Published in final edited form as:

Biochem Biophys Res Commun. 2011 January 28; 404(4): 1083–1087. doi:10.1016/j.bbrc.2010.12.117.

Ferric ions inhibit proteolytic processing of progastrin

Gianni Bramante, Oneel Patel, Arthur Shulkes, and Graham S. Baldwin*

The University of Melbourne Department of Surgery, Austin Health, Heidelberg 3084, Victoria, Australia

Abstract

The gastrointestinal hormone gastrin is generated from an 80 amino acid precursor (progastrin) by cleavage after dibasic residues by prohormone convertase 1. Phosphorylation of Ser₇₅ has previously been suggested, on the basis of indirect evidence, to inhibit cleavage of progastrin after Arg₇₃Arg₇₄. Gastrins bind two ferric ions with high affinity, and iron binding is essential for the biological activity of non-amidated gastrins *in vitro* and *in vivo*. This study directly investigated the effect of iron binding and of serine phosphorylation on the cleavage of synthetic progastrin-derived peptides. The affinity of synthetic progastrin₅₅₋₈₀ for ferric ions, and the rate of cleavage by prohormone convertase 1, were not affected by phosphorylation of Ser₇₅. In contrast, in the presence of ferric ions the rate of cleavage of both progastrin₅₅₋₈₀ and phosphoSer₇₅progastrin₅₅₋₈₀ by prohormone convertase 1 was significantly reduced. Hence iron binding to progastrin may regulate processing and secretion *in vivo*, and regulation may be particularly important in diseases with altered iron homeostasis.

Keywords

ferric; gastrin; iron; processing; progastrin; prohormone convertase

Introduction

Most peptide hormones are synthesised as precursors that undergo proteolytic cleavage and post-translational modification to form biologically active products. All gastrins are processed from a common 80 amino acid precursor progastrin, which undergoes a number of post-translational modifications including tyrosine sulfation, serine phosphorylation and site-specific cleavage after paired basic residues [1]. Amidated gastrin (Gamide, progastrin₅₅₋₇₁amide) (Figure 1) is the primary stimulant of acid secretion from the stomach [2], while glycine-extended gastrin (Ggly, progastrin₅₅₋₇₂) acts as a growth factor for the colorectal mucosa [3,4]. Although the exact mechanisms regulating progastrin processing are not completely understood, the fact that the different gastrins have such distinct actions and such different target organs suggests that subtle changes in processing could have major physiological effects.

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*To whom correspondence should be addressed: Professor Graham Baldwin, University of Melbourne Department of Surgery, Austin Health, Heidelberg 3084, Victoria, Australia Tel.: +61 (0)3 9496 5592 Fax: +61 (0)3 9458 1650 grahamsb@unimelb.edu.au.

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Progastrin is phosphorylated on Ser₇₅ *in vivo* [5]. The location of Ser₇₅ immediately adjacent to the initial progastrin cleavage site, C-terminal to the dibasic pair Arg₇₃Arg₇₄ (Figure 1), suggested that serine phosphorylation might delay cleavage of progastrin. Site-directed mutagenesis of both Ser₇₅ and Glu₇₇, which are key components of the kinase recognition sequence, resulted in reduced phosphorylation and increased cleavage *in vitro* [6]. A subsequent study suggested that the serine phosphorylation site might act in synergy with the penta-glutamate domain (progastrin₆₀₋₆₄) to influence the ratio of secreted to intracellular progastrin cleavage products. Glu to Ser substitution and C-terminal truncation resulted in an increase in the secretion ratio of progastrin [7]. However, the effect of serine phosphorylation on progastrin cleavage has not been directly tested and the exact mechanism involving the penta-glutamate region has not been determined.

Progastrin and its derived peptides bind metal ions. Detailed spectrophotometric analyses of the gastrin-ferric ion complex previously demonstrated that Ggly and Gamide bind two ferric ions with high affinity in aqueous solution [8]. Iron specifically bound Glu₆₁-Glu₆₃ of the penta-glutamate domain of Ggly [9], and iron binding was essential for biological activity *in vitro* and *in vivo* [9,10,11]. However, the effect of binding of ferric ions to progastrin on progastrin processing has not been reported. Furthermore, the observation that phosphorylation of tyrosine residues enhanced the binding of ferric ions to the related hormone cholecystokinin [12] suggested that phosphorylation of Ser₇₅ might also influence iron binding. Here two synthetic N-terminally truncated forms of progastrin which differed in serine phosphorylation state have been used to investigate directly the effect of serine phosphorylation on ferric ion binding, and the effects of serine phosphorylation and ferric ions on the cleavage of progastrin. The study was designed in two parts, firstly with trypsin as a model dibasic cleaving enzyme to permit identification of the reaction products, and secondly with the physiologically relevant human enzyme prohormone convertase 1 (PC1).

Materials and methods

Peptides and Reagents

Synthetic Ggly (progastrin₅₅₋₇₂, ZGPWLEEEEEAYGWMDFG, where Z represents pyroglutamic acid) (86% purity) was purchased from Auspep (Melbourne, Australia). The synthetic progastrin derivatives (Figure 1) progastrin₅₅₋₈₀ (pGlu-GPWLEEEEEAYGWMDFGRRSAEDEN) (84% purity) and phosphoSer₇₅progastrin₅₅₋₈₀ (81% purity) were purchased from Mimotopes (Clayton, Australia). The following enzymes and protease inhibitors were from the indicated suppliers: recombinant human prohormone convertase 1 (R&D Systems, Minneapolis, MN), tosylphenylalanyl-chloromethylketone (TPCK)-treated trypsin (Worthington, NJ), the furin inhibitor decanoyl-Arg-Val-Lys-Arg-chloromethylketone (Enzo Life Sciences, Plymouth Meeting, PA) and phenylmethylsulphonyl fluoride (PMSF) (Sigma-Aldrich, St. Louis, MO).

Absorbance Spectroscopy

Progastrin peptides were dissolved in DMSO at a concentration of approximately 0.5 mM before dilution with 49 vols 10 mM sodium acetate buffer (pH 4.0) containing 100 mM NaCl and 0.005% Tween 20 to give a final concentration of approximately 10 M. Absorbance spectra of peptides in the presence of increasing concentrations of ferric ions were measured against a buffer blank, in 1 mL quartz cuvettes thermostatted at 25°C, with a Cary 5 spectrophotometer (Varian, Mulgrave, Australia).

Trypsin Assay

The enzymatic activity of trypsin in the presence of various concentrations of FeCl₃ was measured by absorbance spectroscopy as described previously [13]. The trypsin substrate 2-

N-benzoyl-arginine ethyl ester (Sigma-Aldrich) (4.0 mM in 10 mM sodium acetate buffer (pH 4.0) containing 100 mM NaCl and 0.005% Tween 20) was added to an equal volume of 5 μ M FeCl₃ in the same buffer. The pH was adjusted to 8.0 by addition of 50 mM ammonium bicarbonate (Sigma-Aldrich) prior to the addition of trypsin to start the reaction. The change in absorbance over 2 h was measured at 253 nm against a buffer blank, in 1 mL quartz cuvettes thermostatted at 25°C, with a Cary 5 spectrophotometer (Varian, Mulgrave, Australia).

Prohormone Convertase 1 Assay

The enzymatic activity of recombinant human PC1 was measured by fluorescence spectroscopy with a FLUOstar OPTIMA microplate reader (BMG Labtech, Offenburg, Germany), thermostatted at 37°C, with the excitation and emission wavelengths set at 390 nm and 460 nm, respectively. Equal volumes of various concentrations (0-28.6 M) of FeCl₃ in 25 mM 2-(N-morpholino)ethanesulfonic acid (MES) buffer (pH 6.0) containing 5 mM CaCl₂ and 0.005% Brij-35 and the fluorogenic substrate pyroGlu-Arg-Thr-Lys-Arg-7-amino-4-methylcoumarin (200 M, R&D Systems) in the same buffer were mixed and PC1 was added to a final concentration of 4 g/mL. Fluorescence intensity was measured at regular intervals over 2 h.

Cleavage of Progastrin Peptides with Trypsin

Progastrin peptides were dissolved in DMSO at a concentration of approximately 1 mM before dilution with 39 vols 10 mM sodium acetate buffer (pH 4.0) containing 100 mM NaCl and 0.005% Tween 20 to give a final concentration of approximately 25 M. Progastrin peptides were loaded with ferric ions by addition of an equal volume of 50 M FeCl₃ in the same buffer to give a Fe:progastrin ratio of 2:1. After 10 min at 25°C the peptide solutions were diluted with 4 vols 50 mM ammonium bicarbonate, mixed and a zero time sample removed. TPCK-treated trypsin was then added to a final concentration of 0.5 g/mL. Aliquots were taken from the reaction mixture at 60 min intervals and subsequent cleavage was inhibited by addition of PMSF to 9.9 M. The products of digestion at each time-point were separated by reverse-phase HPLC.

Cleavage of Progastrin Peptides with PC1

Progastrin peptides (approximately 1 mM in DMSO) were diluted with 69 vols 25 mM MES buffer (pH 6.0) containing 5 mM CaCl₂ and 0.005% Brij-35 with or without 28.6 M FeCl₃ to give a final progastrin concentration of approximately 14.3 M and a Fe:progastrin ratio of 2:1. After 10 min at 37°C a zero time sample was removed, and recombinant human PC1 was added to a final concentration of 5.5 g/mL. Aliquots were taken from the reaction mixture at 40 min intervals and subsequent cleavage was inhibited by addition of decanoyl-Arg-Val-Lys-Arg-chloromethylketone to 10 M. The products of digestion at each time-point were separated by reverse-phase HPLC.

Reverse-Phase High-Performance Liquid Chromatography

Samples were applied to a C18 column (8 × 100 mm, Waters Associates, Milford, MA, USA), which had been equilibrated with filtered 50 mM ammonium bicarbonate (Sigma-Aldrich) using a Hewlett Packard 1100 series HPLC (Hewlett Packard, Waldbronn, Germany). Progastrin and progastrin-derived peptides were eluted with a gradient from 20%-50% acetonitrile in 50 mM ammonium bicarbonate at a flow rate of 1 mL/min. Fractions of 1 mL were collected, and aliquots were dried on a Speed Vac (Savant, Hicksville, NY) for mass spectrometry.

Mass Spectrometry

Electrospray ionisation mass spectrometry was performed on a Sciex API-300 triple quadrupole mass spectrometer (Perkin-Elmer, Foster City, CA) fitted with a micro-ion spray ion source (flow rate: 0.2 L/min), previously calibrated to an accuracy of $\pm 0.01\%$ using singly-charged poly(propyleneglycol) reference ions.

Curve Fitting and Statistical Analysis

Absorbance data for the binding of ferric ions to progastrin-derived peptides were fitted to a two-site ordered model with the program BioEqs [14,15]. All statistics were analysed with the program SigmaStat (Jandel Scientific, San Rafael, CA, USA). Results are expressed as the means $\pm$ standard error of the mean (SEM). Data were analysed by Student's t-test. If there was a statistically significant difference in the data set, individual values were compared using Student's t-test with Bonferroni correction. P values < 0.05 were considered statistically significant.

Results

Binding of ferric ions by progastrin₅₅₋₈₀ and phosphoSer₇₅progastrin₅₅₋₈₀

Ggly has previously been shown by fluorescence spectroscopy to bind two ferric ions in aqueous solution at pH 4.0 with an affinity of 0.62 ± 0.19 M [8]. The changes in absorbance (Figure 2) on titration of progastrin₅₅₋₈₀ with ferric ions in 10 mM sodium acetate buffer (pH 4.0) containing 100 mM NaCl and 0.005% Tween 20 were well fitted by a two-site ordered model with the following dissociation constants: $Kd_1 = 0.54$ M; $Kd_2 = 2.15$ M. The observation that the dissociation constants for the binding of ferric ions to phosphoSer₇₅progastrin₅₅₋₈₀ ($Kd_1 = 1.01$ M; $Kd_2 = 0.97$ M) were very similar to the values observed for the non-phosphorylated form indicated that phosphorylation of Ser₇₅ did not significantly affect ferric ion binding.

Progastrin processing by trypsin

To investigate the effect of ferric ions and serine phosphorylation on progastrin processing, experiments were first performed with the serine protease trypsin, which cleaves C-terminal to arginine or lysine residues. Initially progastrin₅₅₋₈₀ was treated with trypsin at pH 8, and the reaction products were separated by reverse-phase HPLC. Progastrin₅₅₋₇₄, which eluted as a discrete peak 0.8 min after progastrin₅₅₋₈₀ (data not shown), was identified by mass spectrometry, consistent with cleavage of progastrin₅₅₋₈₀ at the Arg₇₄-Ser₇₅ bond.

The effect of iron binding to progastrin on progastrin processing was then investigated. Progastrin₅₅₋₈₀ was pre-incubated with ferric ions at pH 4.0 and the iron-loaded complex was then treated with trypsin at pH 8. The reaction products were separated by reverse phase HPLC and the amount of residual progastrin₅₅₋₈₀ was determined from the area of its absorbance peak. The presence of bound ferric ions significantly inhibited cleavage, with 20% less cleavage of progastrin₅₅₋₈₀ over 2 h (Figure 3). The difference was not due to direct inhibition of trypsin by iron, as hydrolysis of 2-N-benzoyl-arginine ethyl ester by trypsin was not affected by the presence of the same concentration of ferric ions (data not shown).

The presence of a phosphate group on Ser₇₅ also significantly inhibited cleavage by trypsin, with approximately 60% less cleavage of phosphoSer₇₅progastrin₅₅₋₈₀ than progastrin₅₅₋₈₀ over 2 h (Figure 3). In contrast to progastrin₅₅₋₈₀, the presence of ferric ions had no significant effect on cleavage of phosphoSer₇₅progastrin₅₅₋₈₀ by trypsin (Figure 3).

Progastrin processing by PC1

To assess the physiological relevance of the results obtained with trypsin the protease assay was repeated with recombinant human PC1. In the presence of ferric ions there was a significant reduction in the extent of cleavage of progastrin₅₅₋₈₀ by PC1 (Figure 4). In contrast to the results obtained with trypsin, there was no significant difference in the rate of cleavage of progastrin₅₅₋₈₀ and phosphoSer₇₅progastrin₅₅₋₈₀ by PC1. This result clearly establishes that phosphorylation of Ser₇₅ does not significantly impede PC1 activity. However, in the presence of ferric ions the cleavage of both progastrin₅₅₋₈₀ and phosphoSer₇₅progastrin₅₅₋₈₀ was significantly slower than in the absence of ferric ions (Figure 4). The difference was not due to direct inhibition of PC1 by iron, as iron did not affect PC1 protease activity in a standard fluorometric activity assay using the PC1 substrate pyroGlu-Arg-Thr-Lys-Arg-7-amino-4-methylcoumarin (data not shown).

Discussion

This study investigated the relationship between progastrin processing, ferric ion binding and serine phosphorylation *in vitro* using synthetic progastrin₅₅₋₈₀ and phosphoSer₇₅progastrin₅₅₋₈₀. The study aimed firstly to determine the importance of phosphorylation of Ser₇₅ to processing of the C-terminal region of progastrin, and secondly to investigate whether or not ferric iron binding by progastrin influenced processing.

In humans, progastrin has been shown to exist with Ser₇₅ in both phosphorylated and unphosphorylated states [5]. Serine phosphorylation sites are conserved amongst a number of prohormones including the related peptide procholecystokinin (proCCK), chromogranin A, proenkephalin A and proneuropeptide Y [5,16,17,18,19]. In the case of progastrin serine phosphorylation may be biologically important as the phosphorylated Ser₇₅ lies immediately adjacent to the dibasic cleavage site Arg₇₃Arg₇₄. Previous studies had suggested that serine phosphorylation of progastrin might delay cleavage, as replacement of Ser₇₅ by Ala resulted in reduced phosphorylation and increased cleavage [6]. Indeed, our trial experiments with trypsin initially supported such a hypothesis, since the phosphate group impeded tryptic cleavage. However, when the hypothesis was tested directly with the physiological progastrin-cleaving enzyme PC1, the observation that cleavage was not inhibited suggested that phosphorylation does not regulate C-terminal progastrin processing. This conclusion is in agreement with unpublished data from the Rehfeld group (quoted in [20]) which indicate that substitution of Ser₇₅ in progastrin with Ala did not change the production of gastrin under steady state conditions. Similarly, in proCCK processing phosphorylation of the serine adjacent to the corresponding dibasic cleavage site has no effect on cleavage [17]. The difference between PC1 and trypsin highlights the importance of using the actual physiological enzyme rather than a structurally unrelated model enzyme with similar specificity.

The crucial importance of ferric ion binding for the biological activity of non-amidated gastrins has been demonstrated *in vitro* and *in vivo* [9,10,11]. The absorbance data presented in Figure 2 indicated that both progastrin₅₅₋₈₀ and phosphoSer₇₅progastrin₅₅₋₈₀ bound 2 ferric ions with M affinities, as has previously been reported for Ggly and progastrin [8,21]. A previous study with progastrin₆₋₈₀ suggested that the progastrin-iron complex once formed was extremely stable, with a half-life of 117 days at pH 7.6 and 25°C [21]. Therefore, metal ion binding to progastrin during processing represents a possible regulatory mechanism that could influence the nature of the progastrin-derived peptides produced.

The biological relevance of our observation that iron binding can inhibit cleavage of progastrin *in vitro* should also be considered. In the cell, progastrin processing initially occurs within immature vesicles that have formed from the trans-Golgi network and is

known to be regulated by pH and by calcium, both of which modulate PC activity [6,22]. However, there are no reports describing a direct effect of iron on processing, such as we have described *in vitro*. Although there is little information about the distribution of iron amongst organelles of the secretory pathway, including the Golgi or secretory vesicles, previous studies have shown the presence of the iron-loaded iron storage molecule, holoferritin, in the rough endoplasmic reticulum, Golgi and nucleus, especially under iron-overload conditions [23,24,25]. Localised expression of the transferrin receptor on the endoplasmic reticulum, Golgi and secretory vesicles might account for the presence of iron within these membrane-enclosed structures [26]. Therefore, in conditions of iron-overload the reported shift in the ratio of different progastrin-derived peptides in the gastric antral mucosa towards less completely processed forms of gastrin [27] may be a consequence of such a metal binding regulatory mechanism.

The concept that distinct post-translational modifications of progastrin are responsible for differential sorting and secretion of progastrin-derived peptides is not a new one. Bundgaard and coworkers have begun to unravel by mutational studies the interconnections between tyrosine sulfation, serine phosphorylation and the acidic metal ion binding domain of progastrin as a potential regulatory mechanism for differential progastrin secretion. The acidic penta-glutamate domain and the dibasic cleavage sites of progastrin have been proposed to synergise in the sorting of the maturing peptides to regulated, constitutive or constitutive-like pathways [7,28]. In the present study we provide direct evidence that metal ion binding to progastrin is likely to have a significant role in determining the cleavage pattern of progastrin as it traverses the secretory pathway. The flow-on effect of changes in iron status on the sorting of progastrin-derived peptides to distinct secretory pathways should therefore be the focus of future investigations.

In conclusion, the results of this study have highlighted the importance of the acidic penta-glutamate metal ion binding domain of progastrin to endoproteolytic processing of progastrin. Serine phosphorylation of progastrin is not likely to contribute directly to the regulation of cleavage of progastrin as both progastrin and phosphoSer₇₅progastrin were cleaved at similar rates by the physiologically relevant enzyme PC1. These results are consistent with other studies suggesting that other C-terminal domains influence the sorting of progastrin and its derivatives to distinct secretory pathways [28]. Furthermore, the impact of metal ion binding on the final secretion products of progastrin processing may be a contributing factor to pathologies such as the increased risk of colorectal cancer seen in patients with iron overload [29] and should be further investigated.

Research Highlights

In contrast to previous reports based on indirect evidence, serine phosphorylation does not influence cleavage of progastrin by prohormone convertase 1.

Binding of ferric ions by progastrin significantly delays cleavage by prohormone convertase 1.

Metal ion binding may represent a novel regulatory mechanism for prohormone processing.

Acknowledgments

This work was supported in part by grants from the National Health and Medical Research Council of Australia (400062, 454322; GB) and the National Institutes of Health (5R01GM065926-08; GB, AS). Mass spectrometry was kindly performed by Professor Gert Talbo of the Baker Heart Research Institute, Melbourne, Victoria.

Abbreviations

CRC	colorectal carcinoma
Ggly	glycine-extended gastrin
PC1	prohormone convertase 1

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Fig. 1.

Structure of progastrin and related peptides. The structures of progastrin₅₅₋₈₀, serine phosphoSer₇₅progastrin₅₅₋₈₀, Ggly (progastrin₅₅₋₇₂) and Gamide (amidated progastrin₅₅₋₇₁) are shown. A pyroglutamic acid residue (Z) protects the N-terminus of all four peptides.

Fig. 2.

Binding of ferric ions by progastrin₅₅₋₈₀ and phosphoSer₇₅progastrin₅₅₋₈₀. Addition of aliquots of ferric chloride to 9.43 μ M progastrin₅₅₋₈₀ (●) or 9.66 μ M phosphoSer₇₅progastrin₅₅₋₈₀ (■) in 10 mM sodium acetate buffer (pH 4.0) containing 100 nM NaCl and 0.005% Tween 20 resulted in an increase in absorbance at 280 nm. Data were corrected for volume added during the titration, and expressed as a percentage of the absorbance of that peptide without ferric ions. Points are means of at least four separate experiments and bars represent the SEM. The lines represent the best fit to a two-site ordered model with the program BioEqs. The best fit values for the dissociation constants are given in the text.

Fig. 3.

Effect of iron binding and serine phosphorylation on cleavage of progastrin by trypsin. The amount of residual progastrin₅₅₋₈₀ (●, ○) or phosphoSer₇₅progastrin₅₅₋₈₀ (■, □) after treatment with trypsin was determined in the absence (●, ■) or presence (○, □) of bound ferric ions by measurement of peak areas after HPLC separation of the cleavage products. Pre-incubation with ferric ions significantly reduced tryptic cleavage of progastrin₅₅₋₈₀ (*, $p < 0.05$). Ser₇₅ phosphorylation also significantly reduced tryptic cleavage of progastrin (#, $p < 0.01$). In contrast, pre-incubation with ferric ions did not inhibit tryptic cleavage of phosphoSer₇₅progastrin₅₅₋₈₀.

Fig. 4.

Effect of iron binding and serine phosphorylation on cleavage of progastrin by PC1. The amount of residual progastrin₅₅₋₈₀ (●, ○) or phosphoSer₇₅progastrin₅₅₋₈₀ (■, □) after treatment with recombinant human PC1 was determined in the absence (●, ■) or presence (○, □) of bound ferric ions by measurement of peak areas after HPLC separation of the cleavage products. Pre-incubation with ferric ions significantly reduced cleavage of progastrin₅₅₋₈₀ (#, $p < 0.001$) or phosphoSer₇₅progastrin₅₅₋₈₀ by PC1 (*, $p < 0.05$). In contrast Ser₇₅ phosphorylation did not affect cleavage of progastrin by PC1.