Sorting of Three Secretory Proteins to Distinct Secretory Granules in Acidophilic Cells of Cow Anterior Pituitary

Sadamitsu Hashimoto,*‡ Guido Fumagalli,* Antonia Zanini,* and Jacopo Meldolesi,*‡

*CNR Center of Cytopharmacology, Department of Pharmacology, and ‡S. Raffaele Scientific Institute, University of Milan, Milan, Italy

Abstract. The distribution of three proteins discharged by regulated exocytosis—growth hormone (GH), prolactin (PRL), and secretogranin II (SgII)—was investigated by double immunolabeling of ultrathin frozen sections in the acidophilic cells of the bovine pituitary. In mammotrophs, heavy PRL labeling was observed over secretory granule matrices (including the immature matrices at the *trans* Golgi surface) and also over Golgi cisternae. In contrast, in somatotrophs heavy GH labeling was restricted to the granule matrices; vesicles and tubules at the *trans* Golgi region showed some and the Golgi cisternae only sparse labeling. All somatotrophs and mammotrophs were heavily positive for GH and PRL, respectively, and were found to contain small amounts of the other hormone as well, which, however, was almost completely absent from granules, and was more concentrated in the Golgi complex, admixed with the predominant hormone. Mixed somatomammotrophs (~26% of the acidophilic cells) were heavily positive for both GH and PRL. Although admixed within Golgi cisternae, the two hormones were stored separately within distinct granule types. A third type of granule was found to contain SgII. Spillage of small amounts of each of the three secretory proteins into granules containing predominantly another protein was common, but true intermixing (i.e., coexistence within single granules of comparable amounts of two proteins) was very rare. It is concluded that in the regulated pathway of acidophilic pituitary, cell mechanisms exist that cause sorting of the three secretory proteins investigated. Such mechanisms operate beyond the Golgi cisternae, possibly at the sites where condensation of secretion products into granule matrices takes place.

In glandular cells, secretory granules form at the *trans* face of the Golgi complex (GC)[1] as a consequence of two processes that are still poorly understood. These two processes are (*a*) the sorting of the specific secretory proteins discharged intermittently in response to appropriate stimuli (regulated pathway) from the products destined to remain in the GC lumen or to be discharged extracellularly continuously (constitutive pathway); and (*b*) the concentration of these specific products to yield the typical dense granule core (5, 15, 21). For convenience, this second process will be referred to herein as condensation. The two properties, to be sorted into the regulated pathway and to undergo condensation, appear to be dictated by the nature of secretory proteins, as they are maintained even when one such protein is expressed in a heterologous cell system (19, 26). Yet by all criteria so far considered, the various secretory proteins of the "regulated type" appear extremely heterogeneous, with no obvious common structural features or sequence homology, so that to date no mechanism has been identified with certainty to account for their common intracellular destination (5, 15).

To obtain precise, although indirect, clues about the processes underlying the formation of secretory granules, we have chosen an immunocytochemical approach (double immunolabeling of ultrathin frozen sections) and investigated in the acidophilic cells of the bovine anterior pituitary the distribution of three secretory proteins targeted to the regulated pathway: growth hormone (GH), prolactin (PRL), and secretogranin II (SgII). GH and PRL are the typical hormones expressed in high amounts by somatotrophs and mammotrophs, respectively. Moreover, the two hormones are coexpressed in comparable amounts by a population of mixed somatomammotrophs (7, 8, 17), where they are known to be preferentially stored separately within two distinct types of secretory granules (8). To date, little information has been available about the distribution of the two hormones in the other portions of the secretory pathway, in particular in the GC, the subcellular structure where assembly of secretory granules takes place (5, 6, 15). The other protein investigated, SgII, is an acidic, secretory, tyrosine-sulfated phosphoprotein (4, 25), initially discovered in the pituitary gland where it is localized primarily within gonadotropic and thyrotropic cells, but also present in the acidophilic cells (24). Its subcellular distribution in relation to that of GH and PRL had not been investigated in detail yet.

1. *Abbreviations used in this paper*: GC, Golgi complex; GH, growth hormone; PRL, prolactin; SgII, secretogranin II.

Materials and Methods

A rabbit antiserum against ovine PRL, kindly provided by Dr. C. H. Li (Hormone Research Laboratory, University of California, San Francisco, CA), was characterized by immunoblotting as reported in reference 8. Antibodies against bovine GH and SgII were raised in rabbits and purified by affinity chromatography. Preparations thus obtained were monospecific for their own antigen as described in detail in references 8 and 24. Purified rabbit antibodies against bovine brain cholinesterase were kindly donated by Dr. C. Gotti (CNR Center of Cytopharmacology, University of Milan, Milan, Italy). Gold particles, 5 and 15 nm in diameter, were prepared using white phosphorus and citrate as reducing agents (27). After conjugation with staphylococcal Protein A (Sigma Chemical Co., St. Louis, MO) the particles were purified by centrifugation on a 10–30% linear sucrose gradient.

Anterior pituitaries from two 3-y-old Holstein Friesian nursing cows were collected immediately after slaughter and fixed for 2 h at 4°C with 4% paraformaldehyde and 0.2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.3). Small pieces were immersed in 2.3 M sucrose in PBS for 1 h, mounted on specimen holders, and quickly frozen in liquid nitrogen. Silver-to-gold thin frozen sections were cut at −100°C on a microtome (Reichert Scientific Instruments, Buffalo, NY) equipped with a FC4 cryochamber. The sections were transferred to Formvar-coated nickel grids and then processed for immunostaining. After incubation in the first antibody and staining, (usually with the large, 15-nm gold particles) the grids were rinsed in PBS, and then floated on a drop of unconjugated Protein A (0.05 mg/ml in PBS) for 15 min to block any Protein A-binding site remaining in the section (10). This was followed by incubation with the second antibody and labeling with the other type of gold particles. The sections were then osmicated, dehydrated, and embedded in LR White resin as described in reference 14.

The specificity of the immunostaining was tested by (*a*) omitting either the first or the second antibody; (*b*) substituting the specific antibodies with unrelated (anti-cholinesterase) antibodies or with the preimmune serum. For quantitation, pictures were chosen at random and printed at a final magnification of 60,000. Particles over various subcellular compartments—secretory granules, the Golgi area (including peri Golgi vesicles and tubules), mitochondria, and nuclei—were counted and their density (number per unit area) determined by using an MOP Digiplan 01 apparatus (Kontron Analytical, Kontron AG, Evrett, MA) for surface measurements. For quantitation of weak signals, labeling with small gold particles was preferred because of its greater sensitivity.

Results

Double Immunolabeling of Pituitary Acidophilic Cells

To obtain information on the relative distribution of the three antigens investigated it was essential that the immunolabeling be carried out under adequate experimental conditions. In particular, high signal-to-background ratios were needed in order to prove the specificity of not only the heavy, but also the weak signals observed. As is illustrated in the large field image shown in Fig. 1 *A*, the difference in the signal intensity (observed in acidophilic cells immunolabeled for GH and PRL) between secretory granules and Golgi elements on the one hand, and the other intracellular structure on the other, was indeed very large. In addition, even part of the cytoplasmic labeling, in particular the labeling over tubules and cisternae (likely the endoplasmic reticulum), was probably specific because newly synthesized hormones are known to be segregated within the endoplasmic reticulum. To quantitate the background, measurements were therefore carried out over structures that were certainly free of secretory proteins; e.g., mitochondria and nuclei. The results obtained in 40 cells (total analyzed areas: 13.7 and 13.6 μm^2) yielded very similar results: in mitochondria 1.3 and 5.9 and in nuclei 1 and 4.5 large and small gold particles per μm^2 were observed, respectively. Very similar values were obtained by quantitating the labeling over nonacidophilic cells (i.e., cells that express neither GH nor PRL) included in the sections immunolabeled for the two hormones (see Fig. 4, *E* and *F*).

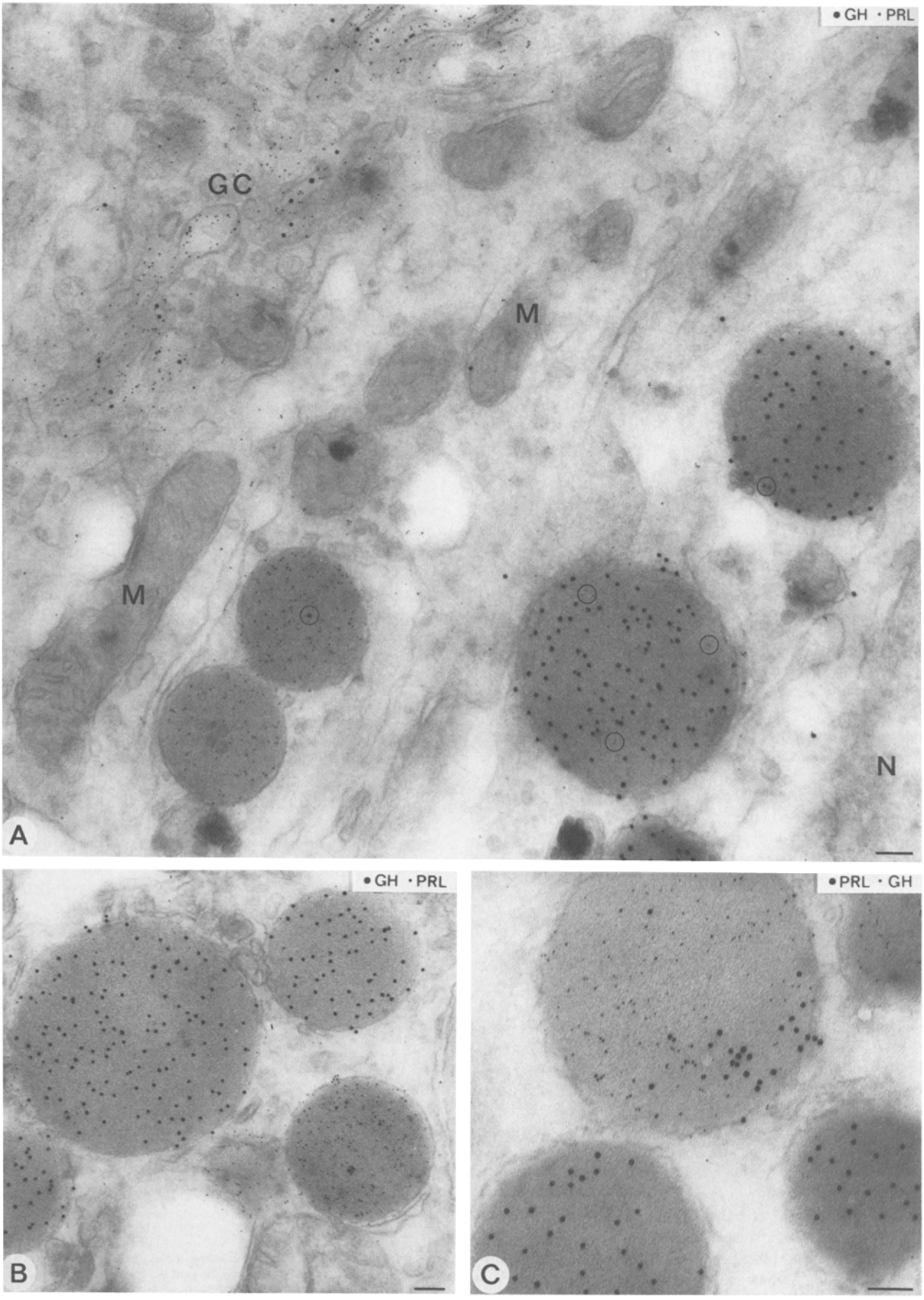
An additional concern was the possibility of cross-contamination in dual labeling due to dissociation of a small fraction of the large Protein A–gold particles bound during the first immunolabeling, that would create new binding sites available for the binding of the second small Protein A–gold particles. A process of this type could affect weak signals, e.g., the weak immunolabeling observed with one hormone over granules heavily positive for the other (see below). We believe however that artifactual dissociation had negligible effect on granule labeling because (*a*) analogous patterns were observed independently of whether the heavy labeling was due to the first or the second gold particles; (*b*) granule labeling for the minority hormone was observed also in single-labeled sections, and in sections dually labeled for one hormone and SgII (that is preferentially addressed to another class of granules; Fig. 4, *A* and *B*), whereas (*c*) it was not observed when an unrelated antibody (anti-cholinesterase) was used in the second immunolabeling (not shown). We conclude, therefore, that under the conditions of our experiments, any labeling clearly exceeding the background values indicated above is specific both for the first and for the second applied antibody.

Distribution of GH and PRL

The distribution of GH and PRL in bovine acidophilic anterior pituitary cells is illustrated in Figs. 1–4. In agreement with the results of our previous study, three types of positive cells were found: those heavily labeled by the antibodies to either one of the two hormones (20.1 and 53.8% of the total labeled cells for GH and PRL, respectively) and the cells heavily labeled by both (26.1%; number of analyzed cells = 303). Unexpectedly, however, a distinct immunoreactivity for the second hormone was found to be present at definite sites even in the first two types of cells.

Mature Secretion Granules. Examples of the distribution of GH and PRL in mature secretion granules are given in Fig. 1. In *A*, two adjacent cells, one predominantly expressing GH and the other PRL, are shown. Even in these granules, which contain high concentrations of one hormone, a weak immunolabeling for the other hormone was consistently observed (encircled in *A*). Counting over 70 PRL-rich and 65 GH-rich granules from 10 mammotrophs and 10 somatotrophs, respectively, revealed that the immunolabeling for the minority hormone (54 and 38 small gold par-

Figure 1. GH and PRL immunolabeling of anterior pituitary acidophilic cells. *A* shows two adjacent cells, one predominantly expressing PRL (*left*), the other GH (*right*) (mammotroph and somatotroph, respectively). Notice the marked difference in the immunolabeling intensity of secretory granules and GC with respect to the rest of the cytoplasm (particularly the mitochondria [*M*] and the nucleus [*N*]). The strong immunolabeling of the GC of the mammotroph is due to both PRL and GH, which appear intermixed, whereas in the two granules only one GH gold particle (encircled) is visible. Likewise, only a small degree of PRL labeling (encircled) is visible over the granules of the somatotroph. *B* shows a somatomammotroph with three GH-rich granules and one electron-dense PRL-rich granule. *C*, again from a somatomammotroph, shows a GH-rich granule which includes a localized region rich in PRL. Bar, 0.1 μm.


• GH · PRL
GC
M
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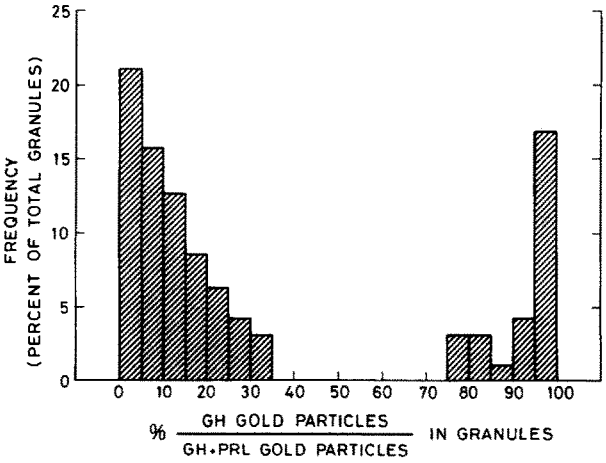

Figure 2. Frequency distribution of the GH and PRL immunolabeling in secretory granules of somatomammotropic cells. Values in abscissa were computed by counting gold particles over individual granule profiles in sections labeled first with anti-PRL (large gold particles) and then with anti-GH (small gold particles). Total number of analyzed granules: 95 from 10 cells.

ticles/μm^2) exceeded the background by several fold, and was therefore specific.

B and *C* come from somatomammotrophs that accumulate GH and PRL predominantly within separate granules. Granules that were heavily positive for PRL were distinctly denser and usually smaller than those heavily positive for GH. However, large PRL-rich granules could also occur. In most granules of somatomammotrophs the labeling for the minority hormone was weak, comparable to that observed in somatotrophs and mammotrophs. In a few, however, the intermixing of GH and PRL was greater and sometimes appeared in the form of heterogeneous granule matrices composed by adjacent regions enriched in either one of the two hormones (Fig. 1 *C*; see also reference 8). As the degree of hormone intermixing in the secretory granules of somatomammotrophs is expected to reflect the efficacy of the mechanism(s) of GH and PRL sorting from each other, a thorough quantitation of the data was carried out. Fig. 2 illustrates granules gathered together in two distinctly separated peaks, one (71%) containing predominantly PRL, the other (29%) GH. In no case did the labeling for the minority hormone approach that of the predominant hormone.

Golgi Complex (GC). The distribution of the two hormones in the GC was investigated in all types of acidophilic pituitary cells: those containing predominantly GH or PRL and the mixed somatomammotrophs. In PRL-rich cells (Fig. 3, *A–C*) the Golgi elements were heavily labeled by anti-PRL antibodies, but GH immunoreactivity was always present. In the lumen of Golgi cisternae differently sized gold particles marking GH and PRL appeared usually intermixed (Fig. 3, *A–C*). With respect to the cisternae, the immature granules (caught in the process of budding or located immediately adjacent to the Golgi stacks) appeared already more enriched in PRL and similar to the mature granules discussed above (Fig. 3, *A* and *B*). Counting gold particles over the Golgi area and secretion granules of the same cell confirmed that the ratio between the minority and the predominant hormone was greater in the first than in the second compartment. Table I illustrates values obtained in 12 PRL-rich cells in which GH was labeled with small, and PRL with large gold particles. GH/PRL immunolabeling ratio was over fivefold greater over the Golgi area than over granules.

In somatotrophs (Fig. 3 *D*) the stacked cisternae of the GC were labeled only sparsely, but the GH immunoreactivity appeared more prominent over a system of the interconnected tubules that might correspond to the *trans* Golgi network (11). Some PRL immunoreactivity was found over the cisternae and small vesicles and tubules at the Golgi periphery (arrows in Fig. 3 *D*). Finally, in the somatomammotrophs heavily positive for both hormones (Fig. 3, *E* and *F*) the Golgi cisternae showed dual intermixed labeling, with a predominance, however, of PRL. In contrast, in the condensed granule matrices of various size located adjacent to the Golgi stack (primarily immature granules), either one of the two hormones was found to greatly predominate over the other.

Distribution of SgII

Our previous studies demonstrated that in the bovine anterior pituitary SgII is concentrated together with the corresponding hormones in the small granules of gonadotrophs and thyrotrophs, and is present only in much smaller concentration within cells that express PRL (24). The results now obtained (Fig. 4) confirm these previous findings and, in addition, demonstrate that somatomammotrophs concentrate SgII in a third type of secretion granule, distinct from those containing either GH or PRL, characterized by small size and by a density similar to or even lighter than that of GH-rich granules (Fig. 4, *A–C*, and *E*). Some degree of colocalization of SgII with GH and PRL could also occur, as revealed in some cases by the apparently random distribution of a few gold particles over granules matrices positive for either one of the hormones (Fig. 4, *A–D*). In other cases the SgII immunolabeling was concentrated over small portions of the granule matrix beneath the limiting membrane, continuous with the rest occupied predominantly by one hormone (Fig. 4, *C* and *D*).

SgII immunolabeling in the GC was extremely rare in somatomammotropic cells, while in the cells containing large numbers of small SgII-positive granules (presumably, gonadotropic and/or thyrotropic cells [24]), a low degree of labeling was common over both the cisternae and vacuoles, some of which contained material of intermediate electron density (Fig. 4, *E* and *F*).

Discussion

The ability of individual somatomammotrophs to segregate

Table I. GH/PRL Immunolabeling Ratio of the Golgi Area and Secretory Granules in Pituitary Mammotrophs Doubly Immunolabeled with Anti-GH and Anti-PRL Antibodies

	Golgi area	Granules	Golgi area/ granules ratio
GH/PRL ratio	0.456 ± 0.142	0.088 ± 0.027	5.38 ± 1.64

GH, small gold particles; PRL, large gold particles. Values (expressed as averages ± SD) were computed by counting gold particles over Golgi areas and secretory granules from 12 cells. Total analyzed areas were 18.6 and 9.9 μm^2 for Golgi areas and secretory granules, respectively.

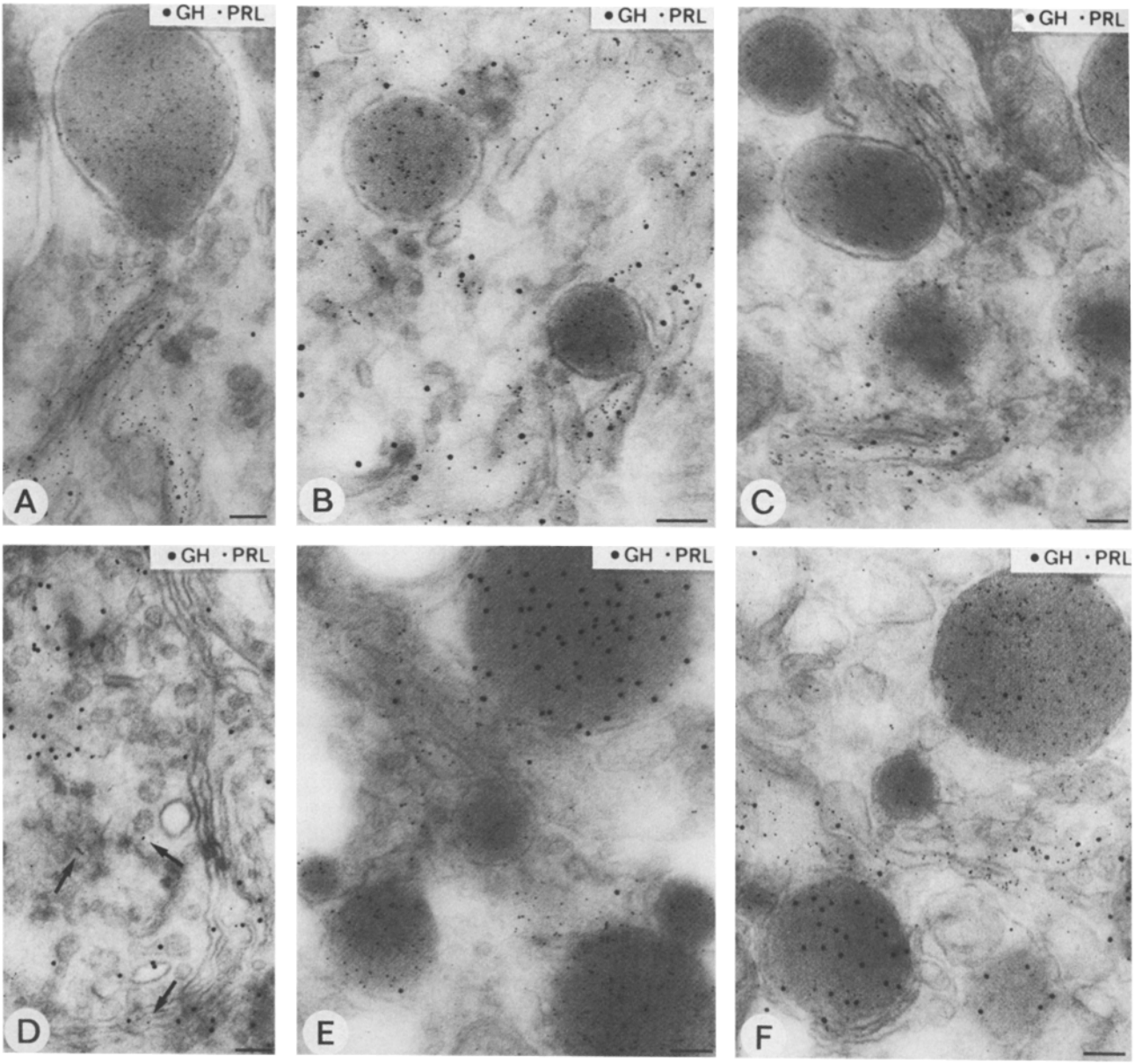

Figure 3. Double GH and PRL immunolabeling of Golgi areas in mammotrophs, somatotrophs, and somatomammotrophs. In the GC cisternae of mammotrophs (*A–C*) marked and intermixed labeling for both PRL and GH is evident, whereas in the adjacent, mostly immature granules the PRL immunolabeling greatly predominates. In somatotrophs (*D*) GH immunolabeling is weak over GC cisternae, and more pronounced over a system of interconnected tubules that might be the *trans* Golgi network. Some PRL immunolabeling is marked by arrows. In somatomammotrophs (*E* and *F*) the mixed immunolabeling of the GC resembles that of mammotrophs, but two populations of immature granules, one enriched in GH, the other in PRL, are visible in the adjacent cytoplasmic areas. Bar, 0.1 μm.

the hormones they express (GH and PRL) within distinct secretory granules had already been described in a previous report from our laboratory (8). In that study, however, the possibility was not excluded that the different subcellular localization was due to an asynchronous synthesis of the two hormones. The present results, on the one hand, demonstrate the existence in somatomammotrophs of a third class of secretory granules, specifically enriched with SgII; on the other hand, they show that GH and PRL are cosegregated within the lumen of the GC. Such a compartment is known to be rapidly drained of its content, and is expected therefore to contain primarily recently synthesized secretory proteins. Cosegregation in Golgi cisternae appears therefore to rule out the hypothesis of an asynchronous synthesis of the two hormones, and indicates that their distribution within distinct granules (and, most probably, the distribution of SgII as well) is due to a process of sorting.

Sorting of the proteins segregated within the lumen of cytoplasmic organelles after synthesis by the membrane-bound polysomes has attracted a great deal of interest in recent years (5, 11, 15, 28). However, only two processes have been considered in detail so far: (*a*) the sorting of lysosomal enzymes (16); and (*b*) the sorting of the secretory proteins targeted to secretory granules (the regulated pathway) from the products destined to remain in the lumen of GC elements, or to be discharged extracellularly by the constitutive path-

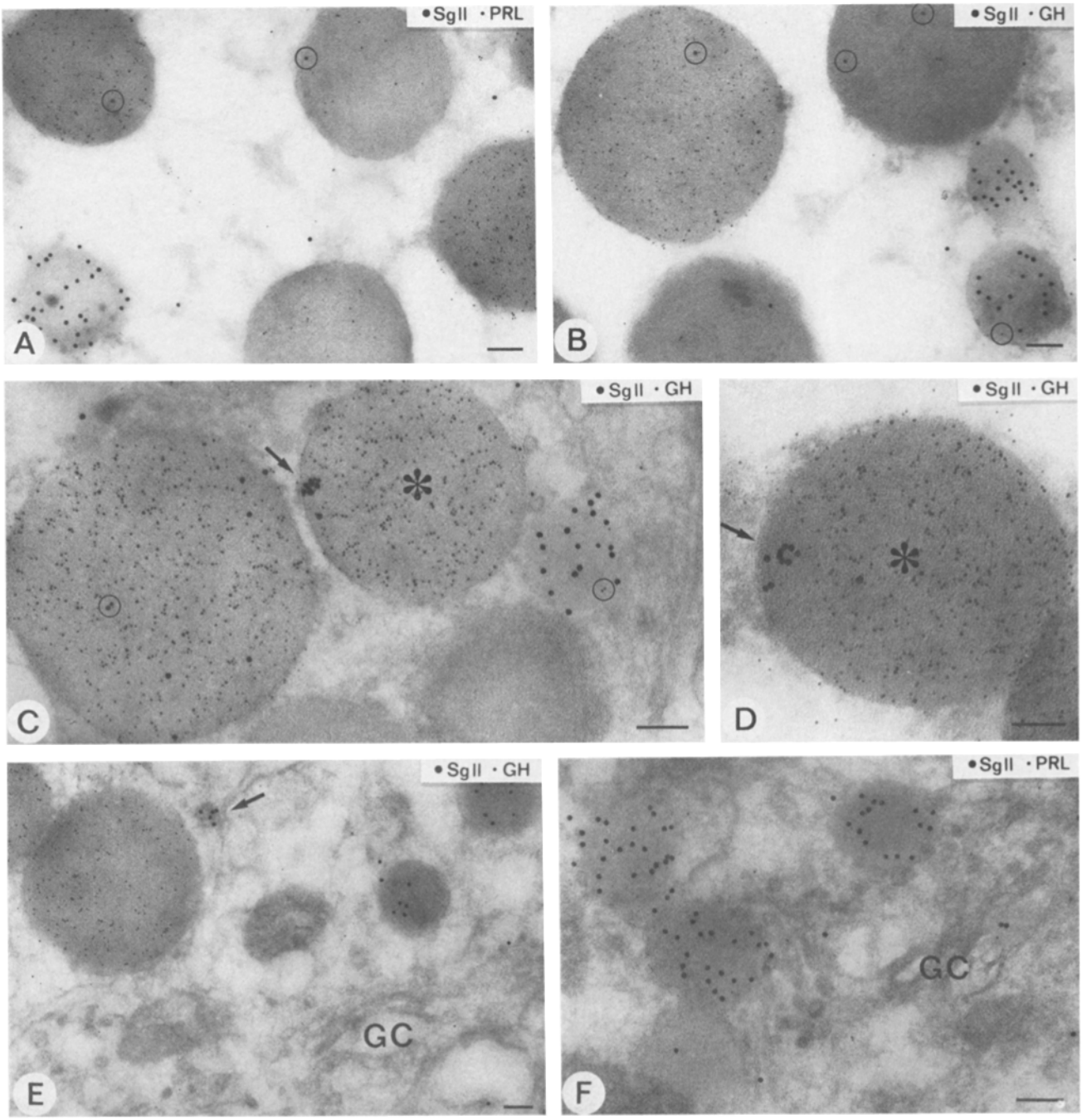

Figure 4. Double immunolabeling for SgII and a hormone (either GH or PRL) in somatomammotrophs and other pituitary cells. Of the three types of granules shown in *A*, one (of small size and light density) is heavily positive for SgII, the second for PRL, the third, only slightly positive for PRL, contains most probably GH, as it seems to correspond to the GH positive granules of *B*. A small degree of cross distribution of SgII and hormones within the granules is marked (encircled). The phenomenon is particularly evident in the GH-rich granules of *C* and *D* (*) where SgII immunolabeling is preferentially concentrated at one edge (*arrows*). *E* and *F* illustrate the Golgi area of SgII-rich cells (most probably gonadotrophs and thyrotrophs [24]). Labeling is mostly over granules but a few gold particles also appear over Golgi cisternae (*GC*). The cell to the left in *E*, predominantly a somatotroph, shows a small SgII-rich granule (*arrow*). Bar, 0.1 μm.

way (15). The results that we have now obtained emphasize that the regulated pathway cannot be considered as a single unit, but can include various classes of granules destined to be discharged in parallel. The existence of multiple secretory granules appears not to be a property of pituitary somatomammotrophs only, but of other cells as well (9, 13, 20), including human blood neutrophils (3). In the latter cells, two types of typical secretory organelles (specific granules and secretory vesicles) have been recently reported to be differently sensitive to intracellular second messengers (18).

The mechanisms by which different proteins of the regulated type are targeted to distinct secretory granules have not been elucidated yet. In our pituitary somatomammotrophs, sorting of GH and PRL appears to occur concomitantly with

their condensation. In fact, in the GC, where the two hormones are known to be in a diluted state, they were found to be intermixed; when integrated into the dense granule matrices, even at the stage of immature granules, they appeared segregated from each other. In this respect it might be interesting that a correlation between sorting and condensation had already been suggested (the budding model of Kelly [15]). Unfortunately, the mechanism underlying the process of condensation is also poorly understood at the present time.

The results obtained with SgII deserve a specific comment. SgII is a secretory sulfo-phosphoprotein known to be widely distributed in endocrine cells (12, 22, 23, 25). Recently, its occurrence has been demonstrated in the central and peripheral nervous system as well (2, 25). To date, SgII had been mostly found to be stored together with other secretory products (hormones and neurotransmitters). The possibility of its involvement in granule assembly, in particular in the processes leading to the condensation of secretory products in the granule matrix, had therefore been considered. In the pituitary, such a possibility appears still open for gonado- and thyrotropic cells, where SgII is stored in high concentration within the specific hormone-containing granules (24). The existence of pituitary cells where SgII is stored in a subpopulation of secretory granules had been mentioned in an earlier report (24). Now these cells are conclusively identified as the acidophilic cells and SgII-containing granules are shown to be distinct from the granules containing GH or PRL. A role of SgII in GH and PRL packaging appears therefore impossible in these cells.

The cells expressing predominantly either GH or PRL, that is somato- and mammotropic cells, were found to express small amounts of the other hormone as well. Although unexpected, this result appears to correlate with previous immunofluorescence data on the bovine anterior pituitary. Those data demonstrate the existence (together with a relatively small population of highly GH-positive cells) of other cells, weakly fluorescent for the hormone that because of their large number could only be mammotrophs (24). The clear-cut demonstration of the coexistence of GH and PRL in most, if not all, acidophilic cells that we have now obtained, is due to the greater resolution of the ultrathin cryosection technique, which revealed the preferential accumulation of the minority hormones in the GC. The present observation, particularly in the mammotropic cells, that the immunolabeling ratio of the minority versus predominant hormone is distinctly greater in the GC than in secretory granules suggests that in these cells the two hormones are transported to the surface by different organelles. The nature of the transporting organelle for minority hormones remains to be elucidated.

Finally, mention should be made of the possible physiological relevance of our findings. The existence of more than one type of secretory granule could enable the cell to modify the nature of its released proteins depending on the stimuli. If discharge of the various granules was regulated by different second messengers (as it might be predicted for GH and PRL granules, based on the present knowledge of hormone release from the glandular tissue or mixtures of dissociated cells [1, 29]) different stimuli could yield secretion of different (sets of) protein(s) from the same cell. Alternatively, if the various types of granules were differently sensitive to the same messenger (as it has already shown to be the case with human blood neutrophils [18]), the cell could be able to modify the proportion of the various released proteins depending on the intensity of one applied stimulus. Experimental testing of these hypotheses in our experimental model obviously requires the isolation of somatomammotrophic cell populations.

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