Lumenal protein sorting to the constitutive secretory pathway of a regulated secretory cell

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Summary

Newly synthesized secretory granule content proteins are delivered via the Golgi complex for storage within mature granules, whereas constitutive secretory proteins are not stored. Most soluble proteins traveling anterograde through the trans-Golgi network are not excluded from entering immature secretory granules, whether or not they have granule-targeting signals. However, the 'sorting-for-entry' hypothesis suggests that soluble lumenal proteins lacking signals enter transport intermediates for the constitutive secretory pathway. We aimed to investigate how these constitutive secretory proteins are sorted. In a pancreatic β-cell line, we stably expressed two lumenal proteins whose normal sorting information has been deleted: alkaline phosphatase, truncated to eliminate its glycosylphosphatidylinositol membrane anchor (SEAP); and $Cab45_{361}$, a Golgi lumenal resident, truncated to eliminate its intracellular retention (Cab_{308}Myc). Both truncated proteins are efficiently secreted, but whereas SEAP enters secretory granules, Cab_{308}Myc behaves as a true constitutive marker excluded from granules. Interestingly, upon permeabilization of organelle membranes with saponin, SEAP is extracted as a soluble protein whereas Cab_{308}Myc remains associated with the membrane. These are among the first data to support a model in which association with the lumenal aspect of Golgi and/or post-Golgi membranes can serve as a means for selective sorting of constitutive secretory proteins.

Key words: Trans-Golgi network, Granule maturation, Constitutive-like secretory pathway

Introduction

Lumenal protein trafficking in regulated secretory cells may include protein exit from the Golgi complex via transport intermediates destined for constitutive secretion, the endosomal system, immature secretory granules or retrograde transport (Arvan et al., 2002). Lumenal protein sorting for storage in secretory granules is the subject of ongoing investigation (Arvan and Halban, 2004). For secretory granules in pancreatic β-cells, studies have tended to support 'sorting by retention' (Kuliawat and Arvan, 1994), which suggests that lumenal protein entry into newly forming secretory granules is not especially selective, and the combination of selective protein removal from maturing granules (Feng and Arvan, 2003; Kuliawat et al., 1997) along with polymeric assembly of regulated secretory proteins (Dannies, 2001) both contribute to achieving a satisfactory efficiency of intragranular retention of selected secretory contents during mature granule biogenesis. In addition to 'sorting by retention', some lumenal proteins also use 'sorting for entry' to gain initial access to secretory granules at the time of their formation at the TGN (Tooze et al., 2001). In the 'sorting for entry' model, constitutive secretory proteins are generally portrayed as being excluded from capture into granules and therefore traveling to the cell surface by default (Cool et al., 1997; Dhanvantari and Loh, 2000; Glombik et al., 1999; Krömer et al., 1998; Moore and Kelly, 1986; Moore and Kelly, 1985; Tooze et al., 1990).

Studies in recent years describe proteins with no apparent granule sorting-for-entry signals in which the lumenal product is delivered abundantly to endocrine secretory granules (El Meskini et al., 2001; Feng and Arvan, 2003; Molinete et al., 2000). Indeed in pancreatic β-cells, no bona fide marker secretory protein of the constitutive pathway (i.e. a lumenal protein that is not also found to significantly enter immature secretory granules) has yet been identified. We have therefore endeavored to find such a constitutive secretory pathway marker. Our general strategy has been to examine, after delivery to the ER lumen, the trafficking of tester proteins in which specific sorting information for trafficking to various intracellular destinations has been deleted. Up to now, however, we found that newly synthesized lysosomal proenzymes en route to the endosomal system (Turner and Arvan, 2000) enter immature secretory granules in regulated secretory cells (Klumperman et al., 1998; Kuliawat and Arvan, 1994) and when these proenyzmes are defective for specific mannose 6-phosphate receptor-mediated recognition, their entry into granules is, if anything, even more abundant (Kuliawat and

Arvan, 1992). We have now extended this strategy to two additional genetically modified lumenal proteins: secretory alkaline phosphatase (SEAP, produced by truncation of the signal for addition of the glycosylphosphatidylinositol membrane anchor) and Cab_{308}Myc (produced by replacement of the C-terminal 53 residues of full-length $Cab45_{361}$ with a single Myc epitope tag). SEAP has been described as a constitutive secretory protein in the pancreatic INS-1 cell line (Molinete et al., 2000) although we find that SEAP abundantly enters the immature granules of these cells. However, we describe Cab_{308}Myc as the first bona fide secretory marker protein of the constitutive pathway, not entering granules in these cells. Interestingly, from saponin-permeabilized organelles, SEAP behaves as a typical soluble secretory protein whereas Cab_{308}Myc clearly binds to the lumenal aspect of secretory pathway membranes. Binding to membranes that ultimately become transport intermediates of the constitutive secretory pathway may serve as at least one means of capturing secretory proteins, preventing their entry into secretory granules, and ensuring their constitutive secretion.

Results

Entry of SEAP into the regulated secretory pathway of INS-1 cells

In the secretory pathway, acquisition of endoglycosidase H (endo H) resistance occurs shortly after arrival of N-linked glycoproteins in the Golgi complex (Rothman and Fine, 1980). In constitutively secreting cells, because export from the endoplasmic reticulum (ER) is generally rate limiting in the overall pathway of intracellular transport of any particular secretory glycoprotein to the extracellular space (Lodish, 1988), the predominant intracellular pool of that protein tends to be endo H sensitive. However, for glycoproteins stored within the lumen of secretory granules, an endo-H-resistant pool of secretory protein generally becomes increasingly evident, with the size of this pool at steady state approximating the fraction of molecules stored in secretory granules (Stahl et al., 1996). With that in mind, we expressed in INS-1 cells a cDNA encoding SEAP, an N-linked glycoprotein and we examined stably-transfected clones by western blotting after endo H digestion of cell lysates. Fig. 1A shows that an endo-H-resistant pool of SEAP was detected by immunoblotting in transfected cells (clone 12), which represented roughly half of all intracellular SEAP molecules. (In some blotting experiments such as Fig. 1A, recombinant endo H itself produces a nonspecific band of variable intensity, also found in controls cells.) When clone 12 was compared with two independently selected INS-1 cell clones with greater or lesser expression levels (Fig. 1B), 55±4% of SEAP was endo H resistant in these clones at steady state, as quantified by densitometry, indicating accumulation in a Golgi and/or post-Golgi compartment. Such accumulation may appear surprising because in PC12 cells and GH4C1 cells, SEAP has been reported to be a selective marker of the constitutive secretory pathway (Gorr, 1996; Harrison et al., 1996), which should result in the majority of SEAP molecules undergoing rapid unstimulated release rather than intracellular accumulation. To explore this further in INS-1 cells, we examined the fraction of intracellular molecules acquiring endo H resistance within 2 hours of synthesis. As shown on the right of Fig. 2, after 2 hours of chase, less than half of total newly synthesized SEAP (lane T) remained endo H sensitive, little more than one-third was already released to the medium (lane M) and 22±5% detected intracellularly in an endo-H-resistant form (lane C). Thus, although a fraction of labeled SEAP molecules does undergo rapid unstimulated secretion (discussed below), the results of Figs 1 and 2 suggest that a cohort of labeled SEAP molecules enters a post-Golgi compartment such that ⩾50% of cellular SEAP is ultimately contained in this endo-H-resistant pool at steady state.

Despite the fact that INS-1 cells are rounded and tend to be suboptimal for secretory pathway immunofluorescence, we wished to use this methodology to examine the steady-state Golgi/post-Golgi distribution of SEAP further. For this purpose, cells were first treated for 1 hour with cycloheximide to minimize signal derived from ER pools of SEAP and

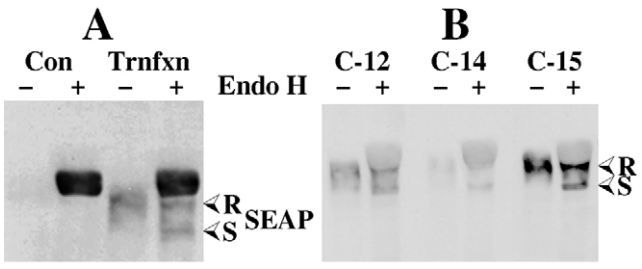

Fig. 1. Western blotting of secretory alkaline phosphatase (SEAP) expressed in INS-1 β-cells. (A) A stably transfected clone of INS-1 cells (clone 12, Trnfxn) immunoblotted for alkaline phosphatase next to untransfected control cells (Con). In addition, a faster-migrating specific band is detected in the stably transfected cells. Upon glycan digestion (+), the steady state distribution of SEAP is divided into endo-H-sensitive (S) and endo-H-resistant (R) forms, the latter indicative of a significant Golgi and/or post-Golgi pool of the protein in these cells. (B) A comparison of SEAP expression level in the clone shown in panel A (C-12) to two other independently selected INS-1 clones (C-14 and C-15), each of which maintains a similar proportion of endo-H-resistant SEAP.

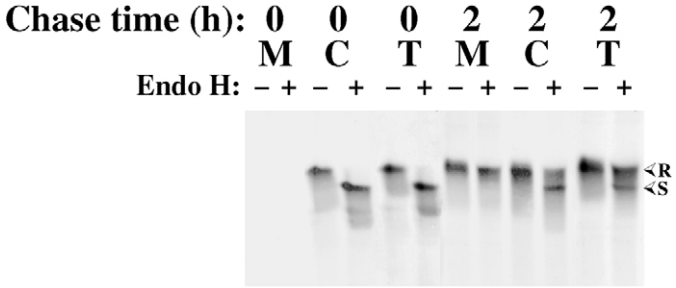

Fig. 2. Acquisition of endo H resistance and secretion of SEAP at 2 hours of chase in INS-1 β-cells. Cells were metabolically labeled for 30 minutes and the media (M), cells (C) or total (T, media + cells) were immunoprecipitated with anti-alkaline-phosphatase antibody. At the zero chase time, SEAP had not yet appeared in the medium and all intracellular SEAP was sensitive to digestion with endo H (S). At 2 hours of chase, more than half of labeled SEAP had acquired endo H resistance (R); 35% of labeled SEAP reached the medium (all of which is endo H resistant), and 25% of intracellular SEAP accumulated as an endo-H-resistant form. The data are representative of three experiments.

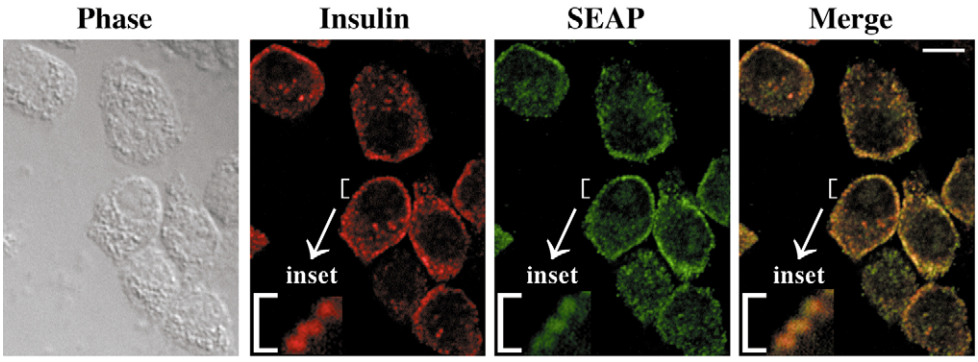

Fig. 3. Phase-contrast microscopy and confocal indirect immunofluorescence distribution of insulin and SEAP in transfected INS-1 β-cells. Insulin secretory granules tend to concentrate in a subplasmalemmal distribution and are not found in the stippled areas seen by phase contrast. All cells in the clonal population express SEAP (green), which exhibits large areas of overlapping distribution with that of insulin (red). A small bracket in each fluorescence image encloses three puncta that are thought to represent subplasmalemmal secretory granules or granule clusters. The bracketed area is reproduced as an inset at higher magnification in the lower left corner of the merged image to highlight the degree of colocalization of SEAP and insulin. Bar, 10 μm.

proinsulin. Upon single-antibody labeling for SEAP and scanning low-power fields, all cells in the clonal population expressed the heterologous gene product. When double labeled for SEAP and insulin, there was substantial (albeit imperfect) signal overlap by confocal immunofluorescence (Fig. 3). This degree of colocalization is consistent with significant SEAP entry into insulin secretory granules (in contrast to a constitutive secretory protein, below).

To examine the possibility of long-term SEAP storage in the regulated secretory pathway of INS-1 cells, metabolic labeling experiments were performed up to 7 hours of chase, exploiting stimulated exocytosis of granule contents during prolonged stimulation (Neerman-Arbez and Halban, 1993). Specifically, unstimulated bathing medium was removed and replaced after 1 hour and 3 hours of chase to allow sufficient time for newly synthesized secretory proteins to undergo storage in granules, and a further 4 hours (3-7 hours of chase) of labeled protein secretion was then collected under unstimulated or stimulated conditions, respectively. All chase media were then analyzed by immunoprecipitation for SEAP (Fig. 4B) followed by a second round of immunoprecipitation with anti-insulin (Fig. 4A). From these analyses, three points were clear: (1) As expected, during the 3- to 7-hour chase interval (lanes marked 7), each of three independent clones exhibited negligible unstimulated but strong stimulated secretion of insulin; (2) during that same 3- to 7-hour chase interval, regardless of SEAP expression level in the individual clones, SEAP also showed stimulus-dependent exocytosis, in parallel with insulin, with corresponding depletion of intracellular pools of SEAP; (3) each of the clones exhibited a comparatively large release of newly synthesized proinsulin during the first unstimulated hour of chase, which is unusual for INS-1 cells (Kuliawat et al., 1997). Although this could reflect passage number or clonal effects (Hohmeier et al., 2000), it is unequivocal from these data that SEAP is not excluded from entry into the stimulus-dependent secretory pathway.

The Golgi lumenal-resident protein, Cab45, is endogenously expressed and not secreted from the INS-1 pancreatic β-cell line

Cab45, the first resident protein of the Golgi lumen to be described (Scherer et al., 1996), is a ubiquitously expressed member of the CREC family (multiple EF-hand, low-affinity calcium binding proteins localized within the lumen of the secretory pathway) (Honore and Vorum, 2000), which includes calumenin, ERC-55, reticulocalbin, and other members. Reticulocalbin and ERC-55 exhibit an exclusive steady-state distribution in the ER whereas calumenin is primarily in ER but in some cultured cells may also be localized to the Golgi lumen (with a further fraction that may be secreted) (Vorum et al., 1999). A more distantly related protein containing two EF-

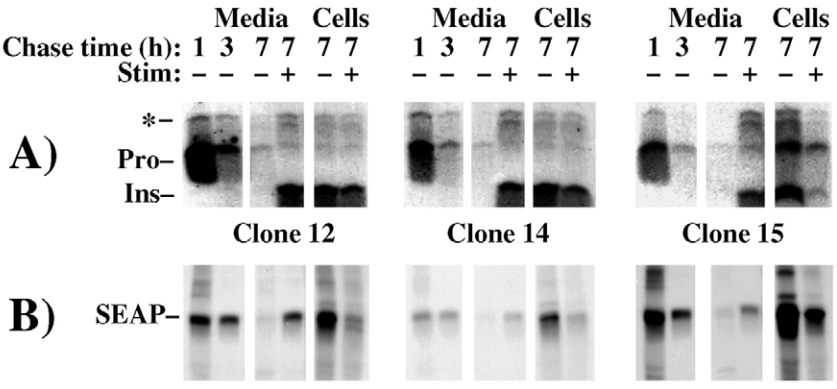

Fig. 4. Similar handling of proinsulin and SEAP during pulse-chase examination of transfected INS-1 β-cells. Each of three independent clones expressing SEAP were pulse labeled for 30 minutes with ^{35}S amino acids and unstimulated media were removed and replaced at 1 hour and 3 hour chase times. A final collection of medium was then performed from 3-7 hours of chase (7) under unstimulated or stimulated (Stim – or +) conditions, respectively. Secretion of insulin-containing peptides was analyzed by immunoprecipitation with anti-insulin (A, proinsulin conversion intermediates highlighted with an asterisk), whereas secretion of SEAP from the identical samples was analyzed by immunoprecipitation with anti-alkaline phosphatase (B). Note that SEAP enters and is stored within the stimulus-dependent secretory pathway, in parallel with insulin, in all clones of INS-1 cells.

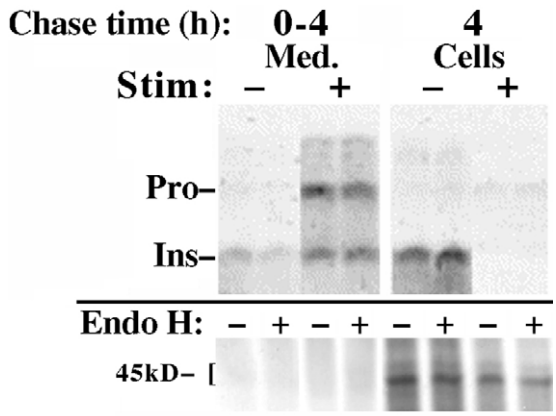

Fig. 5. Endogenous expression of Cab45, a Golgi lumenal-resident protein, in INS-1 cells. Cells were pulse labeled for 30 minutes with ^{35}S amino acids and chased continuously for 4 hours in the absence (–) or presence (+) of secretagogue (Stim). In duplicate, media (Med.) were collected and cells lysed, and the samples were then analyzed by sequential immunoprecipitation with mAb anti-Cab45 (lower panel) and polyclonal anti-insulin (upper panel). The Cab45 immunoprecipitates were further divided either for mock digestion (–) or endoglycosidase H (Endo H) digestion (+). At 4 hours of chase, Cab45 appears as two intracellular endo-H-resistant bands; the position of the 45 kDa molecular mass marker is shown.

hand motifs, CALNUC, also exists in two intracellular pools, one of which resides within the Golgi lumen where it associates with the lumenal aspect of Golgi membranes (Lin et al., 1998; Lin et al., 1999).

Cab45, a protein of 361 amino acids, can acquire N-linked glycans in the ER and rapidly accumulates in an endo-H-resistant form within the Golgi complex, which also represents its steady-state distribution by immunofluorescence (Scherer et al., 1996). A monoclonal anti-Cab45 antibody recognizes an antigen in the region of residues 68-109. After a 30 minute pulse labeling and 4 hours of chase in the absence and presence of secretagogue, we followed (by immunoprecipitation) the fate of both (pro)insulin (Fig. 5, upper panel) and endogenous Cab45 (Fig. 5, lower panel). In the absence of stimulation proinsulin was converted to insulin and stored intracellularly, whereas in the presence of stimulation proinsulin and insulin were secreted. Cab45 was detected intracellularly both as a slower-migrating minor species and a faster-migrating major species, both of which appeared endo H resistant. Endogenous Cab45 of β-cells was not detectably secreted under any conditions (Fig. 5, lower panel); similar results were obtained (not shown) using the previously described polyclonal anti-Cab45 (Scherer et al., 1996). Thus, Cab45 is not normally a secretory protein of β-cells.

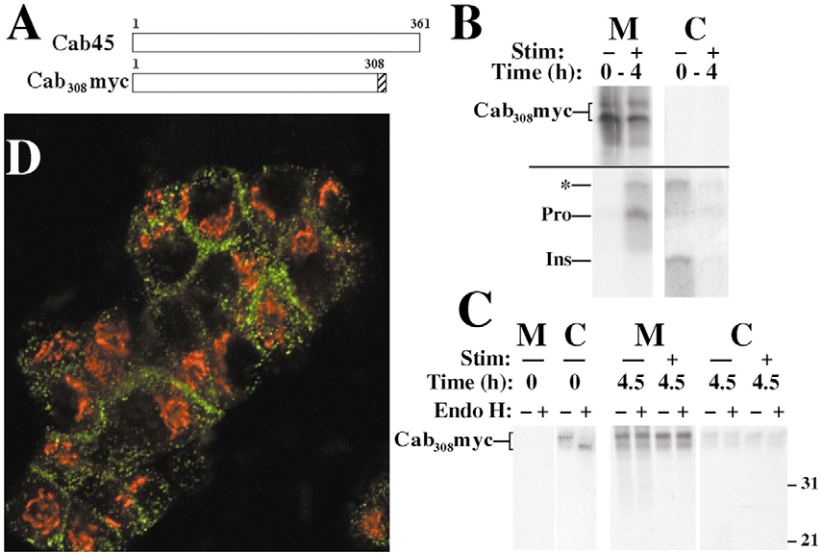

Fig. 6. Secretion of Cab_{308}Myc from transfected INS cells. (A) Scheme of the primary structure of Cab45 and the Cab_{308}Myc construct. (B) Cells were pulse labeled for 30 minutes with ^{35}S amino acids and chased continuously for 4 hours in the absence (–) or presence (+) of secretagogue (Stim). Media (M) were collected and cells lysed (C), and the samples were then analyzed by sequential immunoprecipitation with anti-Myc (upper panel) and anti-insulin (lower panel; proinsulin conversion intermediates highlighted with an asterisk). (C) Cells were labeled as in B and then either lysed immediately without chase (Time 0) or chased for 4.5 hours in the absence (–) or presence (+) of secretagogue (Stim). Media (M) were collected and cells lysed (C), and the samples were then analyzed by immunoprecipitation with anti-Myc followed by mock digestion (–) or endoglycosidase H (Endo H) digestion (+). By SDS-PAGE, Cab_{308}Myc migrates as two bands ranging from 33 to 35 kDa. (D) Double-labeled immunofluorescence distribution of Cab_{308}Myc in transfected INS-1 cells (using polyclonal rabbit anti-Myc, green) relative to the (Golgi) distribution of GM130 (in red).

Secretion of Cab_{308}Myc from INS-1 cells and its failure to enter the regulated secretory pathway

The C-terminal HDEF motif has been shown to prevent the secretion of calumenin in mouse cells (Yabe et al., 1997), and a similar HDEL motif confers intracellular retention of reticulocalbin and ERC-55; evidently, the upstream domains of CREC family members (which consist largely of the EF-hands plus flanking sequences) do not contain sufficient information for efficient intracellular retention (Honore and Vorum, 2000). In order to generate a secretable version of Cab45, we sought to mutagenize the cDNA encoding full-length $Cab45_{361}$, truncating from the C-terminus with the intention to study the longest Cab45-derived construct consistent with good protein expression after transfection. (Two additional constructs were prepared that did not contain C-terminal Myc-epitope tags, which were designed to delete 4 or 17 amino acid residues, respectively, from the C-terminus. The former construct was expressed but not secreted from COS cells, whereas transfection of the latter construct yielded no detectable protein; thus we proceeded with the various Myc-tagged truncations described in the text.) To unequivocally distinguish such constructs from endogenous Cab45-derived proteins that are expressed in the same cells, the truncation constructs were C-terminally tagged with a Myc epitope. The following constructs were prepared and screened by transient transfection in COS cells: Cab_{128}Myc, Cab_{167}Myc,

Cab_{226}Myc, Cab_{263}Myc, Cab_{308}Myc and Cab_{361}Myc. Although protein expression was not detected for the first and last of these constructs (and Cab_{167}Myc was barely detectable in cells), the Cab_{263}Myc and Cab_{308}Myc constructs were well expressed and well secreted as endo-H-resistant proteins, whereas the 226-Myc construct was well expressed but presumably misfolded as it was retained intracellularly in an endo-H-sensitive form. We therefore elected to introduce the Cab_{308}Myc cDNA (Fig. 6A) into INS-1 cells and selected stably transfected clones. When INS-1 cells expressing Cab_{308}Myc were pulse labeled for 30 minutes and then chased continuously for 4 or 4.5 hours, respectively, in the absence or presence of secretagogue, the protein acquired endo H resistance and the vast majority was secreted (Fig. 6B,C; the two Cab_{308}Myc bands differ in the extent of N-glycosylation and they collapse to a single band after PNGase F digestion, not shown). Consistent with this lack of intracellular retention, Cab_{308}Myc, unlike endogenous Cab45, was no longer a Golgi-resident protein as it did not colocalize with GM130, but rather, was distributed to other vesicular organelles at the cell periphery (Fig. 6D). However, whereas secretagogue stimulation caused augmented release of labeled proinsulin (and conversion intermediates), there was no detectable enhancement of newly synthesized Cab_{308}Myc secretion (Fig. 6B,C). Using western blotting or continuous radiolabeling for one day in order to follow all (new and old) molecules, there was still no increase of Cab_{308}Myc in the medium after 2 hours of secretagogue stimulation over that recovered under unstimulated conditions, whereas insulin recovered by radioimmunoassay was increased six- to sevenfold (data not shown).

To examine the generality of this behavior, we also transiently transfected Cab_{308}Myc into the AtT20 cell line. Cells were then radiolabeled and chased for two sequential intervals of 3 hours, and 30 minutes, respectively, in the absence of secretagogue, and then 1 mM Ba^{2+} was either not added or added for the final 30-minute, third chase interval (to stimulate exocytosis). As expected during the first 3 hours of chase (Dumermuth and Moore, 1998), dramatic constitutive-like secretion of the POMC processing intermediate was observed (asterisk, Fig. 7 lanes labeled 1). Subsequent addition of Ba^{2+} elicited stimulus-dependent release of newly-synthesized ACTH (upper panels, lanes labeled Stim +). This could be demonstrated (in untransfected or transfected cells) by comparing the increased ACTH secretion in the third chase period with that in the second period from the same cells (i.e. an internal control – also confirmed by a parallel external control in which the third chase interval lacked Ba^{2+} addition, shown at far left). Although our Myc immunoprecipitations from the lysates of AtT20 cells were essentially uninterpretable owing to high background (not shown), Cab_{308}Myc in the medium was detected exclusively and specifically from transfected cells, and this was observed only during the initial unstimulated chase interval such that no further secretion could be observed even as ACTH release was elicited by subsequent Ba^{2+} treatment (Fig. 7, lower panels). These data were reproduced in three independent experiments, indicating that lack of storage of Cab_{308}Myc in secretory granules is not a phenomenon restricted to pancreatic β-cell lines.

The foregoing behavior of newly synthesized Cab_{308}Myc – a large fraction of the protein released into unstimulated secretion within the first 4 hours of synthesis, and consequently no apparent secretagogue-stimulated secretion over a long stimulation interval – appears reminiscent of results recently reported for α1-antitrypsin (AAT) in both INS-1 and Min6 cells (Feng and Arvan, 2003; Ohkubo et al., 2003). However, AAT entry into ISGs prior to rapid and near-quantitative unstimulated secretion could be demonstrated when secretagogue exposure was confined to only the first 30-60 minutes of chase – maximizing sensitivity by catching the first wave of labeled secretory protein traversing the intracellular transport pathway (Feng and Arvan, 2003). We elected to explore this more sensitive stimulation protocol on the secretion of Cab_{308}Myc in our clones of INS-832/13 cells. Although such cells have a more robust exocytotic response to high glucose than ordinary INS-1 cells (Hohmeier et al., 2000), we could not stimulate Cab_{308}Myc secretion with high glucose as sole secretagogue at any chase time (not shown). We therefore used a combination secretagogue cocktail that elicits a higher fraction of insulin granule exocytosis (Neerman-Arbez and Halban, 1993).

Using a 30-40 minute pulse-labeling period followed

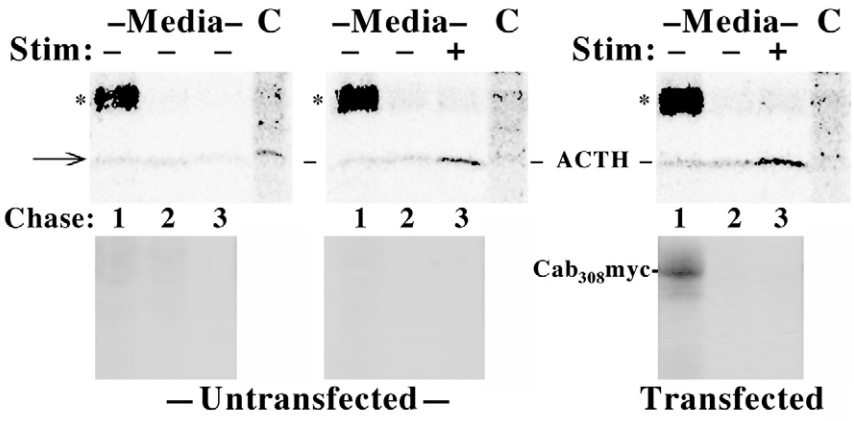

Fig. 7. Secretion of Cab_{308}Myc from AtT20 cells. Two days after transient transfection, untransfected control cells (left panels) or transfected cells (right panel) were pulse-labeled as in Fig. 6B, and then chased sequentially for period 1 (3 hours), period 2 (30 minutes) and period 3 (30 minutes). Periods 1 and 2 were in the absence of secretory stimulus whereas period 3 included either the absence (–) or presence (+) of secretagogue (Stim) as indicated. The upper panels show immunoprecipitation with anti-ACTH from media and from cells (C) at the end of the experiment. A large quantity of constitutive-like release of POMC processing intermediate (asterisk) is seen in period 1 (Dumermuth and Moore, 1998). Stimulus-dependent secretion in period 3 is evident by increased ACTH secretion over that recovered in the medium of period 2 from the same cells, which serves as an internal control. From transfected AtT20 cells, Cab_{308}Myc (lower panels) was immunoprecipitated only from the secretion during period 1, and no further release occurred upon subsequent stimulation of granule exocytosis. The data shown are representative of three independent experiments.

immediately (at the zero chase time) by a 40-minute stimulation in both INS-832/13 cells and Cab_{308}Myc-expressing (clone 2) cells, we observed a strong stimulus-dependent secretion of newly-synthesized insulin (Fig. 8A, middle panels). AAT also exhibited stimulus-dependent release when transiently transfected into these cells (Fig. 8A, lower panels), although this behavior is confined to a narrow chase interval that maximally detects entry into immature secretory granules (Feng and Arvan, 2003). However, even at the earliest, most-sensitive chase intervals, there was no apparent stimulation of Cab_{308}Myc secretion upon secretagogue addition (Fig. 8A, upper panels). To be sure that this was not a consequence of clonal selection or great overexpression of Cab_{308}Myc, two additional clones of INS-832/13 cells (clones 34 and 24) that have decreased relative expression of Cab_{308}Myc were examined. Indeed, the results are quite comparable: good stimulus-dependent release of newly synthesized insulin (Fig. 8B, lower panels) without commensurate stimulus-dependent release of Cab_{308}Myc (upper panels). Thus, unlike SEAP, or even AAT, Cab_{308}Myc is the first newly synthesized secretory protein we have found that does not exhibit appreciable stimulus-dependent release from INS-1 cells.

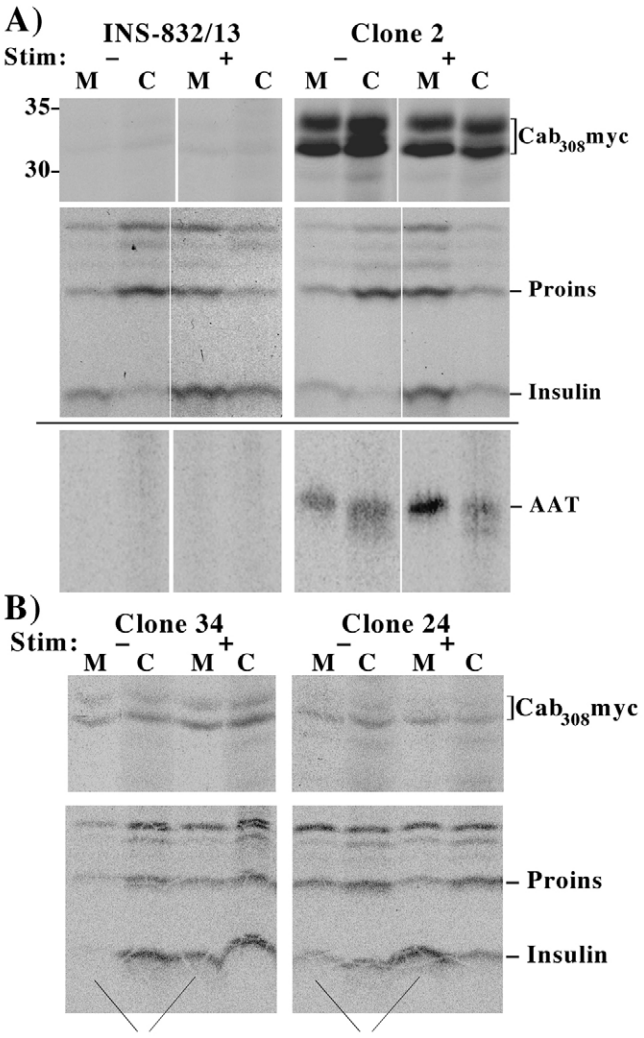

Fig. 8. Short-term secretion of newly synthesized Cab_{308}Myc and AAT from untransfected or transfected INS-832/13 cells. (A) Cells were pulse labeled for 30 minutes with ^{35}S amino acids and chased for 40 minutes in the absence (–) or presence (+) of secretagogue (Stim). Media (M) were collected and cells lysed (C), and the samples were then analyzed by sequential immunoprecipitation with anti-Myc (upper panels) and anti-insulin (middle panels), or anti-AAT (lower panels). A representative clone transfected with the Cab_{308}Myc construct (Clone 2) is shown on the right. Untransfected INS-832/13 cells (left) serve as a negative control for the anti-Myc and anti-AAT immunoprecipitations. The positions of proinsulin (Proins) and insulin are shown; higher bands represent proinsulin conversion intermediates. (B) Two additional independent clones transfected with the Cab_{308}Myc construct (Clones 34 and 24) are shown with less Cab_{308}Myc expression. The experimental protocol was identical to A except that cells were pulse labeled for 40 minutes. For clarity, lines have been added at bottom indicating the stimulus-dependent secretion of newly synthesized insulin. As shown, stimulus-dependent secretion of Cab_{308}Myc was zero in these clones. These experiments have been repeated and confirmed.

We also examined the immunofluorescence localization of Cab_{308}Myc relative to that of insulin, after reducing the ER pools of both proteins by a 60-minute pre-treatment with cycloheximide. Under these conditions, Cab_{308}Myc is not detectable in the ER by immunofluorescence (immunolabeled with anti-calnexin), and primarily exists in the cell periphery in organelles that are non-overlapping with anti-insulin (Fig. 9A-D; highly magnified inset in Fig. 9E).

To differentiate the extent to which the Cab_{308}Myc immunofluorescence might derive from immature versus mature insulin granules, we capitalized on the GSA8 mAb against proinsulin whose immunoreactivity is lost upon processing to insulin such that mature granules (which have little residual proinsulin) remain unlabeled (Orci et al., 1987). Most proinsulin labeling (secondary antibody fluorescing in red, Fig. 9F) concentrated in the juxtanuclear area, but even here there was very clear segregation from Cab_{308}Myc (secondary antibody fluorescing in green, see inset). The data in Fig. 9 contrast with results obtained for SEAP (Fig. 3), and are consistent with the conclusion that Cab_{308}Myc does not appreciably enter immature granules of the regulated secretory pathway.

Membrane-binding properties of SEAP and Cab_{308}Myc

Investigators have often stated or implied that in regulated secretory cells, secretory proteins travel by default via the constitutive pathway by virtue of being soluble, i.e. being excluded from sorting for entry into granules or clathrin-coated vesicles (Dannies, 1999; Glombik and Gerdes, 2000; Halban and Irminger, 1994; Moore et al., 1989; Thiele et al., 1997; Tooze, 1998). This view is at least superficially at odds with the sorting-by-retention hypothesis which suggests that specific sorting information is not required for entry into forming secretory granules (Arvan and Castle, 1998). Indeed, the majority of endogenous proinsulin is recovered in the soluble phase prior to its conversion to insulin within secretory granules (Kuliawat and Arvan, 1994). Thus, it seemed useful to examine both SEAP (which enters immature granules) and Cab_{308}Myc (which does not) for their solubility after permeabilization of organelle membranes with saponin and extraction of contents under either ‘aggregative’ or ‘non-aggregative’ conditions (Chanat and Huttner, 1991). Using this assay, SEAP was primarily released into the soluble

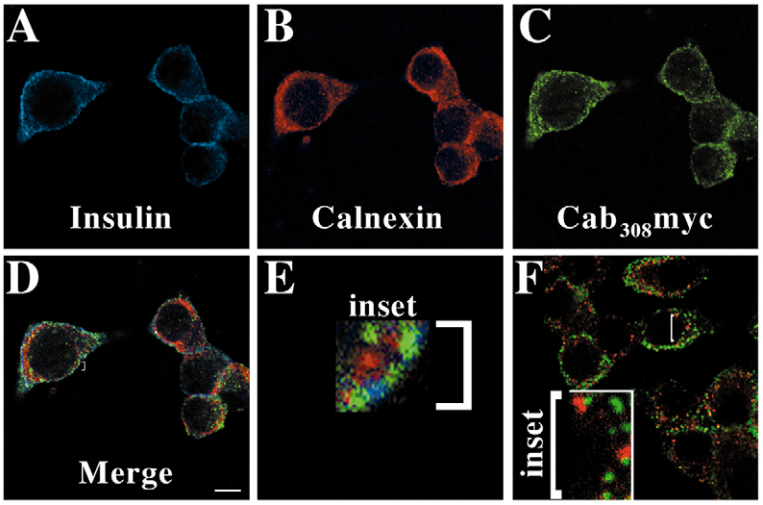

Fig. 9. Indirect immunofluorescence distribution of Cab_{308}Myc in transfected INS cells. Triple immunofluorescence labeling with guinea pig anti-insulin (A, blue), rabbit anti-calnexin (B, red) and mouse anti-Myc (Cab_{308}Myc, C, green) after 60 minutes of cycloheximide treatment. In the cell periphery, Cab_{308}Myc primarily exists in organelles that are non-overlapping with anti-insulin (merge, D; an additional tenfold zoom of the inset from D is shown in E). (F) A different experiment using the same cells double labeled for Cab_{308}Myc (with anti-rabbit conjugate in green) and the mAb GSA8 directed against the intact proinsulin cleavage site (with anti-mouse conjugate in red), which marks immature rather than mature secretory granules. Bar, 10 μm.

supernatant under either condition, and a small fraction of SEAP remaining with the membrane pellet was detected only upon substantial overexposure (Fig. 10A, left panel). By contrast, western blotting of Cab_{308}Myc showed that it was recovered almost quantitatively in the membrane pellet under both conditions (Fig. 10A, right panel). Identical results were obtained for Cab_{308}Myc that had been newly synthesized during a pulse labeling (Fig. 10B). After dissolving organelle membranes with 1% Triton X-100, Cab_{308}Myc was recovered in the supernatant (Fig. 10B). This behavior is similar to that observed for the endogenous Cab45 parent protein (Fig. 10C).

Is capture of Cab_{308}Myc by membranes destined for the constitutive secretory pathway a process that first begins in the TGN? To examine this question, pulse-labeled Cab_{308}Myc was examined for membrane association in cells treated with brefeldin A, which prevents anterograde egress of newly synthesized proteins from the ER. Although SEAP remained saponin extractable under these conditions (not shown), newly synthesized Cab_{308}Myc still became associated with the membrane (Fig. 10D). Thus the process of Cab_{308}Myc sorting to the constitutive secretory pathway begins more proximally, with membrane association initiated at the level of the ER. Despite this, Cab_{308}Myc eventually becomes a fully soluble protein at the time of exocytosis, insofar as the secreted protein does not pellet even in the absence of detergents. Nevertheless, we conclude that membrane association of Cab_{308}Myc precedes its intracellular sorting as a selective marker of the constitutive secretory pathway.

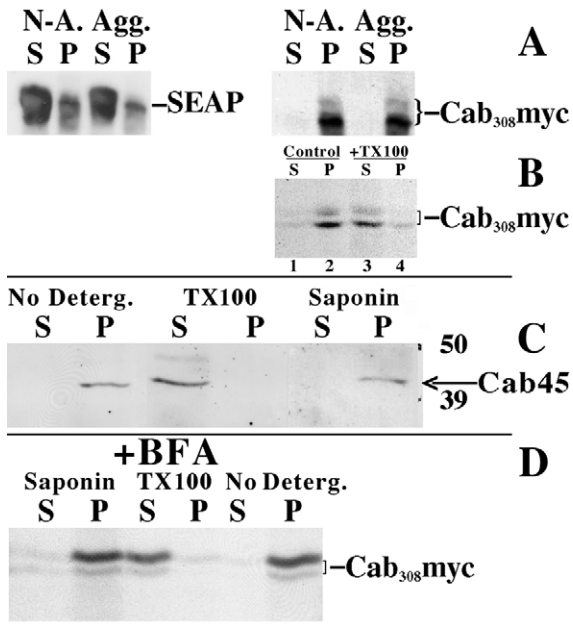

Fig. 10. Behavior of SEAP, Cab_{308}Myc and Cab45 after saponin permeabilizing intracellular membranes and sedimenting the resultant extract. Both non-aggregative (N-A.; neutral pH, low calcium) and aggregative (Agg.; low pH, high calcium) conditions were used for extraction as described in the Materials and Methods. (A) Western blotting of SEAP and Cab_{308}Myc after saponin permeabilization of organelle membranes. SEAP, which enters secretory granules, appears largely (>90%) soluble under both non-aggregative and aggregative conditions, whereas Cab_{308}Myc is almost completely (>95%) sedimentable under these conditions. (B) Immunoprecipitation of newly synthesized Cab_{308}Myc after saponin permeabilization or Triton X-100 solubilization, followed by sedimenting the resultant extracts. INS-Cab_{308}Myc cells were pulse labeled with ^{35}S-amino acids for 100 minutes without chase. Organellar membranes from these cells were saponin permeabilized under non-aggregative conditions (control, lanes 1 and 2), or dissolved with 1.5% Triton X-100 (lanes 3 and 4). After centrifugation, the supernatants (S) and solubilized pellets (P) were immunoprecipitated with anti-Myc and analyzed by SDS-PAGE and fluorography. (C) Western blot of Cab45 as in A, using non-aggregative conditions in the absence (No Deterg.) or presence of detergents as shown. The data shown are representative of three independent experiments. (D) INS-Cab_{308}Myc cells were pulse labeled as in panel B in the presence of brefeldin A (BFA, 10 μg/ml). Organellar membranes from these cells were saponin permeabilized under non-aggregative conditions, or dissolved with 1.5 % Triton X-100 (TX100), or mock treated without detergent (No deterg.). After centrifugation, the supernatants (S) and solubilized pellets (P) were immunoprecipitated with anti-Myc and analyzed by SDS-PAGE and fluorography.

Discussion

Some lumenal proteins of the regulated secretory pathway have biophysical behaviors that suggest sorting for entry at the level

of the TGN (Blazquez et al., 2001; Blazquez et al., 2000; Dhanvantari and Loh, 2000; Zhang et al., 2003), and perturbation of membrane organization appears to impair formation of immature secretory granules (Wang et al., 2000). Curiously, such perturbation also inhibits secretory protein trafficking via the constitutive pathway (Wang et al., 2000), raising the hypothesis that certain lumenal proteins of the constitutive secretory pathway may also be selectively captured for entry into post-TGN transport intermediates (Rustom et al., 2002). In polarized MDCK epithelial cells where all newly synthesized secretory proteins transported to apical and basolateral media are thought to use constitutive secretion pathways, there is a precedent that at least some of these proteins may undergo selective capture by membranes of post-TGN transport intermediates (Caplan et al., 1987; Cheong et al., 1999; Kuhn et al., 2000; Martin-Belmonte et al., 2000; Martin-Belmonte et al., 2001; Urban et al., 1987; Zhang et al., 2002). Indeed in general, the same degree of attention paid to the trafficking of secretory granule content proteins in regulated secretory cells has not been given to constitutive secretory proteins in these cells, and most views have tended to fall back on models of constitutive protein trafficking as occurring by exclusion from entry into granules (Bauerfeind and Huttner, 1993; Thiele and Huttner, 1998). However the interpretation of some candidate proteins as markers of the constitutive secretory pathway (Chanat et al., 1993; Glombik et al., 1999; Matsuuchi and Kelly, 1991; Moore and Kelly, 1985) has been confounded by slow ER exit (Stahl et al., 1996) or rapid unstimulated protein secretion occurring only after entry into immature granules of the regulated secretory pathway (Castle et al., 1998) or by results applicable within one model cell line but not another (Feng and Arvan, 2003; Gorr et al., 1999). Indeed, until now, we are unaware of an unequivocal candidate protein marker of the constitutive secretory pathway in pancreatic β-cells.

Molinete et al. followed secretion of SEAP expressed in INS-1 cells (Molinete et al., 2000), the same cell line used in the present study. Although those authors monitored SEAP exocytosis only by secreted enzyme activity, the results obtained were fully consistent with those obtained herein (Fig. 4), including a nearly fourfold overall stimulation of SEAP secretion upon secretagogue exposure. We therefore would not agree with their description of this protein as a constitutive secretory protein marker in INS-1 cells (Molinete et al., 2000), especially because we find that the cells maintain a significant post-Golgi storage pool in the steady state (detected by endo H resistance, Fig. 1) and an overlapping immunofluorescence distribution with that of insulin (Fig. 3). Quite possibly, much of the rapid unstimulated secretion of SEAP (Figs 2, 4), which contributes to its high basal secretion rate (Molinete et al., 2000), is derived from constitutive-like secretion rather than constitutive secretion (Arvan and Castle, 1998), as has recently been suggested for AAT (Feng and Arvan, 2003). We do not claim that SEAP is as good a granule storage marker as insulin, but there are no data indicating that initial entry of SEAP into β-secretory granules is any less efficient than that of proinsulin, the endogenous protein that enters newly forming β-secretory granules at the TGN (Arvan and Halban, 2004).

By contrast with SEAP, Cab_{308}Myc offers a fresh perspective of secretory protein transit through the constitutive pathway of regulated secretory cells. Loss of information encoded in the C-terminal region of the parent protein Cab45, similar to other members of the CREC family (Honore and Vorum, 2000) causes the protein to lose its intracellular retention within the Golgi complex and become rapidly secreted (Fig. 6). Moreover, Cab_{308}Myc does not show apparent stimulus-dependent secretion in either INS-1 cells (Fig. 6) or AtT20 cells (Fig. 7). In β-cells, this remains true even when performing our most sensitive assay that picks up the ‘first-wave’ of newly synthesized molecules having an opportunity to enter immature granules – the only biochemical assay that can detect AAT entry into immature granules (Fig. 8). Thus we conclude that unlike AAT, Cab_{308}Myc does not acquire stimulus competence even transiently. The inability to stimulate Cab_{308}Myc release does not reflect atypical intracellular transport. Indeed several features establish that Cab_{308}Myc follows the prototypical constitutive secretory pathway, including the fact that Cab_{308}Myc clearly advances to and through the Golgi complex en route to secretion as demonstrated by its acquisition of endo H resistance (Fig. 6C). In the cell periphery, Cab_{308}Myc primarily exists in organelles that are non-overlapping with anti-insulin (Fig. 9A-E), and Cab_{308}Myc avoids entry into immature granules rich in proinsulin (Fig. 9F). Cab_{308}Myc also does not colocalize with EEA1 (not shown); colocalization studies with other markers are ongoing. The data identify Cab_{308}Myc as the first marker protein that appears dedicated to the constitutive secretory pathway in pancreatic β-cells.

How does Cab_{308}Myc avoid entry into immature granules of the regulated secretory pathway when SEAP and so many other soluble proteins cannot avoid such entry? One clear way for an intracellular secretory protein to avoid entry into immature granules is to be associated within secretory pathway membranes destined to become transport intermediates for the constitutive secretory pathway. A radiolabeled heparan sulfate proteoglycan (HSPG) that exhibits tight membrane association intracellularly was one of the first proteins reported to be constitutively secreted in PC12 cells (Chanat and Huttner, 1991). Although a cDNA or peptide sequence has never been reported that encodes the particular constitutively secreted HSPG – these older findings appear to suggest such a possibility. In the present study we demonstrate that unlike SEAP, Cab_{308}Myc does not behave as a soluble protein while within the secretory pathway. Recent findings suggest that EF-hand-containing proteins bind to basic and/or hydrophobic clusters in membrane-associated protein partners (McLaughlin et al., 2005; McLaughlin and Murray, 2005). The EF-hand is a feature already encoded in endogenous Cab45, which adheres to the lumenal aspect of Golgi membranes (Fig. 10C), rather than a feature that we have introduced by mutagenesis. These data represent nearly a complete turnaround from earlier models which held that the constitutive pathway is a default route for soluble species, whereas entry into granules occurs only for proteins with active membrane binding.

We also consider TGN membrane binding to the lumenal aspect of forming secretory granules as a viable and potentially important mode of lumenal protein entry into the regulated secretory pathway (Colomer et al., 1996; Tooze et al., 2001). Obviously, regardless of the secretory pathway, at the time of exocytosis, the membrane associations of membrane-bound secretory proteins such as Cab_{308}Myc must be reversed to account for their free release to the extracellular environment,

and good precedent exists for this (Schlegel et al., 2001). Based on the foregoing studies, we conclude that membrane binding does not seem to be required for entry into ISGs at the level of the TGN (as lumenal proteins completely in the soluble phase also abundantly enter newly forming granules), whereas still other proteins could bind to TGN membranes destined for the constitutive pathway, for endosomes, or for retrograde transport. Binding to the lumenal aspect of TGN membrane domains would clearly be the most efficient way to create selective targeting to the constitutive pathway in the face of the large volume of luminal protein traffic bound for secretory granules.

Materials and Methods

Antibodies and other materials

A rabbit polyclonal anti-alkaline-phosphatase (IgG fraction) was from Rockland (cat. no. 200-4135); for western blotting this was used at a dilution of 1:500. A rabbit polyclonal antibody against the last 15 residues of Cab45 (GSKLMDYARNVHEEF, conjugated as multiple antigenic peptides on a polylysine backbone) has been used previously (Scherer et al., 1996). A mouse anti-Cab45 mAb (clone 30, cross-reacting with residues 68-109 of mouse, rat, and human Cab45) was from BD Transduction Laboratories. Guinea pig polyclonal anti-insulin was from Linco Research (St Charles, MO). Polyclonal anti-Myc was obtained either from Santa Cruz Biotechnology (used at 1:200 for western blotting) or from Immunology Consultants Laboratory (Newberg, OR; used at 1:1000 for western blotting); mouse monoclonal anti-Myc (used in immunofluorescence experiments) was from Clontech. The proinsulin cleavage site antibody (mouse mAb GSA8) was kindly provided by O. Madsen, Hagedorn Institute, Copenhagen, Denmark. Secondary antibody-peroxidase conjugates were from Jackson ImmunoResearch Laboratories (West Grove, PA) and were used at a dilution of 1:2000. [^{35}S]Methionine/cysteine (Trans-Label) was from ICN Radiochemicals. Methionine/cysteine-deficient and complete RPMI, and stock chemicals were from Sigma (St Louis, MO). The RIA kit against rat insulin was from Linco.

Cell culture

INS-1 β-cells were cultured in RPMI-1640 medium supplemented with 30 mM sodium bicarbonate, 1 mM sodium pyruvate, 10 mM HEPES pH 7.35, 50 μM β-mercaptoethanol, 10% fetal bovine serum and 0.1% penicillin-streptomycin (Gibco BRL) at 37°C with 5% CO_2 as described (Neerman-Arbez and Halban, 1993). The INS-832/13 subclone of INS-1 cells (Hohmeier et al., 2000) was obtained from the laboratory of C. Newgard (Duke University, Durham, NC) and was grown in the same medium.

Transfection

The SEAP construct subcloned into pcDNA3 was obtained from S. Gorr (University of Louisville, KY) and has been described elsewhere (Molinete et al., 2000). The Cab_{308}Myc construct was prepared by PCR mutagenesis of Cab45b (Genbank cDNA accession number U45978; protein accession number AAB01813) in which the C-terminal 53 residues were deleted and replaced with a single Myc epitope tag followed immediately by a stop codon. This construct was confirmed by DNA sequencing and was then subcloned into the pCB7 expression vector that independently confers hygromycin resistance. For both constructs, expression is under control of the CMV promoter. Plasmid DNA was transfected into INS-1 cells with Lipofectamine (Gibco-BRL). SEAP cells were selected with 150 μg/ml G418 (Gibco BRL) beginning 48 hours after transfection, and individual drug-resistant cell clones were maintained in the presence of 100 μg/ml G418. Cab_{308}Myc-expressing INS-1 cells were selected with 50 μg/ml hygromycin and individual drug-resistant cell clones (and all subsequent passages) were maintained at the same dose of antibiotic. Additional Cab_{308}Myc clones were also prepared using INS-832/13 cells (Hohmeier et al., 2000). Cab_{308}Myc-expressing AtT20 cells were studied 48 hours after transient transfection.

Metabolic labeling

INS-1 cells or INS-832/13 cells were cultured in the absence of selection antibiotics for at least 2 days before experiments. Cells were pre-incubated for 30 minutes in methionine and cysteine-free DMEM, and then pulse labeled with [^{35}S]met/cys for 30 minutes in the same medium.

Analysis of unstimulated and stimulated secretion

After pulse labeling, the cells were washed in PBS before being chased for various times in RPMI containing 3 mM glucose. Insulin secretory granule exocytosis was then stimulated for the times indicated in complete growth medium containing a combination secretagogue (Neerman-Arbez and Halban, 1993) including 16.7 mM glucose, 1 μM phorbol 12-myristate 13-acetate, 1 mM isobutylmethylxanthine and 1 mM tolbutamide. Granule exocytosis in AtT20 cells was stimulated with 1 mM $BaCl_2$. 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-HCl, pH 7.4 (Turner and Arvan, 2000). Cell lysates and chase media were routinely treated with a proteinase inhibitor cocktail (Roche Applied Science, Indianapolis, IN). These samples were pre-cleared and then immunoprecipitated with zysorbin as the secondary immunoabsorbent (Zymed Laboratories, South San Francisco, CA).

Endo H or PNGase F digestion

After immunoprecipitation, zysorbin-bound Cab_{308}Myc was eluted by boiling for 5 minutes in 2% SDS and 5% β-mercaptoethanol in 62.5 mM Tris-HCl, pH 6.8, followed by centrifugation at 12,000 ***g*** for 4 minutes. The supernatants were diluted to 0.4% SDS and 1% β-mercaptoethanol and then mock-digested or digested with Endo H or PNGase F (New England Biolabs, Beverly MA) as per the manufacturer's instructions. In some instances, cells were lysed directly in denaturing buffer provided by the manufacturer, prior to Endo-H digestion and analysis by SDS-PAGE and western blotting.

Immunofluorescence

To minimize immunofluorescence staining of secretory proteins within the ER, in selected experiments, cells were treated with cycloheximide (10 μg/ml) for 60 minutes before fixation. Cells were then fixed with 4% formaldehyde and permeabilized with 0.1% Triton X-100. After fixation the cells were incubated for 30 minutes in 5% newborn bovine serum in PBS containing 0.02% sodium azide (wash) and then processed for immunodetection. Primary antibodies were diluted in wash, anti-insulin antibodies were used at 1:250, mouse mAb anti-Myc at 1:250, mouse mAb anti-proinsulin at 1:1000, and anti-SEAP antibodies at 1:2500, and incubated with the cells for 30 minutes at RT. To assess background staining, anti-Myc and anti-SEAP antibodies were incubated with untransfected cells whereas guinea pig IgGs served as negative control for the insulin antibody. Bound antibodies were detected with secondary antibodies that were either Alexa Fluor 546-tagged (Molecular Probes) or FITC-tagged (Dako). Fluorescence was monitored with a Leica TCS-NT confocal laser-scanning microscope (Heidelberg, Germany), using standard filter settings and sequential scanning to avoid overlap of emission from the fluorophores. The thickness of the optical section was calculated with the help of the Leica TCS-NT software and was set to 0.486 mm.

Solubility assay

The permeabilization and protein extraction protocol was adapted from Chanat and Huttner (Chanat and Huttner, 1991). Briefly, INS-1 cells were either unlabeled or pulse labeled for 30 minutes and chased for 1 hour. Cells were then scraped from the dish in ice-cold PBS in the absence of detergents, followed by centrifugation at 500 ***g*** for 5 minutes. The cell pellet was resuspended in 1 ml PBS and homogenized by passage (up-and-down) eight times through a 25 g needle. Cell debris was pelleted by centrifugation at 500 ***g*** for 5 minutes and the supernatant re-centrifuged at 690,000 ***g***. The membrane pellet was washed once with 1 ml PBS and re-pelleted as before. The washed pellet containing membrane-enclosed secretory protein was then resuspended in 500 μl 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 minutes, or Triton X-100 (1.5% final concentration) and incubated at room temperature for 5 minutes. The membrane extract was finally subjected to centrifugation at 690,000 ***g***, and both supernatant and pellet fractions were collected for subsequent analysis.

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