells

Cells were fixed for 4–6 h in McLean and Nakane's fixative (46) as in the case of pituitary tissue. Fixation and labeling were carried out in the tissue culture dishes as described previously (49). Briefly, the cells were permeabilized (0.005% saponin in 0.1 M PBS, pH 7.4), incubated for 2 h at room temperature with one of the antibodies described above (diluted in 0.1 M PBS containing 1% ovalbumin and 0.005% saponin), followed by incubation for 2 h in Fab fragments of sheep anti-rabbit IgG conjugated to HRP (1:50 dilution in 0.1 M PBS +1% ovalbumin +0.005% saponin). After washing the cells were fixed in glutaraldehyde and reacted with diaminobenzidine as done for pituitary tissue.

Immunofluorescence Labeling of Semithin Frozen Sections

Semithin frozen sections (0.5-1 μm) were cut from pituitary tissue or pellets of AtT-20 cells at −70 C on an Ultracut E ultramicrotome equipped with the FC-4E cryoattachment (Reichert Scientific Instruments, Buffalo, NY) and transferred to glass slides coated with poly-L-lysine (mol wt, 40,000; 1 mg/ml). They were then incubated for 2 h at 25 C with one of the affinity-purified antibodies directed against different regions of POMC (in PBS, 1% BSA, pH 7.4), followed by incubation for 1 h with FITC-conjugated sheep anti-rabbit F(ab′)$_2$ (1:50). Stained sections were mounted in 90% glycerol in PBS containing 0.1% p-phenylenediamine (to retard fading), examined by epifluorescence illumination and photographed using a Zeiss Photomicroscope III and Kodak Tri-X Pan (ASA 400) film.

Preparation and Immunogold Labeling of Ultrathin Frozen Sections

Ultrathin cryosectioning of AtT-20 cell pellets and rat pituitary tissue was carried out on a Reichert Ultracut E equipped with a cryoattachment at −110 C following the techniques of Tokuyasu (48, 50). Sections were transferred to hexagonal nickel grids (200 mesh) which had been coated with formvar and carbon. Subsequent incubations and washing steps were carried out by floating the grids on droplets of the filtered solutions. After quenching with 10% fetal bovine serum (FBS) containing 0.01 M glycine (to block free aldehyde groups), the sections were incubated for 1 h with one of the above-described primary antibodies (diluted in PBS with 10% FBS), followed by 5 nm colloidal gold conjugated to goat anti-rabbit IgG (diluted 1:50). For double immunolabeling the sections were first incubated for 1 h with Kathy (specific for processed ACTH) followed by incubation in 10 nm protein A gold conjugate. Sections were then quenched with free protein A (0.1 mg/ml) and incubated with Danielle (recognizes the precursors as well as the processed products) or with Jamie (specific for the α-amidated C-terminal of JP) followed by 5 nm protein A-conjugate. After labeling, the sections were postfixed in 2% glutaraldehyde in PBS (10 min), stained with 2% osmium tetroxide (15 min), followed by 2% acidic uranyl acetate (15 min), and finally absorption stained (5 min) with 0.002% lead citrate in 2.2% polyvinyl alcohol as described by Tokuyasu (50).

Acknowledgments

We thank Mary Bronson and Ann Whitehouse for their help in the preparation of the figures and Beth Dudeck for her assistance in the preparation of the manuscript.

Received May 4, 1989. Revision received May 18, 1989. Accepted May 18, 1989.

Address requests for reprints to: Dr. Marilyn Farquhar, Yale University School of Medicine, Department of Cell Biology, 333 Cedar Street, New Haven, Connecticut 06510.

This work was supported by NIH Research Grants DK-17780 (to M.G.F.) and DK-32948 and DA-00097 (to R.E.M.). Preliminary results of the work have been presented at the American Society of Cell Biology/American Society for Biochemistry and Molecular Biology Joint Meeting in San Francisco, CA, 1989.

* Recipient of Fellowship 1986–1989 from the German Research Foundation.

REFERENCES

1. Steiner DF, Kemmler W, Tager HS, Peterson JD 1974 Proteolytic processing in the biosynthesis of insulin and other proteins. Fed Proc 33:2105–2115
2. Steiner DF, Michael J, Houghten R, Mathieu M, Gardner PR, Ravazzola M, Orci L 1987 Use of a synthetic peptide antigen to generate antisera reactive with a proteolytic processing site in native human proinsulin: demonstration of cleavage within clathrin-coated (pro)secretory vesicles. Proc Natl Acad Sci USA 84:6184–6188
3. Orci L, Ravazzola M, Storch MJ, Anderson RGW, Vassalli JD, Perrelet A 1987 Proteolytic maturation of insulin is a post-Golgi event which occurs in acidifying clathrin-coated secretory vesicles. Cell 49:865–868
4. Brownstein MJ, Russell JT, Gainer H 1980 Synthesis, transport, and release of posterior pituitary hormones. Science 207:373–378
5. Loh YP, Gritsch HA 1981 Evidence for intragranular processing of proopiocortin in the mouse pituitary intermediate lobe. Eur J Cell Biol 26:177–183
6. Cullen EI, Mains RE 1987 Biosynthesis of amidated joining peptide from pro-adrenocorticotropin-endorphin. Mol Endocrinol 1:583–594
7. Mains RE, Eipper BA 1979 Synthesis and secretion of corticotropins, melanotropins, and endorphins by rat intermediate pituitary cells. J Biol Chem 254:7885–7894
8. Eipper BA, Mains RE 1978 Existence of a common precursor to ACTH and endorphin in the anterior and intermediate lobes of the rat pituitary. J Supramolecular Structure 8:247–262
9. Eipper BA, Park L, Keutmann HT, Mains RE 1986 Amidation of joining peptide, a major pro-ACTH/endorphin-derived product peptide. J Biol Chem 261:8686–8694
10. Mains RE, Eipper BA 1983 Phosphorylation of rat and human adrenocorticotropin-related peptides: physiological regulation and studies of secretion. Endocrinology 112:1986–1995
11. Eipper BA, Mains RE 1980 Structure and biosynthesis of proadrenocorticotropin/endorphin and related peptides. Endocr Rev 1:1–27
12. Tooze J, Tooze SA 1986 Clathrin-coated vesicular transport of secretory proteins during the formation of ACTH-containing secretory granules in AtT20 cells. J Cell Biol 103:839–850
13. Gumbiner B, Kelly RB 1982 Two distinct intracellular pathways transport secretory and membrane glycoproteins to the surface of pituitary tumor cells. Cell 28:51–59

14. Burgess TL, Kelly RB 1987 Constitutive and regulated secretion of proteins. Annu Rev Cell Biol 3:243–293
15. Kelly RB 1985 Pathways of protein secretion in eukaryotes. Science 230:25–31
16. Sato SM, Mains RE 1988 The synthesis and processing of pro-ACTH-endorphin in the developing rat pituitary. Ann NY Acad Sci 512:286–299
17. Smith RE, Farquhar MG 1966 Lysosome function in the regulation of the secretory process in cells of the anterior pituitary gland. J Cell Biol 31:319–347
18. Farquhar MG 1971 Processing of secretory products by cells of the anterior pituitary gland. In: Heller H, Lederis K (eds), Subcellular Structure and Function in Endocrine Organs. Cambridge University Press, Cambridge, England, pp 70–122
19. Farquhar MG, Palade GE 1981 The Golgi apparatus (complex)—(1954–1981)—from artifact to center stage. J Cell Biol 91:(no 3, pt 2), pp 77s–103s
20. Willingham MC, Pastan IH 1984 Endocytosis and exocytosis: current concepts of vesicle traffic in animal cells. Int Rev Cytol 92:51–92
21. Rambourg A, Clermont Y, Hermo L 1981 Three-dimensional structure of the Golgi apparatus. In: Hand AA, Oliver C (eds) Basic Mechanisms of Cellular Secretion. Methods in Cell Biology. Academic Press, Inc., NY 23:155–166
22. Roth J, Taatjes DJ, Lucocq JM, Weinstein J, Paulson JC 1985 Demonstration of an extensive trans-tubular network continuous with the Golgi apparatus stack that may function in glycosylation. Cell 43:287–295
23. Griffiths G, Simons K 1986 The trans Golgi network: sorting at the exit site of the Golgi complex. Science 234:438–443
24. Mitchell WM, Moses HL, Orth DN, Korinek 1971 Murine leukemia C-type virus associated with functional murine carcinomas of endocrine origin. Nature 231:99–100
25. Salpeter M, Farquhar MG 1981 High resolution autoradiographic analysis of the secretory pathway in mammotrophs of the rat anterior pituitary. J Cell Biol 91:240–246
26. Eipper BA, Mains RE, Herbert E 1986 Peptides in the nervous system. Trends Neurosci 9:463–467
27. Weber E, Martin R, Voigt KH 1979 Corticotropin/β-endorphin precursor: Concomitant storage of its fragments in the secretory granules of anterior pituitary corticotropin/endorphin cells. Life Sci 25:1111–1118
28. Vaudry H, Pelletier G, Guy J, Leclerc R, Jegou S 1980 Immunohistochemical localization of γ-endorphin in the rat pituitary gland and hypothalamus. Endocrinology 106:1512–1520
29. Mains RE, Eipper BA 1981 Coordinate, equimolar secretion of smaller peptide products derived from pro-ACTH/endorphin by mouse pituitary tumor cells. J Cell Biol 89:21–28
30. Habener JF, Potts JT, Rich A 1976 Pre-proparathyroid hormone: Evidence for an early biosynthetic precursor of proparathyroid hormone. J Biol Chem 251:3893–3899
31. Glembotski CC 1981 Subcellular fractionation studies on the posttranslational processing of pro-adrenocorticotropic hormone/endorphin in rat intermediate pituitary. J Biol Chem 256:7433–7439
32. Tooze J, Hollinshead M, Frank R, Burke B 1987 An antibody specific for an endoproteolytic cleavage site provides evidence that pro-opiomelanocortin is packaged into secretory granules in AtT20 cells before its cleavage. J Cell Biol 105:155–162
33. Lodish HF 1988 Transport of secretory and membrane glycoproteins from the rough endoplasmic reiculum to the Golgi. J Biol Chem 263:2107–2110
34. Farquhar MG 1985 Progress in unraveling pathways of Golgi traffic. Annu Rev Cell Biol 1:448–488
35. Fisher JM, Sossin W, Neucomb R, Scheller RH 1988 Multiple neuropeptides derived from a common precursor are differentially packaged and transported. Cell 54:813–822
36. Davidson HW, Rhodes CJ, Hutton JC 1988 Intraorganellar calcium and pH control proinsulin cleavage in the pancreatic B-cell via two distinct site-specific endopeptidases. Nature 333:93–96
37. Orci L, Ravazzola M, Amherdt M, Perrelet A, Powell SK, Quinn DL, Moore H-PH 1987 The trans-most cisternae of the Golgi complex: a compartment for sorting of secretory and plasma membrane proteins. Cell 51:1039–1051
38. Novikoff AB, Novikoff PM 1977 Cytochemical contributions to differentiating GERL from the Golgi apparatus. Histochem J 9:525–551
39. Anderson RGW, Orci L 1988 A view of acidic intracellular compartments. J Cell Biol 106:539–543
40. Merisko EM, Fletcher MA, Palade GE 1986 The reorganization of the Golgi complex in anoxic pancreatic acinar cells. Pancreas 1:95–108
41. Griffiths G, Fuller SD, Back R, Hollinshead M, Pfeiffer S, Simons K 1989 The dynamic nature of the Golgi complex. J Cell Biol 108:277–297
42. Kornfeld R, Kornfeld S 1985 Assembly of asparagine-linked oligosaccharides. Annu Rev Biochem 54:631–664
43. Sato SM, Mains RE 1988 Plasticity in the ACTH-related peptides produced by primary cultures of neonatal rat pituitary. Endocrinology 122:68–77
44. Chung KN, Walter P, Aponte GW, Moore HPH 1989 Molecular sorting in the secretory pathway. Science 243:192–197
45. Slot JW, Geuze HJ 1985 A new method of preparing gold probes for multilabeling cytochemistry. Eur J Cell Biol 38:87–93
46. Brown WJ, Farquhar MG 1984 The mannose-6-phosphate receptor for lysosomal enzymes is concentrated in cis Golgi cisternae. Cell 36:295–307
47. McLean W, Nakane PF 1974 Periodate-lysine-paraformaldehyde fixative. A new fixative for immunoelectron microscopy. J Histochem Cytochem 22:1077–1083
48. Tokuyasu KT 1986 Application of cryomicrotomy to immunocytochemistry. J Micro 143:139–14
49. Brown WJ, Constantinescu E, Farquhar MG 1984 Redistribution of mannose-6-phosphate receptors induced by tunicamycin and chloroquine. J Cell Biol 99:320–326
50. Tokuyasu KT, Use of polyvinylpyrrolidone and polyvinyl alcohol for cyroultramicrotomy. Histochem J, in press