Clathrin-coated Vesicular Transport of Secretory Proteins during the Formation of ACTH-containing Secretory Granules in AtT20 Cells

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Abstract. We have studied by electron microscopy and immunocytochemistry the formation of secretory granules containing adrenocorticotropic hormone (ACTH) in murine pituitary cells of the AtT20 line. The first compartment in which condensed secretory protein appears is a complex reticular network at the extreme *trans* side of the Golgi stacks beyond the TPPase-positive cisternae. Condensed secretory protein accumulates in dilated regions of this *trans* Golgi network. Examination of *en face* and serial sections revealed that "condensing vacuoles" are in fact dilations of the *trans* Golgi network and not detached vacuoles. Only after presumptive secretory granules have reached an advanced stage of morphological maturation do they detach from the *trans* Golgi network. Frequently both the dilations of the *trans* Golgi network containing condensing secretory protein and the detached immature granules in the peri-Golgi region have surface coats which were identified as clathrin by immunocytochemistry. Moreover both are the site of budding (or fusion) of coated vesicles, some of which contain condensed secretory protein. The mature granules below the plasma membrane do not, however, have surface coats. Immunoperoxidase labeling with an antiserum specific for ACTH and its precursor polypeptide confirmed that many of the coated vesicles associated with the *trans* Golgi network contain ACTH. The involvement of the *trans* Golgi network and coated vesicles in the formation of secretory granules is discussed.

THE extent to which clathrin and coated vesicles play an essential role in the exocytic pathway in animal cells remains to be established. In electron microscopic studies of conventionally fixed and embedded cells, coats with the appearance of clathrin have repeatedly been reported on the rims of Golgi cisternae and peri-Golgi vesicles (reviewed in reference 5). Aggeler et al. (1) have established that in critical point-dried macrophages the coats on vesicles budding off Golgi cisternae have the structure of clathrin cages; and with different antisera against clathrin Griffiths et al. (9) and Louvard et al. (17) have established, by immunocytochemical methods, that the coats associated with the rims of at least some Golgi cisternae and peri-Golgi vesicles do indeed consist of clathrin. Some of these clathrin-coated vesicles associated with the Golgi stacks are, however, known to be involved in the transport of lysosomal enzymes from the Golgi apparatus to lysosomes (6, 28, 29, 32), rather than in exocytosis.

In cells with regulated exocytic pathways that involve secretory granules as a storage station during the exocytosis of secretory protein, conventional electron microscopy has revealed coats and coated vesicles associated with the so-called condensing vacuoles in which precipitated secretory protein accumulates (reviewed in reference 5). Recently Orci et al. (23), using antiserum against clathrin (17), showed that clathrin is concentrated at the *trans* pole of the Golgi apparatus in several types of secretory cells. They (22) also obtained data consistent with the idea that a clathrin-coated, post-Golgi compartment matures into uncoated secretory granules concomitant with the proteolytic maturation of proinsulin in pancreatic B cells. The coated immature granules are the major, if not exclusive, site of this proteolysis (24). However, these authors did not comment on the role of the clathrin coats at this site.

The mouse pituitary tumor cell line, AtT20 (13, 18, 26), provides a model system in which the role of clathrin and coated vesicles in the formation of secretory granules can be studied. In these cells, there are two distinct exocytic pathways, one is constitutive and the other, involving secretory granules, is regulated and inducible with secretagogues (14). About 80% of the adrenocorticotropic hormone (ACTH)[1] is diverted into the regulated pathway in which the cleavage of ACTH from its precursor polypeptide, proopiomelanocortin (POMC), begins concomitant with the packaging of the hormone into secretory granules (13). Here we report elec-

1. *Abbreviations used in this paper*: ACTH, adrenocorticotropic hormone; POMC, proopiomelanocortin.

tron microscopic, cytochemical, and immunocytochemical studies of the formation of secretory granules containing ACTH in these cells and the distribution of clathrin and the involvement of coated vesicles. In AtT20 cells, as in some other endocrine cells in vivo (3, 15), the extreme *trans* compartment of the Golgi apparatus is the site at which condensing secretory proteins first occur. Here we follow the terminology proposed recently by Griffiths and Simons (12) for this extreme *trans* compartment and refer to it throughout as the *trans* Golgi network. Several of our observations complement previous biochemical studies (13, 14) and are consonant with current ideas about the formation of secretory granules (16).

Materials and Methods

Cell Culture

AtT20D16V cells were grown in Falcon plastic ware in Dulbecco's modified Eagle's medium supplemented with 3.5 g/liter of glucose and 10% fetal calf serum or horse serum (Gibco, Grand Island, NY). The cells were replated after dispersion of clumps with 0.25% trypsin and 0.05% EDTA every 4–5 d. Under these culture conditions, the cell population doubles in ~40 h (35).

Antisera

We are very grateful to Dr. D. Louvard (Pasteur Institute, Paris) for the affinity-purified antiserum against polymerized clathrin (17) and to Dr. R. Kelly (University of California at San Francisco) for the affinity-purified serum against ACTH which also recognizes the ACTH precursor polypeptide POMC (13, 14).

Electron Microscopy

Cells growing in 35-mm dishes were washed with Ca^{++}- and Mg^{++}-free PBS and fixed at room temperature for 1 h in 2–3% glutaraldehyde and 2% formaldehyde in 0.1 M sodium cacodylate buffer at pH 7.2. They were then washed three times for 10 min each wash in 0.1 M sodium cacodylate buffer, pH 7.2, and postfixed in 2% OsO_4 in 0.1 M cacodylate buffer, pH 7.2, for 1 h. After three further washes in the buffer, the cells were incubated overnight at 4°C in 0.5% magnesium uranyl acetate (B.D.H. Ltd., Poole, England) in water. They were then dehydrated in ethanol, detached from the dishes with propylene oxide, pelleted, and embedded in Epon. Thin sections were stained in lead citrate for 1 min before examination in Philips 301 or 400 microscopes.

Immunoelectron Microscopy Immunogold Labeling

Cells were fixed in 3% formaldehyde and 0.1% glutaraldehyde in Mg^{++}- and Ca^{++}-free PBS. They were then washed in PBS, scraped from the dishes and either infiltrated with sucrose and frozen in liquid nitrogen (10, 34) or infiltrated and embedded in Lowicryl K4M (2). We are grateful to Dr. J. Roth (Biozentrum, Basel) for embedding the material in Lowicryl K4M. Thin sections of frozen or Lowicryl-embedded cells were cut and labeled according to published procedures (10, 27, 34) with either the affinity-purified rabbit anti-clathrin antiserum (17) or with the affinity-purified rabbit antiserum against ACTH (12, 13). After incubation with these antisera, sections were incubated with Protein A conjugated to 7–10-nm gold. Finally the sections were contrasted with uranyl acetate or, in the case of the Lowicryl sections, uranyl acetate and lead citrate, before being examined.

Immunoperoxidase Labeling

Cells were fixed in the same fixature as used for immunogold labeling. We then followed the protocol for immunoperoxidase labeling given by Reggio et al. (25) using the affinity-purified antiserum against ACTH.

Enzymecytochemistry

Thiamine pyrophosphatase labeling was done according to the method of Novikoff and Goldfischer (21).

Results

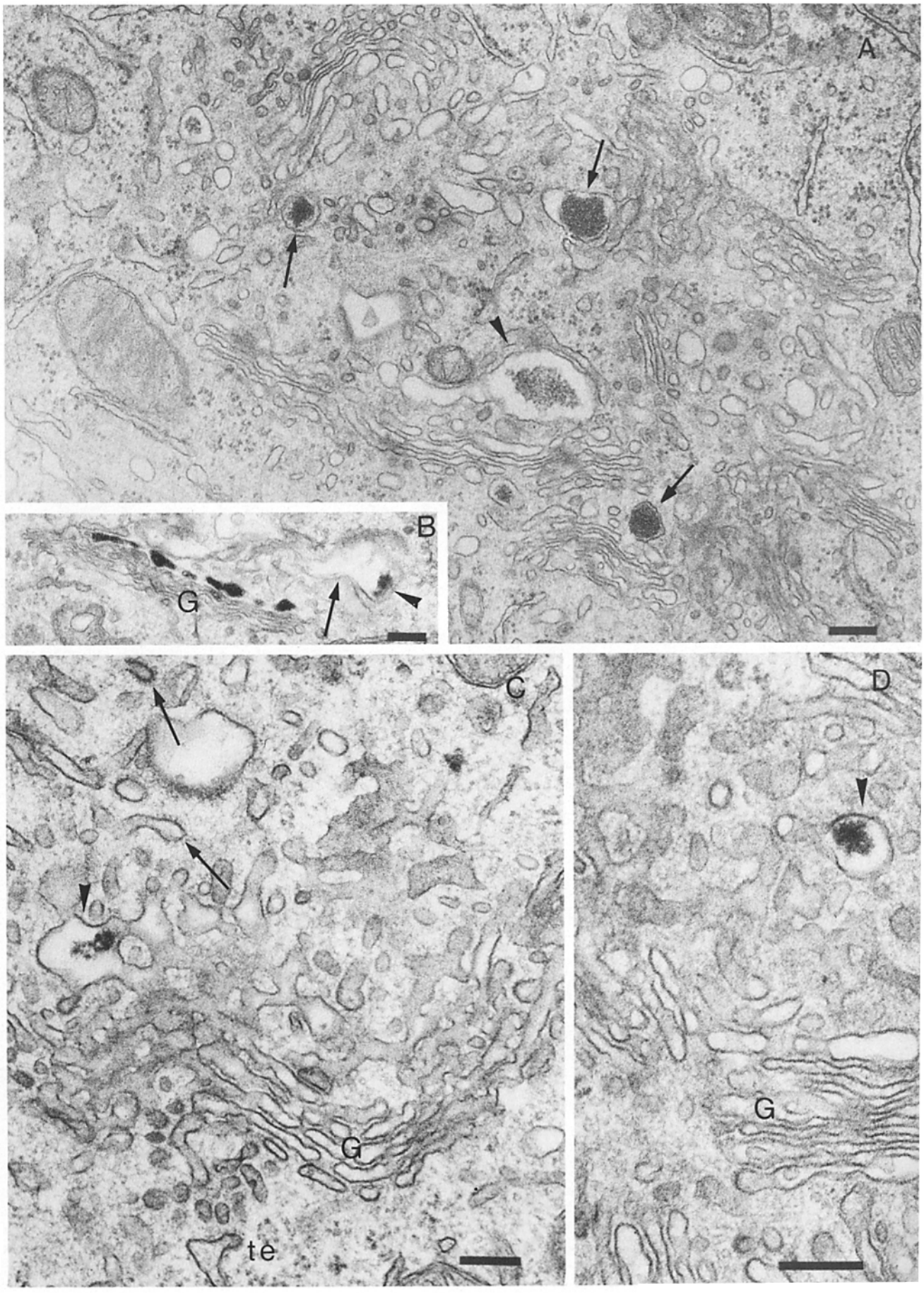
Condensed Secretory Proteins in the Trans Golgi Network

We examined thin sections of Epon-embedded AtT20 cells to determine the compartment in which condensed secretory proteins first appear. As Fig. 1, *A* and *B*, shows, in these cells the stacked Golgi cisternae, including those which are TPPase positive, do not contain aggregates of secretory protein. Condensed secretory protein is first seen in cisternae that lie beyond, and often clearly separated from the remainder of the Golgi stack (Fig. 1, *A* and *B*). Occasionally these cisternae are rather straight with aggregated secretory protein at their distended rims (not shown) and are reminiscent of GERL cisternae, as described by Novikoff (20). Normally, however, they are partially or wholly dilated, have irregular profiles, and contain various amounts of secretory protein, most of which is much less condensed than that in mature granules.

Transverse sections (Fig. 1, *A* and *B*) establish that concentration and condensation of secretory protein does not begin before the *trans*-most region of the Golgi apparatus, beyond the TPPase-positive cisternae. However, such sections conceal the reticular nature of this extreme *trans* compartment which is immediately apparent in *en face* sections (Fig. 1, *C* and *D*). These show a network of interconnecting tubular elements with dilated regions containing the first aggregations of secretory proteins. Depending on the angle of sectioning of the *trans* Golgi network, one obtains the range of images shown in Fig. 1.

Examination of ribbons of transverse sections of Golgi stacks provides further evidence that the dilated extreme *trans* Golgi cisternae containing condensing secretory proteins are indeed part of the *trans* Golgi network and not detached "condensing vacuoles." In all the serial sections examined, it proved possible to trace the profiles of these extreme *trans* Golgi cisternae through depths of several thousands of Å until eventually they linked up with tubular elements of the *trans* Golgi network (for example Fig. 2, *E* and *F*). In other words, in AtT20 cells we found no evidence of detached condensing vacuoles. The extreme *trans* Golgi cisternae in which condensing secretory proteins first accumulate are specialized but integral parts of the extensive and complex *trans* Golgi network.

Figure 1. A is a survey micrograph of part of the Golgi region of an AtT20 cell. It shows a large dilated extreme *trans* Golgi cisterna (*arrowhead*) containing partially condensed protein and elsewhere more-condensed secretory protein beginning to resemble the cores of secretory granules (*arrows*). Note that the stacked Golgi cisternae do not contain condensed secretory protein. *B* shows a Golgi stack labeled for TPPase. The extreme *trans* cisterna (*arrow*) with condensing secretory protein (*arrowhead*) are not labeled and lie beyond the TPPase-positive *trans* cisternae of the Golgi stack (*G*). *C* and *D* show the reticular structure of the extreme *trans* Golgi region as seen in *en face* sections. Secretory protein is precipitated and aggregated in dilated regions (*arrowheads*) of the *trans* Golgi network, which is revealed as a complex network of tubular elements (*arrows*). The stacked Golgi cisternae are labeled *G* and coated surfaces of the *trans* Golgi reticulum are marked with arrows in *C*. Note also in *C*, on the *cis* side of the Golgi stack, a transitional element (*te*). Bars, 0.2 μm.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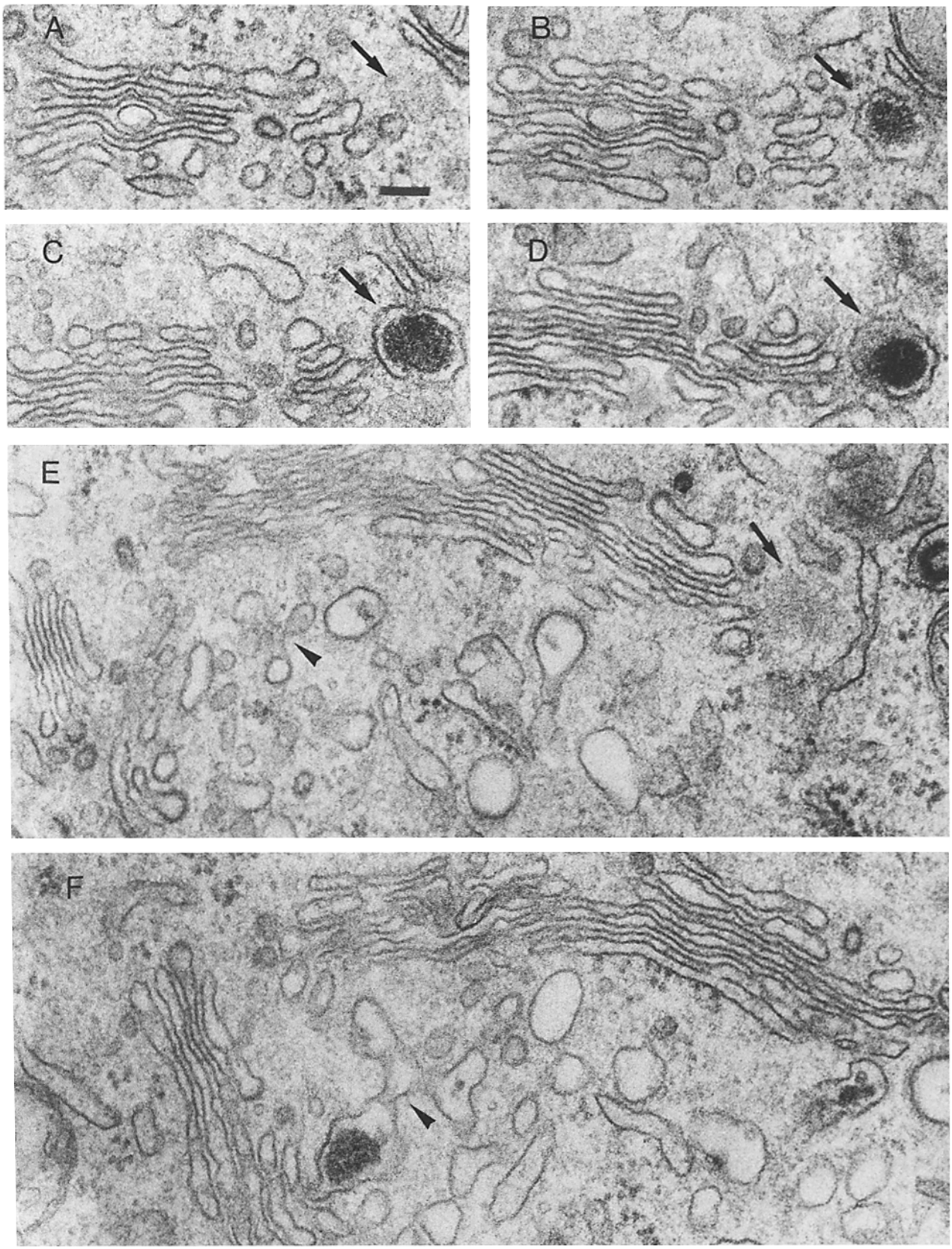


Figure 2. Serial sections of a Golgi region. The micrographs are of sections in a ribbon of 14 sections. *A–E* show in consecutive sections a detached immature secretory granule (*arrows*) at the edge of a Golgi stack. The region marked by an arrowhead in *E* contains elements of the *trans* Golgi reticulum. Notice in particular the tripartite structure next to the arrowhead. *F* is a micrograph of the next but one section after *E*. The tripartite structure has enlarged and comprises three dilated regions with a large clump of condensing secretory proteins in one of them. Secretory granules (*A–E*) arise by condensation of secretory proteins into granule cores followed by detachment from the *trans* Golgi network. Bar, 0.1 μm.

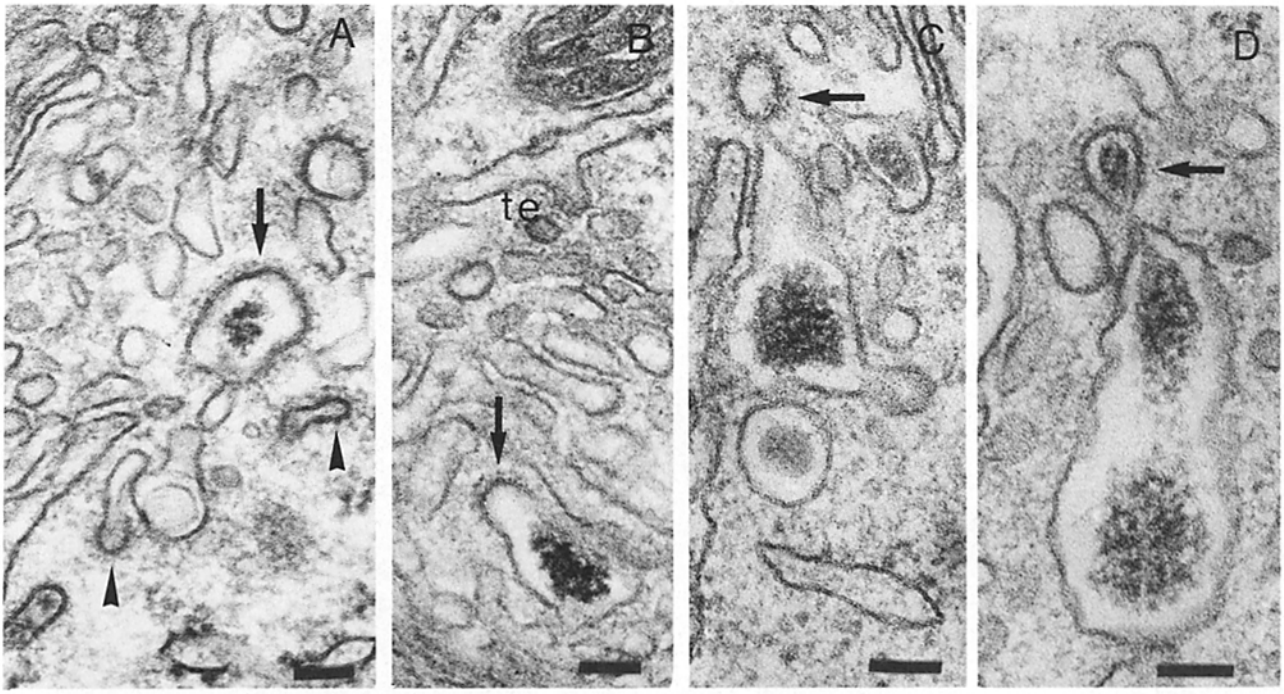


Figure 3. Coated vesicles and coated surfaces at the *trans* Golgi network. *A* shows a clathrin-coated dilation (*arrow*) of the *trans* Golgi network, containing condensing secretory proteins. Note also coated tubular element (*arrowheads*). *B* shows a similar dilation of the *trans* Golgi network with clathrin coating a region of membrane that might be at an early stage of vesicle budding (*arrow*). A transitional element is labeled *te*. *C* shows a dilation of the *trans* Golgi network containing a clump of secretory proteins. A clathrin-coated vesicle, without particulate contents, is budding from (or fusing with) this dilation (*arrow*). *D* shows a dilation of the *trans* Golgi network, with condensing secretory proteins, which is giving rise to a coated vesicle containing secretory proteins (*arrow*). The distribution of secretory proteins between the coated vesicle and the dilated compartment clearly indicates that this is a budding event. Bars, 0.1 μm.

Clathrin Coats and Coated Vesicles on the Trans Golgi Network

Dilations of the *trans* Golgi network containing secretory protein frequently have coated surfaces (Fig. 3). The coated regions range from the entire surface of the dilation (Fig. 3 *A*) to typical peri-Golgi coated vesicles ~1,000 Å in diameter in the process of budding or fusing (Fig. 3, *B–D*). While most of these coated vesicles do not have particulate contents (Fig. 3, *B* and *C*), after searching carefully some can be found that contain condensed secretory protein (Fig. 3 *D*).

To establish unequivocally that the coats on the surfaces of the *trans* Golgi network, as well as those on the surface of detached immature secretory granules (see below), consist of clathrin we labeled thin frozen sections with an affinity-purified antiserum specific for polymerized clathrin (17) using the immunogold procedure. The result was that expected. As an example, Fig. 4, *A–C*, shows regions of the surfaces of the *trans* Golgi network labeled for clathrin. In addition, the 1,000 Å cytoplasmic vesicles associated with this compartment are also labeled.

ACTH in Coated Vesicles and Condensing Vacuoles

Most of coated vesicles associated with the *trans* Golgi network that can be seen in thin sections of conventionally fixed and embedded cells lack particulate contents; for example Fig. 3, *B* and *C*. To assess whether or not some of these coated vesicles without particulate contents at this site also contain ACTH, or its precursor POMC, we labeled AtT20 cells with an affinity-purified antiserum against ACTH, which recognizes and immunoprecipitates both POMC and mature ACTH (3, 13).

After immunoperoxidase labeling, which amplifies any signal because of the enzymatic reaction involved and also allows examination of large section areas, we detected labeling of the entire regulated secretory pathway from the rough endoplasmic reticulum to the mature secretory granules (Fig. 5 *A*). Labeling of the endoplasmic reticulum, including the nuclear envelope (not shown), was the least intense. In the Golgi stacks, sometimes all, but sometimes only some of the cisternae were labeled. The *trans* Golgi reticulum was labeled and in favorable sections its extensive branching was revealed (Fig. 5 *B*). Several of the small coated vesicles budding from (or fusing with) dilated regions of the *trans* Golgi reticulum, were also clearly labeled by the anti-ACTH antiserum (Fig. 5, *C–G*). This establishes unequivocally that a significant number of the coated vesicles associated with the *trans* Golgi reticulum transport ACTH. These ACTH-immunolabeled coated vesicles were observed more frequently than the rare coated vesicles, containing condensed secretory proteins in conventional thin sections of the *trans* Golgi reticulum (for example, Fig. 3 *D*). This implies that some of the coated vesicles at this site carry ACTH or its precursor as soluble proteins.

After immunogold labeling of both cryosections and Lowicryl K4M sections of cells fixed identically to those used for immunoperoxidase labeling, only condensed secretory protein in the *trans* Golgi network and the cores of immature and mature secretory granules were labeled (Fig. 6, *A–C*). Neither the endoplasmic reticulum nor the stacked Golgi cister-

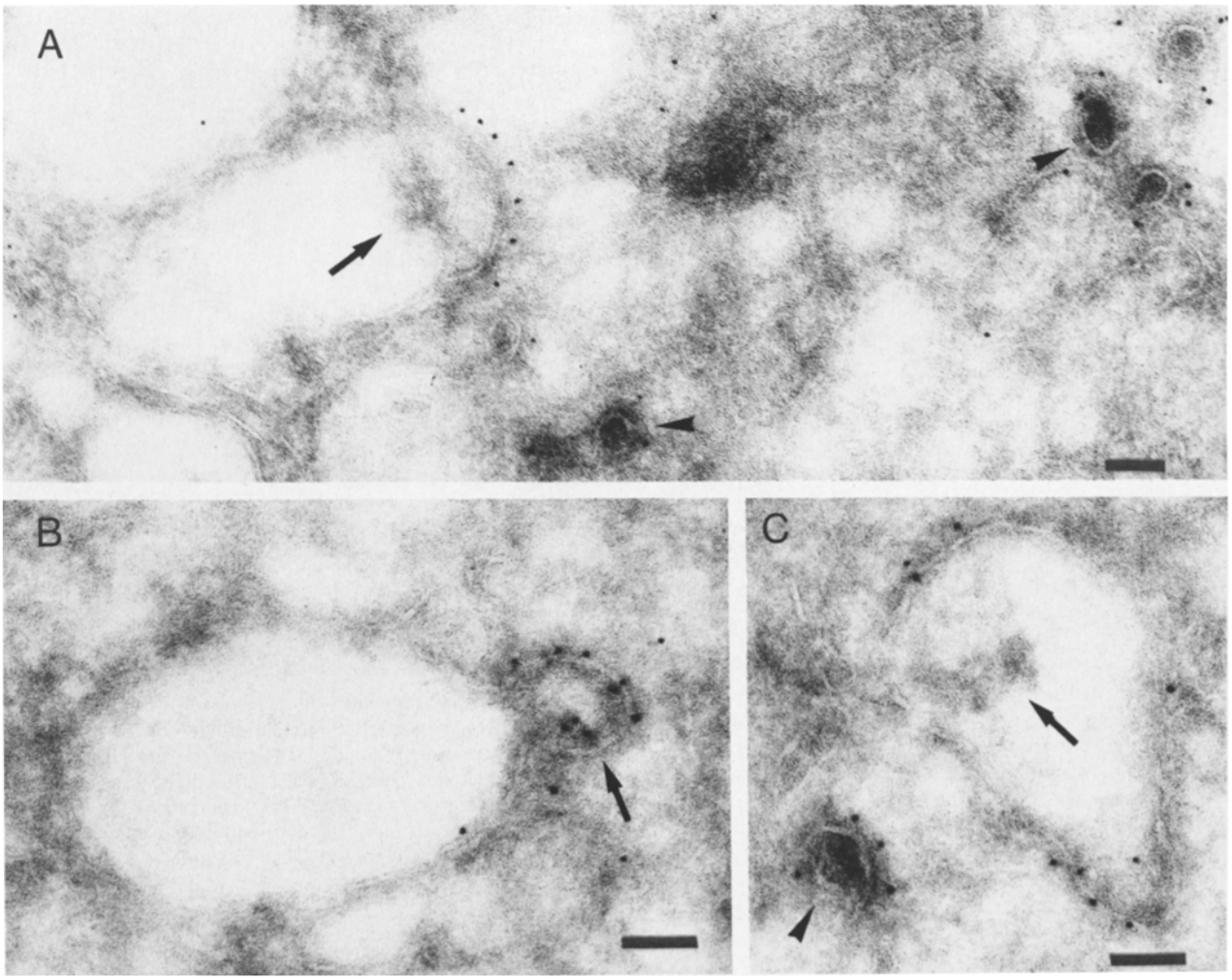


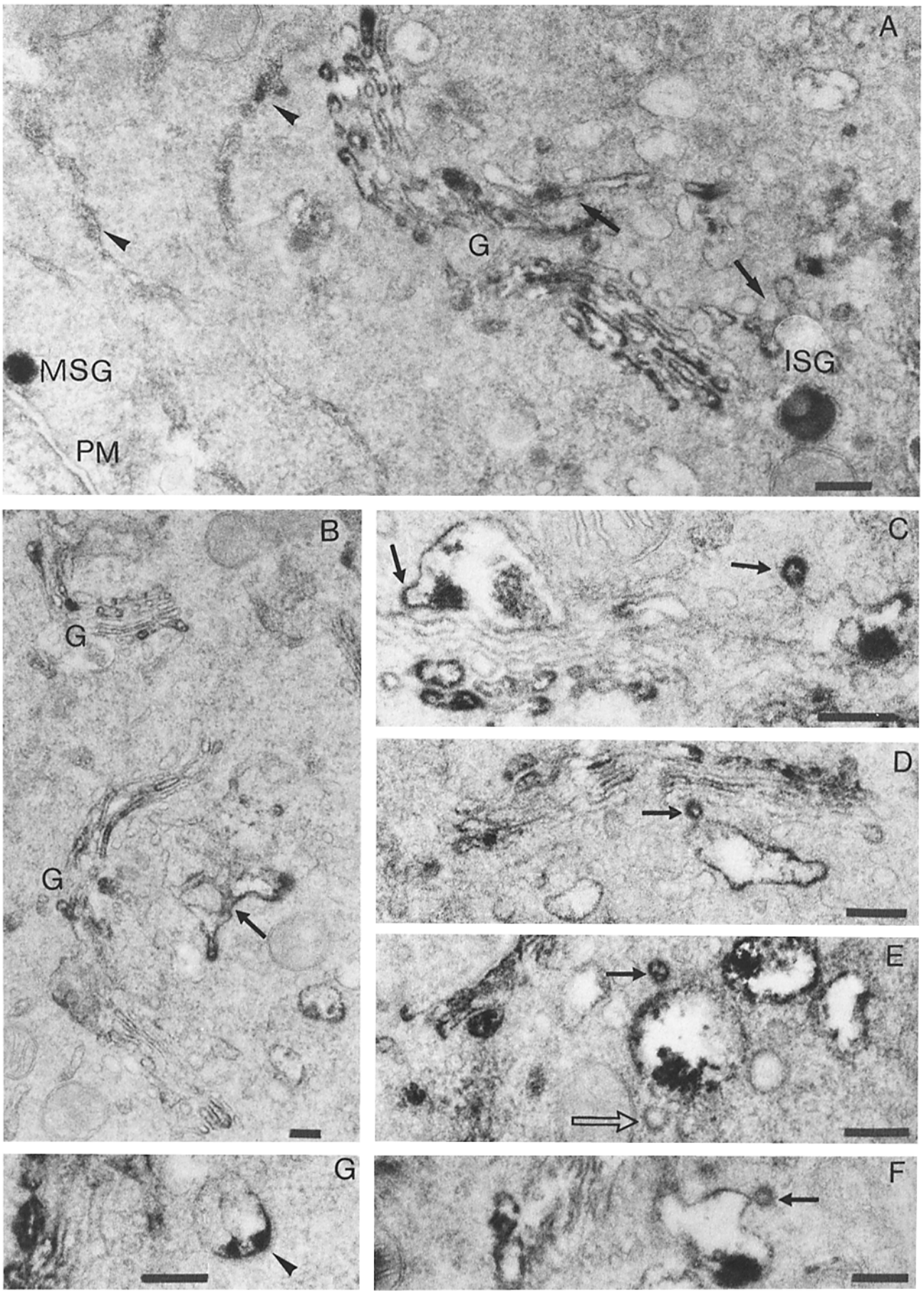
Figure 4. A–C show dilations of the *trans* Golgi network, labeled for clathrin by the immunogold procedure. Parts of the surfaces of these compartments are labeled indicating the presence of polymerized clathrin; the budding (or fusing) vesicle in *B* is also clathrin coated (*arrow*). The arrows in *A* and *C* point to condensing secretory protein. In *A* and *C*, small clathrin coated vesicles in the cytoplasm are also labeled (*arrowheads*). Immunogold labeling of the ACTH in the condensing secretory proteins within the *trans* Golgi network is shown in Fig. 6. Bars, 0.1 μm.

nae were consistently labeled above background. Labeling was least over the *trans* Golgi network and heaviest over the cores of discharged granules still close to the cell surface (Figs. 6, *D* and *E*); over some extracellular granule cores, there were more than twice as many gold particles as over the most heavily labeled intracellular granules. We interpret these observations to indicate (*a*) that the concentration of ACTH and the other proteins of the core of secretory granules leading to their condensation first occurs in the *trans* Golgi network; (*b*) that the concentration of ACTH in the endoplasmic reticulum and Golgi stacks is below the threshold of detection by the immunogold method, and (*c*) that as the cores of secretory granules dissolve in the medium there is a decrease to the steric hindrance to immunogold labeling.

Detached Immature Granules in the Peri-Golgi Region

To determine at what stage in their maturation developing granules detach from the *trans* Golgi network to become separate compartments, we examined serial sections of the Golgi region of AtT20 cells. Detachment occurs at a late stage in the maturation of the core, after it has become highly condensed and is separated from the membrane by a halo of fairly uniform width, which is a characteristic of mature

Figure 5. Immunoperoxidase labeling of AtT20 cells for ACTH. *A* shows elements of the entire secretory pathway labeled for ACTH. All cisternae of the Golgi stack (*G*) are labeled as is the *trans* Golgi reticulum (*arrows*). There is a detached immature secretory granule (*ISG*) in the Golgi region which is larger than a mature peripheral secretory granule (*MSG*). The profiles of the rough endoplasmic reticulum are also labeled (*arrowheads*). The plasma membrane is indicated (*PM*). *B* shows in addition to labeled Golgi stacks (*G*), a labeled *trans* Golgi network (*arrow*) sectioned *en face*. Compare this figure with Fig. 1. *C–G* are examples of coated vesicles (*arrows*) labeled for ACTH that are budding from (or fusing with) elements of the *trans* Golgi network containing secretory protein. A coated surface on a dilation of the *trans* Golgi network can be seen in *G* (*arrowhead*). There is a coated vesicle not labeled for ACTH in *E* (*open arrow*). Bars, 0.2 μm.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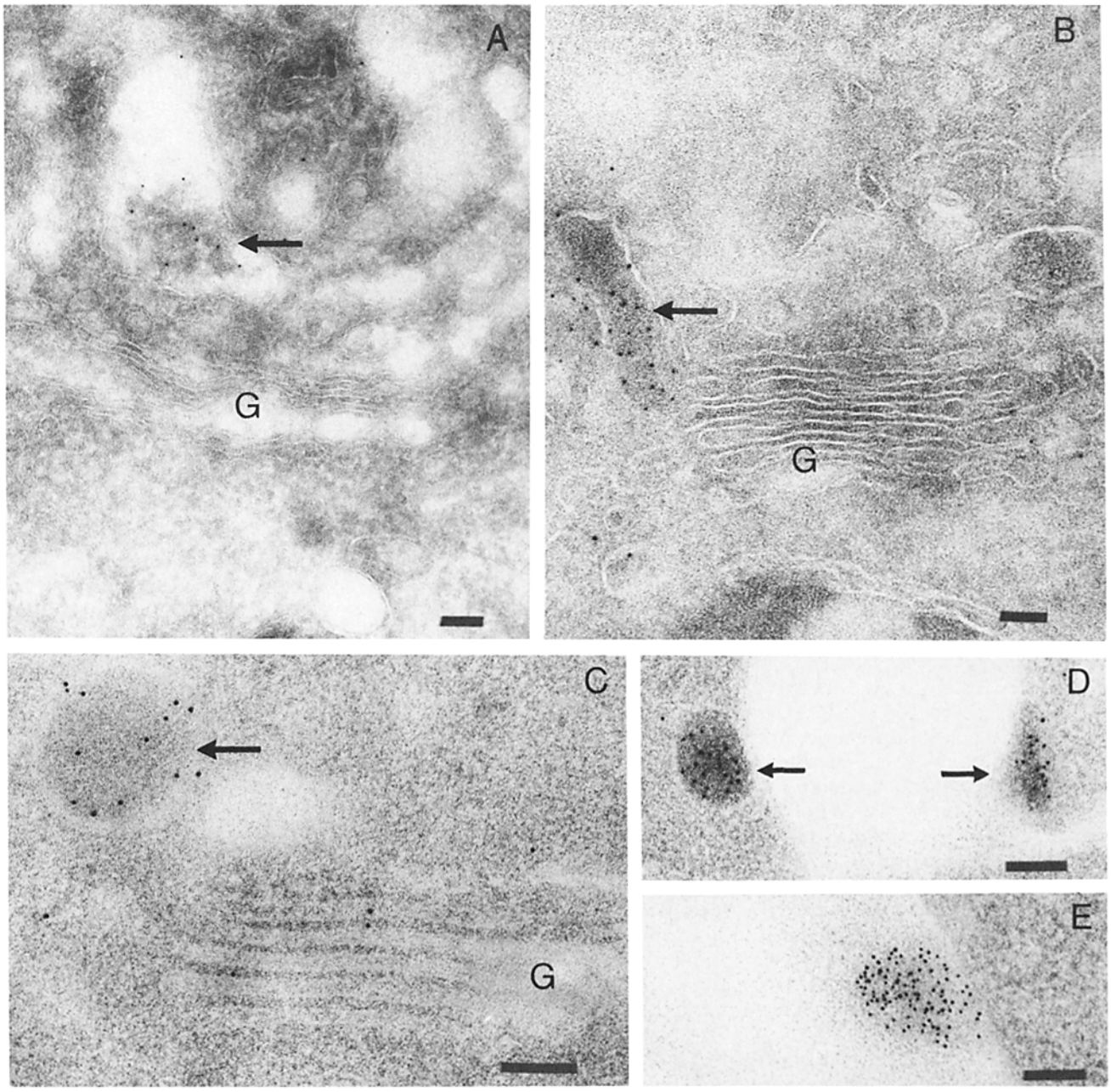

Figure 6. Immunogold labeling of AtT20 cells for ACTH. *A* and *B* show cryosections of AtT20 cells in which labeling is restricted to the condensing secretory proteins in the *trans* Golgi network (*A*) and an immature secretory granule (*B*) (*arrows*). The Golgi stacks (*G*) were not labeled above background in these sections, neither was the rough endoplasmic reticulum (not shown). *C–E* are thin sections of cells embedded in Lowicryl K4M and immunogold labeled for ACTH. In *C*, as in *A* and *B*, labeling was restricted to the immature secretory granule (*arrow*). The stacked Golgi cisternae (*G*) were not labeled above background. *D* shows labeled secretory granules (*arrows*) at the periphery of two cells. *E* shows an extracellular granule core dissolving in the medium in another area of the same section. Note the heavier labeling of the extracellular granule compared to the intracellular granules. Bars, 0.1 μm.

granules (see Figs. 2, 7–10). In many cases, the core had also assumed a spherical or ovoid shape and was ~1,800–2,000 Å in diameter as in mature peripheral granules. Fig. 7 shows an example of an immature granule, with a condensed almost spherical core, which appears to be still attached to, or very intimately associated with tubular elements of the *trans* Golgi reticulum. Fig. 8 is another example of a peri-Golgi granule still apparently attached to a tubular element. By contrast, the granule in Fig. 2 with a morphologically mature core, while still being close to a Golgi stack, is without detectable connections to the *trans* Golgi network. Clearly developing granules reach an advanced stage of morphological maturation before detaching from the *trans* Golgi network.

Coated Surfaces and Coated Vesicles Associated with Peri-Golgi Immature Granules

Peri-Golgi granules often have clathrin coats on parts of their surface (Fig. 9, *A* and *B*) and many also have cores which

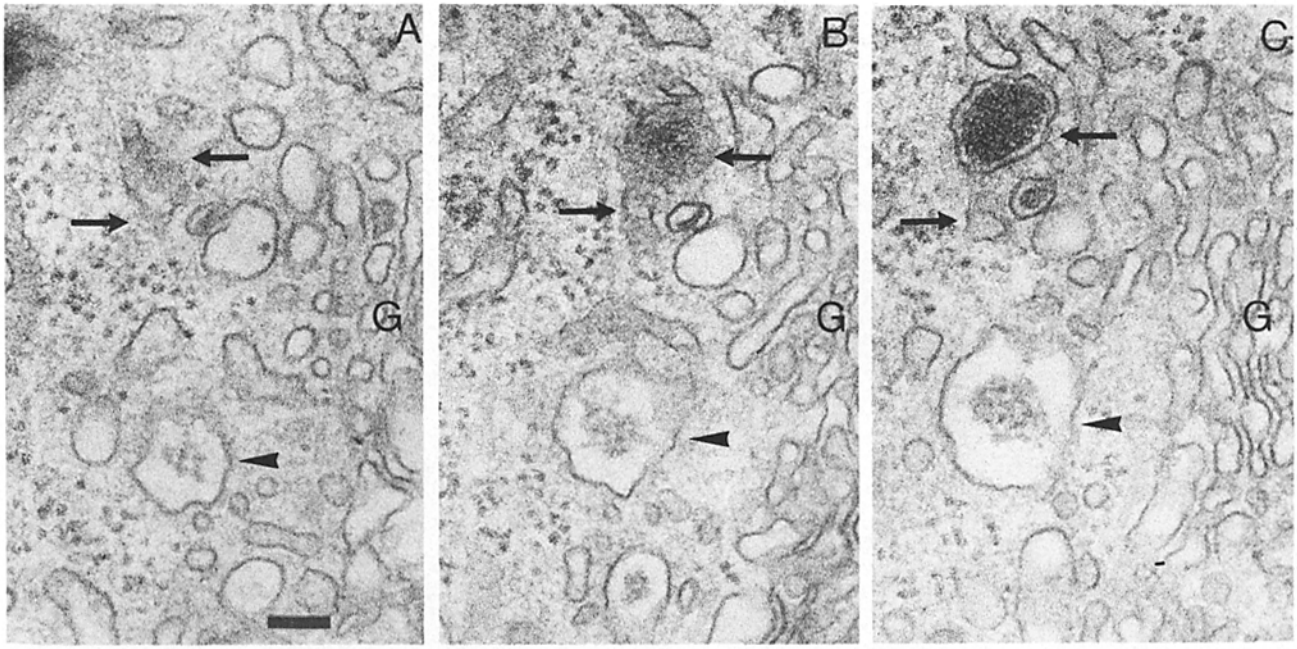

Figure 7 (A–C) Serial sections of an immature peri-Golgi secretory granule which appears to be still attached to a tubular region of the *trans* Golgi network (*arrow*). Also note a large dilation of the *trans* Golgi network containing condensing secretory protein (*arrowhead*) that extended throughout the ribbon of sections. The Golgi stack is labeled *G*. Bar, 0.1 μm.

are larger and more irregularly shaped than those of mature granules (Fig. 9, *A* and *B*). It is usually difficult to distinguish vesicular budding from vesicular fusion events in electron micrographs; however, the distribution of condensed secretory proteins sometimes allows this distinction to be made with some confidence. For example Fig. 9, *C* and *D*, apparently shows the budding of coated vesicles, each ~1,000 Å in diameter and containing highly condensed granule core proteins, from maturing peri-Golgi secretory granules, while Fig. 10 shows micrographs of six serial sections containing a peri-Golgi secretory granule. In Fig. 10 *C*, the granule appears to have a simple spherical core, but the following sections reveal that condensed secretory protein is being removed from the granule by a budding coated vesicle (Fig. 10, *D* and *E*).

Fig. 9, *E–G*, shows another example of a coated vesicle (dimensions 1,110 Å × 980 Å) associated with a detached peri-Golgi granule. In this case, the membrane of the coated vesicle is continuous with that of the granule, however the ball of condensed secretory protein (~860 Å × 640 Å) within the lumen of the vesicle is clearly separated from the core of the granule. This series of micrographs shows either a late stage in a budding event, after the separation of the condensed secretory protein in the coated vesicle from that remaining in the core, or possibly a fusion event and the delivery of secretory proteins. One striking feature of the coated structures associated with peri-Golgi secretory granules is that they contain condensed secretory proteins. By contrast at the surface of *trans* Golgi network, most coated vesicles do not have particulate contents (see above).

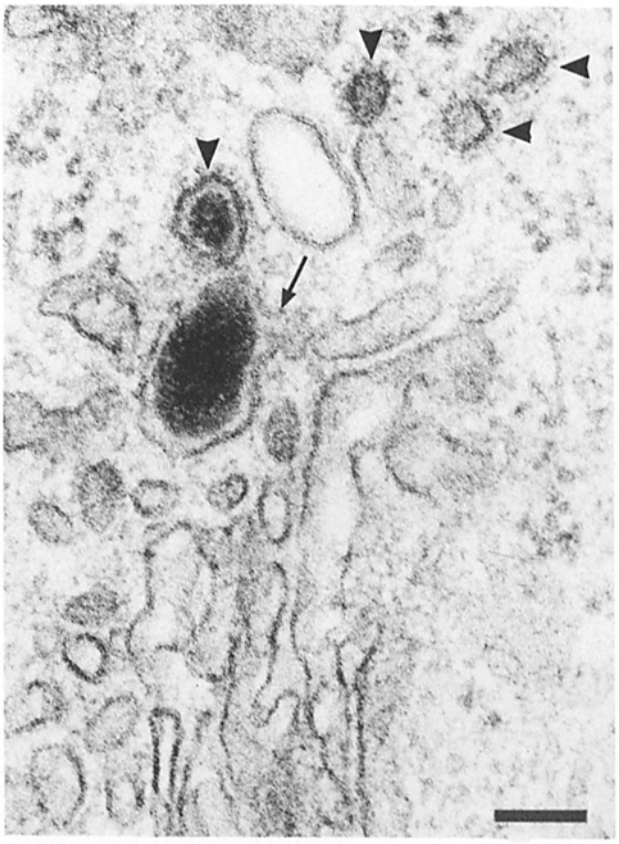

Figure 8. A peri-Golgi immature secretory granule. Note that the membrane of the granule appears to be continuous with a tubular element (*arrow*). Note also a coated vesicle with condensed secretory protein and other coated vesicles without particulate contents (*arrowheads*). Bar, 0.1 μm.

Discussion

Of the many previous studies of the formation of secretory granules in endocrine and exocrine cells (reviewed in refer-

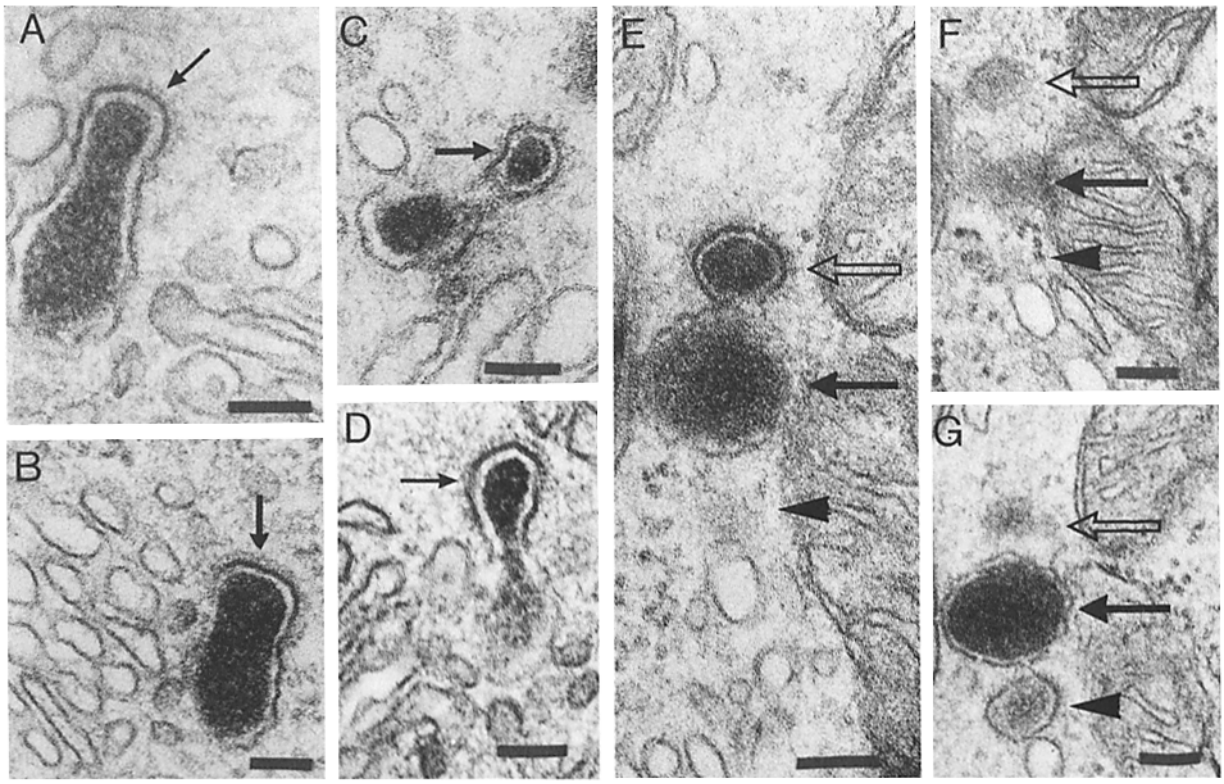

Figure 9. *(A–G)* Clathrin-coated surfaces of immature secretory granules. *A* and *B* show detached granules close to Golgi stacks; the cores are highly condensed and separated from the membrane by a uniform halo; part of the membrane is coated (*arrows*). *C* and *D* show granules close to Golgi stacks giving rise to a clathrin-coated vesicle, ~1,000 Å in diameter, that contains condensed secretory protein (*arrows*). *E–G* show three serial sections. In *E* the membrane of a granule is seen to be in continuity with that of a coated vesicle ~1,000 Å in diameter. The condensed secretory protein of the granule core is, however, separated from the ball of condensed secretory proteins in the vesicle. *F* and *G* are micrographs of the immediately preceding and following thin sections. Open arrows indicate the coated vesicle, arrows indicate the granule, and the arrowheads indicate a second coated vesicle containing less condensed secretory proteins. Bars, 0.1 μm.

ence 5), those of Hand and Oliver (15) and Broadwell and Oliver (3) are particularly germane to our observations. They showed that in vivo in both acinar cells of the rat exorbital lacrimal gland and in vasopressin-producing neurons the extreme *trans* Golgi compartment, the GERL cisternae, is the predominant site of secretory granule formation. Under normal conditions, condensed secretory protein was not found in the stacked Golgi cisternae in these cells. We find the same situation in AtT20 cells, a clonal line of pituitary tumor cells. Moreover, in AtT20 cells the extreme *trans* Golgi compartment is seen to be complex; it comprises tubular elements linked in a network with dilated regions in which condensed secretory proteins first accumulate.

The *trans* Golgi network is the site of budding (or fusion) of clathrin-coated vesicles, some of which contain secretory protein. What is the function of this coated vesicular transport of ACTH? From the published data on other cell types there is good reason to believe that the *trans* Golgi network is the compartment in which proteins destined for different sites in the cell are sorted from one another. In MDCK cells, viral membrane proteins destined for the apical or basolateral surfaces do not part company before they reach the extreme *trans* side of the Golgi stacks (7) in which sialyl transferase is localized (28). When these cells are incubated at 20°C, vesicular stomatitis virus and influenza virus membrane proteins fail to reach their appropriate domains of the plasma membrane and instead accumulate at the *trans* side of the Golgi apparatus (19). This 20°C compartment has recently been identified in baby hamster kidney cells as the acid phosphatase-positive *trans* Golgi network (11). In all probability, in AtT20 cells the constitutive and regulated exocytic pathways diverge from the *trans* Golgi network. The coated vesicles that contain ACTH and bud from the *trans* Golgi network could, therefore, be destined for the constitutive pathway, while the aggregates of precipitated secretory proteins remaining in the dilations of the network would mature into the cores of the secretory granules of the regulated pathway. Within the immature secretory granules, the ACTH precursor could then be proteolytically cleaved to liberate the mature hormones characteristic of the regulated pathway (16, 24).

However at present there is no unequivocal evidence that clathrin is involved in constitutive exocytosis in any cell type (reviewed in 12); coated vesicles leaving the *trans* Golgi network and containing ACTH might be unrelated to either exocytic pathway. For instance, the receptor-mediated transport of hydrolytic enzymes from the Golgi apparatus to lysosomes occurs in clathrin-coated vesicles (6, 29, 30, 32, and references therein). If this transport occurs at the level of the *trans* Golgi network rather than earlier, which is currently disputed (4, 8), it is possible that ACTH is sometimes adventitiously incorporated into coated vesicles destined for lysosomes. However, peri-Golgi lysosomes are not labeled by the immunoperoxidase reaction with the anti-ACTH antiserum

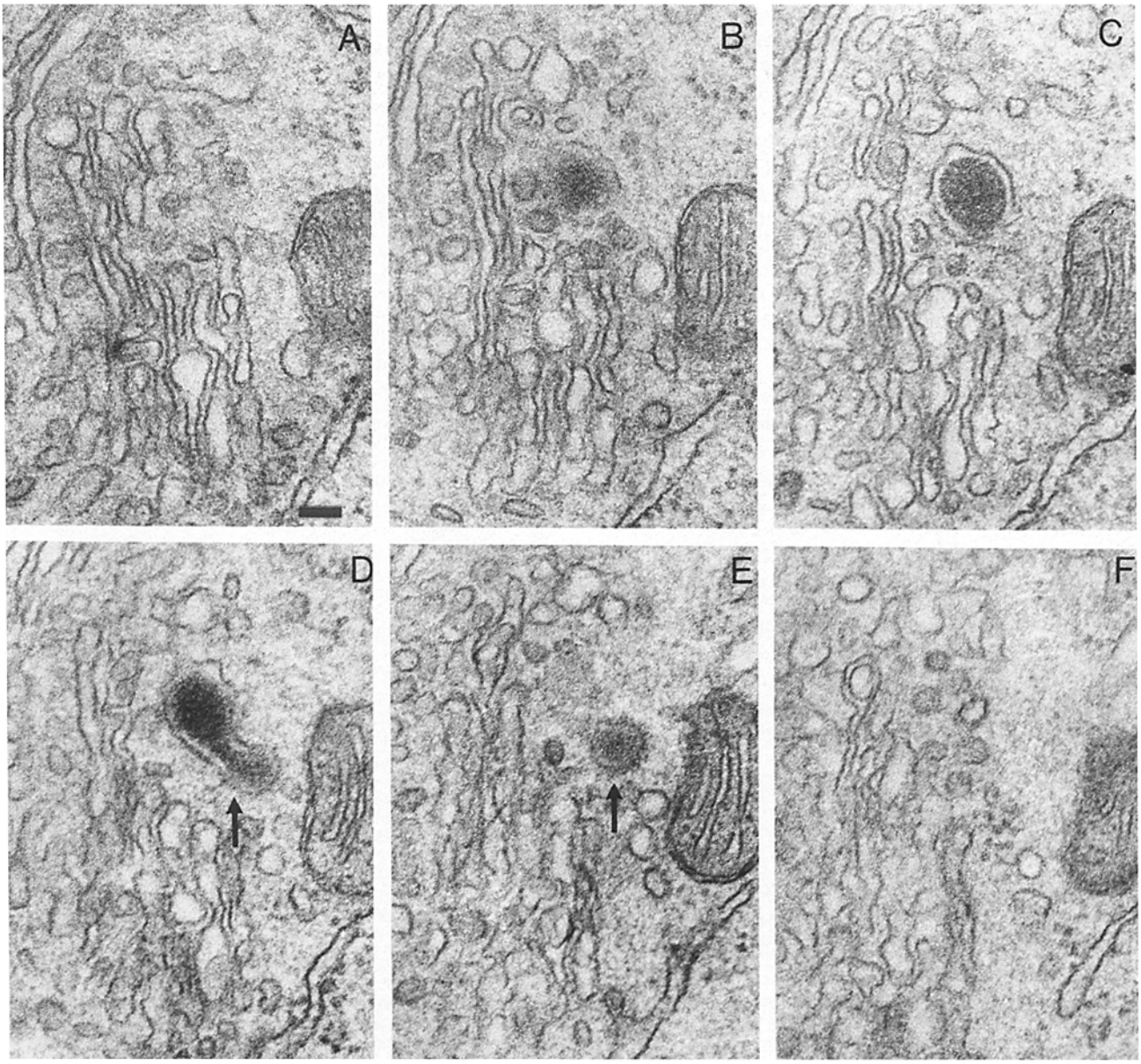

Figure 10. Micrographs of serial sections of an immature secretory granule lying close to a Golgi stack. In *C*, the granule appears to have a regular, almost spherical shape with a diameter of ~2,200 Å. *D* and *E*, however, reveal that condensed secretory protein is being withdrawn from the granule as a coated vesicle buds from it (*arrows*). Bar, 0.1 μm.

(data not shown), so if any ACTH reaches lysosomes from any source, it must be rapidly degraded.

Compared with the *trans* Golgi network, the peri-Golgi immature secretory granules are a structurally, biochemically, and functionally simpler compartment, destined to become mature granules. The coated vesicles associated with the immature granules are characterized by their content of highly condensed secretory protein. Pulse-chase experiments indicate that the proteolytic conversion of precursor to mature ACTH begins concomitant with the packaging of the hormone into secretory granules (13). We envisage, therefore, that the peri-Golgi immature granules contain ACTH that has been or is in the process of being cleaved from its precursor. If so, the coated vesicles containing condensed secretory proteins, which bud from the immature secretory granules of the regulated pathway, are unlikely to enter the constitutive pathway, which primarily externalizes the uncleaved ACTH precursor (14). We are trying to raise antibodies to a synthetic peptide corresponding to the sequence of the cleavage site in POMC from which ACTH is liberated by proteolysis. Such antibodies would allow us to distinguish compartments containing POMC from those containing mature ACTH.

One of the striking characteristics of mature secretory granules of endocrine cells, revealed both by electron microscopy (31) and sedimentation analysis (13), is their uniform size. The size of the granules is so characteristic that it can be used to distinguish one type of endocrine cell from another (31). How is such cell-type specific uniformity achieved, especially since the aggregates of condensed secretory protein in the *trans* Golgi network vary greatly in size and shape? Apparently the secretory granule contents self-

assemble into a core (perhaps by some phase separation process) whose characteristic size must be determined by the particular set of proteins. Kelly (16) has recently drawn the analogy between the budding of secretory granules from the *trans* Golgi cisternae and the budding of enveloped viruses at the cell surface. He proposed that any excess proteins or membrane not destined for the mature granules might be removed by vesicular transport and recycled back up the pathway. We interpret our micrographs in precisely that way, since they apparently show withdrawal of excess condensed secretory proteins in coated vesicles budding from maturing secretory granules. This removal in coated vesicles of excess condensed secretory proteins might be part of the mechanism that ensures the uniform size as well as appropriate composition of mature granules. The condensed secretory proteins removed by the coated vesicles could then be returned to less mature granules or to the *trans* Golgi network as Kelly proposes (16). Delivery to lysosomes in a pathway analogous to that in pituitary mammotrophs (33) seems less likely since lysosomes are not labeled by immunoperoxidase cytochemistry with anti-ACTH antisera.

The budding of clathrin-coated vesicles from immature granules would also serve to remove those molecules to which clathrin binds from the granule membrane by concentrating them in the membrane of the budding coated vesicle. This would be an example of Kelly's proposal (16) that vesicular transport removes inappropriate proteins from immature granules; since the membrane of mature secretory granules is never coated it must lack functional clathrin-binding molecules.

In summary, both the *trans* Golgi network and peri-Golgi immature secretory granules are the site of clathrin-coated vesicular transport of ACTH. In the case of the peri-Golgi granules, we are confident that secretory proteins are withdrawn from the granule cores by this mechanism. In other words, coated vesicular transport plays a direct role in the maturation of secretory granules of the regulated exocytic pathway in AtT20 cells.

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