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Fc fusion to Glucagon-like peptide-1 inhibits degradation by human DPP-IV, increasing its half-life in serum and inducing a potent activity for human GLP-1 receptor activation

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The short *in vivo* half-life of GLP-1 prevents it from being used clinically. This short half-life occurs because GLP-1 is rapidly degraded by dipeptidyl peptidases such as DPP-IV. To overcome this obstacle, a GLP-1/Fc was constructed and evaluated to determine if it was degraded by DPP-IV and in serum. When the degradation of GLP-1/Fc by human DPP-IV and rabbit serum was compared with that of GLP-1 it was found to be reduced by approximately 5- and 4-fold, respectively. Furthermore, GLP-1/Fc showed a potent activity for human GLP-1 receptor activation (EC_{50} approximately 6 nM). Taken together, these results indicate that GLP-1/Fc may have an extended half-life *in vivo* that occurs as a result of inhibition of degradation by human DPP-IV. Due to the extended half life, GLP-1/Fc may be useful for clinical treatments. [BMB reports 2009; 42(4): 212-216]

INTRODUCTION

GLP-1 is involved in insulin secretion and anti-apoptosis in pancreatic beta cells via PKB/Akt activation. In addition, GLP-1 increases the proliferation of beta-cell mass, especially in diabetic patients (1-7). GLP-1 binds to and activates the GLP-1 receptor, which is a type of GPCR that is expressed at particularly high levels in pancreatic beta and delta cells. GLP-1 receptor then couples to a stimulatory G-protein (Gs), which subsequently activates adenylate cyclase, thereby increasing intracellular cyclic AMP levels and activating CREB-responsive genes for insulin signaling (8, 9). These effects eventually regulate the level of glucose in blood. As a result, GLP-1 is now recognized as a promising agent for the treatment of diabetes (10-12).

Dipeptidyl peptidase IV (DPP-IV) is a member of the DPPs that is commonly found in the membrane-bound form in a number of mammalian tissues, including human and rabbit tissues. The soluble form of DPP-IV, which is present in serum, is derived from the membrane form by proteolytic processing. DPP-IV is a serine protease that cleaves two amino acids from the N-terminus of GLP-1 to inactivate GLP-1 activity, which results in GLP-1 having a half-life of less than 5 min *in vivo* (13). This short half-life prevents the clinical use of GLP-1. However, it was recently reported that gene therapy using a GLP-1/mouse IgG1-Fc fusion construct lowered the glucose levels in db/db mice, which indicates that the fusion protein is functional *in vivo* (14).

Despite these findings, the biochemical properties and *in vivo* stability of GLP-1/Fc-fusion constructs have not yet been compared with those of GLP-1. Therefore, in this study, we constructed a GLP-1/Fc containing a human IgG1 secretion peptide at the N-terminus for secretion. We then demonstrated that GLP-1/Fc is protected from rapid degradation by human DPP-IV *in vitro* and has a prolonged half-life in serum. In addition, the results of a luciferase reporter assay demonstrated that GLP-1/Fc had a potent activity for human GLP-1 receptor activation in an established CHO cell line.

RESULTS

GLP-1/Fc has a potent biological activity for human GLP-1 receptor activation *in vitro*

GLP-1/Fc was prepared by overlapping PCR strategy (Fig. 1). Next, GLP-1/Fc and 8x His-Human DPP-IV were expressed and purified from CHO and SF21 insect cells, respectively, after which their purity was assessed by SDS-PAGE (Fig. 2A, B). The activity of the purified human DPP-IV was then confirmed by using Gly-Pro-AFC as a fluoro-substrate (Fig. 2C). The luciferase activity of an established human GLP-1 receptor/CRE-luciferase-co-expressed CHO cell line was then evaluated. The results of the assay revealed that the maximum expression of luciferase occurred within 2-3 h when 1 nM of GLP-1 was used (data not shown), which demonstrates that GLP-1/Fc is functional for human GLP-1 receptor activation. In addition, when the cell line was treated with GLP-1/Fc for 2 h, potent luciferase expression was observed (EC_{50}; $\cong$ 6 nM) (Fig. 3).

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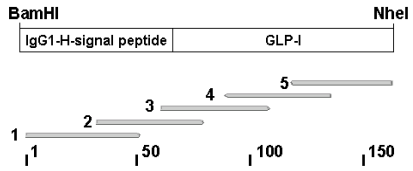
A.



B.

1	BamH1-Sig-F1	TC*GGATCC*ATGGGATGGAGCTATATCATCCTCTTTTTGGTAGCAACAGCTA
2	Sig-F2	CTTTTTGGTAGCAACAGCTACAGATGTCCACTCCCACGCCGAGGGCAC
3	Sig-F3	ACTCCCACGCCGAGGGCACCTTCACCAGCGACGTGAGCAGCTACCTGG
4	GLP-1-R1	GCGATGAACTCCTTGGCGGCCTGGCCCTCCAGGTAGCTGCTCACGTCG
5	NheI-GLP-1-R2	TC*GCTAGC*CCGGCCCTTCACCAGCCAGGCGATGAACTCCTTGGCGG

Fig. 1. Construction of the GLP-1/Fc containing a signal peptide (19 amino acids) comprised of a human IgG1-heavy chain at the N-terminus for secretion. (A) Cloning of cDNA encoding a signal peptide comprised of a human IgG1-heavy chain/GLP-1 fusion protein was generated using an overlap PCR strategy as described in the Materials and Methods section. (B) Primers used to construct the fusion protein by overlap PCR.

These findings demonstrate that, although GLP-1/Fc is not as potent as GLP-1, it still strongly activates human GLP-1 receptor and may be useful as a physiological ligand.

GLP-1/Fc is protected from degradation by human DPP-IV *in vitro* and has an increased half-life in rabbit serum

Next, we evaluated Fc-fusion to GLP-1 to determine if it inhibits the degradation of GLP-1 by human DPP-IV. To accomplish this, we used non-saturated concentrations of the ligands (GLP-1; 0.4 nM, GLP-1/Fc; 16 nM) used for luciferase expression (Fig. 3). The two ligands were incubated at 37°C for 2 h with an equal amount of human DPP-IV *in vitro* in a reaction mixture with a total volume of 40 µl. Next, 11 µl of each reactant was added to 96-well culture plates containing human GLP-1 receptor/CRE-luciferase-co-expressed CHO cells. After 2 h of incubation, the amount of GLP-1 or GLP-1/ Fc that was not degraded by human DPP-IV was measured using a luciferase reporter assay as described in the Materials and Methods section. The results revealed that approximately 5-fold more human DPP-IV was required to degrade GLP-1/Fc than GLP-1, which indicates that the degradation of GLP-1/Fc by DPP-IV is inhibited by approximately 5-fold when compared with that of GLP-1 *in vitro*. (Fig. 4A). To clarify that GLP-1/Fc had a prolonged half-life in serum, we performed an *ex vivo* study using New Zealand white rabbit serum, which is known to contain DPP activity including DPP-IV. Interestingly, as shown in Fig. 4B, the biological activity of GLP-1/Fc was sustained for approximately 4-times longer than that of GLP-1, which was rapidly degraded and inactivated in the serum. These findings indicate that GLP-1/Fc may have a prolonged half-life *in vivo*.

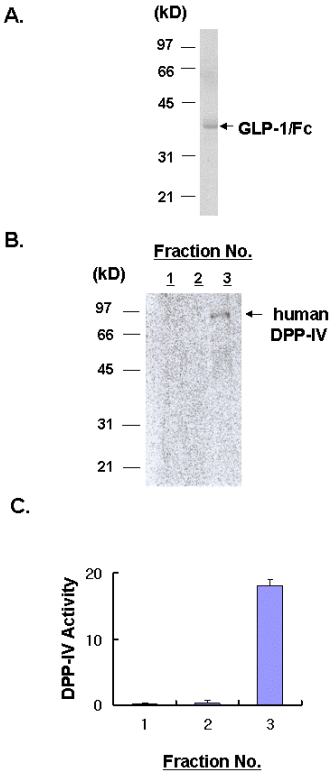


Fig. 2. Expression and purification of GLP-1/Fc and 8x His-human DPP-IV from the CHO cell line and insect SF21 cells, respectively. (A) GLP-1/Fc fusion protein was expressed by the established CHO cell lines as the secreted form in serum-free α-MEM and then purified as a single band using a protein-G sepharose column equilibrated with PBS. (B) Human DPP-IV containing 8x His-tags at the C-terminus was expressed by insect SF21 cells and then purified as a single band using His-Bind Resin (Qiagen). (C) The activity of the DPP-IV was confirmed using 2.5 mM Gly-Pro-AFC as a fluoro-substrate in 50 mM Hepes (pH 7.4), as described in the Materials and Methods section. The data are presented as means ± standard error (n = 3).

DISCUSSION

Here, we report that GLP-1/Fc has a potent activity for human GLP-1 receptor activation (the EC_{50}; about 6 nM *in vitro*). These findings are based on the results of a luciferase reporter assay of the human GLP-1 receptor/CRE-luciferase-co-expressed CHO cell line. These findings demonstrate that GLP-1/Fc is functional as a human GLP-1 receptor ligand. In addition, we demonstrated that GLP-1/Fc is protected from degradation by human DPP-IV *in vitro*, and that it has an increased half-life in serum.

It is well known that GLP-1 exerts anti-diabetic effects; however, its very short half-life, which is primarily caused by degradation by DPP-IV, inhibits its clinical application (15-17). The results of this study revealed that the degradation and inhibition effects of human DPP-IV on GLP-1/Fc were approximately five-fold lower than those on GLP-1 *in vitro*, which in-

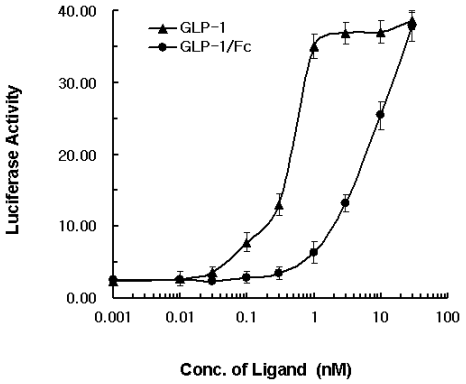


Fig. 3. Biological evaluation of GLP-1 and GLP-1/Fc for human GLP-1 receptor activation using a luciferase reporter assay system in a human GLP-1 receptor/CRE-Luciferase-co-expressed CHO cell line. Cells in a 96-well plate were treated with GLP-1 or GLP-1/Fc for 2 h and then assessed using a luciferase reporter assay, as described in the Materials and Methods section. The data are presented as means ± standard error (n = 3).

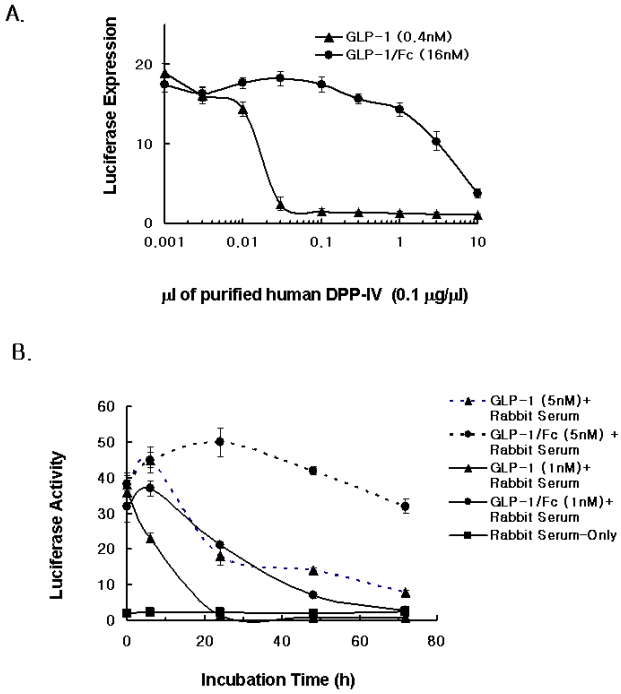


Fig. 4. Comparison of GLP-1 and GLP-1/Fc degradation by human DPP-IV *in vitro*, and in New Zealand white rabbit serum (ex vivo). (A) To compare the degradation of GLP-1 and GLP-1/Fc by human DPP-IV *in vitro*, purified human DPP-IV stock (0.1 μg/μl in 20 mM Hepes (pH 7.4), 50 mM NaCl) was serially diluted with PBS containing 1 mg/ml BSA and then added to GLP-1 (0.4 nM) and GLP-1/Fc (16 nM). Next, the samples were incubated overnight, after which the levels of non-degraded GLP-1 or GLP-1/ Fc were measured using a cell-based luciferase assay, as described in the Materials and Methods section. (B) Both GLP-1 and GLP-1/Fc were diluted to 10 and 50 nM in a total 40 μl of 50% rabbit serum for the indicated times. Next, 11 μl of each sample were added to 100 μl of culture media in 96-well plates and then incubated for 2 h to measure the luciferase expression by non-degraded GLP-1 or GLP-1/Fc in rabbit serum. The data are presented as means ± standard error (n = 3).

dicates that the fusion form may be more stable than GLP-1 *in vivo*. These findings were supported by an ex vivo study conducted using New Zealand white rabbit serum, which contains DPPs at sufficient levels to degrade GLP-1.

It was recently reported that GLP-1/mouse IgG1/Fc exerted an anti-diabetic effect in db/db mice (14), which demonstrates that GLP-1/Fc exerts biological activity *in vivo*. However, this is the first study to compare the biochemical properties of GLP-1/Fc, such as its degradation by human DPP-IV and in serum, to those of GLP-1.

In this study, we used a luciferase reporter assay system in an established CHO cell line to compare the biological potencies of GLP-1/Fc and GLP-1, as well as their degradation by human DPP-IV. The results revealed that, in addition to biological potency, GLP-1/Fc is protected from degradation by human DPP-IV. Specifically, the degradation and inhibition of the activity of the GLP-1/Fc fusion protein by human DPP-IV was approximately five-fold lower than that of native GLP-1 *in vivo*. Furthermore, the inactivation of the GLP-1/Fc-fusion protein was approximately four-fold lower than that of the native GLP-1 in rabbit serum, which suggests that the *in vivo* half-life of the fusion protein may be increased.

These data indicate that GLP-1/Fc may have an extended half-life *in vivo*, due to inhibition of the degradation by human DPP-IV. These findings indicate that GLP-1/Fc may be useful in the treatment of diabetes.

MATERIALS AND METHODS

Cloning of human GLP-1 receptor

To express human GLP-1 receptor (NM-002062) in mammalian cells, the coding sequence was amplified by PCR from a human kidney cDNA library (Clontech) using specific primers (BglII-GLP-1R-F: 5′-CTCT*AGATCT*ATGGCCGGCGCCCCCGG CCC; XhoI-GLP-1R-R: 5′-CTCT*CTCGAG*TCAGCTGCAGGAGG CCTGGC) followed by digestion with BglII and XhoI. The PCR product was then subcloned into the pCDNA3 DHFR+ vector and subsequently digested by BamHI and XhoI.

Cloning of GLP-1/Fc

To construct an expression vector for the GLP-1/Fc fusion protein, we used primers for the signal peptide (19 amino acids) of the human IgG1-heavy chain and the coding sequence of the active GLP-1 peptide (Fig. 1B). The signal peptide of the human IgG1-heavy chain was introduced to the N-terminus of GLP-1 to induce secretion of the fusion protein. GLP-1 containing the signal peptide was then amplified using an overlap PCR strategy (Fig. 1A), after which it was digested by BamHI and NdeI. This fragment was then ligated into pCDNA3-Fc

DHFR that had been modified from pCDNA3 (Invitrogen Inc.) to give pCDNA3-GLP-1/Fc DHFR.

Cloning of human DPP-IV for baculoviral expression

Construction of expression vector for human DPP-IV was conducted as follows: the full-length human DPP-IV cDNA (NM-001935) was amplified by PCR amplification using a 5′-primer containing the BamH1 restriction enzyme site (5′-CTCT*GGAT CC*ATGAAGACACCGTGGAAGGTTC) and a 3′-primer containing His tags (5′-GATGGTGATGATGAGGTAAAGAGAAAC ATTGTTTTATG) and a human placenta cDNA library (Clontech) as a template. An additional PCR amplification was then conducted to insert the XhoI site and 8x His-tags at 3′ using the 5′-primer; BamH1-DPP-IV, and the 3′-primer; XhoI-His-DPP-IV (5′-CTCG*CTCGAG*CTAATGATGATGATGATGATGT). Following digestion with BamHI and XhoI, the PCR product was subcloned into pBacPAK8 digested by BamHI and XhoI.

Expression and purification of GLP-1/Fc from CHO cell lines

For mammalian expression of the GLP-1/Fc, stable CHO (DHFR -/-) cell lines that over-express the fusion proteins were established using lipofectamine (Invitrogen) according to manufacturer's instructions. Briefly, 1.5×10^6 cells were plated on 100-mm culture dishes in α-MEM containing 10% (v/v) heat-inactivated FBS, hypoxanthine (Sigma Chemical Co.) and antibiotics (Gibco BRL). After 24 h, the media was exchanged with serum-free α-MEM and 4 μg of pCDNA3-GLP-1/Fc was then transfected into the cells. At 5 h after transfection, the media was exchanged with α-MEM containing 5% d-FBS without hypoxanthine (Sigma Chemical Co.). The samples were then incubated overnight, after which G418 with a final concentration of 800 μg/ml of G418 and 300 nM MTX (Sigma Chemical Co.) were added to select only transfected cells. The culture media was exchanged with the same fresh media every 2-3 days until colonies appeared. Next, the colonies were selected and subcultured in 150-mm culture dishes in selection medium containing 5% d-FBS to produce a large quantity of GLP-1/Fc proteins. After the cells were confluent, the culture media was exchanged with serum-free media to facilitate purification of the secreted proteins. The samples were then incubated for an additional 3 days, after which the media was harvested, diluted two-fold with PBS and then loaded onto a protein-G sepharose column that had been equilibrated with PBS. The bound proteins were then washed with PBS, eluted with 0.1 M glycine (pH 2.1) and neutralized by the addition of 1 M Tris-HCl (pH 8.0).

Expression and purification of human DPP-IV from SF21 insect cells

The baculoviral vector, pBacPAK8, carrying human DPP-IV was expressed with the same method as described (18). Briefly, the recombinant viral vector was infected into SF21 insect cells using 10 MOI in TNM-FH medium containing 10% FBS according to the manufacturer's instructions (Clontech), after which the cells were incubated for an additional 2 days. Next, the cells were harvested by centrifugation and then resuspended in hypotonic buffer comprised of 20 mM Hepes (pH 7.4), 5 mM NaCl and protease inhibitor cocktail (Roche). The sample tubes were then kept on ice for 10 min, after which the cells were disrupted with a dounce homogenizer (B pestle). Next, the protein extracts were collected by removing the cell debris by centrifugation at 12,000 rpm for 20 min at 4°C. The concentrations of the extracts were then adjusted by the addition of 50 mM Hepes (pH 8.0) and 500 mM NaCl, after which the samples were loaded onto a His-bind resin column (Qiagen). The column was then washed with the same buffer containing 60 mM Imidazole, after which the absorbed proteins were eluted using the same buffer containing 200 mM Imidazole. Finally, the active fractions were collected and dialyzed against 20 mM Hepes (pH 7.4) and 50 mM NaCl and stored as aliquots (0.1 μg/μl) at -70°C.

***In vitro* assay of human DPP-IV**

The activity of DPP-IV was confirmed using Gly-Pro-AFC (2.5 mM) as a fluoro-substrate in 50 mM Hepes (pH 7.4) in a total of 50 μl in a non-transparent 96-well plate. Briefly, DPP-IV was added to a plate containing the fusion protein, which was then incubated at 37°C. The fluorescence was then read by reading the absorbance at an excitation level of 405 nm and an emission of 505 nm in a luminometer (Molecular Probe).

Establishment of human GLP-1R/pCRE-luciferase-coexpressed CHO cell lines

For biological evaluation of GLP-1/Fc for human GLP-1R activation, GLP-1R/pCRE-Luciferase-coexpressed CHO cell lines were established using the lipofectamine method. Briefly, 5 μg of pCDNA3-human GLP-1 receptor and 10 μg of pCRE-luciferase (Stratagene) were co-transfected into CHO (DHFR -/-) cells using the lipofectamine method. After 5 h, the media was exchanged with α-MEM containing 10% d-FBS, 30 nM MTX and antibiotics and then incubated overnight. On the next day, G418 (final 200 μg/ml) was added to the samples to select the transfected cells. The culture media was then changed with fresh media every 2-3 days using a step-wise increase of G418 up to a final concentration of 1,000 μg/ml. When the colonies appeared, colonies expressing both human GLP-1 receptor and CRE-luciferase were selected using a luciferase assay system (Promega) following treatment with 1 μM of GLP-1 for 24 h in 96-well plates.

Cell-based evaluation of biological activity and degradation of GLP-1 and GLP-1/Fc

The established GLP-1R/CRE-luciferase-co-expressed CHO cell line was used for biological evaluation of both GLP-1 and GLP-1/Fc for human GLP-1R activation. Briefly, the cells were plated at a density of $0.5 \sim 1 \times 10^4$ cells/well in 100 μl of α-MEM containing 5% d-FBS, 30 nM MTX, 800 μg/ml G418 and antibiotics. The next day, the cells were treated with either

GLP-1 or GLP-1/Fc for 2 h after serial dilution with PBS containing 1 mg/ml of BSA. To compare the degradation of GLP-1 and GLP-1/Fc by human DPP-IV and in serum, the two ligands were serially diluted with PBS containing 1 mg/ml BSA (Sigma Chemical Co.) and then incubated overnight in a total of 50 µl of BSA in the presence of human DPP-IV or New Zealand white rabbit serum. Next, 11 µl aliquots of the samples were added to culture media in 96-well plates, which were subsequently incubated for 2 h at 37°C in 5% CO_2 to induce luciferase expression by the non-degraded GLP-1. The samples were then washed twice with 100 µl of PBS. Next, 50 µl of 1× reporter lysis buffer (Promega) was added to the culture plates and 20 µl of protein extracts were transferred to a non-transparent 96-well plate. The luciferase activity was then measured using a lumat model LB953 luminometer (Berthold, Germany).

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