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Definition of the Residues Required for the Interaction between Glycine-extended Gastrin and Transferrin *In Vitro*

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Abstract

Transferrin is the main iron transport protein found in the circulation, and the level of transferrin saturation in the blood is an important indicator of iron status. The peptides amidated gastrin17 (Gamide) and glycine-extended gastrin17 (Ggly) are well known for their roles in controlling acid secretion and as growth factors in the gastrointestinal tract. Several lines of evidence, including the facts that transferrin binds gastrin, that gastrins bind ferric ions, and that the level of expression of gastrins positively correlates with transferrin saturation, suggest the possible involvement of the transferrin-gastrin interaction in iron homeostasis. In the present work the interaction between gastrins and transferrin has been characterized by surface plasmon resonance and covalent crosslinking. Firstly, an interaction between iron-free apo-transferrin and Gamide or Ggly was observed. The fact that no interaction was observed in the presence of the chelator EDTA suggested that the gastrin-ferric ion complex was the interacting species. Moreover, removal of ferric ions with EDTA reduced the stability of the complex between apo-transferrin and gastrins, and no interaction was observed between Gamide or Ggly and diferric-transferrin. Secondly, some or all of glutamates at positions 8–10 of the Ggly molecule, together with the C-terminal domain, were necessary for the interaction with apo-transferrin. Thirdly, monoferric transferrin mutants incapable of binding iron in either the N- or C-terminal lobe still bound Ggly. These findings are consistent with the hypothesis that gastrin peptides bind to non-ligand residues within the open cleft in each lobe of transferrin and are involved in iron loading of transferrin *in vivo*

Keywords

Ferric; gastrin; iron; transferrin

Introduction

Iron plays a central role in cellular processes because of its ability to accept or donate electrons readily, and to cycle between ferric (Fe^{3+}) and ferrous (Fe^{2+}) forms. Iron is essential for DNA synthesis, respiration and metabolic processes as a key component of cytochromes, oxygen-binding molecules such as haemoglobin and myoglobin, and iron-sulfur clusters in many enzymes. Because of its crucial biological functions, iron must be readily available throughout the body.

Transferrin is the main iron transport protein in the circulation. The biological importance of transferrin is shown by the fact that hypotransferrinemic *hpx* mice [1] die from severe anaemia within 14 days *post partum* [2]. Transferrin is able to bind two Fe^{3+} ions with very high affinity and can then donate iron to cells throughout the body via the transferrin receptor 1 (TfR1). The crystal structure of the single transferrin polypeptide chain (comprised of 680–690 amino acid residues) has been determined in both diferric [3] and iron-free (apo-transferrin) forms [4]. The chain is folded into two lobes, the N-lobe and C-lobe, derived from the N-terminal and C-terminal halves of the protein, respectively. The two lobes share 60% homology and are presumed to have arisen by gene duplication and fusion [5]. Each lobe is folded into two subdomains which come together to form a cleft which provides a binding site for one ferric ion [6]. *In vitro* studies have shown that the two lobes are kinetically and thermodynamically distinct and that cooperativity between the lobes is required for iron release [7,8]. Transferrin adopts a “closed” (holo) conformation when iron enters the cleft and an “open” (apo) conformation when iron is released. In healthy humans, although the concentration of transferrin in the serum is 25–50 mM, only approximately 30% is saturated with iron. The proportions of the four possible forms are: 27% diferric, 23% monoferric N-lobe, 11% monoferric C-lobe and 39 % apo-transferrin [9]. Transferrin saturation is an important indicator of iron status, since it modulates the concentration of hepcidin, the peptide responsible for regulation of iron release from cells which store iron.

The gastrointestinal peptide hormone gastrin (amidated gastrin17, Gamide) is well known as a stimulant of gastric acid secretion, and as a growth factor for the gastric mucosa [10]. More recently non-amidated precursor forms, such as progastrin and glycine-extended gastrin17 (Ggly), have also been shown to stimulate proliferation and migration of cell lines derived from a variety of gastrointestinal tumours although, in contrast to Gamide, stimulation of growth by Ggly *in vivo* is restricted to the colorectal mucosa [10]. Fluorescence quenching data have revealed the presence of two Fe^{3+} ion binding sites in both Ggly and Gamide with a K_d of 0.6 μM in aqueous solution [11]. Glu7 serves as a ligand for one ferric ion, and Glu8 and Glu9 bind a second ferric ion in both Ggly [12] and Gamide [13]. Although both Ggly and Gamide bind iron, only in the case of Ggly is biological activity dependent on ferric ion binding [12], while Gamide is fully active in the absence of metal ions [13].

Evidence for a connection between gastrins and iron homeostasis was first provided in a search for gastrin-binding proteins in porcine gastric mucosa [14]. An interaction between Gamide and transferrin was identified by covalent cross-linking assays [14], and subsequently a more detailed ultracentrifugal study revealed that at pH 7.4 apo-transferrin bound 2 molecules of gastrin with a K_d of 6.4 μM [15]. Importantly, no significant binding of Gamide to diferric-transferrin was detected. The observations that circulating gastrin concentrations are increased in the iron-loading disorder hemochromatosis [16], and that circulating Gamide concentrations were correlated with transferrin saturation in both mice and humans [17], suggest that the interaction between gastrins and transferrin may be important in the regulation of iron homeostasis. Independent evidence for a connection between gastrins and iron status has been provided by a microarray comparison of gene expression profiles in the stomachs of gastrin-deficient and wild-type mice. The concentration of gastric hepcidin mRNA in gastrin-deficient mice was only 40% of the value in wild-type mice, and Gamide infusion restored the hepcidin mRNA concentration to 130% of the wild-type value [18].

The biochemical basis of the gastrin-transferrin interaction is still unknown. Knowledge of the regions of transferrin required for the binding of gastrin, and of the regions in gastrin required for the interaction with transferrin, is obviously essential to a full understanding of

the interaction. The independent implication of iron (see references in [17]) and non-amidated gastrins such as Ggly [10] in the development of colorectal cancer make it particularly important to establish whether or not Ggly also interacts with transferrin. Here surface plasmon resonance and covalent crosslinking have been used to explore whether Ggly interacts with transferrin *in vitro*, to investigate whether iron is required for the Ggly-transferrin interaction, to define the domains/residues of Ggly involved in the interaction using Ggly mutants, and lastly to determine the regions of transferrin required for the interaction with gastrins.

Results

Both Gamide and Ggly interact with apo-transferrin but not holo-transferrin

An interaction between immobilized Gamide or Ggly peptides and apo-transferrin was clearly observed using surface plasmon resonance (Figure 1A), whereas no binding was found for holo-transferrin (Figure 1B). The apparent rate constants for association (k_a) and dissociation (k_d) for Gamide were: $k_a = 5.94 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ and $k_d = 8.06 \times 10^{-4} \text{ s}^{-1}$, and for Ggly: $k_a = 5.20 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ and $k_d = 1.06 \times 10^{-3} \text{ s}^{-1}$. The data are consistent with the hypothesis that gastrins bind within the iron-binding cleft, which needs to be in the open (apo) conformation for the association between gastrins and transferrin to occur.

Covalent cross-linking experiments confirmed that Ggly interacts with apo-transferrin but not with holo-transferrin (Figure 1C). Thus two different approaches demonstrate that transferrin must be in the open (iron-free) conformation to be able to interact with Ggly, as was previously found for Gamide [14,15]. To measure the affinity of apo-transferrin for Ggly, a titration curve using unlabelled Ggly was constructed (Figure 1D). The IC_{50} for binding of Ggly to apo-transferrin was found to be $39 \pm 1 \mu\text{M}$.

Importance of Fe^{3+} ions for the gastrin/apo-transferrin interaction

Since both Gamide and Ggly bind 2 ferric ions [11], the iron chelator EDTA was co-injected with apo-transferrin into the BIAcore channel to determine whether the ferric ions were required for the interaction between gastrins and apo-transferrin. In the presence of EDTA no interaction between apo-transferrin and either Gamide or Ggly was observed (Figure 2A). Therefore, Fe^{3+} ions must be present for formation of the complex between apo-transferrin and Ggly or Gamide.

The effect of Fe^{3+} ions on the stability of the gastrin/apo-transferrin complex was then investigated. After formation of the gastrin/apo-transferrin complex, EDTA was injected into the BIAcore to chelate any available iron. As soon as the EDTA was injected the association between gastrins and apo-transferrin was disrupted, indicating that Fe^{3+} ions were essential for the stability of the gastrin/apo-transferrin complex (Figure 2B).

Characterisation of Ggly domains involved in the interaction with apo-transferrin

We have previously demonstrated that Glu7 acts as a ligand for the first Fe^{3+} , and that Glu8 and Glu9 act as ligands for the second Fe^{3+} in the gastrin-ferric ion complex for both Ggly [12] and Gamide [13]. To characterize the involvement of the glutamates in the interaction of the peptide with apo-transferrin, Ggly mutants in which alanine was substituted for glutamate at positions 7 and 8–10 (E7A and E8–10A, respectively) were used (Table 1). As the residual crosslinking of apo-transferrin to ^{125}I -labelled Ggly in the presence of $100 \mu\text{M}$ unlabelled Ggly was less than 35% of the value in its absence, Ggly mutants were also tested at this concentration. Mutant E7A significantly competed with radiolabelled Ggly for the binding to apo-transferrin (66.5% relative density; $p < 0.001$), although the extent of competition was significantly less than with the parental Ggly peptide (Figure 3A). The

triple mutant, E8–10A, did not compete with Ggly for apo-transferrin binding. Thus, the lack of interaction between apo-transferrin and the E8–10A peptide suggests that either some or all of Glu8–10, are involved in the interaction with apo-transferrin. Alternatively, these results could indicate that the ferric ion bound to Glu8 and Glu9 itself binds to transferrin.

To determine whether the N- or C-terminus of Ggly is also required for the interaction between Ggly and apo-transferrin, short N- and C-terminal fragments of Ggly with or without the polyglutamate region (Table 1) were included as unlabelled competitors in the crosslinking experiments (Figure 3B). Although the peptide Ggly_{1–11} did not interact with apo-transferrin, the fragment Ggly_{5–18}, which contains both the glutamate region and the C-terminal portion, interacted with apo-transferrin with similar potency (30.5% relative density, $p < 0.05$) to the parental Ggly peptide (36.6% relative density, $p < 0.05$). However, the peptide Ggly_{12–18} with the C-terminal portion alone (i.e. lacking the pentaglutamate sequence) did not interact with apo-transferrin. Thus, neither the pentaglutamate sequence nor the C-terminal portion is alone sufficient for interaction with apo-transferrin to occur.

Mutation of the N- or C-terminal iron-binding sites of transferrin does not prevent interaction with Ggly

N- and C-lobe transferrin mutants were used to investigate the effect of loss of either iron-binding site on the affinity of transferrin for Ggly (Figure 4). The transferrin mutants contained mutations that completely disrupted iron binding to either the N-lobe (Mono C, Y95F/Y188F) or the C-lobe (Mono N, Y426F/Y517F), and hence each bound only one ferric ion [19]. The affinity of full length recombinant apo-transferrin for Ggly ($31 \pm 1 \mu\text{M}$) (Figure 4A) was nearly identical to the affinity of commercially available apo-transferrin ($39 \pm 1 \mu\text{M}$) (Figure 1C). Although the two transferrin mutants (Mono N and Mono C) each bound Ggly, and the intensity of the radioactive cross-linked band was not significantly different in either case from that observed for apo-transferrin, the affinity in each case was lower than the affinity of wild type apo-transferrin for Ggly (Figure 4B, C). The IC_{50} values for the interaction between Ggly and the Mono N and Mono C transferrins were $96 \pm 1 \mu\text{M}$ and $64 \pm 1 \mu\text{M}$, respectively.

Discussion

The *in vitro* formation of a complex between Gamide and apo-transferrin was first demonstrated over 20 years ago [14,15]. Although evidence was obtained for a complex between two molecules of Gamide and apo-transferrin, no association was observed between Gamide and iron-loaded transferrin (holo-transferrin). Our observation that the iron saturation of serum transferrin was correlated with circulating Gamide concentrations in both mice and humans strongly suggested that the interaction between Gamide and transferrin is physiologically relevant. Thus serum transferrin saturation was reduced in agastrinemic gastrin-deficient mice at 4 weeks, and was increased in hypergastrinemic CCK2 receptor-deficient mice at 4 weeks. Similarly, in patients with multiple endocrine neoplasia type 1, approximately 40% of whom develop hypergastrinemia, there was a significant correlation between serum transferrin saturation and serum Gamide concentrations [17]. On the basis of this data we suggested a mechanism, based on the well-known fact that efficient loading of apo-transferrin requires an anion (such as bicarbonate) or an anionic chelator (such as nitrilotriacetate), to explain the correlation between circulating Gamide concentrations and serum transferrin saturation. The model proposed that, following export of ferrous ions from the enterocyte by ferroportin and their oxidation to ferric ions by hephaestin, circulating Gamide or Ggly might act as chaperones for the uptake of ferric ions by apo-transferrin. The failure to detect significant binding of Gamide to diferric-transferrin [14,15] suggested that Gamide dissociates after iron transfer has

occurred, and hence plays a catalytic role consistent with the difference in the circulating concentrations of Gamide and transferrin. In the present study, we explored further the interaction between Gamide and transferrin, and characterised the interaction between Ggly and transferrin for the first time. Using two different *in vitro* techniques, namely surface plasmon resonance and covalent crosslinking, we observed that Ggly, like Gamide, only interacts with apo-transferrin (Figure 1). Based on the facts that the signals observed on interaction of Gamide and Ggly with apo-transferrin in the surface plasmon resonance study were of similar magnitude, and that Gamide and Ggly differ by a single amino acid, it is very likely that two molecules of Ggly will also bind to one molecule of apo-transferrin.

Ggly has previously been reported to bind two ferric ions, the first via Glu7 and the second via Glu8 and Glu9 [12]. In order to determine whether both of these iron-binding sites are involved in the interaction with transferrin, we used Ggly mutants in which the glutamates had been mutated to alanines (Table 1, Figure 3). Analysis of the Ggly mutants revealed that the Ggly E7A peptide still bound to apo-transferrin. Therefore neither Glu7 nor the first ferric ion is directly involved in the interaction with apo-transferrin. Additionally, the first ferric ion is unlikely to be transferred to apo-transferrin. The second ferric ion binding site is formed by Glu8 and Glu9 [12]. The observation that the Ggly E8–10A peptide no longer bound to apo-transferrin in the crosslinking assays suggests either that binding to transferrin occurs through one or more of Glu8–10, or that the binding of the second ferric ion to Glu8 and Glu9 is crucial in the recognition of Ggly. Clearly in the latter case the second ferric ion is likely to be involved in loading apo-transferrin.

The role of the N- and C-termini of Ggly in the interaction with transferrin was investigated by cross-linking experiments (Figure 3), using the Ggly fragments listed in Table 1. The fact that Ggly1–11 did not significantly inhibit the interaction of [¹²⁵I]-Ggly with transferrin suggested that the N-terminal domain of Ggly is not involved in the association with transferrin. However the observations that Ggly5–18 was as effective as Ggly as a competitor, and that Ggly12–18 was ineffective, indicated that both the C-terminus of Ggly as well as the pentaglutamate sequence are critical to the interaction with apo-transferrin. Thus, one or more of the seven C-terminal amino acids of Ggly is necessary for the formation of the complex.

Since it is well established that each lobe of transferrin binds one ferric ion, the cross-linking analysis was extended to transferrin mutants in which the iron-binding tyrosines in either the N- or C-lobe had been replaced by phenylalanines. This experiment allowed determination of whether or not the iron-binding residues in either lobe were required for the interaction with Ggly. The affinity of Ggly for each of the two authentic monoferric transferrins was similar and only slightly weaker than the affinity for recombinant wild-type apo-transferrin (which is capable of binding iron in both lobes) (Figure 4). The simplest explanation for this result is that there is no direct involvement of the iron-binding residues in either lobe in the interaction with Ggly. However since each molecule of apo-transferrin binds two molecules of gastrin (presumably with one molecule of gastrin bound to each lobe), the possibility remained that mutation of the iron-binding residues did affect gastrin binding, and that the observed binding was to the unmutated lobe. The observation that the extent of cross-linking was the same to MonoN and MonoC transferrin as to wild-type apo-transferrin (Figure 4A) strongly suggests that both mutant transferrins still bound two molecules of gastrin, and hence that the first explanation was correct. Further studies of the binding of gastrin to a transferrin with the iron-binding residues in *both* lobes mutated, or to the individually expressed N- or C-lobes with and without the iron-binding residues mutated, would conclusively disprove the second alternative explanation.

Our data also provide some information on the mechanisms of iron transfer from gastrin to transferrin. The fact that no interaction was observed between apo-transferrin and either Gamide or Ggly in the presence of EDTA (Figure 2A) shows that gastrin peptides must bind ferric ions in order to interact with apo-transferrin. Furthermore the pre-formed complex between apo-transferrin and either Gamide or Ggly dissociates immediately upon addition of EDTA (Figure 2B). One attractive possibility is that this dissociation is triggered by the transfer of a ferric ion from one of the relatively low affinity binding sites on gastrin to one of the relatively high affinity binding sites on transferrin, since our data clearly indicate that holo-transferrin does not bind gastrins (Figure 1C). As discussed above the study with Ggly mutants supports the second iron binding site on gastrin as the more likely iron donor.

In conclusion, the current work provides a much better understanding of the complex formed between gastrin peptides and apo-transferrin. Taken together the data are consistent with our hypothesis [17] that gastrin peptides catalyse the loading of iron onto transferrin, and hence gastrins should be considered as part of the rapidly expanding network of molecules that play a role in iron homeostasis. Moreover the demonstration of an interaction between Ggly and transferrin suggests that the stimulatory effects of Ggly and iron on the development of colorectal carcinoma may be linked, perhaps through a Ggly-dependent increase in transferrin saturation with a concomitant increase in the availability of iron to the tumour cells.

Experimental procedures

Peptides

Ggly₂₋₁₇ was obtained from Mimotopes, while all other gastrin peptides and fragments (Table 1) were from Auspep Pty. Ltd. (Melbourne, Australia). All Ggly peptides were used at 100 μ M and were made up in dimethylsulphoxide. Apo-transferrin was from Sigma-Aldrich (St Louis, MO, USA). The transferrin mutants “Mono C transferrin” with the mutations Y95F/Y188F, “Mono N transferrin” with the mutations Y426F/Y517F, and full-length recombinant human transferrin, were prepared as described previously [19].

Iron removal from transferrins

Prior to crosslinking or surface plasmon resonance analysis iron was removed from the transferrin mutants using a previously reported procedure [20]. Briefly, solutions of Mono C and Mono N transferrin were placed in Centricon 10 microconcentrators (Millipore, North Ryde, Australia) together with 2 ml of buffer containing 0.5 M sodium acetate, pH 4.9, 1 mM EDTA and 1 mM nitrilotriacetic acid. Sample volumes were reduced to 100 μ l by centrifugation at 6500 rpm for 2 hours, during which period the characteristic salmon-pink colour of iron-loaded transferrin disappeared. The samples were subsequently washed once with 2 ml of 100 mM KCl, once with 2 ml of 100 mM sodium perchlorate, three times with 2 ml of 100 mM KCl, and five times with 2 ml of 100 mM NH_4HCO_3 .

Labeling of peptides with I^{125}

Ggly₂₋₁₇ (2 mg/ml) was iodinated using the iodogen method and the mono-iodinated peptide was separated from di-iodinated and unlabeled peptide by reverse phase high performance liquid chromatography as previously described [14].

Crosslinking

The radiolabeled Ggly₂₋₁₇ was reacted with the bivalent crosslinker disuccinimidyl suberate (0.6 mM) via the single N-terminal amino group in 50 mM Hepes buffer, pH 7.6 for 15 minutes at 4°C. Apo-transferrin (113 μ g/ml) was mixed with unlabelled Ggly and the crosslinked ^{125}I -labelled Ggly₂₋₁₇ was added. In order to find the regions of Ggly necessary

for transferrin interaction, Ggly mutants with alanines substituted for glutamates or short Ggly fragments were used in the cross-linking experiments instead of the unlabelled Ggly. The reaction was stopped by addition of reduced 2X SDS loading dye and the samples were boiled for 5 minutes at 100°C.

SDS polyacrylamide gel electrophoresis

The apo-transferrin-Ggly complex (2 µg of protein) was separated from unreacted Ggly by SDS polyacrylamide gel electrophoresis. Subsequently the gel was stained with Coomassie blue and de-stained overnight with a solution containing 7% acetic acid, 5% methanol and 2% glycerol. The extent of incorporation of radioactivity was determined by phosphorimager (FujiBAS 1800 II, Fujifilm, Melbourne, Australia) and densitometric analysis using Multigauge software (Fujifilm, Melbourne, Australia). A reduction in intensity of the radioactive signal indicated binding of the unlabelled peptide to apo-transferrin. Data are expressed as a percentage of the density observed with apo-transferrin and ¹²⁵I-labelled Ggly₂₋₁₇ only, after correction for variation in protein loading.

Surface Plasmon Resonance

The kinetics of transferrin binding to immobilized Gamide and Ggly were measured with a BIAcore 3000 biosensor instrument (BIAcore, Uppsala, Sweden). Binding of transferrin to immobilized peptides was measured in resonance units (RU) (1000 RU = 1 ng of protein bound/mm² of flow cell surface). The running buffer was Hanks' balanced salt buffer with no added iron salts, and the same buffer was used for diluting samples before injection. Synthetic biotinylated Gamide (Biotin-QGPWLEEEEEAYGWMDFAmide) and Ggly (Biotin-QGPWLEEEEEAYGWMDFG) peptides were immobilized onto streptavidin-coated carboxymethylated dextran chips. To measure binding interactions, the transferrins, at a concentration of 10 µg/ml, were passed over the immobilized peptides at a flow rate of 20 µl/min at 25°C. After each binding assay, flow cells were regenerated by short pulses of 5 µl 0.01% SDS.

Statistical Analysis

Statistics were analysed by Student's t test using the program SigmaStat (Jandel Scientific, San Rafael, CA, USA). Values of the concentration required for 50% inhibition (IC₅₀) were determined by fitting crosslinking data to the equation for one-site competition

$$f = \min + (\max - \min) / (1 + 10^{(x - \log IC_{50})})$$

and dose-inhibition curves were plotted using Sigmaplot (Jandel Scientific). Data are presented as mean ± SEM from 3 separate experiments.

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Abbreviations

CCK2 receptor	cholecystokinin2 receptor
Gamide	amidated gastrin ₁₇

Ggly	glycine-extended gastrin ₁₇
IC₅₀	concentration required for 50% inhibition
TfR-1	transferrin receptor-1

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Figure 1. Both Gamide and Ggly interact with apo-transferrin but not holo-transferrin

A. Following injection of apo-transferrin (10 μ g/ml) into the BIAcore channel an interaction was observed with both Gamide (red line) and Ggly (blue line) by surface plasmon resonance. After removal of apo-transferrin from the running buffer (thick arrow) the interaction between Ggly/Gamide and apo-transferrin gradually declined. **B.** Upon injection of holo-transferrin (10 μ g/ml) into the BIAcore channel no interaction was observed with Gamide (red line) or Ggly (blue line). **C.** The interaction between Ggly and apo-transferrin was also detected using covalent cross-linking. [125 I]-Ggly₂₋₁₇ was pre-reacted with the bivalent crosslinker disuccinimidyl suberate before mixing with apo-transferrin in 50mM Hepes buffer, pH 7.6 in the absence or presence of increasing concentrations of unlabelled Ggly. The apo-transferrin Ggly complex was separated from the unreacted Ggly by SDS polyacrylamide gel electrophoresis, and the extent of incorporation of radioactivity was determined by phosphorimager and densitometric analysis. Unlabelled Ggly inhibited the interaction in a dose-dependent manner. Lack of interaction between Ggly and holo-transferrin was also confirmed. **D.** The IC₅₀ for binding of Ggly to apo-transferrin was found

to be $39 \pm 1 \mu\text{M}$ by curve-fitting, with an intercept of 92.3%. Data points are means $\pm$ SEM, where $n=3$.

Figure 2. Fe^{3+} ions are important for both the formation and stability of the gastrin/apo-transferrin complex

A. Injection of the iron chelator EDTA (3mM) into the BIAcore channel at the same time as apo-transferrin prevented the association between the apo-transferrin and either Ggly (blue line) or Gamide (red line). **B.** Following injection of apo-transferrin into the BIAcore channel, a complex was formed between apo-transferrin and Ggly (blue line) or Gamide (red line). After addition of the iron chelator EDTA to the flow buffer, the gastrin/apo-transferrin complexes dissociated.

Figure 3. Both glutamates 8–10 and the C-terminal portion of the Ggly peptide are important for the interaction between Ggly and apo-transferrin

A. Binding of glutamate→alanine mutants of Ggly to apo-transferrin was assessed by competition with radiolabelled Ggly in a covalent cross-linking assay. A representative analysis of the interaction between apo-transferrin and Ggly glutamate mutants (100 μ M) by SDS polyacrylamide gel electrophoresis is shown, followed by densitometric quantification of the data. Mutant E7A (coarse hatched bar) significantly competed with radiolabelled Ggly for binding to apo-transferrin (66.5% of control (grey bar) with no unlabelled peptide; ***, $p < 0.001$), although with reduced potency compared to the parental Ggly peptide (fine hatched bar). The triple mutant E8–10A (cross hatched bar) did not compete with Ggly for apo-transferrin binding. **B.** Short N- and C- terminal fragments of Ggly with or without the polyglutamate region were used to determine whether the Nor C-terminus of Ggly is

required for the interaction between Ggly and apo-transferrin. A typical analysis of the interaction between apo-transferrin and Ggly fragments (100 μ M) by SDS polyacrylamide gel electrophoresis is shown followed by densitometric quantification of the data. Ggly₁₋₁₁ (medium hatched bar) did not interact with apo-transferrin, while the fragment Ggly₅₋₁₈ (coarse hatched bar), which contains both the glutamate region and the C-terminal portion, interacted with apo-transferrin with greater potency (30% of control (grey bar) with no unlabelled peptide, *, $p < 0.05$) than the parental Ggly peptide (fine hatched bar). Peptide Ggly₁₂₋₁₈ (cross hatched bar) which lacks the polyglutamate region did not interact with apo-transferrin. Data are means $\pm$ SEM where $n=3$.

Figure 4. Both the N- and C- terminal lobes of transferrin can interact with Ggly

A. Apo-transferrin and apo-transferrin mutants were crosslinked to radiolabelled Ggly₂₋₁₇ in the presence or absence of 100 μ M unlabelled Ggly, and the samples were separated by SDS polyacrylamide gel electrophoresis to remove the unbound radiolabel. The extent of cross-linking was not significantly different between recombinant wild-type apo-transferrin (WT), apo-transferrin which only binds iron in the N lobe (Mono N), and apo-transferrin which only binds iron in the C lobe (Mono C). Data are the means $\pm$ SEM from three independent experiments. **B.** The interaction between Ggly and recombinant wild-type apo-transferrin. The amount of radioactivity associated with transferrin in the presence of increasing concentrations of unlabelled Ggly was determined by densitometric scanning and was

expressed as a percentage relative to sample with no unlabelled Ggly. The line of best fit was drawn with an IC_{50} of $31 \pm 1 \mu M$ and an intercept of 101%. **C.** The interaction between Ggly and apo-transferrin which only binds iron in the N lobe (Mono N). The line of best fit was drawn with an IC_{50} of $96 \pm 1 \mu M$ and an intercept of 115%. **D.** The interaction between Ggly and apo-transferrin which only binds iron in the C lobe (Mono C). The line of best fit was drawn with an IC_{50} of $64 \pm 1 \mu M$ and an intercept of 134%.

Table 1

Gastrin peptides used for the crosslinking studies

Peptide	Amino Acid Sequence			
	1	6	10	18
Gamide	ZGPW LEEEEE AYGWMD _{NH2}			
Ggly	ZGPW LEEEEE AYGWMD _{OH}			
Ggly1–11	ZGPW LEEEEE A _{OH}			
Ggly12–18	YGWMD _{OH}			
Ggly5–18	LEEEEE AYGWMD _{OH}			
GglyE7A	ZGPW LE <u>A</u> EEEE AYGWMD _{OH}			
GglyE8–10A	ZGPW LEE <u>AAA</u> AYGWMD _{OH}			

The pentaglutamate sequence of gastrins is shown in bold. Amino acids that differ from the naturally occurring sequence are underlined.