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# Carboxypeptidase-B-like processing of the C-terminus of glucagon-like peptide-2 in pig and human small intestine

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We developed specific, C-terminal radioimmunoassays for three proglucagon (PG) fragments: PG 151–158, PG 151–160 and PG 126–159 (glucagon-like peptide-2 (GLP-2)) in order to determine the exact C-terminal sequence of the newly isolated GLP-2 in man and pig. The antigens and the antisera showed no mutual cross-reactivity. By gel filtration of extracts of pig and human small intestine, the immunoreactivity eluting at the position of GLP-2 was identified by the radioimmunoassays for glucagon-like peptide-2 (PG 126–159) and for PG 151–158, whereas the assay for PG 151–160 was completely negative. We conclude that the C-terminal amino acid residue of pig and human ileal GLP-2 is PG 158. Thus the basic residues, PG 159 and 160 are removed during its processing in the small intestine.

Proglucagon; Posttranslational processing; Glucagon; Enteroglucagon; Gastrointestinal hormone

## 1. INTRODUCTION

The structure of human proglucagon (PG) was predicted at first in 1983 by Bell and co-workers, from the nucleotide sequence of a single gene encoding glucagon. PG was suggested to consist of 159 amino acid residues (with a C-terminal arginine residue) [1]. In 1984, however, Heinrich and co-workers, who sequenced a structurally similar gene encoding for glucagon in the rat, suggested that human proglucagon, like rat proglucagon, consisted of 160 amino acids (with an additional C-terminal basic residue, lysine [2]). The proglucagon sequence consists of three glucagon-like sequences flanked by dibasic potential cleavage sites: glucagon itself and two C-terminal amino acid sequences with approximately 50% sequence homology with glucagon, glucagon-like peptide-1 (GLP-1) and glucagon-like peptide-2 (GLP-2) (fig.1). Both GLP-1-immunoreactivity (IR) and GLP-2-IR are found in gel filtrations of mammalian small intestinal extracts (e.g. human and pig [3,4]). The elution position of GLP-2-IR corresponds to a peptide of a molecular mass of about 3 kDa. As deduced from the PG sequence post-translational cleavage of proglucagon to obtain GLP-2 would be expected to occur, N-terminally, at the site of the dibasic amino acids at residue numbers 124–125. For the C-terminus, three possibilities might be considered: (i) no modification of the C-terminus of PG; (ii) post-translational cleavage of PG with a trypsin-like endoprotease or (iii) C-terminal cleavage of PG first with a trypsin-like endoprotease and subsequently

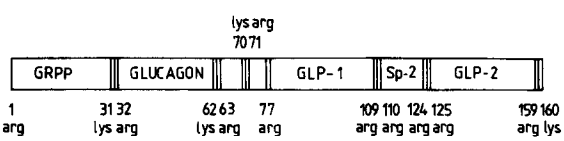


Fig.1. Proglucagon in mammals is a 160 amino acid peptide precursor. It consists of an N-terminal peptide which is homologous to glicentin-related pancreatic peptide, GRPP (PG 1–30), and three glucagon-like sequences: glucagon (PG 33–61), glucagon-like peptide-1 (PG 78–107 amide), and glucagon-like peptide-2 (PG 126–158), the two latter peptides separated by an intervening sequence of 13 amino acids, the so-called spacer peptide-2 (Sp-2) (PG 111–122 amide).

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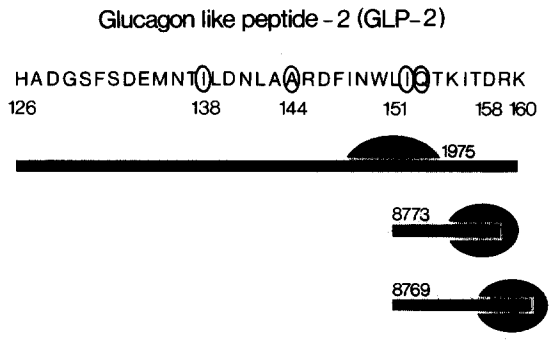


Fig.2. Amino acid sequence of the C-terminus of human proglucagon containing the GLP-2 sequence. Peptide fragments employed in this study are indicated according to their size and position. Crescents represent the antigenic sites of the antisera employed. Encircled amino acids represent residues in human PG differing from pig PG.

with a carboxypeptidase-B-like exoprotease. The three conceivable C-terminal residues would thus be: residues 160, 159 or 158. We recently isolated GLP-2 from pig small intestine and found that the majority of porcine GLP-2 corresponded to proglucagon 126–158 and that human GLP-2 corresponded exactly in size to the porcine peptide [5]. However, although no traces of further residues (in particular basic residues) were found, some uncertainty about the exact C-terminal sequence of this rather long peptide remained. Thus, to determine the exact C-terminal structure of GLP-2, we investigated the immunoreactive behaviour of GLP-2 in extracts of small intestine from man and pig with specific C-terminal radioimmunoassays (RIA) against the PG fragments: PG 151–158, PG 151–160 and a previously developed radioimmunoassay for PG 126–159 (GLP-2) (fig.2).

## 2. MATERIALS AND METHODS

PG 151–158, PG 151–160, cys$^{150}$PG 150–158 and cys$^{150}$PG 150–160 were synthesized on an Applied Biosystems 430A peptide synthesizer, using a standard BOC-chemistry procedure. Resin-bound amino acids and protected amino acids were purchased from Applied Biosystems (Warrington, England). Peptides were cleaved from the resin and deprotected by liquid HF by a standard procedure [6]. Free peptides were purified on a low pressure RP-18 column using acetic acid (2%)/water/ethanol (0–40%) as eluent. Purity was ascertained by amino acid, sequence and HPLC (high pressure liquid chromatography) analyses. Synthetic PG 151–158 and PG 151–160 were used as standards and for preparation of labelled peptides.

For the production of antisera cys$^{150}$PG 150–158 and cys$^{150}$PG 150–160 (5 mg each) were coupled to keyhole limpet hemocyanin (4 mg) through the cysteine residues using *m*-maleimido-benzoyl-*N*-hydroxysuccinimide [7]. Rabbits were boosted five times each at two week intervals with immunogen corresponding to 250 $\mu$g of peptides and were bled ten days after the last immunization.

Synthetic PG 151–158 and PG 151–160 were iodinated with $^{125}$I-labelled Bolton-Hunter reagent according to the manufacturer's instructions (Amersham, Buckinghamshire, England) and purified by gel chromatography before use, as previously described [8].

For the assay of GLP-2, we used synthetic GLP-2 (PG 126–159, human sequence, code 7167, Peninsula, Belmont, CA, USA) as the standard, $^{125}$I-labelled GLP-2 and antiserum 1975 raised against synthetic GLP-2, as described in [8]. The antigenic site for this antiserum has previously been found to be located in the C-terminus of the peptide between PG residues 146 and 158 [8].

Standard or sample and antiserum (ab) were incubated (final dilution (fd.) of ab 8773 (against PG 151–158) 1:2500; fd. of ab 8769 (against PG 151–160) 1:5000; and fd. of ab 1975 1:10000 (against PG 126–159)) for 48 h at 4°C. After another 48 h of incubation with labelled peptide at 4°C, free and bound peptides were separated by adsorption to plasma-bound charcoal as previously described [8].

All possible combinations of the three different antisera, labelled peptides and synthetic peptides were studied to reveal possible cross-reactions between them.

2 nmol of synthetic GLP-2 (PG 126–159) in 1.5 ml of saline was incubated with 15 $\mu$l of carboxypeptidase B (art. 2300, batch 9623 230, Merck, Darmstadt, FRG) for 2 h at room temperature and subsequently applied on a K16/100 column packed with G-50 SF (Pharmacia, Sweden), equilibrated and eluted in 0.04 mol/l sodium phosphate buffer containing in addition 0.1% human serum albumin (reinst, trocken, Behringwerke, Marburg, FRG), 2.9 g/l NaCl, 0.04 mmol/l thiomersal and 20 mmol/l EDTA at a flow rate of 19 ml/h. All fractions were subjected to radioimmunoassay using antisera 1975 and 8773. The amount of and the elution position of immunoreactive material were compared with those of 2 nmol of undigested synthetic GLP-2 applied to the same column. $^{125}$I-labelled albumin and $^{22}$NaCl were added to all samples before gel filtration for internal calibration. The measured immunoreactivity was then plotted against $K_d$, the coefficient of distribution (calculated as $(V_e - V_o)/(V_t - V_o)$, where $V_e$ is the volume of the fraction in question, $V_o$ the position of $^{125}$I-labelled albumin and $V_t$ the position of $^{22}$NaCl).

Pieces of normal human ileum were obtained during surgery on the gastrointestinal tract (approved by Local Ethical Committee) and immediately frozen. Pieces of pig ileum were removed from anaesthetized pigs (strain LYY). Tissues were subsequently extracted in acid-ethanol (method II in [9]) and subjected to gel filtration on a 50 × 1000 mm column (K 50/100, Pharmacia, Sweden) packed with Sephadex SF (Pharmacia), equilibrated and eluted with 0.5 mol/l acetic acid at a flow rate of 60 ml/h. All samples were then subjected to RIAs for GLP-2 (PG 126–159), PG 151–158 and PG 151–160. The measured immunoreactivity was then plotted against $K_d$, the coefficient of distribution, calculated as described above.

## 3. RESULTS AND DISCUSSION

In this study we employed specific, C-terminal RIAs for each of the three proglucagon fragments: PG 151–158, PG 151–160 and PG 126–159, to determine the C-terminal residue of GLP-2 in pig and human small intestine.

The experimental detection limit (lowest concentration to be distinguished from zero) was 80 pmol/l for both PG 151–158 and PG 151–160. The intraassay coefficient of variation was below 10% for both assays. The specificities of the antisera are illustrated in tables 1 and 2. As shown in table 1, ab 1975 strongly bound labelled synthetic GLP-2 (PG 126–159) but not labelled PG 151–158 or PG 151–160. Ab 8773 strongly bound labelled PG 151–158 but not labelled GLP-2 or PG 151–160. Ab 8769 strongly bound labelled PG 151–160 but not labelled GLP-2 or PG 151–158. Table 2 shows that synthetic GLP-2 (PG 126–159) displaced labelled GLP-2 from ab 1975, whereas PG fragments, PG 151–158 and PG 151–160, were inactive. PG 151–158 displaced labelled PG 151–158 from ab 8773, whereas PG 151–160 and GLP-2 did not react. PG 151–160 displaced labelled PG 151–160 from ab 8769, whereas PG 151–158 and GLP-2 did not react.

Upon gel filtration of the extracts, an immunoreactive moiety coeluting with synthetic GLP-2 was identified with the assays for PG 126–159 and PG 151–158, whereas the PG 151–160 assay was negative. Furthermore the PG 151–158 assay reacted neither with PG 151–160 nor with PG 126–159, but did react with PG 126–159 after removal of residue 159 with carboxypeptidase B (figs 3 and 4). In gel filtrations of extracts of pig small intestine, ab 8773 measured smaller amounts of GLP-2-IR compared to ab 1975, probably due to the fact that ab 8773 was raised against a human PG fragment from a region in which 2 residues differ from the pig sequence (PG 152 and 153) [5]. From these data we conclude

Table 1

Antiserum specificity

| | $^{125}$I-PG 126–159 | $^{125}$I-PG 151–158 | $^{125}$I-PG 151–160 |
|---|---|---|---|
| Ab 1975 | + | – | – |
| Ab 8773 | – | + | – |
| Ab 8769 | – | – | + |

The table shows reactions between antisera (1975, 8773 and 8769) and the three $^{125}$I-labelled peptides. + represents binding of ab to labelled peptides and – represents no binding

Table 2

Antiserum specificity

| | Ab 1975 + $^{125}$I-PG 126–159 | Ab 8773 + $^{125}$I-PG 151–158 | Ab 8769 + $^{125}$I-PG 151–160 |
|---|---|---|---|
| GLP-2 | | | |
| PG 126–159 | + | – | – |
| PG 151–158 | – | + | – |
| PG 151–160 | – | – | + |

The table shows displacement of labelled peptides from antisera with unlabelled peptides. + represents displacement of labelled peptide and – represents no displacement

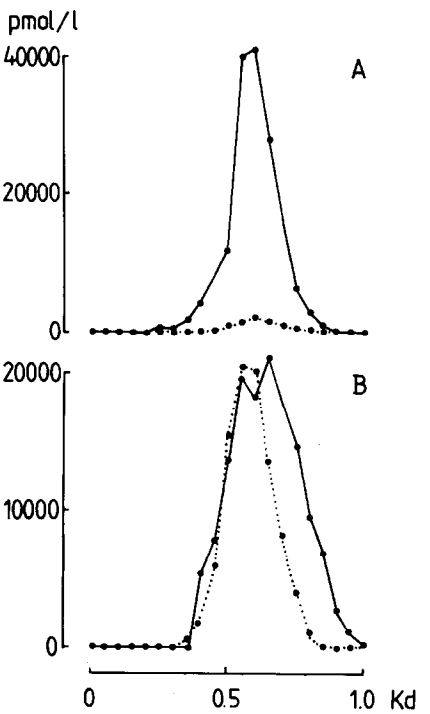


Fig.3. Gel filtration profile of (A) 2 nmol of synthetic GLP-2 (PG 126–159) and (B) 2 nmol of synthetic GLP-2 after treatment with carboxypeptidase-B. As measured with antiserum 1975 (——). As measured with antiserum 8773 (– – –). GLP-2-immunoreactivity is plotted against $K_d$, the coefficient of distribution, calculated as described.

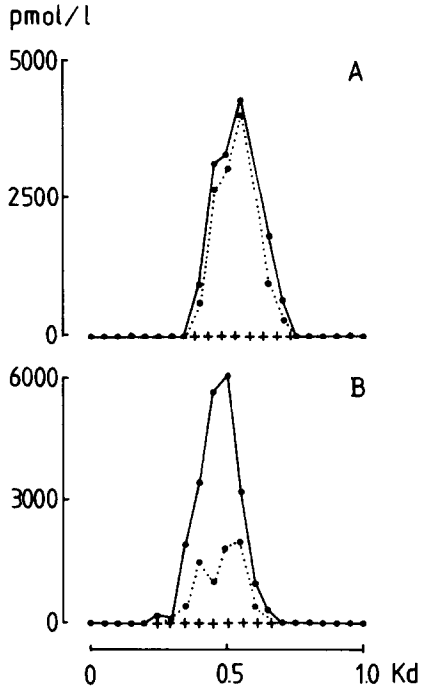


Fig.4. Gel filtration profile of (A) extract of human and (B) extract of pig small intestine, as measured with antiserum 1975 (——), antiserum 8773 (·····) and antiserum 8769 (+ + +). GLP-2-immunoreactivity is plotted against $K_d$, the coefficient of distribution, calculated as described.

that the amino acid sequence of GLP-2 in pig and human small intestine is PG 126–158.

The GLP-2 sequence is less conserved among species than glucagon and GLP-1; human GLP-2 differs from pig, ox and hamster GLP-2 in 4, 4 and 3 positions, respectively [5,10,11], and is not found in fish PG at all [12]. Thus, studies of the physiological effects of GLP-2 must be carried out with a peptide, the structure of which corresponds to that of GLP-2 from the chosen experimental animal.

Synthetic 'GLP-2' (= human sequence PG 126–159) which is commercially available has been completely inert except in one study. Hoosein and Gurd [13] reported that synthetic 'GLP-2' stimulated adenylate cyclase in hypothalamus of rats, though they were unable to identify receptors for GLP-2 in this tissue. Whether the naturally occurring GLP-2 has similar effects in hypothalamus remains to be investigated.

GLP-2-IR has been shown to be produced and co-secreted with the insulinotropic peptide GLP-1 and glicentin [4] from small intestine of pig, and to be released into the circulation in man after a mixed meal [8]. Further studies with synthetic replicas of species-specific GLP-2 will allow the delineation of its eventual hormonal functions.

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