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Contribution of site-specific PEGylation to the dipeptidyl peptidase IV stability of glucose-dependent insulinotropic polypeptide

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Abstract—The effects of PEGylation of glucose-dependent insulinotropic polypeptide (GIP) on potency and dipeptidyl peptidase IV (DPPIV) stability are reported. N-terminal modification of GIP(1–30) with 40 kDa polyethylene glycol (PEG) abrogates functional activity. In contrast, C-terminal PEGylation of GIP(1–30) maintains full agonism and reasonable potency at the GIP receptor and confers a high level of DPPIV resistance. Moreover, the dual modification of N-terminal palmitoylation and C-terminal PEGylation results in a full agonist of comparable potency to native GIP that is stable to DPPIV cleavage. The results provide the basis for the development of long acting, PEGylated GIP, GIP variants, or GIP-based hybrid peptide therapeutics.

The incretin glucose-dependent insulinotropic polypeptide (GIP) is a 42-residue peptide involved in the regulation of fat and glucose metabolism.[1–3] The use of GIP or GIP variants holds promise as a peptide therapeutic for conditions such as diabetes and obesity.[2,4] For example, modified GIP peptides improve glucose tolerance in rodents,[4] and intravenous administration and continuous infusion of GIP causes an acute stimulation of insulin secretion in type II diabetic patients.[5,6] The use of unmodified GIP as a therapeutic, however, is limited by the short in vivo lifetime of the native peptide.[2]

Two mechanisms largely account for the elimination of GIP in vivo. The serine protease dipeptidyl peptidase IV (DPPIV) provides one major mechanism for GIP inactivation via cleavage of the two N-terminal amino acids,[7,8] which abrogates GIP receptor activation and nullifies the insulinotropic property of native GIP.[9] DPPIV proteolysis of GIP in humans occurs very rapidly with a half-life of 5–7 min.[10,11] A second mechanism of GIP elimination, common to all low molecular mass peptides, is renal clearance.[10,11] The DPPIV-digested product of GIP is cleared in humans with a half-life of around 20 min.[10,11] These metabolic fates of GIP need to be circumvented to realize the therapeutic potential of the peptide.

One approach to improve the in vivo half-life of GIP is the use of N-terminal GIP variants of improved DPPIV stability.[4] Indeed, N-terminal variants of GIP that are resistant to DPPIV cleavage improve glucose disposal in normal and obese rodents.[4] The use of peptide variants, however, carries the risk of an immune response and such variants are still subject to renal clearance. A second approach to extending the duration of action of peptide therapeutics is conjugation with fatty acids, which reduces renal clearance by inducing association with plasma proteins. For example, N-terminal palmitoylation of GIP improves DPPIV resistance in vitro,[12] and N-terminal and internally palmitoylated variants of GIP offer improved glucose disposal over native GIP in mice.[12,13]

Conjugation of GIP with polyethylene glycol (PEG) offers a third approach to increase the in vivo lifetime of GIP. The pharmacokinetic properties of proteins are improved by PEGylation, which decreases renal clearance and increases stability to proteolysis.[14,15] PEGylation also offers the potential for reduced immunogenicity,[14,15] which is advantageous in the use of peptide

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variants or hybrids. Finally, PEG conjugation can limit blood–brain barrier penetration[16] and hence reduce potential side effects.

GIP is a 42-residue peptide that exhibits both insulinotropic and somatostatinotropic properties.[1–3] The insulinotropic activity is localized to residues 1–30,[17] which is the region of GIP used here. The N-terminus was derivatized with the fatty acid palmitate or with 40 kDa PEG, and the C-terminus was derivatized with 40 kDa PEG (Table 1).[18] Site-specific PEGylation was performed at a non-native Cys residue introduced at either N- or C-terminus. In addition, the dual modification of N-terminal palmitoylation and C-terminal PEGylation was performed (Table 1).

The N- and C-terminal modifications have different effects on the activation of the GIP receptor (Fig. 1).[19] GIP(1–30) is a full agonist with an apparent EC_{50} value of 0.4 ± 0.1 nM (Table 1). N-terminal PEGylation is functionally very detrimental and increases the apparent EC_{50} value at least 250-fold to over 100 nM (Table 1). This is not simply due to modifying the N-terminus, since N-terminal palmitoylation has minimal effect on receptor stimulation: palm-GIP has an apparent EC_{50} of 0.8 ± 0.1 nM (Table 1). Thus, N-terminal PEGylation of GIP(1–30) is not tolerated functionally.

In contrast to N-terminal PEGylation, C-terminal PEGylation only modestly affects functional activity: GIP–PEG is a full agonist with an EC_{50} value of 2.6 ± 0.1 nM (Table 1). Moreover, the dual modification of N-terminal palmitoylation and C-terminal PEGylation has an effect comparable to N-terminal palmitoylation alone on function (EC_{50} = 0.7 ± 0.1 nM) (Table 1). Thus, C-terminal PEGylation of GIP(1–30) is functionally tolerated and certain N-terminal modifications (e.g., palmitoylation) can be combined with C-terminal PEGylation and maintain functional activity.

Studies evaluating GIP(1–30) competitive binding give similar results (Fig. 2).[20] A small increase in the apparent IC_{50} is observed upon C-terminal PEGylation (Table 1). In contrast, N-terminal PEGylation leads to a substantial loss of binding potency (IC_{50} > 1 μM; Table 1), implying that N-terminal PEGylation affects GIP receptor stimulation through direct disruption of the binding interaction.

The stability to DPPIV proteolysis of the functionally active PEGylated analogs GIP(1–30)–PEG and palm-

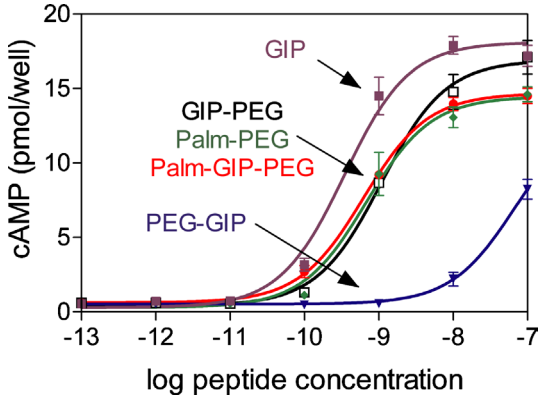

Figure 1. cAMP production in a CHO cell line expressing the human GIP receptor stimulated by GIP(1–30) (■), GIP(1–30)–PEG (□), palm-GIP (♦), palm-GIP–PEG (●), and PEG–GIP(1–30) (▼). PEGylation is functionally tolerated at the C-terminus, but not at the N-terminus. In addition, the combination of C-terminal PEGylation with the N-terminal modification of palmitoylation is functionally tolerated.

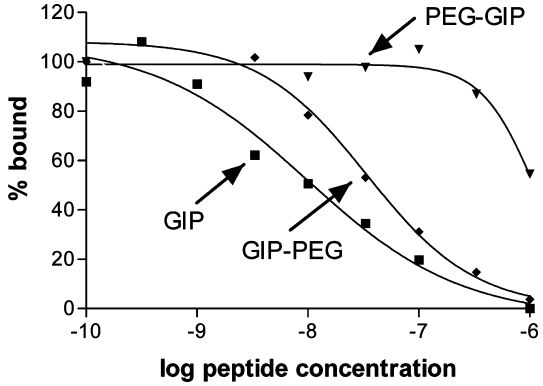

Figure 2. Competition-binding displacement from the human GIP receptor of ^{125}I-GIP by GIP(1–30), PEG–GIP(1–30), and GIP(1–30)–PEG. Binding activity is preserved upon PEGylation at the C-terminus but not at the N-terminus.

GIP(1–30)–PEG was evaluated with N-terminal Edman sequencing.[21] If proteolysis occurs, the appearance of DPPIV products with a free N-terminal α-amino group will be detected with Edman sequencing.

Upon incubation of GIP(1–30)–PEG with DPPIV, N-terminal sequences corresponding to the appearance of proteolytic products beginning at Glu 3 and Thr 5 are observed with Edman sequencing (Fig. 3). The stability of GIP(1–30)–PEG (50% intact after 7 days) is,

Table 1. Peptide sequences, modifications, and in vitro activities

Peptide	Sequence[a]	EC_{50} (nM)[b]	IC_{50} (nM)[b]
GIP(1–30)	YAEGTFISDYSIAMDKIHQQDFVNWLLAQK	0.4 ± 0.1	8 ± 1
Palm-GIP(1–30)	Palm-YAEGTFISDYSIAMDKIHQQDFVNWLLAQK-C	0.8 ± 0.1	28 ± 1
PEG–GIP(1–30)	C(PEG)-YAEGTFISDYSIAMDKIHQQDFVNWLLAQK	>100	>1000
GIP(1–30)–PEG	YAEGTFISDYSIAMDKIHQQDFVNWLLAQK-C(PEG)	2.6 ± 0.1	34 ± 1
Palm-GIP(1–30)–PEG	Palm-YAEGTFISDYSIAMDKIHQQDFVNWLLAQK-C(PEG)	0.7 ± 0.1	19 ± 1

[a] The peptide core corresponds to residues 1–30 of human GIP. Palm denotes that the N-terminus is palmitoylated. C(PEG) denotes a non-native Cys residue at either the N- or C-terminus that is conjugated with 40 kDa mPEG2-MAL. All peptides are amidated at the C-terminus.

[b] Values are means of three experiments ± the standard error of the mean.

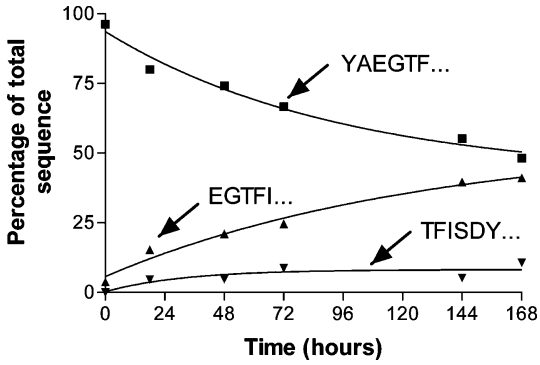

Figure 3. DPPIV proteolysis of GIP(1–30)–PEG. The appearance of peptides corresponding to proteolytic products of GIP–PEG beginning at residue Glu 3 (EGTFI...) and Thr 5 (TFISDY...), and the loss of the full-length peptide (YAEGTFI...) were monitored with Edman sequencing. The same experiment with palm-GIP–PEG showed no appearance of proteolysis products, indicating that N-terminal palmitoylation stabilizes against DPPIV cleavage.

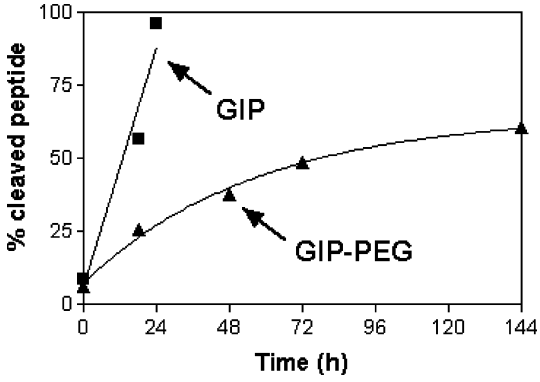

Figure 4. Relative rates of DPPIV proteolysis of GIP(1–30) and GIP(1–30)–PEG shown as the appearance of proteolytic products with time.

however, significantly higher than GIP(1–30) (complete degradation after 24 h under the conditions used here; Fig. 4). C-terminal PEGylation, therefore, confers significant but not complete resistance to DPPIV proteolysis.

The stability of the C-terminally PEGylated analog to DPPIV proteolysis is made essentially complete by N-terminal palmitoylation. No degradation products of palm-GIP(1–30)–PEG are observed by Edman sequencing over a period of 7 days.

PEGylation often increases protease resistance,[15] and this is seen here for GIP(1–30) upon C-terminal PEGylation. However, the protection afforded by C-terminal PEGylation is not sufficient to completely preclude DPPIV proteolysis. Modification of the N-terminus of GIP is also required. The N-terminal amino group of DPPIV-bound peptides forms binding interactions with DPPIV, as seen in the crystal structures of DPPIV complexes,[22,23] and hence modifications such as acetylation or palmitoylation that modify the N-terminal amino group would be expected to protect DPPIV substrates to proteolysis. Indeed, such modifications do confer DPPIV resistance.[12,24] In addition, N-terminal amino-acid variations also impart DPPIV resistance,[4] and such changes could be combined with C-terminal PEGylation.

PEGylation can decrease the in vitro potency of a protein, which is compensated for in vivo by the extended lifetime and associated prolonged in vivo efficacy of the PEGylated protein.[14] However, C-terminal PEGylation of GIP(1–30) has a strikingly less effect on in vitro function than is seen with other PEGylated proteins.[14] This finding suggests that the C-terminus of GIP(1–30) is quite tolerant of changes in structure–function terms. More generally, the results illustrate the importance of site-specific PEGylation in maintaining activity, as opposed to the use of non-specific PEGylation that can impair the in vitro activity of the derivatized protein.[25–27]

We conclude that GIP(1–30) can be modified at the N-terminus (to impart DPPIV resistance) and PEGylated at the C-terminus (to improve lifetime, impart DPPIV resistance and reduce immunogenicity) whilst maintaining potency and full agonism of the GIP receptor. The results provide the basis for the development of long acting, PEGylated GIP, GIP variants GIP-based hybrid peptide therapeutics of improved pharmacokinetic properties, improved in vivo efficacy, and extended duration of action in vivo.

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18. Peptides were synthesized with solid-phase Fmoc/HBTU chemistry with an ABI 433A synthesizer. Palmitoylated peptides were prepared by coupling HBTU-activated palmitic acid to the N-terminal amine prior to cleavage. Final purification was by C_{18} HPLC using a linear H_2O/CH_3CN gradient containing 0.1% (v/v) TFA. Purity (>99%) was confirmed with analytical C_{18} HPLC, and identity was confirmed with electrospray mass spectrometry. Peptides were PEGylated with 40 kDa branched PEG derivatized with maleimide (Nektar Therapeutics mPEG2-MAL) and purified with reversed phase phenyl HPLC (TosaHaas Phenyl-5PW) using a linear H_2O/CH_3CN gradient containing 0.1% (v/v) TFE. The absence of free peptide was confirmed with analytical C_{18} HPLC and PEGylation confirmed with SDS–PAGE.
19. Receptor activation experiments were performed in triplicate with a stable CHO cell line expressing the GIP receptor. Samples contained 0.001–100 nM peptide in 10 mM HEPES, pH 7.4, 150 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 0.1 mM 3-isobutyl-1-methylxanthine, and 1% (w/v) BSA. Following a 15 min incubation under CO_2, cAMP content was determined with a scintillation proximity assay based on competition between cAMP and ^{125}I-labelled cAMP (Amersham Biosciences RPA559). Data were fit to a single-site-binding model with Prism 3.03 (GraphPad Software).
20. Competitive-receptor-binding reactions contained 15–25 μg membrane isolated from a stable CHO cell line expressing the GIP receptor and peptide in 20 mM Tris–HCl, pH 7.4, 1 mM EDTA, 0.5% (w/v) BSA, and 0.1% (w/v) bacitracin. ^{125}I-labeled GIP (Amersham IM303, 2000 Ci/mmol) was added to a final concentration of 50 pM in the absence and presence of 0.3–1000 nM GIP(1–30), PEG–GIP(1–30) or GIP(1–30)–PEG. Plates were incubated for 2 h at room temperature. The bound from free ^{125}I-labeled GIP was separated with vacuum. Plates were washed with ice-cold PBS containing 0.1% (w/v) BSA, dried at room temperature, 30 μl Ultima Gold (Perkin-Elmer 6013329) were added and ^{125}I-labeled GIP levels determined with a Wallac Microbeta counter. Data were fit to a single-site-binding model with Prism 3.03 (GraphPad Software).
21. GIP, PEG–GIP, or palm-GIP–PEG at 0.4 mM were digested with 2 mU/100 μl of human DPPIV in 20 mM Tris, pH 8.0, at room temperature, quenched with 10% TFA and stored at −80 °C. Edman degradation (10–15 cycles) of samples (1 nmol peptide in 10 μl of 10 mM HEPES, pH 7.4, 5% TFA) was performed with an ABI Procise 494HT protein sequencer using the pulsed-liquid method. Palm-GIP slowly precipitated under these conditions, precluding a quantitative study.
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