Intermediates in the Constitutive and Regulated Secretory Pathways Released In Vitro from Semi-intact Cells

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Abstract. Regulated secretory cells have two pathways that transport secreted proteins from the Golgi complex to the cell surface. To identify carrier vesicles involved in regulated and constitutive secretion, PC12 pheochromocytoma cells were labeled with \[^{33}\text{S}\]sulfate to identify markers for the two secretory pathways, then mechanically permeabilized and incubated in vitro. Small constitutive secretory vesicles, containing mostly sulfated proteoglycans, accumulated during an in vitro incubation with ATP. In the presence of GTP\(_\gamma\)S, the constitutive vesicles became significantly more dense, suggesting that a coated intermediate was stabilized. Larger immature regulated secretory granules, enriched in sulfated secretogranin II, also escaped from the permeabilized cells in vitro. During granule maturation, their density increased and the amount of cofractionating proteoglycans diminished. The data suggest that sorting continues during secretory granule maturation.

Membrane traffic between compartments involves carrier vesicles that bud from a donor compartment and fuse with the acceptor compartment. To explain how membrane proteins are sorted to different organelles, we need to know how the carrier vesicle selects a subset of the proteins present in the donor membrane compartment and how it fuses exclusively with the acceptor. With these questions in mind, considerable effort has been expended in identifying carrier vesicles involved in ER to Golgi transport, intra-Golgi transport and post-trans-Golgi network (TGN)\(^3\) transport. Unfortunately, because intracellular transport is efficient in vivo, the carrier vesicles are short-lived, and so are present in very small amounts. Isolation of the carrier vesicles has been facilitated by using mutants in the secretory pathway that accumulate vesicles, or by generating vesicles in vitro under conditions in which fusion with the acceptor compartment is less probable. From such studies, we know that the carrier vesicles involved in transport to the Golgi network (Groesch et al., 1990; Segev, 1991; Rexach and Schechman, 1991; Paulik et al., 1988) and to the cell surface (Walworth and Novick, 1987; Holcomb et al., 1987; de Curtis and Simons, 1989) are small (~50 nm in diameter). Intra-Golgi carrier vesicles also accumulate in the presence of the nonhydrolyzable analogue GTP\(_\gamma\)S (Melançon et al., 1987). These vesicle are larger (80 nm) and have a coat consisting of \(\beta\)-COP and other proteins associated into coatamers (Malhotra et al., 1989; Duden et al., 1991; Serafini et al., 1991; Waters et al., 1991).

In regulated secretory cells, there are two types of carrier vesicles that leave the TGN and fuse with the plasma membrane, the constitutive secretory vesicle and the regulated secretory granule (for review see Kelly, 1991). The two carrier vesicles differ in the types of protein they transport and whether or not their fusion with the cell surface is regulated by cytoplasmic second messengers. Isolation of the two types of carrier vesicles ought to help clarify how protein sorting and regulated fusion are mediated. The pioneering study of Tooze and Huttner (1990) suggested that isolation of the two classes of vesicles might be possible. To label secreted proteins in PC12 cells, they were pulse labeled with \[^{33}\text{S}\]sulfate. Radioactive sulfate is incorporated into the intermediate phosphoadenosine phosphosulfate (PAPS), which is transported into the lumen of the Golgi network (Milla and Hirschberg, 1989) and transferred to carbohydrate chains (Rodén, 1980) and to tyrosine residues of proteins (Huttner, 1982). Since tyrosine sulfation of proteins occurs concomitant with or after sialic acid addition in hybridoma cells, it is thought to occur in a late trans-Golgi compartment (Baeuerle and Huttner, 1987). The site of sulfation of proteoglycans is less well localized. Tooze and Huttner (1990) showed that intact cells, labeled briefly with \[^{33}\text{S}\]sulfate, secreted only three major \(^{33}\text{S}\)-labeled species. Two tyrosine sulfated proteins, secretogranin II and chromogranin B, are markers for the regulated pathway while sulfated proteoglycans are a marker for the constitutive. When isolated membranes were incubated in the presence of ATP, sulfated-labeled vesicles budded from the Golgi fractions. Although vesicles containing the constitutive marker largely overlapped vesicles containing the regulated protein markers on equilibrium density gradients, enough separation was achieved to suggest that sorting was indeed occurring in vitro.

1. **Abbreviations used in this paper:** PAPS, phosphoadenosine phosphosulfate; TGN, trans-Golgi network.
In principle it should be possible to explore both the formation of secretory vesicles and their docking and fusion with the plasma membrane using gently permeabilized, semi-intact cells. In semi-intact cells from the growth hormone–secreting GH line, mature secretory granules of the regulated pathway do not leave the cells, but release their contents when calcium levels are raised (Martin and Walent, 1989). To use semi-intact cells to study vesicle formation, it is essential that post-Golgi carrier vesicles be able to leak from the cells (Bennett et al., 1988). To determine if intermediates in the secretory pathway would escape from semi-intact cells, we examined the release of \([35S]\) sulfate-labeled vesicles from PC12 cells. We obtained evidence that two classes of vesicles escaped, constitutive secretory vesicles and precursors of mature secretory granules. Their formation and properties were dependent on ATP, GTP, and the time between labeling and permeabilizing the cells. The small constitutive vesicles resembled the carrier vesicles released from yeast and other cells in their homogeneous sedimentation behavior. The precursors in the regulated pathway, free to diffuse out of the permeabilized cells, were much larger and may still be engaged in the sorting process as has been suggested for exocrine cells (von Zastrow and Castle, 1987).

**Materials and Methods**

**Materials**

ATP, GTP, GTP\(\gamma\)S, creatine phosphate, creatine kinase, PAPS, and anti-synaptophysin (SY38) were purchased from Boehringer Mannheim Corp. (Indianapolis, IN). \(\mathrm{Na}_{2}{35}\mathrm{~S}\) was purchased from Amersham Corp. (Arlington Heights, IL). All other reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

**Cell Culture, Labeling, and Pulse-Chase Experiments**

Five separate clones of the rat pheochromocytoma (PC12) cell line (Green and Tischler, 1976) were tested during the course of these experiments, and gave similar results. PC12 cells were cultured in 10% CO\(_2\) in DME H21 containing 10% horse serum, and 5% FCS. Six to eight 15-cm dishes of confluent PC12 cells were washed in warm PBS, then dislodged from the plates in 10–12 ml PBS by pipetting, and transferred to a 15-ml polypropylene test tube. Cells were centrifuged 2 min at 200 g, then washed once more in PBS and centrifuged as above. The cells were resuspended in 5 ml \(\mathrm{Na}_{2}{35}\mathrm{~S}\)-labeling medium (DME H21 without cold sulfate containing 1–2 enz test tube). Cells were centrifuged 2 min at 200 g, then washed once more in warm PBS, thendislodged from the cell culture dish using a 2.25 M sucrose pad. After centrifuging 1 h at 218,000 g, gradient fractions were collected, TCA precipitated, fractionated by PAGE, transferred to a0.4-ml 2 M sucrose pad. After centrifuging 1 h (model SW55TI; Beckman Instruments, Inc., Palo Alto, CA). The supematant was decanted and the pellet (P2) was resuspended in 0.25 ml 0.25 M buffered sucrose by passing 10 times through a 25-gauge needle. This homogenate was centrifuged 10 min at 70 g to give a supernatant of "cell ghost" membranes (PIM) and a white nuclear pellet.

In some experiments (see Figs. 2 A and 6 A) the membranes in the SI fraction were diluted to 1 ml with cold 0.25 M buffered sucrose. This suspension was layered over a 0.4 ml pad of 0.3 M buffered sucrose (Bennett et al., 1988). The samples were centrifuged at 100,000 g (32.5 k rpm) for 1 h (model SW55TI; Beckman Instruments, Inc., Palo Alto, CA). The supernatant was decanted and the pellet (P2) was resuspended in 0.25 ml 0.25 M buffered sucrose by passing 10 times through a 25-gauge needle. In agreement with Bennett et al. (1988), we found no difference in sucrose gradient profiles of galactosyl transferase activity or TCA-precipitable \(\mathrm{35}^\mathrm{S}\) when membranes were concentrated and resuspended in this manner, except that soluble protein and free label were reduced. Alternatively, \(\mathrm{SI}\) was applied directly to sucrose velocity or equilibrium gradients.

**Gradients**

In preliminary experiments, centrifugal force \(\times\) time was varied in sucrose velocity gradients to resolve different organelles. Approximately 0.2 ml was layered over a 0.3–1.5 M buffered sucrose gradient with a 0.4–ml 2 M sucrose pad at the bottom and centrifuged 100,000 g (32.5 k rpm) for 1 h (except where noted) in a SW55TI rotor. Gradients were collected from the bottom, 200 \(\mu\) l per fraction. A 25,000-g, 1-h gradient was found to separate well different populations of immature secretory granules from immature secretory granules in agreement with Tooze and Hutner (1990), but did not allow the smallest sulfate-containing vesicles to enter the gradient sufficiently. A 100,000-g, 1-h sucrose velocity gradient resolved these vesicles from free label at the top of the gradient. For equilibrium gradients, \(\sim 0.2\) ml of sample was applied to a 0.5–1.5 M sucrose gradient with 0.4–ml 2 M sucrose gradient with 0.4–ml 2 M sucrose pad at the bottom and centrifuged 100,000 g (32.5 k rpm) for 1 h (except where noted) in a SW55TI rotor. Gradients were collected from the bottom, 200 \(\mu\) l per fraction. A 25,000-g, 1-h fraction was used to separate the populations of immature secretory granules in agreement with Tooze and Hutner (1990), but did not allow the smallest sulfate-containing vesicles to enter the gradient sufficiently. A 100,000-g, 1-h sucrose velocity gradient resolved these vesicles from free label at the top of the gradient.

**Galactosyl transferase assays** were done as described in Brew et al. (1974).

**Gels**

Gradient fractions were brought to 0.9 ml with water and precipitated by
the addition of 100 μl 100% TCA, 1 mg/ml deoxycholate, and an incubation at 4°C for 12 h. After a 5-min centrifugation at 10,000 g, pellets were washed with cold acetone and resuspended in 50 μl 7 M urea, 2% SDS, 125 mM Tris, pH 6.95, 20 mM DTT, 1 mM EDTA, 0.1% Bromophenol blue, and heated (55°C for 15 min). 10 μl of each sample was counted in scintillation fluor (Ecolume; ICN Biochemicals, Irvine, CA) for quantitation of radioactivity. The remainder was applied to acrylamide gels that were run as in Laemmli (1970) except that the amount of crosslinker was increased (8% acrylamide, 0.4% bisacrylamide). Gels were soaked in 0.3 M sodium salicylate before drying for fluorography.

To quantify synaptophysin in the same gel (see Figs. 3 and 4), the bottom part was cut, transferred to nitrocellulose, and probed with SY38 antibody as described above. In two experiments, quantitation of fluorograms was done with a densitometer (Bio-Rad Laboratories, Cambridge, MA) (Figs. 5A and 6B). A PhosphorImager (Molecular Dynamics, Sunnyvale, CA) was used for the remainder. For densitometric quantitation, an adjacent scan below the chromogranin B and secretogranin II bands was subtracted as background. For PhosphorImager quantitation, pixel volume from the broad region of top of the gel well above the chromogranin B band, where proteoglycans were most concentrated, was quantified as proteoglycans. Chromogranin B and secretogranin II were quantified by subtracting as background the average pixel volume from equal areas above and below each band, to subtract contributions from rapidly migrating proteoglycans.

Results

Separation of Unattached Vesicles from Other Membranes

When PC12 cells are incubated with radioactive sulfate the major labeled secretory products are secretogranin II, chromogranin B, and proteoglycans (Tooze and Huttner, 1990). We confirmed that secretogranin II is secreted from intact cells mainly by the regulated pathway and proteoglycans (Tooze and Huttner, 1990). When PC12 cells are incubated with radioactive sulfate the major labeled secretory products are secretogranin II, chromogranin B, and proteoglycans (Tooze and Huttner, 1990). We confirmed that secretogranin II is secreted from intact cells mainly by the regulated pathway and proteoglycans (Tooze and Huttner, 1990). When PC12 cells are incubated with radioactive sulfate the major labeled secretory products are secretogranin II, chromogranin B, and proteoglycans (Tooze and Huttner, 1990). We confirmed that secretogranin II is secreted from intact cells mainly by the regulated pathway and proteoglycans (Tooze and Huttner, 1990). When PC12 cells are incubated with radioactive sulfate the major labeled secretory products are secretogranin II, chromogranin B, and proteoglycans (Tooze and Huttner, 1990). We confirmed that secretogranin II is secreted from intact cells mainly by the regulated pathway and proteoglycans (Tooze and Huttner, 1990).

Formation and Release of Sulfate-labeled Vesicles

Although galactosyl transferase activity was not released

Figure 1. Outline of experimental procedure giving rise to the different cell fractions that were analyzed on velocity or equilibrium gradients.
Figure 2. (A) Galactosyl transferase activity in the cell ghost membranes fractionated on a sucrose velocity gradient (0.3-1.5 M sucrose, 25,000 g, 1 h) after permeabilization (-o-, PIM). Gradients were collected from the bottom of the tube and fast sedimenting membranes are in the low numbered velocity gradient fractions. Also given is the galactosyl transferase activity (-o-, P2) in vesicles that leaked from the permeabilized cells when they were warmed to 37°C for 15 min with ATP regenerating system. A P2 was prepared and analyzed on a 0.3-1.5 M sucrose gradient, 100,000 g, 1 h. The amount of [3H]galactose incorporated is plotted without subtraction of the assay background (1,000 ± 500 cpm). (B and C) TCA-precipitable 35S cpm in vesicles (-o-, SI) released from semi-intact cells during 15 min at 37°C or in the ghost membranes (-o-, PIM) that remained behind. In B, no ATP was added, while in C, from semi-intact cells, sulfate-labeled vesicles were released from pulse-labeled cells, especially after incubation in vitro in the presence of ATP. When PC12 cells were pulse labeled 5 min with [35S]sulfate, permeabilized, and incubated 15 min at 37°C, 35% of the total TCA-precipitable radioactivity was released into the SI from the permeabilized cells if the cells were incubated with an ATP regenerating system (Fig. 2 C, SI). When SI was analyzed by velocity sedimentation at higher g force than that used by Tooze and Huttner (1990), most of the membrane-associated TCA-precipitable 35S radioactivity entered the gradient and sedimented in a single peak (fractions 16-22) near the top. The homogeneity is because of uniform sedimentation properties rather than the vesicles reaching equilibrium density, since the distance sedimented was proportional to centrifugation time and force (data not shown). The radioactivity at the very top of the gradients (fractions 24-26) was probably associated with soluble protein since it could be removed if membranes in the SI were isolated by centrifuging through a small sucrose pad (Bennett et al., 1988) before application to sucrose velocity gradients (see below).

The vesicles released in the presence of ATP are not likely to be fragmented Golgi membranes since galactosyl trans-

Figure 3. Equilibrium density separation of vesicles that escaped from cells labeled and permeabilized as in Fig. 2. TCA-precipitable 35S radioactivity (-o-, SI cpm) of membranes that escaped from semi-intact cells, during an incubation with ATP, after separation on a sucrose equilibrium density gradient. Unlabeled synaptophysin (-o-, p38) released from the cell was detected using a Western blot (see Materials and Methods) and quantified by phosphorimaging. Also shown is TCA-precipitable 35S radioactivity from cell ghost membranes from the same experiment after incubation at 37°C with ATP (-o-, PIM). The density of each fraction was measured by refractometry and the quantities plotted as a function of density. The samples were centrifuged for 16 h at 32,500 rpm.
Figure 4. Analysis of the constitutive marker, $^{35}$S-proteoglycans, in vesicles that escaped from permeabilized cells on sucrose velocity gradients (A–C) and equilibrium gradients (D–F). Gradients were run as in Figs. 2 and 3, gradient fractions 3–22 were fractionated by SDS-PAGE and proteoglycans were quantified by phosphorimaging. The position of the peak of synaptophysin immunoreactivity (synaptic vesicles) released from cells fractionated without in vitro reactions is indicated by an arrow in A–C. (A) Quantitation of the proteoglycans on the gradients of SI material shown in Fig. 2B and C, from 15 min in vitro reactions without (-a, warm - ATP) or with (o, warm + ATP) ATP. (B) Quantitation of the proteoglycans on velocity gradients of membranes released (SI) from cells pulsed 5 min as in Figs. 2 and 3 and chased for 15 min in vivo before permeabilization. Cell fractions were analyzed before (-o, no reaction) and after (o, warm + ATP) incubation 15 min at 37°C + ATP. (C) Quantitation of the proteoglycans from an experiment identical to that described in B, but with a 60-min chase time in vivo. (D) Quantitation of the membrane-associated proteoglycans on the equilibrium density gradient shown in Fig. 3. (E) Quantitation of the proteoglycans in vesicles from the same in vitro incubation as in B, but fractionated on an equilibrium density gradient. Only the vesicles released in the presence of ATP are shown. (F) Quantitation of the proteoglycans in vesicles from the same in vitro incubation as in C, fractionated on a sucrose equilibrium gradient. Only the vesicles released in the presence of ATP are shown. The scales used are arbitrary. The amounts of material on each gradient are shown in the next figure.

Constitutive Secretory Vesicles

To determine if the sulfate-containing vesicles released from the semi-intact cells were derived from the regulated pathway, the constitutive pathway, or both, we examined the distribution of sulfated markers in the velocity and equilibrium gradients. Gradient fractions were analyzed by SDS-PAGE and each sulfated marker, as well as synaptophysin, was quantified. The distribution of proteoglycans across the velocity and equilibrium gradients (Fig. 4) was very similar to that of radioactivity (Fig. 2, B and C, and Fig. 3), indicating that proteoglycans incorporate the majority of $^{35}$S sulfate. The major peak of proteoglycans in fractions 16–22 on su-
crosse velocity gradients sedimneted more rapidly than synaptic vesicles (Fig. 4 A) and had an equilibrium density of 1.13-1.14 g/ml (Fig. 4 D). Since proteoglycans are secreted mainly by the constitutive pathway, we tentatively conclude that the small proteoglycan-rich vesicles that accumulate in vitro are constitutive secretory vesicles.

Constitutive secretory vesicles derived from the TGN have been difficult to detect in vivo, presumably because they fuse with the cell surface soon after synthesis. If the vesicles were indeed constitutive in origin then their content of labeled proteoglycans should disappear rapidly during a chase. However, we found that incorporation of $^{35}$SO$_4^{2-}$ into the major labeled secretory proteins, proteoglycans, chromogranin B, and secretogranin II (the sum of the amount of each marker in the media and cells) increased linearly for the first 15 min of the in vivo chase (Fig. 5 A). 15 min is probably the time that is taken to deplete intracellular pools of $^{35}$S-PAPS. Consistent with this possibility, when cells were permeabilized without chasing, the addition of cold PAPS to the in vitro reaction reduced by 20% the amount of TCA-precipitable radioactivity in ATP-released membranes as well as in the Golgi fractions associated with the cell ghost membranes. Incorporation into proteoglycans and secretogranin II was equally reduced by cold PAPS.

To examine the disappearance of label from the released vesicles, cells were incubated in vivo for 15 min to deplete $^{35}$S-PAPS pools and then permeabilized or chased a further 45 min in vivo before permeabilization. After permeabilization a 15 min in vitro reaction was carried out as in the above experiments. The total $^{35}$S-labeled proteoglycans in constitutive vesicles (velocity gradient fractions 16-22) released when semi-intact cells were incubated with ATP was highest after a 15 min in vivo chase (Fig. 5 B). This is probably attributable to continued sulfate incorporation during the chase (Fig. 5 A) before the bulk of proteoglycans is secreted. After a 60-min chase, the total amount of radioactivity in the constitutive vesicles diminished rapidly (Fig. 5 B). Since 80% of proteoglycans are secreted by a 60-min chase (data not shown), fewer labeled proteoglycans are expected to be present in a carrier vesicle traveling between the Golgi complex and the cell surface. Velocity and density gradient analysis showed (Fig. 4) that the small light vesicles were still generated in the presence of ATP with an efficiency that was independent of chase time, averaging ~23% (Fig. 5 B).

Secretory vesicles containing $^{35}$S-labeled proteoglycans escaped from the permeabilized cells to a small extent, even in the absence of incubation in vitro. The sedimentation properties of vesicles released without ATP did not resemble those generated during incubation in the presence of ATP. Their sedimentation rate gradually increased with time (Fig. 4 B and C, no reaction).

The nonhydrolyzable GTP analogue, GTPyS, has major effects on in vitro membrane traffic, affecting both the formation and fusion of carrier vesicles. The presence of GTPyS during the in vitro reaction reduced the yield of constitutive secretory vesicles by about a third (63% ± 3 in five experiments), and those that were produced migrated with increased sedimentation velocity (Fig. 6 A) which is probably because of increased density (Fig. 6 B). The proteoglycans in the less dense peak (Fig. 6 B) had approximately the same density as those in the more dense peak (Fig. 6 A).
A  P2 Velocity Gradients

B  Equilibrium Gradient

Figure 6. GTPγS affects budding and the velocity and density of constitutive secretory vesicles. (A) 35S-labeled vesicles (P2) that escaped from cells labeled for 5 min in vivo before permeabilization were analyzed by velocity sedimentation as before. After permeabilization, samples were incubated for 15 min in vitro without (−, warm − ATP) or with ATP (−, warm + ATP) or with ATP and 20 μM GTPγS (−, warm + ATP + GTPγS). TCA-precipitable radioactivity is plotted. (B) After velocity gradient separation as in A, fractions 10–23 were diluted 1:2.4 in 20 mM MOPS, 1 mM EGTA, and centrifuged 1 h 42 k rpm. The pellet was resuspended in 0.25 M buffered sucrose and applied to sucrose equilibrium gradients. −, TCA-precipitable cpm. Fractions were pooled in pairs for PAGE. −−, Densitometric quantitation of proteoglycans. GTPγS depleted secretogranin II in the least dense fractions in these experiments, but otherwise had little effect on the distribution of the regulated secretory marker (not shown).

density as constitutive secretory vesicles (Fig. 4). The new peak that appeared in GTPγS had a density close to 1.19 g/ml, suggesting coated intermediates similar to those purified by Malhotra et al. (1989).

We conclude that three separate classes of vesicles containing proteoglycans can be detected. (a) A homogeneous population of small vesicles whose accumulation depends on an in vitro incubation with ATP, which we call constitutive secretory vesicles; (b) a small, dense class that accumulated in vitro in the presence of ATP and GTPγS, tentatively classified as coated constitutive vesicles; and (c) a minor class of heterogeneous rapidly sedimenting vesicles that were released without an in vitro incubation with ATP and that changed their sedimentation rate with time. This third class of vesicles has properties in common with immature regulated secretory granules.

Immature Regulated Secretory Granules

Secretogranin II, a marker for the regulated secretory pathway, was also quantified in the experiments described above. After a 5-min pulse labeling, there was an ATP-dependent release of secretogranin II—containing vesicles from the permeabilized cells during the in vitro incubation (Fig. 7 A). In contrast to proteoglycans, most of the secretogranin II was broadly distributed across the velocity gradients with a much greater fraction (55–85%) in more rapidly sedimenting membranes. After equilibrium centrifugation the regulated secretory marker was found in vesicles more dense than the proteoglycan-containing constitutive secretory vesicles (Fig. 7 D) in agreement with Tooze and Huttner (1990). By the criteria of sedimentation rate and density, therefore, the secretogranin II–containing vesicles differ from constitutive secretory vesicles.

The selective sorting of secretogranin II into heterogeneous vesicles larger than the constitutive vesicles suggests that the large vesicles are precursors of regulated secretory granules. The putative immature secretory granules differ from constitutive vesicles in several ways. The addition of GTPγS to the in vitro incubation only slightly reduced the amount of secretogranin II–containing vesicles formed in vitro, and did not have a dramatic effect on their density (data not shown). The release of immature secretory granules from the semi-intact cells thus appeared to be less sensitive to GTPγS than was the release of constitutive secretory vesicles. A second difference is that after in vivo chases of 15 and 60 min, release of secretogranin II–rich vesicles was largely ATP-independent. Their velocity and equilibrium density characteristics were determined during the same pulse-chase experiment used to characterize constitutive vesicles. We found that after 15- and 60-min in vivo chases, permeabilized cells released secretogranin II–labeled vesicles without need of an in vitro incubation (Fig. 7, B and C, no reaction). With or without an in vitro incubation, the released vesicles had a sedimentation velocity that increased with time of chase (Fig. 7 A–C). The immature secretory granules released from the cells also increased in density with time of chase (Fig. 7 D–F and Fig. 8 A). To determine the density of mature secretory vesicles, cells were labeled with [35S]sulfate for 12 h and chased for an additional 12 h. The secretogranin II–containing vesicles were associated with the cell ghosts (PIM) and had an average density of 1.20 g/ml (Fig. 8 A). Thus, the secretory granule precursors that are released by semi-intact cells did not reach the density of mature granules by 60 min. Despite the differences between constitutive vesicles and immature secretory granules, the radioactivity recovered in both vesicle types peaked at 15 min of chase.
Comparison of Regulated and Constitutive Vesicles

Because of the major difference in their sedimentation velocities, it has been possible to resolve experimentally constitutive vesicles from immature secretory granules and compare their properties. Constitutive vesicles were rich in sulfated proteoglycans and not detectable unless permeabilized cells are incubated with ATP. Their size, density, and ratio of secretogranin II to proteoglycans remained constant, independent of chase time (Fig. 8). The sulfate-labeled Golgi membranes from which they may arise do not leak out of the permeabilized cells. The density of the Golgi peak fractions (1.178 ± 0.002 g/ml) and the ratio of secretogranin II to proteoglycans (0.08 ± 0.01) also remained constant during the chase.

Immature secretory granules containing secretogranin II only showed ATP-dependent release immediately after pulse labeling. As the chase time increased, any ATP-dependent release was presumably concealed by the release of pre-existing immature secretory granules from cytoplasmic pools. The density of the immature granules was less than that of the Golgi membranes immediately after the pulse (Fig. 8 A). The immature secretory granule fractions (4–12, Fig. 7, A–C) contained 55–85% of the released 35S-secretogranin II, but also a minor fraction (30–40%) of the released 35S-proteoglycans. The ratio of secretogranin II to proteoglycans (0.05 ± 0.02) immediately after the pulse was not significantly different from that of the Golgi fractions. Unlike the Golgi fractions, however, or the constitutive vesicles, both the density and the ratio of secretogranin II to proteoglycans in the immature granule fraction increased during an in vivo chase (Fig. 8). Mature secretory vesicles, identified in the cell ghost membrane fractions (PIM) after a 12 h [35S]sulfate label and a 12–h in vivo chase, contained very

Figure 7. Analysis of the regulated marker secretogranin II in vesicles that escape from permeabilized cells. The fractions are from the experiments described in Fig. 4. (A–C) Sucrose velocity gradients; (D–F) equilibrium gradients after 0 (A and D), 15 (B and E), or 60 (C and F) min in vivo chases. The average equilibrium density of constitutive secretory vesicles and mature secretory granules is pointed out in D–F. The secretogranin II in fractions with density <1.1 g/ml (D–F) most probably represents soluble protein released from the immature secretory granules. –o–, (A) warm ATP, (B and C) no reaction; –o–, warm + ATP as in Fig. 4.
interest in regulated secretory cells since there are at least two types of carrier vesicles that leave the Golgi region carrying different classes of secretory protein. Carrier vesicles containing the two classes of protein can be resolved by density gradient centrifugation (Tooze and Hutner, 1990). Using a mechanically permeabilized cell system that preserved regulated exocytosis in vitro (Martin and Walent, 1989) we found that sulfate-containing vesicles escaped from the cells. These vesicles were generated from a biosynthetic compartment that remained inside the cell ghosts, presumably the TGN. The vesicles that were released were of two types, both lighter in density than the membranes from which they were derived. One population of small, relatively homogeneously sized vesicles contained 60–70% of the released proteoglycans, consistent with its involvement in the constitutive secretory pathway. Their size, density, and the ratio of secretogranin II to proteoglycans (Fig. 8) remain relatively constant late after a pulse label. They are slightly enriched in sulfated proteoglycans relative to the Golgi membranes.

Constitutive vesicles released in the presence of ATP contained ~23% of the total labeled proteoglycans in the cell (Fig. 5 B). The ability to recover constituent vesicles so efficiently is presumably because vesicles are more likely to escape permeabilized cells rather than fuse with the cell surface. Constitutive secretory vesicles, also called exocytotic transport vesicles, have been generated in vitro from liver Golgi membranes bound to magnetic beads (Salameo et al., 1990), mechanically permeabilized polarized epithelia (Bennett et al., 1988; Bomsel et al., 1990; Pobyclewicz and Mellman, 1990) and cell-free TGN membranes from BHK cells (de Curtis and Simons, 1989). They also accumulate in yeast cells defective in the GTP-binding protein, sec4 (Holcomb et al., 1987; Walworth and Novick, 1987). PC12 constitutive vesicles have properties in common with the vesicles described in these studies, being relatively uniform in sedimentation velocity and density, which implies a uniformity in size.

GTP is involved in both the budding of vesicles and their fusion. In yeast, the GTP-binding protein Sar1 is required for budding from the ER and the nonhydrolyzable analogue, GTPγS, slows but may not block the budding completely (Oka et al., 1991; Rexach and Schekman, 1991). In mammalian cell preparations, GTPγS inhibited the formation of both regulated and constitutive carrier vesicles, but only by ~50% (Tooze et al., 1990). The fusion process also involves GTP and can usually be completely inhibited by GTPγS in ER to Golgi, intra-Golgi, and Golgi to cell surface transport (Baker et al., 1988; Ruohola et al., 1988; Beekers and Balch, 1989; Melançon et al., 1987; Miller and Moore, 1991; Rexach and Schekman, 1991; Segow, 1991). Non-clathrin-coated vesicles that are involved in intercisternal Golgi transport accumulate in the cell in the presence of GTPγS (Malhotra et al., 1989). When budding from the TGN of PC12 cells was examined in the presence of GTPγS, fewer constitutive vesicles were formed and their sedimentation velocity and equilibrium density were increased, consistent with the retention of a coat. The partial inhibition of constitutive secretory formation (Tooze et al., 1990) (Fig. 6 A) could be explained if GTPγS prevents uncoating of a coated intermediate, sequestering the proteins required for the next round of budding. The complete inhibition by GTPγS of secretion from permeabilized fibroblasts may imply that

**Figure 8.** Summary of the properties of vesicles tentatively identified as constitutive vesicles, and immature secretory granules. (A) Peak equilibrium densities of the sulfate-labeled constitutive vesicles and immature secretory granules, plotted against in vivo chase time for several experiments. Individual determinations are plotted for 35S-proteoglycans in constitutive vesicles (Δ), 35S-proteoglycans made in the presence of GTPγS (●), and 35S-secretogranin II in immature secretory granules (○). The density of mature vesicles, labeled 12 h, then chased for 12 h is included for comparison (●). Average values ± SEM for each group are indicated on the y-axis. (B) The ratio of secretogranin II to proteoglycans increased with time of chase in regulated but not constitutive vesicles. The ratio of secretogranin II to proteoglycans was measured in velocity gradient fractions 4–12 (regulated vesicles) and fractions 16–22 (constitutive vesicles) and is plotted vs in vivo chase times. The 24-h point (12-h labeling, 12-h chase) represents PIM vesicles of equilibrium density 1.196–1.250 g/ml. Individual determinations are plotted as (○), immature regulated vesicles; (●), mature granules; or (Δ) constitutive vesicles. Average ± SEM are indicated on the y-axis. All quantifications for this figure were done by phosphor-imaging.

little labeled proteoglycans and were denser than immature granules (Fig. 8).

The constitutive vesicles and the immature secretory granules can therefore be distinguished readily from each other and from the Golgi membranes from which they arose by several criteria, including association with cell ghosts after permeabilization, sedimentation velocity, density, and ratio of soluble sulfated proteins.

**Discussion**

Traffic between the TGN and the cell surface is of particular
constitutive vesicle fusion has an absolute requirement for GTP hydrolysis (Miller and Moore, 1991).

The second class of vesicles released from permeabilized cells contained secretogranin II, a marker for the regulated secretory pathway. Although the secretogranin II–rich and proteoglycan–rich vesicles could be separated by density, velocity sedimentation allowed more complete resolution (Figs. 4 and 7). This resolution has revealed several aspects of the sorting process. Immediately after a pulse label, the release of the vesicles is ATP dependent, consistent with their formation in vitro during the incubation. They are significantly lighter than Golgi fractions and have a secretogranin II to proteoglycan ratio 2–3 times the constitutive vesicles (Fig. 8 B). After a long chase, any ATP-dependent formation of the secretogranin II–rich vesicles is obscured by ATP-independent release of pre-existing secretogranin II–rich vesicles. The density of the vesicles that accumulate during the chase is greater than that of vesicles generated in vitro, about the same as the donor Golgi membranes (Fig. 3), but still not as dense as mature secretory vesicles. Their content, however, has a secretogranin II to proteoglycan ratio that is twice that of the Golgi at 15 min chase and four times that of the Golgi at 60 min chase. The latter ratio is 15 times greater than constitutive vesicles, and approaches that of mature vesicles (Fig. 8 B). Such properties would be predicted for immature secretory granules but are less easy to reconcile with other possibilities such as fragmented Golgi membranes.

The increase in density of the secretogranin-rich vesicles with time is consistent with models in which vesicles of low density bud from immature secretory granules, increasing the ratio of the proteinaceous core to surrounding membrane (Kelly, 1985; von Zastrow and Castle, 1987; von Zastrow et al., 1989). If small vesicles bud off, they should contain soluble proteins excluded from the proteinaceous core (Fig. 9), and the ratio of the regulated marker, secretogranin II, to the constitutive one, sulfated proteoglycan should increase in the granules during granule maturation. Consistent with the latter prediction, the ratio of the two markers does increase during a chase (Fig. 8 B). Although the model outlined in Fig. 9 is consistent with the data, alternative explanations cannot be eliminated. In particular, it is important to show that newly synthesized proteoglycans and secretogranin II are actually in the same membranous intermediate.

von Zastrow and Castle (1987, 1989) have suggested that early phase secretion of secretory granule components is because of the budding of vesicles from immature granules. From their analysis of the types of protein secreted in the early phase they concluded that sorting involves condensation and continues after the formation of immature granules from the TGN. Both their model and Fig. 9 raise the issue of whether the late sorting compartments should be considered part of the TGN, or an immature secretory granule. We are experimentally classifying components that stay with the cell ghosts as Golgi associated and those intermediate vesicles that escape as immature or precursor secretory granules.

Chung et al. (1989) have suggested that sorting of regulated proteins involves a receptor that recycles between the TGN and a low pH compartment that is analogous in some ways to the prelysosomal or late endosome compartment. The fraction we call immature secretory granules could in principle be such a sorting compartment in which ligand dissociates from receptor at low pH. The crucial difference be-
tween the two models is whether condensation precedes sorting (Fig. 9) or vice versa (Orci et al., 1987; Tooze et al., 1987).

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