Trafficking of non-regulated secretory proteins in insulin secreting (INS-1) cells

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Abstract

Aims/hypothesis. Sorting of proinsulin to the regulated secretory pathway of pancreatic beta cells and retention of insulin in dense-core granules of this pathway is remarkably efficient. To monitor the specificity of these events, the secretion of two exogenous secretory proteins not known to carry information for sorting or retention in the regulated pathway was investigated in INS-1 cells.

Methods. SEGFP, a fusion protein consisting of a signal peptide N-terminal to EGFP (mutant green fluorescent protein with enhanced fluorescence) and secreted alkaline phosphatase (SEAP) were expressed in INS-1 cells by transfection and by infection with recombinant adenovirus, respectively. Secretion of SEGFP was monitored by quantitative western blotting and that of SEAP by its activity.

Results. Secreted alkaline phosphatase showed high basal secretion (6.6% total) but only modest (3.6-fold) stimulation of secretion by secretagogues, in keeping with secretion largely through the constitu-

tive pathway. By contrast SEGFP had a secretory pattern similar to insulin, with low basal secretion (0.8% total) and 16-fold stimulation by secretagogues. Granular localization of SEGFP was confirmed by high resolution electron microscopy immunocytochemistry. Pulse-chase experiments indicated retention of SEGFP in granules at least 24 h after synthesis. The secretory SEGFP, but not cytosolic EGFP, formed disulphide-linked oligomers. This could be implicated in its regulated secretion.

Conclusion/interpretation. These data indicate that in INS-1 cells SEGFP, but not SEAP, is unexpectedly handled as a regulated secretory protein and stored along with insulin in granules. This raises questions about the specificity and mechanism of the sorting of proteins to granules in INS-1 cells or their retention therein or both. [Diabetologia (2000) 43: 1157–1164]

Keywords GFP, insulin, regulated secretory pathway, secretory granules, secretory alkaline phosphatase.

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Abbreviations: GFP, Green fluorescent protein; EGFP, enhanced green fluorescent protein; SEGFP, secreted EGFP; SEAP, secreted alkaline phosphatase; KRBB, Krebs-Ringer bicarbonate buffer; EM, electron microscopy; TGN, Trans Golgi Network.

Pancreatic beta cells, in common with other neuroendocrine cells, have two secretory pathways. The constitutive pathway, used for membrane renewal and secretion of proteins, is not subject to regulation by secretagogues [1]. The regulated pathway is used to store insulin (and other proteins) in dense core granules and subsequently release it in response to appropriate stimuli [2–4]. The secreted proteins (constitutive or regulated) share a common route from synthesis in the rough endoplasmic reticulum to the trans Golgi network (TGN). There they are segregated to different secretory vesicles by as yet a poorly defined mechanism (“sorting for entry”) [5]. The contents of

dense-core granules of the regulated pathway are further refined by two other processes, "sorting by retention" [6] and "sorting for exit" [7], believed to be dependent upon differential solubility of granule proteins and binding to granule membrane proteins, respectively. Removal of proteins by both processes is believed to arise in clathrin-coated vesicles, which bud from maturing granules. In beta cells the combined consequence of these sorting events is that more than 99% of all proinsulin is directed to granules, converted therein to insulin, and retained in these secretory vesicles of the regulated pathway [8].

It has been assumed that these sorting events must, at least in combination, be specific to allow for selective enrichment of appropriate proteins in granules. It follows that if a foreign secretory protein not normally destined for the regulated pathway is expressed in a neuroendocrine cell it should either be channelled directly to the constitutive pathway or cleared from granules shortly after entry into this compartment. To test this hypothesis, two exogenous secretory proteins not known to carry any specific structural information for regulated secretion were expressed in INS-1 cells.

Materials and methods

Materials. Tissue culture reagents were obtained from Gibco (Gibco Life Technologies, Basel, Switzerland). Polyclonal and monoclonal antibodies against enhanced green fluorescent protein (EGFP) were obtained from Clontech (Clontech Laboratories, Heidelberg, Germany) and the monoclonal antibodies M1 and M2 against the FLAG epitope from Kodak (Kodak, Scientific Imaging System, New Haven, Conn., USA). All others reagents were purchased from Sigma (Sigma, St. Louis, Mo., USA) unless stated otherwise.

Cell culture and transfection. The INS-1 cells [9] were cultured in RPMI 1640 supplemented with 10% fetal calf serum and 10 mmol/l HEPES, 2 mmol/l glutamine, 1 mmol/l Na-pyruvate and 50 μ mol/l β -mercaptoethanol. The INS-1 cells were transfected with the different constructs using the polyethylenimine (PEI) method [10] and 2 days later 100 μ g/ml of G418 was added to the medium and changed every 2 days. After 3 weeks the clones were pooled and analysed by fluorescence microscopy for EGFP expression. Studies on transiently transfected cells were done 2 days after transfection as above.

Plasmids

All the constructs were ultimately cloned into pcDNA3 (Invitrogen, Groningen, The Netherlands) allowing for transient or stable expression in mammalian cells. We used EGFP for all green fluorescent protein (GFP) constructs.

Secreted EGFP (SEGFP) with FLAG epitope. The sequence encoding EGFP was cloned into the plasmid pcDNA3 containing the sequence of preproinsulin signal peptide and the FLAG epitope resulting in a junction of 93 bp between the FLAG epitope and the EGFP.

Secreted EGFP (SEGFP) without FLAG epitope. A *Bam*HI was introduced by site-directed mutagenesis using the Stratagene method (QuikChange site-directed mutagenesis kit, Stratagene, Heidelberg, Germany) in front of the FLAG sequence with primer 5' GCCGCAGCGGATCCCAAGGAC-GAC (sequence of the signal peptide in 5', *Bam*HI site underlined and start of FLAG sequence in bold) and primer 5' **GTCGTCCTTGGGATCCGCTGCGGC**. A deletion between the *Bam*HI sites was made resulting in a junction between signal peptide and EGFP of 18 bp. Note that the sequence of this 6-amino-acid junction peptide (DPPVAT) is identical to the last 6 residues of the 31-residue junction peptide introduced in the fusion protein with FLAG described above.

Secretion assay. The INS-1 cells were seeded 2 days before use. For the experiments, cells were washed twice with a modified Krebs-Ringer bicarbonate buffer (134 mmol/l NaCl, 4.8 mmol/l KCl, 1 mmol/l CaCl_2 , 1.2 mmol/l MgSO_4 , 1.2 mmol/l KH_2PO_4 , 5 mmol/l NaHCO_3 , 2.8 mmol/l glucose, 10 mmol/l HEPES, pH 7.4: KRBB-HEPES), then incubated for 1 h at 37°C with KRBB-HEPES (basal secretion), followed by 1 h at 37°C with KRBB-HEPES supplemented with 10 mmol/l glucose, 10 mmol/l leucine, 10 mmol/l glutamine, 1 mmol/l isobutylmethylxanthine (IBMX) 10 μ mol/l forskolin, 0.1 μ mol/l phorbol 12-myristate 13-acetate (PMA) (stimulated secretion) [11]. The two secretion media (basal and stimulated) were centrifuged at 2000 rpm for 5 min to remove any cells which could have detached from the dish during incubations. For western blot, cells were made soluble in 2 $\times$ SDS-PAGE loading buffer and sonicated 10 s and media proteins were first precipitated with 5% ice-cold trichloroacetic acid (TCA) for 30 min and then resuspended in 2 $\times$ SDS-PAGE loading buffer. For the secreted alkaline phosphatase (SEAP) assay, cells were extracted in KRBB-HEPES containing 0.2% Triton X-100, sonicated 10 s and centrifuged 5 min at 8000 rpm. For radioimmunoassay, cells were washed with PBS and then extracted in 1 mol/l acetic acid, 0.1% BSA. The cells were scraped and freeze-thawed twice to ensure complete extraction of cellular proteins.

SEAP assay. For SEAP (from Clontech) experiments, cells were infected the day before study with a replication defective recombinant adenovirus expressing SEAP. The virus was generated using standard techniques as described previously [12] with SEAP under the control of the cytomegalovirus (CMV) promoter. Infected cells were then incubated as above to monitor basal and stimulated secretion of SEAP. Samples (media and cell extracts) were diluted 1/20 to 1/400 with diethanolamine 100 μ mol/l. The SEAP substrate buffer (diethanolamine 100 μ mol/l, 1 mg/ml p-nitrophenyl phosphate) was added to the samples and incubated 10 min at 37°C in a 96-well plate. The plates were read at 405 nm in an ELISA microplate reader.

Western blot analysis. We did SDS gel electrophoresis under reducing conditions unless otherwise stated, using 12% polyacrylamide gels according to Laemmli [13] and the proteins were then transferred to nitrocellulose. The nitrocellulose membranes were blocked for 1 h at room temperature in TRIS-buffered saline (TBS), 5% dry milk (Bio-Rad Laboratories, Glatbrugg, Switzerland) before incubation with the first antibody in TBS, 5% dry milk, 0.5% Tween-20 overnight at 4°C. The polyclonal and monoclonal antibodies against EGFP were diluted 1:1000. The primary antibody was then visualized using a horseradish peroxidase coupled second antibody diluted 1:1000 in TBS, 5% dry milk, 0.5% Tween-20 combined with the ECL detection procedure from Amersham

International (Amersham, Bucks, UK). After scanning, the density of the bands was quantified using NIH Image 1.59 software, Bethesda, Md., USA.

Radioimmunoassay (RIA). Insulin in media and extracts was measured by radioimmunoassay using the charcoal separation technique as described [14]. Rat insulin was used as the standard with a guinea-pig anti-porcine insulin antibody and ^{125}I -insulin as the tracer.

Electron microscopy. For thin section immunocytochemistry, stably transfected INS-1 cells were fixed with 1% glutaraldehyde buffered in 0.1 mol/l phosphate, pH 7.4, infused with 2.3 mol/l sucrose, frozen in liquid nitrogen and sectioned by cryoultramicrotome. Double immunolabelling was carried out as described before [15]. The primary antibodies were mouse monoclonal anti-insulin mAb3 (culture supernatant, diluted 1:5, [16] and polyclonal anti-GFP (diluted 1:10). Goat anti-mouse and goat anti-rabbit IgG coupled to gold particles of 10 and 15 nm in diameter, respectively were from Aurion (Aurion, Wageningen, The Netherlands).

Pulse-chase and immunoprecipitation. Stably transfected INS-1 cells were seeded 2 days before the experiment. For the experiments, cells were washed twice with KRBB-HEPES buffer, incubated for 10 min at 37°C with KRBB-HEPES containing 3.7 Mbq/ml of [^{35}S]Met then chased for the appropriate time in INS-1 medium. The cells were then treated as for the "secretion assay". Before immunoprecipitation, all samples (media and cell extracts) were adjusted to 0.2% Triton X-100 and 100 $\mu\text{mol/l}$ phenylmethylsulphonyl fluoride (PMSF). The monoclonal antibodies M1 and M2 against the FLAG epitope (final dilution 1/500) were added for overnight incubation at 4°C with mild agitation. Anti-mouse sepharose beads (Cappel Organon Teknika, Turnhout, Belgium) were added for 1 h at 4°C with mild agitation. The beads were centrifuged and washed 5 $\times$ with KRBB-HEPES/0.2% Triton X-100 and finally resuspended in SDS-PAGE loading buffer. After electrophoresis (12% SDS-PAGE), the gel was dried and exposed to XAR Kodak film (Kodak, Scientific Imaging System, New Haven, Conn., USA). Band density was quantified using NIH Image 1.59 software.

Statistical analyses. Data are compared using Student's *t* test for unpaired groups. When *p* was ≤ 0.05 it was considered as statistically significant.

Results

Secretion of SEAP, SEGFP and insulin from INS-1 cells. The INS-1 cells, stably transfected with SEGFP, were infected with recombinant adenovirus expressing SEAP. It was then possible to monitor secretion of the two exogenous proteins, as well as insulin, from the same cells. To this end, cells were incubated for 1 h under basal conditions, for a second hour in the presence of secretagogues (1 h stimulation) and then extracted. The SEAP was quantified by its enzyme activity, SEGFP by western blot and insulin by radioimmunoassay. Secretion of SEAP amounted to 6.6% of total (activity in basal and stimulated media plus cell extracts) per hour under basal conditions and was increased 3.6-fold upon stimulation (Fig. 1). The secre-

Fig. 1 A, B. Secretion of SEAP, SEGFP and insulin from INS-1 cells. INS-1 cells were stably transfected with EGFP with a signal peptide and the FLAG epitope (SEGFP) and subsequently infected with a recombinant adenovirus expressing SEAP (secretory alkaline phosphatase). Cells were incubated under basal conditions for 1 h followed by a second 1-h incubation in the presence of a cocktail of secretagogues. Cells were extracted at the end of the second incubation. **A** Basal secretion, expressed as a per cent of total (basal + stimulated + cell content); **B** fold stimulation (stimulated/basal). Data are for insulin (radioimmunoassay, $\square$), SEGFP (western blotting, hatched) and SEAP (alkaline phosphatase assay, $\blacksquare$). Data are means $\pm$ SEM, *n* = 5–8. * *p* < 0.01 for differences between SEAP and both insulin and SEGFP. Differences between insulin and SEGFP were not statistically significant

tory pattern for SEGFP was very similar to that of insulin, but quite different to that of SEAP. Basal release for SEGFP and insulin was only 0.8%/h and as such much lower than for SEAP but the fold stimulation elicited by secretagogues was much higher (16.0-fold and 25.3-fold for SEGFP and insulin, respectively; this difference not being statistically significant) (Fig. 1).

In light of the unexpected finding of apparently well regulated secretion of SEGFP, a number of control experiments were done (data not shown). The INS-1 cells, which had been transfected with the empty vector (pcDNA3) neither contained nor secreted any detectable EGFP, confirming that the western blot analysis was specific. Although expression of SEGFP did not alter the secretory pattern of SEAP, there was an increase in fold stimulation of insulin secretion from the cells stably transfected with SEGFP but we believe that this reflects variability of INS-1 cells (and more specifically different passage number) rather than a specific effect of SEGFP on insulin secretion kinetics. Finally, expression of SEAP did not influence SEGFP or insulin secretion. It should be noted that SEGFP secretion was also characterized after transient transfection. In all cases, the same regulated secretory pattern was observed, indicating that this was not an aberration of the cells which had been stably transfected with SEGFP.

For the purposes of immunoprecipitation (see below) it was found necessary to include the FLAG epitope in the SEGFP fusion construct (see Materials

Fig. 2. Western blot of the secretion media of cells expressing SEGFP with or without the FLAG epitope. Cells expressing SEGFP with (lanes 1 and 3) or without FLAG epitope (lanes 2 and 4) were incubated under basal conditions for 1 h (lanes 1 and 2) followed by a 1-h incubation in the presence of a cocktail of secretagogues (lanes 3 and 4). The proteins in the media were loaded on a 10% SDS-PAGE, transferred to nitrocellulose and visualized using a monoclonal anti-GFP. Mobility of molecular weight markers is shown to the right

and methods) used for all experiments described. Given that this epitope is highly acidic (with 5 acidic amino acids in the 8-residue sequence) it was possible that it might have influenced the intracellular fate of SEGFP and be responsible for the regulated secretion we observed. To control for this, the EGFP secretory pattern of INS-1 cells expressing SEGFP with or without the FLAG epitope was compared and found to be indistinguishable (Fig. 2). In all cases, there was a low level of basal secretion of SEGFP with pronounced stimulation by secretagogues (compare lanes 1 and 3, and 2 and 4).

High resolution electron microscopy (EM) immunocytochemistry shows co-localization of EGFP and insulin in granules of INS-1 cells expressing SEGFP. The secretion experiments suggested that SEGFP was being handled by the regulated secretory pathway. Given that alternate regulated secretory pathways have been described in various cells, we felt it was essential to confirm routing by the major regulated secretory pathway by localizing EGFP to dense-core granules of this pathway. In a first series of experiments, cells were examined by confocal fluorescence microscopy. The EGFP was visualized by its direct fluorescence and insulin by immunofluorescence (data not shown). The images were suggestive of co-localization of the two proteins to the same compartment with both having an overlapping punctate staining pattern commonly regarded as typical of secretory granules. It was, however, our opinion that light microscopy did not provide the fine resolution required for identification of subcellular compartments and these confocal images are therefore not presented here. Rather, granular localization of EGFP was confirmed by EM immunocytochemistry. To this end, thin sec-

tions were incubated with polyclonal anti-GFP in combination with monoclonal anti-insulin serum and staining revealed by differentially sized colloidal gold particles. Immunoreactivity of EGFP was distinctly and specifically located over dense core secretory granules (Fig. 3) and both EGFP and insulin were found co-localized within the same granules.

SEGFP forms disulphide-linked oligomers. The INS-1 cells expressing SEGFP were incubated for 1 h in the presence of secretagogues (1 h stimulation), extracted with SDS-PAGE loading buffer with or without β -mercaptoethanol and then subjected to SDS-PAGE and western blot using monoclonal anti-GFP antibody. Under reducing conditions, SEGFP in the cell extract or in the secretion medium showed a single band at 33,000 M_r (Fig. 4, lanes 1 and 2). Under non-reducing conditions, SEGFP, both in the cell extract and in the medium, showed additional bands of higher molecular weight consistent with the formation of disulphide-linked oligomers (Fig. 4, lane 3 and 4). Whereas two such bands were seen in the extracts, only one was seen for SEGFP secreted to the medium. The EGFP (cytosolic form) did not form cross-linked oligomers (not shown).

Regulated secretion of SEGFP is not the result of transient passage through granules. Both the measurement of SEGFP secretion as described and its co-localization with insulin in granules by morphology can only testify to the predominant cellular localization of the protein in the steady state. It was therefore important to find out whether SEGFP was stored in granules or alternatively in transit to another cellular destination (i.e. endosomes and ultimately lysosomes) consequent to refinement of granule contents.

To follow the time-course of SEGFP entry to granules, INS-1 cells expressing SEGFP were pulse-labelled (10 min with [35 S]Met) and then chased for 1 h under basal conditions followed by a second hour with secretagogues. Cells were then extracted. The two 1-h incubation buffers and the extracts were immunoprecipitated using anti-FLAG antibody (the FLAG epitope being present in the SEGFP fusion protein as discussed above). This was necessary given that immunoprecipitation using the anti-GFP antibody was not sufficiently quantitative or specific, de-

Fig. 3 A–C. Electron microscopic immunolabelling of EGFP and insulin in INS-1 cells transfected with SEGFP. The INS-1 cells were stably transfected with SEGFP (see Legend to Fig. 1). Ultrathin cryosections were double immunolabelled for EGFP with 15 nm gold and for insulin with 10 nm gold. In **A** a region at the periphery of an INS-1 cell containing numerous labelled secretory granules is seen. Labelling is also present on enlarged cisternae of rough endoplasmic reticulum (ER). In **B** and **C** labelled granules are seen at higher magnification to show the co-localization of large and small gold particles (encircled) revealing EGFP and insulin respectively. Bars, 200 nm

spite its being found to be effective for western blot. The immunoprecipitated products were then subjected to SDS-PAGE and autoradiography. There was pronounced stimulation of secretion of labelled SEGFP (Table 1) indicating that at least some of the newly synthesized labelled SEGFP had been transferred to granules (and was thus available for regulated secretion) within 1–2 h of synthesis. As less labelled SEGFP was secreted in response to secretagogues at this early time of chase than that observed after longer times (see below and Table 1), some of such labelled material could still have been in a compartment proximal to secretory granules and as such not available for secretion.

Immature granules are known to mature within approximately 2 h of their formation. It is during this

time that granule contents are refined (by the sorting by retention and sorting for exit processes mentioned above). To determine whether SEGFP which had entered granules was removed with time, the transfected INS-1 cells were again pulse-labelled (10 min) but then incubated for 24 h under basal conditions before the 1-h basal and 1-h stimulated secretion periods. These two 1-h incubation media and the extracts were immunoprecipitated and analysed as above. It was again evident (Table 1) that the secretion of labelled SEGFP was stimulated by secretagogues even though 24 h had elapsed before the start of the stimulatory incubation. In parallel experiments, we found that approximately 50% of the labelled SEGFP

Table 1. Secretion and storage of radiolabelled SEGFP in transfected INS-1 cells

	Basal secretion	Stimulated secretion	Cell content
2-h chase	0.5 %	9.7 %	89.8 %
24-h chase	0.7 %	18.6 %	80.7 %

INS-1 cells transfected with SEGFP (see Legend to Fig. 1) were pulse-labelled (10 min) and chased for 2 h or 24 h followed by 1-h incubation under basal conditions and 1 h with secretagogues. Basal secretion, stimulated secretion and cell content of labelled SEGFP (immunoprecipitation using anti-FLAG) are expressed as a per cent of total (basal + stimulated + cell content). Data are for one representative experiment out of two independent experiments

Fig. 4. Western blot of SEGFP in reducing and non-reducing conditions. The INS-1 cells expressing SEGFP were incubated in the presence of a cocktail of secretagogues for 1 h then extracted with $2 \times$ SDS-PAGE loading buffer with (lanes 1 and 2) or without (lanes 3 and 4) β -mercaptoethanol. The proteins in the cell extract (lane 1 and 3) or in the secretion medium (lane 2 and 4) were run on a 10% SDS-PAGE, transferred to nitrocellulose and revealed with a monoclonal anti-GFP. Mobility of molecular weight markers is shown to the left

(10 min pulse-label) was lost from INS-1 cells during the 24-h incubation. It was not possible from these experiments to establish the cause of this loss of SEGFP, which could have been due to secretion from granules during the 24-h chase or to intracellular degradation. In any event, the loss of SEGFP was similar to that of insulin over this same 24-h period (data not shown).

Taken together, these results indicate that SEGFP is present in granules and available for regulated secretion at least 24 h after its synthesis. Studies carried out in the steady-state thus truly reflect storage of SEGFP in these organelles of the regulated pathway.

Discussion

The results of this study indicate that EGFP, if expressed as a secretory protein with an N-terminal signal peptide (which is removed shortly after synthesis – data not shown), is handled like the resident regulated secretory protein insulin in INS-1 cells, with storage in granules. As a consequence, this foreign protein without any appropriate credentials as a regu-

lated secretory protein is secreted from INS-1 cells in response to secretagogues. The presence or absence of a FLAG epitope was without any observable effect on EGFP secretion. To construct the SEGFP fusion proteins, it was, however, necessary to include a junction peptide. It is important to note that the last 6 residues of the longer junction peptide used for the construct with FLAG were identical to the hexapeptide junction of the construct without FLAG. Although this sequence (DPPVAT) presents no obvious features suggesting it is responsible for targeting the fusion protein to granules, it cannot be excluded that it alone was responsible for the unexpected behaviour of SEGFP. Given that SEGFP forms disulphide-linked oligomers and that the common junction sequence contains no cysteines, and based on the hypothesis that such cross-linking could be responsible for SEGFP localization to granules, we predict that the junction sequence is not in itself responsible for the regulated secretion of the fusion protein.

One explanation for the regulated secretion of SEGFP from granules might be that all secretory proteins in INS-1 cells are handled in this fashion. It is known that transformed cells can suffer from perturbation to secretory trafficking pathways. Such was not the case given that the secretory pattern for SEAP was quite different to that for SEGFP. We therefore conclude that storage of SEGFP in INS-1 cell granules is intrinsic to the protein itself rather than merely reflecting a leaky, non-discriminatory sorting system.

Previous studies in numerous neuroendocrine cell types have led to the hypothesis that trafficking and sorting of proteins to the regulated secretory pathway is an active process dependent upon interplay between specialized features of the sorted protein and the cellular sorting machinery [2]. These protein features, whether they be true structural sequences or domains recognized by a receptor-like entity {for the example of pro-opiomelanocortin (POMC) [17]} or physico-chemical properties allowing for condensation under appropriate circumstances (for the example of chromogranin B [18]) remain poorly defined for the vast majority of regulated secretory products. The sorting machinery itself is equally poorly characterized, with the recent identification of carboxypeptidase E as a sorting receptor [19, 20] being questioned both in general [21] as well as in the specific context of insulin secreting cells [22]. Constitutive secretion, by contrast, is believed to be largely a default pathway, at least at the level of movement between the TGN and the plasma membrane [23]. The experiments with SEAP confirm that INS-1 cells are able to discriminate between proteins destined for one or the other secretory pathway, albeit not as effectively as has been assumed previously. There was, thus, a stimulation of SEAP secretion by the secretagogue cocktail but much less than that observed for SEGFP or

insulin. When considered with the relatively high rate of basal secretion, we believe that some SEAP enters secretory granules and is available for regulated secretion but the percentage of SEAP molecules entering granules and being retained therein is much lower than for SEGFP or insulin itself. Although SEAP has been shown previously to be constitutively secreted in endocrine and neuroendocrine cells, this protein is sorted to the regulated pathway in exocrine cells [7, 24–26].

Others have reported that GFP is targeted to INS-1 cell secretory granules when fused to the proinsulin sequence but not when expressed with only an N-terminal signal peptide [27]. This is in apparent contradiction to the present findings. Discrete differences in the fusion constructs or INS-1 cells could explain the contrasting findings (and notably the 6-residue junction peptide common to both SEGFP constructs used in the present study), as could the use of EGFP rather than GFP in the present study. It must be stressed, however, that in the other report the conclusions regarding the cellular fate of signal peptide-GFP were based on confocal microscopy alone [27]. As mentioned in the Results, we do not regard light microscopy as providing sufficient resolution to discriminate between vesicular compartments, which accounts for our dependence on the high resolution of EM immunocytochemistry in combination with a biochemical assessment of regulated secretion. This combination did allow for unambiguous localization of EGFP in granules after expression of SEGFP.

Sorting from the TGN to granules is just one model to account for selective packaging and concentration of specific proteins in these secretory vesicles. Granule contents are known to be refined as the organelles mature. It is thus conceivable that some secretory proteins find their way to immature granules by default (particularly in the context of the important flux of proinsulin to granules which is known to arise in beta cells). Under normal circumstances, such unwanted proteins should, however, be extracted from maturing granules as their contents are refined [28] (so-called “sorting for exit” [25]). The results of this study suggest that if such removal of SEGFP from granules occurs it is limited in its extent. This in turn suggests that SEGFP is selectively retained in mature granules (possibly as a result of condensation allowing for sorting by retention) or that it is not a favoured ligand for “sorting for exit” or both.

Green fluorescent protein has been used in real-time trafficking of hormones to the secretory granules of neuroendocrine cells including insulin-secreting cells [29–32]. In such experiments, GFP was shown to be correctly targeted to secretory granules when fused to cargo proteins of the regulated secretory pathway or to a dense-core granule membrane protein [27, 30, 33]. For such an experimental approach to be interpretable it must be shown that the

intracellular fate of GFP is determined by the protein to which it is fused and that it has no effect on the trafficking of this protein. In our study this first condition was not satisfied because SEGFP was directed to secretory granules of the regulated pathway of INS-1 cells. Such spontaneous and aberrant sorting of SEGFP to this pathway precludes its use as a marker for trafficking in the secretory pathways of this particular cell type, in turn raising more general questions as to the specificity and mechanism of the process responsible for sorting to the regulated pathway. Similar results have been found in yeast, with spontaneous trafficking of secretory GFP to the vacuole [34]. Our findings suggest that in addition to its intrinsic fluorescence, SEGFP must have, quite by chance, special features common to proteins normally sorted to or retained within INS-1 cell granules or both. We have observed that the SEGFP forms disulphide-linked oligomers in the secretory pathway. Although these experiments do not allow for differentiation between self-association of SEGFP or cross-linking to other protein(s), oligomerization could allow for sorting to or retention in granules. It is important to note, however, that not all SEGFP in granules appear cross-linked. The precise role of oligomerization in the intracellular trafficking of SEGFP deserves further study and may in turn shed light on the sorting/retention mechanisms in regulated secretory cells.

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