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The human gastrin precursor

Characterization of phosphorylated forms and fragments

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There is a potential phosphorylation site in the *C*-terminal region of the precursor for the acid-stimulating hormone gastrin, which is immediately adjacent to an important cleavage point. In the present study we have sought to identify, separate, quantify and characterize phosphorylated and unphosphorylated forms of human progastrin and its fragments. Identification was made by two radioimmunoassays: (*a*) a novel assay employing an antibody raised to intact human progastrin; and (*b*) an assay using antibody reacting with the *C*-terminal tryptic fragment of human progastrin, as well as progastrin itself. Two forms of human progastrin isolated from a gastrinoma were separated by ion-exchange h.p.l.c., and had similar elution positions on reverse-phase h.p.l.c. and on gel filtration. The more acidic peptide contained close to equimolar amounts of phosphate. On trypsinization, peptides were released that co-eluted on ion-exchange h.p.l.c. with, and had the immunochemical properties of, naturally occurring *C*-terminal fragments of progastrin. One of the latter was isolated and shown by Edman degradation after derivatization with ethanethiol to have the sequence Ser (P)-Ala-Glu-Asp-Glu-Asn. Similar peptides occur in antral mucosa resected from ulcer patients. The unphosphorylated forms of progastrin predominated, whereas the phosphorylated forms of the *C*-terminal fragments were predominant. This distribution could be explained by preferential cleavage of phosphorylated progastrin. We conclude that in human progastrin, Ser-96 can occur in the phosphorylated form; this residue immediately follows a pair of basic residues (Arg-Arg) that are cleaved during synthesis of the biologically active product.

INTRODUCTION

Conversion of the precursors of peptide hormones or neurotransmitters to active molecular species often involves limited proteolysis, together with possible modification of individual residues by amidation, sulphation, glycosylation, *N*-terminal acetylation or phosphorylation [1]. Many examples are now known of cell-specific patterns of biosynthetic processing. In some instances these are attributed to alternative pathways of mRNA splicing [2,3], but in others there are different pathways of post-translational processing. The mechanisms involved in determining and regulating post-translational pathways are still poorly understood. In the case of the human gastric-acid-stimulating hormone, gastrin, it is known from cDNA sequencing that the initial precursor consists of 101 residues [4–6]. In pyloric antral mucosa, cleavage of progastrin at pairs of basic residues, followed by *C*-terminal amidation, generates the main biologically-active form of the hormone, the heptadecapeptide (G17) and three flanking peptides [7–10] (Fig. 1). In other tissues expressing the gastrin gene, the major products may include a 34-residue *N*-terminal extended form of G17 (G34) and, in the case of certain gastrinomas, the intact precursor [11,12]. We recently reported that the extreme *C*-terminal flanking peptide of progastrin occurred in pig antral mucosa in both phosphorylated and unphosphorylated forms [13]. The phosphorylated serine was of special interest since it was located immediately following the sequence Gly-Arg-Arg that is subsequently processed to yield the *C*-terminal amide of G17. The possibility arises that phosphorylation plays a part in determining post-translational processing of the precursor. If this idea is correct, it should be possible to identify phosphorylated forms of the intact gastrin precursor. We report here the identification of intact human progastrin in phosphorylated and unphosphorylated forms, and localization of a phosphoserine residue to the *C*-terminal tryptic fragment.

MATERIALS AND METHODS

Peptides

The hexapeptide Tyr-Ala-Glu-Asp-Glu-Asn (YAEDEN) was purchased from Peninsula Laboratories, Belmont, CA, U.S.A., and purified by h.p.l.c. [11]. Natural human progastrin was isolated as described previously (see below); this preparation is a mixture of phosphorylated and unphosphorylated peptide. Peptides were standardized by absorbance at 280 nm on the basis of the molar absorption coefficient for Trp ($5377\ M^{-1}\cdot cm^{-1}$) and Tyr ($1507\ M^{-1}\cdot cm^{-1}$). Thus progastrin (3 Trp, 1 Tyr) has a molar absorption coefficient of $17639\ M^{-1}\cdot cm^{-1}$.

Abbreviations used: TFA, trifluoroacetic acid; PTH, phenythiohydanitoin.

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Extraction and isolation

The starting material for the preparative isolation of both human progastrin and its *C*-terminal flanking peptide was a large liver metastasis obtained at surgery from a patient with gastrinoma. It was extracted in boiling water as previously described [11,12]. Briefly, tissue was boiled in water (0.1 g·ml^{-1}) for 10 min, homogenized, and the extract centrifuged. The supernatant was extracted with chloroform and methanol, and the aqueous layer lyophilized and subsequently fractionated by gel filtration on Sephadex G50. Further purification is described below. In addition, analytical studies were made on boiling-water extracts of gastrinoma tumour tissues from four other patients, and on extracts of human antral mucosa obtained at resection for duodenal ulcer ($n = 11$). These tissues were extracted by boiling water (0.1 g·ml^{-1}), homogenized and centrifuged (2000 *g*, 10 min). Extracts were fractionated by gel filtration on Sephadex G50 (2.5 cm × 100 cm) equilibrated and eluted with 0.05 M-ammonium bicarbonate. Radioimmunoassay of the eluates using antibody L214 (Fig. 1 and see below) was initially employed to detect intact progastrin and its *C*-terminal tryptic peptide.

Tubes in Sephadex G50 eluates corresponding to progastrin were further purified on h.p.l.c. using a Z-module C_{18} (μ Bondapak) cartridge (Waters Associates) and a Techsil 5 μm C_{18} (4 mm × 250 mm) column in an Altex h.p.l.c. system eluted with a gradient from 0.1% aqueous trifluoroacetic acid (TFA) to acetonitrile. The phosphorylated and unphosphorylated progastrins were separated on anion-exchange h.p.l.c. using an Accell QMA (Waters Associates) column (4 mm × 100 mm), and gradient elution from 0.01 M-triethylamine carbonate, pH 5.6, to 0.75 M, pH 7.6, in methanol/H_2O (v/v; 1:2). The triethylamine was freshly redistilled over ninhydrin and was gassed with CO_2 immediately before use. It was anticipated that ion-exchange chromatography might separate progastrin variants differing both in sulphation (of tyrosine) and phosphorylation states. To simplify the analysis, samples were digested with aryl sulphatase prior to reverse-phase h.p.l.c. to convert sulphated variants to the unsulphated form. Thus, progastrin-containing tubes in Sephadex G50 eluates were incubated with 400 μg·ml^{-1} aryl sulphatase (Sigma, Poole, Dorset, U.K., 30 units·mg^{-1}) for 2.5 h at 37 °C, in sodium acetate (pH 5.7, 0.1 M). The samples were then acidified with TFA and purified by reverse-phase h.p.l.c.

Tubes in Sephadex G50 eluates corresponding to the extreme *C*-terminal tryptic peptide of progastrin were further purified by anion-exchange h.p.l.c. on Accell QMA using a triethylamine carbonate gradient as described above. Material for structural studies was finally desalted on Sephadex G10 (1 cm × 10 cm) eluted with distilled water.

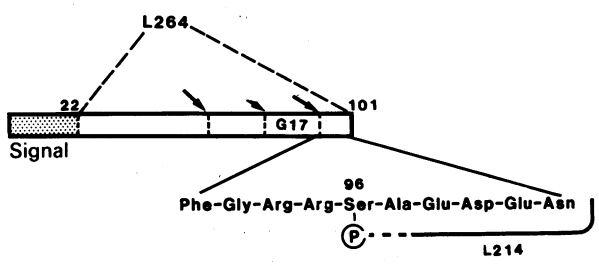

Fig. 1. Schematic representation of human preprogastrin

Cleavage at pairs of basic residues (arrows) generates the main biologically active form, G17 (allowing for subsequent formation of a *C*-terminal amide), together with two flanking peptides to the *N*-terminus of the G17 region and one to the *C*-terminus. The anitbodies used in the present study react with (a) the *C*-terminal flanking peptide of progastrin (L214) and (b), intact progastrin (L264). The signal peptide corresponds to preprogastrin 1–21. Note the putative phosphorylation site in the *C*-terminal flanking peptide.

Radioimmunoassay

Two assays were used in the present series of experiments (Fig 1). One employed an antibody specific for the extreme *C*-terminus of progastrin (L214), that reacts both with the intact precursor and with its *C*-terminal fragments. This antibody was raised to a synthetic analogue (YAEDEN) of the *C*-terminal hexapeptide fragment of progastrin in which Ser-96 is substituted with Tyr. The same peptide was labelled with ^{125}I as previously described [11,12]. In addition, we have developed a second radioimmunoassay using an antibody specific for intact human progastrin isolated from gastrinoma tissue. Progastrin recovered from the reverse-phase h.p.l.c. steps (see above), and corresponding to a mixture of phosphorylated and unphosphorylated peptide (60 nmol), was coupled to bovine thyroglobulin (1.25 mg) by addition of 25 μl of glutaraldehyde (5%) in 0.1 M-phosphate buffer, pH 7.4. The mixture was incubated at 22 °C for 60 min, and then dialysed against distilled water (5 litres, 4 °C, 36 h). The incorporation of peptide into conjugate was 85% based on the recovery of a small quantity of ^{125}I-labelled progastrin added to the reaction mixture. Two rabbits received 10 nmol of peptide, emulsified in Freund's complete adjuvant and injected at multiple intradermal sites. They were boosted at 6–8 week intervals with the equivalent of 5 nmol of peptide and bleedings were taken from an ear vein 8–10 days after each boost. Both rabbits produced antibodies binding labelled progastrin, but only one of them (L264) was adequate for radioimmunoassay studies. Radiolabelled peptide for assays was prepared by the chloramine T method as follows: 10 μl of 0.25 M-phosphate buffer, pH 7.5, was added to 500 pmol of peptide followed by 5 μl of Na^{125}I (18.5 MBq, Amersham) and 10 μl of 1 mg of chloramine T/ml. The reaction was stopped after 10 s with 10 μl of 2.5 mg of sodium metabisulphite/ml and the reaction mixture purified on DEAE-cellulose columns (1 cm × 10 cm) eluted with a gradient from 0.1% to 5% ammonium carbonate. The peak of labelled peptide was diluted in assay buffer and stored at −40 °C. Assays were incubated in 1.0 ml of phosphate buffer (0.02 M, pH 7.5) containing 0.1% (w/v) bovine serum albumin. Antibody L264 was used at a titre of 1:3000 with 2000 c.p.m. label; using natural human progastrin as standard the concentration needed for 50% inhibition of binding of label was 318 pmol·l^{-1} (mean ± S.E.M., $n = 9$). Antibody-bound and free label were separated by addition of 100 μl of suspension of NORIT charcoal (10 g), dextran (1 g) and filtered human plasma (5 ml) in water (100 ml) followed by centrifugation at 2000 *g* for 5 min at 4 °C. Antibody specificity was determined by comparison of the inhibition of binding by natural intact progastrin and other progastrin-derived peptides. All

assays included control tubes for non-specific binding and standard curves of natural intact progastrin. Extracts of antral mucosa were routinely assayed with a *C*-terminal-specific G17 antibody [14].

Structural studies

The extreme *C*-terminal flanking peptide was characterized by microsequence analysis carried out on 200 pmol using an Applied Biosystems gas-phase microsequenator and h.p.l.c. detection of phenylthiohydantoin (PTH) derivatives. The peptide was derivatized with ethanethiol for identification of Ser phosphate as *S*-ethylcysteine as described by Meyer *et al.* [15]. Sequence information was also obtained for progastrin recovered from reverse-phase h.p.l.c. of two of the tumours. Phosphate was determined by using the Malachite Green method for small sample volumes (0.2 ml) [16]. Both KH_2PO_4 and *O*-phosphoserine (Sigma, Poole, Dorset, U.K.) were used as standards. Trypsinizations were carried out with tosyl-phenyl-CH_2Cl (TPCK)-trypsin (10 μg, Worthington, Diagnostics Div.) in ammonium bicarbonate buffer (0.1 M, pH 8.0), and samples were then separated on anion-exchange h.p.l.c. as described above.

RESULTS

Identification of intact progastrin by specific radiommunoassay

Progastrin was assayed by a newly developed radioimmunoassay based on a rabbit polyclonal antibody (L264) raised to human progastrin 22–101 and the same peptide as label. Extracts of human antral mucosa and of gastrinoma tissue inhibited binding of label to antibody L264 in parallel with the natural standard peptide, whereas peptides derived from progastrin by cleavage, e.g. the natural *C*-terminal flanking peptide, G17, G34, and the synthetic analogue YAEDEN were inactive in the assay (Fig. 2). Digestion of standard progastrin with trypsin, or treatment of tissue extracts with trypsin, abolished immunoreactivity with antibody L264. Gel filtration of extracts of pyloric antrum or gastrinomas on Sephadex G50 revealed a single major peak of activity measured with L264 eluting in the position of progastrin

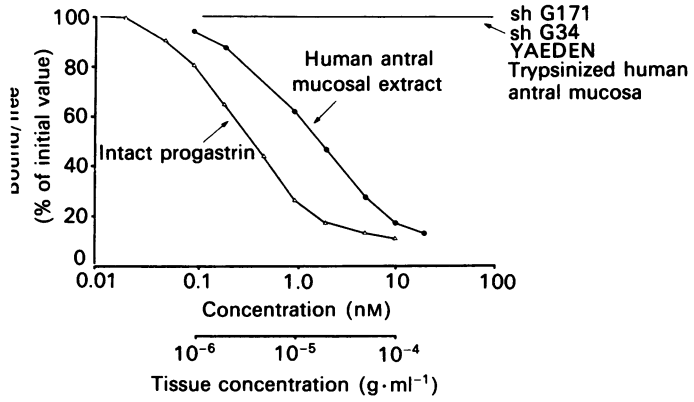

Fig. 2. Inhibition of binding of label to antibody L264 by natural progastrin isolated from a gastrinoma, and by a human antral extract

Note that YAEDEN, G17, G34, and the human antral extract digested with trypsin do not react.

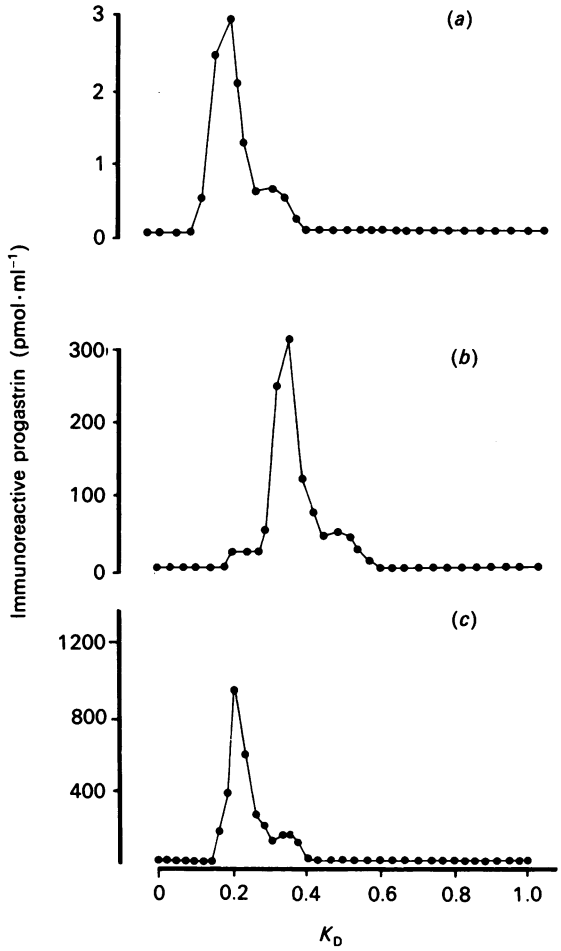

Fig. 3. Separation on Sephadex G50 eluted with ammonium bicarbonate of extracts of gastrinomas, and pyloric antral mucosa

(*a*) and (*b*), gastrinoma extracts; (*c*) a pyloric antral mucosal extract. A single major peak of progastrin immunoreactivity and a shoulder of immunoreactive material on the descending side is characteristic of antral extracts and most gastrinomas. In a single gastrinoma (*b*), however, a later eluting variant predominated.

(Figs. 3*a* and *c*); in some extracts there was a minor component that appeared as a shoulder on the descending side of the peak. In one gastrinoma (Fig. 3*b*) the latter peak (identified here as progastrin variant) was the major immunoreactive component.

Separation of progastrin forms

For further characterization of progastrin, studies were made on material isolated from gastrinomas where starting concentrations were high. Separation by reverse-phase h.p.l.c. of the appropriate tubes from Sephadex G50 columns revealed a single major peak of immunoreactivity (Fig. 4*a*). The component designated progastrin variant in gel filtration eluates had a lower retention time compared with authentic progastrin on h.p.l.c. (Fig. 4*b*). The variant awaits full structural characterization, and in the present work has not been studied further. The major peak on reverse-phase h.p.l.c. (Fig. 4*a*) was identified as progastrin 22–101 on the basis of co-elution with standard, and partial sequence data obtained from

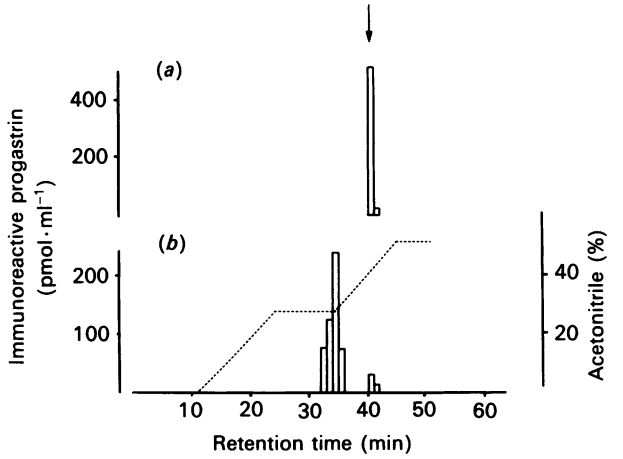

Fig. 4. Separation on reverse-phase h.p.l.c. of gastrinoma extracts previously run on Sephadex G50

(*a*) Shows authentic progastrin from Fig. 3(*a*), and (*b*) shows the pattern obtained with the variant (retention time 34 min) obtained from the Sephadex run shown in Fig. 3*b*. The arrow indicates the elution position of progastrin 22–101

samples of progastrin isolated from two other tumours confirmed this assignment. When this material was further separated on an Accell QMA ion-exchange h.p.l.c. column two peaks of immunoreactivity (designated progastrin peak I and peak II in order of elution) were obtained (Fig. 5*a*); the less acidic peak (progastrin I) was the predominant one, but refractionation of both confirmed their similar retention times on reverse-phase h.p.l.c. (Figs. 5*b* and *c*).

Characterization of progastrin forms

Microphosphate analysis indicated close to equimolar amounts of phosphate in progastrin II and no detectable phosphate in progastrin I (Table 1). Evidence for the site of phosphorylation was obtained by digestion of a mixture of the two progastrins with trypsin which liberated two peptides (designated flanking peptide peak I and peak II in order of elution) that were separated by ion-exchange chromatography and reacted with the *C*-terminal-specific progastrin antibody (L214), indicating that they correspond to the extreme *C*-terminal fragment (Fig. 6). Trypsinization of unphosphorylated progastrin alone yielded a single peak corresponding to flanking peptide peak I. The retention times on ion-exchange chromatography of the two tryptic peptides were the same as those of the naturally occurring *C*-terminal flanking peptides in gastrinoma or pyloric antral mucosal extracts when these were run on the same system (see below) (Fig. 6). The ratio of A_{280} to immunoreactivity with L214 and L264 was similar for the phosphorylated and unphosphorylated forms of progastrin; after digestion with trypsin there was quantitative recovery (> 70%) of both phosphorylated and unphosphorylated *C*-terminal flanking-peptide immunoreactivity measured with L214. The results suggest that phosphorylation does not influence immunoreactivity of either progastrin or its *C*-terminal fragment with the present antibodies.

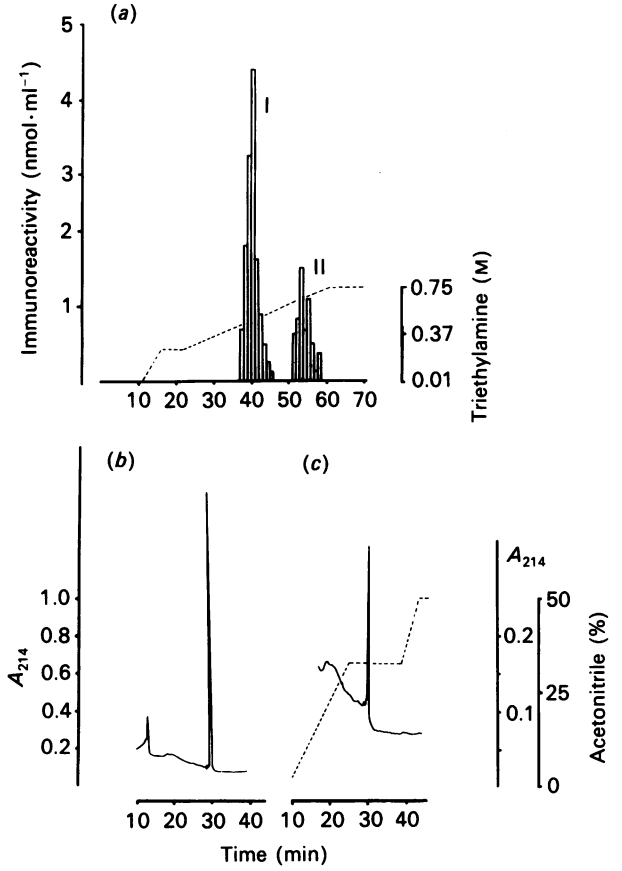

Fig. 5. Separation of progastrin

(*a*) Separation of intact human progastrin on Accell QMA anion-exchange h.p.l.c. column eluted with a gradient (broken line) of triethylamine carbonate. The two peaks are identified as I and II. (*b*) and (*c*) Separation of progastrin I and II respectively on reverse-phase h.p.l.c. A Techsil C_{18} 5 μm column (4 mm × 250 mm) was used in an Altex h.p.l.c. system. Gradient elution was made from 0.1% TFA to acetonitrile (broken line). Detection was by A_{214}. Note in (*b*) and (*c*) that the less acidic material from (*a*) (I) runs identically on reverse-phase h.p.l.c. with the more acidic material (II). Immunoassay revealed 10 nmol of the first peak and 1.5 nmol of the second.

Characterization of the *C*-terminal flanking peptide

To characterize unequivocally the *C*-terminal progastrin fragments they were isolated in semi-preparative studies by gel filtration on Sephadex G50 followed by ion-exchange h.p.l.c., and finally gel filtration on Sephadex G10 (Fig. 7). Pilot studies using a number of reverse-phase systems, e.g. C_{18}, phenyl or cyano-columns, and elution with TFA or heptafluoroacetic acid in acetonitrile, failed to identify conditions in which the *C*-terminal fragments were retained. We attribute this to the fact that these peptides are small and highly acidic. We were fortunate, however, that anion-exchange h.p.l.c. followed by gel filtration on Sephadex G10 to remove salts recovered (> 75% at each step) material in good yield and in a form suitable for structural analysis.

Structural studies

Estimation of inorganic phosphate in the two *C*-terminal flanking peptides revealed no detectable

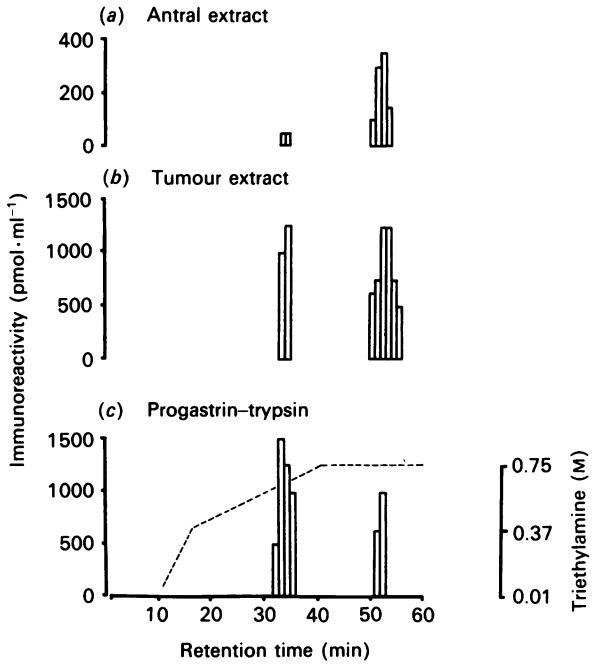

Fig. 6. Separation on ion-exchange h.p.l.c. of the two *C*-terminal flanking peptides

An Accell QMA column (4 mm × 100 mm) was used in an Altex h.p.l.c. system. Gradient elution was made from 0.01 M-triethylamine carbonate, pH 5.6, to 0.75 M-triethylamine carbonate, pH 7.6. (*a*) Human antral mucosa extract previously separated on Sephadex G50; (*b*) gastrinoma extract previously run on Sephadex G50; (*c*) trypsinization of a mixture of 75% phosphorylated and 25% unphosphorylated intact progastrin. Note that the two *C*-terminal tryptic peptides were released in proportions matching those of the two forms of intact progastrin.

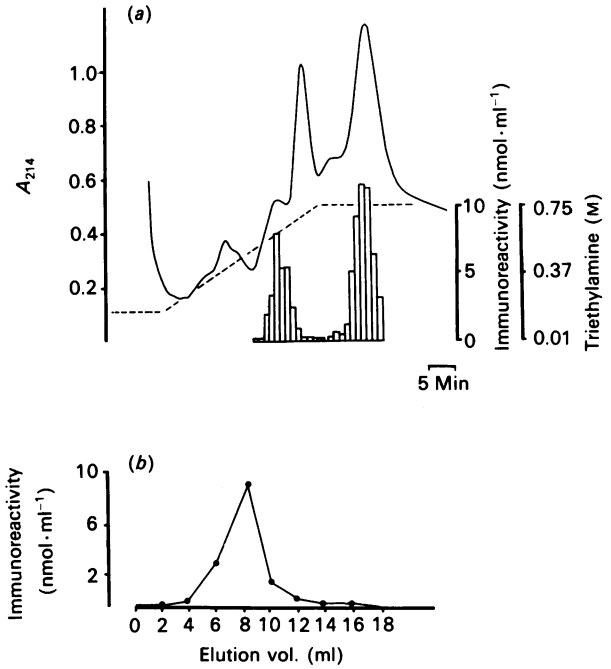

Fig. 7. Isolation of the naturally occurring *C*-terminal flanking peptide of human progastrin from a gastrinoma extract

(*a*) Separation on Accell QMA anion-exchange h.p.l.c. column of imunoreactive material (L214) corresponding to the progastrin *C*-terminal fragment obtained from preparative Sephadex G50 eluates. Elution with a gradient (broken line) of triethylamine carbonate (see Fig. 4). (*b*) Refractionation of the more acidic immunoreactive material from the ion-exchange column on a Sephadex G10 (1 cm × 10 cm) column eluted with distilled water. The single major immunoreactive peak was taken for analytical studies.

phosphate from 450 pmol of peak I, and close to equimolar amounts of inorganic phosphate in peak II (Table 1). Amino acid analysis of the latter indicated the composition: Ser (0.4), Ala (1.01), Glu (2.91), Asp (1.81); the low recovery of serine is characteristic of phosphoserine. Sequence analysis of peak II gave an unequivocal structure identical to that of human progastrin 96–101 predicted from the cDNA sequence (Fig. 8), except that in the first cycle of Edman degradation the serine residue was identified only as the DTT adduct and PTH serine itself could not be identified. In this system serine normally yields two products: PTH-serine and the DTT adduct of PTH serine in the approximate peak height ratio of 1:3. A shift in ratio towards the DTT adduct is characteristic of phosphoserine residues [15]. Direct evidence for the presence of phosphoserine was obtained by derivatization of the peptide with ethanethiol following β-elimination with base to generate *S*-ethylcysteine. The latter has a characteristic retention time on h.p.l.c. and was unequivocally identified in the first cycle of Edman degradation of the phosphorylated fragment.

Table 1. Phosphate determination of the two forms of naturally occurring *C*-terminal-flanking peptide of progastrin and of intact progastrin

For both intact progastrin and the flanking peptide, peaks I and II refer to order of elution from ion-exchange h.p.l.c.: flanking peptide was detected by radioimmunoassay using L214 (standard YAEDEN), and progastrin by radioimmunoassay using L264 (standard, progastrin), and by A_{280}.

Peptide		Inorganic phosphate (pmol)	Immunoreactivity (pmol)
Flanking peptide	Peak I	< 150.0	450.0
	Peak II	715.0	680.0
Progastrin	Peak I	< 150.0	345.0
	Peak II	240.0	350.0

Tissue concentrations

In the progastrin-specific assay concentrations of immunoreactive material in human antral extracts were 0.89 ± 0.30 nmol·g^{-1} compared with 9.2 ± 2.3 nmol·g^{-1} measured by *C*-terminal G17 specific antibody (mean ± S.E.M., $n = 11$). These data therefore suggest that intact progastrin occurs in approximately 10% of the concentration of G17 (the main secretory product) in human pyloric antral G-cells, and this compares well with previous estimates of the relative concentrations of

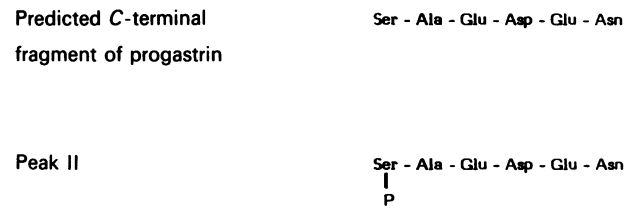

Fig. 8. Amino acid sequence of the *C*-terminal-flanking peptide of progastrin (see Fig. 7)

For comparison the amino acid sequence predicted from cDNA sequencing is shown. The presence of phosphorylated Ser was confirmed by (*a*) a shift in the ratio of PTH-Ser to the DTT adduct in the first cycle on sequencing the underivatized peptide, (*b*) sequencing of the peptide derivatized with ethanethiol gave *S*-ethylcysteine on the first cycle, and (*c*) inorganic phosphate analysis after ashing which gave 715 pmol of phosphate from 680 pmol of peptide.

progastrin and G17 in antral extracts based on assays using a *C*-terminal progastrin antibody (L214) and gel filtration to separate progastrin and immunoreactive fragments [11]. Concentrations of progastrin in antral extracts were too low for reliable estimates of relative proportions of phosphorylated and unphosphorylated forms. In four tumours, however, the phosphorylated form of progastrin accounted for $19.7 \pm 3.8\%$ (mean $\pm$ S.E.M.) of total progastrin immunoreactivity; in two tumours it was possible to establish that a mean of 73.8% of *C*-terminal flanking peptide was phosphorylated. Moreover, in antral mucosal extracts $90.2 \pm 1.2\%$ ($n = 4$) of total *C*-terminal-fragment immunoreactivity was in the phosphorylated form.

DISCUSSION

The present study provides evidence for the occurrence of human progastrin in two forms differing in the presence or absence of a phosphorylated serine in the extreme *C*-terminal region. These results extend a previous brief report of possible phosphorylated human progastrin variants [12] by providing evidence for the precise site of phosphorylation and by quantification of phosphorylated and unphosphorylated forms. The site of the phosphoserine is of interest because it immediately follows the tripeptide Gly-Arg-Arg which is processed to give the functionally important *C*-terminal amide of the biologically active forms of gastrin. The co-product of cleavage at this site is a *C*-terminal progastrin fragment which we have identified in human antrum and isolated and sequenced from tumour extracts in the phosphorylated form. We have previously described phosphorylation of the *C*-terminal fragment of porcine progastrin and noted that potential phosphorylation sites occur adjacent to cleavage sites in several precursors, e.g., pro-vasoactive intestinal polypeptide, -gastrin-releasing peptide and -neuropeptide Y [13]. However, in the pig, unlike human gastrinomas, it is difficult to identify intact progastrin and so it was not possible to examine the relationships between phosphorylated precursor (progastrin) and cleavage product (*C*-terminal-flanking peptide). The close proximity of cleavage and phosphorylation sites in progastrin raises the possibility that phosphorylation influences processing during regulatory peptide biosynthesis. The finding that the intact gastrin precursor occurs in a phosphorylated form contributes directly to this idea.

The present studies have been facilitated by the fact that in certain gastrinomas there are high concentrations of intact progastrin [11,12]. In human antral mucosa, progastrin appears to occur in concentrations that are about 10% those of G17. It is interesting to note that in the gastrinoma material examined in the present study, the unphosphorylated form of progastrin predominated, whereas in both antral mucosa and gastrinomas the phosphorylated form of the *C*-terminal tryptic peptide predominated. The present methods have not yet proved sufficiently sensitive to characterize the phosphorylation state of pyloric antral progastrin. The different ratios of phosphorylated and unphosphorylated progastrin compared with the *C*-terminal fragment could be explained by preferential cleavage of the phosphorylated precursor. These findings are therefore directly relevant to the idea that phosphorylation plays a regulatory role in cleavage of the precursor. At present, however, we cannot exclude the possibility that intact progastrin and its *C*-terminal fragment show different susceptibilities to the action of phosphatases.

Previous studies have established that several peptide hormones are substrates for cAMP-dependent kinase, e.g., atrial natriuretic peptide, B human chorionic gonadotropin, prolactin and glicentin [17–20]. Moreover, the heptadecapeptide form of gastrin can itself be phosphorylated on the tyrosine by epidermal growth factor-stimulated tyrosine kinase [21]. For the most part, the natural occurrence of these phosphorylated peptides *in vivo* is either uncertain or unlikely. In other instances, notably adrenocorticotropic hormone (ACTH), growth hormone, human alpha chorionic gonadotropin, and an opioid-related peptide incorporation of ^{32}P has been demonstrated in cell cultures [22–27]; naturally occurring phosphorylated variants of prolactin [19], growth hormone [28], and proenkephalin-derived peptides [29] have also been identified. In the case of gastrin, ACTH and the opioid peptides, the phosphorylated residue occurs in the sequence Ser-X-acidic residue, which is a substrate for the action of physiological casein kinase [23,30]. Progastrin could, however, also be a substrate for casein kinase II. It remains to be shown whether or not phosphorylation influences the primary activity of these peptides. The finding of a phosphorylation site immediately adjacent to a cleavage site in progastrin is interesting in view of evidence that in other systems phosphorylation might influence proteolysis. In the case of certain enzymes [31,32] and cytoskeletal proteins [33], it appears that phosphorylation precedes proteolytic degradation and might tag the protein as destined for breakdown. Similar mechanisms would provide the opportunity to regulate the biosynthetic processing of regulatory peptides, and might in turn provide the capacity for cell-specific patterns of processing of a particular precursor.

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