Insulin targeting to the regulated secretory pathway after fusion with green fluorescent protein and firefly luciferase

Aristea E. POULI, Helen J. KENNEDY, J. George SCHOFIELD and Guy A. RUTTER¹

Department of Biochemistry, School of Medical Sciences, University Walk, University of Bristol, Bristol BS8 1TD, U.K.

We have prepared recombinant cDNAs encoding chimaeras between human preproinsulin (sp.B.C.A., for B-, Connecting- and A-peptides) and a thermostable mutant of green fluorescent protein (GFP^{S65T, V163A}, GFP*). The subcellular localization of the expressed chimaeras was monitored in living insulin-secreting INS-1 β -cells by laser scanning confocal microscopy. When GFP* was fused at the immediate N-terminus of the B-chain (sp.[GFP*].B.C.A.myc) two distinct patterns of fluorescence were apparent. In 1530/1740 cells examined, fluorescence was confined to a reticular, exclusively extranuclear structure, and closely co-localized with the endoplasmic reticulum marker, calreticulin. However, 210/1740 (12.1%) of cells displayed punctate fluorescence, which partially co-localized with the *trans*-Golgi network marker, TGN 38, and with the dense core secretory granule marker, phogrin. Since secretion of GFP* fluorescence into the medium could not readily be measured, we prepared a chimaera in which firefly luciferase was fused at the C-terminus of proinsulin (sp.B.C.A.myc.[Luc]). This chimaera displayed a dis-

tribution closely similar to that of sp.[GFP*].B.C.A.myc, but with a lower proportion (15/310, 4.8%) of the cells showing clear punctate distribution. At substimulatory glucose concentrations (3 mM) secretion of sp.B.C.A.myc.[Luc] could not be detected (rate of release into the medium identical with that of the cytosolic *Renilla reniformis* luciferase), indicating that the chimaera did not enter the constitutive secretory pathway. However, elevated (30 mM) glucose stimulated the release of the sp.B.C.A.myc.[Luc] luciferase chimaera, without a detectable effect on *R. reniformis* luciferase release. These data suggest that fusion of insulin, and the much larger photoproteins GFP* and luciferase, leads predominantly to misfolding and retention in the endoplasmic reticulum. However, the properly folded chimaeras are apparently still correctly targeted to the regulated, rather than the constitutive, secretory pathway. These chimaeras should therefore be valuable tools to monitor the exocytosis of insulin in real time.

INTRODUCTION

Understanding of the mechanisms which underlie the regulated release of insulin would be greatly aided by a means of imaging these processes in real-time in single living islet β -cells. Fusion and expression of cDNAs encoding proinsulin and a photoprotein, such as firefly luciferase [1] or green fluorescent protein (GFP) [2], offers an attractive approach to achieving this goal. Indeed, GFP has recently been shown to be partially targeted to the regulated secretory granules of neuroendocrine PC12 cells by peptide hormones which undergo less extensive processing than proinsulin (neuropeptide Y and chromogranin B) [3–5].

In order to extend these studies to islet β -cells we have therefore examined the ability of proinsulin to direct the targeting of modified forms of either GFP or firefly luciferase to the secretory granules, in order to visualize regulated insulin secretion. *A priori* it is uncertain whether such a strategy would result in the correct targeting of these chimaeras to the secretory granules. Both luciferase (64 kDa) and GFP (27 kDa) are considerably larger proteins than either proinsulin (9 kDa, consisting of B-, C- and A-chains) [6] or mature insulin (6 kDa, lacking the connecting or C-peptide) [7]. In particular, it has been postulated that co-crystallization of proinsulin or mature insulin molecules, via dimerization and formation of hexamers [7], may be responsible for the correct packaging of insulin into immature secretory granules during sorting in the *trans*-Golgi network

(TGN) [8]. It seems uncertain whether, after the addition of the large photoproteins, the aggregation of insulin to dimers, hexamers, and ultimately a crystalline lattice, could occur. As alternative models of insulin targeting, it has been suggested that other proteins which are localized to secretory granules, including chromogranins and secretogranins [9] or carboxypeptidase E ([10]; but see also [11]), may act as anchors for proinsulin ('receptor-mediated targeting'). Finally, the processive expulsion of proteins other than insulin and proinsulin during granule formation may also be important [12].

We demonstrate here that successful targeting of photoprotein reporters to the regulatory secretory pathway as chimaeras with proinsulin can be achieved after fusion of the corresponding cDNAs, although with low efficiency. This approach should form the basis of convenient new methods to analyse the control of regulated insulin secretion from both islet β -cells populations and single living cells.

MATERIALS AND METHODS

Materials

The combined firefly and *Renilla reniformis* luciferase quantification kit ('Stop-and-Glo') was obtained from (Promega Corp, Madison, WA, U.S.A.) and the expression plasmid for

Abbreviations used: CMV, cytomegalovirus; GFP, green fluorescent protein; EGFP, enhanced green fluorescent protein; ER, endoplasmic reticulum; TGN, *trans*-Golgi network; CMV, cytomegalovirus; TRITC, tetramethylrhodamine isothiocyanate.

¹ To whom correspondence should be addressed (e-mail g.a.rutter@bristol.ac.uk).

Renilla luciferase (pCMV.LUC_{ren}) was a gift from Dr. Bruce Sherf (Promega). All other reagents were obtained from Sigma (Poole, Dorset, U.K.) or BDH (Poole, Dorset, U.K.). Tissue culture reagents were obtained from Gibco (Paisley, U.K.). Anti-*c-myc* monoclonal antibody 9E:10 [13] was provided by Dr. G. Evan (ICRF, Lincoln's Inn Fields, London). cDNA encoding phogrin under cytomegalovirus (CMV) promoter control (pCDNA3.phogrin) [14] and a polyclonal antibody to the N-terminal luminal domain of the protein, were provided by Dr. John Hutton (University of Colorado, Denver, CO, U.S.A.).

Methods

Construction of cDNAs encoding chimaeras between insulin and GFP* and firefly luciferase

sp.[GFP*]. Fragment *sp*, a 99-base pair cDNA corresponding to amino acids 1–29 of human preproinsulin, i.e. the signal peptide plus five amino acids of the proinsulin B-chain, was generated by PCR amplification from the plasmid pT7-1 (provided by Professor K. Docherty, University of Aberdeen, U.K.) using primers 5'-TTTAAAGCTTATGGCCCTGTGGATGCGC-3' (primer #1; *Hind*III site underlined) and a primer 5'-TTTGGTACCGTGTTGGTTCACAAAGGC (primer #2; *Asp*718 site underlined) in the reverse direction. The product was subcloned into a CMV-based plasmid, pCMX.GFP*, provided by Dr J. Pines (Laboratory of Molecular Biology, University of Cambridge, U.K.).

sp.B.C.A.[GFP*]. To generate a construct in which GFP* was located at the C-terminus of proinsulin, the entire coding region of human preproinsulin cDNA was amplified using primer #1 and primer #3: 5'-TTTGGTACCGGTGCAGTAGTTCTC-CAG (*Asp*718 site underlined). The resulting product was cleaved and subcloned into vector pCMX.GFP*.

sp.[GFP*].B.C.A.*myc*. Fragment *sp* was generated as described above and subcloned into a plasmid bearing GFP^{S65T} (pCMX.[GFP^{S65T}]; Dr J. Pines) to generate a plasmid encoding *sp*.[GFP^{S65T}]. Proinsulin cDNA was amplified to produce fragment B.C.A with primer #4: 5'-TTTGAATTCATTGTGA-ACCAACACCTG (*Eco*R1 site underlined) complementary to codons 25–30 of human preproinsulin (amino acids 1–6 of the B-chain), plus two additional bases to provide in-frame fusion with GFP^{S65T}; and primer #5: 5'-TTTGGATCCTTACTTCAGGT-CCTCCTCGGAGATCAGCTTCTGCTCCATGTTGCAGT-AGTTCTCCAG (*Bam*HI site underlined). Primer #5 was complementary to amino acids 105–110 of human preproinsulin (the last six amino acids of the A-chain), plus a 12 amino acid *c-myc* epitope. The B.C.A. fragment was ligated into plasmid encoding *sp*.[GFP^{S65T}] to generate *sp*.[GFP^{S65T}].B.C.A.*myc*. cDNA encoding GFP* (mutations, S⁶⁵T, and V¹⁶³A) was amplified with primer #6: 5'-TTTGGTACCGTAAAGGAGAACCT (*Asp*718 site underlined) corresponding to nucleotides 29–43 of the wild-type GFP sequence (GeneBank no. M62653) and primer #7: 5'-TTTGAATCCTTTGTATAGTTCATC (*Eco*R1 site underlined) was shuttled into *sp*.[GFP^{S65T}].B.C.A. to generate construct *sp*.[GFP*].B.C.A.*myc*.

sp.B.C.A.*myc*.[Luc]. The entire coding region of human preproinsulin cDNA (including the 24 amino acid signal peptide, *sp*) was amplified by PCR from the corresponding cDNA using primer #1 and 5'-TTTCCATGGACTTCAGGTCCTCCTCG-GAGATCAGCTTCTGCTCCATGGTCGCACTCCCACC-3' (primer #8; *Nco*I site underlined); the latter primer included a 42 nucleotide sequence encoding the *c-myc* epitope tag [13]. The resulting *Hind*III/*Nco*I fragment was subcloned into plasmid pGL3-basic (Promega) to provide in-frame fusion at the 5' end of cDNA encoding a modified version of firefly luciferase, which

Figure 1 Linear maps of constructs

(a) *sp*.[GFP*]; (b) *sp*.B.C.A.[GFP*]; (c) *sp*.[GFP*].B.C.A.*myc*; (d) *sp*.B.C.A.*myc*.[Luc]. All inserts were subcloned into vector pCMX under the control of the CMV immediate early gene promoter (see Methods section).

lacks coding sequence for the three C-terminal amino acids (Ser-Lys-Leu) responsible for targeting the wild-type protein to peroxisomes. In addition, codon usage within this modified luciferase cDNA has been optimized for expression in mammalian cells [15]. The entire preproinsulin–luciferase chimaera was subcloned via flanking *Xba*I and *Xho*I sites into plasmid pCDNA1neo (Invitrogen).

The sequence of all plasmid inserts (see Figure 1) was verified by automated sequencing (Molecular Recognition Centre, University of Bristol, U.K.) using appropriate oligonucleotide primers.

Culture and transfection of cells for live imaging and immunocytochemistry

INS-1 cells (passage no. 86–110) were cultured as previously described [16] on glass coverslips and transfected using lipopolyamine Tfx-50TM or TransfectamTM (Promega), or by microinjection as previously described [19,20]. For transfection, cells were incubated for 2 h in 1.0 ml of serum-free INS-1 medium [16] containing Tfx-50 (2 µg of DNA/well; 3:1 charge ratio with respect to DNA, as per the manufacturer's instructions) and for a further 24 h in the additional presence of 1.0 ml of complete (serum-containing) medium. Post-transfection/-injection, cells were cultured for 24–36 h at 37 °C in an atmosphere of 5% CO₂ before examination.

Glucose-regulated secretion of insulin–luciferase chimaera and total insulin

Cells were cultured in 24-well microtitre plates and transiently transfected with plasmid encoding sp.B.C.A.*myc*.[Luc]. Incubations were then performed for 2 h or 4 h at 37 °C in 0.2 ml of complete medium supplemented with either 3 or 30 mM glucose. The medium was then removed, and detached cells were sedimented by brief (30 s) centrifugation (1000 *g*). The activities of firefly and *Renilla* luciferases were then assayed in 10 or 50 μ l of the medium using the 'Stop-and-Glo' Kit (Promega) and a photon-counting luminometer (LB-9501, EG & G. Berthold, Bad Wildbad, Germany). Release of immunoreactive rat insulin was assayed by a broad-specificity ELISA [17,18].

Cell fixation and immunocytochemistry

Transfected cells were fixed and permeabilized using 4% (v/v) paraformaldehyde plus 0.2% Triton X-100. The c-*myc* epitope was probed with a mouse monoclonal antibody 9E:10 [13] (1:100) and developed with fluorescein-conjugated goat anti-mouse immunoglobulin G (Sigma). A polyclonal rabbit anti-serum (Promega) was used at a final concentration of 5 μ g/ml to probe luciferase, and developed with tetramethylrhodamine isothiocyanate (TRITC)-conjugated anti-rabbit immunoglobulin G. Insulin was probed with a broad-specificity guinea pig anti-insulin antiserum (Dako Ltd., Bucks., U.K.) and revealed with fluorescein-conjugated rabbit anti-guinea antibody (Dako). Confocal images were obtained using a Leica TCS 4D/DM IRBE confocal microscope with 488 and 568 nm excitation lines, and analysed off-line using a Silicon Graphics workstation. Hard images were subsequently generated with Adobe Photoshop and Freelance Graphics For Windows.

Statistics

Data are presented as the means $\pm$ S.E.M. Statistical significance was calculated by Student's *t*-test assuming equal variances.

RESULTS

Expression of chimaeras between GFP* and insulin

We first explored the ability of INS-1 β -cells [16] to synthesize and express detectable levels of different forms of (untargeted) GFP. We introduced, by microinjection [19,20] or transient transfection, plasmids encoding either wild-type, S⁶⁵T or (S⁶⁵T, V¹⁶³A)GFP (GFP*) under CMV immediate early gene promoter control. Whereas the S⁶⁵T mutation increases both the intrinsic fluorescence and folding of GFP [21], V¹⁶³A mutation enhances thermostability [22]. We were able to observe a detectable fluorescence signal only with cells expressing the doubly mutated GFP*, and with commercially available, enhanced GFP (EGFP) mutants (bearing the chromophore mutations S⁶⁵T, F⁶⁴L, and silent mutations in codon usage). Although brighter, expressed EGFP showed a slightly greater tendency to photobleach than GFP* (results not shown). These data are in contrast with observations on the expression of different forms of GFP in non-neuroendocrine-derived cell lines, including CHO.T [20–23] and 3T3-L1 adipocytes [24], where expression of all of these forms of GFP produces a detectable signal, with intensity ascending in the order GFP < GFP(S⁶⁵T) < GFP* < EGFP. We therefore chose GFP* as the backbone of the constructs subsequently used as the basis of the proinsulin chimaeras. These were constructed using PCR-based strategies using human insulin cDNA as template, as described in the Methods section.

Figure 2 Localization of insulin–GFP chimaeras within living INS-1 β -cells

Optical slices of cells incubated in Krebs–Ringer bicarbonate, comprising 135 mM NaCl/3.6 mM KCl/10 mM Na-Hepes, pH 7.4/2 mM NaHCO₃/0.5 mM NaH₂PO₄/0.5 mM MgSO₄/1.5 mM CaCl₂/5.5 mM glucose, equilibrated with O₂/CO₂, 95:5 (37 °C), were obtained using a 40 $\times$ /1.0 NA PL FLUOTAR objective and the inverted laser scanning microscope (DM IRBE/TCS NT) with argon laser excitation at 488 nm. Images were obtained as the combination of 30 separate scans; no photobleaching was apparent during this procedure. Cells were transfected with: (a) untargeted GFP*; (b) sp.[GFP*]; (c) sp.B.C.A.[GFP*]; and (d–f) sp.[GFP*].B.C.A.*myc*. Note the reticular localization (pattern 1, see text for details) in (d) compared with clear punctate localization (pattern 2) in (e) and (f); (g–i) motion of vesicular structures present in cells displaying pattern 2 fluorescence, recorded by time-lapse confocal microscopy. Images were recorded at 5 min intervals. Note the movement of the individual granules. Bar = 2 μ m (a–f) or 1 μ m (g–i).

Figure 2 compares the distribution of the expressed chimaeras in living INS-1 β -cells [16] examined in single optical slices (0.5 μ m depth). Wild-type GFP* was present throughout the cell cytosol and nucleus (Figure 2a). As expected, addition of cDNA encoding the preproinsulin secretory signal peptide immediately 5' to GFP* (sp.[GFP*], Figure 1a) caused relocalization to a reticular structure, likely to represent the endoplasmic reticulum (ER; Figure 2b). Cycloheximide treatment (10 μ M for 90 min) of cells expressing construct sp.[GFP*] resulted in a shift of fluorescence towards the perinuclear region, and a decrease in overall fluorescence (results not shown), consistent with the release of this chimaera through unregulated secretion. In contrast, construct sp.B.C.A.[GFP*] (Figure 1b) showed a closely similar distribution to wild-type (untargeted) GFP* (compare Figures 2c and 2a). The presence of strong fluorescence in the cell nucleus is most compatible with exclusion of the protein from the lumen of

the secretory pathway and its free diffusion in the cytosol and nucleus.

When the fusion of the insulin secretory peptide and GFP* was extended to include cDNA encoding the whole of proinsulin, the resultant chimaera (sp.[GFP*].B.C.A.myc, Figure 1c) displayed one of two distinct patterns of fluorescence (Figure 2, d–f). In either case, fluorescence was entirely excluded from the nuclear region, indicating transport into the secretory pathway. In pattern 1 (Figure 2d), the distribution of fluorescence closely resembled that which was apparent upon expression of sp.GFP*, i.e. reticular localization (Figure 2b). This pattern was observed in 1530/1740 (87.1 %) of cells examined. In pattern 2 (observed in 210/1740, 12.1 % of cells) the fluorescence was instead punctate, with the majority located in smaller, vesicular structures (Figures 2e and 2f). These structures possessed dimensions (200–1000 nm diameter) compatible with the dimensions of secretory granules [25], were distributed randomly throughout the cytosol, and were mobile, as monitored by time-lapse confocal microscopy (Figures 2, g–i).

Immunolocalization of chimaeras between GFP* and insulin

In order to determine more precisely the localization of the chimaeric constructs in INS-1 cells, we performed immunocytochemical analyses of fixed and permeabilized cells using markers of three different compartments of the regulated secretory pathway. The first of these was the ER marker, calreticulin. Comparison of the distribution of calreticulin with a polyclonal anti-calreticulum antibody (revealed with a TRITC-conjugated secondary antibody) revealed essentially complete co-localization with that of the incorporated c-myc epitope tag of sp.[GFP*].B.C.A.myc (revealed with monoclonal antibody 9E:10; Figure 3, a–c); intrinsic GFP* fluorescence from the chimaera was lost under the permeabilization and fixation conditions used in these experiments (results not shown). In these cells, colocalization with the TGN marker, TGN38 (revealed with fluorescently conjugated secondary antibodies) was poor (Figure 3, d–f). In contrast, in cells displaying more punctate GFP* fluorescence *in vivo* (pattern 2, Figures 2e and 2f), subsequent permeabilization and fixation revealed greatly increased co-localization with the TGN (Figure 3, g–i). Since direct co-localization with endogenous insulin was not possible, due to the presence of the insulin sequence within the recombinant chimaera, we next probed cells displaying pattern 2 fluorescence with the dense-core secretory protein marker, phogrin. Phogrin (phosphatase on the granule of insulinoma cells) [14] was localized in INS-1 cells exclusively to insulin-containing vesicles (Figure 3, j–l). In sp.[GFP*].B.C.A.myc-expressing cells displaying punctate (pattern 2) fluorescence, typically 40–50 % of the chimaera was co-localized with phogrin (either endogenous or overexpressed; Figure 3, m–o), demonstrating correct targeting to the regulated secretory pathway. Nevertheless, phogrin-positive, sp.[GFP*].B.C.A.myc-negative, and sp.[GFP*].B.C.A.myc-positive, phogrin-negative, vesicular structures were also apparent (Figure 3o). The nature of these vesicles is uncertain, but they may represent vesicles of the constitutive secretory pathway.

In an attempt to determine whether chimaera sp.[GFP*].B.C.A.myc entered the constitutive, as well as the regulated, secretory pathway, we endeavoured to measure release of GFP* fluorescence into the medium. No fluorescence over background could be detected, even after 12 h incubation, in the presence of stimulatory concentrations (30 mM) of glucose, indicating that neither constitutive nor regulated secretion could be detected by this method. These measurements are limited, however, by: (i) the unavoidable presence of fluorophores in the

Figure 3 Localization of proinsulin chimaeras in distinct compartments

Cells were transiently transfected with the constructs shown and fixed with 4% paraformaldehyde as described in the Methods section. After probing with the primary antibodies indicated, localization was revealed with FITC-conjugated (a, d, g, j, m, b, s), or TRITC-conjugated (b, e, h, k, n, p, q, t) secondary antibodies. Optical slices (0.5 μ m in depth) were obtained by confocal analysis with an upright confocal microscope (Leica TCS 4D/DM IRBE; see under 'Methods') using a 40 $\times$ oil-immersion objective (Leica PL APO 1.32 NA). Digital overlays of the images were performed using on-board software (Leica TCS-NT). Yellow coloration indicates close co-localization of the two proteins examined. (a–c) Cells were transfected with sp.[GFP*].B.C.A.myc before probing with antibodies: (a) 9E:10, 1:1000 dilution; (b) anti-calreticulin antibody (1:100); (c) overlay of (a) and (b); (d–f) as (a–c), probing with: (d) 9E:10; (e) anti-TGN38

Table 1 Effect of glucose on the release of firefly and *R. reniformis* luciferase activities from INS-1 cell populations

Cells were incubated in Krebs–Ringer bicarbonate buffer (see legend to Figure 2) for the indicated times before the assay of firefly and *R. reniformis* luciferase activities, as given in the Methods section. Data are the mean of eight separate experiments. * $p < 0.05$ for the effect of glucose. Release of total immunoreactive insulin was determined as given in the Methods section and was the mean of two experiments which agreed to within 10%.

[Glucose] (mM)	Time ... 2 h			4 h		
	Ratio†		Insulin release (ng/well)	Ratio†		Insulin release (ng/well)
	Medium	Cells		Medium	Cells	
3	0.76 ± 0.27	1.34 ± 0.29	11.1	0.71 ± 0.19	1.18 ± 0.19	17.0
30	1.17 ± 0.29*	1.11 ± 0.31	36.3	1.02 ± 0.270	1.18 ± 0.18	39.2

† $10^2 \times$ ratio of firefly:*R. reniformis* luciferase activity.

medium used for the incubations; and (ii) the fact that only a small proportion of the total cell population (typically 5–10%) expressed significant levels of fluorescence.

Expression of chimaeras between firefly luciferase and insulin

In order to monitor the secretion of a chimaera between proinsulin and a GFP* surrogate, we prepared an analogous chimaera between preproinsulin and a modified version of the photoprotein luciferase, which lacked a peroxisomal targeting motif [15]. This offered the possibility of detecting very few molecules of the resultant chimaera (< 2000) released into the medium, by using bioluminescence as opposed to fluorescence measurements. Since modification of the C-terminus of firefly luciferase can interfere with the luminescence properties of this protein [26] we fused preproinsulin at the C-terminus, via a 12-amino-acid *c-myc* epitope tag (sp.B.C.A.*myc*[Luc]; Figure 1d). In contrast to the modified (cytosolically targeted) luciferase (Figure 3p) and to the analogous GFP* chimaera (sp.B.C.A.[GFP*]; Figure 2c), the sp.B.C.A.*myc*[Luc] displayed either clear reticular localization (and nuclear exclusion; 295/310 cells examined; pattern 1; Figure 3r) or the punctate distribution (Figure 3q) similar to that observed in cells expressing the GFP* chimaera, sp.[GFP*].B.C.A.*myc* (see Figures 2e and 2f; pattern 2, 15/310, 4.8%). Essentially identical results were also obtained using monoclonal antibody 9E:10 to the *c-myc* epitope tag (results not shown), confirming that the fusion protein remained intact. Figure 3(s–u) revealed the partial co-localization of luciferase immunoreactivity with that of phogrin in a cell displaying pattern 2 distribution. With cells showing either pattern 1 or pattern 2 distribution, correct folding of luciferase was apparently preserved in intact cells, since these displayed easily detectable luminescence (imaged according to [19]) in the presence of 1 mM luciferin (results not shown). In order to determine whether this chimaera was targeted to the constitutive or regulated secretory pathway, we co-transfected INS-1 cells with sp.B.C.A.*myc*[Luc]

and with cDNA encoding the distinct, cytosolically targeted and coelenterazine-utilizing luciferase from the sea pansy, *R. reniformis* [27]. By this approach, measurement of released firefly luciferase activity, using the substrate luciferin, could be normalized for cell leakage by measuring the release of *R. reniformis* into the medium. In cells maintained at low (3 mM) glucose, the ratio of firefly:*R. reniformis* luciferase activities was lower in the culture medium than in the cells (Table 1), with the release of either protein into the medium at either 2 h or 4 h corresponding to less than 5% of the amount of either in the cells (results not shown). These data indicated that, at low glucose concentrations (3 mM), firefly luciferase was not released into the medium via the constitutive secretory pathway. However, a significant increase in the ratio between the two proteins was apparent in the medium when cells were maintained at a high concentration of glucose (30 mM; Table 1). In contrast, there was no change in the ratio of firefly:*R. reniformis* luciferase activity in the cell fraction compared with cells maintained at 3.0 mM glucose (Table 1). The release of total insulin (measured by ELISA) was 3.5-fold (at 2 h) and 2.1-fold (at 4 h) higher in cells maintained at 30 mM glucose than in cells maintained in 3 mM glucose for the same period (Table 1). These data indicate that the high glucose concentration stimulated release of the sp.B.C.A.*myc*[Luc] chimaera via the regulated secretory pathway.

DISCUSSION

We describe here the targeting of a thermostable mutant of GFP, and of enhanced firefly luciferase, to insulin-containing granules of the islet β -cell line INS-1. Optimally, up to 50% of the phogrin-positive dense-core granules within a single cell could be shown also to contain the insulin–GFP chimaera, sp.[GFP*].B.C.A.*myc* (Figure 3o), or the luciferase chimaera, sp.B.C.A.*myc*[Luc] (Figure 3u). This proportion is comparable with, or better than, that observed with chromograninB.GFP in PC12 cells [4]. By using the fusion construct between preproinsulin and the GFP mutant we have been able to perform dynamic imaging of insulin secretory granules in real time and in single living cells (Figure 2, g–i). Similarly, by fusing preproinsulin and firefly luciferase we have been able to record stimulated exocytosis by the release of the chemiluminescent chimaera (Table 1).

With both the GFP* and luciferase chimaeras, targeting to the regulated, rather than the constitutive, secretory pathway, was observed. This was demonstrated either by co-localization with the dense-core secretory granule marker, phogrin (sp[GFP].B.C.A.*myc*, Figure 3o; sp.B.C.A.*myc*[Luc], Figure

(1:100); (f) overlay of (d) and (e); (g–i) as (d–f), but with a cell displaying pattern 2 (punctate) fluorescence; (j–l) transfection with pCDNA3.phogrin, probing with: (j) anti-insulin (1:100) and (k) anti-phogrin (1:100) antibodies; (l) overlay of (j) and (k); (m–o) sp.[GFP*].B.C.A.*myc*, probing with (m) 9E:100 and (n) anti-phogrin; (o) overlay of (m) and (n); (p) modified (untargeted) luciferase, probing with anti-luciferase antibody; (q, r) sp.B.C.A.*myc*[Luc], probing with anti-luciferase; (q) pattern 2 and (r) pattern 1 distributions; (s–u) sp.B.C.A.*myc*[Luc] and pCDNA3.phogrin, probing with (s) 9E:10; (t) anti-phogrin antibody; (u) overlay of (s) and (t).

3u), or by regulated secretion firefly luciferase bioluminescence into the medium (sp.B.C.A. *myc*.[Luc], Table 1). In the latter case, the absence at low glucose concentrations of release in excess of that of a cytosolic protein indicated exclusion from the constitutive secretory pathway. In contrast, the ability of glucose to provoke release of sp.B.C.A.*myc*.[Luc] was consistent with the localization of the protein to the regulated pathway. In the presence of stimulatory glucose concentrations, the rate of release of sp.B.C.A. *myc*.[Luc] appeared greater during a 2 h rather than a 4 h incubation, in contrast with the sustained release of native insulin. These data presumably indicate the more rapid depletion of the luciferase chimaera relative to total insulin, consistent with the limited co-localization of the chimaera with the native hormone.

All of the above data indicate that the extended insulin molecules are still able to interact with the targeting machinery of the regulated secretory machinery, provided the chimaeras fold properly and escape the ER. Whether this machinery consists of a receptor localized on the inner surface of the TGN [10], or simply other co-aggregating proinsulin molecules [8], cannot be readily distinguished by these data in insulin-secreting cells. Thus, it seems possible that incorporation of the insulin/photoprotein chimaeras into larger aggregates, containing predominantly native proinsulin, could occur, whereas aggregates containing only the chimaera would be unfeasible. By this argument, other data suggest that aggregation may indeed be an important requirement for correct targeting to the regulated pathway in other cell types. In particular, we have so far failed to observe co-localization of chimaera sp.[GFP*].B.C.A.*myc* and the dense-core granule marker, dopamine β -hydroxylase, after expression in PC12 cells (results not shown). In these non-insulin producing cells, co-aggregation between individual sp[GFP*].B.C.A.*myc* molecules would seem unlikely (see above).

With each of our chimaeric reporters, correct targeting to secretory granules after fusion with insulin was a relatively rare event (see Figures 2 and 3). Thus reticular distribution, shown with the GFP* chimaera to correspond to ER localization (Figure 3c), was observed in 87 % of transfected cells. The most likely explanation is the misfolding of the insulin moiety of the fusion construct, and therefore retention in the ER by interaction with molecular chaperones, including BiP/Kar2p. At present we have no clear explanation for the fact that the targeting was essentially quantal, i.e. within individual cells the chimaeras were confined either predominantly to a reticular (ER) location, or largely escaped retention in the ER and entered the distal part of the secretory pathway including the TGN and secretory granules (Figures 3i and 3o). We have considered the possibility that this may be a cell-cycle-related phenomenon. However, this seems unlikely given that in six out of 58 separate, non-synchronized, cultures of sp.[GFP*].B.C.A.*myc*-transfected INS-1 cells, essentially 100 % of the fluorescent cells displayed entirely punctate fluorescence. An alternative possibility may be that the level of expression of the chimaeras, with respect to other cellular proteins involved in folding and disulphide-bond formation in the ER, is critical.

The reason for the complete mistargeting of the chimaera sp.B.C.A.[GFP*] to the cytosol and nucleus is unclear. One possibility is that this may result from severe misfolding of the protein, causing its expulsion from the ER by a reversal of the normal protein-import mechanism [28]. That this mistargeting did not occur with the corresponding luciferase chimaera (sp.B.C.A.*myc*.[Luc]) may be attributable to the presence of the *c-myc* epitope tag between the end of the insulin A-chain and luciferase. The encoded 12-amino-acid sequence may well act as an effective 'spacer', allowing folding and disulphide-bond

formation within the proinsulin moiety, (relatively) unhindered by the bulky photoprotein. It might be predicted that the presence of such a spacer peptide, C-terminal to GFP*, may enhance the probability of correct targeting of the sp.[GFP*].B.C.A.*myc* construct to distal parts of the secretory pathway (beyond the ER) and to regulated secretory granules. Similarly, the luciferase chimaera sp.B.C.A.*myc*.[Luc] would seem to be amenable to further engineering, including the lengthening (or shortening) of the *c-myc* spacer, as well as the alteration of its amino acid composition and sequence. Such further engineering of the GFP* chimaeras may therefore lead ultimately to their exclusive localization within regulated secretory granules.

Conclusions

Use of secretory-granule targeting of firefly luciferase and GFP* to monitor secretion

At the level of cell populations, the insulin-luciferase chimaera described in this study should provide a convenient system in which secretion can be monitored selectively from cells co-transfected with cDNAs encoding proteins which may be involved in the control of secretion. The secreted luciferase-insulin can be assayed rapidly and inexpensively with the use of commercially available kits, and does not require lengthy immunoadsorption steps as demanded by ELISA or radioimmunoassays.

The ability to monitor the position of individual secretory granules in single living cells using GFP*-proinsulin chimaeras should provide a powerful *in vivo* system with which to examine the molecular basis of regulated exocytosis. This will offer several advantages over methods that are currently employed to measure secretion from single cells, such as the use of lipophilic fluorescent dyes [29], the reverse haemolytic plaque assay [30] and capacitance measurements [31]. Importantly, the ability to follow secretion at the level of individual living cells provides a means by which an almost unlimited array of molecular species (antibodies, inhibitory peptides, dominant negative cDNAs or antisense RNAs, buffered Ca^{2+}) can be used to study the process, since these can be microinjected directly into the cell cytosol [19]. For example, this should enable a careful analysis of the role of numerous proteins (syntaxin, synaptobrevin, SNAP25, etc.) which have been implicated in the control of vesicle fusion with the plasma membrane [32]. Furthermore, the ability to monitor the fate of an individual granule as it approaches and fuses with the plasma membrane should also illuminate the kinetic details of the process, and allow examination of the extent to which individual granules respond during the activation of secretion.

Use of secretory-granule targeting of firefly luciferase to measure intragranular ATP concentration in living cells

Localization of firefly luciferase to secretory granules may in principle provide a convenient means of imaging changes in ATP concentration in this compartment of living cells. This is likely to be of particular importance in the β -cell where (cytosolic) ATP levels are likely to play a crucial role in coupling nutrient metabolism and regulated secretion [33]. However, measurements with isolated secretory granules suggest that ATP concentrations are likely to be relatively high in this compartment (i.e. in the low mM range) [34], whereas native luciferase has a K_m for ATP of approximately 10 μM [35]. Under normal physiological conditions such *in vivo* ATP measurements are therefore likely to require the use of an engineered luciferase, with a K_m for ATP considerably higher than that of the native enzyme.

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