Peptide production and secretion in GLUTag, NCI-H716, and STC-1 cells: a comparison to native L-cells

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Abstract

GLUTag, NCI-H716, and STC-1 are cell lines that are widely used to study mechanisms underlying secretion of glucagon-like peptide-1 (GLP-1), but the extent to which they resemble native L-cells is unknown. We used validated immunoassays for 14 different hormones to analyze peptide content (lysis samples; $n = 9$ from different passage numbers) or peptide secretion in response to buffer (baseline), and after stimulation with 50 mM KCl or 10 mM glucose + 10 μM forskolin/3-isobutyl-1-methylxanthine ($n = 6$ also different passage numbers). All cell lines produced and processed proglucagon into GLP-1, GLP-2, glicentin, and oxyntomodulin in a pattern (prohormone convertase (PC)1/3 dependent) similar to that described for human gut. All three cell lines showed basal secretion of GLP-1 and GLP-2, which increased after stimulation. In contrast to freshly isolated murine L-cells, all cell lines also expressed PC2 and secreted large amounts of pancreatic glucagon. Neurotensin and somatostatin storage was low and secretion was not consistently increased by stimulation. STC-1 cells released more glucose-dependent insulinotropic polypeptide than GLP-1 at baseline ($P < 0.01$) and KCl elevated its secretion ($P < 0.05$). Peptide YY, which normally co-localizes with GLP-1 in distal L-cells, was not detected in any of the cell lines. GLUTag and STC-1 cells also expressed vasoactive intestinal peptide, but none expressed pancreatic polypeptide or insulin. GLUTag contained and secreted large amounts of CCK, while NCI-H716 did not store this peptide and STC-1 contained low amounts. Our results show that hormone production in cell line models of the L-cell has limited similarity to the natural L-cells.

Key Words
- GLP-1-producing cell lines
- peptide production
- L-cells

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Introduction

Enteroendocrine cells are derived from a common pluripotent stem cell that also gives rise to enterocytes, goblet cells, and Paneth cells (Roth *et al.* 1992), but there are pronounced differences with respect to the expression pattern of the different gut hormones along the intestine. The gut hormone, glucagon-like peptide-1 (GLP-1), is a key hormone in the regulation of blood glucose and satiety in humans. It is produced by enteroendocrine L-cells located in the gut epithelium, and is particularly known for its incretin actions , that is, the enhancement

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of meal-stimulated insulin secretion. This serves to limit postprandial blood glucose levels (Holst 2007), and therefore, GLP-1-based drugs are used to treat type II diabetes. Many studies investigating the secretion of GLP-1 have utilized animal models and while these are useful for studying physiological processes in the broad sense, they are not always ideal for distinguishing direct vs indirect effects on the L-cell, or for dissecting the underlying molecular mechanisms coupling stimulus to secretion. Therefore, it was not until the development of three GLP-1-producing cell lines, namely GLUTag, STC-1, and NCI-H716, in the 1990s that the specific molecular mechanisms leading to the secretion of GLP-1 began to be unveiled. Naturally, the physiological relevance of studies with these cell lines crucially depends on their degree of resemblance to natural L-cells, but this has not been characterized in any detail. All three cell lines are derived from carcinomas; GLUTag and STC-1 cells originated from tumors of the large bowel and small intestine of mice, respectively, while NCI-H716 cells were derived from ascites fluid from a human with adenocarcinoma of the colon (Bruïne *et al.* 1992, Drucker *et al.* 1994, Grant *et al.* 1991, Lee *et al.* 1992, Rindi *et al.* 1990).

In the L-cell, prohormone convertase (PC)1/3 processing of the glucagon precursor, proglucagon, results in the formation of GLP-1, together with GLP-2 and glicentin (Holst 2007). Glicentin may be processed further into oxyntomodulin (OX) and glicentin-related pancreatic peptide. The pancreatic α-cells also produce proglucagon, but here the precursor is predominantly processed by PC2 to generate glucagon and the major proglucagon fragment (Holst 2010, Holst *et al.* 1994). Recently, however, several groups have provided evidence that some populations of L-cells exist, in which GLP-1 is co-localized with other hormones not derived from proglucagon. Thus, proximal L-cells may also contain CCK and neurotensin (NT), whereas distal L-cells typically also produce peptide YY (PYY) (Egerod *et al.* 2012, Habib *et al.* 2012, Mortensen *et al.* 2003, Svendsen *et al.* 2015). None, however, seems to contain somatostatin (SST) (Egerod *et al.* 2012).

As GLUTag, NCI-H716, and STC-1 cells all are derived from carcinomas, we hypothesized that they may, depending on their respective differentiation state, express, process, and secrete hormones that normally are not produced by native L-cells. Furthermore, all of these cells lines are immortal, which also distinguishes them from the natural L-cells. We, therefore, undertook this study to explore to what extent these 'L-cell models' actually resemble natural L-cells by examining their expression and secretion of a panel of intestinal and pancreatic hormones.

Materials and methods

Cell work

Cells were cultured following previously described protocols (Kuhre *et al.* 2014*a*,*b*). In brief, cells were kept at 37°C, 5% CO_2 until 80–85% confluent, then split and transferred to fresh nucleon-coated T75 flasks (Cat. No. EW-01930-54, Thermo Fisher Scientific) for GLUTag and STC-1 cells, or anti-attachment coated T25 flasks (Cat. No. C6731, Sigma-Aldrich) for NCI-H716 cells. Cell medium composition was based on publications from several groups (Drucker *et al.* 1994, Eiki *et al.* 2009, Katsuma *et al.* 2005, Reimann & Gribble 2002, Reimer *et al.* 2001), and comprised i) for GLUTag: low glucose (1 g/L) DMEM (Cat No. 6046, Sigma-Aldrich) supplemented with 10% (vol/vol) fetal bovine serum (FBS), 1% (vol/vol) penicillin (10,000 U/mL)/streptomycin (10,000 µg/mL) (p/s), and GlutaMAX (200 mM; Cat. No. 35050061, Gibco, Life Technologies); ii) for NCI-H716: low glucose (2.0 g/L) RPMI 1640 (Cat. No. R8750, Sigma-Aldrich) supplemented with 10% (vol/vol) FBS and 1% (vol/vol) p/s; and iii) for STC-1: high glucose (4.5 g/L) DMEM (Cat No. 6429, Sigma-Aldrich) supplemented with 15% (vol/vol) horse serum, 2.5% (vol/vol) FBS.

For secretion studies, cells were plated onto 24-well plates precoated with matrigel basement membrane (Cat. No. 354234; BD Biosciences) 18–24 h before the study. On the following day, cells (~80% confluent) were incubated for 2 h with buffer (baseline secretion) or buffer to which was added 50 mM KCl or 10 mM glucose and 10 µM forskolin/3-isobutyl-1-methylxanthine (FSK/IBMX, stimulants). The buffer consisted of 138 mM NaCl, 4.5 mM KCl, 4.2 mM $NaHCO_3$, 1.2 mM NaH_2PO_4, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, and 10 mM HEPES supplemented with 0.1% (wt/vol) fatty acid-free BSA (Cat. No. A-603-10G, Sigma-Aldrich) (pH = 7). Supernatants were obtained and centrifuged (1500 ***g***, 4°C, 5 min) to remove any floating cells or debris. The resulting supernatants were transferred to fresh Eppendorf tubes and samples were either directly processed for hormone quantification while kept on ice or stored at −20°C until analysis. In either case, samples were assayed non-extracted.

Lysis samples were obtained by incubating the cells with ice-cold lysis buffer for 5 min while kept on ice, followed by thorough mechanical disruption by scraping, as described previously (Kuhre *et al.* 2014*a*). Lysis buffer consisted of: 1% Igepal CA-630 (Cat. No. I8896, Sigma-Aldrich), 0.12 M sodium deoxycholate monohydrate (Cat. No. D5670, Sigma-Aldrich), 0.05 M Tris–HCl (Cat. No. A1087, AppliChem GmbH, Darmstadt, Germany), 0.15 M NaCl, and one tablet

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EDTA-free protease inhibitor cocktail/50 mL (Cat. No. 05056489001, F. Hoffmann-La Roche, Basel, Switzerland). Samples were stored at −80°C, after which peptides were purified using Sep-Pak C18 cartridges (Cat. No. WAT036810, Waters, Milford, MA, USA), eluted with 70% ethanol + 0.1% trifluoroacetic acid and dried under a gentle stream of compressed air overnight. The purified samples were reconstituted in sodium phosphate buffer supplemented with 0.1% (wt/vol) human serum albumin (Cat. No. 12666, Merck). Total protein content was quantified by a BCA Protein Assay Kit (Cat. No. 71285-3, Millipore).

Gel filtration analysis

Pooled extracted cell lysis samples ($n = 9$) were centrifuged (4°C, 4 min, 4500 ***g***) and the supernatants were fractionated by gel filtration on a Sephadex G-50SF-packed K16-100 column (Pharmacia), equilibrated, and eluted with the same sodium phosphate buffer as above. Gel filtration effluents were collected automatically in fractions corresponding to approximately 1/50 of the fractionation volume of the column. The column was precalibrated using ^{125}I-labeled albumin (V_0), ^{22}Na (V_i), and unlabeled proglucagon-derived peptides: glucagon, GLP-1, GLP-2, OX, and glicentin. Coefficients of distribution were calculated as: $K_d=(V_e-V_0)/V_i$, where V_e is the elution volume for the substance in question, V_0 is the exclusion volume, and V_i is the available inner volume determined as the difference between the elution volumes of the albumin and sodium calibrators (Holst 1983). Peptide concentrations were analyzed as described previously. In all runs, ^{22}Na and ^{125}I-labeled albumin were added as internal controls. Between runs, the column was washed with buffer; no immunoreactive moieties were detected in fractions from any on these control runs (data not shown).

Biochemical measurements

Protein content in lysis samples was measured using BCA Protein Assay Kit according to the manufacturer's instructions. Hormone contents of lysis and secretion samples were measured using well-characterized in-house RIA, in-house sandwich ELISA, or validated commercially available ELISA. Samples were diluted in the respective assay buffers so that all positive measurements were within the sensitive part of the standard curves. Information regarding assay type (RIA or ELISA), antibody codes, epitopes, sensitivity, specificity, and linear range for the assays is provided in Supplementary Table 1 (see section on supplementary data given at the end of this article).

Proglucagon-derived gut peptides GLP-1 (intact) was measured using an in-house two-site sandwich ELISA involving two monoclonal antibodies: GLP-1 F5 as catching antibody (COOH-terminally directed) and Mab26.1 as a detecting antibody (NH2-terminally directed) (Wewer Albrechtsen *et al.* 2015*a*). Total GLP-1 was measured using an in-house RIA (antibody 89390) utilizing a C-terminal antibody that binds amidated (x-36$_{amide}$) but not glycine (x-37) extended GLP-1 isoforms (Orskov *et al.* 1994). GLP-2 (intact) was measured using an in-house RIA (antibody 92160), which employs an antibody that targets N-terminally GLP-2 (Hartmann *et al.* 2000). For measurements of intact GLP-1 and GLP-2, valine pyrrolidide (a generous gift from Novo Nordisk) was added to the samples (final concentration 0.01 mM) to prevent any DPP-4-mediated degradation. Glicentin/OX was measured using a C-terminally directed in-house RIA with equal affinity for OX and glicentin (antibody 645) (Wewer Albrechtsen *et al.* 2015*b*).

Gut peptides not derived from proglucagon CCK was measured using an in-house RIA (antibody 92128), which measures all bioactive CCK forms (i.e. amidated and tyrosyl-*O*-sulfated CCK-58, -33, -22, and -8) while displaying no cross-reactivity with gastrin (Rehfeld 1998). ^{125}I-labeled sulfated CCK-8 was used as tracer and CCK-8 as standard (Rehfeld 1998). Chromogranin A (CgA) was assayed by in-house RIA (antibody 95058) (Børglum Jensen *et al.* 1999) targeting the N-terminus of sequence 340-348 in CgA. Gastrin was measured using an in-house RIA (antibody 2604), which is directed against the C-terminus of gastrin-17 and binds all bioactive (i.e. amidated) gastrins (gastrin-71, -34, -17, and -14) in plasma with equimolar potency without cross-reactivity with CCK peptides (Stadil & Rehfeld 1973). GIP$_{total}$ was measured using a C-terminally directed antiserum (code: 80867-5), which reacts fully with intact GIP and the N-terminally truncated metabolite GIP (3–42) (Krarup & Holst 1984). Intact, bioactive GIP was measured using in-house RIA (antibody 98171), which reacts with the N-terminal part of intact GIP (1–42), and cross-reacts <0.1% with GIP (3–42) or the structurally related peptides: GLP-1 (7/9–36) amide, GLP-2 (1–33), or glucagon (Deacon *et al.* 2000). NT was measured using an in-house N-terminal RIA, thus measuring total NT (antibody 3D97) (Kuhre *et al.* 2015). For GLUTag and STC-1, PYY$_{total}$ was measured using a side-viewing antibody (Cat. No. T-4093, Bachem, Bubendorf, Switzerland) that binds murine PYY, with murine/porcine PYY standards and ^{125}I-labeled porcine

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PYY (Cat. No. NEX 240, PerkinElmer) (Svendsen *et al.* 2015). For NCI-H716, PYY_{total} was quantified by in-house RIA (code: MAB8500), reacting with the mid part of human PYY (Torang *et al.* 2015). Vasoactive intestinal polypeptide (VIP) was measured using in-house RIA previously described (Fahrenkrug & Schaffalitzky de Muckadell 1977).

Pancreatic peptides For content analysis (Fig. 2), glucagon was measured using a commercially available sandwich ELISA (Cat. No. 10-1271-01, Mercodia, Uppsala, Sweden), targeting both the N- and C-terminal of glucagon (thus only measuring fully processed pancreatic glucagon and neither C- nor N-terminally elongated or truncated forms (Wewer Albrechtsen *et al.* 2014)). For gel filtration data, glucagon was measured using an in-house RIA employing an antibody directed against the C-terminal of pancreatic glucagon (antibody 4305) (Orskov *et al.* 1991). Insulin was measured using in-house RIAs, reacting with either murine (GLUTag and STC-1) or human (NCI-H716) insulin (antibody 2006-3 and 2004-3, respectively) as described previously (Brand *et al.* 1995, Orskov *et al.* 1991). Pancreatic polypeptide (PP) (total) was measured using in-house RIAs employing antibodies that bind human (antibody 7198) or murine (antibody HYB 347-07) PP (Dirksen *et al.* 2013). SST was measured using in-house RIA employing a side-viewing antibody (code: 1758-5), which reacts with all molecular forms of SST that possess the mid sequence, including gut-derived SST 1–28 (Holst & Bersani 1991).

Microarray

GLU-Venus transgenic mice, expressing the fluorescent protein YFP-Venus under control of the proglucagon promoter (Reimann *et al.* 2008), were used to obtain L-cell-positive and L-cell-negative epithelial cell populations. K-cell populations were isolated from YFP-Venus mice (Parker *et al.* 2009) directed by the prepro GIP-promoter. In both cases, mice aged 2–6 months were killed by cervical dislocation and the gut was collected into ice-cold Leibovitz-15 (L-15) medium. For K-cell population tissue from the upper small intestine (incorporating 10–15 cm of gut, immediately distal to the pylorus was collected, whereas tissue for L-cell isolation was collected at sites in proximal and distal small intestine and large intestine as described previously. Tissues were chopped into 1–2 mm piece and digested twice for 30 min with 1 mg/mL collagenase XI in calcium-free Hanks balanced salt solution at 37°C (Sigma-Aldrich). Cell suspensions were filtered through 70 μM nylon strainers (BD Biosciences) and centrifuged at 300 ***g*** for 5 min, and pellets were resuspended in L-15 supplemented with 10% (vol/vol) FBS. Cytomation MoFlo cell sorter (488 nm excitation; Beckman Coulter, High Wycombe, UK) was used to separate populations of >95% pure Venus-positive or -negative cells (presumably dominated by enterocytes) as described previously (Habib *et al.* 2012, Parker *et al.* 2009, Reimann *et al.* 2008). The α-, β-, and δ-cells were isolated as described previously. In brief, Venus positive α-cells and eYFP-positive δ-cells were isolated from mice in which the expression of fluorescent proteins was controlled by the proglucagon and somatostatin promoter, respectively (Adriaenssens *et al.* 2015). The β-cells were isolated on the basis of their size, using forward and side scatter characteristics distinguishing them from other nonfluorescent islet cells in single cell preparations from mice with glucagon-driven Venus expression. PC1/3 and -2 expression was determined by microarray as described previously (Habib *et al.* 2012, Reimann *et al.* 2008).

Data analysis

Cell content and secretion data (Figs. 2 and 3) were presented as mean±S.E.M., microarray expression data (Fig. 1) as means, and gel filtration data (Fig. 4) as peptide concentrations (pM) in each of the collected fractions (expressed as K_d values). For cell content data,

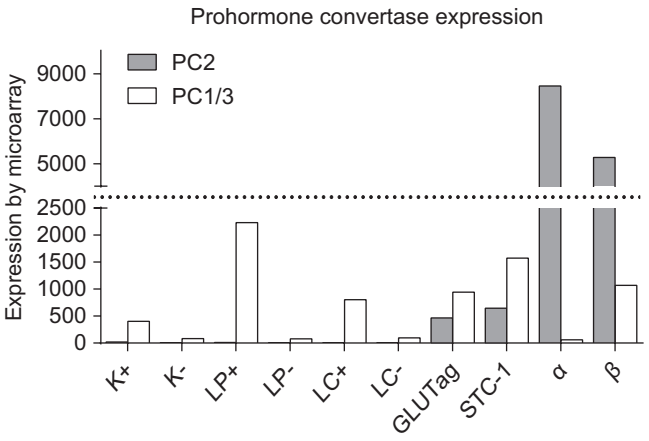

Figure 1
Microarray expression of PC1/3 and PC2 in GLUTag and STC-cells, isolated murine K- and L-cells, murine none K- and L-cells, pancreatic α- and β-cells. Expression levels of PC1/3 and PC2 are shown for FACS-sorted mouse K-cells (K+) and proximal L-cells (LP+) collected from the small intestine, and colonic L-cells (LC+), reference tissue (K–, LP–, and LC–) collected from identical anatomical sites as K- and L-cells were isolated from pancreatic mouse α- and β-cells and GLUTag and STC-1 cells. PC, prohormone convertase.

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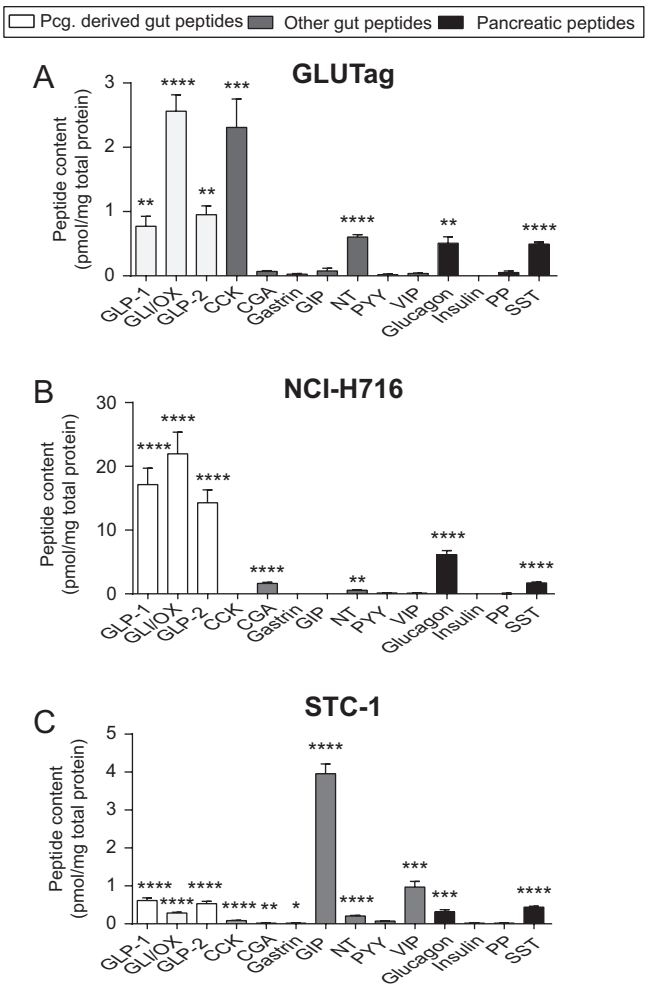

Figure 2
Gut and pancreatic hormone contents in GLUTag, NCI-H716, and STC-1 cells. Data are shown as mean ± S.E.M. relative to baseline. Level of significance between baseline and stimulant are indicated with stars; *$P<0.05$, **$P<0.01$, ***$P<0.001$, ****$P<0.0001$. $n = 6$. CgA, chromogranin A; GIP (total), gastric inhibitory peptide; GLI/OX, glicentin/oxyntomodulin; GLP-1, glucagon-like peptide-1; GLP-2, glucagon-like peptide-2; NT, neurotensin (total); PP, pancreatic polypeptide; PYY, PYY (total); SST, somatostatin (total); VIP, vasoactive intestinal polypeptide.

peptide concentrations (pM) were normalized to protein concentration (mg/L) to adjust for intragroup variation in confluence. Secretion data were expressed as absolute concentration (nonnormalized) values (pM) for cell contents, statistical significance was tested by one-sample *t*-test, for each peptide testing the measured concentrations against the respective assay's lower limit of detection (2–10 pM, depending on the assay). For secretion, statistical significance was assessed by paired *t*-test, testing stimuli values against baseline. A *P* value of <0.05 was considered to be significant. Graphs were constructed in GraphPad Prism 5 and edited in Adobe Illustrator.

Results

Expression of PC2 and PC1/3 in primary K- and L-cells, GLUTag and STC-1 cells, and α- and β-cells

PC1/3 was expressed by primary murine K-cells and by primary murine L-cells from the small intestine and large intestine, respectively but not in reference tissue collected from same anatomical sites (Fig. 1). PC2 expression was not detected in K- or L-cells or in reference gut tissue. GLUTag and STC-1 cells expressed PC1/3 but, in addition, both expressed PC2 to about half the level of PC1/3. Primary α-cells from the mouse expressed very high levels of PC2 but no PC1/3, while primary β-cells expressed both PC1/3 and PC2, as expected.

Hormone content of GLUTag, NCI-H716, and STC-1 cells

Overall, the three cell lines varied considerably with respect to the peptides they contained, as well as the relative amounts of each peptide. All three cell lines contained the gut-derived proglucagon products GLP-1, GLP-2, and glicentin/OX. GLP-1 and GLP-2 were present in a 1:1 ratio in all cell lines, but only in NCI-H716 cells were glicentin and OX present in comparable amounts; in the other cells, glicentin dominated. All three cell lines also stored glucagon and SST, but neither insulin nor PP. GLUTag cells contained high levels of CCK and NT. NCI-H716 cells also contained low levels of CgA and NT. STC-1 cells contained very high levels of total GIP (Fig. 2) and similar levels of intact GIP (data not shown), both significantly higher than the levels of GLP-1 ($P<0.001$). In addition, STC-1 cells contained comparatively low, but significant concentrations of CCK, gastrin, NT, and relatively high concentrations of VIP. All three cell lines were PYY negative.

Secretory repertoire of GLUTag, NCI-H716, and STC-1 cells

GLUTag cells secreted GLP-1, GLP-2, and glucagon in high concentrations and their secretion was enhanced by both stimulants (50 mM KCl and 10 mM glucagon + 10 μM FSK/IBMX) (Fig. 3A). CCK was secreted in high concentrations and showed near significant increases after both stimulants were compared with baseline ($P=0.054$, baseline vs KCl; $P=0.086$, baseline vs glucagon + FSK/IBMX). CgA was secreted in low concentrations, but KCl significantly enhanced the secretion compared with baseline. The secretion of NT and SST was not altered by

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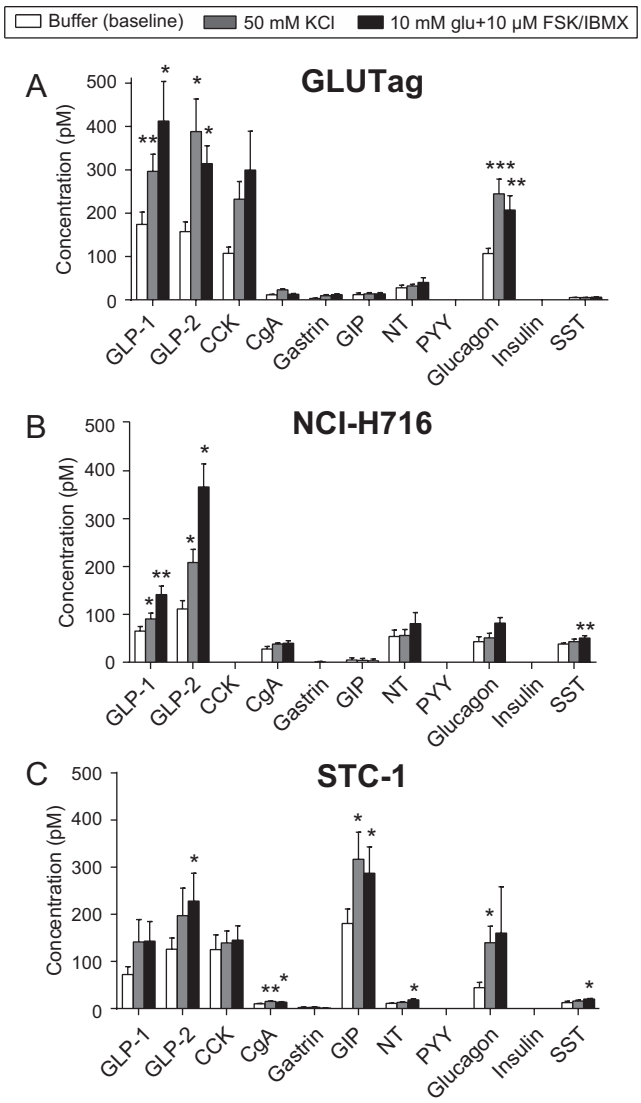

Figure 3
Secretory repertoire of GLUTag, NCI-H716, and STC-1 cells. Data are shown as mean ± S.E.M. Level of significance between baseline and stimulant are indicated with stars; *P<0.05, **P<0.01, ***P<0.001, n = 6. CgA, chromogranin A; FSK, forskolin; GIP, gastric inhibitory peptide; GLI/OX, glicentin/oxyntomodulin; GLP-1, glucagon-like peptide-1; GLP-2, glucagon-like peptide-2; glu, glucagon; IBMX, 3-isobutyl-1-methylxanthine; NT, neurotensin; PP, pancreatic polypeptide; PYY, peptide YY; SST, somatostatin.

the stimuli. GIP was not secreted in the basal state or in response to the stimuli. NCI-H716 cells secreted GLP-1 and GLP-2 and their secretion was enhanced by both stimulants (Fig. 3B). NCI-H716 also secreted relatively large amounts of NT, glucagon, and SST, but only the secretion of SST was significantly elevated by stimulation and only by glucagon + FSK/IBMX. CgA was secreted in low concentrations and its secretion was enhanced by both stimuli. GIP secretion was not detected in any of the treatment groups. STC-1 cells released GLP-1 and GLP-2, but the stimulants only caused a borderline significant elevation of the secretion of GLP-1 compared with basal secretion (P=0.054, baseline vs KCl; P=0.13, baseline vs glucagon + FSK/IBMX), while the GLP-2 response to the positive control reached significance (Fig. 3C). However, STC-1 cells actually secreted more GIP (measured using both GIP assays) than GLP-1 at baseline (P<0.01), and its secretion was elevated by both stimulants. CCK was secreted in concentrations similar to GLP-1 and GLP-2, but neither stimulus elevated its secretion compared with baseline. High concentrations of glucagon and low levels of SST and CgA were also secreted by the STC-1 cell at the basal state, and for each, secretion was enhanced by one or both of the stimulants. None of the cell lines secreted PYY.

Processing patterns of proglucagon-derived peptides

Gel filtration pattern of extractable glucagon showed that, for all the three cell lines, the majority of immunoreactive material was eluted at the position of unlabeled pancreatic glucagon ($K_d \approx 0.85$), although small amounts of larger immunoreactive moieties were also found, particularly in the NCI-H716 cells (Holst 1983). For GLP-1 and GLP-2, the majority of immunoreactive material was also found at elution positions close to that previously described for synthetic unlabeled GLP-1 and GLP-2 (Ørskov *et al.* 1991) ($K_{dGLP\text{-}1} \approx 0.55$ and $K_{dGLP\text{-}2} \approx 0.50$ (Buhl *et al.* 1988). Single peaks for glicentin and OX were identified for all three cell lines at elution positions close to previously reported K_d values for unlabeled synthetic glicentin and OX (≈0.35 and ≈0.66, respectively) (Holst 1983).

Discussion

According to data obtained from immunohistochemical studies of L-cells from human, porcine, and rat tissues and expression analysis on FACS-sorted mouse L-cells, two subpopulations of L-cells seem to exist: a proximal small intestinal population that, in addition to the proglucagon peptides derived from PC1/3 processing, often co-express CCK and/or GIP to some degree, and a more distal L-cell population that co-express PYY (Habib *et al.* 2012, Mortensen *et al.* 2003, Svendsen *et al.* 2015, Theodorakis *et al.* 2006). Supporting this, cell-specific ablation of intestinal L-cells also resulted in dramatic reductions in CCK and PYY expression (mRNA) in proximal and distal small intestine, respectively (Pedersen *et al.* 2013).

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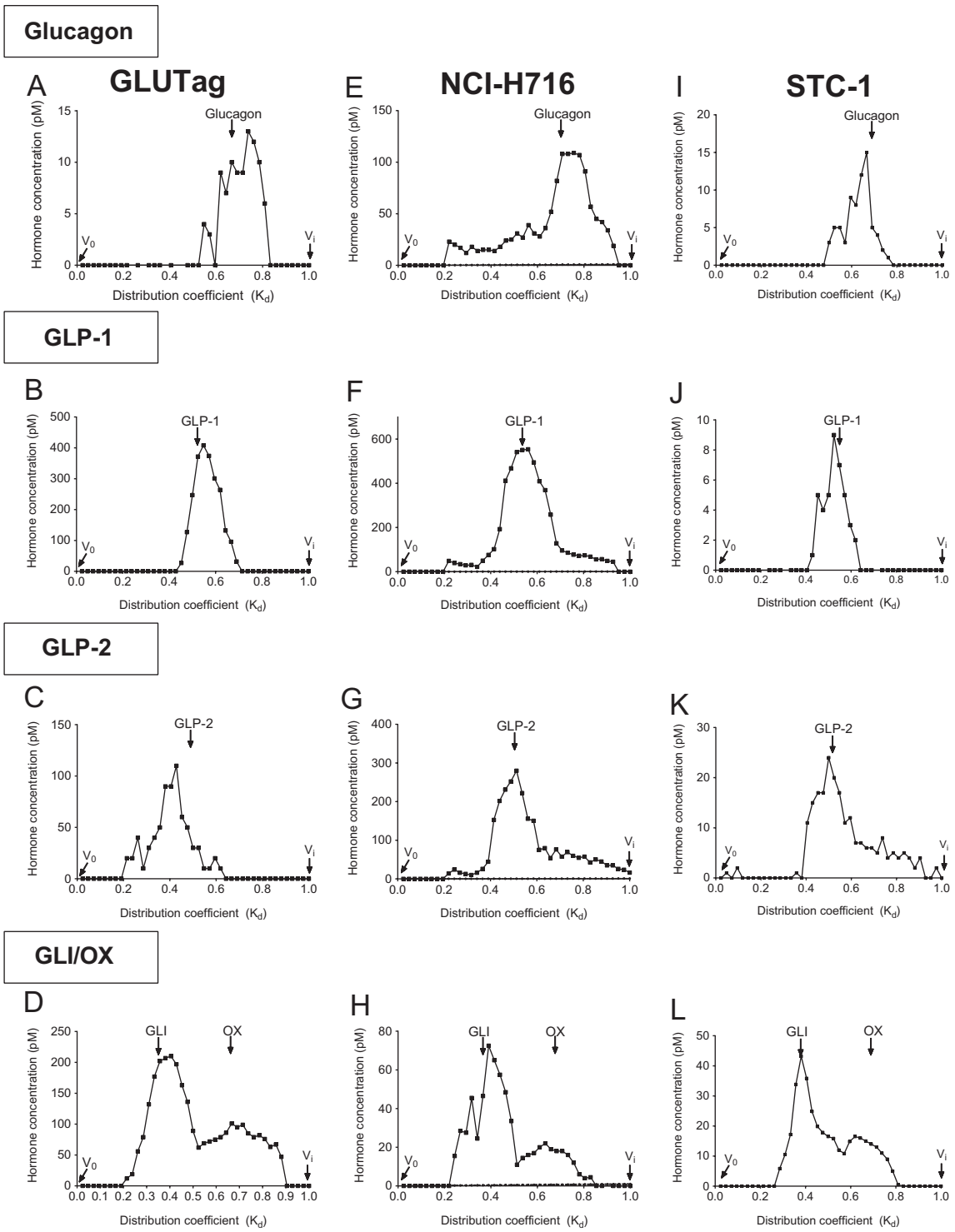

Figure 4

Processing patterns of proglucagon-derived gut peptides. Gel filtration-based characterization of the processing pattern of proglucagon. Eluted immunoreactivity (pM) is plotted against coefficient of distribution (K_d). Elution fraction containing the calibrators ^{22}Na and ^{125}I-labeled albumin were defined as V_0 and V_i, respectively (indicated on graphs). Representative K_d values for each of the measured peptides are indicated on top of graphs. For each cell line, nine extracted cell-lysis samples were pooled.

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Similarly, in sorted murine K-cells, PYY was detected by expression analysis in addition to GIP (Parker *et al*. 2009). Therefore, it appears that L-cells are more plurihormonal than previously thought.

The GLUTag, NCI-H716, and STC-1 cell lines are frequently used as models to study L-cell physiology, mostly because they are easy to maintain in culture and because no method for culture of pure primary L-cells has been established. Such cell lines are useful because they are suitable for cell biological studies, for instance, live monitoring of the intracellular concentration of free calcium and patch-clamp studies (Reimann & Gribble 2002, Reimann *et al*. 2004). However, these cell lines also have their limitations, in particular with respect to their lack of the polarization (apical vs basolateral surfaces), which is characteristic of native L-cells. Furthermore, the translational value of results generated with these cell lines naturally depends on whether the peptide contents and secretory patterns reflect those of natural L-cells. Nevertheless, the peptide profiles of the various cell lines have only sporadically been investigated previously.

We, therefore, characterized the peptide content and secretory repertoire of the three cell lines by assaying for 10 gut and 4 pancreatic peptides. The major finding is that despite producing GLP-1 and GLP-2 as expected, all three cell lines produce and secrete several other peptides that are not 'classical' L-cell products.

The detection of SST in both the extracts and incubation medium of all three cell lines was entirely unexpected, since natural L-cells are not thought to express SST (Egerod *et al*. 2012), as SST-producing cells result from an early lineage commitment that solely results in expression of SST. A later lineage commitment then results in cells producing ghrelin/motilin and substance P, while a yet later lineage commitment results in cells expressing CCK, GIP, GLP-1/2, NT, and PYY (Egerod *et al*. 2012).

Unexpectedly, we did not detect any PYY, which often co-localizes with GLP-1 in distal L-cells, in either the lysates or secretion samples from any of the cell lines. A few studies have reported measurable PYY storage in and secretion from STC-1 cells (Geraedts *et al*. 2009, Hand *et al*. 2013), but the study by Geraedts *et al*. (2009) reports measured concentrations that either are below or at the lower level of detection as reported by the assay manufacturers. However, given that the other peptides measured in our study were present at very high levels, combined with the lower limit of detection sensitivity (<1 pM) of the applied assays for PYY measurement in this study, we consider it unlikely that our results are erroneous. Instead, this cell line may show variability with respect to PYY expression.

Surprisingly, the peptide that was stored and released in the highest amounts from the STC-1 cells was GIP, with equivalent concentrations being measured using both N- and C-terminally directed assay (data only shown for intact GIP). Expression of GIP in STC-1 cells has been demonstrated previously, although only ~7% of the cells in the tumors gave rise to the cell line stained for GIP (Rindi *et al*. 1990). Subsequently, a GIP-enriched subclone (STC-6-14) was established, exhibiting glucose-dependent GIP-secretion, which was further enhanced when co-secreted somatostatin was immune-neutralized (Kieffer *et al*. 1995). Other STC-1-derived cell lines engineered to express insulin under control of the GIP-promoter secreted insulin in response to a number of stimuli, although the cell lines differed in their glucose responsiveness (Cheung *et al*. 2000, Kieffer *et al*. 1995, Ramshur *et al*. 2002). GIP secretion has also been reported from GLUTag cells in response to the artificial sweetener sucralose (Margolskee *et al*. 2007), although sucralose failed to stimulate GIP secretion from mixed epithelial cultures (Parker *et al*. 2009). In this study, we did not detect significant amounts of stored or secreted GIP in GLUTag and NCI-H716 cells, in agreement with our previously reported failure to detect GIP mRNA in GLUTag cells (Habib *et al*. 2012).

All three cell lines also contained and secreted glucagon in relatively high amounts, consistent with the observation that GLUTag and STC-1 cells, like the pancreatic α-cell (but unlike natural L-cells), express PC2. The finding was not due to cross-reactivity of glicentin in the glucagon assay (the entire glucagon sequence is contained in the middle part of glicentin), but the assay employed is highly specific for fully processed pancreatic glucagon, and detects neither glicentin nor OX (nor any elongated or truncated splice variants of glucagon) (Wewer Albrechtsen *et al*. 2014). In accordance with a previous study in GLUTag and STC-1 cells (Drucker *et al*. 1994), insulin was not detected in any of the cell lines. Our results also confirm data from other studies regarding the presence of GLP-2 in GLUTag and STC-1 cells, and the ability of GLUTag and in particular STC-1 cells, to produce and secrete CCK (Cordier-Bussat *et al*. 1997, Hand *et al*. 2010, Hand *et al*. 2013, Nemoz-Gaillard *et al*. 1998, Sidhu *et al*. 2000).

A major strength of this study is that secretion studies were included, since cellular storage of peptide hormones does not necessarily correlate to secretory

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output, as peptide secretion, for instance, depends on activation of the exocytotic machinery by molecular sensing mechanisms and intracellular sorting of the peptide into secretory vesicles, which then have to be recruited to the rapidly releasable vesicle pool. Emphasizing this, the cell lines differed considerably in the amounts of peptides stored (6–20 times higher for NCI-H716 cells), whereas the amounts actually secreted differed only two- to three-fold. Another strength of this study is that we also analyzed the processing pattern of the proglucagon-derived peptides. In summary, our analysis indicates that all three cell lines have the same PC1/3-dependent processing pattern of the proglucagon precursor into GLP-1 and GLP-2, glicentin, and OX as that described for human and pig (Buhl *et al*. 1988, Holst 1983, Orskov *et al*. 1987, Ørskov *et al*. 1991), although unlike native L-cells, all three cell lines also stored and secreted glucagon.

Comparing the three cell lines, GLUTag and STC-1 cells (derived from mice) contain and secrete more different peptides than the (human) NCI-H716 cells, while NCI-H716 cells contain and secrete larger amounts of the expressed peptides. Of the three cell lines tested, STC-1 cells resembled L-cells the least, since GLP-1 secretion was only weakly elevated by the stimulants, whereas GIP was both expressed and secreted in high concentrations. Therefore, this cell line may be less suitable for GLP-1 secretion studies. GLUTag cells also express and secrete high levels of CCK but not PYY, which suggest that they could be used as a model for proximal rather than distal native L-cells. NCI-H716 showed the most restricted peptide pattern, expressing neither CCK nor PYY, but storing and secreting SST. Therefore, NCI-716 cells seem to resemble neither proximal nor distal L-cells. Supporting that NCI-H716 cells represent an aberrant stage of differentiation, another study concluded that the expression of proglucagon in this cell line does not seem to be regulated by the same mechanisms as those activating human and rat proglucagon promoters (Cao *et al*. 2003). It would be of immense scientific interest to study the secretory products from isolated native L-cells in response to different stimuli, but unfortunately, attempts to culture isolated (mouse) L-cells have been unsuccessful. In conclusion, results obtained with the cell lines investigated here should be interpreted with some caution and preferably validated in more physiological models including *in vivo* studies, or studies using isolated perfused organs, where hormone secretion can be assessed directly from the relevant organ, while vasculature, paracrine relationships, and innervations are preserved.

Supplementary data
This is linked to the online version of the paper at http://dx.doi.org/10.1530/JME-15-0293

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
R E K, N J W A, and J J H were responsible for idea and design of the study. R E K, N J W A, E B M, J F R, F R, and F M G produced and analyzed data. REK prepared figures and drafted the manuscript. N J W A, C F D, E B M, F R, F M G, J F R, and J J H edited and revised manuscript. R E K, C F D, E B M, F R, F M G, J F R, and J J H approved final version of the manuscript.

Prior presentation
Some of the findings of this study were presented at the 75th Scientific Sessions of the American Diabetes Association, Boston, MA, 5–9 June 2015.

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