# Novel DPP-IV-resistant Analogs of GLP-1: The *N*-terminal Extension of GLP-1 by a Single Amino Acid

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Glucagon-like peptide-1 (GLP-1) is an incretin hormone produced from intestinal L cells of the gut and plays an important role in glucose-dependent insulin secretion, suppression of glucagon secretion, proliferation of pancreatic β-cells, and inhibition of food intake.[1] The short half-life of GLP-1 ($t_{1/2}$ < 2 minutes) resulted from degradation by dipeptidyl peptidase IV (DPP-IV), however, has stymied the application of GLP-1 for the treatment of insulin-resistant type 2 diabetes.[2] Exendin-4, which is derived from saliva of a lizard Gila monster with ~53% sequence homology with GLP-1, has Gly in position 2 rather than Ala, as is the case in GLP-1. Thus, exendin-4 is resistant to the cleavage by DPP-IV and has a half-life of approximately 30 minutes after intravenous administration and 2 - 3 hours after subcutaneous administration in humans.[3,4]

Previous research has been focused on the development of long-acting GLP-1 mimetics or DPP-IV inhibitors. A number of studies were reported on DPP-IV-resistant GLP-1 analogs with chemical modification[5,6] or amino acid substitution.[7-12] in which the latter largely relied upon substitutions of amino acid in the N-terminal region in GLP-1 to evade the proteolytic degradation by DPP-IV. In addition, efforts to prolong the half-life of GLP-1 by attaching macromolecules such as albumin or immunoglobulin heavy chains (IgG-Fc), which are widely found in serum, have been sought. For example, albumin fusion to GLP-1 was shown to activate GLP-1 receptor-dependent signaling pathways in baby hamster kidney cells.[13] The fusion of mouse IgG-Fc to human GLP-1 (GLP-1/IgG-Fc) increased the half-life of GLP-1 in mouse.[14]

In this study we sought to elucidate whether the N-terminal extension of GLP-1 by a single amino acid, Ala or Gly, evades the degradation by DPP-IV using 7-amino-4-methylcoumarin (AMC)-conjugated tripeptide (Ala-His-Ala-AMC or Gly-His-Ala-AMC). We further examined whether the N-terminal modified GLP-1/IgG-Fc fusion proteins could induce the expression of insulin receptor substrate-2 (IRS-2) in rat pancreatic β cells.

Many previous attempts to synthesize DPP-IV-resistant GLP-1 analogs have been focused on mutations of amino acids in the GLP-1 sequence. We constructed GLP-1/IgG-Fc fusion constructs with the N-terminal extension of GLP-1 by a single amino acid, Ala or Gly, in the pSGHV0 vector. 6×His tag followed by an enterokinase recognition sequence, Asp-Asp-Asp-Asp-Lys, were localized prior to the N-terminal end of GLP-1. The recombinant GLP-1/IgG-Fc fusion protein transiently expressed in CHO-K1 cells were secreted into medium, suggesting that the protein is folded properly. The recombinant GLP-1/IgG-Fc fusion protein was successfully purified using a 5-mL HisTrap column on FPLC (see Fig. 1A). The GLP-1/IgG-Fc was eluted in 100 - 200 mM imidazole

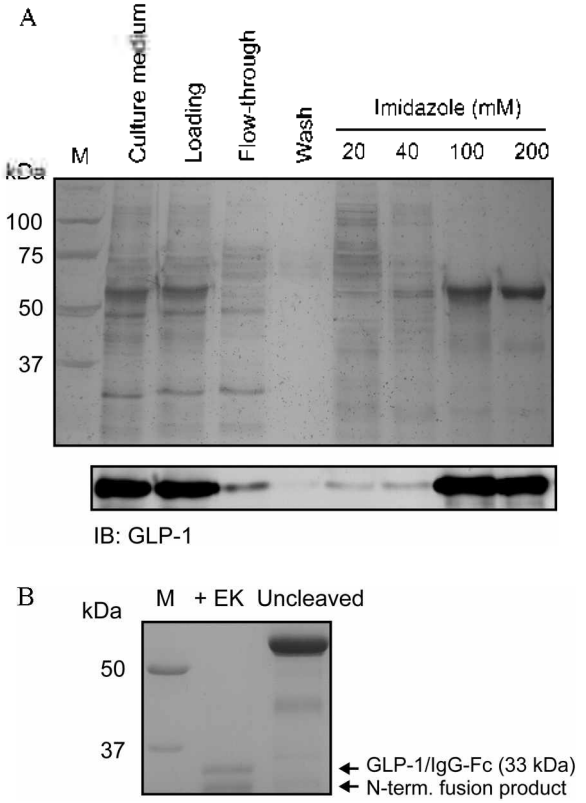


**Figure 1.** Expression and purification of GLP-1/IgG-Fc fusion protein. (A) GLP-1/IgG-Fc fusion proteins were expressed in CHO-K1 cells. The 3, 6, 10-days conditioned medium were pooled and purified using a 5-mL HisTrap column on FPLC. Proteins separated by SDS-PAGE were stained with silver nitrate (upper panel) and identified by immunoblotting with anti-GLP-1-antibody (lower panel). M, marker. M.W. of fusion protein is 58 kDa. (B) Cleavage of the fusion protein by enterokinase. The purified fusion proteins were pooled and concentrated followed by treatment with enterokinase for 16 hour at 25 °C. M, marker, Fusion protein appears as a 33 kDa after enterokinase treatment.

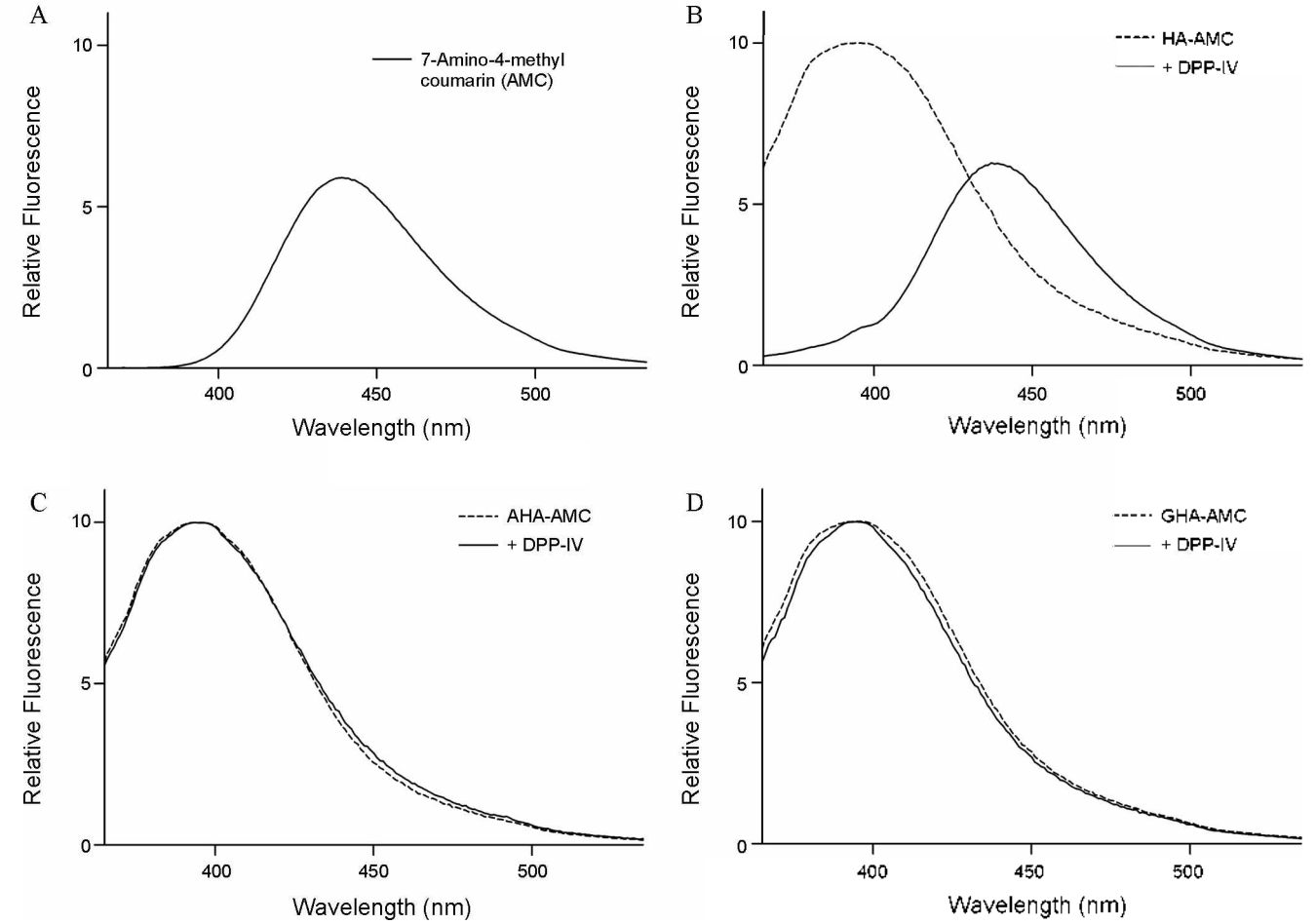


**Figure 2.** DPP-IV degradation assay. His-Ala (HA)-, Ala-His-Ala (AHA)- and Gly-His-Ala (GHA)-conjugated AMC were synthesized, respectively. AMC-conjugated peptides were incubated in the absence or presence of DPP-IV for 1 hr at 37 °C. The fluorescence change was measured between 365 - 535 nm upon excitation at 350 nm. (A) AMC only, (B) HA-AMC, (C) AHA-AMC, (D) GHA-AMC.

concentration, suggesting that the fusion protein bound to the nickel resion with a high affinity. The fusion protein appeared on the SDS gel with a molecular weight of 58 kDa. Following buffer exchange, the enterokinase recognition sequence was cleaved off by enterokinase at 25 °C (see Fig. 1B). Enterokinase cleaves the C-terminal side of the recognition sequence allowing complete removal of affinity tag sequences. Recombinant GLP-1/IgG-Fc fusion protein upon treatment of enterokinase appeared on the SDS gel as a 33 kDa band. The enterokinase enzyme was removed by protein A affinity column chromatography.

Previously high-performance liquid chromatography and mass spectrometry have been used for the identification of cleaved GLP-1 fragments upon DPP-IV treatment.[2,15] In this study, we utilized the fluorescence change of AMC-conjugated peptides as a measure to examine if the N-terminal extension of GLP-1 by a single amino acid confers resistance to degradation by DPP-IV. The enzyme DPP-IV cleaves the N-terminal dipeptide of substrates, especially after amino acid Pro or Ala.[16] Native His-Ala-AMC and N-terminal extended Ala-His-Ala-AMC and Gly-His-Ala-AMC were synthesized, respectively. It is expected that there would be no change of fluorescence if either of the tripeptides is resistant to DPP-IV. AMC itself showed its maximal emission at 440 nm upon excitation at 350 nm (see Fig. 2A). The fluorescence emission from AMC-conjugated peptides was blue-shifted compared to AMC. Upon treatment of DPP-IV for 1 hour at 37 °C, the fluorescence emission from His-Ala-AMC was restored to the normal spectrum of AMC (see Fig. 2B), suggesting that the peptide bond between His-Ala and AMC was cleaved off by DPP-IV. However, the treatment of DPP-IV had no effect on the fluorescence emission of Ala-His-Ala-AMC or Gly-His-Ala-AMC (see Fig. 2C and 2D), indicating that the peptide bond between the tripeptides and AMC is resistant to DPP-IV action.

Next, we examined the expression of IRS-2 in insulin-producing rat INS-1 cells to see the GLP-1/IgG-Fc fusion protein has retained its biological activity. The IRS-2 branch of insulin and insulin-like growth factor signaling pathways strongly promote β-cell growth and survival, which is essential for compensatory β cell function during physiological or metabolic stress.[17] GLP-1 induced 2-fold (2.0 ± 0.4) increase of IRS-2 protein expression (see Fig. 3). Upon treatment of wild-type GLP-1/IgG-Fc fusion protein, the expression of IRS-2 protein was increased 1.6-fold (1.6 ± 0.1), 20% lower than the induction of IRS-2 by GLP-1. The modified A- and

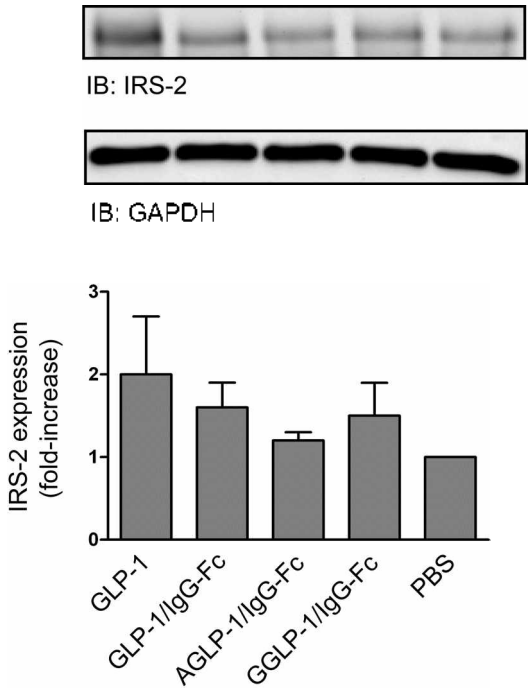


**Figure 3.** Induction of IRS-2 protein. Rat insulinoma INS-1 cells grown in 24-well plate were treated with native GLP-1, GLP-1/IgG-Fc, Ala-GLP-1/IgG-Fc and Gly-GLP-1/IgG-Fc, respectively for 7 hours. Fusion protein was identified by polyclonal antibody to IRS-2. Experiments were repeated three times. Bar graph shows the band intensity of IRS-2 over PBS-treated sample as mean ± S. D.

G-GLP-1/IgG-Fc fusion protein both showed 1.2-fold (1.2 ± 0.1) and 1.5-fold (1.5 ± 0.2) increase of IRS-2 expression, respectively. Addition of Ala to the dipeptide led to 20% lower IRS-2 expression than the addition of Gly. Our data suggests that the N-terminal extension of GLP-1/IgG-Fc fusion protein by a single amino acid is functionally active. Ligand binding to the GLP-1 receptor occurs in two steps: the C-terminal region of GLP-1 initially binds to the receptor and a secondary contact occurs between the N-terminal region of GLP-1 and the transmembrane and extracellular loops of the GLP-1 receptor for receptor activation.[18,19] Thus, it is believed that the decreased IRS-2 expression compared to native GLP-1 is in part due to structural hindrance in GLP-1 binding to the GLP-1 receptor by Fc fusion.

In conclusion, we demonstrated that the AMC-conjugated tripeptides were resistant to degradation by DPP-IV. Furthermore, both of the N-terminal extended GLP-1/IgG-Fc fusion proteins by a single amino acid, Ala or Gly, were functionally active by inducing the expression of IRS-2 in rat insulinoma INS-1 cells. Mechanisms of fusion protein binding to the GLP-1 receptor and signal generation need to be further elucidated in the future.

## Experimental Section

**Materials.** Human glucagon cDNA was purchased from OriGene Technologies, Inc. (USA). Human GLP-1 was purchased from Sigma (USA). The pSGHV0 vector was kindly provided by Jong-Bok Yoon (Yonsei University, Seoul, Korea).[20] The pOPGFc plasmid containing human IgGγ-Fc was kindly provided by Young-Chul Sung (Pohang University of Science and Technology, Pohang, Korea). AMC-conjugated three peptides (His-Ala-AMC, Ala-His-Ala-AMC, and Gly-His-Ala-AMC) were synthesized at Peptron (Korea). Enterokinase was purchased from Invitrogen (USA). Monoclonal antibody to GLP-1 and polyclonal antibody to IRS-2 were purchased from Santa Cruz Biotechnology, Inc (USA). All other reagents, unless stated otherwise, were from Sigma (USA).

**GLP-1/IgG-Fc fusion plasmid construction.** The human IgGγ-hinge Fc sequence encoding 234 amino acids was amplified from the pOPGFc plasmid by polymerase chain reaction (PCR) with the following primers: the forward primer was 5'-GGATCCgctagc**GAGCCCAAATC**-3' (BamHI site underlined, linker sequence in small letters, and the N-terminal region of IgG-Fc in boldface type) and the reverse primer was 5'-GCGGCCGC**TCATTTACCCGGAGA**-3' (NotI site underlined and the C-terminal region of IgG-Fc in boldface type). The PCR product was sequenced and subcloned into the BamHI and NotI sites of the pSGHV0 vector, generating a construct of pSGHV0-Fc. Human GLP-1 was amplified from human glucagon cDNA by PCR with the following primers; the forward primer was 5'-GGATCCgacgatgacgataag**CATG CTGAAGGGACC**-3' (BamHI site underlined, enterokinase recognition sequence in small letters, and the N-terminal region of GLP-1 in boldface type), and the reverse primer was 5'-GA AATCTCGCTAGC**TCCTCGGCC**-3' (NheI site underlined and the C-terminal region of GLP-1 in boldface type). GLP-1 with an N-terminal Ala was amplified with the forward primer 5'-GGATCCgacgatgacgataaggctGCT**CATGCTGAAGGGA CC**-3' (BamHI site underlined, enterokinase recognition sequence in small letters, Ala double underlined, and the N-terminal region of GLP-1 in boldface type). GLP-1 with an N-terminal Gly was amplified with the forward primer 5'-GGATCCgacgatgacgataagGGA**CATGCTGAAGGGACC**-3' (BamHI site underlined, enterokinase recognition sequence in small letters, Gly double underlined, and the N-terminal region of GLP-1 in boldface type). The PCR product digested with BamHI and NheI was subcloned into the pSGHV0-Fc vector. Accuracy of the fusion constructs in the expression vector was confirmed by DNA sequence analysis.

**Expression of GLP-1/IgG-Fc fusion proteins.** Chinese hamster ovary (CHO)-K1 cells were transfected with wild-type or modified GLP-1/IgG-Fc plasmid using polyethyleneimine according to the manufacturer's instructions. The 3, 6, 10-days conditioned medium were combined for protein purification.

**Purification of GLP-1/IgG-Fc fusion proteins.** Protein purification was carried out using AKTA Prime (GE Healthcare). 5-mL HisTrap FF column was equilibrated with buffer A (20 mM sodium phosphate, 0.5 M NaCl, pH 8.0). Following sample loading, the column was washed with buffer A and eluted with Buffer B (1 M imidazole) with 2 - 50% gradient at 5 mL/min. After elution, proteins were concentrated using Vivaspin 20 (GE Healthcare). The fusion proteins were incubated with enterokinase (EKMax, Invitrogen) for 16 hours at 25 °C, and the enzyme was removed with EKAway affinity resin (Invitrogen). Cleaved fusion proteins were separated by Protein A

column chromatography (GE Healthcare), and neutralized with 1 M Tris·Cl, pH 8.0. Fusion protein in each fraction was separated by SDS-PAGE and stained by Coomassie staining solution and silver nitrate, respectively. The expression of fusion protein was also identified by immunoblotting with monoclonal antibody to GLP-1 followed by detection with ChemiDoc XRS (Bio-Rad). Each band was analyzed using Quantity One software (Bio-Rad).

**Degradation by DPP-IV enzyme.** AMC-conjugated peptides were synthesized, buffer exchanged with acetate buffer, and lyophilized. 100 μM of AMC-conjugated peptide in 500 μL of 50 mM Tris·HCl, 1 mM EDTA, pH 8.0 was incubated with DPP-IV for 1 hr at 37 °C. The fluorescence change was measured using AMICO-Bowman Series 2 (AB2) Spectrofluorometer between 365-535 nm upon excitation at 350 nm. Data were plotted using GraphPad Prism software.

**IRS-2 induction.** INS-1 cells were grown in RPMI 1640 with 10% FBS at 37 °C with 5% $CO_2$. INS-1 cells were treated with 50 nM GLP-1 or GLP-1/IgG-Fc fusion proteins. Cells were washed with cold PBS and lysed in lysis buffer at 4 °C for 1 hour. Cells were harvested at 12,000 × g for 10 min and the supernatant was collected. 30 μg of cell lysates were separated by SDS-PAGE in a 10% separation gel and transferred onto nitrocellulose membrane. The expression of IRS-2 was identified by immunoblotting using polyclonal antibody to IRS-2.

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