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Cell-Free Protein Sorting to the Regulated and Constitutive Secretory Pathways

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Summary

To elucidate the mechanism of secretory granule formation, we here identify the first intermediate in this process, the immature secretory granule, in the neuroendocrine cell line PC12 and demonstrate the packaging of a regulated secretory protein, secretogranin II, to immature secretory granules in a cell-free system. The formation of immature secretory granules was as fast ($t_{1/2} \approx 5$ min) as that of constitutive secretory vesicles identified by the presence of a rapidly secreted heparan sulfate proteoglycan. Using the cell-free system, the formation of post-Golgi secretory vesicles was found to be dependent upon ATP. Two distinct populations of vesicles were formed: immature secretory granules containing secretogranin II and constitutive secretory vesicles containing the heparan sulfate proteoglycan. These results show that in a cell-free system, a constitutive and a regulated secretory protein are sorted upon exit from the *trans*-Golgi network.

Introduction

Protein secretion is a basic function of all animal cells. In the constitutive secretory pathway, which is common to all cells, proteins are continuously secreted without intracellular storage. In the regulated secretory pathway, which is present, in addition to the constitutive pathway, only in certain cells such as exocrine cells, endocrine cells, and neurons, a subset of secretory proteins is sorted from the constitutive secretory proteins to highly specialized storage organelles, secretory granules, from which they are released only upon stimulation of the cell (for review, see Burgess and Kelly, 1987).

Morphological studies (for review, see Farquhar and Palade, 1981) have revealed several steps in the formation of secretory granules. These are the formation of a dense-core aggregate in the *trans*-Golgi network (TGN), envelopment of the aggregate by membrane, budding of the enveloped aggregate from the TGN, which yields a dense-core immature secretory granule, and maturation of the immature granule into a dense-core mature secretory granule. Immature secretory granules are usually found in the vicinity of the TGN, whereas mature secretory granules characteristically are located in the periphery of the cell and are competent to fuse with the plasma membrane.

The intracellular transport route of constitutive and regulated secretory proteins is thought to be the same until these proteins reach the TGN (for reviews, see Farquhar, 1985; Griffiths and Simons, 1986; Burgess and Kelly, 1987; Pfeffer and Rothman, 1987). As to the site of segregation of the constitutive and regulated secretory pathway, different conclusions have been reached. Electron microscopical studies on neuroendocrine cells have suggested that segregation occurs upon exit from the TGN; markers for the constitutive and regulated pathway were found to be colocalized in this compartment, but only the regulated secretory proteins were detected in immature and mature secretory granules (Orci et al., 1987; Tooze et al., 1987). On the other hand, biochemical studies on exocrine cells have suggested that sorting of constitutive and regulated secretory proteins also occurs in immature secretory granules (von Zastrow and Castle, 1987). It is unclear whether these different conclusions reflect the fact that in the electron microscopical studies, viral particles and membrane proteins rather than secretory proteins were used as markers for the constitutive pathway, that differences exist between neuroendocrine and exocrine cells, or that different methodologies were used.

While mature secretory granules have been extensively characterized not only morphologically (for review, see Farquhar and Palade, 1981) but also biochemically (e.g., chromaffin granules; for review, see Winkler et al., 1986), little is known about immature secretory granules beyond their morphological appearance (Farquhar et al., 1978; Tooze and Tooze, 1986). Immature secretory granules, however, are the first distinct post-TGN compartment en route to the mature secretory granule (Farquhar et al., 1978; Tooze and Tooze, 1986). Understanding the formation of immature granules is, therefore, a prerequisite to understanding the biogenesis of secretory granules. In addition, immature secretory granules may well be a sorting station; the stringency of segregation of constitutive secretory proteins or of Golgi-resident proteins from regulated secretory proteins during the formation of immature secretory granules is unknown, and removal of the former proteins from immature granules would improve the overall fidelity of the sorting process.

To obtain information on the biogenesis of secretory granules, we biochemically identified immature secretory granules and developed a cell-free system in which these organelles, as well as constitutive secretory vesicles, are formed from the TGN. The cell-free system has been used to study the sorting of regulated and constitutive secretory proteins from one another and from Golgi-resident proteins upon exit from the TGN and to investigate some of the factors involved in the biogenesis of secretory granules.

Results

Our approach to study the formation of immature secretory granules and constitutive secretory vesicles was based on the following strategy. First, we decided to use markers for the constitutive and regulated pathway that are subject to *trans*-Golgi-specific posttranslational mod-

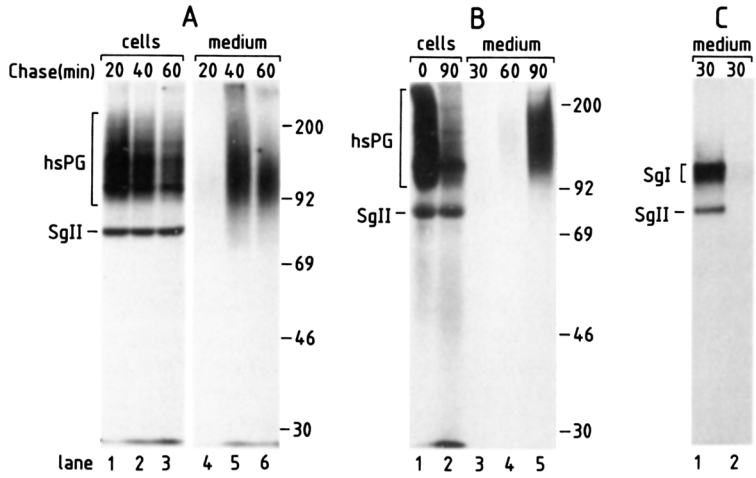

Figure 1. Characterization of the Constitutive Secretory hsPG and the Regulated Secretory Protein SgII

(A) Sulfate-labeled hsPG is rapidly secreted from PC12 cells. PC12 cells were pulse-labeled for 10 min with [^{35}S]sulfate and chased for 20 min (lanes 1, 4), 40 min (lanes 2, 5), or 60 min (lanes 3, 6). After each time point, the cells (lanes 1–3) and medium (lanes 4–6) were collected.

(B) The secretion of the hsPG is blocked by incubation at 20°C. PC12 cells were sulfate-labeled for 10 min at 20°C and either harvested (lane 1) or chased for 60 min at 20°C and 30 min at 37°C and harvested (lane 2). During the chase period, the medium was removed from the cells after 30 min (lane 3) and 60 min (lane 4) at 20°C, and after the final 30 min at 37°C (lane 5).

(C) SgII is released via the regulated pathway. PC12 cells were labeled overnight with [^{35}S]sulfate and chased for 30 min in medium containing either 55 mM K^+ (lane 1) or 5 mM K^+ (lane 2), which was collected. In (A)–(C), cells and media were analyzed by SDS–PAGE followed by fluorography. The positions of molecular weight markers (in kd), the hsPG (long bracket), and SgII are indicated. The short bracket in (C) indicates SgI, which is also secreted upon depolarization (Rosa et al., 1985b) but was not analyzed as a marker for the regulated pathway because of its overlapping migration with some of the constitutively secreted hsPG.

ifications. This provided a means of selectively labeling only those marker molecules present in the *trans*-Golgi and monitoring their exit from the TGN, since marker molecules in pre-*trans*-Golgi and post-Golgi compartments were not labeled. Second, the formation of post-TGN vesicles containing the labeled markers was monitored by physically separating these vesicles from the donor compartment, the TGN.

Sulfated Marker Proteins of the Constitutive and Regulated Secretory Pathways

Sulfation of proteins on tyrosine is an established *trans*-Golgi-specific posttranslational modification (Baeuerle and Huttner, 1987). Cells of the neuroendocrine line PC12 contain two major tyrosine-sulfated proteins, secretogranins I and II (SgI and SgII), that are very efficiently (~95%) targeted to secretory granules (Lee and Huttner, 1983; Rosa et al., 1985b, 1989; Gerdes et al., 1989; S. A. Tooze and W. B. Huttner, unpublished data) and thus are ideal markers to follow the transport of regulated secretory proteins from the TGN to secretory granules. PC12 cells also contain sulfated proteoglycans (Schubert et al., 1988; Gowda et al., 1989), and the sulfation of the carbohydrate chains of proteoglycans, too, is a *trans*-Golgi-specific event (Kimura et al., 1984).

Figure 1 shows that in PC12 cells, sulfated proteoglycans are excluded from secretory granules and can be used as markers for the constitutive pathway of secretion. Incubation of PC12 cells for 10 min with [^{35}S]sulfate resulted in the labeling of both SgI and SgII as well as a component with diffuse electrophoretic mobility between the top of the gel and the SgI region (Figure 1A); this component proved to be sensitive to heparinase II (data not shown) and thus was a heparan sulfate proteoglycan (hsPG), consistent with previous studies showing the presence of one major hsPG in PC12 cells (Schubert et al., 1988; Gowda et al., 1989). Sulfate-labeled hsPG was secreted within 40 min of chase while virtually no SgII appeared in the medium (Figure 1A, lanes 4–6). (The "SgI region" of the gel could not be interpreted with respect to regulated vs. constitutive secretion; this region contained not only sulfate-labeled SgI but also some of the sulfate-labeled hsPG, as shown by the analysis of sulfate linkage and two-dimensional PAGE.)

To define more precisely the pathways of secretion of the hsPG and SgII, two criteria were applied: the inhibition of constitutive secretion at 20°C and the stimulation of regulated secretion from PC12 cells by K^+ depolarization. Viral membrane proteins that reach the cell surface via the constitutive pathway are arrested in the TGN when the temperature is lowered to 20°C (Matlin and Simons, 1983). We tested whether this temperature block also inhibited the intracellular transport and secretion of the hsPG (Figure 1B). When PC12 cells were pulse-labeled with [^{35}S]sulfate for 10 min at 20°C and then chased at 20°C, the secretion of the hsPG into the medium was inhibited over a 60 min chase period (Figure 1B, lanes 3 and 4). However, when the temperature was then raised for 30 min from 20°C to 37°C, the hsPG was rapidly secreted (Figure 1B, lane 5). SgII was not secreted during the chase at either 20°C or 37°C (Figure 1B).

SgI and SgII are stored in secretory granules in a wide variety of neuroendocrine cells (Rosa et al., 1985a, 1985b). In PC12 cells, the fusion of secretory granules with the plasma membrane can be induced by depolarization of the cells with high K^+ in the presence of external Ca^{2+}. Consistent with previous observations (Rosa et al., 1985b), stimulation of sulfate-labeled PC12 cells with 55 mM K^+ resulted in the release of SgI and SgII into the medium (Figure 1C, lane 1), which was Ca^{2+}-dependent (data not shown). In contrast, the hsPG was not secreted upon stimulation, showing that it was not accumulated in secretory granules.

Thus, in PC12 cells, SgII and the hsPG are specific

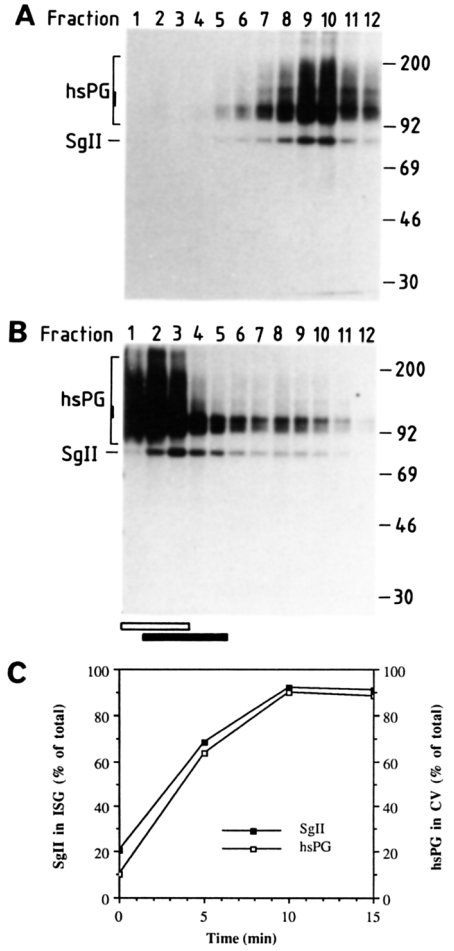

Figure 2. Immature Secretory Granules Are Formed In Vivo from the TGN Very Rapidly

PC12 cells were pulse-labeled for 5 min with [^{35}S]sulfate or pulse-labeled and chased for 5, 10, or 15 min. The PNS from each time point was subjected to velocity gradient centrifugation. Fractions from each gradient (1, top; 12, bottom) were analyzed by SDS–PAGE, followed by fluorography and quantitation of the radioactivity in the hsPG (bracket; the thick portion indicates the area used for quantitation) and SgII. The fluorograms show the distribution of the hsPG and SgII across the gradients after the 5 min pulse (A) and the 5 min pulse followed by a 15 min chase (B). (C) Time course of appearance of the labeled hsPG and SgII in post-TGN secretory vesicles. Fractions containing constitutive secretory vesicles (CV) and immature secretory granules (ISG) were identified from the autoradiograms (see the open and closed bar in [B], respectively), and the [^{35}S]hsPG and the [^{35}S]SgII in these fractions was quantitated and is expressed as percent of the total (sum of fractions 1–12) [^{35}S]hsPG and [^{35}S]SgII.

markers for the regulated and constitutive secretory pathway, respectively, and their secretory route from the TGN to the cell surface can be selectively monitored by sulfate labeling.

Separation of the TGN and Post-TGN Secretory Vesicles

From morphological data, the formation of secretory granules typically begins in the TGN by membrane envelopment of an aggregate of regulated secretory proteins and budding to yield an immature secretory granule (for review, see Farquhar and Palade, 1981). Similarly, the vesicles that transport secretory proteins via the constitutive pathway to the cell surface are thought to form from the TGN (for review, see Griffiths and Simons, 1986).

To identify biochemically the immature secretory granules and constitutive secretory vesicles and to separate them from the putative donor compartment, the TGN, the following protocol was employed. PC12 cells were pulse-labeled for 5 min with [^{35}S]sulfate, with or without a subsequent chase in vivo, and then homogenized. Because sulfate uptake, sulfate activation, and translocation of activated sulfate are known to take ~2 min (Baeuerle and Huttner, 1987), the effective labeling time was ~3 min. Organelles in the postnuclear supernatant (PNS) were then fractionated on a velocity-controlled continuous sucrose gradient designed to separate the TGN from post-TGN secretory vesicles. After a 5 min pulse, a single peak of labeled proteins was detected in the bottom half of the gradient (peak in fractions 9 and 10), which contained both SgII and the hsPG (Figure 2A). This peak coincided with that of the TGN marker enzyme sialyltransferase (Roth et al., 1985; see Figure 3D below), confirming that these proteins were present in the TGN at the end of the 5 min pulse. After a 15 min chase, most of the labeled SgII and the hsPG was found in the top half of the gradient (fractions 1–5, Figure 2B), suggesting that the labeled proteins had left the TGN during this time period and were now present in post-TGN vesicles. (The finding that the [^{35}S]SgII-containing post-TGN vesicles sedimented more slowly than the TGN in these velocity-controlled gradients, although they would be expected to be at least as dense, probably reflected the smaller size of these vesicles compared with the TGN [see Figure 6, below].) Thus, pulse–chase labeling of SgII and the hsPG in PC12 cells with [^{35}S]sulfate followed by velocity gradient centrifugation of the PNS is a means of biochemically identifying, and separating from one another, the TGN and post-TGN vesicles.

The formation of the [^{35}S]SgII-containing post-TGN vesicles from the TGN was very rapid: within 5 min after a 5 min pulse, more than half of the labeled SgII had left the TGN (Figure 2C). In fact, the kinetics of SgII exit from the TGN were as rapid as that of the constitutive marker, the hsPG (Figure 2C).

Cell-Free Formation of Post-TGN Secretory Vesicles

To gain further insight into the formation of immature secretory granules and the sorting of constitutive and regulated secretory proteins, we developed a cell-free system. Based on the ability to monitor biochemically the transport of SgII and the hsPG from the TGN to post-TGN vesicles, we decided to test whether SgII and the hsPG labeled in vivo in the TGN by a 5 min pulse with sulfate could be packaged into post-TGN vesicles in vitro after homogenization of PC12 cells. A PNS was prepared from pulse-labeled cells in a low ionic strength, sucrose-containing buffer at neutral pH and then supplemented with ATP, an ATP-regenerating system, and unlabeled PAPS (the co-substrate for sulfation; this was added to prevent radioactive sulfate incorporation in vitro). When this PNS was kept at 0°C, both sulfate-labeled SgII and hsPG remained in

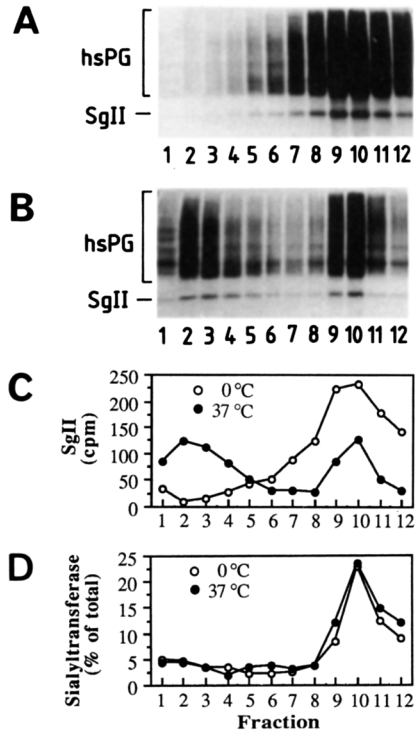

Figure 3. Cell-Free Formation of Post-TGN Secretory Vesicles from the TGN

A PNS was prepared from PC12 cells that had been either labeled for 5 min with [^{35}S]sulfate (A–C) or mock-labeled (D) in vivo. After in vitro incubation for 60 min at either 0°C (A) or 37°C (B), the PNS was subjected to velocity gradient centrifugation. Fractions from the gradients (1, top; 12, bottom) were analyzed by SDS–PAGE. The fluorograms in (A) and (B) show the distribution of the hsPG (long brackets) and SgII across the gradients. (C) shows the quantitation of [^{35}S]SgII across the gradients shown in (A) (open circles) and (B) (closed circles). (D) shows the distribution of the endogenous sialyltransferase activity across the gradients after in vitro incubation of the PNS at either 0°C (open circles) or 37°C (closed circles), expressed as the percent of total activity (sum of fractions 1–12).

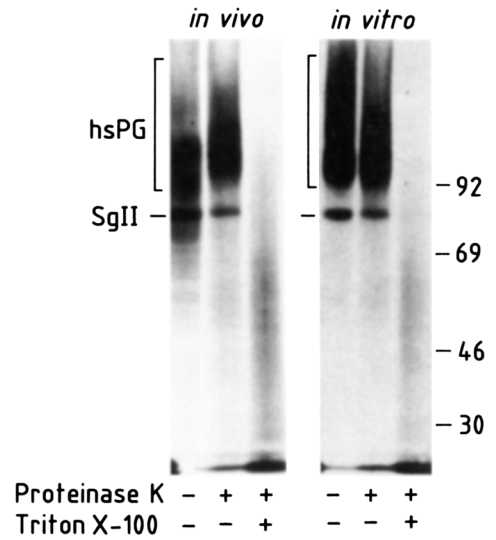

Figure 4. The hsPG and SgII Are Packaged during the In Vitro Incubation into Membrane-Enclosed Vesicles

In vitro: Fractions 2–5 from the velocity gradient of a PNS incubated in vitro at 37°C as described in Figure 3 were pooled and incubated in the absence (–) or presence (+) of proteinase K without (–) or with (+) Triton X-100, as indicated. Samples were then analyzed by SDS–PAGE and fluorography.

In vivo: For comparison, proteinase K treatment was performed on fractions 2–5 from the velocity gradient of a PNS prepared from PC12 cells pulse-labeled and chased for 15 min in vivo as described in Figure 2.

the bottom half of the velocity gradient in the fractions containing TGN (Figures 3A and 3C), as identified by the peak of sialyltransferase activity (Figure 3D). In contrast, incubation of this PNS at 37°C for 60 min resulted in the ATP-dependent appearance (see Figure 7, below) of both labeled SgII and hsPG in the top half of the gradient (Figures 3B and 3C). In fact, the labeled SgII and hsPG were found at the same position of the gradient (fractions 1–5) as the post-TGN vesicles identified after a 15 min chase in vivo (compare Figure 3B with Figure 2B).

We verified that the change in sedimentation of sulfate-labeled SgII and hsPG after in vitro incubation of the PNS at 37°C was not due to lysis of the TGN and release of soluble SgII and hsPG. After an in vitro incubation of the PNS and velocity gradient centrifugation, fractions 2–5 were pooled and incubated with proteinase K, either in the absence or presence of Triton X-100. In the absence of detergent, both SgII and the hsPG were protected from degradation, while the addition of detergent rendered the proteins completely sensitive to proteinase K digestion (Figure 4, in vitro). This indicated that the sulfate-labeled SgII and hsPG found at the top of the gradient after in vitro incubation of the PNS were in sealed membrane vesicles impermeable to proteinase K. Similar protection from proteinase K digestion was observed for sulfate-labeled SgII and hsPG in the post-TGN vesicles formed in vivo, identified after a 15 min chase (Figure 4, in vivo).

Retention of Membrane Proteins in the TGN after Cell-Free Formation of Post-TGN Vesicles

It was important to determine whether the SgII-containing and the hsPG-containing vesicles found in the top half of the velocity gradient after in vitro incubation of the PNS were formed by selective budding events from the TGN rather than by fragmentation of the TGN. As shown in Figure 3D, the distribution of sialyltransferase activity, a membrane protein of the TGN (Roth et al., 1985), across the velocity gradient was unaffected by in vitro incubation at 37°C. Similar observations were made with tyrosylprotein sulfotransferase (data not shown), another membrane protein of the TGN (Baeuerle and Huttner, 1987; C. Niehrs and W. B. Huttner, submitted). Thus, the budding of vesicles that contained SgII and the hsPG in vitro was selective, resulting in the cell-free segregation of these secretory proteins from TGN-resident proteins.

Cell-Free Sorting of Constitutive and Regulated Secretory Proteins

The final intracellular destination of regulated secretory proteins is the secretory granule, while that of constitutive

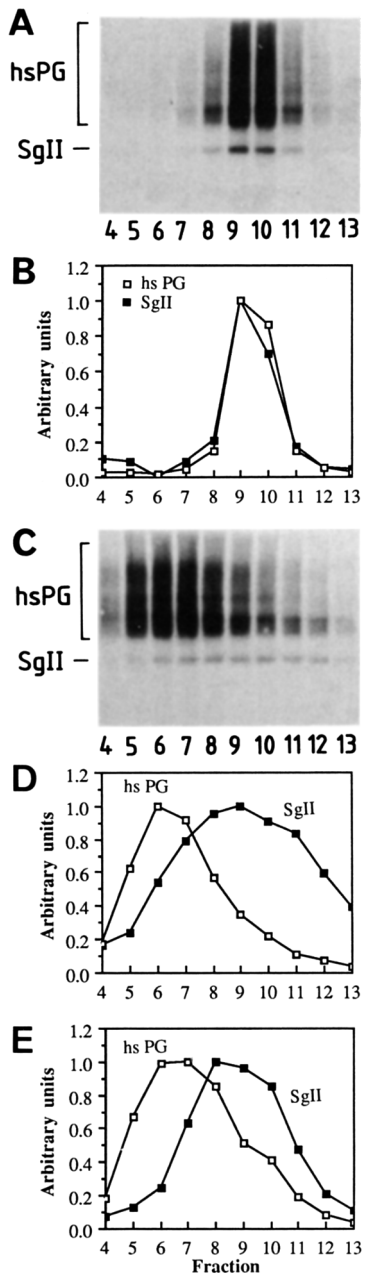

Figure 5. Both Constitutive Secretory Vesicles and Immature Granules Bud from the TGN during the In Vitro Incubation

(A) and (B) A PNS prepared from PC12 cells labeled for 5 min with [^{35}S]sulfate in vivo was kept for 60 min at 4°C in vitro and subjected to velocity gradient centrifugation. Fractions 8–10 were pooled, loaded onto a second sucrose gradient, and centrifuged to equilibrium.

(C) and (D) A PNS prepared from PC12 cells labeled for 5 min with [^{35}S]sulfate in vivo was incubated for 60 min at 37°C in vitro and subjected to velocity gradient centrifugation. Fractions 1–4 were pooled and subjected to equilibrium centrifugation. Fractions (0.8 ml) from the equilibrium gradients (4, top; 13, bottom) were analyzed by SDS–PAGE followed by fluorography (A, C) and quantitation of the hsPG and SgII. Only the fractions containing significant radioactivity are shown.

(E) Distribution of the hsPG-containing constitutive secretory vesicles and the SgII-containing immature secretory granules formed in vivo across the equilibrium gradient. A PNS prepared from cells pulse-labeled for 5 min with [^{35}S]sulfate in vivo and chased for 15 min in vivo was subjected to velocity gradient centrifugation, and fractions 1–4 were subjected to equilibrium centrifugation followed by analysis as in (D). In (B), (D), and (E), the peak value of each curve was arbitrarily set to 1.0.

secretory proteins is the constitutive vesicle. If these two different classes of proteins are respectively packaged directly into either immature secretory granules or constitutive secretory vesicles upon exit from the TGN, then SgII and the hsPG should be in two different vesicle populations after an in vitro budding reaction. In contrast, if some of the constitutive and the regulated secretory proteins were transiently colocalized in post-TGN vesicles and then segregated from one another, a portion of SgII and the hsPG should be in the same vesicle population after an in vitro budding reaction. To investigate whether the sulfate-labeled SgII and hsPG were present in one or more post-TGN vesicle populations, various fractions from the velocity gradient were subjected to a second, equilibrium sucrose gradient centrifugation.

Equilibrium centrifugation of the TGN (fractions 8–10 from a velocity gradient of a PNS kept at 4°C) showed that the hsPG and SgII cosedimented (Figures 5A and 5B) as expected, since both proteins labeled during the 5 min sulfate pulse remained in the TGN at this temperature. In contrast, equilibrium centrifugation of the post-TGN vesicles formed during an in vitro incubation of the PNS at 37°C (fractions 1–4 of the velocity gradient) resolved these into two distinct populations, one containing the sulfate-labeled hsPG (peaking in fraction 6, density 1.098 g/ml) and another containing sulfate-labeled SgII (peaking in fraction 9, density 1.131 g/ml), each characterized by a bell-shaped distribution across the gradient without detectable shoulders (Figures 5C and 5D). This indicated that during the in vitro incubation these two proteins had been packaged into different vesicles. The post-TGN vesicles formed in vivo, found in the top half of the velocity gradient after a 5 min sulfate pulse followed by a 15 min chase, were also resolved into two distinct populations (Figure 5E). The sedimentation behavior of the two vesicle populations formed in vitro was very similar to that of the two post-TGN vesicle populations formed in vivo (compare Figures 5D and 5E).

From these data we conclude that a regulated secretory protein, SgII, and a constitutive secretory protein, the hsPG, are sorted directly into two distinct vesicle populations upon exit from the TGN and that this sorting occurs faithfully in the cell-free system.

SgII Is Packaged into Dense-Core Immature Secretory Granules

The post-TGN vesicles containing SgII that were formed in vivo or in vitro were significantly denser than the constitutive secretory vesicles containing the hsPG (see Figures 5D and 5E), as expected, since immature secretory granules are known to contain dense-core aggregates of secretory proteins (Farquhar et al., 1978; Tooze and Tooze, 1986). To characterize morphologically the SgII-containing post-TGN vesicles obtained from the second, equilibrium gradient and to establish directly that they contained membrane-enclosed dense-core aggregates of secretory protein, electron microscopy was performed. For compari-

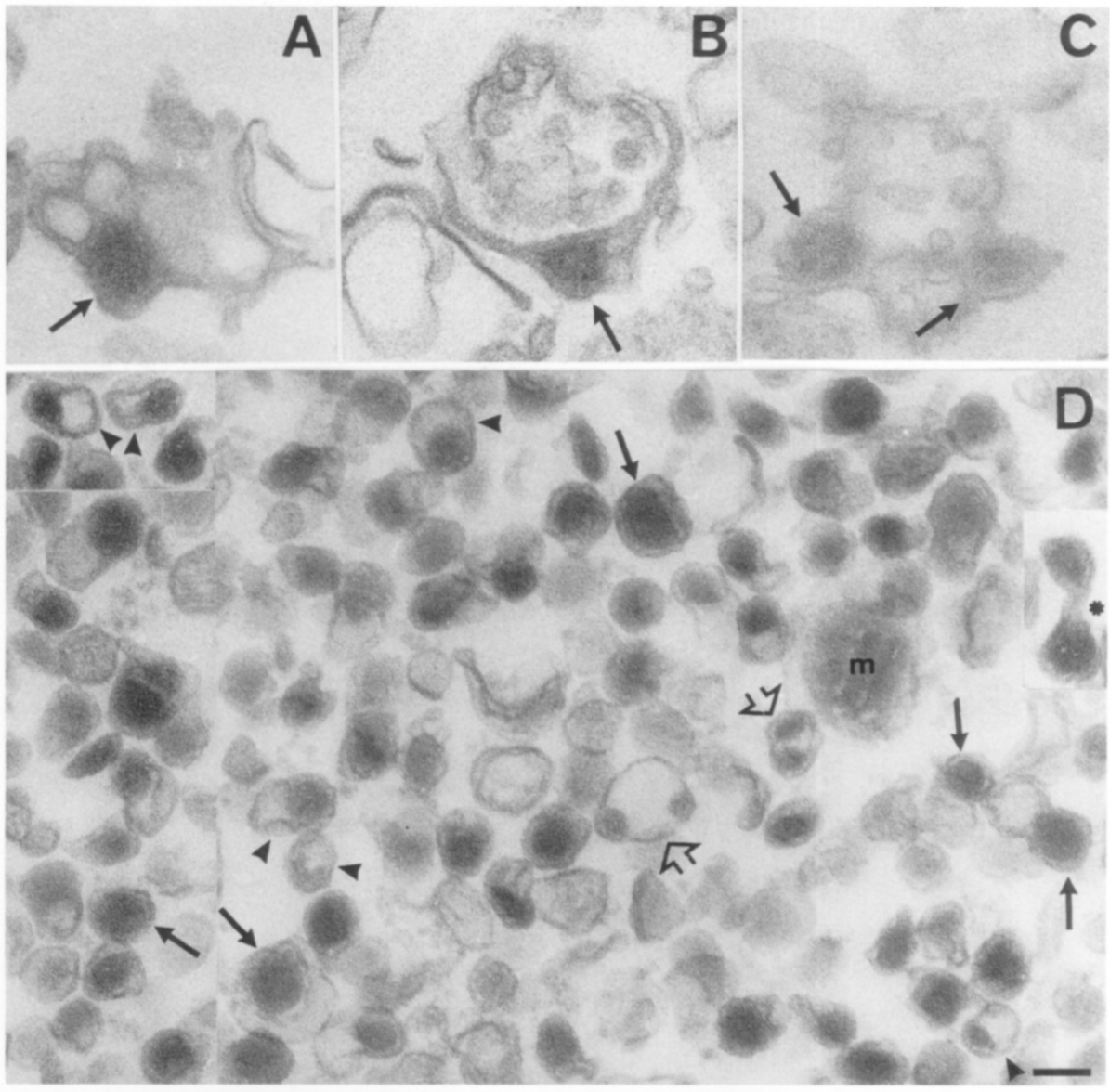

Figure 6. Thin Section Electron Microscopy of the TGN and Immature Secretory Granules of PC12 Cells

A crude fraction containing membranes derived from the TGN was obtained from a velocity gradient (see Figure 2A, fraction 9). Fractions enriched in immature secretory granules were obtained by sequential velocity gradient centrifugation (see Figure 2B, fraction 2) and equilibrium gradient centrifugation (see Figure 5E, fractions 8–11). (A), (B), and (C): Dense-core aggregates (arrows) in cisternal and tubular structures characteristic of the TGN. (D) Composite showing a representative field and selected images of immature secretory granules. Arrows indicate dense-core aggregates, arrowheads denote granules with loosely surrounding membranes, and open arrows show granules with two dense-core aggregates. The asterisk indicates two immature secretory granules that appear to be connected; m, mitochondrion. Note that the contrast of the dense-core aggregates varies in both the TGN and immature secretory granules, depending upon the plane of sectioning. All panels are of the same magnification; the bar in (D) = 100 nm.

son, TGN-derived membranes in the crude fraction obtained after velocity centrifugation were analyzed.

The morphological appearance of the TGN-derived membranes (Figures 6A–6C) was characterized by the presence of large, cisternal and tubular membrane structures similar to those observed for the TGN in vivo (for reviews, see Farquhar and Palade, 1981; Griffiths and Simons, 1986). In several instances, dense-core aggregates were seen in the lumen of these membrane-enclosed compartments, and these closely resembled aggregates in the TGN of fixed cells (Tooze and Tooze, 1986). Analysis of the SgII-containing post-TGN vesicle fraction (Figure 6D) showed that it was highly enriched in membrane-enclosed dense-core aggregates whose appearance closely resembled that of immature secretory granules (Tooze and Tooze, 1986). The membrane of these vesicles was often irregularly shaped and loosely surrounded the core. Occasionally, multiple small dense cores were found in a single membrane-enclosed structure. All of these morphological data are consistent with the conclusion that the

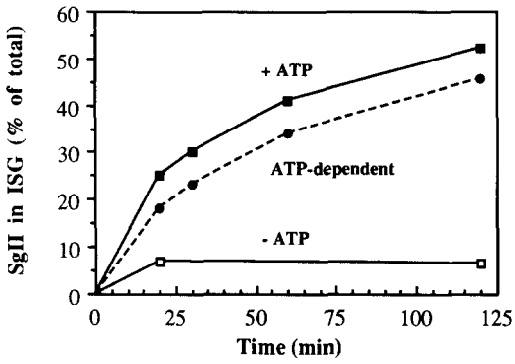

Figure 7. Effect of ATP on the Cell-Free Formation of Immature Secretory Granules

The PNS prepared from PC12 cells labeled for 5 min with [^{35}S]sulfate in vivo was incubated at 37°C in vitro with (+) or without (−) ATP for periods up to 120 min. After velocity gradient centrifugation, aliquots of the fractions were analyzed by SDS–PAGE. [^{35}S]SgII in the cell-free formed immature secretory granules (ISG) is expressed as the percent of total [^{35}S]SgII in the TGN. ATP-dependent immature granule formation was calculated by subtraction of the percent values obtained in the absence of ATP from those obtained in the presence of ATP.

SgII-containing post-TGN vesicles we have identified are newly formed immature secretory granules.

Quantitation of the size of the dense cores of the immature secretory granules from PC12 cells showed that they had an average profile diameter of 80 ± 19 nm (n = 120). This was significantly less than the profile diameter of 115 ± 15 nm (n = 118) observed for the core of mature PC12 cell secretory granules (data not shown), which were isolated under the same conditions of sequential velocity and equilibrium sucrose gradient centrifugation and processed for electron microscopy as the immature secretory granules, as will be described elsewhere (S. A Tooze, J. Tooze, and W. B. Huttner, unpublished data).

Formation of Immature Secretory Granules Is ATP-Dependent

Using the cell-free system, we investigated whether the formation of immature secretory granules from the TGN requires ATP. The time course of immature granule formation was studied by following the appearance of sulfate-labeled SgII in these vesicles over 120 min of incubation of the PNS at 37°C, either with ATP or in the presence of hexokinase and glucose to deplete ATP (Figure 7). This showed that the formation of immature granules in vitro was dependent upon ATP. The ATP-dependent cell-free formation of immature secretory granules proceeded fairly linearly for about 30 min. Thereafter, the rate of appearance of sulfate-labeled SgII in the immature granules decreased, indicating a reduction in either the rate of granule formation or the specific activity of labeled SgII by mixing with unlabeled SgII in the TGN. Analysis of the hsPG revealed that the formation of the constitutive secretory vesicles was also ATP-dependent (data not shown), consistent with previous observations on the formation of post-Golgi secretory vesicles in other cells (Bennett et al., 1988; de Curtis and Simons, 1989).

We tested whether the formation of immature secretory granules and constitutive secretory vesicles from the TGN was affected by the nonhydrolyzable analog of GTP, GTPγS. Addition of 10 μM GTPγS to the PNS prior to the in vitro incubation decreased the formation of both types of post-Golgi secretory vesicles to ~50% of the control value (data not shown).

We attempted to determine whether soluble proteins are required for the cell-free formation of immature secretory granules. However, these experiments were hampered by the fact that the in vitro incubation of TGN-derived membranes in the absence of cytosol affected their sedimentation upon velocity gradient centrifugation in a manner that did not allow the reliable quantitation of cell-free granule formation (data not shown).

Discussion

Identification of Immature Secretory Granules and Constitutive Secretory Vesicles

The results presented here were based on: the selective labeling of the markers for the regulated and constitutive secretory pathways, SgII and the hsPG, respectively, in the TGN with radioactive sulfate, i.e., shortly before their packaging into post-TGN vesicles; and the separation of the TGN, immature secretory granules, and constitutive secretory vesicles from one another by sequential velocity and equilibrium gradient centrifugation. With this approach, we demonstrated that regulated and constitutive secretory proteins are colocalized in the TGN and identified two distinct post-TGN vesicle populations, immature secretory granules and constitutive secretory vesicles, respectively, into which these proteins are packaged. Electron microscopy of the TGN and the immature secretory granule fractions showed the characteristic morphological features observed for these organelles in vivo (Farquhar et al., 1978; Tooze and Tooze, 1986; for review, see Farquhar and Palade, 1981).

As monitored by the appearance of sulfate-labeled SgII, the formation of immature secretory granules from the TGN occurred rapidly, with a half-time of ~5 min, and was essentially complete within 15 min. Monitoring the sulfate-labeled SgII over longer chase times revealed that these immature secretory granules are subject to maturation (S. A. Tooze, J. Tooze, and W. B. Huttner, unpublished data) and thus are a vesicular intermediate en route to mature secretory granules. These were key observations in the development of the cell-free system, because they underscored the necessity of monitoring directly the formation of immature secretory granules, rather than mature secretory granules, from the TGN. To this end, an assay involving the physical separation of these vesicles from the TGN was likely to be more sensitive and accurate than following, for example, the proteolytic processing of SgII, which is a characteristic but relatively slow intragranule process (Rosa et al., 1985b; Fischer-Colbrie et al., 1987; Huttner et al., 1988a).

Cell-Free Sorting of Secretory Proteins

The biochemical identification of constitutive secretory

vesicles in addition to immature secretory granules allowed us to develop a cell-free system in which the exit of both regulated and constitutive secretory proteins from the TGN could be investigated. Several lines of evidence indicated that the two vesicle populations formed in vitro, containing either sulfated SgII or sulfated hsPG, were immature secretory granules and constitutive secretory vesicles, respectively. First, the cell-free formed vesicles were indistinguishable in their sedimentation behavior upon sequential velocity and equilibrium centrifugation from the immature secretory granules and constitutive secretory vesicles formed in vivo. Second, the cell-free formed vesicles containing hsPG had a similar density to that recently reported for constitutive secretory vesicles from BHK cells (de Curtis and Simons, 1989), and the cell-free formed SgII-containing vesicles had a significantly greater density, as expected for immature secretory granules. Third, the cell-free formed vesicles lacked sialyltransferase activity, and their formation was ATP-dependent, indicating that they were post-TGN vesicles resulting from specific budding events rather than vesicles resulting from the fragmentation of the TGN.

The finding that in the cell-free system, constitutive and regulated secretory proteins can be segregated from one another and from Golgi-resident proteins and packaged into distinct post-TGN secretory vesicles implies that all of the components required for these specific sorting, budding, and retention events, i.e., intrinsic components of the TGN and/or soluble factors, were functioning after homogenization of cells. In this context, it has been hypothesized that the selective aggregation of regulated secretory proteins is involved in their sorting from constitutive secretory proteins (Kelly, 1985; Huttner et al., 1988b; Gerdes et al., 1989; Tooze et al., 1989), and this might provide part of the driving force for budding of an immature secretory granule from the TGN. The presence of dense-core aggregates in the TGN-like membrane structures and immature granules obtained after sucrose gradient centrifugation does not prove, but certainly is consistent with, this possibility.

The Site of Sorting of Regulated Secretory Proteins

By monitoring the dynamics of secretory protein transport from the TGN to post-TGN vesicles in vivo and in the cell-free system, we provide direct evidence that most, if not all, of the segregation of constitutive and regulated secretory proteins in neuroendocrine cells occurs upon exit from the TGN. The distinct, bell-shaped distribution of the hsPG-containing constitutive secretory vesicles and the SgII-containing immature secretory granules across the equilibrium gradient did not support the existence of post-TGN vesicles containing significant amounts of both markers. This distribution was observed in vivo as well as in the cell-free system and was in striking contrast to the comigration of these two markers upon equilibrium centrifugation of the TGN-derived membranes. Because the distribution of the hsPG and SgII across gradients was distinct at the earliest studied time point after exit from the TGN, after 5 min of chase in vivo, these two markers appear to be largely sorted from one another when leaving the TGN via constitutive secretory vesicles and immature secretory granules, respectively. Our direct demonstration of the TGN as the major sorting station for constitutive and regulated secretory proteins in neuroendocrine cells is consistent with previous results of morphological studies (Orci et al., 1987; Tooze et al., 1987) and differs from the conclusion reached in a study with exocrine cells (von Zastrow and Castle, 1987).

The Fate of Immature Secretory Granules

One of the unexpected findings was the size of the immature granules (80 nm core), which was significantly smaller than that reported by others (Greene and Tischler, 1976; Watanabe et al., 1983) and also observed by us (S. A. Tooze, J. Tooze, W. B. Huttner, unpublished data) for mature secretory granules of PC12 cells (115 nm core). The small size of the immature granules was not an artifact resulting from prolonged exposure to hypertonic sucrose, since the same size was observed after 15 min of sedimentation into approximately isotonic sucrose (data not shown). In line with the observation of double-cored granules, one possible explanation would be that in PC12 cells, mature secretory granules originate by fusion of two or more immature granules, as was previously suggested for secretory granules in endocrine cells of the anterior pituitary (Farquhar et al., 1978). This would imply removal of the excess membrane present after fusion of immature granules. It is tempting to speculate that this membrane removal may be a means of recycling membrane proteins, involved in the sorting of regulated secretory proteins and the formation of immature granules, back to the TGN. Although the recycling of such membrane proteins could in principle occur from the mature secretory granules via the plasma membrane, the recycling from immature granules to the TGN would render the formation of immature granules independent of the exocytosis of mature granules. Whatever the fate of the membrane retrieved after fusion of immature granules may be, it is likely that immature secretory granules are not just carrier vesicles, as constitutive secretory vesicles are thought to be, but may well be a branching point for membrane traffic.

Experimental Procedures

Cells and Reagents

PC12 cells (clone 251, Heumann et al., 1983), originally obtained from Dr. H. Thoenen (Martinsried), were grown in 10% horse serum, 5% fetal calf serum in DMEM at 10% CO_2. The cells were routinely passaged once a week. Fine chemicals were obtained from Boehringer-Mannheim (Mannheim, FRG) and Sigma (Munich). Carrier-free [^{35}S]sulfate was purchased from Amersham Buchler (England).

[^{35}S]Sulfate Labeling of PC12 Cells

Prior to labeling, PC12 cells were depleted of endogenous sulfate by incubation for 30 min at 37°C in sulfate-free medium (DMEM lacking sulfate with 1% of the level of normal methionine and cysteine, plus 1% dialyzed horse serum and 0.5% dialyzed fetal calf serum). They were then either pulse-labeled or continuously labeled in sulfate-free medium as described in the figure legends. Typically, 0.8–1.0 mCi/ml of [^{35}S]sulfate was used for pulse labeling, while 0.2–0.5 mCi/ml was used for longer labeling times. To chase the sulfate label, the normal growth medium was supplemented with twice the normal concentration of sulfate. In the 20°C block experiments, the cells were prein-

cubated, labeled, and chased at 20°C as described above. For stimulated release, PC12 cells were sulfate-labeled overnight and then chased for 30 min in DMEM containing either normal or elevated (55 mM) concentrations of KCl. At the end of the pulse or the chase, either aliquots of the cells and medium were analyzed by SDS–PAGE followed by fluorography as described (Lee and Huttner, 1983) or a PNS was prepared from the cells and subjected to velocity centrifugation followed by SDS–PAGE and fluorography (see below).

Cell-Free Formation of Post-TGN Secretory Vesicles

Pulse-Labeling of PC12 Cells

Usually, two 15 cm^2 dishes of confluent cells were used per experimental condition. PC12 cells were depleted of endogenous sulfate and pulse-labeled with [^{35}S]sulfate without subsequent chase as described above. They were then immediately placed on ice to prevent any intracellular transport.

Preparation of Postnuclear Supernatant

Unless otherwise indicated, all steps were performed at 4°C. PC12 cells, either labeled or unlabeled, were washed twice with TBSS (Tris-buffered saline plus sulfate: 4.5 mM KCl, 137 mM NaCl, 0.7 mM Na_2HPO_4, 1.6 mM Na_2SO_4, 25 mM Tris–HCl [pH 7.4]) and once with TBSS plus protease inhibitors (pi; 0.5 mM PMSF and 10 μg/ml Trasylol). Each 15 cm^2 dish of cells was scraped off in 10 ml of TBSS/pi using a cut silicone stopper attached to a disposable pipette. Typically, after this step ~20% of the cells were permeable to trypan blue. Cells from two dishes were pooled and pelleted by centrifugation for 5 min at 700 × g, washed once in homogenization buffer plus sulfate containing protease inhibitors (HBS/pi: 0.25 M sucrose, 1 mM EDTA, 1 mM Mg acetate, 1.6 mM Na_2SO_4, 10 mM HEPES–KOH [pH 7.2] pi as above), and pelleted by centrifugation for 5 min at 1700 × g. Each cell pellet was resuspended in 5 times its volume of HBS/pi (typically 1 ml) and passed through a 22-gauge needle until a single-cell suspension was obtained. The cell suspension was then homogenized in the cell cracker originally designed by Balch et al. (1984) and manufactured by the EMBL workshop, using an 18 μm clearance. The cell cracker was rinsed with 1 ml of HBS/pi, and the rinse was pooled with the initial homogenate, yielding a total volume of approximately 1.6 ml. This homogenization procedure resulted in >98% of the cells being broken, with intact nuclei as assessed by light microscopy. The nuclei and unbroken cells were pelleted by centrifugation at 1000 × g for 10 min. The resulting PNS routinely contained 3–4 mg/ml of protein as measured by the modified Lowry assay (Markwell et al., 1978) using bovine serum albumin as standard.

In Vitro Incubation of the PNS

The standard condition developed to obtain cell-free formation of immature secretory granules and constitutive secretory vesicles was as follows: 1.25 ml of the PNS in HBS/pi prepared from sulfate-labeled PC12 cells was supplemented at 4°C with 25 μl of 1 mM PAPS to inhibit additional radioactive sulfate incorporation during the incubation, 25 μl of 10 mM Mg acetate, and 50 μl of ATP and an ATP-regenerating system prepared according to Davey et al. (1985) (corresponding to 16.7 μl of 100 mM ATP, 16.7 μl of 800 mM creatine phosphate, and 16.7 μl of 4 mg/ml creatine phosphokinase in 50% [w/v] glycerol). In some experiments, 50 μl of 10 mg/ml of hexokinase in 250 mM D-glucose (Davey et al., 1985) was added instead of ATP and the ATP-regenerating system. In other experiments, the PNS in addition received 10 μM GTPγS. The PNS was then placed at 37°C (t = 0) and incubated without further agitation for 20 min to 2 hr. Controls were supplemented as above and kept on ice for the same times. After the incubation at either temperature, the PNS was subjected to velocity centrifugation. In time course experiments, samples after incubation at 37°C were transferred to and kept on ice until the last time point and then subjected to velocity centrifugation.

Velocity and Equilibrium Centrifugation

For the separation of post-TGN secretory vesicles (immature granules and constitutive vesicles) and the TGN, velocity centrifugation was performed using a linear sucrose gradient prepared from 0.3 M (5.5 ml) to 1.2 M (6.0 ml) sucrose in 10 mM HEPES–KOH (pH 7.2). Approximately 1.3 ml of the PNS was loaded onto the gradient, which was then centrifuged at 25,000 rpm in a Beckman SW40 rotor for 15 min (after having reached the final speed), with the brake applied at the end of the run. Fractions (1 ml) were collected from top to bottom of the gradient. The fractions were subjected to equilibrium centrifugation or aliquots of them were subjected to acetone precipitation, and the proteins were analyzed by SDS–PAGE followed by fluorography.

For the separation of immature granules and constitutive vesicles, fractions 1–4 from the top of the velocity gradient were pooled, and 3 ml was loaded on a second linear sucrose gradient made from 0.5 M (4.5 ml) to 2.0 M (5.0 ml) in 10 mM HEPES–KOH (pH 7.2). In addition, fractions 8–10 from the velocity gradient, containing the TGN, were pooled, diluted with an equal volume of HBS/pi, and loaded onto the second gradient. This gradient was centrifuged to equilibrium for 5 hr at 25,000 rpm in a Beckman SW40 rotor (centrifugation times up to 16 hr gave similar results). Fractions (0.8–1.0 ml) were collected from the top of the gradient. Aliquots of these fractions were subjected to acetone precipitation, and the proteins were analyzed by SDS–PAGE followed by fluorography.

Quantitation of Radioactivity in SgII and the hsPG

SgII

The radioactivity contained in the SgII band was quantitated by scintillation counting after elution of the protein with pronase as described previously (Friederich et al., 1988), using Ready-safe (Beckman Instruments) as scintillation fluid. In some experiments, radioactive SgII was quantitated by densitometric scanning of the fluorograms, using the LKB gel scanner and software.

hsPG

The sulfate-labeled smear migrating between 95–200 kd was demonstrated to be a heparan sulfate proteoglycan by digestion with heparinase II as described by Schubert et al. (1988) with the following modifications. Sulfate-labeled PC12 cells were solubilized in 20 mM Tris–HCl (pH 7.4), 150 mM NaCl, 0.3% Tween-20, 5 mM EDTA, the lysate was centrifuged for 3 min at 13,000 × g, and aliquots of the supernatant as well as aliquots of the medium were adjusted to 100 mM Tris–HCL (pH 7.0) and 1 mM $CaCl_2$. After addition of heparinase II (0.7 U/ml), samples were incubated overnight at 37°C and subjected to acetone precipitation, and the proteins were analyzed by SDS–PAGE followed by fluorography. This revealed that the ~200–95 kd sulfate-labeled smear was sensitive to heparinase II digestion. In cells, the 200–95 kd region contained, in addition to the hsPG, sulfate-labeled bands such as SgI and a 130 kd glycoprotein, which were not sensitive to heparinase II digestion (data not shown).

To quantitate the radioactivity contained in the hsPG, a representative area of the gel between SgI and a 130 kd sulfated band (shown as the thick portion of the bracket in Figures 2A and 2B), which contained the hsPG but no other sulfate-labeled proteins, was swollen in 10% acetic acid, 30% methanol to remove the salicylate used for fluorography. The gel piece was dried by lyophilization after extensive washing with H_2O. The dried gel piece was reswollen into 10 mM Tris–HCl (pH 7.0), 1 mM $CaCl_2$ containing 0.5 U/ml heparinase II, and incubated overnight at 37°C. The eluate was collected and the gel piece was washed 3 times with the digestion buffer. All washes were pooled with the original eluate and counted in Ready-safe. Alternatively, the radioactivity in the hsPG, from the same region of the gel as above, was quantitated by densitometric scanning of the relevant area of the fluorograms.

Quantitation of Cell-Free Immature Secretory Granule Formation

[^{35}S]SgII in the fractions of the velocity gradient was quantitated as described above. "Total [^{35}S]SgII in the TGN" was defined in the sample kept at 4°C as the sum of [^{35}S]SgII in fractions 9–12, which contained the peak of sialyltransferase (see Figure 3D). "[^{35}S]SgII in cell-free formed immature secretory granules" was defined in the samples incubated at 37°C as the sum of the increases in [^{35}S]SgII in the fractions at the top of the gradient above the corresponding values of the sample kept at 4°C (typically fractions 1–4, see Figure 3C). [^{35}S]SgII in cell-free formed immature secretory granules was then expressed as the percent of total [^{35}S]SgII in the TGN.

Protease Protection

Fractions 2–5 from the top of the velocity gradient were pooled, and aliquots (100 μl) were incubated with 1 mM dibucaine (Scheele et al., 1980) for 5 min at 4°C before the addition of 0.1 mg/ml proteinase K and 0.3% Triton-X 100, as indicated. After incubation at 4°C for 30 min, the digestion was stopped by the addition of 2 mM phenyl methylsul-

fonylfluoride followed by boiling in sample buffer, and the samples were analyzed by SDS–PAGE followed by fluorography.

Electron Microscopy of TGN and Immature Secretory Granules

Sucrose gradient fractions containing TGN or enriched in immature secretory granules, prepared from PC12 cells as described above, were collected and mixed 1:1 in a modified Karnovsky's fixative (Tooze and Tooze, 1986) at 4°C. After fixation overnight at 4°C, the samples were diluted 5-fold with a 1:1 mixture of HBS (without sulfate) and fixative and pelleted for 60 min at 36,000 rpm in a Beckman SW40 rotor. The supernatant was removed and the samples were processed and embedded in Epon as previously described (Tooze and Tooze, 1986). Ultrathin sections were cut and viewed in a Zeiss EM10 electron microscope. The size of the immature granules was quantitated by measuring the diameter of the dense core of the granules from negatives taken at a magnification of 17,000×. Only those profiles were chosen where the limiting granule membrane was clearly visible. Immature granules with multiple cores were not included in the quantitation.

Miscellaneous

For the analysis of the sulfate linkage to macromolecules in the "SgI region" of the gel, fractions from the equilibrium gradient containing either constitutive secretory vesicles or immature secretory granules were subjected to SDS–PAGE, and the relevant area of the gel, containing either sulfate-labeled hsPG or sulfate-labeled SgI, was subjected to tyrosine sulfate analysis as described (Huttner, 1984). Sialyltransferase activity was assayed in the fractions of the velocity gradient as described (Brändli et al., 1988), using asialofetuin as the exogenous acceptor.

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