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# Linker engineering for fusion protein construction: Improvement and characterization of a GLP-1 fusion protein

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ABSTRACT

Protein engineering has been successfully applied in protein drug discovery. Using this technology, we previously have constructed a fusion protein by linking the globular domain of adiponectin to the C-terminus of a glucagon-like peptide-1 (GLP-1) analog. Herein, to further improve its bioactivity, we reconstructed this fusion protein by introducing linker peptides of different length and flexibility. The reconstructed fusion proteins were overexpressed in *Escherichia coli* and purified using nickel affinity chromatography. Their agonist activity towards receptors of GLP-1 and adiponectin were assessed *in vitro* by using luciferase assay and AMP-activated protein kinase (AMPK) immunoblotting, respectively. The effects of the selected fusion protein on glucose and lipid metabolism were evaluated in mice. The fusion protein reconstructed using a linker peptide of AMGPSSGAPGGGGS showed high potency in activating GLP-1 receptor and triggering AMPK phosphorylation *via* activating the adiponectin receptor. Remarkably, the optimized fusion protein was highly effective in lowering blood glucose and lipids in mice. Collectively, these findings demonstrate that the bioactivity of this GLP-1 fusion protein can be significantly promoted by linker engineering, and indicate that the optimized GLP-1 fusion protein is a promising lead structure for anti-diabetic drug discovery.



## 1. Introduction

Glucagon-like peptide-1 (GLP-1) is a classical hormone responsible for promoting insulin secretion in a glucose-dependent manner [1]. GLP-1-based therapies have exhibited potential therapeutic roles in diabetes [2]. However, native GLP-1 is limited in pharmacotherapy investigation due to its short half-life.

To extend the circulating half-life of native GLP-1, fusion protein technologies have been used to enhance pharmacotherapy. Fusing GLP-1 to a large 'carrier' moiety, such as albumin and Fc, improved its effects by slowing its clearance *in vivo* [3,4]. Intriguingly, a novel approach of pharmacotherapy has been generated which combined GLP-1 analogs with other metabolic hormones into a single molecule, glucagon for example, in order to simultaneously activate multiple receptors to improve glucose metabolism and increase energy expenditure [5,6]. By linking GLP-1 to estrogen, the co-agonist took advantage of the beneficial effects of both GLP-1 and estrogen on energy balance while avoiding adverse effects on other tissues [7]. Recently, we fused mutated GLP-1 (A2G, K28R) to the globular domain of adiponectin (gAd) which is involved in regulation of blood glucose as well as lipid metabolism [8]. The fusion protein GAD was proved to be superior to native GLP-1 in anti-diabetic activity [8]. However, simple linking of the two moieties by a short peptide AMG decreased the effect of gAd. Likewise, Zhao et al. constructed a fusion protein consisting of GLP-1 and gAd, which showed prominent potency in lowering blood glucose, but it remains unclear whether both domains were functional [9].

To our knowledge, the linker plays an important role in maintaining the intrinsic bioactivity of the fusion protein. In this study, with the aim to create optimal linkers capable of efficiently separating the domains, linker peptides of different length and flexibility were introduced into the construction of the fusion proteins. These variants were compared the ability of receptor activation *in vitro*. Subsequently, the effects of the optimized fusion protein on glucose and lipid metabolism were evaluated *in vivo*.

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## 2. Materials and methods

### 2.1. Plasmid construction and protein purification

The fusion proteins with linkers composed of AMGPSSGAPGGGGS, AMG and AMGGGGSGGGGS were designated as GAD1, GAD2 and GAD3, respectively. The synthetic fusion genes were digested with *BglII* and *HindIII* restriction enzymes (Takara Biotechnology, Otsu, Shiga, Japan) and subsequently cloned into pET-32a expression vector. All constructs were expressed in *Escherichia coli* BL21 (DE3). Expressed fusion proteins were purified using nickel affinity chromatography (GE Healthcare, Uppsala, Sweden) as described previously [8]. After nickel affinity chromatography, the thioredoxin (Trx) tagged fusion protein (Trx-GAD) was cleaved using enterokinase under optimized conditions (0.25 mg fusion protein per unit enzyme at 37 °C for 8 h). The released Trx fusion partner was removed using nickel affinity chromatography and the final GAD product protein was concentrated on an Amicon Centricon Filter (Millipore, Billerica, MA, USA) with a molecular weight cutoff of 10,000 kDa. The purity of protein was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Then, the purified proteins were identified by western blot with a rabbit polyclonal antibody against adiponectin (Abcam, Cambridge, UK).

### 2.2. Reporter gene assay

CHO-K1-GLP-1R-CRE-Luc$^+$ cells were used to detect GLP-1 receptor (GLP-1R) agonists, as they stably expressed GLP-1R. The cells were suspended in DMEM/F12 culture medium (Gibco, Carlsbad, CA, USA) with 0.25% FBS (Gibco). Cells were seeded onto a 96-well plate with a density of $3 \times 10^5$ cells per well and incubated at 37 °C and 5% $CO_2$. Meanwhile, the indicated doses of GAD variants were added. After 4-h incubation, cells were lysed and then quantified by luciferase assay reagent (Promega, Madison, WI, USA).

### 2.3. Phosphorylation of AMP-activated protein kinase (AMPK)

The effect of fusion proteins on the phoshorylation of AMPK was investigated as described previously [10,11]. Briefly, C2C12 myoblasts were cultured in DMEM containing 10% FBS. After differentiation by horse serum (Gibco), cells were treated with vehicle (DMEM containing 2% horse serum), GLP-1 or GAD at 150 nM for 15 min. Then the cells were lysed and subsequently phospho-AMPK and AMPK were detected by western blot.

### 2.4. Animal maintenance

Eight-week-old male C57BL/6 mice purchased from Yangzhou University were maintained individually with a 12 h light-dark cycle and fed a normal chow. All animal experiments were performed in accordance with the guidelines for the Laboratory Animal Management Regulations in China and approved by the Institutional Animal Care and Use Committee of China Pharmaceutical University (SCXK 2012 (SU)-0004).

### 2.5. Oral glucose tolerance test (OGTT)

After overnight fasting, saline, GLP-1 (60 nmol/kg) or GAD1 (60 nmol/kg) was injected *via* intraperitoneal (*i.p.*) into mice 30 min prior to oral administration of 2 g glucose/kg body weight ($t = 0$). Blood samples were collected at 0, 15, 30, 60, 120 min after gavage. Blood glucose was measured with a glucometer (Sannuo, Changsha, China).

### 2.6. Lipid tolerance test

Lipid tolerance test was carried out according to a method described previously [12]. After 16-h fasting, saline, GLP-1 (60 nmol/kg) or GAD (60 nmol/kg) was injected (*i.p.*) into mice 20 min prior to 200 μL of olive oil gavage. Twenty minutes after gavage, Triton WR-1339 (0.5 g/kg body weight, 15% solution prepared in saline) was injected *via* tail vein (*i.v.*) to block lipid clearance ($t = 0$). Blood samples were collected at 0, 30, 60, 90 min for plasma total cholesterol (TC), triglyceride (TG) and apolipoprotein B-100 (ApoB-100) assay. TC and TG loading test kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The ApoB-100 ELISA kits were obtained from BD Biosciences (Franklin Lakes, NJ, USA).

### 2.7. Free fatty acid clearance

Mice fasted overnight were injected (*i.v.*) with 30 μL of lipid emulsion (Intralipid-20%) half-an-hour after treatment with saline, GLP-1 (60 nmol/kg) or GAD (60 nmol/kg), while normal group was only treated with saline (without Intralipid-20%, GLP-1 or GAD) as blank control [10]. Plasma FFA was isolated at 60 min after Intralipid-injection and free fatty acid (FFA) was measured by colorimetry. The FFA kits were obtained from Nanjing Jiancheng Bioengineering Institute.

### 2.8. Statistical analysis

All values are expressed as mean ± SEM. Significant differences were compared by ANOVA. A $p$-value of less than 0.05 was considered statistically significant.

## 3. Results

### 3.1. Recombinant protein production and characterization

The fusion proteins with different linkers were shown in Fig. 1A. Each Trx-GAD recombinant protein has an apparent molecular mass of ~38 kDa, which was mainly expressed as soluble form. After removal of the Trx tag, GAD variants migrated with an apparent molecular mass of ~20 kDa, which produced single bands as analyzed by SDS-PAGE (Fig. 1B). As shown in Fig. 1C, these purified proteins have strong reactivity with specific anti-adiponectin polyclonal antibody. This set of data demonstrates that GAD variants were successfully obtained.

### 3.2. Effect of GAD on GLP-1R activation

To investigate the influence of different linkers on GLP-1R activation, the luciferase reporter gene assay was performed. As indicated in Fig. 2A, all of GAD variants dose-dependently elicited luciferase responses. GAD1 displayed the strongest potency with an $EC_{50}$ value of 25.2 nM among the variants. Whereas, the $EC_{50}$ values of GAD2 (74.86 nM) and GAD3 (111.3 nM) were about 3-fold and 4-fold respectively higher than that of GAD1. In addition, only GAD1 appeared to be a full agonist of GLP-1R.

### 3.3. Effect of GAD on adiponectin receptor activation

Next, we evaluated the efficiency of GAD on adiponectin receptor 1 (AdipoR1) activation by determining the phosphorylation of AMPK, as gAd could raise the fatty acid oxidation through promoting the phosphorylation of AMPK at Thr-172. Treatment C2C12 myoblasts with GAD1 or GAD3 caused significant increases in the phosphorylation of AMPK (GAD1, $p < 0.001$; GAD3, $p < 0.01$; Fig. 2B), whereas GAD2 only modestly induced AMPK phosphorylation.