The trafficking of alpha₁-antitrypsin, a post-Golgi secretory pathway marker, in INS-1 pancreatic beta cells

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Running Title: Constitutive-like secretion of alpha₁-protease inhibitor

Key Words: protein trafficking, granule biogenesis, immature secretory granule, secretory protein sorting, trans-Golgi network, endosomal system

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SUMMARY

A sulfated alpha₁-antitrypsin (AAT), thought to be a default secretory pathway marker, is not stored in secretory 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 secretagogue-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 while a smaller fraction (~10%) goes on to lysosomes. Secretagogue-stimulated ISG exocytosis re-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.
INTRODUCTION

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 prohormone 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 with the sorting by retention model above, describe a subpopulation of luminal proteins that have already been sorted upon exit from the TGN (2), thereby contributing to the distinct 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) could 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 towards secretory granule biogenesis (17). Interestingly however, from studies of NIT-1 insulinoma cells, Rindler and colleagues have argued that soluble secretory proteins are largely excluded from entry into beta cell ISGs (18). Further, Glombik and Gerdes 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 (2). On the other hand, recent data indicate that a green fluorescent protein targeted to the secretory pathway only by a cleavable signal sequence [ie, with no apparent sorting-for-entry
signal] is able to abundantly enter insulin secretory granules (19), and similar phenotypes are observed for entry into ACTH secretory granules (20, 21). Moreover, these are but the latest in a series of observations of luminal proteins not expected to be selectively sorted for entry into ISGs that nevertheless do enter the regulated secretory pathway [for review, see (17)]. For example, newly-synthesized hydrolases in 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 try to find such a marker. In PC12 cells, Glombik et al reported that a sulfated alpha1-antitrypsin [AAT, also known as alpha1-protease inhibitor (26)] is not targeted to secretory granules, and granule storage of this protein is conferred only after appending specific structural information from the granule protein, chromogranin B (27); indicating 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 on to the endosome/lysosome system, implicating endosomes as an intermediate in the constitutive-like secretory pathway.
**EXPERIMENTAL PROCEDURES**

*Antibodies and other Materials* — A rabbit polyclonal anti-human AAT was from Roche Applied Science (Indianapolis, IN); 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-caveolin-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 ImmunoResearch Laboratories (West Grove PA). \[^{35}S\]Methionine/Cysteine (Express\[^{35}S\] or \[^{35}S\]S) was from New England Nuclear (New Bedford, MA). Methionine/Cysteine-deficient and complete RPMI, tunicamycin, brefeldin A, leupeptin, pepstatin, and stock chemicals were from Sigma (St. Louis, MO).

*Cell culture* — INS-1 cells were cultured in RPMI-1640 medium supplemented with 30 mM sodium bicarbonate, 1 mM sodium pyruvate, 10 mM HEPES pH 7.35, 50µM beta-mercaptoethanol, 10% fetal bovine serum and 0.1% Penicillin-streptomycin (Gibco BRL) at 37°C with 5% CO\(_2\) as described (28). The INS-832/13 subclone of INS-1 cells (29) was obtained from the laboratory of Dr. C. Newgard (Duke U., Durham NC) and was grown in the same medium.

*Transfection* — The wild type human AAT cDNA (obtained from Dr. R. Sifers, Baylor College of Medicine, Houston TX) was cloned into the pcDNA3 expression vector (InVitrogen) in which expression is under control of the CMV promoter. Plasmid DNA was transfected into INS-1 cells with the Effectene Transfection Reagent Kit (Qiagen, Valencia CA) following the manufacturer’s protocol. Cells were selected with 150 µg/ml G418 (Gibco BRL) beginning at 48 h after transfection. Individual drug-resistant cell clones obtained by serial dilution were picked manually and maintained in the presence of 100 µg/ml G418. In INS-832/13 cells the plasmid was transiently transfected and the entire cell pool used for studies at 48 h after transfection (Fig. 4B).

*Metabolic Labeling* — INS-AAT cells or INS-832/13 cells were cultured in the absence of G418 for at least 2 d before experiments. Cells were pre-incubated for 30 min in methionine and cysteine-free RPMI-1640, and then pulse labeled with \[^{35}S\]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 contains only 2 mM glucose). In other experiments, INS cells were labeled with \[^{35}S\]met/cys in complete growth medium for extended periods to approach steady-state labeling conditions. Where indicated, brefeldin A (BFA) was included during labeling and chase at a concentration of 5 µg/ml. Tunicamycin (20 µg/ml), when employed, was at was added 1 h before and during the pulse labeling but was not present not during chase incubations. Where indicated,
pepstatin (100 uM) and leupeptin (200 uM) were included in the during preincubation and pulse labeling periods.

Stimulation of granule exocytosis — Secretory granule exocytosis was stimulated for the times indicated in complete growth medium containing a combination secretagogue including 10 mM glucose, 1 uM phorbol 12-myristate 13-acetate, 1mM isobutylmethylxanthine, 1 uM 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 secretagogue employed was complete chase medium containing a final glucose concentration of 15 mM.

Immunoelectron microscopy — We employed the method of Orci and colleagues (30), using INS-AAT cells fixed with 2% paraformaldehyde, 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. Ultrathin sections were mounted on nickel grids and then immunolabeled as follows: Sections were floated on blocking solution (Aurion, The Netherlands), incubated overnight at 4°C with the proinsulin cleavage site antibody (mouse mAb GSA8 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% BSA-c (Aurion). Sections were rinsed in PBS with BSA-c 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% gluteraldehyde in PBS. The immunolabeled sections were examined and images captured at a magnification of 40,000x in a JEOL 1200EX transmission electron microscope at 80kv, performed in the Albert Einstein College of Medicine Analytical Imaging Facility.

Cell fractionation — We employed the method of Rindler and colleagues (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 gently scraped from the plate, followed by centrifugation at 2000 x 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 cocktail. The cells were then passed 10 (up-and-down) times through a 25-gauge needle followed by 10 (back-and-forth) passes through a ball bearing homogenizer with 40 um clearance. After removal of nuclei and cell debris by centrifugation at 2000 x g for 4 min, the post-nuclear supernatant was collected and a 400 ul aliquot of this loaded atop a 2 ml discontinuous sucrose gradient [sucrose layers 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), 0.8M (0.3 ml)]. After centrifugation in a Sorvall RP55-S swinging bucket micro-ultracentrifuge rotor at 200,000 x g av for 2 h, 10 fractions of 200 ul were collected from the top for analysis by immunoprecipitation.
**Immunoprecipitation** — Cell lysates and chase media were routinely treated with a proteinase inhibitor cocktail (Roche Applied Science, Indianapolis, IN). These samples were pre-cleared with zysorbin (Zymed Laboratories, South San Francisco CA) and then subjected to immunoprecipitation. Zysorbin-bound immunocomplexes were sedimented at 12,000 g for 4 min and pellets were washed twice with cell lysis buffer and once 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 zysorbin was eluted by boiling for 5 min in 2% SDS and 5% beta-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% beta-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 Chanat and Huttner (31). Briefly, AAT-expressing INS-1 cells were pulse labeled for 30 min and chased for 1 h. Cells were then scraped from the dish in ice-cold PBS, followed by centrifugation at 500 g for 5 min. The cell pellet was resuspended in 1 ml PBS and homogenized by passage 8 (up-and-down) times through a 25 g needle. Nuclei and cell debris were pelleted by centrifugation at 500 g for 5 min. Membranes in the postnuclear supernatant were then pelleted at 23,000 g for 30 min and the membranes washed once with 1 ml PBS and re-pelleted as before. The washed pellet containing membrane-enclosed AAT was then resuspended in 500 ul aggregative milieu (10 mM MES-NaOH, pH 6.4, 10 mM CaCl$_2$ plus 1.2 mM leupeptin) or nonaggregative milieu (10 mM MES-NaOH, pH 7.4, 30 mM KCl plus 1.2 mM leupeptin) with or without saponin (1 mg/ml) and incubated on ice for 15 min. The membrane extract was finally subjected to centrifugation at 23,000 g for 30 min, and both supernatant and pellet fractions were collected for quantitative immunoprecipitation of AAT and Cab45.

**Raft assay** — Fractionation of Triton X-100 insoluble rafts was performed by standard procedure (32; 33). Briefly, confluent cells pulse labeled for 30 min and chased for 1 h were rinsed with twice with PBS and lysed in for 20 min on ice in 1.4 ml 1% Triton X-100, 150mM NaCl, 5mM EDTA and 25 mM Tris-HCl, pH 7.5. The lysate was scraped from the dish, passed 10 (up-and-down) times through a 22 g needle, and then mixed with 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, and 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. Six 350 ul gradient fractions were collected manually from the top and these were processed for immunoprecipitation of AAT and immunoblotting of caveolin-1.
**Western blotting** — 100 ug total cell lysate protein, or immunoprecipitated protein samples, were subjected to 10% SDS-PAGE and electrotransferred to nitrocellulose (semi-dry transfer, 150 mA for 1 hour). The membrane was then blotted with the primary antibody against AAT or caveolin-1 at 1:1000 dilution and a secondary IgG-peroxidase conjugate at 1:1000 dilution, followed by enhanced chemiluminescent detection (ECL, Amersham Biosciences, Piscataway NJ).
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 nonexpressing clones were obtained (Fig. 1). Most subsequent experiments were performed with clone #3 (to which this manuscript will refer to 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 \(^{[35}S\)met/cys pulse-labeling of INS-AAT cells without chase (not shown), a labeled 56 kDa intracellular AAT glycoprotein was predominant and 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 pre-treated and co-treated with brefeldin A (BFA) during pulse labeling to block ER exit, a faster migrating newly-synthesized AAT\(_{ER}\) band was clearly apparent (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 weight of 44.5kD, 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 2h chase, described further, below.]

The foregoing and additional data (see below) indicate that AAT rapidly exits the ER in INS-AAT cells. Since 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 AAT\(_{mature}\) was released during the first 4 h under unstimulated conditions (Fig. 3 left panel) so that there was relatively little intracellular AAT\(_{mature}\) remaining at 4 h chase. Consequently, during the next period from 4-8 h of chase, only a small fraction of labeled AAT\(_{mature}\) could be released under any conditions (Fig. 3 left panel) despite that stored intracellular insulin could be significantly depleted from the cells under stimulation conditions (Fig. 3, right panel). Together, the data in Figures 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 AAT\textsubscript{mature} is released into the unstimulated secretion during the first 4 h period, and because so little intracellular AAT\textsubscript{mature} 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 AAT\textsubscript{mature} secretion (Fig. 3) despite that a 4 h incubation with the secretagogue stimulates exocytosis of ~50% of labeled secretory granules from these cells (28). In this respect, AAT\textsubscript{mature} behaves as one would expect if AAT\textsubscript{mature} were a constitutive secretory marker protein. However, a modest stimulation of AAT\textsubscript{mature} secretion upon secretagogue addition could in fact be observed during both of these 4 h chase periods (Fig. 3), raising the question of whether newly-synthesized AAT\textsubscript{mature} 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 (lower 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 (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 AAT\textsubscript{mature} 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) and our unpublished data]. Finally by 60 min of chase, all AAT had exited the ER (Fig 4A lower panel), a significant fraction was released 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 to a 60 min stimulus-dependent secretion of insulin of 20% of the granule pool [(28) and our unpublished data]. From these data we calculate that ≥ 90% of newly-synthesized AAT enters the stimulus-dependent secretory pathway, despite that AAT\textsubscript{mature} is not stored but is instead routed into relatively rapid unstimulated secretion; features which 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 that which 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 CMV 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\textsubscript{mature} 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 mAb against proinsulin whose immunoreactivity is lost upon processing to insulin (30). Thus, mature granules which have little residual proinsulin remain unlabeled, while ISGs of different ages contain varying degrees of labeling. This mAb 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 (upper granule, Fig. 4C). By contrast, we could observe proinsulin-positive ISGs (arrows, Fig. 4C) that were also positive for AAT (arrowheads, Fig. 4C). Taken together, the data in Fig. 4 indicate that AAT substantially enters ISGs in the INS-1 pancreatic beta-cell line.

A fraction of newly-synthesized AAT reaches lysosomes, indicating routing from the biosynthetic pathway into the endosomal system

In the course of preliminary experiments, we noted small quantities of an intracellular labeled AAT-immunoprecipitable band that appeared at an M\textsubscript{r} \sim 40 kDa; moreover, the labeled band did not appear in cells treated with BFA, or even in the absence of BFA until 1 h of chase, becoming more intense at later chase times with little apparent turnover. Because the band was not apparent immediately upon pulse labeling and was smaller than completely deglycosylated AAT\textsubscript{mature} (indeed, its molecular mass is further diminished to \sim 35 kDa upon PNGase F-mediated deglycosylation, eg, Figs. 2B and 5A, B), it evidently reflected a post-translational proteolysis in which the AAT\textsubscript{frag} product contains at least 300 of the 394 residues of full-length AAT. Normally, AAT is N-glycosylated at N46, N83, and N247 (36); the smaller molecular weight loss upon
PNGase F-mediated removal of N-glycans from AAT\textsubscript{frag} than that observed for the full-length AAT\textsubscript{mature} (eg, Fig 2B) suggests that AAT\textsubscript{frag} is lacking at least one N-glycan and therefore probably comprises the C-terminal ~300 amino acids of AAT lacking N46. Because such a fragment contains 8 of the 9 AAT methionines plus the single AAT cysteine, the intensity of this \[^{35}\text{S}]\text{met/cys-labeled band in pulse-chase experiments is likely to very closely reflect the stoichiometry of its production. By quantifying band intensities from pulse-chase experiments like that shown in Fig. 3, we determined that ~10% of AAT is cleaved intracellularly in INS cells. The fact that AAT\textsubscript{frag} is endo H resistant (eg, Fig 2B) and yet is never secreted under any conditions (see Figs. 2, 3) indicates that cleavage occurs within a post-Golgi compartment that is beyond all secretory pathway branchpoints.

The fact that recovery of the labeled AAT\textsubscript{frag} from unstimulated INS-AAT cells was not decreased at 8 h chase suggested production of a protease-resistant fragment. Since the unproteolyzed AAT\textsubscript{mature} is rapidly released from cells, the production of a nonsecreted protease-resistant AAT\textsubscript{frag} 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 that only ~1 in 10 newly-synthesized AAT\textsubscript{mature} molecules undergoes cleavage, as demonstrated by both assays, the unsecreted AAT\textsubscript{frag} was found to be the predominant intracellular form of the protein in INS cells at steady state (Fig 5 A, B).

We observed considerable EM-immunogold labeling of AAT over lysosomes (not shown), and Glickman and Kornfeld reported that proteolysis of newly-synthesized luminal proteins delivered to lysosomes could be blocked by pre-incubating cells with a combination of pepstatin and leupeptin (37). Following their protocol in pulse-chase experiments, a preincubation of live INS cells with the pepstatin/leupeptin combination effectively abolished the intracellular formation of AAT\textsubscript{frag}. Pepstatin/leupeptin treatment did not block secretion of AAT\textsubscript{mature}, moreover, inhibition of AAT\textsubscript{frag} production was also observed in cells treated with ammonium chloride (these data not shown). Together, these findings establish that a fraction of AAT\textsubscript{mature} is delivered from the secretory pathway to the endosome/lysosome system. To see if this delivery was routed through ISGs, we examined the effect of incubation with secretagogue on the intracellular appearance of AAT\textsubscript{frag}. As shown in Fig. 3 (left panel), in INS-AAT cells stimulated between 0 - 4 h of chase to increase AAT\textsubscript{mature} secretion (second lane), the recovery of AAT\textsubscript{frag} in the cells was decreased (fourth lane). This does not reflect nonspecific intracellular proteolysis in response to the secretagogue because when cells were similarly stimulated from 4 - 8 h of chase (ie, after AAT\textsubscript{frag} had already formed in lysosomes), there was no longer any effect on the recovery of the intracellular AAT\textsubscript{frag} (Fig 3 left
panel, eighth lane). Thus, most if not all of the \( \text{AAT}_{\text{frag}} \) was derived from molecules that were initially in the stimulus-dependent secretory pathway but \textit{en route} to the endosomal system.

\textit{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 and colleagues (18), see \textit{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 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 \( \text{AAT}_{\text{mature}} \) band had a shifted distribution with a concentration in fractions 2-4. After a 30 min chase, \( \text{AAT}_{\text{mature}} \) was the predominant form recovered, with a similar distribution to that found for \( \text{AAT}_{\text{mature}} \) at 15 min including an increasing concentration in fraction 4 (Fig. 6, middle gradient). At this chase time, analysis of proinsulin-derived peptides across the gradient faintly showed the first appearance of newly-synthesized insulin which, along with proinsulin conversion intermediates, were recovered in fractions 3 and 4 (Fig. 6, fifth gradient). After long-term labeling and chase, mature insulin was no longer confined to fractions 3 and 4 but also had migrated broadly down into denser regions of the sucrose gradient, while AAT could no longer be recovered as the ER form or \( \text{AAT}_{\text{mature}} \); instead \( \text{AAT}_{\text{frag}} \) was now recovered in dense fractions (Fig. 6, bottom two gradients). In conjunction with the previous findings (Figs 3-5), these results support the notion that newly-synthesized \( \text{AAT}_{\text{mature}} \) enters ISG-enriched fractions and that a portion of \( \text{AAT}_{\text{mature}} \) goes on to be delivered to lysosomes where it is recovered as \( \text{AAT}_{\text{frag}} \).

\textit{The entry step into ISGs does not require AAT N-glycosylation, raft association, or polymerization}

The N-glycans on AAT are known to facilitate its early folding (36), and upon tunicamycin treatment, loss of N-glycans on AAT causes the protein to accumulate in the ER (38). However, this does not represent a permanent block in ER exit; rather, the rate of newly-synthesized unglycosylated AAT secretion is only slowed (Fig. 7A). However, the ability to detect protein targeting to granules becomes impaired for proteins with slow ER exit, partly because new secretory proteins no longer transit in a tightly synchronous wave (which impairs detection in pulse-chase analyses), and partly because accumulation of labeled granules in the storage pool is reduced when the rate-limiting storage step is preceded by another slow (ER exit) step in the secretory pathway.
(39; 40). Indeed, upon tunicamycin treatment, the ability to detect any stimulated AAT secretion within the first 2 h of chase was compromised (not shown); nevertheless, we could still detect stimulated release during the 2-4 h chase interval (Fig. 7B). Thus, N-linked glycans do not appear to be required for AAT entry into ISGs in INS-1 cells.

Recent studies of secretory products found in AtT20 and 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). Despite that AAT\textsubscript{mature} is not stored in granules, nevertheless, 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 \[^{35}\text{S}]\text{met/cys} and chased for 60 min at which time all intracellular AAT is recovered as AAT\textsubscript{mature} and stimulus-dependent secretion is 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 \textit{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 Huttner 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") (31). 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 panels). 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 set of panels). Unglycosylated AAT behaved similarly to AAT\textsubscript{mature} (Fig. 9, middle set of panels). 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 soluble proteins (including AAT\textsubscript{mature}) from Golgi/post-Golgi organelles. These data strongly suggest that the abundant entry of AAT\textsubscript{mature} 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 lumenal 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, AAT\textsubscript{mature} abundantly enters ISGs although virtually none is stored in granules (Figs. 3, 4); thus, despite that 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 in mind, INS-AAT cells either untreated or pre-treated 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 remained intracellularly where it was converted to mature cathepsin B, while virtually none could be released upon secretagogue exposure the next day (media lane marked "S"). In tunicamycin pre-treated cells (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 AAT\textsubscript{mature} remaining in control cells after overnight chase and thus effectively no subsequent AAT\textsubscript{mature} secretion under unstimulated or stimulated conditions (Fig. 10, lower left panel). In tunicamycin pre-treated cells after overnight chase (lower right panel), there was some continued emergence of labeled unglycosylated AAT into the secretion; 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 and Gerdes have demonstrated that a sulfated form of AAT is not stored in secretory granules (27); 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 AAT\textsubscript{ER} to AAT\textsubscript{mature} (Fig. 2); shortly thereafter, AAT\textsubscript{mature} begins to appear in large quantities in the culture medium (Figs 3, 4, and 7B) until intracellular AAT\textsubscript{mature} is depleted (Figs 4, 7B, 10). Thus, we can in part confirm findings reported in PC12 cells, insofar as essentially no AAT\textsubscript{mature} is ultimately stored in secretory granules.

Nevertheless, under short pulse-chase conditions where 10-20% of granules are triggered to undergo stimulated exocytosis during 30-60 min in the presence of secretagogue, we observe very significant secretagogue-enhanced discharge of AAT\textsubscript{mature} (Fig. 4A, 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 where 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 AAT\textsubscript{mature} is elicited with high glucose as the sole added secreagogue (Fig. 4B). Together these data argue strongly that newly-synthesized AAT\textsubscript{mature} abundantly enters newly-made (pro)insulin secretory granules.

Chanat and Huttner have suggested that an aggregative 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 (31). 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 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 and colleagues that constitutive-like secretion does not importantly involve immature granules in insulinoma cells (18); 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 outbound pathways from the TGN is likely to vary between different 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 proved 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 alpha_1-protease inhibitor is processed to a stable, protease-resistant fragment which accumulates intracellularly (Figs 5A, 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_frag population, and this effect is most dramatic for cells stimulated beginning at the zero chase time (Fig. 3, third and fourth lanes), somewhat less apparent for cells stimulated between 2 and 4 h of chase (Fig. 7B, last two lanes), and not at all evident for cells stimulated between 4 and 8 h of chase (Fig. 3, seventh and eighth lanes). These results indicate that AAT_frag produced in the endosome/lysosome system is selectively derived from AAT_mature molecules initially contained in ISGs. Theoretically, AAT_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\textsuperscript{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\textsuperscript{mature} is so efficiently removed from maturing beta cell granules, when soluble C-peptide is not (6). One possibility is that AAT\textsuperscript{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 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\textsuperscript{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 (11) 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. While 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 undetected). Because some cell types have co-opted regulated exocytotic 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) that may need to be considered in mapping the stimulus-dependent pathways traversed by secretory proteins (62; 63).
Acknowledgements

This work was supported by grants NIH DK48280, American Diabetes Association Mentor-Based Postdoctoral award, and a grant from the AlphaOne Foundation. We are indebted to Dr. R. Kuliawat (Albert Einstein College of Medicine, NY) for help and instruction in use of the INS cell system. We also thank Drs. R. Sifers (Baylor College of Medicine, Houston TX), M. Brantly (U. Florida, Gainesville), P. Scherer (Albert Einstein College of Medicine, NY), and A. Chang (Albert Einstein College of Medicine, NY), as well as members of the Arvan laboratory for helpful discussions.
References

Figure legends:

Figure 1. Selection of INS cell clones that secrete human alpha1-antitrypsin. Media bathing the indicated G418-resistant clones were immunoprecipitated after continuous metabolic labeling for 1 day, and subjected to SDS-PAGE and fluorography. The lane marked "U" represents untransfected INS-1 cells. The positions of molecular weight markers are indicated on the left.

Figure 2. ER, Golgi and post-Golgi forms of AAT in INS-1 cells. (A) Cells were pulse labeled for 30' and either unchased (first lane) or chased for 1 h with (+) or without (-) 5 ug/ml BFA. AAT was then immunoprecipitated from cells and media. (B) Cells were pulse labeled for 30' and chased for 2 hours. AAT from cells and media, as indicated, was then immunoprecipitated. An undigested control sample ("C") or that digested with Endo H ("E") or PNGase F ("P") was analyzed by SDS-PAGE and fluorography.

Figure 3. Kinetics of AAT secretion from INS-1 cells. The cells were pulse labeled for 45' 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 were then incubated in fresh chase medium in the absence (-) or presence (+) of secretagogue for the next 4 h before cell lysis. Immunoprecipitated AAT (left panel, last four lanes) and insulin (panel at right, insulin marked with *) from the media and cell lysates were analyzed by SDS-PAGE and fluorography.

Figure 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 times indicated. 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 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 in 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).

Figure 5. Massive accumulation of a proteolytically cleaved but stable 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 metabolically labeled 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 leupetin plus pepstatin A during a 30 min methionine/cysteine starvation and 30 min pulse labeling period ("L/P") or were untreated controls ("C"). 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.

**Figure 6.** Trafficking of newly-synthesized AAT as measured by cell fractionation of INS-AAT cells. INS cells were pulse labeled either for 5 min (second gradient from the top) or 1 h plus a 15 min chase (top gradient) or 15 min plus various chase times (third, fourth and fifth gradients), or labeled continuously overnight and then chased for 2 h (two gradients at the bottom) — all under unstimulated conditions. In each case the cells were lysed and loaded atop discontinuous sucrose gradients and spun for only 2 h (ie, not equilibrium density conditions) as described in Experimental Procedures and collected in 10 fractions, with fraction 1 at the top. Each fraction was immunoprecipitated for the protein indicated and analyzed by SDS-PAGE. Note in the fifth gradient (30 min chase) a peak in the appearance of proinsulin conversion intermediates (marked with asterisks), indicating ISG-containing fractions in which proinsulin processing is beginning.

**Figure 7.** Loss of AAT N-glycans slows its intracellular transport but does not block entry into ISGs. (A) INS cells were untreated (Control) or treated with tunicamycin as described in Experimental Procedures. Cells were pulse labeled for 30’ and chased in the absence of the drug for various times as indicated. Immunoprecipitated AAT from the media and cell lysates was analyzed by SDS-PAGE and fluorography. Immunoprecipitated AAT secreted from control cells was either undigested or subjected to PNGase F digestion before the SDS-PAGE. (B) Parallel wells of INS cells untreated (Control) or treated with tunicamycin were pulse labeled for 30 min and chased for 2 h in the absence of secretagogue, and then chased further for 2h in either the absence (-) or presence (+) of secretagogue. Immunoprecipitated AAT from the media and cell lysates were analyzed by SDS-PAGE and fluorography. The positions of molecular weight markers are indicated.

**Figure 8.** AAT is not recovered in the Triton X-100 insoluble raft fraction of INS-1 cells. Cells were pulse labeled for 30 min and chased for 1 h, before Triton X-100 extraction and raft analysis as described in Experimental Procedures. The fractions were analyzed for labeled AAT by
immunoprecipitation, SDS-PAGE and fluorography, as well as unlabeled caveolin-1 by SDS-PAGE and immunoblotting.

**Figure 9.** *AAT does not polymerize in the secretory pathway of INS-1 cells.* Cells untreated or treated with tunicamycin were pulse labeled for 30 min and chased for 1 h. A post-nuclear supernatant was obtained and the organelles then either untreated or permeabilized with saponin in the presence of aggregative ("Agg." ) or nonaggregative ("N.A." ) buffer before analysis by sedimentation (see *Experimental Procedures*). Both AAT and endogenously expressed Cab45 were immunoprecipitated from supernatant ("S") and pellet ("P") with specific antibodies and analyzed by SDS-PAGE and fluorography. Cab45 was analyzed from cells not treated with tunicamycin.

**Figure 10.** *Unlike procathepsin B (ProB), AAT does not use its N-glycans for exit from maturing secretory granules.* Either untreated control cells or cells treated with tunicamycin were pulse labeled for 1 hour and chased in complete media overnight in the absence of secretagogues ("O/N"), followed by further chase under unstimulated (U) or secretagogue-stimulated (S) conditions for 6 h. Immunoprecipitated AAT and ProB from the media and cell lysates were analyzed by SDS-PAGE and fluorography.
Clone: 2 3 5 6 7 U 9 10 12

AAT
50

Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 7
Figure 8
Figure 9
Figure 10

Control
Media

Cells

Chase: O/N U S

+ Tunicamycin
Media

Cells

- ProB
- ProB-
(unglycosylated)

- mature B
- mature B-
(unglycosylated)

- AAT_mature
- AAT-
(unglycosylated)
- AAT_frag
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J. Biol. Chem. published online June 7, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M305690200

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