Distinct Molecular Mechanisms For Protein Sorting within Immature Secretory Granules of Pancreatic $\beta$-cells

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Abstract. In the $\beta$-cells of pancreatic islets, insulin is stored as the predominant protein within storage granules that undergo regulated exocytosis in response to glucose. By pulse-chase analysis of radiolabeled protein condensation in $\beta$-cells, the formation of insoluble aggregates of regulated secretory protein lags behind the conversion of proinsulin to insulin. Condensation occurs within immature granules (IGs), accounting for passive protein sorting as demonstrated by constitutive-like secretion of newly synthesized C-peptide in stoichiometric excess of insulin (Kuliawat, R., and P. Arvan. J. Cell Biol. 1992. 118:521-529). Experimental manipulation of condensation conditions in vivo reveals a direct relationship between sorting of regulated secretory protein and polymer assembly within IGs. By contrast, entry from the trans-Golgi network into IGs does not appear especially selective for regulated secretory proteins. Specifically, in normal islets, lysosomal enzyme precursors enter the stimulus-dependent secretory pathway with comparable efficiency to that of proinsulin. However, within 2 h after synthesis (the same period during which proinsulin processing occurs), newly synthesized hydrolases are fairly efficiently relocated out of the stimulus-dependent pathway. In tunicamycin-treated islets, while entry of new lysosomal enzymes into the regulated secretory pathway continues unperturbed, exit of nonglycosylated hydrolases from this pathway does not occur. Consequently, the ultimate targeting of nonglycosylated hydrolases in $\beta$-cells is to storage granules rather than lysosomes. These results implicate a post-Golgi mechanism for the active removal of lysosomal hydrolases away from condensed granule contents during the storage process for regulated secretory proteins.

The past decade has witnessed considerable debate regarding the mechanism(s) used by specialized neuroendocrine and exocrine cells for sorting polypeptide hormones and other content proteins into storage (secretory) granules. Protein sorting during intracellular transport is well established for newly synthesized lysosomal enzymes, which are recognized by mannose-6-phosphate receptors and conveyed via clathrin-coated vesicles from the TGN to an endosomal compartment, en route to lysosomes (Kornfeld and Mellman, 1989; Trowbridge et al., 1993). By analogy, the "sorting for entry" model of protein targeting to storage granules proposes that through specific mechanisms, regulated secretory proteins (and not other proteins) are selected from the mixed contents of the TGN (Griffiths and Simons, 1986; Orci et al., 1987a; Tooze et al., 1987a; Moore et al., 1989; Tooze and Huttner, 1990) and conveyed to immature granules (IGs), the first organelle in the biosynthetic pathway able to undergo stimulus-dependent exocytosis (Arvan et al., 1991; Tooze et al., 1991b). It has recently been recognized that regulated secretory protein condensation into insoluble aggregates plays a role in the sorting process (Gerdes et al., 1989; Wagner et al., 1991). Consequently, Huttner and colleagues have hypothesized that sorting receptors in the TGN (Bauerfeind and Huttner, 1993) may direct selected protein aggregates (Chanat and Huttner, 1991) from TGN to IGs, while denying similar access to nongranule content proteins.

A conceptual difficulty with condensation of regulated secretory proteins in the TGN is that it may sterically hinder the catalyzed cleavage (by prohormone convertases; Steiner et al., 1992), of proinsulin, proopiomelanocortin, and certain other regulated secretory proteins whose proteolytic maturation is completed only after arrival in IGs (Orci et al., 1987b; Tooze et al., 1987b; Kuliawat and Arvan, 1992; Zhou et al., 1993; Huang and Arvan, 1994). As an alternative to sorting for entry, the "sorting by retention" model proposes the following sequence of events: soluble proteins are largely unsorted upon entry into IGs; receptors mediate efficient vesicular removal of selected molecules from IGs, along with a modest sampling of proteins in the fluid phase.

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domains" within regulated secretory polypeptides; however, van et al., 1991); while the condensation of regulated secretory proteins favors their efficient retention within IGs (Kulawat and Arvan, 1992). Both hypotheses embrace "sorting domains" within regulated secretory polypeptides; however, since sorting by retention is accomplished by passive (self-associating; Arvan and Castle, 1992) rather than active (receptor-binding; Moore et al., 1989) protein interactions, the retention hypothesis predicts that sorting domains may not be highly conserved between different regulated secretory proteins.

To distinguish (TGN-based) sorting for entry from (IG-based) sorting by retention, we have posed four related questions. (a) Can secretory proteins enter IGs in the soluble phase? (b) Is the efficiency of protein storage within the regulated secretory pathway influenced by polymer assembly that occurs after export from the TGN? (c) If secretory proteins are not sorted for entry into IGs, how does the cell keep substantial quantities of nonsecretory proteins, such as newly synthesized lysosomal enzymes, from entering IGs? (d) If nonsecretory proteins do enter IGs, how do these proteins avoid becoming permanent members of the regulated secretory pathway? In this report, we have put these questions to the test by examining the β-cells of isolated pancreatic islets.

**Materials and Methods**

**Isolation and Pulse Radiolabeling of Rodent Pancreatic Islets**

Islets from 250 g Sprague-Dawley rats or 25 g CD-1 mice were isolated similarly to that previously described (Arvan et al., 1991) after pancreatic digestion by perfusion with collagenase (Boehringer Mannheim Biochemicals, Indianapolis, IN); flotation on a histopaque gradient (Sigma Chemical Co., St. Louis, MO); picking individual islets; preincubation overnight in 10% calf serum containing DME in the presence of 28 mM glucose; and transferring the islets to fresh DME containing 3.5 mM glucose "recovery medium" for 4 h before labeling. This protocol yields efficient proinsulin synthesis and lysosomal biogenesis (Landstrom et al., 1988). Mouse islets were routinely prepared for the analysis of lysosomal enzymes because of the superior immunoprecipitations obtained with available antibodies. When tunicamycin (Sigma Chemical Co., St. Louis, MO) was used, isolated islets were preexposed to the drug at a dose of 20 μg/ml during the 4 h before labeling. Islets were pulse-labeled for up to 30 min in deficient DME containing up to 100 μCi of [35S]sulfate and methionine mixture, or [3H]leucine (Dupont-NEN, New Bedford, MA). In preparation for in vitro analysis of assembly of regulated secretory protein aggregates, rat islets were chased for various times in fresh DME, before lysis.

In experiments labeling newly synthesized lysosomal enzymes, after short initial incubation in chase medium (DME containing 3.5 mM glucose), a 60-min period of proinsulin and insulin secretion was collected from islets bathed in fresh chase medium or that plus a high glucose-containsing secretagogue (22 mM glucose, 1 mM tolbutamide, 1 mM isobutylmethylxanthine, and 1 μM phorbol 12-myristate, 13 acetate). The islets were lysed in 1% boiling SDS, and then diluted 10-fold into a buffer yielding final concentrations of 100 mM NaCl, 10 mM EDTA containing 1% Triton X-100, 0.2% Na deoxycholate, and 25 mM Tris, pH 7.5. The same protocol was followed for prolonged chase experiments, except that the overnight chase was in recovery medium, before the 60-min period of collected secretion. An anti-protease cocktail containing aprotenin (1 μM), leupeptin (0.1 mM), pepstatin (10 μM), and diisopropylfluorophosphate (1 mM) was added finally to all collected chase media and islet lysates.

**Sucrose Velocity Gradient Centrifugation**

At each chase time, the media was removed and the islets were snap-frozen in liquid nitrogen, and subsequently thawed in 0.2 ml of a lysis buffer containing 0.3% NIKKOL (a nonionic detergent; Nikko Chemicals, Tokyo, Japan), 1 mM CaCl2, 0.5 mM ZnCl2, 0.4 M NaCl, 10 mM Mes, pH 6.0, and the anti-protease cocktail listed above plus 10 mM iodoacetamide. All experiments shown included 0.4 M NaCl to minimize the sedimentation of high molecular weight protein complexes unrelated to hormones, preliminary studies (not shown) indicated the use of 0.4 M NaCl did not alter the relative sedimentation of proinsulin or insulin. The islets were lysed at 4°C using continuous, cup-horn sonication (model 450; Branson, Danbury, CT) for 15 s at setting 2 (~80 W). All samples were spun through a 1.5 ml ultracentrifuge rotor in a TLA100 (Beckman Instruments, Inc., Palo Alto, CA). Throughout the lower 2.0 ml, a concentration of 0.1% Nikkol, 1 mM CaCl2, 0.5 mM ZnCl2, 10 mM Mes, pH 6.0 was maintained. The 2.18-M gradients were generally collected from above in five fractions, including the pellet. The proinsulin plus insulin in each fraction was then rapidly concentrated using a 1.3:1 ratio of Pro-Cipitate (Affinity Technology, New Brunswick, NJ), except in the case of C-peptide (see Fig. 2) for which the protein was immunoprecipitated using a specific antiserum (Linco, St. Louis, MO). The precipitates were then dissolved in SDS gel sample buffer and analyzed by tricine-urea-SDS-PAGE (Schagger and vonLagehov, 1987), followed by Coomassie staining, fluorography, or phosphorimaging. Although insulin was undetectable by conventional Coomassie blue staining, sensitivity by colloidal Coomassie (Sigma Chemical Co.) was >0.5 μg insulin per lane. Regardless whether the islets were untreated or treated with zinc or chloroquine, recovery of insulin from the gradient was >85%.

**Labeled C-Peptide/Insulin Ratios**

The C-peptide/Insulin (C/I) ratios from media collected during the 60-120-min chase period from control islets or those treated with zinc or chloroquine, were measured by specific immunoprecipitation using antisera to insulin and rat C-peptide as described previously (Kulawat and Arvan, 1992). Data from the supplier (Linco) and our own preliminary studies indicate that the anti-C-peptide precipitation may under-recover rat and mouse C-peptides, especially C-peptide-I, so that the C/I ratios >unity are nevertheless likely to be underestimates. However, neither chloroquine nor zinc treatment of islets influences the subsequent immunoprecipitation step per se (data not shown).

**Analysis of Lysosomal Enzymes in Pulse-Chase Experiments**

Several different sources of antisera were initially tested for quantitative immunoprecipitation of lysosomal hydrolases from rodent islets. Based on these trials, experiments using antisera to cathepsin L (from Dr. C. Gabel, Pfizer Research, Groton, CT), cathepsin B (from Dr. A. Saluja, Beth Israel Hospital, Boston, MA), and cathepsin D (from Dr. S. Diment, New York University, New York) were performed in mouse islets. β-glucuronidase, which was too low for reliable detection in mouse islets, was immunoprecipitated from rat islets (antiserum from Dr. R. Ganschow, University of Cincinnati, OH). The precipitation reagent used was either protein A agarose or Zysorbin (Zymed, San Francisco, CA). Immunoprecipitates were dissolved in SDS gel sample buffer and analyzed by 12% acrylamide SDS-PAGE, followed by fluorography.

**Results**

**Selective Protein Storage in the Secretory Granule Compartment**

A central feature of the β-cells of pancreatic islets is the synthesis and maintenance of a very large population of insulin storage granules. To test whether heavy biosynthetic traffic into the regulated secretory pathway results in inefficient sorting of stored granule contents, isolated islets were pulse-labeled with 35S-labeled amino acids, and chased overnight to allow time for all labeled proteins to be targeted to their final destinations. The targeting of β-glucuronidase, a representative lysosomal hydrolase, was distinct from that of insulin in that only the latter could be released upon addi-
the islets were divided and a 60-min period of secretion was collected either in medium containing 3.5 mM glucose or a high glucose-containing secretagogue. The chase media and islet lysates were immunoprecipitated with antibodies to $\beta$-glucuronidase (top) or insulin (bottom). To illustrate the lack of stimulated $\beta$-glucuronidase secretion compared to ($\sim$20%) insulin secretion, the SDS-PAGE of insulin was underloaded while the fluorography of mature $\beta$-glucuronidase ($\sim$74 kD) was intentionally overexposed 15-fold.

The assembly of insoluble aggregates is important for protein targeting to storage granules (Chanut and Huttner, 1991), and in pancreatic $\beta$-granules, insulin is packaged in insoluble dense cores (Michael et al., 1987). However, there have been no attempts to demonstrate biochemically when and where these insoluble polymers first form. To examine polymer assembly, sedimentation on linear sucrose velocity gradients was examined after solubilization of islet organelle membranes by sonication in a cell lysis buffer including 0.3% Nikkol, 1 mM CaCl$_2$, and 500 $\mu$M ZnCl$_2$ at pH 6.0. Mildly acidic conditions are thought to approximate the luminal environment of IGs, as opposed to the more acidic internal pH of mature granules (Orci et al., 1986, 1987b); while calcium and zinc were included because these ions play a role in protein assembly within the $\beta$-granule core (Howell et al., 1978). Centrifugation speed and time (see Materials and Methods) were adjusted to distinguish polymeric assemblies (hundreds-to-thousands of molecules) from soluble proinsulin hexamers (Grant et al., 1972) that are believed to form within the endoplasmic reticulum (Emdin et al., 1980).

A solubilized insulin standard analyzed by velocity gradient did not exit the load fraction (Fig. 2, top gradient). By contrast $\sim$60% of Coomassie-stainable islet insulin penetrated the gradient (Fig. 2, second gradient). To determine whether the sedimentable insulin represented intact granules or granule cores, we examined the sedimentation of soluble C-peptide which is co-sequestered within $\beta$-granules. Since C-peptide does not stain with Coomassie blue or silver, pulse-labeled islets were first chased overnight to maximize the fraction of labeled insulin and C-peptide stored in mature granules. After cell lysis as described above, the fraction of total radiolabeled insulin which pelleted was also $\sim$60% (Fig. 2, third gradient). However, the soluble C-peptide was quantitatively retained in the load fraction (Fig. 2, last gradient). These data indicate that granule membranes were indeed disrupted (Michael et al., 1987). We conclude that although the vigorous sonication procedure does cause a partial solubilization of granule insulin, the sedimentable "GC fraction" (Fig. 2) exhibits the behavior expected of polymeric assemblies of granule core protein.

Next, using this in vitro assay, the kinetics of polymer assembly of regulated secretory protein was examined. By 60 min, a time when virtually all labeled proinsulin has arrived in IGs (Orci, 1982), there was still very little sedimentable material; however, at later chase times significant polymer assembly was readily demonstrable (Fig. 3 A). A quantitative comparison of the kinetics of proinsulin-to-insulin conversion and recovery in the GC fraction graphically demonstrated that formation of insoluble aggregates lagged behind the appearance of labeled insulin (Fig. 3 B). If islet insulin is formed in IGs (Orci et al., 1987b; Kuliawat and Arvan, 1992; Huang and Arvan, 1994), then these data suggest that formation of insoluble aggregates occurs after proinsulin enters the regulated secretory pathway.
Figure 3. In vitro assay of secretory protein polymerization in pulse-labeled pancreatic islets. (A) Islets were pulse-labeled in batch with [35S]cysteine and methionine, lysed by sonication in the presence of 0.3% Nikkol at the times indicated, and analyzed by sucrose-velocity gradient centrifugation as in Fig. 2. Gradient fractions were analyzed by SDS-PAGE and phosphorimaging. Note that the top of the gradients are to the right. (B) When quantified, the conversion to labeled insulin at 30 min (15%), 60 min (60%), 120 min (81%), and 210 min (88%) was normalized to that seen after overnight chase (96%). The sedimentation of labeled proinsulin to the GC fraction was indistinguishable at 30 min from that seen at zero chase time (not shown), while progressive increments in sedimentation of labeled insulin at 60 min (18%), 120 min (30%) and, 210 min (40%) were normalized to that seen maximally after overnight chase (62%).

The Relationship between Protein Condensation in IGs and the Efficiency of Insulin Sorting

Constitutive-like membrane traffic, which originates in vesicles that bud from IGs, leads to the secretion of newly synthesized C-peptide in stoichiometric excess of insulin (Arvan et al., 1991). This elevated C/I ratio serves as a convenient assay that reflects the solubility of C-peptide vs. insolubility of insulin, altering their relative probability for departure from IGs in budding vesicles (Kulawiak and Arvan, 1992). A moderately acidic pH is thought to make an important contribution to the environment favoring intragranular conversion of proinsulin to C-peptide and insulin (Orci et al., 1986, 1987b; Rhodes et al., 1987) as well as to the subsequent condensation of insulin (Michael et al., 1987). Cellular exposure to 200 µM chloroquine, an agent which raises the pH inside acidic organelles, perturbs the storage of proteins normally targeted to secretory granules and enhances secretion by a pathway that exhibits constitutive characteristics (Moore et al., 1983) (although these studies were controversial because the dose, 200 µM, and several hour duration of chloroquine exposure caused other effects such as profound inhibition of prohormone processing [Mains and May, 1988]). These earlier studies led us to reconsider whether it might be possible to selectively manipulate the efficiency of insulin polymer assembly in vivo without disturbing prohormone processing.

Two maneuvers were attempted for this purpose. First, exposure to chloroquine (100 µM) was re-examined, except that importantly, the period of islet exposure was delayed until well after pulse-labeling was completed (60-120 min of chase). This modification allowed unperturbed intracellular conversion of newly synthesized proinsulin to insulin. Thus under these conditions, effects of chloroquine could not be ascribed to sorting for entry, since the arrival of proinsulin in IGs (as measured by processing to insulin) was quantitatively unaffected. Nevertheless, polymeric assembly of nascent insulin to the sedimentable GC fraction was reduced markedly (Fig. 4 A). Further, the 60-120-min chase medium from chloroquine-treated islets contained an increased amount of newly synthesized insulin while C-peptide enrichment over insulin was eliminated (i.e., C/I ratio = 1.0, see Fig. 5). These data suggest that by inhibiting insulin condensation, the two polypeptides were no longer spatially segregated within the IG lumen, so that sorting between them could no longer take place.

In the second maneuver, zinc was added to the medium bathing pulse-labeled islets. Zinc uptake by tissues in vivo and in culture is well documented (Johnson and Evans, 1982; Raffaelli et al., 1992); its uptake by pancreatic islets (Ludvigsen et al., 1979) leads to accumulation in β-granules (Figelewicz et al., 1980); and it is known to stabilize insulin crystals (Baker et al., 1988). When ZnSO4 (100 µM) was added to islet chase media, labeled proinsulin-to-insulin conversion at 120 min was not enhanced, but the insulin content of the GC fraction was clearly increased (Fig. 4 B). In conjunction with improved condensation, constitutive-like membrane traffic from 60-120 min exhibited a reduced insulin content with an elevation of C/I ratio (Fig. 5). These data bolster the suggestion that divalent cations facilitate the passive assembly of insoluble protein polymers which are im-
important for peptide hormone storage in the regulated secretory pathway, and support a direct link between this process and the protein sorting which occurs within IGs. By contrast, these data (Figs. 3–5) do not support the idea that assembly of insoluble aggregates is important for the "entry" into IGs of the regulated secretory protein, proinsulin, which appears to be fully soluble.

**Newly Synthesized Hydrolases En Route to Lysosomes Pass through Immature Granules**

If regulated secretory proteins can enter IGs in the soluble phase, then it is difficult to conceive of a means by which soluble proteins intended for destinations other than storage granules can be excluded from IGs, particularly in cell types where a large fraction of volume flow is directed into the regulated secretory pathway. Because IGs are competent for stimulus-dependent exocytosis (Arvan et al., 1991; Tooze et al., 1999b), any nonsecretory proteins that enter IGs must at least transiently behave as members of the regulated secretory pathway. Since in pancreatic β-cells, mature lysosomal hydrolases have at most, limited overlap in their steady-state distribution with regulated secretory protein (Fig. 1), we examined the trafficking of newly synthesized lysosomal enzymes in these cells.

As expected, when pulse-labeled pancreatic islets were chased overnight to allow labeled proteins to reach their final destinations, there was quantitative intracellular processing of cathepsin B from precursor to mature lysosomal form; secretagogue addition at this time induced a stimulated discharge of labeled insulin without detectable release of mature cathepsin B (Fig. 6A). By contrast, stimulated secretion of the labeled ~39-kD cathepsin B precursor was evident at 20–80 min of chase, and was declining during the 60–120 min chase period (Fig. 6A). Stimulus-dependent hormone secretion during these early chase periods represented mostly unprocessed proinsulin and conversion intermediates, indicating an origin from IGs (Kuliawat and Arvan, 1992; and data not shown). By comparing the fractional stimulated release of procathepsin B to that measured from anti-insulin immunoprecipitation in the same samples, the labeled hydrolase was at least ~73% as efficient as labeled proinsulin for entry into the stimulus-dependent secretory pathway.

This finding was confirmed in nine paired samples that were similarly analyzed. The absolute magnitude of the stimulus-dependent secretion (i.e., stimulated minus control) of procathepsin B varied between experiments, resulting in a large standard deviation (Table I). Nevertheless, between different pairs as well as by overall averaging, the stimulus-dependent fraction of labeled procathepsin B tended to parallel the regulated exocytosis of proinsulin/insulin (Table I). This regulated secretory behavior suggested that the secretion of cathepsin B precursor originated from IGs. Since procathepsin B enters the stimulus-dependent secretory pathway nearly as efficiently as proinsulin (which enters IGs with >99% efficiency; Rhodes and Halban, 1987), the present data suggest that hydrolase precursors enter the IG compartment in great abundance. While one could conceive of the possibility that stimulated secretion of newly synthesized hydrolases might originate from endosomes or endosome-derived vesicles rather than IGs, this secretion was not at all diminished even when lysosomal enzyme traffic to endosomes was markedly inhibited—moreover, it was secretion was collected. The chase media and islet lysates were then analyzed by immunoprecipitation for both proinsulin plus insulin, and cathepsin B. Prolonged secretory collection helped to minimize effects of differences in ER exit rates for proinsulin and cathepsin B. In the experiment shown, the stimulus-dependent release of labeled (pro)insulin present in islets at each chase time averaged 17.3%, which was not inhibited as the chase progressed. By contrast, the stimulus-dependent fraction of cathepsin B precursor, which was 73% of the stimulus-dependent fraction of proinsulin secreted from 20–80 min, declined rapidly as a function of chase time. A ~39-kD precursor (upper arrow) was the only form secreted into the medium, while a ~35-kD form (lower arrow) accumulated in a nonsecretory compartment, presumably lysosomes. Representative data are shown from one of nine such experiments (see Table I). (B) After tunicamycin exposure, islets were labeled as in A and chased 45 min before the secretion period. A ~34-kD nonglycosylated cathepsin B precursor (asterisk) exhibited regulated exocytotic discharge.
possible to trap labeled lysosomal enzymes within the storage granules (described further, below).

Thus, at least one newly synthesized lysosomal enzyme behaves like a transient member of the regulated secretory pathway. To determine whether this behavior of cathepsin B, a moderately abundant β-cell hydrolase, is a special case or is generally representative of lysosomal enzyme trafficking, the stimulus-dependent secretion was also measured for newly made cathepsin D and cathepsin L, whose expression levels range ~100-fold in these cells. For these hydrolases as well, secretagogue addition early led to obvious stimulus-dependent secretion, while secretion of labeled hydrolases at later chase times was greatly attenuated (Fig. 7). When normalized to the release of labeled proinsulin and insulin, an extremely high fraction of these newly synthesized hydrolases were found to enter the stimulus-dependent pathway: 87% for cathepsin D; >95% for cathepsin L; and a comparably high value for β-glucuronidase (data not shown). Nevertheless, over time, little lysosomal enzyme persisted in the stimulus-dependent secretory pathway (Figs. 6 and 7), suggesting that these hydrolase precursors were in transit to their normal, lysosomal destination.

**Table 1. Secretion of Newly Synthesized Procathepsin B from Isolated Mouse Pancreatic Islets**

| Antibody            | Cathepsin B | Insulin  |
|---------------------|-------------|----------|
| Stimulated          | 33.6 ± 15.9 | 34.6 ± 9.1 |
| Unstimulated        | 11.8 ± 11.5 | 9.6 ± 2.8 |
| Stimulus-dependent  | 21.8 ± 10.8 | 24.9 ± 11.3 |

Islets were isolated and recovered overnight as described in Materials and Methods. Each preparation of islets was radiolabeled and divided into pairs of approximately equal numbers. The islets were chased ~30 min before an additional 60-min collection from unstimulated or stimulated islets. The media and cell lysates were each analyzed by immunoprecipitation using an anti-insulin serum as well as an antiserum to cathepsin B. The fraction (%) of labeled cathepsin B secreted into the medium corresponded exclusively to the precursor form (Fig. 6) and was quantitated either by phosphorimaging or scanning densitometry after SDS-PAGE and fluorography. The data shown represent the mean value from nine such pairs (± standard deviation).

**Entry of Lysosomal Enzymes into the Stimulus-dependent Secretory Pathway Does Not Require Mannose-6-Phosphate Recognition**

The observation that different lysosomal enzymes at all levels of expression appear to enter IGs, represents a pattern other than that found in nonregulated secretory cells, where most hydrolases en route to lysosomes bind mannose-6-phosphate (M6P) receptors in the TGN and are relocated via clathrin-coated vesicles to an endosomal compartment (Kornfeld and Mellman, 1989; Trowbridge et al., 1993). Thus, it seemed possible that hydrolase precursors could be sorted for entry into β-cell IGs based on M6P recognition, as has been hypothesized in cytotoxic T lymphocytes (Griffiths and Isaaz, 1993). To test this possibility, we wished to block N-linked carbohydrate addition to new lysosomal enzymes and thereby prevent the formation of M6P recognition signals. Unlike for more complicated glycoproteins, nonglycosylation of biosynthetized lysosomal enzymes (either after mutagenesis or after tunicamycin treatment) does not induce detectable misfolding nor block the ability of these enzymes to bind and ultimately cleave (Smith et al., 1989; Kane, 1993) (as measured for nonglycosylated cathepsin B [Runzi, M. A. Saluja, R. Dawra, M. Saluja, H. Nishino, and M. Steer, 1993. Pancreas 8:771]) substrate peptides. Indeed, intracellular transport of these lysosomal enzymes through the ER and Golgi complex occurs normally; and in nonregulated secretory cells, most (but not all) nonglycosylated hydrolases are constitutively secreted "by default," indicating that they proceed unsorted through the soluble secretory pathway (von Figura et al., 1979; Rosenfeld et al., 1982; Nishimura et al., 1986a,b; Rindler and Traber, 1988; Glickman and Kornfeld, 1993; Kane, 1993).

Remarkably in pancreatic islets treated with tunicamycin, although the radiolabeling between different sets of islets varied to some extent, the fraction of total labeled cathepsin B that entered the stimulus-dependent secretory pathway was not at all inhibited by the absence of N-linked carbohydrate addition (Fig. 6B). An identical result was observed for cathepsin L (Fig. 8). Thus, hydrolase entry into the stimulus-dependent secretory pathway did not require the presence of the M6P signal. This result clearly indicates that transient, regulated release of new lysosomal hydrolases cannot originate from an endosome compartment, since the routing of newly synthesized hydrolases into endosomes is profoundly inhibited in the absence of M6P recognition.

**Relocation of Newly Synthesized Nonglycosylated Cathepsin B Out of the Regulated Secretory Pathway Is Blocked**

Because previous evidence has suggested that clathrin-coated vesicles bud from IGs (Kuliaat and Arvan, 1992), the preceding data raise the possibility that lysosomal enzyme precursors may be removed from IGs in clathrin-coated buds. This proposal is consistent with a recent view of IGs as a functional extension of the TGN (Arvan and Castle, 1992), and could help to explain the well-recognized loss of clathrin that accompanies granule maturation (Orci et al., 1987b). To examine whether the M6P moiety might play a role in the ability of hydrolase precursors to be removed from IGs, pancreatic islets treated with tunicamycin were labeled and chased overnight to allow newly synthesized proteins to reach their final destinations. At this time, cathepsin B from control islets shows a mobility shift upon SDS-PAGE after digestion with endoglycosidase F, while from tunicamycin-treated islets there was no shift upon digestion, confirming an absence of N-linked glycans (Fig. 9A).

As expected after overnight chase, secretion of labeled cathepsin B from control islets was minor (Fig. 9B). Re-
lated or stimulated secretion, as in Fig. 6. Newly synthesized cathepsin L was specifically immunoprecipitated and analyzed by SDS-PAGE and fluorography. In each set of islets, the stimulus-dependent fraction of glycosylated (~40 kD) or nonglycosylated (~37.5 kD) cathepsin L actually exceeded the stimulus-dependent fraction of labeled proinsulin, which ranged from 18.4–21.3% in this experiment. Thus, both forms of cathepsin L behaved as virtually perfect markers for entry into IGs. The data from a representative experiment (of three) are shown.

markably, however, there was a dramatic stimulus-dependent exocytosis of nonglycosylated cathepsin B (~40% in the experiment of Fig. 9 B), indicating that in β-cells, the nonglycosylated lysosomal enzyme was unable to traffic out of the stimulus-dependent secretory pathway. Stimulated secretion of the labeled protein after such a long chase time is consistent with residence of the nonglycosylated hydrolase in storage granules, but is not compatible with protein trafficking through the constitutive secretory pathway or endosomes. Thus, we conclude that without alteration of primary sequence, simple removal of carbohydrate was sufficient to change the ultimate targeting of cathepsin B from lysosomes to storage granules.

**Discussion**

**Sorting by Retention in the Regulated Secretory Pathway**

There is general agreement that insulin and many other regulated secretory proteins are packaged into an insoluble dense core (Giannattasio et al., 1975; Michael et al., 1987), which is a general feature of exocrine and neuroendocrine storage granules (Palade, 1975; Kelly, 1985). Two current models of targeting to storage granules (sorting for entry [Bauerfeind and Huttner, 1993] and sorting by retention [Kuliawat and Arvan, 1992]) differ in whether the condensation of regulated secretory proteins need or need not occur prior to protein entry into IGs. Understanding where condensation first occurs is crucial because if a large number of molecules enter IGs in the soluble phase, then it is difficult to conceive of a sorting mechanism that could prevent other soluble proteins (including nonsecretory proteins) from entering IGs as well. This if of minor concern in most cell culture models of regulated secretion, where the fraction of volume that flows from the TGN into IGs is modest relative to other TGN exit routes (i.e., the constitutive secretory pathway, and clathrin-coated vesicle traffic to endosomes). However, β-cells of pancreatic islets, like many other regulated secretory tissues in vivo, produce an abundant population of storage granules, which represents a significant fraction of the entire cell volume. In these cells, a large majority of volume flowing through the TGN must enter directly into IGs, while the constitutive secretory pathway is likely to be diminutive by comparison. In this case, the question becomes whether soluble nonsecretory proteins can avoid the IG compartment.

In pancreatic β-cells, proinsulin enters IGs with >99% efficiency (Rhodes and Halban, 1987). Postulates concerning the formation of insoluble protein aggregates in the TGN (Chanat and Huttner, 1991) do not seem applicable to proinsulin, which requires efficient proteolytic conversion to insulin after entry into IGs (Orci et al., 1987b; Davidson et al., 1988; Huang and Arvan, 1994). Nevertheless, until now, no biochemical investigations have been made concerning the condensation of proinsulin (or other regulated proteins) while in transit through the intracellular secretory pathway. Using a sedimentation assay, we observed that assembly of regulated secretory protein polymers began shortly after the first appearance of insulin and accumulated progressively thereafter (Fig. 3). The data strongly suggest that in islet β-cells, insulin rather than proinsulin is primarily responsible for the formation of the nascent β-granule core, and IGs serve as the site of this assembly. Thus, we propose that proinsulin enters IGs in the soluble phase in pancreatic β-cells. These data are entirely consistent with earlier biophysical studies indicating that randomly coiled C-peptides interfere with close-packing of soluble proinsulin hexamers (Steiner, 1973; Weiss et al., 1990), thereby rendering proinsulin incapable of forming large, insoluble complexes in an aqueous environment above pH 3 (Fullerton et al., 1970; Grant et al., 1972). While we do not preclude the possibility that some proteins in some cell types might undergo complex pre-treatment with tunicamycin, were chased overnight before a 60-min collection of unstimulated or stimulated secretion. Tunicamycin pre-treatment did not inhibit the stimulus-dependent secretion of labeled insulin, which averaged ~40% in this experiment. (A) Pooled media and cell lysates from control and tunicamycin-treated islets were mock-digested or digested with endoglycosidase F (4,000 U/ml, 3 h at 37°C) (B) Immunoprecipitation of cathepsin B revealed a ~35 kD mature form in the control cells. By contrast, both ~34 plus ~31 kD nonglycosylated forms (arrows) exhibited regulated exocytosis, suggesting that the smaller form was generated by proteolysis within the secretory pathway. The data shown are from a representative experiment (of three).
lated secretory tissues, the importance of IG-based sorting is an important sorting compartment (Grimes and Kelly, 1991; Pimplikar and Hutner, 1992), we note that even in these cells, recent evidence suggests that the IG is an important sorting compartment (Grimes and Kelly, 1992) as appears to be the case in AtT-20 cells (Milgram et al., 1994). For the reasons described above, in "real" regulated secretory tissues, the importance of IG-based sorting is likely to be considerably greater than in these cell culture systems.

Constitutive-like Vesicle Budding from IGs Facilitates Protein Sorting by Retention

If condensation within IGs plays an important role in regulated secretory protein sorting by retention, a mechanism must exist in which other proteins can be removed from the IG compartment. Constitutive-like vesicle budding, which corresponds kinetically to the period shortly after IGs detach from the TGN, was initially discovered in highly granulated tissues as part of a secretory pathway conveying a small fraction of granule content proteins under basal conditions (Arvan and Castle, 1987; Arvan and Chang, 1987; vonZastrow and Castle, 1987). To date, not all of the vesicular transport steps in this pathway have been mapped. Nevertheless, there is reason to believe that initial steps of this pathway involve clathrin-coated vesicles which travel from IGs to endosomes, from which some of the transported cargo is regurgitated to the cell surface (Arvan and Castle, 1992). The constitutive-like vesicles departing from IGs sample a modest fraction of fluid contents enriched in soluble components, accounting ultimately for the unstimulated secretion of newly synthesized C-peptide in stoichiometric excess of insulin (Kuliawat and Arvan, 1992). The fact that the intraluminal environment of IGs can be experimentally manipulated (Fig. 4) has provided a handle by which to explore the interplay between protein condensation and protein storage within granules, or removal therefrom (Fig. 5). Although the data is pharmacological in nature, a picture emerges that while solubility of proinsulin does not interfere with massive entry into IGs, the insolubility of insulin plays an important role in enhancing the efficiency of its targeting to storage granules. These results are consistent with, and may help to explain, older studies analyzing the effect of weak bases on storage granule formation and maturation (Moore et al., 1983; vonZastrow et al., 1989). Moreover, Carroll et al. (1988) have described transgenic mice in which two-thirds of the islet insulin is a mutant that exhibits defective oligomerization with zinc and hence defective condensation. In these β-cells, most secretory granules were morphologically deficient in forming the dense core; and isolated islets exhibited "unregulated" secretion consistent with excessive traffic of uncondensed proteins through the constitutive-like pathway. Thus, we conclude that both the nature of the secretory proteins as well as the intraluminal IG environment play a significant role in enhancing the efficiency of protein storage in regulated secretory granules.

Lysosomal Enzyme Trafficking in Regulated Secretory Cells

In nonregulated secretory cells, lysosomal hydrolase precursors exit the biosynthetic pathway at the level of the TGN (Griffiths and Simons, 1986; Kornfeld and Mellman, 1989). In the β-cells of mouse pancreatic islets, the distributions of mature lysosomal enzymes overlap little with storage granules (Figs. 6–8); nevertheless, lysosomal enzyme precursors enter the stimulus-dependent secretory pathway. While one might postulate that release of lysosomal enzyme precursors could derive from a stimulated endosomal compartment rather than from IGs, the fact that tunicamycin treatment (which interferes with the traffic of newly synthesized hydrolases to endosomes) does not inhibit stimulus-dependent hydrolase secretion (Figs. 6 and 8) effectively eliminates this possibility. Moreover, the persistence of stimulated secretion of nonglycosylated hydrolases after overnight chase (Fig. 9) is incompatible with stimulated hydrolase secretion via the constitutive secretory pathway. In addition, the vesicle fractionation studies have previously demonstrated that procathepsin B is found in isolated β-granules while mature cathepsin B is found exclusively in a lysosome fraction (Docherty et al., 1984). Taken together, these data indicate that the stimulus-dependent secretion of lysosomal enzyme precursors derives predominantly if not exclusively from the regulated secretory pathway. Finally, because the absence of N-linked glycosylation does not reduce the stimulatable fraction, the entrance of lysosomal hydrolases into β-cell IGs must not require the M6P moiety.

In conventional regulated secretory tissues (Brands et al., 1982; Docherty et al., 1984; Taugner and Hackenthal, 1988; Tooze et al., 1991) it has been suggested that lysosomal enzyme entry into storage granules may imply "mis-sorting." Certainly, in our studies of the β-cells of pancreatic islets, at most a minor fraction of newly made hydrolases was actually sorted at the TGN. While various possibilities might theoretically account for the behavior of lysosomal enzymes in β-cells, the simplest and likeliest explanation is that most newly synthesized hydrolases bypass the TGN and enter IGs because they are soluble, and a major fraction of volume in the intracellular transport pathway flows into IGs in these cells. Nevertheless, the fact that lysosomal enzymes may use bulk flow to gain entrance into IGs should not be considered mis-sorting. Rather, the data indicate a temporary residence of these precursors in β-cell IGs as part of the normal transit of hydrolases en route to lysosomes (Fig. 6).

In theory, nonprotein "bulk flow markers" could be used to confirm the magnitude of fluid phase entry into β-cell IGs. In reality, such markers do not presently exist for studying the regulated secretory pathway. Certainly, sulfated glycosaminoglycans (which are excellent markers of rapid secretion, but are unable to distinguish traffic through constitutive [Tooze and Hutner, 1990] vs. constitutive-like vesicles [Grimes and Kelly, 1992]) cannot be used successfully for this purpose. In fact, for most (although perhaps not all) cell types, the preponderance of lysosomal enzymes not sorted via the M6P signal recognition pathway probably represent the best protein markers available to define how unsorted proteins will travel in the soluble secretory pathway (von Figura et al., 1979; Rosenfeld et al., 1982; Nishimura et al., 1988a,b; Rindler and Traber, 1988; Kane, 1993). Thus, based on the solubility of proinsulin and evidence supporting
the bulk flow of lysosomal enzymes into IGs, the sorting for entry model is unlikely to account for the selectivity of insulin targeting to the storage granules of pancreatic β-cells. Although the M6P signal is not required for entry of lysosomal enzymes into IGs, the presence of carbohydrate appears essential for the active sorting of hydrolases out of the regulated secretory pathway (Fig. 9). Although additional work is needed, the fact that more than three-quarters of newly synthesized procathepsin B is transferred to lysosomes during prolonged chase bespeaks an extremely efficient retrieval system from IGs, suggesting a receptor-mediated process, presumably involving M6P receptors. Consequently, inefficiency in retrieving hydrolases from IGs (either because of interaction with condensing secretory proteins or because of poor M6P recognition) would represent mis-sorting of lysosomal enzymes, leaving them stranded in storage granules (Brands et al., 1982; Docherty et al., 1984; Tagner and Hackenthal, 1988; Tooze et al., 1991a). Taken together, the present observations define IGs as an important branchpoint in trafficking of luminal proteins destined for lysosomes and storage granules in a system which represents one of the nearest approximations to physiological protein lysosomes during prolonged chase bespeaks an extremely efficient retrieval system from IGs, suggesting a receptor-mediated process, presumably involving M6P receptors. 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