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Induction of insulin receptor substrate-2 expression by Fc fusion to exendin-4 overexpressed in *E. coli*: a potential long-acting glucagon-like peptide-1 mimetic

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Exendin-4 (Ex-4), a peptide secreted from the salivary glands of the Gila monster lizard, can increase pancreatic β-cell growth and insulin secretion by activating glucagon-like peptide-1 receptor. In this study, we expressed a fusion protein consisting of exendin-4 and the human immunoglobulin heavy chain (Ex-4/IgG-Fc) in *E. coli* and explored its potential therapeutic use for the treatment of insulin-resistant type 2 diabetes. Here, we show that the Ex-4/IgG-Fc fusion protein induces expression of insulin receptor substrate-2 in rat insulinoma INS-1 cells. Our findings therefore suggest that Ex-4/IgG-Fc overexpressed in *E. coli* could be used as a potential, long-acting glucagon-like peptide-1 mimetic. [BMB reports 2010; 43(2): 146-149]

INTRODUCTION

Exendin-4 (Ex-4), a peptide secreted from the salivary glands of the Gila monster lizard (*Heloderma suspectum*), shares 53% sequence homology with human glucagon-like peptide-1 (GLP-1) (1). Activation of the GLP-1 receptor induces the cAMP signaling pathway and enhances glucose-dependent insulin release, thereby promoting β-cell growth and survival (2). Ex-4 also provokes downstream signaling pathways leading to β-cell growth and glucose-dependent insulin secretion upon binding to the GLP-1 receptor (3). While GLP-1 is rapidly degraded by dipeptidyl peptidase IV (DPP-IV) (4), Ex-4 eludes proteolytic degradation by having a Gly in position 2 rather than the Ala, possessed by GLP-1. Exenatide, a synthetic analog of Ex-4, has previously been used as a twice-daily therapeutic drug for the treatment of insulin-resistant type 2 diabetes (5). Klonoff *et al.* showed that exenatide treatment for three years in patients with type 2 diabetes lead to progressive weight reduction with sustained improvements in glycemic control (6).

A number of studies have been reported whose aims were to improve the expression and potency of recombinant GLP-1 or Ex-4 using different expression systems or fusion protein technology. The fusion of macromolecules such as albumin or immunoglobulin heavy chain (IgG-Fc) is an effective strategy for evading renal clearance and for prolonging the half-life of target molecules, which in turn reduces dosing frequencies (7). GLP-1/IgG-Fc fusion protein normalized glucose levels in db/db mice when expressed in COS-7 cells and *E. coli* (8). Expression of the fusion protein in CHO and SF21 cells also attenuated degradation by DPP-IV approximately 4-5 folds (9). The GLP-1/IgG-Fc and Ex-4/IgG-Fc fusion proteins, when expressed in diabetic mice via intramuscular plasmid-based gene transfer, reduced fed blood glucose levels and other symptoms of diabetes (10). Ex-4 conjugated to albumin was shown to have a longer plasma half-life when expressed in *Pichia pastoris* (11). However, the effect of Ex-4/IgG-Fc expression in either mammalian cells or *E. coli* has not yet been reported.

In this study, we sought to confirm whether or not Ex-4/IgG-Fc fusion protein when overexpressed in *E. coli* has bioactivity such as inducing insulin-related gene expression. Although there are certain advantages in expressing Fc fusion proteins in a eukaryotic expression system over a prokaryotic one, they are not critical for ligand-receptor interactions such as the binding of Ex-4/IgG-Fc fusion protein to the GLP-1 receptor. Furthermore, expression of Fc fusion proteins in *E. coli* provides an economical means of producing bioactive peptides in large quantities. Since Ex-4 has been shown to stimulate pancreatic β-cell growth and survival by inducing the expression of insulin receptor substrate-2 (IRS-2) (12, 13), we examined the effect of Ex-4/IgG-Fc fusion protein on IRS-2 induction in rat insulinoma INS-1 cells. We report that Ex-4/IgG-Fc fusion protein induces the expression of IRS-2.

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RESULTS AND DISCUSSION

Expression and purification of Ex-4/IgG-Fc fusion protein in *E. coli*

The plasmid for the expression of Ex-4/IgG-Fc fusion protein was constructed using a pET28a vector containing a His6 tag followed by an enterokinase recognition sequence (Asp-Asp-Asp-Asp-Lys) located just prior to the N-terminal end of the Ex-4/IgG-Fc fusion protein coding region (see Fig. 1). Enterokinase cleaves the target protein at the C-terminal side of the recognition sequence, thereby allowing the complete removal of affinity tag sequences. We overexpressed Ex-4/IgG-Fc fusion protein in *E. coli* BL21 (DE3). Following IPTG induction at mid-log phase, the cell growth temperature was reduced from 37°C to 30°C in order to enhance protein folding. As shown in Fig. 2, Ex-4/IgG-Fc fusion protein was increased in expression in a time-dependent manner, appearing on SDS gel with a molecular weight of approximately 35 kDa. Purification was performed using nickel affinity column chromatography. Ex-4/IgG-Fc was eluted using 200-500 mM imidazole, suggesting that the fusion protein binds to nickel resin with high affinity (see Fig. 3A). Following buffer exchange, the recognition sequence was cleaved at room temperature by enterokinase, which was then removed by protein A affinity column chromatography. This form of recombinant Ex-4/IgG-Fc fusion protein was confirmed by both Coomassie staining and immunoblotting with monoclonal antibody specific to Ex-4 (see Fig. 3B).

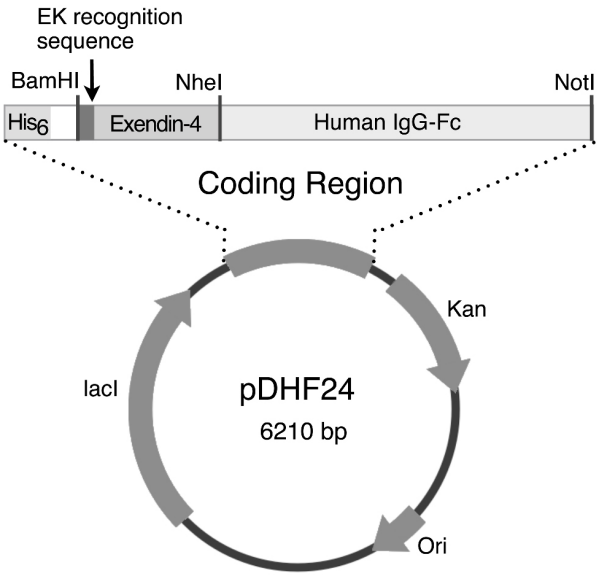

Fig. 1. Construction of Ex-4/IgG-Fc fusion plasmid. The diagram depicts a vector map of Ex-4/IgG-Fc constructed from the pET28 vector, which is named pDHF24 in our laboratory. The His6-tag and enterokinase recognition sequence are located prior to the N-terminus of Ex-4/IgG-Fc fusion protein.

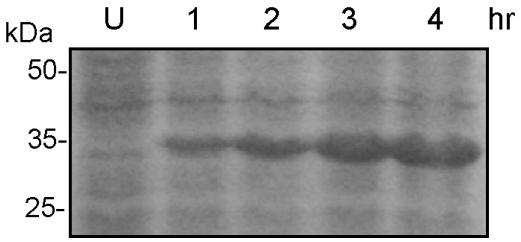

Fig. 2. Expression of Ex-4/IgG-Fc fusion protein in *E. coli*. Ex-4/IgG-Fc fusion protein was expressed in *E. coli* BL21 (DE3) and appeared as a 35 kDa band during SDS gel electrophoresis. *U*, uninduced.

Induction of IRS-2 protein in rat pancreatic β-cells

To elucidate whether Ex-4/IgG-Fc fusion protein retained bioactivity, we treated the INS-1 rat β-cell line with either GLP-1 or purified Ex-4/IgG-Fc fusion protein. Treatment of INS-1 cells with 100 nM GLP-1 or 10 nM Ex-4/IgG-Fc fusion protein increased the expression of IRS-2 4.1-fold and 3.0-fold, respectively (see Fig. 4). Our data therefore indicate that the Ex-4/IgG-Fc fusion protein binds to the GLP-1 receptor on the cell surface, activating downstream signaling pathways leading to the expression of IRS-2. IRS-1 and IRS-2 serve as adaptor molecules between the insulin receptor and signaling proteins containing Src homology-2 domains (14). Specifically, IRS-2 is involved in the regulation of β-cell mass, whereas IRS-1 is responsible for β-cell insulin production (14). Park *et al*. showed that Ex-4 increased cAMP levels in human islets and MIN6

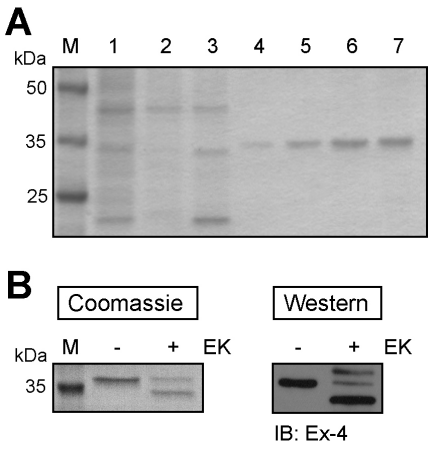

Fig. 3. Purification of Ex-4/IgG-Fc fusion protein. (A) Nickel affinity column chromatography. M, Molecular weight marker; *Lane 1*, Flow-through; *Lane 2*, Binding buffer; *Lane 3*, Wash buffer; *Lane 4*, 200 mM imidazole; *Lane 5*, 300 mM imidazole; *Lane 6*, 400 mM imidazole; *Lane 7*, 500 mM imidazole. (B) Enterokinase treatment; Coomassie stained gel (left) and Western blot (right) are shown. *M*, Molecular weight marker, *IB*, immunoblot.

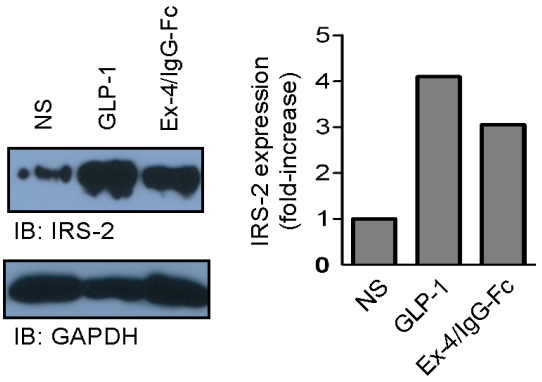

Fig. 4. Induction of IRS-2 protein. Rat insulinoma INS-1 cells were treated with 100 nM GLP-1 or 10 nM Ex-4/IgG-Fc fusion protein for 7 hours. Experiments were repeated three times. A representative blot is shown. Bar graph shows the fold-increase of IRS-2 band intensity over the basal level. *NS*, nonstimulated. *IB*, immunoblot.

cells, resulting in increased IRS-2 expression and Akt phosphorylation (12). Jhala *et al*. also showed that Ex-4 increases IRS-2 protein levels in MIN6 cells (13). Although able to evade degradation by DPP-IV, Ex-4 is still quickly metabolized in vivo with a half-life of 18～41 min (15). Kim *et al*. showed that Fc fusion to GLP-1 attenuated degradation by DPP-IV *in vitro* (9). This ability of Fc fusion to Ex-4 to prolong the half-life of Ex-4 needs to be further determined.

In summary, we expressed the Ex-4/IgG-Fc fusion protein in *E. coli* and examined its effect on GLP-1 receptor activation in rat insulinoma INS-1 cells. Our data shows that the Ex-4/IgG-Fc fusion protein expressed in *E. coli* induces IRS-2 protein expression. The effects of Ex-4/IgG-Fc on the downstream signaling pathways of the GLP-1 receptor that lead to β-cell growth and glucose-dependent insulin secretion therefore need to be further elucidated.

MATERIALS AND METHODS

Materials

GLP-1 was purchased from Sigma (St. Louis, Mo). The pSGHV0 vector was kindly provided by Jong-Bok Yoon (Yonsei University, Seoul, Korea) (16). yT&A cloning vector was purchased from Yeastern Biotech Co. (Taiwan). The pET28a expression vector was purchased from Novagen (Madison, WI). Monoclonal antibody specific to Ex-4 was purchased from Bioporto (Denmark). Polyclonal antibody specific to IRS-2 (H-205) was purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). Monoclonal antibody specific to glyceraldehydes 3-phosphate dehydrogenase (GAPDH) was purchased from Chemicon (Billerica, MA). Horseradish peroxidase (HRP)-labeled goat anti-rabbit secondary antibody was purchased from Upstate (Billerica, MA). All other reagents, unless stated otherwise, were obtained from Sigma (St. Louis, Mo).

Construction of Ex-4/IgG-Fc fusion plasmid

The Ex-4/IgG-Fc fusion plasmid was generated by a two-step polymerase chain reaction (PCR). First, two primers for Ex-4 were synthesized and annealed. The forward primer was 5'-ggatccGACGATGACGATAAG**CATGGCGAAGGAACATTCACCAGCGACTTGTCAAAACAGATGGAAGAGGAGGCAGTGCGG**-3' (BamHI site in small letters, enterokinase recognition sequence underlined, and the N-terminal part of Ex-4 in boldface type). The reverse primer was 5'-gctagc**AGATGGCGGAGGTGCCCCGCTACTTGGTCCGCCGTTCTTAAGCCACTCAATAAATAACCGCACTGCCTCCTCTT**-3' (NheI site in small letters and the C-terminal part of Ex-4 in boldface type). The partially overlapped Ex-4 sequences were amplified using another set of primers to complete the entire Ex-4 sequence. The forward primer was 5'-ggatcc**GATGACGA**-3' (BamHI site in small letters and the N-terminal part of Ex-4 in boldface type). The reverse primer was 5'-gctagc**AGATGGCGGAG**-3' (NheI site in small letters and the C-terminal part of Ex-4 in boldface type). The resulting PCR product was then subcloned into yT&A cloning vector. Next, human IgG-Fc sequence was digested from the pSGHV0 vector and subcloned into the yT&A cloning vector containing the Ex-4 cDNA. The entire Ex-4/IgG-Fc fusion plasmid was digested and subcloned into a pET28a vector. The accuracy of the construct was confirmed by DNA sequence analysis.

Expression and purification of Ex-4/IgG-Fc fusion protein

The Ex-4/IgG-Fc fusion plasmid was transformed into *E. coli* BL21 (DE3). A single colony grown on an LB/kanamycin plate was selected for overnight growth at 37°C, followed by inoculation into 50 ml LB/kanamycin broth for IPTG induction at mid-log phase. Cells were grown for 4 hours, after which 1 ml aliquots of cells were collected to check time-course expression of the fusion protein by SDS-PAGE. Cells were harvested at 10,000 × g for 5 min. The pellet was resuspended in binding buffer (20 mM Tris·Cl, 0.5 M NaCl, 5 mM imidazole, pH 8.0) followed by sonication at 4°C. After centrifugation at 12,000 × g for 10 min, the supernatant was collected. Protein purification procedures were performed using a 1 ml-HisTrap column (GE Healthcare) at 4°C. Bound Ex-4/IgG-Fc fusion protein was eluted using a step-gradient (200-500 mM imidazole in binding buffer).

IRS-2 induction

INS-1 cells were grown in RPMI 1640 medium containing 10% FBS at 37°C in a 5% CO_2 atmosphere. Before ligand treatment, cells were incubated in RPMI 1640 medium without FBS for 30 min. INS-1 cells were treated with 100 nM GLP-1 or 10 nM of Ex-4/IgG-Fc fusion protein for 7 hours. Cells were washed with cold PBS and lysed in 100 μl of lysis buffer (25 mM HEPES, pH 7.2, 150 mM NaCl, 5 mM EDTA,

1% Triton X-100, 1 mM PMSF, 10 mM sodium fluoride, 1 mM sodium orthovanadate, 10% glycerol) for 1 hour at 4°C. The Sigma protease inhibitor mixture (P2714) was then added to the lysis buffer. Cells were harvested at 12,000 × g for 10 min after which the supernatant was collected. Thirty μg of cell lysates were loaded onto a 10% SDS-PAGE separation gel. The protein was transferred to a nitrocellulose membrane and blocked for 1 hour at room temperature in 5% non-fat dry milk and 0.1% Tween-20 in PBS, pH 7.4. The expression of IRS-2 was identified using polyclonal antibody specific to IRS-2 (1 : 1,000) followed by HRP-labeled goat anti-rabbit secondary antibody (1 : 5,000). Detection was performed with enhanced chemiluminescence and the films were subjected to densitometric analysis using Fuji Multiguage V3.0 Software (Fuji Film). Experiments were repeated three times and a representative blot is shown.

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