Glucagon-related peptides in the human gastrointestinal mucosa

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Summary. We studied the chromatographic profile and the distribution of glucagon-related peptides in the human gastrointestinal mucosa, using radioimmunoassays directed against the glucagon 6–15 and 19–29 sequences, and against the glicentin sequences 15–30 and 61–69, and a radioreceptor assay for glucagon. Very small amounts of glucagon-related peptides were found in the gastric mucosa, whereas at least four different components could be identified in the distal intestine. One component (mean concentration 130 pmol/g ileal mucosa) is similar to porcine glicentin for size and C-terminal extension, but differs from the glucagon part of the molecule in the N-terminal extension. A second component (mean concentration 131 pmol/g) is probably identical to porcine peak II enteroglucagon (glicentin 33–69), and a third component (7.9 pmol/g) seems to be identical with glucagon. A fourth component containing the glucagon sequence plus an N-terminal extension was also identified (1.7 pmol/g). Thus the human intestinal mucosa contains large amounts of peptides containing the glucagon sequence; at least one of these probably also possesses glucagon-like bioactivity. The proposed structures of the four components are consistent with the base sequence of the first half of the human glucagon gene.

Key words: Glicentin, proglucagon, gut glucagon-like immunoreactivity, enteroglucagon, oxyntomodulin, glicentin 33–69, radioreceptor assay.

Since the recent elucidation of the chemical structure of the two predominating porcine enteroglucagons, glicentin and peak II glucagon-like immunoreactivity (GLI) (or glicentin 33–69, glucagon 1–37, bioactive enteroglucagon, oxyntomodulin) [1–4], and the demonstration of remarkable biological effects of these peptides [3–5], the interest in this group of peptides has increased greatly [6]. Unfortunately little is known about the structure of the human enteroglucagons; only a few preliminary reports have been published [7–9]. We therefore carried out a systematic investigation of extractable glucagon-related peptides in the human gastrointestinal mucosa. For the chemical characterization, we employed Sephadex chromatography, region-specific radioimmunoassays directed against four different regions of the glicentin molecule and a glucagon radioreceptor assay [2].

Materials and methods

Extraction

Mucosal tissues were obtained from the operating theatre during surgical operations on the gastrointestinal tract. None of the patients were known to have diabetes; most had gastrointestinal cancer. Duodenal mucosa was obtained from kidney donors during transplantation operations. It was confirmed by subsequent microscopy that specimens were taken from apparently normal tissues. The mucosa was scraped off the other layers of the specimen in question immediately after excision, washed in ice-cold saline (0.154 mol/l), and frozen in dry ice. Thus, the time interval between excision and freezing was kept within the range of a few minutes. Colon specimens were studied without separation of the mucosa. Specimen weight ranged from 1 to 50 g. Frequently more than 10 g were extracted. Frozen tissue was stored at $-20\,°C$ until extracted as previously described [method II in 10]. In brief, frozen tissue was homogenized (in a blender) and extracted with acid/ethanol at initially $-20\,°C$ and centrifuged, and the supernatant was mixed with ice-cold diethyl ether. The aqueous proteinaceous phase was isolated at $-50\,°C$, redissolved in dilute HCl with urea at 4–8 mol/l and subjected to gel filtration.

All gel filtrations were carried out at $4\,°C$ on either 50×1000 mm or 16×1000 mm columns (K 50/100 or K 16/100, Pharmacia Fine Chemicals, Uppsala, Sweden), packed with Sephadex G-50, fine grade (Pharmacia) and equilibrated and eluted with acetic acid (0.5 mol/l). Constant flow rates (1 and 0.5 ml/min, respectively) were maintained with precision pumps (Microperpex model 2132, LKB, Bromma, Sweden), and fractions corresponding to approximately 2% of bed volume were automatically collected. Sample size never exceeded 2% of bed volume. The columns were calibrated with unlabelled and labelled glicentin and glucagon, and ^{125}I-labelled albumin and ^{22}NaCl were added in trace amounts to all samples to be filtered for internal calibration. Column effluents were freeze-dried, reconstituted in assay buffer and subjected to radioimmuno- or radioreceptor assay as described previously [2]. Elution positions are referred to by

their coefficient of distribution, $K_d = (V_e - V_o)/V_i$, where V_e is the elution volume of the substance in question, V_o is the exclusion volume of the gel, and V_i is the available inner volume, determined as the difference between the elution volumes of ^{125}I-labelled albumin and $^{22}Na^+$. K_ds for glucagon and glicentin were 0.25 ± 0.02 and 0.69 ± 0.02, respectively (mean ± SD). Recovery of small amounts of glucagon and glicentin on this column system is greater than 65%, and probably still greater with extracts because of the carrier effect of other proteins present in the extracts.

Structural analysis of the column effluent was performed using assays for the glicentin sequence 15–30, the glucagon sequence 6–15, the glucagon sequence 19–29 and glicentin sequence 61–69, and glucagon radioreceptor analysis as described previously [2, 6, 11, 12] (Fig. 1). The assays for the glucagon sequence 19–29, and the radioreceptor assay both require absence of extensions of the C-terminal and the N-terminal sequence of the glucagon molecule, respectively. Standards in the assays for glucagon sequences 6–15 and 19–29 were highly purified porcine glucagon, and standards in the assays for the glicentin sequences 15–30 and 61–69 were highly purified porcine glicentin. The results are given as pmol-equivalents to these standards. Assays for glicentin 61–69 were made with antiserum 4804, which was raised against synthetic glicentin 49–69 [13], glicentin standards and using ^{125}I-labelled glicentin [11] as tracer; incubation and separation methods were similar to those employed for the glucagon assays. The assay sensitivity was below 5 pmol/l, and the intra-assay coefficient of variation in the working range was below 10%; neither intact glucagon nor the glucagon fragment 19–29 cross-reacted in this assay in con-

centrations of up to 1 nmol/l. As previously discussed [14], the detection limit of the combined extraction and chromatography procedures is about 2.5 fmol or 0.2 pmol in 1 g (wet weight) of tissue (sensitivity of the glucagon assays around 1 fmol/ml and fraction size of 2.5 ml gives 2.5 fmol; taking into account dilution inherent in chromatography and extraction and recovery with these procedures, this corresponds to 0.2 pmol/g).

Results

The immunoreactive contents of all extracts determined by the four region-specific assays are given in Table 1. Assays for the glicentin sequence 15–30 were negative in all extracts. Values close to the detection limits were found with the assay for the glucagon sequence 19–29 in extracts from the stomach, duodenum, jejunum and the large intestine, and with the assay for the 6–15 sequence in the fundus and the antrum; with both assays ileum was the predominant source of glucagon-like immunoreactivity. Significant amounts of immunoreactive material were detected with the assay for the glicentin sequence 61–69 in all regions of the distal intestine with the majority in the ileum.
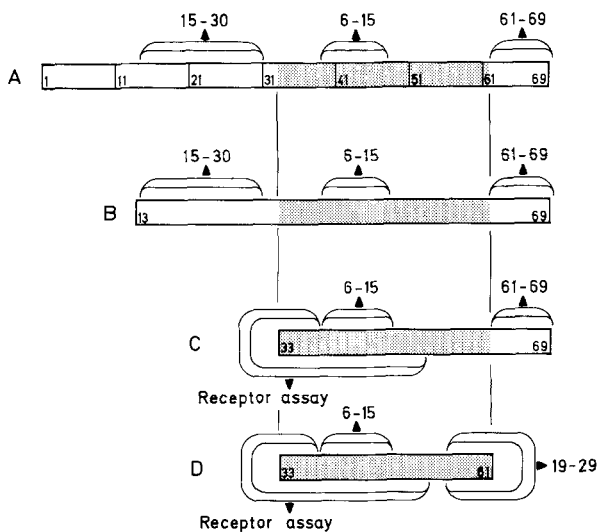
Fig. 1. Binding regions of the assays used. The figure shows the structure of porcine glicentin (A) and the various fragments thereof (B–D, including glucagon (D), the sequence of which is identical with the glicentin region 33–61 shaded area) as they occur in porcine intestinal mucosa [6, 15]. The binding regions of the assays against glicentin 15–30, glicentin 61–69, glucagon 6–15 and glucagon 19–29, as well as the probable binding region of the radioreceptor assay [12] are indicated by parentheses

Fig. 2. Average gel filtration profiles of immunoreactive glucagon-related peptides in extracts from human jejunal, ileal, colonic and rectal mucosa, as determined by a radioimmunoassay against the 6–15 sequence of the glucagon molecule. For each of the gel filtrations the effluent concentrations at the K_d values indicated in the figure were read off from the individual elution curves (by interpolation if necessary) and expressed as percentage of the total amount of immunoreactivity applied to that column; the mean ± SEM fractional outputs were then plotted against K_d. The elution positions of unlabelled glicentin and glucagon are indicated by arrows.
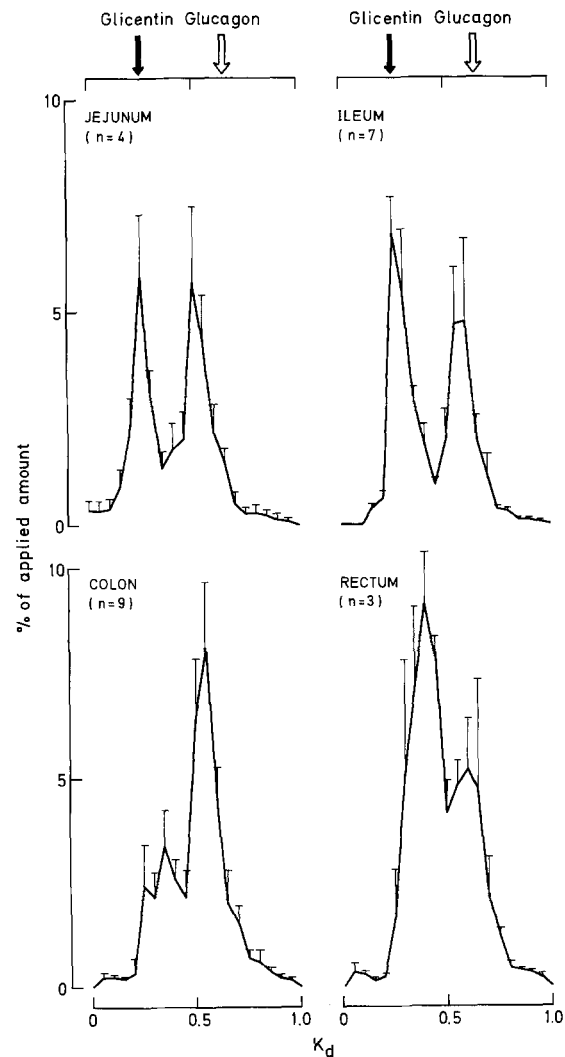
**Table 1.** Gel filtration profile of human gastrointestinal glucagon-like immunoreactivity

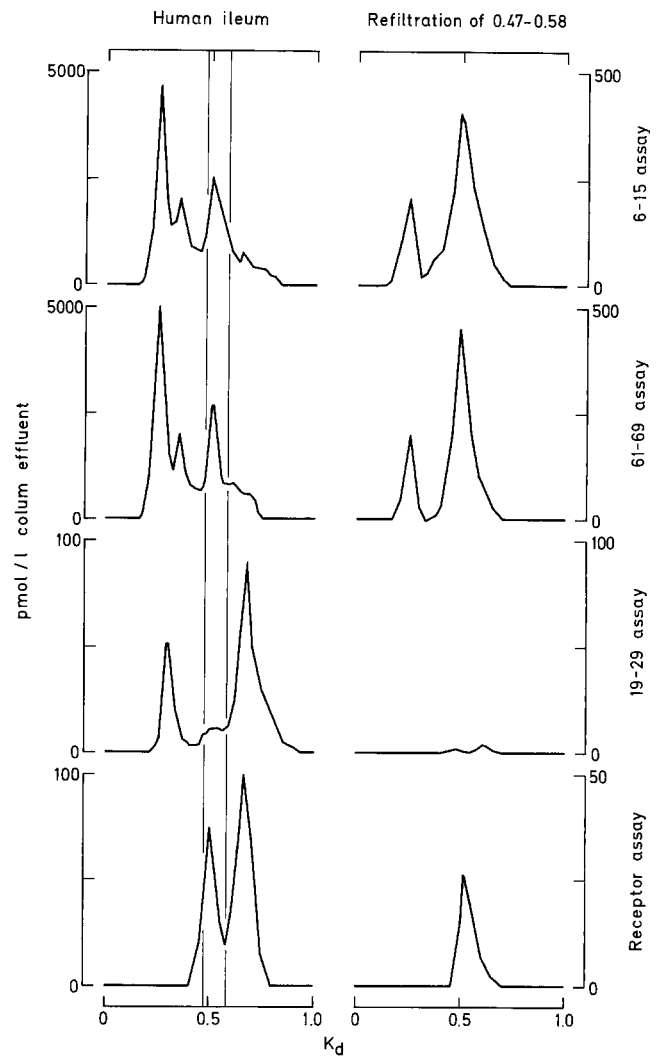| Tissue	Total concentration (pmol/g)	Gel filtration profile: K_d values and peak fraction of total (%)							
		Peak 1		Peak 2		Peak 3		Peak 4	
		K_d	Peak fraction (%)	K_d	Peak fraction (%)	K_d	Peak fraction (%)	K_d	Peak fraction (%)
Glucagon 6–15 immunoreactivity									
Fundus ($n=8$)	<0.15			No peaks identified[a]					
Corpus ($n=3$)	4.2 ± 1.2	0.25±0.01	66.1 ± 0.2			0.54±0.01	33.8± 1.6		
Antrum ($n=3$)	1.5 ± 0.14	0.13±0.02	27.7 ± 0.65			0.52±0.01	8.2± 2.7	0.75±0.01	64.1± 9.9
Duodenum ($n=3$)	1.53± 0.33	0.26±0.01	22.8 ±12.5	0.36±0.01	10.1± 5.5	0.49±0.05	34.2±15.9	0.70±0.02	32.7±21.9
Jejunum ($n=4$)	5.06± 0.44	0.24±0.01	44.0 ±12.0			0.50±0.02	51.4±10.3		
Ileum ($n=7$)	326.06±76.3	0.25±0.02	39.9 ± 7.4	0.39±0.01	11.0± 3.7	0.54±0.03	40.2± 7.7		
Colon[b] ($n=9$)	15.87± 4.25	0.23±0.02	21.2 ± 8.4	0.35±0.01	24.8± 6.4	0.54±0.01	64.7± 7.3		
Rectum ($n=3$)	8.24± 0.46	0.23±0.02	7.04± 3.92	0.40±0.03	58.1± 6.6			0.63±0.01	30.2± 7.2
Glucagon 19–29 immunoreactivity									
Fundus ($n=8$)	<0.15			no peaks identified[a]					
Corpus ($n=3$)	0.80± 0.03			no peaks identified					
Antrum ($n=3$)	0.47± 0.16							0.75±0.01	91.1± 2.1
Duodenum ($n=3$)	0.22± 0.12	0.28±0.04	29.9 ± 4.6					0.67±0.04	68.5± 3.9
Jejunum ($n=4$)	0.4 ± 0.11	0.34±0.02	24.2 ± 2.6					0.73±0.02	65.8± 8.0
Ileum ($n=5$)	10.42± 2.20	0.34±0.02	15.9 ± 1.0					0.72±0.01	75.9± 4.1
Colon[b] ($n=5$)	0.66± 0.19	0.32±0.03	28.7 ± 9.8					0.67±0.07	42.3± 0.5
Rectum ($n=3$)	0.84± 0.47	0.34±0.01	14.7 ± 1.7	0.45±0.02	25.4± 5.1			0.69±0.03	55.8± 3.4
Glicentin 61–69 immunoreactivity									
Jejunum ($n=2$)	2.7 ± 7.5	0.24–0.25	22.9 –45.9	0.35–0.37	10.0–21.5	0.49–0.54	23.8–41.6		
Ileum ($n=6$)	46.3 ±11.7	0.25±0.01	44.6 ± 6.3	0.35±0.01	17.6± 6.3	0.54±0.02	39.2± 4.5		
Colon[b] ($n=4$)	1.36± 0.21	0.33±0.01	27.6 ± 5.3	0.41±0.01	11.8± 2.3	0.55±0.01	53.6± 1.9		

Values are mean ± SEM

[a] Data from reference [14]

[b] Stripping of mucosa omitted; peptide concentration therefore refers to the whole wall. Glicentin 15–30 immunoreactivity was undetectable in all tissues
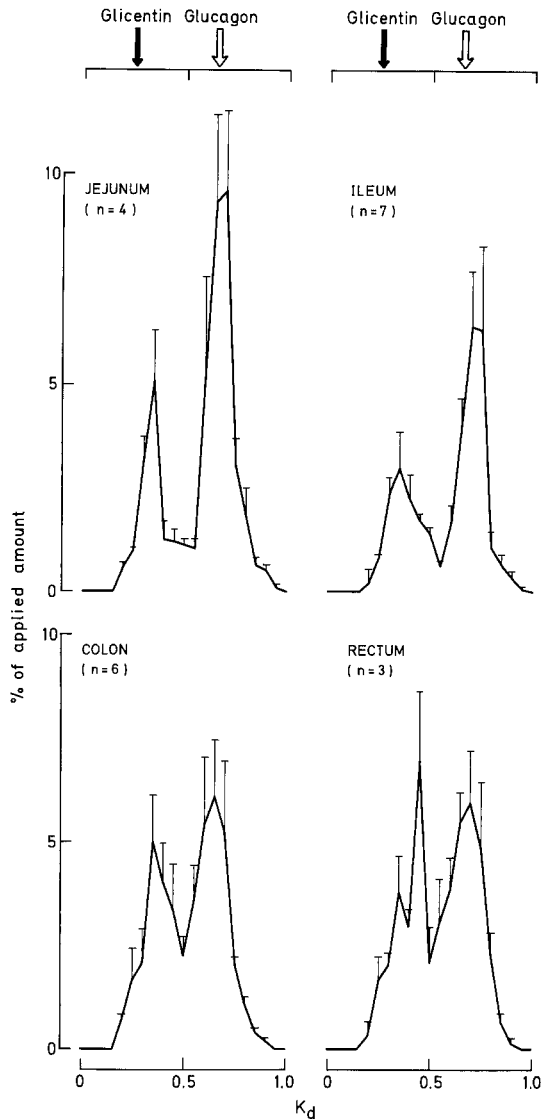
Fig. 3. Average gel filtration profiles of immunoreactive glucagon-related peptides in extracts from human jejunum, ileum, colon and rectum, as determined by a radioimmunoassay against the 19–29 sequence of the glucagon molecule. See Figure 2 for details

Fig. 4. Left panel: Gel filtration profile of an extract of human ileal mucosa as determined with radioimmunoassays against various sequences of glucagon and glicentin (see Fig. 1), and a radioreceptor assay against glucagon. Right panel: the fractions eluting at K_d 0.47–0.58 were pooled and subjected to refiltration and repeated profile analysis

Figure 2 shows the chromatographic profile of extracts from four parts of the human intestinal mucosa as determined by the assay for the glucagon sequence 6–15. In the ileum and jejunum, well defined peaks were seen at K_d 0.25 and 0.5–0.6; the latter peak was also present in the colon and rectum extracts, which were otherwise characterized by the presence of a component at K_d 0.34–0.40. Chromatographic profiles from the same regions determined with the assay against the glucagon 19–29 sequence are shown in Figure 3. The dominating peak was at K_d 0.65–0.7, but all regions also showed a clear peak at K_d 0.30.

Figure 4 shows three representative chromatographic profiles of ileum extracts as determined with the assays for the glucagon regions 6–15 and 19–29, the glicentin 61–69 assay and a glucagon radioreceptor assay.

The profile obtained with the glicentin 61–69 assay closely follows that of the glucagon 6–15 assay. The radioreceptor assay profile consists of a peak, which coincides with the K_d 0.5 peak identified in the glicentin 61–69 and the glucagon 6–15 assays, and a second peak which coincides with the K_d 0.68 peak identified in the glucagon 19–29 assay. Refiltration of the fractions corresponding to the K_d interval 0.47–0.58 resulted in further isolation of the K_d 0.5 peak, which then showed very little glucagon 19–29 immunoreactivity, but reacted strongly with the remaining three assays. The elution position (K_d 0.5) corresponds exactly to the elution position of glicentin 33–69 [2].

The relative distribution of glicentin 61–69 immunoreactivity in extracts from different regions is indicated in Table 1.

Discussion

The chromatographic profiles of the glucagon-related peptides found in this study are similar to those found in porcine tissues [2, 6, 15]; the most remarkable difference is the complete absence of glicentin 15-30 immunoreactivity, showing that this sequence is not found in the human enteroglucagon molecules. Nevertheless the majority of the glucagon-like immunoreactivity, as determined with the glucagon 6-15 assay, was found in a peak with the same K_d and therefore probably the same size as glicentin [1]. This means that in human intestinal mucosa there must be an analogous 'human glicentin' which, however, differs in N-terminal sequence from its porcine counterpart. As judged from the glicentin 61-69 assay, the C-terminal sequence of the 'human glicentin' could very well be similar to the C-terminal sequence of porcine glicentin since this assay identified apparently the same peak at K_d 0.25. Furthermore, a component at K_d 0.5 could be identified similarly by the glucagon 6-15 assay, the glicentin 61-69 assay and the radioreceptorassay, whereas the glucagon 19-29 activity detected disappeared on refiltration and therefore probably represents contamination. This component thus behaves exactly like its porcine counterpart, peak II GLI (or glicentin 33-69 or glucagon 1-37 or 'oxyntomodulin' [2-4], and as discussed earlier [2, 12] this strongly suggests that its structure is identical to the glicentin sequence 33-69. A similar conclusion was reached by Munck et al. [16].

In the extracts of the large intestine, there was less 6-15 immunoreactivity at K_d 0.25, but a prominent peak was observed at K_d 0.35-0.4. The structure of this component is unclear but a similar component is observed in porcine extracts where it probably represents a fragment of glicentin with N-terminal deletions [6, 15]. Possibly proteolytic activity in the specimens of large intestinal mucosa, which were often excised after prolonged interruption of the local circulation (hemicolectomy), could explain the difference.

Assuming that the C-terminal sequence of the 'human glicentin' and the 'human peak II GLI' are identical with the porcine sequence, which seems to be the case (see below), one would have expected equal immunoreactivity of these components and therefore the estimations of these components with the glicentin 61-69 assay and the glucagon 6-15 assay to coincide. This was sometimes the case (Fig. 4), but on average the glicentin assay measured less than the glucagon assay (Table 1). The reason for this is unclear, but suggests some structural difference, which is not large enough to influence elution position detectably. Such modifications might include deamidations or guanylations which might take place to a varying extent during the extraction procedure.

The assay against the glucagon 19-29 sequence identified two components in the intestinal extracts, again with positions similar to those found in porcine extracts. Due to their low concentration and to contamination with other glucagon-related peptides, it was difficult to characterize these components further; however, the elution position of the smaller of the two components, which is exactly coincident with the elution position of glucagon, and its reactivity in the radioreceptorassay strongly suggest that this component is identical to glucagon. Whether or not the larger component is similar to the "large glucagon" found in pancreatic extracts [17, 18] remains to be investigated.

In conclusion, our results show that the glucagon-related peptides of the human gastrointestinal tract comprise at least four molecular forms, one which is similar to porcine glicentin but with a different N-terminal sequence, another which is similar to and probably identical with porcine peak II glucagon-like immunoreactivity (glicentin 33-69), a third which is similar to glucagon itself and finally a component, also found in pigs, which is slightly smaller than glicentin and probably has the entire glucagon sequence at its C-terminus.

After completion of this study, the structure of the human glucagon gene was published [19]. Because a search of a gene library revealed a single gene for glucagon only, this would indicate that the intestinal and the pancreatic glucagon-related peptides are derived from identical precursors which, however, have been subjected to different post-translational processing as already suspected [6]. The base sequence of the human glucagon gene shows that the first half of the precursor sequence shows extensive homology with glicentin, with complete identity of the 29-69 sequences, which include the glucagon and the peak II glucagon-like immunoreactivity moieties. Major differences, however, were found in the 14-28 sequence (six positions). Thus the structural predictions of the present study are entirely consistent with the gene structure, and proves that the post-translational modifications of the intestinal glucagon precursor are very similar in man and pig.

Unlike many experimental animals the human stomach contains very little extractable glucagon-like immunoreactivity. The intestinal mucosa, however, is a rich source of glucagon-related peptides of which at least one probably also possesses glucagon-like bioactivity [3, 4, 8]. This would explain the finding that pancreatectomy in man does not cause complete disappearance of glucagon and related peptides in plasma [20].

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