The sorting of proglucagon to secretory granules is mediated by carboxypeptidase E and intrinsic sorting signals

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Abstract

Proglucagon is expressed in pancreatic alpha cells, intestinal L cells and brainstem neurons. Tissue-specific processing of proglucagon yields the peptide hormones glucagon in the alpha cell and glucagon-like peptide (GLP)-1 and GLP-2 in L cells. Both glucagon and GLP-1 are secreted in response to nutritional status and are critical for regulating glycaemia. The sorting of proglucagon to the dense-core secretory granules of the regulated secretory pathway is essential for the appropriate secretion of glucagon and GLP-1. We examined the roles of carboxypeptidase E (CPE), a prohormone sorting receptor, the processing enzymes PC1/3 and PC2 and putative intrinsic sorting signals in proglucagon sorting. In Neuro 2a cells that lacked CPE, PC1/3 and PC2, proglucagon co-localised with the Golgi marker p115 as determined by quantitative immunofluorescence microscopy. Expression of CPE, but not of PC1/3 or PC2, enhanced proglucagon sorting to granules. siRNA-mediated knockdown of *CPE* disrupted regulated secretion of glucagon from pancreatic-derived alphaTC1–6 cells, but not of GLP-1 from intestinal cell-derived GLUTag cells. Mutation of the PC cleavage site K70R71, the dibasic R17R18 site within glucagon or the alpha-helix of glucagon, all significantly affected the sub-cellular localisation of proglucagon. Protein modelling revealed that alpha helices corresponding to glucagon, GLP-1 and GLP-2, are arranged within a disordered structure, suggesting some flexibility in the sorting mechanism. We conclude that there are multiple mechanisms for sorting proglucagon to the regulated secretory pathway, including a role for CPE in pancreatic alpha cells, initial cleavage at K70R71 and multiple sorting signals.

Key Words
- proglucagon
- carboxypeptidase E
- sorting
- glucagon
- GLP-1
- prohormone processing

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Introduction

Proglucagon is a prohormone that is expressed in the alpha cells of the pancreas, the L cells of the intestine and in select neurons of the brainstem. In the pancreatic alpha cell, PC2 cleaves proglucagon to yield glucagon (Rouille *et al*. 1994, Furuta *et al*. 2001). By contrast, proglucagon is processed to glucagon-like peptide (GLP)-1 and GLP-2 in the intestine and brain by PC1/3 (Dhanvantari *et al*. 1996, Dhanvantari & Brubaker 1998, Damholt *et al*. 1999).

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Glucagon is secreted primarily in response to low blood glucose levels, and maintains euglycaemia by stimulating hepatic glycogenolysis and gluconeogenesis (Jiang & Zhang 2003). By contrast, GLP-1 is secreted in response to nutrient ingestion, and enhances glucose-stimulated insulin secretion (Drucker 2007). Therefore, both hormones are critical in maintaining glucose homoeostasis in response to nutritional status. While mechanisms of exocytosis of glucagon and GLP-1 have been extensively studied (Tolhurst *et al.* 2009, Gonzalez-Velez *et al.* 2012), and the regulation of biosynthesis and processing of proglucagon continue to be investigated (Cawley *et al.* 2011, Helwig *et al.* 2011, Whalley *et al.* 2011), the mechanisms that govern the sorting of proglucagon into secretory granules are yet to be identified.

Endocrine and neuroendocrine cells are characterised by the presence of a regulated secretory pathway, with specific intracellular compartments and associated proteins for the sorting, processing and storage of peptide hormones. Peptide hormones are first synthesised as larger precursors, or prohormones, and are selectively targeted to the regulated secretory pathway via the *trans*-Golgi network (TGN). Proteins destined for the regulated secretory pathway are separated from constitutive and lysosomal proteins and are packaged into budding immature secretory granules. The process of sorting can occur at the TGN through interaction with a membrane-bound sorting receptor, or with membrane lipids, via a sorting signal within the prohormone. Alternatively, sorting can occur by selective retention of prohormones within the immature secretory granule, while other proteins are removed. Prohormones then undergo endoproteolysis to yield their constituent peptide hormones, which are then stored in mature, dense-core secretory granules until secretion is triggered in a Ca^{2+}-dependent manner.

Some clues to how proglucagon is sorted are provided by the proteins expressed in the granules of alpha and L cells, and by the primary and secondary structures of proglucagon. The prohormone processing enzyme carboxypeptidase E (CPE) is expressed in both alpha and L cells, while PC1/3 is found in L cells and PC2 in alpha cells. Several lines of evidence support the hypothesis that the membrane-bound forms of CPE and PC1/3 can direct prohormones to the regulated secretory pathway, thus acting as receptors or tethers. Depletion of CPE disrupts the regulated secretion of proopiomelanocortin (POMC), proenkephalin and proinsulin in cells in culture (Normant & Loh 1998, Dhanvantari *et al.* 2003*b*), and in the CPE^{fat} mouse, an obese mouse model in which CPE is degraded in the pituitary, POMC is secreted constitutively (Cool *et al.* 1997, Shen & Loh 1997). The presence of full-length PC1/3 with its C-terminal region intact is required for the processing of prorenin to renin in granules in AtT-20 cells (Jutras *et al.* 1997). Without its lipid-binding C-terminal region (Jutras *et al.* 2000, Arnaoutova *et al.* 2003), PC1/3 is not sorted to the regulated secretory pathway, and renin is secreted constitutively. PC2 also contains an alpha-helical, lipid-binding C-terminal tail that directs it to the regulated pathway (Assadi *et al.* 2004), thus enabling it to act as a membrane-bound receptor/tether. However, no evidence to date exists to support a role for PC2 in the sorting of prohormones to the regulated secretory pathway.

Additionally, the structures of the major proglucagon-derived peptides predict some known sorting signals. The structure of glucagon is that of an alpha-helix, as revealed by X-ray crystallography (Sasaki *et al.* 1975) and the nuclear magnetic resonance solution structure of GLP-1 also predicts an alpha-helix (Chang *et al.* 2002). Sequence similarity suggests that GLP-2 is also an alpha-helix, and it has been suggested that the structure of the entire proglucagon molecule may form a 'trimer' of alpha helices (Dey *et al.* 2004). Conserved alpha-helix structures within the amino-terminal end of prosomatostatin (Mouchantaf *et al.* 2001) and the C-terminal ends of PC1/3 (Jutras *et al.* 2000, Arnaoutova *et al.* 2003), PC2 (Assadi *et al.* 2004), PC5/6a (Dikeakos *et al.* 2007) and CPE (Dhanvantari *et al.* 2003*a*, Zhang *et al.* 2003) are critical in targeting these proteins to secretory granules. Additionally, the sequence of glucagon contains an internal pair of basic amino acids, Arg17Arg18, which does not serve as a PC1/3 or PC2 cleavage site (Bataille 2007). Sequences of basic residues within prorenin (Brechler *et al.* 1996), proneurotensin (Feliciangeli *et al.* 2001), proneuropeptide Y (Brakch *et al.* 2002) progastrin (Bundgaard *et al.* 2004) and VGF (Garcia *et al.* 2005) have been identified as sorting signals for these prohormones. Therefore, proglucagon may contain a number of sorting signals to direct it into granules. In the present study, we investigated the requirement of the processing enzymes CPE, PC1/3 and PC2, and of intrinsic sorting signals, for the sorting of proglucagon to secretory granules.

Materials and methods

Cell culture, plasmids and transfections

Wild-type Neuro 2a (N2a wt) cells and Neuro 2a cells stably transfected with mouse *Cpe* (N2a-CPE, clone 17) were obtained from Dr Y P Loh (Bethesda, MD, USA). Cells

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were maintained in DMEM containing 10% FBS and stable transfectants were maintained in media containing 400 μg/ml G418. N2a wt cells were also stably transfected with the enzymatically inactive form of CPE, E300Q (Qian *et al*. 1999) using Lipofectamine 2000 (Invitrogen). Stable transfectants were selected in 800 μg/ml G418, pooled and maintained in 400 μg/ml G418. To examine the role of CPE in sorting proglucagon, N2a wt, N2a-CPE and N2a-E300Q cells were transfected with hamster pre-proglucagon (in pcDNA 3.1; a kind gift from Dr D F Steiner, Chicago, IL, USA). To examine the roles of other prohormone convertases, both N2a wt and N2a-CPE cells were transiently transfected with plasmids encoding PC1/3 and PC2 (kind gifts from Dr N G Seidah, Montreal, QC, Canada). To determine possible sorting signals, the sequence of proglucagon was mutated independently at three sites: at the processing site, R70K71; at the dibasic site within glucagon, R17R18; and at two leucines that were postulated to flank the helix structure within glucagon, L14 and L26. The processing site mutant, K71Q, was a kind gift from Dr D F Steiner (Chicago, IL, USA). We generated the mutation at the dibasic site, R18Q (forward primer sequence 5′-AAATACCTGGACTCCCGC-**CAA**GCCCAAGAT-3′), and the double leucine-to-proline mutation was done in two steps; L14P was made using forward primer 1, 5′-TACAGCAAATAC**CCG**GACTCCCG-CCGAGCC-3′; and L26P was subsequently generated using forward primer 2, 5′-CAAGATTTTGTGCAGTGG**CC-G**ATGAACACC-3′. Bold sequences in primers indicate site of mutation. All site-directed mutagenesis reactions were carried out using the QuikChange II Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA, USA) and results were confirmed by sequencing at the London Regional Genomics Facility, University of Western Ontario.

Immunocytochemistry

Cells were grown on coverslips, and processed for immunocytochemistry as previously described (McGirr *et al*. 2005). Slides were incubated with antibodies against glucagon (1:1000; Bachem/Peninsula Laboratories, Torrance, CA, USA) and the Golgi marker p115 (1:50; Transduction Laboratories, San Jose, CA, USA) or the secretory granule marker chromogranin A (CgA; 1:50, Abcam). The anti-glucagon antibody was raised against the entire sequence of glucagon, and thus recognises unprocessed proglucagon, glicentin, oxyntomodulin and glucagon. To examine CPE immunoreactivity, slides were incubated with the CPE 7–6 antibody (kind gift from Dr Y P Loh, Bethesda, MD, USA), which recognises the C-terminus of CPE. Alexa 488-IgG (Invitrogen) was used to visualise the glucagon or CPE antibody, and Alexa 594-IgG for the p115 or CgA antibody.

Image acquisition and analysis

Cells were visualised with an Olympus IX81 widefield fluorescence microscope. Image acquisition was carried out using In Vivo software. Ten optical sections per cell were collected in 0.2 μm steps covering the *z*-axis field, using a 60× oil immersion objective lens. Cell images were processed using a three-dimensional (3D) blind deconvolution algorithm provided in Image-Pro Plus software (Media Cybernetics, Inc., Rockville, MD, USA).

Image analysis using Pearson's correlation coefficient

Image analyses were conducted using FIJI version 1.46 h (Schindelin 2008), a distribution of ImageJ (NIH, Bethesda, MD, USA). Co-localisation was quantified using the Co-localisation 2 plugin within FIJI. Regions of interest were manually drawn around distinct single- or multi-cell bodies from pseudo-coloured red and green fluorescence images, excluding as much background as possible. The segmented region was defined by a binary mask and applied to both red and green images. The plugin uses an iterative Costes' threshold algorithm (Costes *et al*. 2004) to simultaneously determine maximum threshold of intensity for each colour, above which pixels are considered to be statistically correlated. Red and green image pixels were then used to calculate the Pearson's correlation coefficient (PCC) (*r*) according to Equation 1:

$$r = \frac{\sum_j \left((Sc1_j - S1_{av}) \times (Sc2_j - S2_{av})\right)}{\sqrt{\sum_i (S1_i - S1_{av})^2) \times \sum_i (S2_i - S2_{av})^2}}$$

Briefly, i represents the i-th pixel in the region of interest, S_c1 represents signal intensity of the co-localised pixel from the first channel and S_c2 represents the second channel, $S1$ and $S2$ represent signal intensities of all pixels regardless of co-localisation, and $S1_{av}$ and $S2_{av}$ represent the average pixel intensity of each channel. Note the numerator is dependent on pixels above the Costes auto threshold, while the denominator and mean pixel values use all the pixels in the region of interest. Correlation coefficients for each experiment were treated as one experimental dataset and differences were determined using a one-way ANOVA followed by Tukey's multiple comparison test. Significance was set at $P>0.05$. n values ranged from 10 to 25. The PCC was validated in INS-1 832/13 cells (a kind gift from Dr C B Newgard, Raleigh-Durham, NC, USA) using markers with a

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known low degree of co-localisation (insulin and pdx-1) and a high degree of co-localisation (insulin and CgA) (Supplementary Figures 1 and 2, see section on supplementary data given at the end of this article).

Western blot

For western blot analysis, all cells were grown in six-well plates. Cells were lysed, and protein was extracted, quantified and separated on 12% SDS–PAGE gels as described previously (McGirr *et al.* 2005). Proteins were transferred onto nitrocellulose membranes, which were probed with the CPE 4–5 antibody (kind gift from Dr Y P Loh, Bethesda, MD, USA), or antibodies against PC1/3 or PC2 (kind gifts from Dr N G Seidah, Montreal, QC, Canada) and bands were visualised with SuperSignal Chemiluminescent system as described previously (McGirr *et al.* 2005). To detect secreted CPE, media were collected and protein was concentrated through evaporation. The resulting residue was resuspended in loading buffer and the entire sample was loaded into one well. These samples migrated more slowly through the gel, resulting in an apparent M_r of 60 kDa.

Secretion experiments

AlphaTC1–6 cells (a kind gift from C B Verchere, Vancouver, BC, Canada) were maintained in DMEM containing 15% horse serum and 2.5% FBS. GLUTag cells (a kind gift from D J Drucker, Toronto, ON, Canada) were grown in low-glucose DMEM containing 10% FBS. For stimulated secretion experiments, αTC1–6 cells were seeded in 24-well plates in replicates of six and pre-incubated in HBSS containing 25 mM glucose for 1 h. Cells were then incubated in HBSS containing 1 mM glucose and 10 μM each of forskolin and IBMX for 1 h. GLUTag cells were incubated in low-glucose (5.5 mM) DMEM +0.5% FBS without (basal) or with (stimulated) 10 μM each of forskolin and IBMX for 2 h. After all secretion experiments, media were collected and trifluoroacetic acid (TFA) was added to 0.1%. Cells were rinsed twice in HBSS, and scraped in 1 ml homogenisation buffer (1 M HCl, 1 M formic acid, 1% (v/v) TFA, 1% (w/v) NaCl). The cells were sonicated in one 12 s burst, centrifuged and the supernatant was collected, and both media and cell extracts were passed through a Sep-Pak C18 reverse-phase cartridge to elute proglucagon-derived peptides as previously described (Dhanvantari *et al.* 1996, Dhanvantari & Brubaker 1998). Glucagon and GLP-1 content were assessed by RIA using the glucagon and GLP-1 RIA kits (Linco/EMD Millipore, Bedford, MA, USA).

Design of siRNA

To deplete CPE in αTC1–6 and GLUTag cells, siRNAs were synthesised using the *Silencer* siRNA Construction Kit (Ambion/Life Technologies, Carlsbad, CA, USA). Target sequences within the rat *Cpe* mRNA sequence were selected using an algorithm on Ambion's website, and a search of the mouse genome with BLAST showed that seven of the selected targets were not homologous with any other known mouse mRNAs. siRNAs were synthesised against these seven targets and transfected into both cell lines, which were then screened for the extent of CPE depletion by western blot analysis of cell lysates using the CPE 4–5 antibody (a gift from Dr Y P Loh, Bethesda, MD, USA) (Supplementary Figure 3, see section on supplementary data given at the end of this article). One siRNA, spanning nucleotides 1408–1428 (CPE73), significantly and reproducibly decreased CPE levels in both cell lines, and was used in subsequent secretion experiments. The negative control was Ambion Silencer Negative Control #1, with no known targets in the mouse genome. In all subsequent experiments, 200 nM of control siRNA or CPE73 siRNA were transfected using Oligofectamine (Invitrogen) for 72 h prior to secretion experiments or fixation for immunofluorescence microscopy.

Protein modelling

The full-length proglucagon polypeptide sequence was submitted for *de novo* 3D protein structure prediction using the Robetta 3D modelling web server (Kim *et al.* 2004). The 3D models developed by Robetta used the incretin hormone, gastric inhibitory peptide (GIP; PDB:2B4N) as a reference parent. The Ginzu module (Chivian *et al.* 2005) parsed proglucagon into two domains, each using GIP as a reference parent. The N-terminal domain (aa 1–77) (PDB:2B4N) had a confidence of 5.40 by position-specific iterated BLAST (PSI-BLAST; Altschul *et al.* 1990); the C-terminal domain (aa 78–158) (PDB:2OBU) had a confidence of 2.33 by HH-Search (Soding 2005).

Results

The role of CPE in proglucagon sorting

Since CPE has been identified as a sorting receptor for several other prohormones, we investigated a possible role for CPE in sorting proglucagon to secretory granules. There was no CPE immunoreactivity in N2a-wt cells; it was detected only after transfection of CPE (Fig. 1A). In N2a-wt

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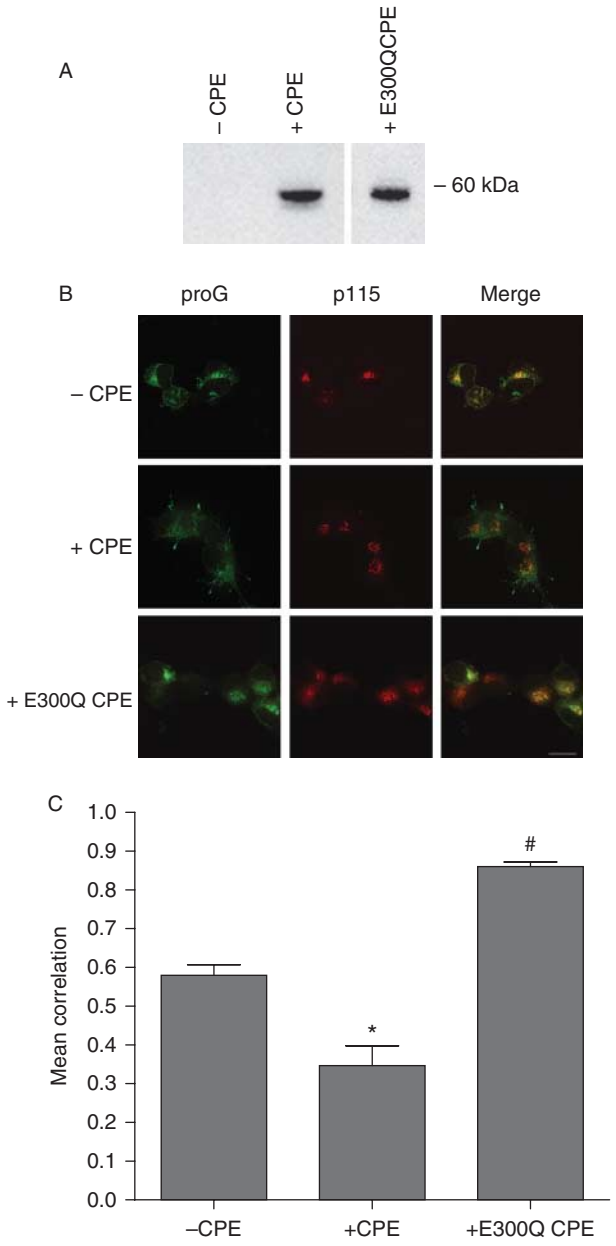

Figure 1
CPE plays a role in the sorting of proglucagon. (A) Western blot analysis of cell extracts from Neuro2A cells lacking CPE expression, and after transfection of wt or E300Q mutant *Cpe*. (B) Full-length proglucagon was transfected into N2a cells, either alone (−CPE), or co-transfected with CPE or E300Q mutant *Cpe*. Cells were processed using antibodies against glucagon (green) and the Golgi protein p115 (red). Representative images are shown. Scale bar represents 10 μm. (C) ImageJ software was used to determine the fluorescence intensity co-variance (PCC) of proglucagon and p115. Proglucagon showed a significant decrease in mean correlation with p115 when co-expressed with CPE, and a significant increase when co-expressed with the E300Q mutant *Cpe*. Values are means ± S.E.M. ($n=10$–25). *$P<0.01$ vs −CPE; #$P<0.01$ vs both cells with and without CPE. Full colour version of this figure available via http://dx.doi.org/10.1530/JOE-12-0468.

cells, proglucagon immunoreactivity associated strongly with that of the Golgi marker, p115 (Fig. 1B). By contrast, overexpression of CPE resulted in more punctate staining of proglucagon in the processes of the cells, and much weaker co-localisation with p115 (Fig. 1B). PCC analysis showed a significant ($P<0.01$) decrease in the co-localisation of proglucagon with p115 in the presence of CPE (Fig. 1C), suggesting that CPE expression was associated with sorting proglucagon to granules. To confirm that proglucagon was actually in the secretory granule compartment, N2a-CPE cells were transfected with proglucagon-EGFP (enhanced green fluorescent protein) and co-immunostained with antibodies against CgA and EGFP (Supplementary Figure 4, see section on supplementary data given at the end of this article). Proglucagon immunoreactivity co-localised with that of CgA in punctate structures in the cell processes, and the PCC was significant ($r=0.84\pm0.01$, $n=6$).

Proglucagon sorting is dependent on properly sorted CPE

In order to further investigate the role of CPE in sorting proglucagon, N2a-wt cells were transfected with the inactive E300Q mutant of *Cpe* (Fig. 1A). In these cells, proglucagon was co-localised with p115 and the PCC was actually significantly ($P<0.01$) greater than that of N2a-wt cells (Fig. 1C). We then examined the trafficking of the *Cpe*-E300Q mutant, as we hypothesised that the sorting of the mutant itself may be impaired, and this could explain its effect on proglucagon sorting. When compared with wt CPE, which had a weak Golgi signal and showed strong immunofluorescence in the tips of the cell processes, the E300Q mutant instead yielded a robust signal in the Golgi (Fig. 2A). Additionally, wt CPE was secreted, while E300Q could only be detected in the cells and not in the media (Fig. 2B), therefore suggesting that E300Q CPE is not properly sorted in N2a cells.

The role of PC1/3 and PC2 in proglucagon sorting

We explored the roles of the other neuroendocrine-specific prohormone convertases, PC1/3 and PC2, in the sorting of proglucagon, since these two enzymes are expressed in the L cell and alpha cell respectively along with proglucagon. We investigated if these other processing enzymes could sort proglucagon alone, or if they could enhance CPE-mediated sorting. Neither PC1/3 nor PC2 were endogenously expressed in N2a-wt cells (Fig. 3A). Cells were transfected with PC1/3 or PC2 alone or in combination with CPE. Figure 3A shows that when PC1/3 was expressed in the absence of CPE, one 84 kDa

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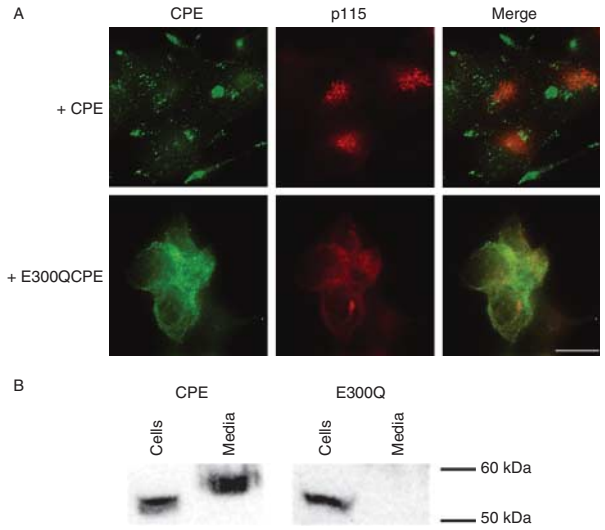

Figure 2
The E300Q mutant *Cpe* is not sorted efficiently to secretory granules. (A) In cells expressing wt *CPE*, punctate staining along the tips of the cell processes indicates presence in secretory granules. In cells expressing E300Q, CPE immunofluorescence largely coincides with that of p115. (B) Wild-type CPE is secreted from N2a cells, while E300Q mutant *Cpe* is not present in the media, further indicating that is it not transiting through the secretory pathway in N2a cells. The apparent increase in molecular weight of the secreted form is due to slower migration through the gel, as stated in Materials and methods. Full colour version of this figure available via http://dx.doi.org/10.1530/JOE-12-0468.

form was present; upon CPE expression, two forms of PC1/3, an 84 kDa form and a 66 kDa form, were detected. The expression and processing of PC2 were not changed in the presence of CPE. When either PC1/3 or PC2 were expressed alone, proglucagon appeared mainly in the Golgi, and the PCC was not significantly different from that of N2a-wt cells (Fig. 3C). Interestingly, proglucagon immunoreactivity appeared in the cell processes, indicative of granule localisation, in the presence of both CPE and PC1/3 (Fig. 3B), and the PCC significantly decreased from the value of cells expressing PC1/3 alone (Fig. 3C). However, the correlation between proglucagon and p115 localisation remained unchanged from N2a wt cells when both CPE and PC2 were expressed (Fig. 3C).

CPE affects proglucagon sorting in alpha cells, but not L cells

In order to examine the role of CPE in proglucagon sorting in a physiological context, we used two proglucagon-expressing cell lines: alphaTC1–6 cells, derived from mouse glucagonoma, and GLUTag cells, derived from mouse enteroendocrine tumours. In alphaTC1–6 cells, when *CPE* expression was knocked down by 61±3% ($n=3$, $P<0.05$) using CPE73 siRNA, normal stimulated secretion of glucagon was inhibited, and basal secretion increased significantly (Fig. 4A and B). Cell content decreased slightly, indicating that the absence of CPE may have affected glucagon synthesis as well as secretion (Fig. 4C). The transfection efficiency, as assessed by EGFP fluorescence, was 36.6±3.7% (calculated from ten images, each with ~50 cells). By contrast, regulated secretion of GLP-1 in GLUTag cells remained intact after knockdown of *CPE* by 74±7% ($n=3$, $P<0.05$) using CPE73 siRNA (Fig. 5).

Initial processing of proglucagon at K70R71 enhances sorting to granules

Although neither PC1/3 nor PC2 are expressed in N2a-wt cells, another processing enzyme, furin, is expressed in all cells (Thomas 2002). Furin cleaves proglucagon at K70R71, which is the initial processing event that precedes further processing in secretory granules. In order to determine if processing at this site is required for sorting, we transfected N2a-CPE cells with proglucagon that harboured the R71Q mutation, which has previously been shown to inhibit processing (Dey *et al.* 2004). Localisation of the R71Q mutant was largely in the Golgi, although there was punctate immunofluorescence in the processes of some cells, and the PCC with p115 was significantly higher than that of wt proglucagon (Fig. 6), suggesting that processing at K70R71 may enhance sorting to granules.

Possible sorting signals are contained within the glucagon sequence

The sequence of glucagon contains two putative sorting signals, based on those described in other prohormones; a dibasic site that is not cleaved by PCs, and significant alpha-helical content. To determine the role of these structures in proglucagon sorting, the dibasic site was mutated to R18Q, and the alpha-helical content of glucagon was decreased by mutating the flanking leucines to prolines (L14P/L26P). Each mutation showed significant co-localisation with p115 (Fig. 6A) and significantly higher PCC with p115 compared with wt proglucagon (Fig. 6B), indicating that the dibasic site and the alpha-helix may each play a role in sorting proglucagon to secretory granules.

Modelling of the structure of proglucagon

We used the entire sequence of proglucagon to generate a 3D model so that clues to the mechanisms of putative sorting signals may be identified. Using the Robetta

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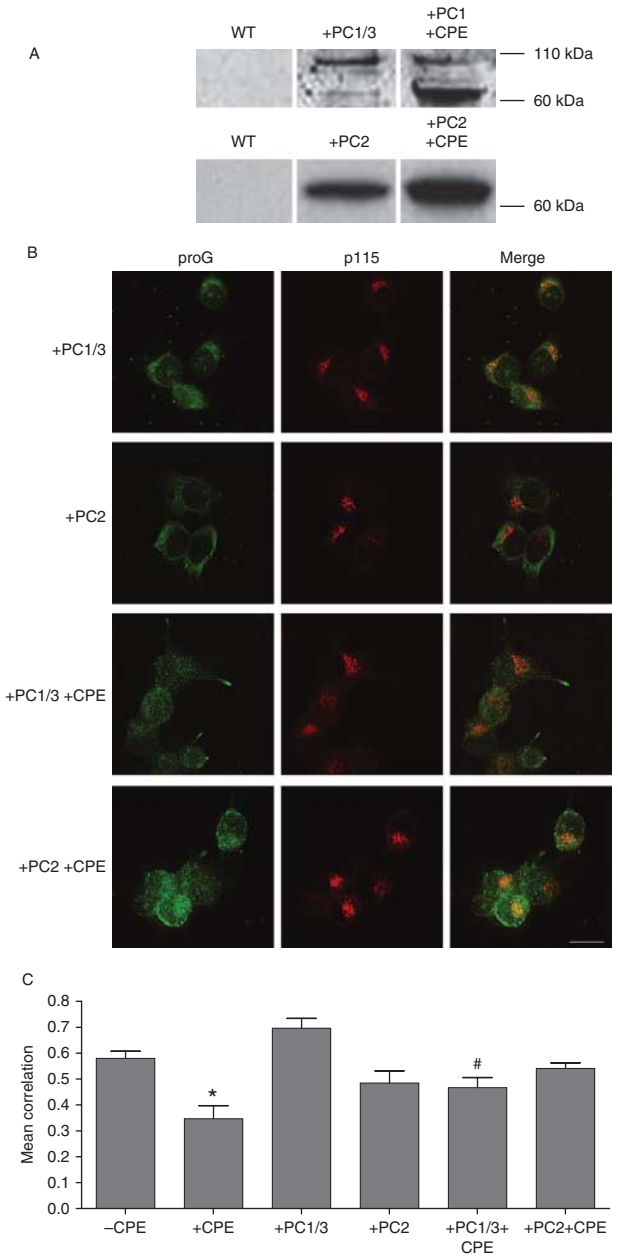

Figure 3

The presence of PC1/3 or PC2 does not enhance sorting of proglucagon. (A) Western blot analysis of PC1/3 (upper panels) and PC2 (lower panels) expression after before and after transfection into N2a cells. Wild-type N2a cells have no detectable expression of either PC. In cells lacking CPE, PC1/3 immunoreactivity is present as a single 84 kDa band, while in the presence of CPE, it is mostly present as a 66 kDa band. PC2 is present as a 66 kDa band with or without CPE. (B) Full-length proglucagon was transfected into N2a cells, either with PC1/3 or PC2 alone or in combination with CPE. Cells were processed using antibodies against glucagon (green) and the Golgi protein p115 (red). Representative images are shown. Scale bar represents 10 μm. (C) Mean correlation of proglucagon and p115 fluorescence in cells expressing PC1/3 or PC2, alone or in combination with CPE, was not significantly different from cells not expressing CPE. Values are means ± S.E.M. ($n = 10–25$). *$P < 0.01$ compared with −CPE, #$P < 0.01$ compared with +PC1 alone. Full colour version of this figure available via http://dx.doi.org/10.1530/JOE-12-0468.

software suite (Seattle, WA, USA. http://robetta.bakerlab.org/), five structures were predicted, one of which is shown in Fig. 7. Three alpha helices, each corresponding to regions within the sequences of glucagon (proglucagon 58–78), GLP-1 (proglucagon 104–124) and GLP-2 (proglucagon 129–152), are randomly arranged within a disordered structure. The prohormone processing site K70R71 is indicated at the N-terminal end of GLP-1. Unlike a previously reported model that predicted a rigid trimeric arrangement of the alpha helices (Dey *et al.* 2004), our model suggests that the spatial orientation of the alpha helices is not fixed, and that the location of the K70R71 site is within a flexible, unstructured region.

Discussion

While many studies have described the tissue-specific, post-translational processing of proglucagon, and elucidated the mechanisms underlying the regulation of glucagon and GLP-1 secretion, the mechanisms by which proglucagon is sorted into secretory granules remain unidentified. Sorting is a critical step in the proglucagon biosynthetic pathway, for two reasons. First, proper sorting ensures final cleavages by PC1/3 and PC2, which must occur in the low-pH, high-[Ca^{2+}] environment of the granules, to yield mature glucagon, GLP-1 and GLP-2. Second, appropriate compartmentalisation within secretory granules is an absolute requirement for stimulated secretion in response to nutritional status. Therefore, we sought to determine some possible mechanisms by which proglucagon is sorted into granules.

We first examined the role of CPE in proglucagon sorting, since it has been shown that CPE can act as a sorting receptor for some prohormones, such as POMC and proinsulin (Cool *et al.* 1997, Dhanvantari *et al.* 2003*b*). We used Neuro 2a cells in our study because we obtained a clone of N2a cells in which CPE expression had been lost (Zhang *et al.* 2003), which provided an ideal control. Sorting efficiency was determined using immunofluorescence microscopy quantified with PCC to determine the extent of co-localisation of proglucagon with the Golgi marker p115. We used a Golgi marker instead of the granule marker, CgA, since we found that CgA gave strong Golgi and granule signals that masked changes in the localisation of proglucagon. PCC has been previously used to determine the localisation of palmitoyltransferases in the endoplasmic reticulum (Gorleku *et al.* 2011) and is thought to be an accurate measure of co-localisation since it is a correlation between fluorescence intensities within the same pixels (Adler & Parmryd 2010).

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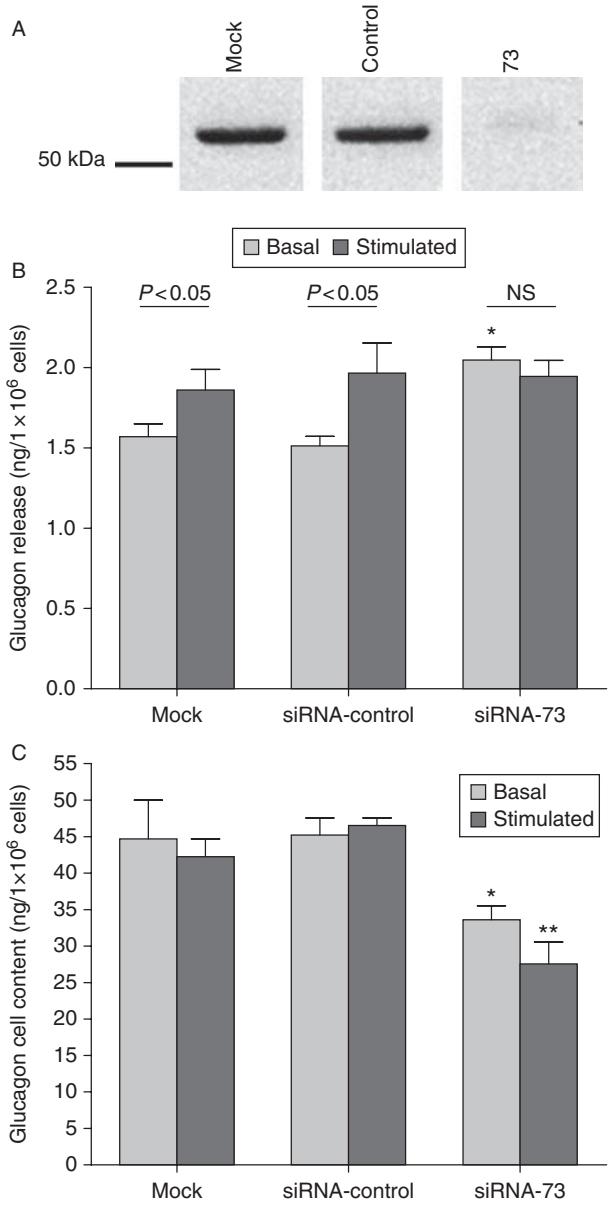

Figure 4
CPE depletion affects regulated secretion of glucagon from alphaTC1–6 cells. (A) Western blot analysis shows siRNA-mediated knockdown of CPE expression in alphaTC1–6 cells. (B and C) AlphaTC1–6 cells were treated with transfection agent alone (mock), or 200 nM of control siRNA or siRNA-73 against *CPE*. (B) There was a significant increase in secretion in response to 10 μM forskolin/IBMX treatment in mock- and control-transfected cells. In cells transfected with siRNA CPE, basal secretion was significantly higher than in control cells, and there was no response to stimulation with forskolin/IBMX. $*P<0.001$ compared with control transfected basal secretion. Values are means±S.E.M. ($n=6$). (C) Glucagon cell content decreased in cells transfected with siRNA-73 compared with control siRNA. $*P<0.05$, $**P<0.01$ compared with control siRNA values. All results are representative of four independent experiments.

In the absence of CPE, the increased presence of proglucagon in the Golgi and the reduced punctate immunofluorescence pattern indicate a reduction in sorting efficiency, as with POMC in these same wt N2a cells (Zhang *et al*. 2003). To confirm that CPE plays a role in sorting proglucagon, we used pancreatic-derived alphaTC1–6 cells, which we have used previously to characterise glucagon secretion under conditions of high glucose (McGirr *et al*. 2005). A 60% decrease in CPE expression resulted in a loss of regulated secretion of glucagon, the extent to which is likely underestimated as CPE levels were not decreased more dramatically. Interestingly, regulated secretion of GLP-1 in GLUTag remained intact after siRNA-mediated reductions in CPE levels. Therefore, CPE plays a role in proglucagon sorting in a cell-specific manner, and different mechanisms of sorting operate in the cell types that express proglucagon.

We found that proglucagon was not targeted to granules in the presence of the E300Q *Cpe* mutant, which itself was retained in the Golgi and not secreted. Therefore, impaired sorting of CPE may prevent the proper targeting of proglucagon to granules. Our finding of

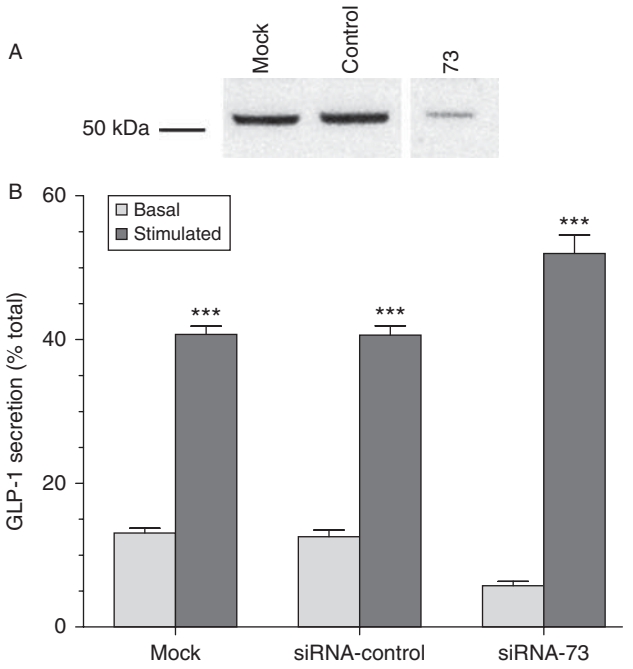

Figure 5
CPE depletion has no effect on regulated secretion of GLP-1 from GLUTag cells. (A) Western blot analysis shows siRNA-mediated knockdown of *CPE* expression in GLUTag cells. (B) GLUTag cells were treated with transfection agent alone (mock) or 200 nM of either control siRNA or siRNA-73 against CPE. There was robust stimulated secretion in response to 10 μM forskolin/IBMX treatment in all groups. Values are means±S.E.M. ($n=6$). $***P<0.001$ compared with basal secretion. All results are representative of four independent experiments.

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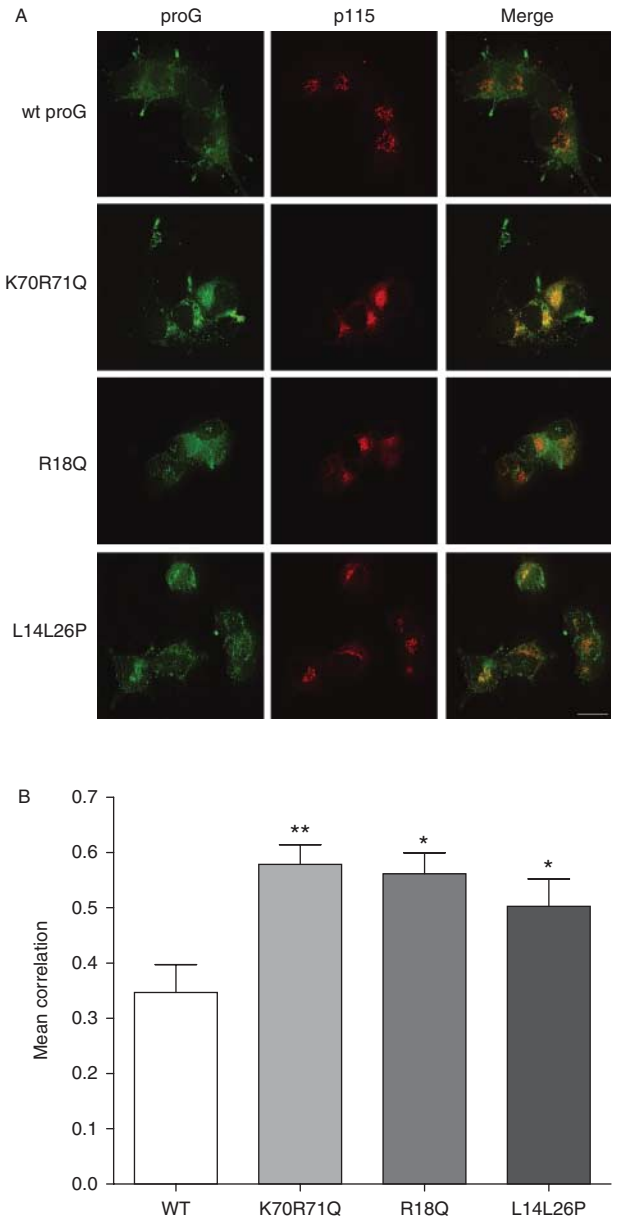

Figure 6

Identification of putative sorting signals in proglucagon. N2a cells expressing *CPE* were transfected with wt proglucagon, or the following mutants: K70R71Q, which disrupts a furin cleavage site; R18Q, which disrupts a dibasic site within glucagon; or L14P/L26P, which reduces the alpha-helical content of glucagon. (A) wt proglucagon shows strong immunoreactivity in the tips of the cell processes, and very weak signal in the Golgi. By contrast, there is strong Golgi signal that co-localised with p115 in cells expressing the K70R71Q mutant proglucagon, as well as signal in the cell processes. Representative images are shown. Scale bar represents 10 μm. The R18Q and L14P/L26P mutants show some Golgi co-localisation and post-Golgi signal. (B) Mean correlation of proglucagon and p115 fluorescence in cells expressing wt proglucagon (wt) or indicated mutants. All mutant constructs showed significantly higher mean correlation with p115 compared with wt proglucagon, indicating some disruption in sorting. $*P<0.05$, $**P<0.01$ compared with wt. Full colour version of this figure available via http://dx.doi.org/10.1530/JOE-12-0468.

impaired sorting of the E300Q mutant is at variance with a previous report that showed that it bound to its substrate and was sorted to granules normally in NIT3 cells (Qian *et al.* 1999). It is not clear how CPE is missorted in N2a cells, since its C-terminal sorting domain (Fricker *et al.* 1990, Zhang *et al.* 2003) is intact in the E300Q mutant, as evidenced by our western blot results showing strong immunoreactivity in cell extracts using a C-terminal antibody. The strong Golgi localisation also demonstrates that E300Q is not degraded, unlike another enzymatically inactive mutant, S202P (Naggert *et al.* 1995); rather, it is the post-Golgi trafficking of the E300Q mutant that is impaired in N2a cells. Although the missorting of E300Q CPE may be unique to N2a cells, nonetheless, these results mirror those showing that improperly sorted PC1/3 prevents the targeting of prorenin to the regulated secretory pathway (Brechler *et al.* 1996). Therefore, there may be a direct interaction between CPE and proglucagon that results in co-targeting to granules. The nature of the interaction between CPE and some other prohormones has been well documented; studies on the structures of POMC, proenkephalin, proinsulin and brain-derived neurotrophic factor showed that exposed acidic amino acid residues on a disulphide-bonded loop confer the ability to bind to two basic residues, K255 and R260, on CPE (Cool *et al.* 1995, 1997, Zhang *et al.* 1999, Lou *et al.* 2005). Our results on modelling the structure of proglucagon predict distinct alpha-helical domains within a largely disordered structure, and no disulphide-bonded loop. However, a CPE binding site may lie in the disordered regions of GRPP (proglucagon 1–30) or IP2 (proglucagon 111–133), which contain acidic amino acids in their sequences. It has been postulated that disordered structures within proteins may mediate protein–protein interactions due to their structural plasticity (Chen 2012). Further characterisation of the cognate domains within CPE that may bind to specific structural sorting determinants of proglucagon will be required to elucidate a potentially distinct molecular mechanism of CPE-mediated proglucagon sorting.

Since other prohormone processing enzymes are also co-targeted to secretory granules with their substrates, we investigated the roles of the proglucagon-processing enzymes, PC1/3 and PC2, in sorting proglucagon, in the absence or the presence of CPE. A possible role of PC1/3 in proglucagon sorting was of interest to us, since: i) PC1/3 may function in the sorting of another prohormone, prorenin; ii) the presence of PC1/3 up-regulates the secretion of the granule markers CgA and SgII, suggesting that PC1/3 may be part of the sorting machinery

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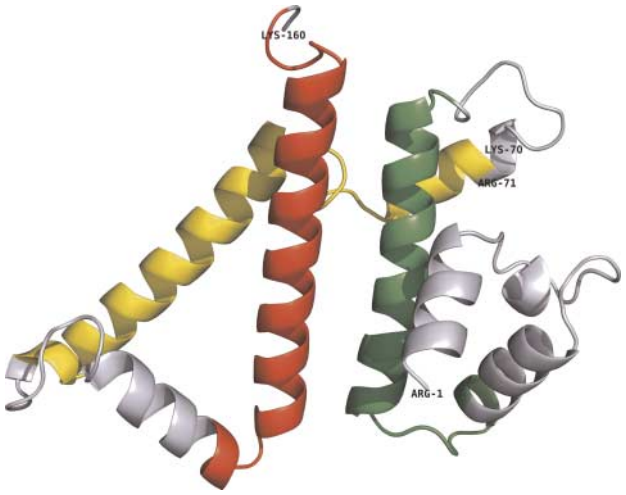

Figure 7
Predictive structural modelling of proglucagon. The Robetta modelling web server was used to generate a *de novo* 3D protein structure prediction for entire sequence of proglucagon. The results are presented in cartoon format, with N- and C-terminal indicated, and the inter-domain cleavage site K70R71 labelled. The sequence of glucagon is coloured green, GLP-1 (1–37) yellow and GLP-2 red. The structure is largely disordered, with alpha helices of glucagon, GLP-1 and GLP-2 arranged randomly within the structure. The K70R71 cleavage site appears to be located in an unstructured region, which may provide flexibility and accessibility to furin. Full colour version of this figure available via http://dx.doi.org/10.1530/JOE-12-0468.

(Lankat-Buttgereit *et al*. 2008); and iii) GLUTag cells were not responsive to *CPE* knockdown, but express PC1/3, which would have been a candidate sorting receptor in these cells. However, our data do not support a role for PC1/3 in proglucagon sorting. Indeed, while there is impaired proglucagon processing in the intestine in both mice (Ugleholdt *et al*. 2004) and a human subject (Jackson *et al*. 2003) lacking PC1/3, GLP-1 was secreted normally in response to feeding, so it may be unlikely that PC1/3 plays a role in proglucagon sorting in the L cell. Therefore, alternative mechanisms operate in the sorting of proglucagon in L cells. This finding lends another level of complexity to the sorting mechanism of proglucagon.

The impaired sorting of the K70Q71 cleavage site mutant of proglucagon suggests that processing at this site enhances sorting to granules. The post-translational processing of proglucagon follows a temporal pattern, with cleavage at K70R71 occurring in the Golgi/immature secretory granules, and further processing by PC1/3 or PC2 into bioactive peptides occurring in mature secretory granules (Dhanvantari *et al*. 1996, Dey *et al*. 2004). Processing at this site is largely through the actions of the enzyme furin, since it occurs in all cell types in which proglucagon has been expressed, regardless of other PCs that may be present, and since proglucagon is processed to glicentin in baby hamster kidney (BHK) cells, a fibroblast cell line lacking secretory granules (Dhanvantari *et al*. 1996). An increase in the sorting efficiency of proglucagon after cleavage at K70R71 in the Golgi would be similar to the observations with prothyrotropin-releasing hormone (pro-TRH), where processing by PC1/3 enhances the sorting of pro-TRH-derived peptides into granules (Mulcahy *et al*. 2005). Future experiments will investigate more closely the role of processing in proglucagon sorting, and the possibility that the products of this initial processing, glicentin and the major proglucagon fragment (MPGF) are sorted into different granule populations, again in a pattern similar to that of pro-TRH sorting (Perello *et al*. 2008).

Finally, we have for the first time identified putative sorting signals within proglucagon. We postulated that an internal dibasic site and the alpha-helical content in the sequence of glucagon could target proglucagon to granules, since both motifs have been identified as sorting signals for a number of regulated secretory proteins (Dikeakos & Reudelhuber 2007). Our results indicate that both motifs may constitute proglucagon sorting signals, and that they may act independently; mutating the dibasic site does not affect alpha-helical content, and altering the helical content leaves the dibasic site intact (Guizzetti and Dhanvantari 2012, unpublished observations). It has been shown that multiple types of sorting signals increase the efficiency of sorting to granules (Lacombe *et al*. 2005), which may then translate into a greater degree of physiological regulation of secretion. In this regard, the sorting efficiency of proglucagon may be similar to that of proinsulin, which also contains a number of different sorting signals (hexamerisation, CPE binding sites and dibasic sites). When these signals are disrupted, the resulting loss of regulated secretion results in the clinical syndrome of hyperproinsulinaemia (Dhanvantari *et al*. 2003*b*). A high degree of sorting efficiency would ensure that the pancreatic alpha and beta cells and intestinal L cells are in a nutrient-responsive state, and would account for the exquisite control of glycaemia exerted by glucagon, insulin and GLP-1.

It is also remarkable that altering the alpha-helical content in glucagon affects sorting, given that there are two other alpha helices, contained within GLP-1 and GLP-2, that could compensate. Either the two other alpha helices do not act as sorting signals, or these results could be consistent with the hypothesis that proglucagon is first processed in the Golgi before entering granules. If the alpha-helix within glucagon is disrupted, while the alpha helices of GLP-1 and GLP-2 stayed intact, the sorting of

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glicentin would be impaired, while the sorting of MPGF would not be affected. Experiments are currently underway to more precisely define the role of the alpha helices within glucagon, GLP-1 and GLP-2, as well as of other possible intrinsic signals, in proglucagon sorting.

Supplementary data

This is linked to the online version of the paper at http://dx.doi.org/10.1530/JOE-12-0468.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

S D designed the experiments, R M completed the experiments and statistical analysis, L G carried out the fluorescence correlation data analysis and protein modelling, and all three authors wrote the manuscript.

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