Post-translational processing of progastrin: inhibition of cleavage, phosphorylation and sulphation by brefeldin A

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The precursor for the acid-stimulating hormone gastrin provides a useful model for studies of post-translational processing because defined sites of cleavage, amidation, sulphation and phosphorylation occur within a dodecapeptide sequence. The factors determining these post-translational processing events are still poorly understood. We have used brefeldin A, which disrupts transport from rough endoplasmic reticulum to the Golgi complex, to examine the mechanisms of cleavage, phosphorylation and sulphation of rat progastrin-derived peptides. Biosynthetic products were detected after immunoprecipitation using antibodies specific for the extreme C-terminus of progastrin, followed by reversed-phase and ion-exchange h.p.l.c. Gastrin cells incorporated [^{3}H]tyrosine, [^{32}P]phosphate and [^{35}S]sulphate into both progastrin and its extreme C-terminal tryptic (nona-) peptide. Ion-exchange chromatography resolved four forms of the C-terminal tryptic fragment of progastrin which differed in whether they were phosphorylated at Ser^{96}, sulphated at Tyr^{103}, both or neither. The specific activity of [^{3}H]tyrosine in the peak that was both phosphorylated and sulphated was higher than in the others. Brefeldin A inhibited the appearance of [^{3}H]tyrosine-labelled C-terminal tryptic fragment but there was an accumulation of labelled progastrin and a peptide corresponding to the C-terminal 46 residues of progastrin. Brefeldin A also inhibited incorporation of ^{32}P and ^{35}S into both progastrin and its C-terminal fragment. Thus phosphorylation of Ser^{96}, sulphation of Tyr^{103} and cleavage at Arg^{94}-Arg^{95} depend on passage of newly synthesized progastrin along the secretory pathway; as brefeldin A is thought to act proximal to the *trans*-Golgi, these processing steps would appear to occur distal to this point. The data also indicate that the stores of unphosphorylated C-terminal tryptic fragment are not available for phosphorylation, implying that this modification occurs proximal to the secretory granule; cleavage is known to occur in the secretory granule which suggests that it occurs after phosphorylation.

INTRODUCTION

Biologically active hormones and neuropeptides are generally produced from inactive precursors by post-translational modifications that typically occur before secretion, and include cleavage of the peptide chain, tyrosine sulphation, serine phosphorylation, glycosylation, C-terminal amidation and N-terminal glutamine cyclization [1–4]. The precursor of the acid-stimulating hormone gastrin, preprogastrin, is a convenient model for the study of post-translational processing mechanisms because it is relatively small (104 residues in the rat), simple (there is a single copy of the sequence defining biological activity) and because several common post-translational modifications, namely cleavage, sulphation, amidation and phosphorylation, occur in a single dodecapeptide sequence which includes the biologically important region of the hormone [5–9].

The phosphorylation site in progastrin is of interest because it is immediately adjacent to a pair of arginine residues (Arg^{94}-Arg^{95}), cleavage at which is an early step in the conversion of precursor to active peptide (Figure 1) [9–11]. The main active products in the rat are peptides of 34, 17 and 16 residues (i.e. G34, G17 and G16) and are produced by further cleavage at Arg^{57}-Arg^{58}, Lys^{74}-Lys^{75} and Gln^{76}-Arg^{77} respectively together with amidation of the C-terminal residue (Phe^{92}). The serine-phosphorylation site is conserved in all members of the gastrin/cholecystokinin family sequenced so far. The kinase responsible for phosphorylation at this site is not known, but it is of interest that acidic residues are invariably located at positions 2 and 3 C-terminal to Ser^{96} (Figure 1); the configuration of serine followed at position 2 to the C-terminus by an acidic residue is also found at phosphorylation sites in casein which raises the possibility that physiological casein kinase or a related enzyme might phosphorylate progastrin [12–14]. Similar potential phosphorylation sites occur immediately adjacent to cleavage points in several other hormone and neuropeptide precursors which are otherwise unrelated to progastrin [10], e.g. provasoactive intestinal polypeptide, progastrin-releasing peptide, proneuropeptide Y and in the region of chromogranin A giving rise to pancreastatin, indicating that this motif is likely to be of significance in several different systems. One possibility is that phosphorylation in some way influences processing at the adjacent cleavage site, but the relationship between phosphorylation and cleavage remains uncertain, and in particular it is not clear whether Ser^{96} in progastrin is phosphorylated before cleavage at the adjacent arginine residue or after (or either).

In the case of gastrin and other peptide precursors that are delivered to the regulated pathway of secretion, there is evidence that important steps of cleavage and amidation occur after segregation of precursor forms into immature secretory granules [15]. The fungal metabolite brefeldin A is known to inhibit the passage of material along the secretory pathway from endoplasmic reticulum to Golgi, but there have been relatively few studies of how brefeldin A influences the post-translational processing of hormonal and neuropeptides [16–20]. We describe here the use of brefeldin A as a tool to investigate the processing of progastrin by phosphorylation of Ser^{96}, sulphation of Tyr^{103} and cleavage at Arg^{94}-Arg^{95}. The data suggest that these events occur at a stage distal to the action of brefeldin A.

Abbreviations used: CFP: C-terminal tryptic flanking peptide of progastrin; G34, G17 and G16, biologically active gastrins of 34, 17 and 16 residues respectively.

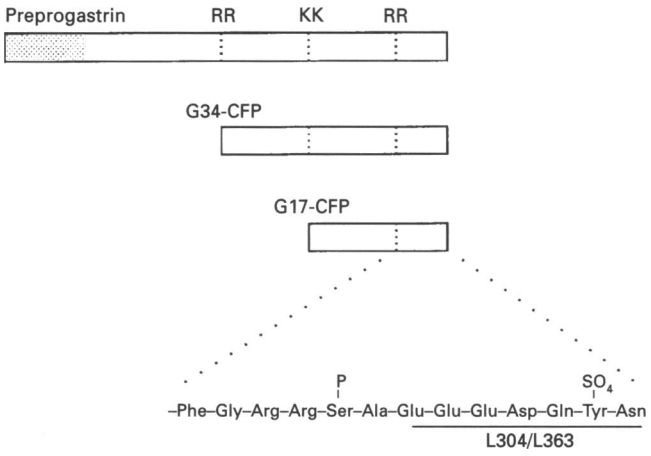

Figure 1 Schematic representation of the structure of rat preprogastrin

The expanded sequence shows the C-terminal tridecapeptide. Note the serine-phosphorylation site immediately adjacent to a pair of arginine residues. The C-terminal tryptic cleavage product of progastrin can also be sulphated at Tyr^{103}. Antibodies L304 and L363 react with progastrin and its C-terminal tryptic fragment. Biologically active gastrins are generated by endo- and carboxy-peptidase cleavage of arginine residues adjacent to the phosphorylation site, and conversion of the glycine-extended product by peptidyl α-amidating mono-oxygenase to peptides C-terminally amidated at Phe^{92}. Further cleavage at Arg^{57}-Arg^{58} (RR) or Lys^{74}-Lys^{75} (KK) generates the active products, G34 and G17 (a third product, G16, is produced by cleavage of the N-terminal residue of G17). Note that cleavage at positions 57/58 or 74/75 without processing of the C-terminal region of the precursor generates putative biosynthetic intermediates (G34-CFP and G17-CFP) which would be expected to react with antibodies specific for the C-terminus of progastrin.

MATERIALS AND METHODS

Tissue and incubations

Studies were made on the gastrin-producing mucosa of the stomach of male Wistar rats (250–330 g) fed *ad libitum* before experiments and receiving omeprazole (400 μmol/kg, for 48 h by gavage) to increase gastrin mRNA levels [21–23]. Mucosal segments of less than 1 mm (total wet weight 100–150 mg) were washed three times in ice-cold Krebs–Ringer bicarbonate and incubated in 3 ml of modified Krebs–Ringer bicarbonate medium containing amino acids (BME 1:50, Sigma, Poole, Dorset, U.K.), vitamins (MEM 1:100, Sigma) and 10 mM Hepes, pH 7.4, at 37 °C, and gassed with 95% O_2/5% CO_2. After 15 min to allow equilibration, either vehicle (30 μl of methanol) or brefeldin A (Cambio Ltd., Cambridge, U.K.) was added (10 μg/ml, from a stock solution of 1 mg/ml in methanol), together with [^{3}H]-tyrosine (50 μCi/ml) or [^{32}P]phosphate (300 μCi/ml). In some experiments, both isotopes were used in combination, and in others [^{35}S]sulphate (100 μCi/ml) was used. All isotopes were obtained from Amersham International, U.K. Tyrosine, phosphate or sulphate was omitted from the incubation medium as appropriate. In order to inhibit phosphatase activity, the inhibitor okadaic acid (1.0 μm) was included in some experiments.

Peptide extraction and separation

Routinely, tissues were incubated for up to 2 h, and then washed in ice-cold Krebs–Ringer containing unlabelled tyrosine, phosphate or sulphate. The tissue was then boiled in 1 ml of distilled water, homogenized and centrifuged. The pellet was re-extracted with 1 ml of water, centrifuged and the supernatants pooled. Progastrin and similar peptides were concentrated by passage of the extract through Sep-Pak C18 cartridges (Waters Associates, Milford, NJ, U.S.A.) and elution with 50% acetonitrile in 0.02 M phosphate buffer, pH 7.4. The organic phase in the eluate was then removed by a stream of N_2. Material that was not retained by the C18 cartridge was diluted with 4 vol. of propan-2-ol and concentrated on a Silica Sep-Pak (Waters Associates) cartridge and eluted with 0.02 M phosphate buffer, pH 7.4. The eluate was dried *in vacuo*, reconstituted with 2 ml of distilled water and pooled with the C18 eluate. This method has previously been shown to give good recovery of intact progastrin and its C-terminal flanking peptide [10,11,24].

Progastrin-derived peptides were further concentrated by immunoprecipitation. Removal of non-specifically precipitated peptides was carried out by a cycle of precleaning in which 50 μl of non-immune rabbit serum was added followed by 200 μl of goat anti-(rabbit IgG). The precipitate was allowed to form at 4 °C overnight and was removed by centrifugation (3000 *g*, 15 min). Progastrin and related peptides were then precipitated from the supernatant by addition of 50 μl of antibody L304 and 200 μl of goat anti-(rabbit IgG). The rabbit antibody is specific for the C-terminal region of rat progastrin and reacts with both intact progastrin and CFP [9]. Precipitates were recovered by centrifuging at 3000 *g* for 15 min, extensively washed with ice-cold 0.02 M phosphate buffer, pH 7.4, and the precipitated gastrin-related peptides were solubilized by boiling with 1 ml of water. The final product was further centrifuged at 100000 *g* for 60 min at 4 °C, and the precipitate discarded.

The progastrin-related peptides recovered after immunoprecipitation were purified by reversed-phase h.p.l.c. on a PRP-LS column (5 μ, 4.6 mm × 250 mm) which was eluted with a gradient from 0 to 28% acetonitrile in 0.05 M NH_4HCO_3, pH 7.9. The reversed-phase h.p.l.c. gradient system was calibrated in separate runs with the synthetic C-terminal nonapeptide of rat progastrin [9], or with intact human progastrin isolated from a gastrinoma [24]. The C-terminal fragments of progastrin in the reversed-phase h.p.l.c. eluates were further separated by ion-exchange h.p.l.c. on a Mono Q column (HR5/5; Pharmacia) equilibrated with 20 mM Tris/HCl, pH 8.2, and eluted with a gradient to 0.5 M NaCl in 20 mM Tris/HCl, pH 8.2. Radioactivity in the column eluates was routinely counted on-line using a Canberra Packard A280 flow radiometer, and a stream splitter was used to recover material for radioimmunoassay or for further chromatography.

Radioimmunoassay

Progastrin-derived peptides in the eluates of reversed-phase and ion-exchange column runs were estimated by radioimmunoassay using antibody L304 or L363 both of which react at the extreme C-terminus of rat progastrin and detect intact progastrin and its C-terminal fragments modified by sulphation of Tyr^{103} or phosphorylation of Ser^{96} (Figure 1) [9]. In some experiments, column eluates were assayed with antibody L66 which reacts at the extreme N-terminus of G34 [25].

RESULTS

Identification of progastrin-derived peptides

Incubation with [^{3}H]tyrosine, [^{32}P]phosphate or [^{35}S]sulphate consistently resulted in the labelling of two peaks in reversed-phase h.p.l.c. eluates (Figure 2 and 3). The major peak co-eluted with the synthetic C-terminal nonapeptide of rat progastrin, i.e. CFP, the second peak, which was always smaller,

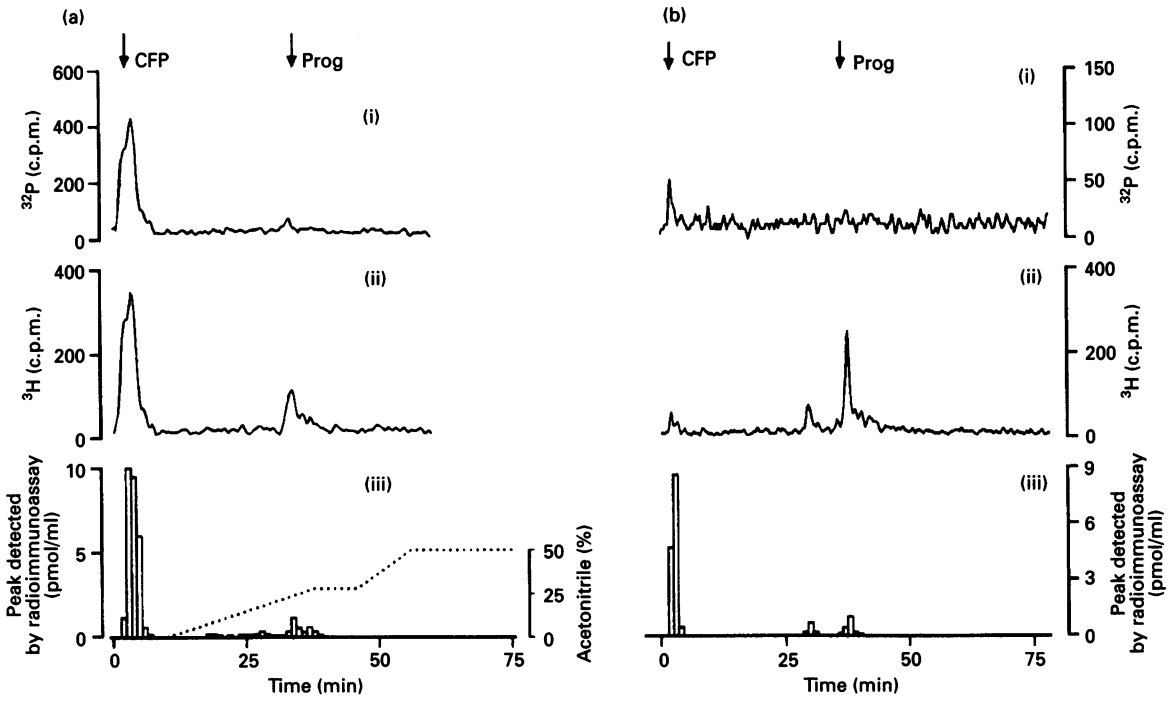

Figure 2 Separation by reversed-phase h.p.l.c. of progastrin-derived peptides incorporating [^{32}P]phosphate and [^{3}H]tyrosine in control and brefeldin A-treated samples

The results of dual-labelling experiments using [^{32}P]phosphate and [^{3}H]tyrosine are shown; peptides are immunoprecipitated with antibody specific for the C-terminus of progastrin. (**a**) Results obtained from control samples; (**b**) samples treated with brefeldin A. In both cases, (i) shows the elution pattern of peptides labelled with [^{32}P]phosphate, (ii) shows labelling with [^{3}H]tyrosine and (iii) shows the results of radioimmunossay of the same column eluates. The broken line indicates the acetonitrile gradient, and arrows at the top show elution positions for synthetic CFP and intact human progastrin (Prog) determined in earlier runs. Note that, in the presence of brefeldin A, there is incorporation of [^{3}H]tyrosine into progastrin, but not into CFP, and that the incorporation of ^{32}P into both progastrin and CFP is inhibited. Flow rate was 1.0 ml/min; 50% of the stream was diverted for on-line counting, and the remainder collected for radioimmunoassay and further chromatography. The up-date time for counting was 15 s.

co-eluted with progastrin isolated from a gastrinoma. In both cases, the peaks of radioactivity corresponded in retention time to peaks of activity detected by radioimmunoassay using antibody specific for the C-terminus of progastrin. A third peak of radioactivity, which was sometimes barely detectable, emerged approx. 7 min before progastrin. In radioimmunoassays, material with a similar retention time reacted with both antibody specific for the N-terminus of G34 and antibody specific for the C-terminus of progastrin, and so was identified as G34-extended to the C-terminus of progastrin (G34-CFP), i.e. progastrin C-terminal fragment generated by cleavage at Arg^{57}-Arg^{58}.

Previous work has established that rat CFP occurs in four forms which are poorly separated on size-exclusion chromatography and reversed-phase h.p.l.c., but readily resolved on ion-exchange chromatography; these peptides have been characterized as CFP variants differing in sulphation of Tyr^{103} and phosphorylation of Ser^{96} [9]. We found that when the eluates of reversed-phase h.p.l.c. corresponding to CFP were further purified by ion-exchange chromatography, four peaks were resolved which were labelled with [^{3}H]tyrosine and also contained material detected by radioimmunossay (Figure 4). The least acidic peptide (I) co-eluted with the synthetic C-terminal nonapeptide of rat progastrin and so was identified as the unmodified peptide. The two most acidic peptides (III and IV) were also labelled in incubations in [^{35}S]sulphate and so were identified as being sulphated on Tyr^{103}, the most acidic peptide (IV) was also labelled with ^{32}P and so was both sulphated and phosphorylated (Figure 4). These assignments are consistent with previous conclusions based on the results of digestion with alkaline phosphatase and aryl sulphatase [9]. Peak II has previously been shown to consist of phosphorylated unsulphated peptide [9]. It was the smallest of the peaks labelled with [^{3}H]tyrosine in the present studies, and labelling with ^{32}P was not observed consistently. Peaks I–IV were the only products of ion-exchange chromatography labelled with [^{3}H]tyrosine, and peaks III and IV were the only products labelled with [^{35}S]sulphate. However, in the case of [^{32}P]phosphate, some labelled material was generally found to emerge before the progastrin-related immunoreactive peptides and we attribute this to non-specific (or gastrin-unrelated) labelling.

The specific radioactivity of [^{3}H]tyrosine in CFP was significantly higher in the sulphated/phosphorylated variant than in the others (Figure 4), suggesting that this form might be generated preferentially. In order to determine whether there might be dephosphorylation (or desulphation) in the present experiments, tissues were incubated first with [^{3}H]tyrosine for 2 h, and then chased with medium containing unlabelled tyrosine. After 3 h chase, there was a 20% reduction in specific radioactivity of peak IV, and increases in specific radioactivity of peaks I–III (Figure 5). Thus some desulphation and dephosphorylation cannot be excluded, but, over the shorter period used for most experiments (2 h), the redistribution of [^{3}H]tyrosine from peptide that was both phosphorylated and sulphated in favour of peptides that were either phosphorylated or sulphated, or neither, is unlikely to have accounted for more than about 10% of total activity.

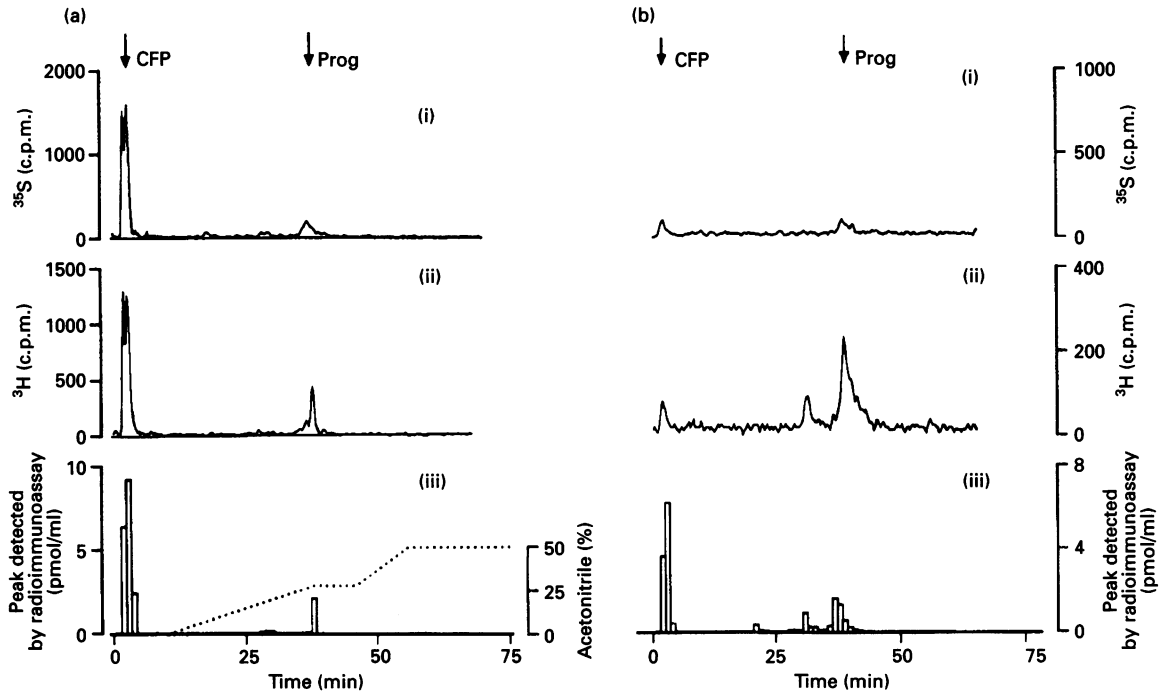

Figure 3 Separation by reversed-phase h.p.l.c. of progastrin-derived peptides incorporating [^{35}S]sulphate and [^{3}H]tyrosine in control and brefeldin A-treated samples

The results of dual-labelling experiments using [^{35}S]sulphate and [^{3}H]tyrosine are shown; see Figure 2 for further details. Note inhibition of incorporation of [^{35}S]sulphate in the presence of brefeldin A.

Effects of brefeldin A

In control experiments, incubation for 2 h resulted in approx. 2-fold higher incorporation of [^{3}H]tyrosine into CFP compared with progastrin, but the specific radioactivity of ^{3}H in progastrin was substantially greater than that in CFP (Tables 1 and 2). In sharp contrast, in the presence of brefeldin A, the incorporation of [^{3}H]tyrosine into CFP was reduced by over 90%, and there was an increase in relative abundance of labelled progastrin (Figure 2 and Table 1). The specific radioactivity of [^{3}H]tyrosine in progastrin after brefeldin A treatment was similar to that in control samples (Table 2). In the presence of brefeldin A, the labelling of G34-CFP increased in parallel with that of progastrin; thus in both control and brefeldin A-treated samples there was about 3-fold higher incorporation of [^{3}H]tyrosine into progastrin compared with G34-CFP (Table 1). The total [^{3}H]-tyrosine counts incorporated into progastrin-related peptides in the presence of brefeldin A were 81 ± 11% of those in control incubations. However, it should be noted that, in the presence of brefeldin A, most [^{3}H]tyrosine was in progastrin rather than CFP, and that there are two tyrosine residues in progastrin and G34-CFP, but only one in CFP. Taking this into account it would appear that the molar incorporation of tyrosine in the presence of brefeldin A was about 50% of that in control samples.

The inhibition by brefeldin A of the incorporation of [^{3}H]tyrosine into CFP cannot be attributed to degradation of the latter, since radioimmunoassays showed the presence of substantial amounts of material that was co-eluted with CFP on reversed-phase and ion-exchange h.p.l.c. There was, however, a statistically significant shift in the ratio of progastrin and CFP detected by radioimmunoassay (Table 1): progastrin increased significantly in the presence of brefeldin and CFP decreased.

The incorporation of [^{32}P]phosphate (Figure 2) and [^{35}S]sulphate (Figure 3) into both progastrin and CFP was inhibited by brefeldin A. Addition of the phosphatase inhibitor okadaic acid to the incubation medium had no effect on the incorporation of ^{32}P in either control or brefeldin A-treated samples. The possibility that brefeldin A in some way produced substantial dephosphorylation of CFP can be discounted because radioimmunoassay of the eluates of ion-exchange columns indicated that the proportions of total CFP in the phosphorylated form in brefeldin A-treated samples (44.3 ± 5.3%) and controls (50.1 ± 4.0%) were similar. This observation also suggests that the apparent inability to incorporate [^{32}P]phosphate into CFP in the presence of brefeldin A cannot be attributed to the presence of already fully phosphorylated substrate. Similarly, the radioimmunoassay data indicate that brefeldin A did not cause desulphation of the previously sulphated CFP which was presumably stored in secretory granules (Figures 2 and 3).

DISCUSSION

The present study shows that gastrin-producing cells incorporate [^{3}H]tyrosine, [^{32}P]phosphate and [^{35}S]sulphate into both progastrin and its C-terminal tryptic (nona-) peptide. Brefeldin A acts differentially on these processes: phosphorylation and sulphation of both peptides is inhibited and there is substantial inhibition of the incorporation of [^{3}H]tyrosine into the C-terminal nonapeptide of progastrin, but the incorporation of [^{3}H]tyrosine into progastrin continues in the presence of brefeldin A. The data are compatible with the idea that phosphorylation, sulphation and cleavage at Arg^{94}-Arg^{95} require delivery of newly synthesized

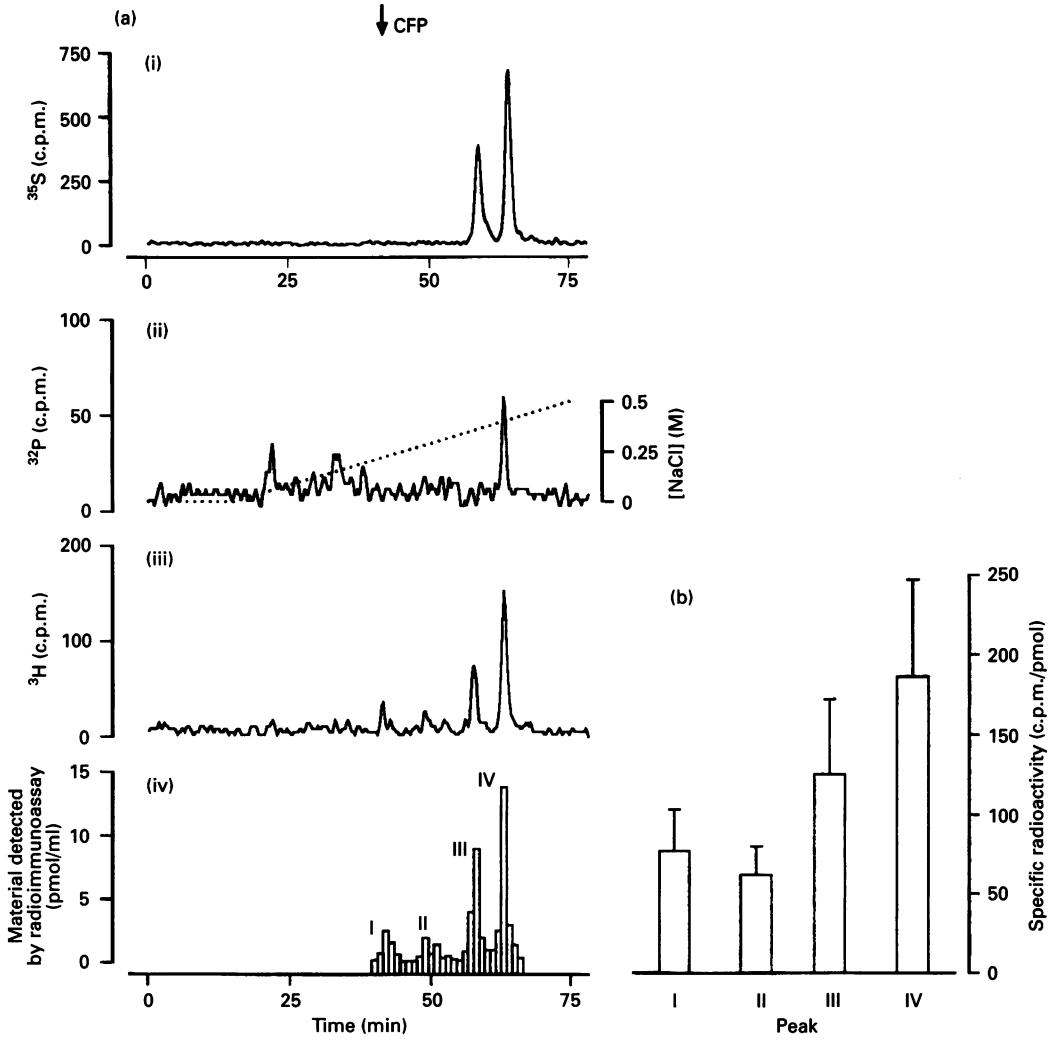

Figure 4 Separation by ion-exchange h.p.l.c. of C-terminal fragments of progastrin labelled with [^{3}H]tyrosine, [^{32}P]phosphate and [^{35}S]sulphate

The combined results of two dual-labelling experiments are shown. The CFP fractions from reversed-phase h.p.l.c. (see Figures 2 and 3) were further separated by ion-exchange chromatography on Mono Q. (**a**) (i) On-line counting of [^{35}S]sulphate. (**a**) (ii)–(iv) [^{32}P]Phosphate, [^{3}H]tyrosine and radioimmunoassay data respectively from a single run. The corresponding data on [^{3}H]tyrosine and radioimmunoassay from the experiment on [^{35}S]sulphate incorporation (**a**) (i) have been omitted for clarity. The radioimmunoassay data show four peaks (I–IV). Note that peak IV is labelled with ^{3}H, ^{32}P and ^{35}S; peak III is labelled with ^{3}H and ^{35}S but not ^{32}P; peaks I and II show incorporation of ^{3}H; from previous work [9], peak II is known to be phosphorylated but not sulphated, and presumably in the present experiments incorporation of radiolabelled phosphate was below the limit of detection. The rate of flow was 1.0 ml/min; 85% of the stream was split for on-line counting and the remainder taken for radioimmunoassay. The up-date time for counting was 20 s. The arrow indicates the elution position of the synthetic C-terminal nonapeptide of rat progastrin identified in a previous run. (**b**) Specific activity of [^{3}H]tyrosine in the four peaks based on estimates of immunoreactivity (mean $\pm$ S.E.M., $n = 5$).

progastrin along the secretory pathway to a point beyond the site of action of brefeldin A.

Brefeldin A causes the collapse of the Golgi complex into the rough endoplasmic reticulum, and so disrupts the forward movement of newly synthesized secretory proteins [16,19,20]. There is evidence for redistribution of membrane proteins from *cis*-, *medial*- and *trans*-Golgi into rough endoplasmic reticulum, but not from the *trans*-Golgi network [18]. Although there are also other potential sites of action of brefeldin A, e.g. interactions between the endosomal compartment and the *trans*-Golgi network [26], the present data are best explained by a failure to transport newly synthesized progastrin from endoplasmic reticulum to Golgi complex.

Previous work has shown that progastrin is converted into its active products in immature secretory granules [15]. The major cleavage sites in progastrin are pairs of basic residues, and it seems likely that endopeptidases of the KEX2 family, e.g. PC2 or PC3, are responsible for cleavage [27–30]. The localization of endopeptidase cleavage to the immature secretory granule implies that endopeptidases must be produced, transported and segregated into the regulated pathway of secretion in parallel with their substrates. It is interesting that there was little or no cleavage of Arg^{94}-Arg^{95} in brefeldin A-treated cells, whereas the cleavage of progastrin that was sometimes observed at Arg^{57}-Arg^{58} was apparently uninfluenced by brefeldin A. Evidently therefore there is a differential effect of brefeldin A on cleavage at the two sites. This observation suggests that there is a strict requirement for access of the precursor beyond the *trans*-Golgi for completion of the functionally important cleavage which generates the biologically active C-terminal part of gastrin, whereas the conditions for cleavage at the N-terminus of G34 are less stringent.

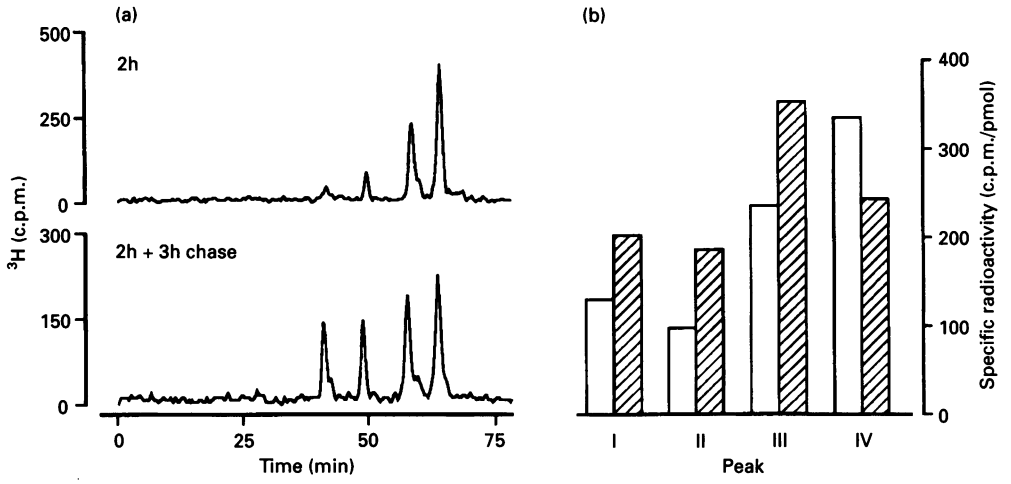

Figure 5 Separation by ion-exchange h.p.l.c. of C-terminal fragments of progastrin labelled with [^{3}H]tyrosine after 2 h incubation, and after a chase for a further 3 h

Samples were separated first by reversed-phase h.p.l.c., and the CFP fraction was taken for ion-exchange chromatography (see Figure 4). (**a**) Elution profiles; (**b**) specific radioactivity of the four peaks after 2 h incubation (open bars) and after a 3 h chase (hatched bars); results are means of two identical experiments. Note the decrease in specific activity of peak IV and the increase in peaks I–III.

Table 1 Influence of brefeldin A on distribution of progastrin-derived peptides labelled with [^{3}H]tyrosine or measured by radioimmunoassay

Results are expressed as percentage of total [^{3}H]tyrosine incorporated into each of the three peaks identified after reversed-phase h.p.l.c., and the relative proportions (percentage of total) of different progastrin-derived peptides determined by radioimmunoassay using antibody specific for the C-terminus of progastrin. Total immunoreactives in the control and brefeldin A-treated samples were similar (control: 18.5 pmol; brefeldin A: 17.8 pmol). Values are means ± S.E.M. (*n* in parentheses). Comparisons were made by the paired *t* test: $^*P < 0.01$, $^*P < 0.05$, $^{***}P < 0.001$, compared with control values.

	Control	Brefeldin A-treated
Labelled peptide		
CFP (6)	66.5 ± 6.7	7.9 ± 2.1***
G34-CFP (6)	8.0 ± 2.2	24.9 ± 6.7
Progastrin (6)	27.0 ± 6.4	67.1 ± 7.0*
By radioimmunoassay		
CFP (7)	89.4 ± 12.9	71.3 ± 12.9*
G34-CFP (7)	3.0 ± 1.3	11.7 ± 4.6**
Progastrin (7)	7.6 ± 2.1	16.8 ± 3.3*

Table 2 Specific radioactivities of ^{3}H, ^{32}P and ^{35}S in progastrin-derived peptides after incubation with and without brefeldin A

Specific radioactivities (c.p.m./pmol) are given for progastrin-derived peptides in experiments where tissues were incubated with [^{3}H]tyrosine, [^{32}P]phosphate or [^{35}S]sulphate in the presence or absence of brefeldin A (10 μg/ml for 2 h). The data for ^{32}P in CFP are based on integration of peak IV in ion-exchange column eluates; all other values are derived from reversed-phase h.p.l.c. eluates. Where there was less than 100 c.p.m. of ^{32}P present in the peak tube in over 50% of the samples, it was not possible to produce reliable estimates of specific radioactivity and this is indicated by ND. Values are means ± S.E.M. (*n* in parentheses). Comparisons were made by the Mann–Whitney *U* test: $^{**}P < 0.05$, $^{***}P < 0.001$, compared with control values.

	Control	Brefeldin A-treated
[^{3}H]Tyrosine (6)		
CFP	180 ± 23	21 ± 6***
G34-CFP	599 ± 232	521 ± 157
Progastrin	1342 ± 569	1161 ± 327
[^{32}P]Phosphate (4)		
CFP	59 ± 20	ND
G34-CFP	ND	ND
Progastrin	430 ± 140	ND
[^{35}S]Sulphate (4)		
CFP	499 ± 197	50 ± 21**
G34-CFP	846 ± 251	130 ± 52**
Progastrin	1301 ± 218	232 ± 78**

Approx. 50% of native CFP occurs in the unphosphorylated form; at least some of the latter may arise by dephosphorylation (Figure 5). In principle, endogeneous kinases could act on either progastrin or CFP. Because brefeldin A inhibited phosphorylation of CFP, it would appear that only newly synthesized peptide is available for the action of kinases, and consequently that these are unable to act on the pool of unphosphorylated CFP that is already contained in secretory granules. Similarly it would appear that sulphation is limited to newly synthesized peptide. In the case of other secretory proteins, e.g. the vitellogenins, there is evidence that phosphorylation occurs just before secretion [31]; vitellogenin is probably secreted via the constitutive pathway which could place the kinase in the distal regions of the Golgi. Recently it has been reported that, in PC12 cells, brefeldin A inhibited sulphation but not phosphorylation of two proteins that are segregated to the regulated pathway of secretion, i.e. chromogranin B and secretogranin II [32]. The present observation that brefeldin A inhibited sulphation of progastrin-derived peptides is consistent with these observations. However, it is not yet clear why brefeldin A acts differently on phosphorylation of the chromogranins compared with the progastrin-derived peptides. There are, however, multiple potential phosphorylation sites in the granins; it is possible that the kinase acting on progastrin-derived peptides is not the same as that acting on the granins and that the two have a differential location.

The results of the present study indicate that phosphorylation, sulphation and Arg^{94}-Arg^{95} cleavage of progastrin occur at a site

distal to that at which brefeldin A acts, i.e. beyond the *trans*-Golgi cisternae. The pool of unphosphorylated CFP in secretory granules is not, however, labelled with [^{32}P]phosphate in the presence of brefeldin A which suggests that phosphorylation occurs proximal to the secretory granule. As cleavage occurs after delivery of progastrin to the secretory granule [15], it would appear that progastrin is phosphorylated before cleavage, probably in the *trans*-Golgi network. This conclusion, together with the observation that the progastrin phosphorylation site is well conserved, suggests a functional role for prohormone phosphorylation in the control of the events associated with precursor maturation.

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