Human colon produces fully processed glucagon-like peptide-1 (7–36) amide

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Abstract The human colon contains many open-type endocrine cells which express the preproglucagon gene and possess glucagon-like peptide-1 (GLP-1) immunoreactivity, but the molecular form of the peptide is unknown. Acid ethanol extracts of human colon ($n = 4$) were subjected to gel filtration and successive purification by high-pressure liquid chromatography, monitored by specific RIAs. A single GLP-1-immunoreactive peak was isolated and identified as GLP-1 (7–36)amide by amino acid sequence analysis and mass spectrometry. We conclude that proglucagon is processed in the large intestine in the same manner as in the small intestine, and results in the formation of fully processed biologically active GLP-1.

Key words: Glucagon-like peptide-1; Proglucagon processing; Human colon

1. Introduction

The preproglucagon gene is expressed in both the pancreatic A-cells and the intestinal L-cells [1], but the primary translational product, proglucagon (PG) undergoes differential posttranslational processing [2,3]. In the pancreas, the major products are glicentin-related pancreatic polypeptide (GRPP; corresponding to PG 1–30), glucagon (PG 33–61) and the major proglucagon fragment (PG 72–158) [4], whilst in the small intestine, glicentin (PG 1–69), GRPP, oxyntomodulin (PG 33–69), glucagon-like peptide-1 (GLP-1; PG 78–107amide) and GLP-2 (PG 126–158) are the main molecular forms [reviewed in 5]. Many L-cells are also found in the large intestine, where the preproglucagon gene is also known to be expressed [1,6] in enteroglucagon producing cells [7,8]. GLP-1 and GLP-2 immunostaining is co-localised with glicentin/enteroglucagon staining in open-type endocrine cells in the ileum of all mammals studied so far [6,9–11], and GLP-1 immunostaining has been shown in both the colon [12] and the rectum [10]. However, although the presence of a GLP-1-immunoreactive moiety has been demonstrated in the large intestine, it is not known how proglucagon is processed in the large intestine, or whether true biologically active GLP-1 (7–36)amide is formed.

This study aimed to isolate and purify the GLP-1-immunoreactive material in the human colon and to ascertain its molecular nature.

2. Materials and methods

2.1. Tissue

Human colonic tissue (whole wall thickness; $n = 4$) was obtained during surgery on the gastrointestinal tract after approval of the local ethical committee. The tissue was immediately frozen and subsequently extracted with acid ethanol as previously described [method for neutral and basic peptides; 13]. Briefly, the frozen tissue was homogenised in 4 vols. of acid ethanol, stirred overnight at 4°C and centrifuged. 5 vols. of cold diethyl ether were added to the supernatant, and the aqueous phase was isolated at −50°C. The precipitate was dissolved in distilled water.

2.2. Purification

The tissue extracts were subjected to gel permeation chromatography on Sephadex G50 Fine grade columns (Pharmacia, Uppsala, Sweden) equilibrated and eluted with 0.5 M acetic acid (16 × 1000 mm at a flow rate of 0.3 ml/min or 50 × 1000 mm at 1 ml/min). Trace amounts of [^{125}I]-labelled albumin and ^{22}NaCl were added to all samples for internal calibration. Fractions were collected and assayed using the RIAs described below.

Fractions containing GLP-1 immunoreactivity were pooled, lyophilised and redissolved in 0.1% trifluoroacetic acid (TFA; Rathburn, Walkerburn, UK). These were subjected to analytical reverse-phase high-pressure liquid chromatography (HPLC) using LKB equipment with an MN cartridge system (Macherey-Nagel, Düren, Germany) fitted with a 4 × 100-mm Nucleosil 300–7 μm C_6H_5 column equipped with a precolumn. The column was eluted with step-wise linear gradients of acetonitrile (AcN; Rathburn) in 0.1% TFA (0% AcN for 12 min followed by 0–75% over 45 min; 1 ml/min), and fractions (1 ml) were assayed as described below. The immunoreactive peak was further purified using a 4 × 100-mm Nucleosil 120–5 μm C_8 column (0% AcN for 8 min followed by 0–30% over 5 min, 30–35% over 15 min and 35–75% over 3 min; 2 ml/min; 500 μl fraction size), a Nucleosil C_6H_5 column as above (0% for 3 min, followed by 0–31% over 5 min, 31–37% over 20 min and 37–75% over 3 min; 1.5 ml/min; 500 μl fraction size) and a C_8 column as before. The final purification was on a Hewlett Packard HP 1090 system using a 2.1 × 150-mm Vydac C_{18} column eluted at a flow rate of 200 μl/min (10–25% AcN over 5 min followed by 25–36% over 55 min and 36–90% over 1 min). The peak eluting at ~33% AcN was collected for mass and sequence analysis.

2.3. Amino acid sequence analysis

Aliquots of the purified peptide were sequenced using an automatic protein sequencer (Procise HT; Applied Biosystems, Foster City, CA) with on-line HPLC detection of the phenylthiohydatoin derivatives. All chemicals and solvents were of sequence or HPLC grade (Applied Biosystems).

2.4. Mass spectrometry

The molecular mass of the purified peptide was determined by matrix-assisted laser desorption ionization mass spectrometry using a Biflex instrument (Bruker, Bremen, Germany). A 0.25-μl aliquot of the purified peptide was spotted on a layer of α-cyano-4 hydroxy cinnamic acid, which was obtained by allowing 0.25 μl of a saturated solution of this matrix in AcN to dry. The samples were analysed in the linear mode with external calibration using dynorphin A and bovine insulin. The accuracy obtained with this method is >0.1%.

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2.5. RIAs

Fractions were assayed using three antisera raised against GLP-1. Antiserum 2135 [11,14] is side-viewing and detects all molecules with the central sequence of GLP-1 (7–36)amide, recognising both N- and C-terminally truncated or extended peptides. Antiserum 91022 [4] is directed towards the N-terminal sequence of GLP-1 (7–36)amide, and cross-reacts fully with N-terminally extended or C-terminally truncated fragments but <0.2% with N-terminally truncated peptides. Antiserum 89390 [15,16] has an absolute requirement for the intact amidated C-terminus of GLP-1 (7–36)amide, and cross-reacts <0.01% with C-terminally truncated or extended forms. For all assays, standard and [^{125}I]-labelled tracer were GLP-1 (7–36)amide, and separation of antibody-bound from free peptide was achieved using plasma-coated charcoal [11,15].

Fractions were also assayed using two antisera raised against glucagon. Antiserum 4304 [17] measures all peptides containing the 6–15 sequence of glucagon, including glucagon itself, and the enteroglucagons, including glicentin and oxyntomodulin. Antiserum 4305 [17] is specific for the C-terminus of glucagon, and only detects peptides with a fully processed C-terminus, and does not cross-react with glicentin or oxyntomodulin.

3. Results

The colonic extracts contained similar amounts of immunoreactive GLP-1 (wet weight; mean ± S.E.M.; n = 4) corresponding to 36 ± 23 pmol/g (side-viewing RIA), 24 ± 15 pmol/g (N-terminal RIA) and 36 ± 25 pmol/g (C-terminal RIA).

Analysis of the tissue extracts by gel filtration revealed a single GLP-1-immunoreactive peak at K_d 0.56 measured with all three GLP-1 antisera (Fig. 1). This corresponds to the elution position of synthetic GLP-1 (1–36)amide and GLP-1 (7–36)amide which, as previously shown, are difficult to distinguish by gel filtration [11].

Measurement of the same fractions with the side-viewing glucagon antiserum (4304) gave two peaks eluting in the positions of glicentin and oxyntomodulin (not shown), as in previous investigations [11]. No immunoreactivity was detected with the C-terminal assay for glucagon.

Analysis of the GLP-1-immunoreactive material from the gel filtration by successive HPLC separations showed that the peptide eluted as a single immunoreactive peak, detected equally by all three GLP-1 assays (Fig. 2). This peak eluted at the position of GLP-1 (7–36)amide, determined by calibration with the synthetic standard after chromatography of the colonic material was complete. The final purification on a Vydac C_{18} column yielded an apparently pure peptide which was subjected to mass and sequence analysis.

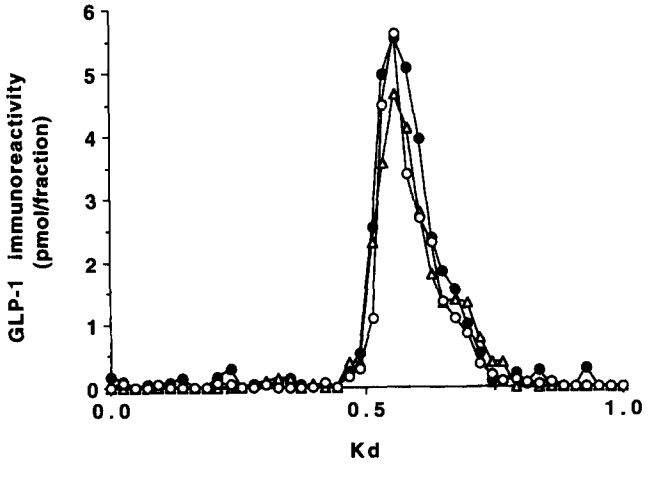

Fig. 1. Gel filtration of an extract of human colon analysed using C-terminal amide-specific (89390; ○-○), N-terminally directed (91022; △-△) and processing-independent (2135; ●-●) RIAs for GLP-1. The concentration is plotted against the coefficient of distribution (K_d).

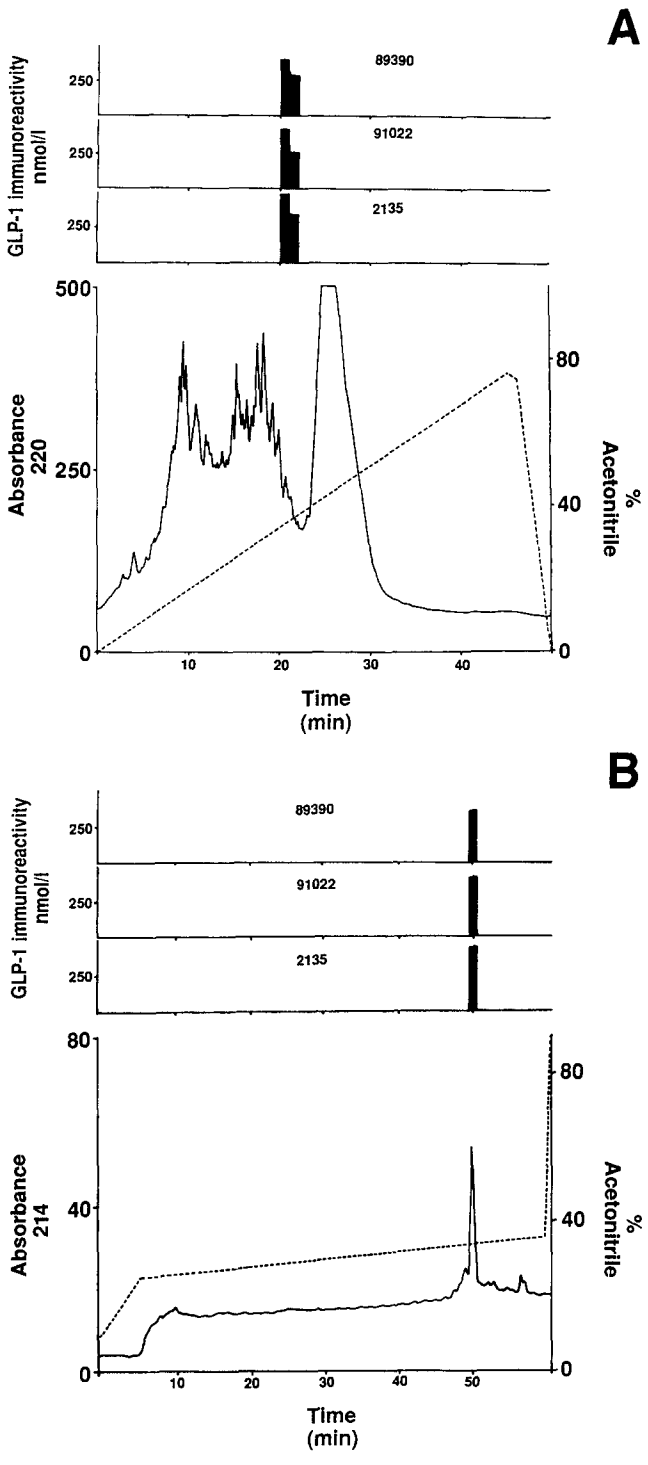

Fig. 2. HPLC analysis of the GLP-1-immunoreactive material eluting from the gel filtration columns showing the first separation on a Nucleosil C_6H_5 column (A) and the final purification on a Vydac C_{18} column (B). The optical density (milli absorbance units) is plotted against retention time, and fractions were assayed using C-terminal amide-specific (89390), N-terminally directed (91022) and processing-independent (2135) RIAs for GLP-1.

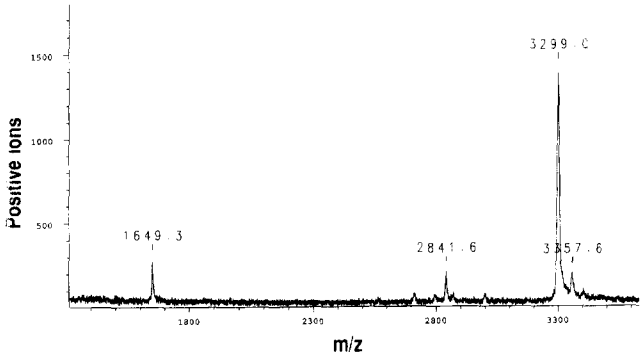

Fig. 3. Mass spectrum of the immunoreactive material eluting from the final HPLC purification on a Vydac C_{18} column. ~0.1 pmol was analysed by laser desorption ionization mass spectrometry. The values given above the peaks indicate the molecular masses of the molecular ions (MH^+).

Protein sequence analysis enabled the entire sequence to be obtained. This was identical with GLP-1 (7–36), with the presence of the C-terminal amide being unequivocally confirmed by positive binding in the C-terminal RIA which has an absolute requirement for an amidated arginine at position 36 [16].

The molecular mass was determined to be 3298.0 compared with a theoretical mass of 3297.7 for GLP-1 (7–36)amide (Fig. 3).

4. Discussion

The ileal L cells express the preproglucagon gene, and it is well-established that posttranslational processing results in the parallel secretion of GLP-1 (7–36)amide with the intestinal enteroglucagon, glicentin [reviewed in 5]. Similar L-cells are found in the large intestine [7,8] and preproglucagon gene expression has been demonstrated in the colon [1,6]. These observations, together with the demonstration of GLP-1-immunoreactive staining co-localising with glicentin in the ileum [6,9,11], colon [12] and rectum [10], and the presence of GLP-1 immunoreactivity in tissue extracts of large and small intestine [6] led to the assumption that processing of proglucagon in these tissues is similar, even though the molecular nature of the GLP-1 immunoreactivity was not identified. In a later study, Mojsov et al. [18] showed that the majority of GLP-1 immunoreactivity in the rat large and small intestine co-eluted on HPLC with synthetic GLP-1 (7–36)amide, although a small proportion eluted in the position of non-amidated, glycine-extended GLP-1 (7–37). The present study has shown that fully processed GLP-1 (7–36)amide is found in the human colon, in concentrations which are similar to those reported for the human small intestine [11]. It should be emphasised that with the employed techniques for extraction and chromatographic analysis, alternative processing products could not have escaped detection, since this methodology has been demonstrated to extract quantitatively all molecular forms of proglucagon processing [4,14,19,20], and the entire gel filtration of the extract was analysed without discarding side fractions. Mass determination revealed a component with a mass of 3356.6, which is close to that of GLP-1 (7–37). This probably represents a minor contaminant, since the HPLC methodology is capable of separating GLP-1 (7–36)amide from GLP-1 (7–37), and a glycine extension was not detected by sequence analysis. An N-terminally truncated metabolite, GLP-1 (9–36)amide, has recently been identified in human plasma [21]. It is noteworthy that this metabolite was not identified in the colonic tissue extracts, indicating that this metabolism occurs outside the tissue, once the peptide has been released.

GLP-1 is released from the small intestine in response to the same physiological stimuli which are known to cause release of glicentin (reviewed by Ørskov [3]). Whilst the release of GLP-1 by the large intestine has not been demonstrated, this is likely to be the case, given that enteroglucagon and GLP-1 are co-secreted in the ileum [5]. Thus, instillation of glucose [22,23] or fermentable fibre [24] into the colon which stimulate the release of enteroglucagon is likely to also cause GLP-1 secretion. GLP-1 secretion is, therefore, likely to be stimulated by the presence of undigested nutrients in the colon, which occurs in certain pathological conditions in which abnormal amounts of unabsorbed nutrients reach the distal intestine and are associated with elevated concentrations of enteroglucagons [5]. A co-secretion of GLP-1, by virtue of its effects on gastric emptying [25] would be beneficial by delaying the supply of further nutrients.

In conclusion, this study has shown that the human colon produces fully processed biologically active GLP-1 in concentrations which are high enough to have a physiological role. Further studies are required to assess the importance of the colon as a source of GLP-1.

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