The Trafficking of α1-Antitrypsin, a Post-Golgi Secretory Pathway Marker, in INS-1 Pancreatic Beta Cells*

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A sulfated α1-antitrypsin (AAT), thought to be a default secretory pathway marker, is not stored in secretive granules when expressed in neuroendocrine PC12 cells. In search of a constitutive secretory pathway marker for pancreatic beta cells, we produced INS-1 cells stably expressing wild-type AAT. Because newly synthesized AAT arrives very rapidly in the Golgi complex, kinetics alone cannot resolve AAT release via distinct secretory pathways, although most AAT is secreted within a few hours and virtually none is stored in mature granules. Nevertheless, from pulse-chase analyses, a major fraction of newly synthesized AAT transiently exhibits secretogogue-stimulated exocytosis and localizes within immature secretory granules (ISGs). This trafficking occurs without detectable AAT polymerization or binding to lipid rafts. Remarkably, in a manner not requiring its glycans, all of the newly synthesized AAT is then removed from granules during their maturation, leading mostly to constitutive-like AAT secretion, whereas a smaller fraction (~10%) goes on to lysosomes. Secretogogue-stimulated ISG exocytosis routes newly synthesized AAT directly into the medium and prevents its arrival in lysosomes. These data are most consistent with the idea that soluble AAT abundantly enters ISGs and then is efficiently relocated to the endosomal system, from which many molecules undergo constitutive-like secretion while a smaller fraction advances to lysosomes.

In regulated secretory cells, anterograde trafficking from the trans-Golgi network (TGN) involves transport intermediates destined for at least three possible destinations: constitutive secretion (contents conveyed rapidly and directly to the cell surface), lysosomes (contents conveyed to the endosomal system), or storage granules of the regulated secretory pathway (contents conveyed by immature storage granules (ISGs)) (1). How luminal proteins (such as peptide hormones) come to be selected for storage in secretory granules potentially involves sorting events that occur both at the TGN and ISGs (2). In the case of insulin (the major peptide hormone secreted by pancreatic beta cells), studies have generally supported a model known as "sorting by retention" (3), which suggests that polymeric assembly of the newly synthesized hormone (4, 5) facilitates its intracellular retention and limits its constitutive-like secretion (6). In addition, endoproteolytic release of a single chain insulin from a fusion protein precursor in the late Golgi of yeast (by Kex2 protease) has been shown to promote intracellular retention and prevent rapid secretion of the single chain insulin (7). However, in mammalian cells, the hormone processing, secretory protein multimerization, and initiation of constitutive-like protein traffic tends to occur substantially within ISGs (8–13).

“Sorting for entry” models, although not mutually exclusive of the sorting by retention model described above, describe a subpopulation of luminal proteins that have already been sorted upon exit from the TGN (2) and thereby contribute to the distinctive compositions of constitutive secretory transport intermediates and ISGs (in addition to TGN-derived clathrin-coated vesicles). The recent observation that some luminal proteins of the regulated secretory pathway may associate with cholesterol-enriched “lipid rafts” (14–16) may be consistent with sorting for entry for such a subpopulation of proteins.

The situation for soluble monomeric secretory proteins within the lumen of the TGN is currently less clear; are such molecules included or excluded from ISGs? Entry of many soluble proteins into ISGs might be expected in endocrine cells in which a large fraction of the luminal volume of the anterograde protein trafficking pathway is directed toward secretory granule biogenesis (17). Interestingly, however, from studies of NIT-1 insulinoma cells, Rindler et al. (18) have argued that soluble secretory proteins are largely excluded from entry into beta cell ISGs. Further, Glombik and Gerdes (2) have pointed out that a key aspect of the sorting by retention model, namely, entrance of constitutive secretory “marker proteins” from the TGN into ISGs, is supported by little direct evidence. On the other hand, recent data indicate that a green fluorescent protein targeted to the secretory pathway only by a cleavable signal sequence (i.e. with no apparent sorting for entry signal) is able to abundantly enter insulin secretory granules (19), and similar phenomena have been observed for entry into ACTH (adrenocorticotropic hormone) secretory granules (20, 21). Moreover, these are but the latest in a series of observations of luminal proteins not expected to be sorted selectively for entry into ISGs that nevertheless do enter the regulated secretory pathway (for review, see Ref. 17). For example, newly synthesized hydrolases en route to lysosomes enter ISGs in pancreatic beta cells (3, 22), and the entry of such proteins into granules does not involve the specific lysosomal targeting signal (23). Further, some of what had been thought to be constitutive secretion of lysosomal procathepsin B from beta cells has been...
demonstrated in fact to represent constitutive-like secretion via an endosomal intermediate (24), although the role of endosomes as intermediates in the release of bona fide secretory proteins is less well studied (25).

Thus far, in most endocrine cells, it has been difficult to identify a soluble secretory protein dedicated to the constitutive pathway, and we have been engaged in a considerable effort to find one, such as a marker for insulin-containing granules (26). Orl et al. (27) reported that a sulfated alpha-1-antitrypsin (AAT) (also known as a protease inhibitor (26)) is not targeted to secretory granules and that granule storage of this protein is conferred only after appending specific structural information from the granule protein, chromogranin B, which indicates that AAT might be a useful constitutive secretory marker. These studies have prompted us to examine the fate of wild-type AAT expressed in the INS-1 pancreatic beta cell line. Indeed, in these cells, we now confirm the finding that AAT exhibits rapid unstimulated secretion and is entirely unstored in secretory granules. However, our evidence indicates that a major fraction of AAT actually enters ISGs and then proceeds to the endosome/lysosome system (28).

**EXPERIMENTAL PROCEDURES**

**Antibodies and Other Materials**—A rabbit polyclonal anti-human AAT was from Roche Applied Science, and a goat polyclonal anti-human AAT was from IGM (Costa Mesa, CA). Guinea pig polyclonal anti-insulin was from Linco Research (St. Charles, MO). Rabbit polyclonal anti-procathepsin B was from Upstate Biotechnologies (Lake Placid, NY). Rabbit polyclonal anti-cavelolin-1 was from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal anti-Cab45 was the kind gift of Dr. P. Scherer (Albert Einstein College of Medicine, Bronx, NY). Secondary antibodies and peroxidase conjugates were from Jackson Immunolaboratories (West Grove, PA). F(ab')2 rabbit anti-human and antiswine AAT was from Upstate Biotechnologies (Lake Placid, NY). F(ab')2 rabbit anti-human AAT was from Roche Applied Science, and a goat polyclonal anti-human AAT was from ICN/Cappel (Costa Mesa, CA). Guinea pig polyclonal anti-insulin was from Linco Research (St. Charles, MO). Rabbit polyclonal anti-procathepsin B was from Upstate Biotechnologies (Lake Placid, NY). Rabbit polyclonal anti-cavelolin-1 was from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal anti-Cab45 was the kind gift of Dr. P. Scherer (Albert Einstein College of Medicine, Bronx, NY). Secondary antibodies and peroxidase conjugates were from Jackson Immunolaboratories (West Grove, PA).

**Individual Drug-Resistant Cell Clones Obtained by Serial Dilution**—Individual drug-resistant cell clones obtained by serial dilution were contained only 2 mM glucose. In other experiments, INS cells were grown in complete growth medium (for INS-832/13 cells the unstimulated chase medium containing a combination secretogogue including 10 mM glucose, 1 mM tolbutamide, 10 mM leucine, and 10 mM glutamine (28). At the end of selected chase periods, media were collected and the cells were lysed in 100 mM NaCl, 1% Triton X-100, 0.1% SDS, 10 mM EDTA, and 25 mM Tris, pH 7.4 (24). For INS-832/13 cells, the secretogogue employed was complete chase medium containing a final glucose concentration of 15 mM.

**Immunoelectron Microscopy**—We employed the method of Orci et al. (30), using INS-AAT cells fixed with 2% paraformaldehyde, and 0.5% glutaraldehyde in 0.1 M phosphate buffer dehydrated through a series of increasing ethanol concentrations, with a progressive lowering of the temperature to −50 °C, embedded in Lowicryl HM-20 monostep resin (Electron Microscopy Sciences), and polymerized using UV light. Ultra-thin sections were mounted on nickel grids and then immunolabeled as follows. Sections were floated on blocking solution (Aurion) incubated overnight at 4 °C with the proinsulin cleavage site antibody (mouse monoclonal antibody G6B8 diluted 1:1000, kindly provided by Dr. O. Madsen, Hagedorn Institute, Copenhagen, Denmark) along with the rabbit polyclonal anti-human AAT diluted 1:100 in PBS with 0.1% bovine serum albumin (BSA−, Aurion). Sections were rinsed in PBS with bovine serum albumin and incubated with 6 nm goat anti-rabbit gold and 15 nm goat anti-mouse gold for 3 h at room temperature. Sections were then rinsed and postfixed with 2% glutaraldehyde in PBS. The immunolabeled sections were examined, and images were captured at a magnification of 40,000× in a JEOL 1200EX transmission electron microscope at 50 kV, processed, and scanned at the Albert Einstein College of Medicine Analytical Imaging Facility.

**Cell Fractionation**—We employed the method of Rindler et al. (18) with certain modifications. Specifically, INS-AAT cells were pulse-labeled and chased for various times. For each sample, the cells were washed once in ice-cold PBS and then scraped gently from the plate followed by centrifugation at 2000 × g for 4 min. The cell pellet was then resuspended in 1 ml of 250 mM sucrose, 1 mM MgCl2, 1 mM EDTA, 10 mM Hepes, pH 7.5, plus a protease inhibitor mixture. The cell pellets were then washed 10 times (up-and-down) through a 25-gauge needle followed by 10 passes (back-and-forth) through a ball-bearing homogenizer with a 40-μm clearance. After removal of nuclei and cell debris by centrifugation at 1000 × g for 20 min, the post-nuclear supernatant was centrifuged at 40,000 × g for 40 min and the resulting supernatant was collected and a 400-μl aliquot of was loaded atop a 2-ml discontinuous sucrose gradient (sucrose fractions from the bottom: 1.8 M (0.2 ml), 1.6 M (0.2 ml), 1.4 M (0.3 ml), 1.2 M (0.3 ml), 1.1 M (0.3 ml), 0.8 M (0.3 ml)). After centrifugation in a Sorvall RPS5-S swinging bucket ultracentrifuge rotor at 200,000 × g for 2 h, 200-μl fractions were collected from the top for analysis by immunoprecipitation.

**Immunoprecipitation**—Cell lysates and chase media were routinely treated with a protease inhibitor mixture (Roche Applied Science). These samples were precleared with Zosbyrin (Zymed Laboratories, South San Francisco, CA) and then subjected to immunoprecipitation. Zosbyrin-bound immunocomplexes were sedimented at 12,000 × g for 4 min. The pellets were washed once with cell lysis buffer in high salt buffer (0.5 M NaCl, 1% Triton X-100, 10 mM EDTA and 25 mM Tris, pH 7.4).

**Endo H or PNGase F Digestion**—After immunoprecipitation, AAT bound to Zosbyrin was eluted by boiling for 5 min in 2% SDS and 5% β-mercaptoethanol in 62.5 mM Tris-HCl, pH 6.8, followed by centrifugation at 12,000 × g for 4 min. The supernates were diluted to 0.4% SDS and 1% β-mercaptoethanol and digested with Endo H or PNGase F (New England Biolabs, Beverly, MA) as per protocol provided by the manufacturer.

**AAT Solubility Assay**—The permeabilization and protein extraction protocol was adapted from Chen and Huttner (21). Briefly, AAT-expressing INS-AAT cells were pulse-labeled for 30 min in methionine- and cysteine-free RPMI 1640 and then pulse-labeled with [35S]Met/Cys for either 15 or 30 min in the same medium. After pulse labeling, the cells were washed three times with PBS before being chased for various times in complete growth medium (for INS-832/13 cells the unstimulated chase medium contained only 2 mM glucose). In other experiments, INS cells were labeled with [35S]Met/Cys in complete growth medium with cell lysates washed once with cell lysis buffer in high salt buffer (0.5 M NaCl, 1% Triton X-100, 10 mM EDTA and 25 mM Tris, pH 7.4). The endo H or PNGase F digests were then run on a 12% SDS-PAGE gel and the AAT was visualized by autoradiography.

**Raft Assay**—Fractionation of Triton X-100 insoluble rafts was per-
formed by standard procedure (32, 33). Briefly, confluent cells pulse-labeled for 30 min and chased for 1 h were rinsed twice with PBS and lysed for 20 min on ice in 1.4 ml of 1% Triton X-100, 150 mM NaCl, 5 mM EDTA, and 25 mM Tris-HCl, pH 7.5. The lysate was scraped from the dish, passed 10 times (up-and-down) through a 22-guage needle, and then mixed with an equal volume of 80% sucrose in the same buffer without Triton X-100. A Triton X-100 extract of unlabeled HEK293 cells containing caveolin-1 was prepared in the same way. Four parts of the labeled extract from INS-1 cells was mixed with one part unlabeled 293 cell extract; this mixed heavy sucrose layer was placed at the bottom of polycarbonate micro-ultracentrifuge tubes and overlaid with 30% sucrose and 5% sucrose layers. Gradients were placed in a Sorvall RP55S rotor and spun at 167,000 × g at 4 °C for 18 h. Sixty-μl gradient fractions, collected manually from the top, were processed for immunoprecipitation of AAT and immunoblotting of caveolin-1.

Western Blotting—100 μg of total cell lysate protein or immunoprecipitated protein samples were subjected to 10% SDS-PAGE and electrophoresed into nitrocellulose (semi-dry transfer, 150 mA for 1 h). The membrane was then blocked with the primary antibody against AAT or caveolin-1 at 1:1000 dilution followed by enhanced chemiluminescent detection (ECL, Amersham Biosciences).

RESULTS

Rapid Intracellular Transit of AAT through the Secretory Pathway of INS Pancreatic Beta Cells—INS-1 pancreatic beta cells were transfected to express the cDNA encoding wild-type human AAT. Multiple clonal isolates selected for G418 resistance were screened for secretion of the 56-kDa AAT, and a variety of positively expressing and non-expressing clones were obtained (Fig. 1). Most subsequent experiments were performed with clone #3 (referred to hereinafter as “INS-AAT” cells), but most of the results presented herein were confirmed with at least two additional independent clones.

In preliminary experiments employing a 45-min [35S]Met/Cys pulse labeling of INS-AAT cells without chase (not shown), a labeled 56-kDa intracellular AAT glycophorin was predominant. A band of identical mobility was also recovered in the subsequent secretion, suggesting that much of the ER export and well established Golgi carbohydrate processing reactions might have already taken place at the zero chase time for molecules made during such a long pulse period (34). To establish this point, when INS-AAT cells were pretreated and cotreated with BFA during pulse labeling to block ER exit, a faster migrating newly synthesized AAT band was clearly visible (Fig. 2A, first lane). If BFA was present only during a 1-h chase, some labeled AAT molecules acquired the higher gel mobility (Fig. 2A, second lane), and when BFA was omitted entirely, the ER molecules were no longer apparent (Fig. 2A, third lane). Without BFA, the mature form of AAT, both intracellularly and in the medium, was PNGase F-sensitive (cleaved to a band migrating at the predicted molecular mass of 44.5 kDa, Fig. 2B, lanes marked “P”) and Endo H-resistant (lanes marked “E”), establishing that this form had undergone Golgi carbohydrate modifications. (A proteolytic fragment of AAT was also observed in cells at 2 h of chase, which is described further below.)

The foregoing and additional data (see below) indicate that AAT rapidly exits the ER in INS-AAT cells. Because ER exit is a rate-limiting step in overall intracellular transport (35), we next examined unstimulated secretion of newly synthesized AAT in two sequential blocks of time up to 8 h. A large majority of AATmature was released during the first 4 h under unstimulated conditions (Fig. 3, left panel) so that there was relatively little intracellular AATmature remaining at 4 h of chase. Consequently, during the next period from 4–8 h of chase, only a small fraction of labeled AATmature could be released under any conditions (Fig. 3, left panel) despite the fact that stored intracellular insulin could be significantly depleted from the cells under stimulation conditions (Fig. 3, right panel). Together, the data in Figs. 1–3 indicate rapid intracellular transit and unstimulated secretion of AAT from INS-1 pancreatic beta cells, with little or no intracellular storage of the protein in mature secretory granules, such as occurs for insulin.

Mass Entry of Newly Synthesized AAT into Beta Secretory Granules—Because so much newly synthesized AATmature is released into the unstimulated secretion during the first 4-h period, and because so little intracellular AATmature remains to be released into the medium during the second 4-h period, the addition of secretagogue during neither of these 4-h chase intervals showed a major effect on AATmature secretion (Fig. 3), despite the fact that a 4-h incubation with the secretagogue stimulates exocytosis of ~50% of labeled secretory granules from these cells (28). In this respect, AATmature behaves as one would expect if AATmature were a constitutive secretory granule protein. However, a modest stimulation of AATmature secretion upon addition of secretagogue could in fact be observed during both of these 4-h chase periods (Fig. 3), raising the question of whether newly synthesized AATmature might significantly enter ISGs but nevertheless fail to be stored within mature granules (17).

To test this point, we attempted to examine more closely the maturation and stimulus-dependent secretion of AAT during the first 60 min after a 15-min pulse labeling (Fig. 4A). At the zero chase time, almost all of the intracellular AAT was the faster migrating ER form (Fig. 4A, upper panel), and there was no AAT secreted (upper panel). At 15 min of chase, less than half of the AAT molecules had acquired Golgi sugars (Fig. 4A, lower panel), and secretion was still barely detectable (upper panel). By 30 min of chase in the absence of stimulation, >50% of the molecules had reached the Golgi complex (suggesting a half-time out of the ER of ~20 min), a small fraction of AATmature was now detected in the unstimulated secretion, and stimulus-dependent secretion of newly synthesized AAT (stimulated minus unstimulated expressed as percent of total) was 10.1 ± 2.1% (n = 3), which is very similar to insulin stimulus-dependent secretion (of ~10%) over the same time (30 min) period (28). Finally by 60 min of chase, all AAT had exited the ER (Fig. 4A, lower panel), a significant fraction was released

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even in the absence of stimulation, and stimulus-dependent secretion of AAT (upper panel) was 19.1 ± 2.6% (n = 6), which can be compared with a 60-min stimulus-dependent secretion of insulin of 20% of the granule pool (28). From these data we calculate that ≥90% of newly synthesized AAT enters the stimulus-dependent secretory pathway, despite that AAT_hom is not stored but instead is routed into relatively rapid unstimulated secretation, features that define the constitutive-like secretory pathway (17).

The standard stimulation of INS-1 cells involves use of a combination of secretagogues (28) that may activate multiple signal transduction pathways. This is done because the amount of secretory granule discharge in response to increased glucose alone (a physiological stimulus of granule exocytosis) is far less in INS-1 cells than we have observed in isolated pancreatic islets (8). However, subclones of INS-1 cells have been isolated that exhibit more robust exocytosis of insulin secretory granules in response to high glucose alone. We therefore obtained one such subclone, called INS-832/13 (29), and transiently transfected these cells with our plasmid encoding the AAT cDNA (driven by the cytomegalovirus promoter). At 48 h after transfection, using a similar metabolic labeling protocol to that described in Fig. 4A, we found that during a 60-min chase in the presence of high glucose as the sole added secretagogue, newly synthesized AAT_hom clearly exhibited stimulus-dependent exocytosis (Fig. 4B). To further examine the intracellular localization of AAT in INS-1 cells, we used a double labeling immunoelectron microscopy approach. To differentiate ISGs from mature insulin granules, we capitalized on the GSA8 monoclonal antibody against proinsulin, in which immunoreactivity is lost upon processing to insulin (30). Thus, mature granules that have little residual proinsulin remain unlabeled, whereas ISGs of different ages contain varying degrees of labeling. This antibody was used in conjunction with the rabbit polyclonal anti-AAT, along with secondary antibodies coupled to large gold particles (anti-mouse, 15 nm) or smaller sized gold particles (anti-rabbit, 6 nm). AAT was found throughout the secretory pathway, with a surprising abundance in lysosome-like structures (see below). However, most mature granules showed little immunoreactivity with either antibody (Fig. 4C, upper granule). By contrast, we could observe proinsulin-positive ISGs (Fig. 4C, arrows) that were also positive for AAT (Fig. 4C, arrowheads). Taken together, the data in Fig. 4 indicate that AAT substantially enters ISGs in the INS-1 pancreatic beta cell line.

**Fig. 3.** Kinetics of AAT secretion from INS-1 cells. The cells were pulse-labeled for 45 min and chased for 4 h in the absence (−) or presence (+) of secretagogue before cell lysis (left panel, first four lanes) and AAT immunoprecipitation. A parallel set of identically labeled cells that had not been stimulated during the first 4 h of chase was then incubated in fresh chase medium (M) in the absence (−) or presence (+) of secretagogue for the next 4 h before cell lysis (next four lanes). Immunoprecipitated AAT (left panel, last four lanes) and insulin (right panel, insulin is marked with an asterisk) from the media and cell lysates were analyzed by SDS-PAGE and fluorography. C, cell lysate.

**Fig. 4.** Granule entry and acquisition of stimulus-dependent secretion of AAT. A, INS-AAT cells were pulse-labeled for 15 min and chased in the continuous presence (+) or absence (−) of secretagogue for the indicated times. AAT immunoprecipitated from cells and media was analyzed by SDS-PAGE and fluorography. Stimulus-dependent secretion of AAT at 30 and 60 min of chase is clearly evident. B, INS-832/13 cells were pulse-labeled for 30 min and chased for 60 min as described in A, except that either 2 mM glucose (unstimulated condition (−)) or 15 mM glucose (Hi glucose (+)) was employed as the sole secretagogue. C, INS-AAT cells were examined by immunoelectron microscopy as described under "Experimental Procedures." The presence of proinsulin in ISGs is indicated by the larger (15 nm) gold particles (arrows), and the presence of AAT is indicated by the presence of the smaller (6 nm) gold particles (arrowheads).
AATfrag was found to be the predominant intracellular form of AAT in lysosomes of INS cells. A, unlabeled cells were lysed and AAT immunoprecipitated with a rabbit polyclonal antiserum. An undigested control sample (C) or that digested with PNGase F (P) was then analyzed by SDS-PAGE and immunoblotting for AAT using a goat polyclonal antiserum and appropriate anti-goat IgG peroxidase secondary antibody. B, cells were labeled metabolically for 2 days to approach steady state, and cellular AAT was immunoprecipitated. An undigested control sample (C) or that digested with Endo H (E) or PNGase F (P) was analyzed by SDS-PAGE and fluorography. C, cells were treated with leupeptin plus pepstatin A during a 30-min methionine/cysteine starvation and a 30-min pulse labeling period (L/P) or were untreated controls (O). Both labeled samples were then chased in complete medium without drugs for 3 h. AAT was immunoprecipitated from cells and analyzed by SDS-PAGE and fluorography.

The production of a protease-resistant fragment. Because the unproteolyzed AATmature is rapidly released from cells, the production of a nonsecreted protease-resistant AATfrag might be expected to result in its intracellular accumulation under steady state conditions. To check this possibility, INS-AAT cells were examined both by AAT immunoblotting (Fig. 5A) and by immunoprecipitation from cells metabolically labeled to approach steady state (Fig. 5B). Remarkably, despite the fact that only ~1 in 10 newly synthesized AATmature molecules undergoes cleavage, as demonstrated by both assays, the unsecreted AATfrag was found to be the predominant intracellular form of the protein in INS cells at steady state (Fig. 5, A and B).

We observed considerable EM-immunogold labeling of AAT over lysosomes (not shown), and Glickman and Kornfeld (3T) reported that proteolysis of newly synthesized luminal proteins delivered to lysosomes could be blocked by preincubating cells with a combination of pepstatin and leupeptin. Following their protocol in pulse-chase experiments, a preincubation of live INS cells with the pepstatin/leupeptin combination effectively blocked the intracellular formation of AATfrag. Pepstatin/leupeptin treatment did not block secretion of AATmature and moreover, inhibition of AATfrag production was also observed in cells treated with ammonium chloride (these data not shown).

Together these findings establish that a fraction of AATmature is delivered from the secretory pathway to the endosome/lysosome system. To see whether this delivery was routed through ISGs, we examined the effect of incubation with secretogogue on the intracellular appearance of AATfrag. As shown in Fig. 3 (left panel), in INS-AAT cells stimulated between 0 and 4 h of chase to increase AATmature secretion (second lane from left), the recovery of AATfrag in the cells was decreased (fourth lane).

This does not reflect nonspecific intracellular proteolysis in response to the secretogogue, because when cells were similarly stimulated from 4 to 8 h of chase (i.e. after AATfrag had already formed in lysosomes), there was no longer any effect on the recovery of the intracellular AATfrag (Fig. 3, left panel, last lane). Thus, most if not all of the AATfrag was derived from molecules that were initially in the stimulus-dependent secretory pathway but en route to the endosomal system.

Analysis of AAT Trafficking in INS Cells by Cell Fractionation—To obtain another view of the routing of newly synthesized AAT through the secretory pathway, we employed a sucrose gradient protocol for beta cell fractionation based primarily on a procedure reported by Rindler et al. (18) (see “Experimental Procedures”). After a 5-min pulse labeling, all of the newly synthesized AAT was recovered exclusively as the Endo H-sensitive ER form, and this was concentrated in fractions 6, 7, and 8, near the peak for newly synthesized calnexin, which was concentrated primarily in fractions 6 and 7 (Fig. 6, upper two gradients). After a 15-min pulse and a 15-min chase (Fig. 6, third gradient), the faster migrating ER form of AAT could still be seen (ranging from fractions 4–8) with a peak in fraction 6, but a more prominent AATmature band had a shifted distribution with a concentration in fractions 2–4. After a 30-min chase, AATmature was the predominant form recovered, with a distribution similar to that found for AATmature at 15 min including an increasing concentration in fraction 4 (Fig. 6, middle gradient). At this chase time, analysis of proinsulin-
other pituitary cells, insulinoma cells, and PC12 cells have suggested the possibility that the entry of luminal proteins into ISGs may require their membrane association (41), more specifically, with cholesterol-sphingolipid rafts in the TGN (14–16; 27). Even though AAT\textsubscript{mature} is not stored in granules, the foregoing data demonstrate that AAT\textsubscript{mature} substantially enters ISGs in INS cells. We therefore wished to test whether this entry involved lipid raft association of AAT\textsubscript{mature}. INS-AAT cells were pulse-labeled for 30 min with [$^3$S]Met/Cys and chased for 60 min, at which time all intracellular AAT was recovered as AAT\textsubscript{mature} and stimulus-dependent secretion was evident (see Fig. 4). At this time, the labeled cells were extracted in buffer containing ice-cold 1% Triton X-100 and mixed with a small quantity of unlabeled cell extract containing caveolin-1 (see “Experimental Procedures”), a raft membrane marker (33). As shown in Fig. 8, labeled AAT\textsubscript{mature} was quantitatively recovered in the load fractions at the bottom of the sucrose gradient (upper panel), whereas caveolin-1 (detected by immunoblot) floated to the opalescent raft fraction near the top of the gradient (lower panel). Thus, using this assay, association of AAT\textsubscript{mature} with cholesterol-sphingolipid rafts is undetectable and appears unlikely to explain the abundant entry of AAT\textsubscript{mature} into secretory granules in pancreatic beta cells. An alternative sorting for entry proposal is that protein polymerization (selective aggregation) within the Golgi stacks or TGN may be required for entry into ISGs insofar as transport intermediates exiting the TGN for other destinations may capture soluble proteins, leaving the polymerized/aggregated proteins for incorporation into secretory granules (42). By contrast we have suggested that in pancreatic beta cells, proinsulin may enter ISGs largely in a soluble state (3) (although the possibility certainly cannot be excluded that a modest fraction of proinsulin and other granule proteins in the Golgi could be membrane associated (14–16; 27; 43)). Chanat and Huttnner (31) demonstrated that certain granule content proteins can be recovered in an insoluble, pelletable state when the Golgi complex and ISGs are permeabilized with saponin, particularly when permeabilization is performed in the presence of millimolar levels of calcium at pH 6.4 (“aggregative buffer”). We employed their assay to examine the assembly state of AAT\textsubscript{mature} at the 1-h chase time. In the absence of saponin, AAT\textsubscript{mature} was fully pelletable, indicating its entrapment within Golgi/post-Golgi compartments (Fig. 9, upper row). In the presence of saponin, AAT\textsubscript{mature} was quantitatively released to the supernatant, regardless of whether the buffer mimicked nonaggregative (N.A.) or aggregative (Agg.) conditions (Fig. 9, upper row). Unglycosylated AAT behaved similarly to AAT\textsubscript{mature} (Fig. 9, middle row). As a control, the endogenously expressed Golgi luminal resident protein, Cab45 (44), was recovered in the pellet both in the presence and absence of saponin, indicating that permeabilization selectively liberates only
s soluble proteins (including AATmature) from Golgi/post-Golgi organelles. These data strongly suggest that the abundant entry of AATmature into ISGs in INS-1 cells does not require its polymerization.

Unexpected Efficiency of Removal of AAT from Maturing Pancreatic Beta-secretory Granules—The first evidence suggesting that luminal protein removal from maturing secretory granules leading to constitutive-like secretion was for C-peptide, a proteolytic product of proinsulin cleavage within pancreatic beta cell ISGs (8). After proteolytic processing, insulin becomes insoluble within granules whereas C-peptide remains completely soluble; nevertheless, the majority of granule C-peptide is not removed from islet ISGs but remains behind in mature granules (6). On the other hand, procathepsin B (ProB) (the soluble lysosomal enzyme precursor, which also abundantly enters ISGs) is virtually quantitatively removed during granule maturation. In this case, efficient removal from maturing granules is directly attributable to ProB binding to mannose 6-phosphate receptors for egress via clathrin-coated vesicles (3, 23). However in the present studies, AATmature abundantly enters ISGs although virtually none is stored in granules (Figs. 3 and 4). Thus, although it is not a lysosomal proenzyme, it must be extremely efficiently removed from maturing granules. Although N-glycans are not required for entry of AAT into ISGs, the foregoing reasoning led us to consider that AAT glycans might be required for a lectin-mediated removal of AAT from maturing granules.

With this possibility in mind, INS-AAT cells, either untreated or pretreated, and pulse-labeled with tunicamycin were then chased overnight in complete medium (without drugs) to try to ensure sufficient time for secretory proteins to leave the ER and proceed into Golgi/post-Golgi compartments. As shown in Fig. 10, some newly synthesized ProB from control cells (upper left panel) was recovered in the unstimulated overnight chase medium (lanes marked “O/N”), although most of it remained intracellularly, where it was converted to mature cathepsin B, whereas virtually none could be released upon secretagogue exposure the next day (media lane marked “S”). In tunicamycin-pretreated cells (Fig. 10, upper right panel), there was very little intracellular recovery of mature unglycosylated cathepsin B, and a large fraction of unglycosylated ProB was released into the unstimulated overnight chase medium. In addition a fraction of mis-sorted unglycosylated ProB remained entrapped within mature secretory granules where a clear stimulated exocytotic release could be demonstrated the next day upon secretagogue addition. This is consistent with previously reported findings (3, 23). When AAT was examined in parallel, there was essentially no labeled AATmature remaining in control cells after overnight chase and thus effectively no subsequent AATmature secretion under unstimulated or stimulated conditions (Fig. 10, lower left panel). In tunicamycin-pretreated cells after overnight chase (Fig. 10, lower right panel), there was some continued emergence of labeled unglycosylated AAT into the secretory granule; however, there was little evidence that unglycosylated AAT was entrapped within mature secretory granules as, unlike for unglycosylated ProB, there was little stimulus-dependent secretion. These data do not provide strong support for the notion that AAT uses its glycans for efficient removal from maturing secretory granules.

DISCUSSION

Wild-type AAT is known to be a soluble monomeric secretory protein (36, 45), and from studies in PC12 cells, Glombik et al. (27) have demonstrated that a sulfated form of AAT is not stored in secretory granules. Therefore, we have endeavored to employ AAT to study soluble secretory protein egress from the TGN in transfected INS-1 pancreatic beta cells. We find that the protein rapidly proceeds from AATEN to AATmature (Fig. 2), and shortly thereafter, AATmature begins to appear in large quantities in the culture medium (Figs. 3, 4, and 7B) until intracellular AATmature is depleted (Figs. 4, 7B, 10). Thus, we can confirm, in part, findings reported in PC12 cells, insofar as essentially no AATmature is stored ultimately in secretory granules.

Nevertheless, under short pulse-chase conditions in which 10–20% of granules were triggered to undergo stimulated exocytosis during 30–60 min in the presence of secretagogue, we observed very significant secretagogue-enhanced discharge of AATmature (Fig. 4, A and B); and we can calculate from these data that 90–100% of newly synthesized AAT has entered the stimulus-responsive secretory pathway. In conjunction with the temporary acquisition of stimulus-dependent secretion, newly synthesized AAT shifts its distribution to a peak in fractions 3 and 4 of our discontinuous sucrose gradient (Fig. 6), which coincide with the fractions in which newly synthesized proinsulin is first being converted to insulin, a feature that defines the ISG compartment (4, 30, 46). AAT immunogold labeling can also be found over ISGs and not mature granules (Fig. 4C). Moreover, in INS-832/13 cells, stimulated exocytosis of newly synthesized AATmature is elicited with high glucose as the sole added secretagogue (Fig. 4B). Together these data argue strongly that newly synthesized AATmature abundantly enters newly made (pro)insulin secretory granules.

Chanat and Huttner (31) have suggested that an aggregative
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milieu in the TGN creates the physical segregation between secretory proteins entering the constitutive secretory pathway and those entering granules, by polymerization/aggregation of the latter proteins. However, this may not be a general finding (see below), as AAT\textsubscript{mature} abundantly enters ISGs in INS cells without polymer/aggregate formation (see Fig. 9; note that polymerization/aggregation has been observed for mutant AAT but not for native wild-type AAT (47)). This distinguishes these results from recent reports demonstrating excellent granule entry and storage of enhanced green fluorescent protein (EGFP, a protein that is not normally targeted to the secretory pathway but that can be introduced therein merely by virtue of the presence of a cleavable signal sequence) in which the luminal EGFP was found to form disulfide-linked oligomers that were suggested to affect the outcome of these experiments (19).

Importantly, in direct response to previous concerns (2), our current findings with AAT\textsubscript{mature} provide one of the first solid pieces of evidence for a key aspect of the sorting by retention model, namely, that while there is a direct constitutive secretory pathway from the TGN to the cell surface that bypasses ISGs of pancreatic beta cells (48), there is nevertheless substantial entrance of soluble secretory proteins from the TGN into ISGs (17). This entry into ISGs requires neither N-glycans (Fig. 7B) (49) nor lipid raft association (Fig. 8). Moreover, our data would seem to conflict with the recent claim of Rindler et al. (18) that constitutive-like secretion does not importantly involve immature granules in insulinoma cells; instead, we support the idea that a large fraction of soluble luminal volume enters beta cell ISGs. However, it must be acknowledged that the partitioning of luminal volume between the various out-bound pathways from the TGN is likely to vary between differently regulated secretory cell types (17, 50), and between cell lines (18) as well as their cognate cell types in vivo.

The selection of AAT as a soluble marker has proven a fortunate choice for demonstrating a second novel aspect of the sorting by retention hypothesis. Specifically, we have long suspected that secretory proteins exiting from maturing secretory granules proceed through the constitutive-like pathway via an endosomal intermediate, and that from these endosomes, one fraction of secretory protein (such as C-peptide) could be channeled to lysosomes for degradation (28) with another fraction conveyed to the extracellular space, creating constitutive-like secretion (6). Indeed, even in yeast it is now postulated that one branch of the secretory pathway transits through endosomes before reaching the cell surface (51). However, this hypothesis has been extremely difficult to test in pancreatic beta cells, because it is challenging to accumulate or pharmacologically capture secretory proteins in endosomes and, unlike for lysosomal enzymes (24), any fraction of soluble secretory proteins subsequently delivered to lysosomes tends to be degraded and therefore is never recovered.

Remarkably in INS-1 cells, as a consequence of intracellular transport through the secretory pathway, a fraction of α\textsubscript{1}-protease inhibitor is processed to a stable, protease-resistant fragment, which accumulates intracellularly (Fig. 5, A and B) in a post-Golgi compartment (Fig. 2B) that can be identified as the endosome/lysosome system (Fig. 5C and data not shown). Moreover, stimulation of granule exocytosis specifically depletes the pool of molecules that generates the AAT\textsubscript{frag} population; this effect is most dramatic for cells stimulated beginning at the zero chase time (Fig. 3, third and fourth lanes), is somewhat less apparent for cells stimulated between 2 and 4 h of chase (Fig. 3B, last two lanes), and not at all evident for cells stimulated between 4 and 8 h of chase (Fig. 3, last two lanes in left panel). These results indicate that AAT\textsubscript{frag} produced in the endosome/lysosome system is selectively derived from AAT\textsubscript{mature} molecules initially contained in ISGs. Theoretically, AAT\textsubscript{frag} could be produced by crinophagy (direct granule-lysosomal fusion (52)), but this would require that ISGs rather than mature secretory granules be degraded by crinophagy, whereas published evidence seems to support the opposite point of view (53–57). Therefore, we believe that the simplest explanation of the foregoing results is that the entire population of AAT\textsubscript{mature} contained in ISGs is removed from granules to the endosomal system, wherein a small proportion of the molecules reaches lysosomes while a larger fraction is secreted.

From our analysis, the greatest mystery is why AAT\textsubscript{mature} is so efficiently removed from maturing beta cell granules when soluble C-peptide is not (6). One possibility is that AAT\textsubscript{mature} utilizes a receptor protein for its selective capture and egress from maturing granules to the endosomal system, similar to the mannose phosphate receptor-mediated egress of ProB from maturing beta cell secretory granules. If this is the case, then all we can say at present is that we have no strong evidence that any such exit receptor is likely to be a lectin, as unglycosylated AAT also enters ISGs (Fig. 7B) but does not appear to become entrapped within mature secretory granules to the extent that unglycosylated ProB does (Fig. 10). We cannot exclude the possibility that there could be a granule exit receptor that only recognizes features of the AAT polypeptide. A second possibility is that relative to the finite period of membrane trafficking out of maturing granules, the kinetics of proinsulin processing may not be sufficiently fast so that much of the C-peptide has not yet been produced (and therefore is unavailable for egress) at a time when the constitutive-like pathway is already carrying away most AAT\textsubscript{mature}. However, we do not favor this view but rather a third possibility. We suspect that the trafficking of soluble proteins out of maturing granules in INS-1 cells and other endocrine cell lines is likely to be somewhat more active than that occurring within “real” pancreatic beta cells, as the size of endocrine granules in cell lines is notably smaller than the granules found in their cognate cells in vivo. Although this could reflect a diminution of homotypic fusion of progranules (58–60), it might also reflect “over-active” constitutive-like membrane trafficking. We think this is likely to explain why a vastly greater fraction of intragranular C-peptide is actually degraded in INS cells than in beta cells contained within rat islets (28).

In conclusion, the foregoing data provide evidence to support the hypotheses that soluble secretory proteins are not readily excluded from entry into beta cell ISGs and that the constitutive-like secretory pathway involves two limbs: one from the ISGs (and the TGN) to the endosomal system followed by a second from endosomes to the cell surface (24) (with a nonsecretory branch leading to lysosomes that typically goes unidentified). Because some cell types have co-opted regulated exocytic secretion from specialized endosome-derived vesicles (25, 61), the co-existence of such routes in cells that also produce classical secretory granules could create additional possibilities (and complexities), which may need to be considered in mapping the stimulus-dependent pathways traversed by secretory proteins (62, 63).

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