



## Engineered $\beta$ -cells secreting dipeptidyl peptidase IV-resistant glucagon-like peptide-1 show enhanced glucose-responsiveness

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### Abstract

Type 2 diabetes is a polygenic disorder characterized by increased insulin resistance, and impaired insulin secretion leading to abnormalities of glucose and lipid metabolism. Reduced responsiveness of the  $\beta$ -cells to glucose is a critical feature of this syndrome. Glucagon-like peptide 1, a product of the pro-glucagon gene makes  $\beta$ -cells competent and has many other anti-diabetic properties. We speculated whether GLP-1-based gene therapy could be an approach for treatment of type 2 diabetes. We started with a clone of rat insulinoma cells (S4 cells), which showed reduced responsiveness to glucose in terms of insulin secretion. We transfected these cells with a plasmid encoding a mutated form of GLP-1 (GLP-1-Gly8), which is resistant to the degrading enzyme dipeptidyl-peptidase IV. Activity of secreted GLP-1-Gly8 was assayed using Chinese hamster lung fibroblasts (CHL) cells that expressed cloned GLP-1 receptor and that were transfected with CRE-Luc. Stable cell lines (Glipsulin cells) obtained by this means produced and stored immunoreactive GLP-1-Gly8. In addition to insulin, the Glipsulin cells secreted the GLP-1-Gly8. The secreted GLP-1-Gly8 was active as evidenced by the ability of the conditioned media to elevate cAMP levels in CHL cells expressing GLP-1 receptors. Glipsulin cells responded to glucose with a 6.8 fold increase in insulin secretion compared to a 2.2 fold increase in the control cells. Our results demonstrate that prolonged exposure to GLP-1-Gly8 secreted by increases glucose-

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responsiveness of these cells. We speculate that engineering GLP-1-Gly8 secretion by  $\beta$ -cells is a potential gene therapeutic strategy to treat diabetes.

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*Keywords:* Diabetes; Pancreatic beta-cells; Insulin secretion; Gene therapy

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## Introduction

Normal pancreatic  $\beta$ -cells secrete insulin briskly in response to a modest increase in the concentrations of glucose and incretin hormones in the plasma. In type 2 diabetes this response becomes sluggish. From a therapeutic viewpoint, an important issue is how to restore normal responsiveness of  $\beta$ -cells to glucose so that normal brisk insulin secretion can take place. In this respect, the incretin hormone glucagons-like peptide-1 has attracted much attention. GLP-1 makes  $\beta$ -cells competent (Holz et al., 1993), sensitises the diabetic  $\beta$ -cells to glucose, and thereby restores normal insulin secretion (Gutniak et al., 1992). GLP-1 is derived from proglucagon. Proglucagon is the translational product of the glucagon gene and contains two glucagon-like peptides (GLP-1 and GLP-2) at the C-terminus of glucagon. Pancreatic  $\alpha$ -cells process the proglucagon and subsequently secrete glucagon, some GLP-1 (in different forms e.g. GLP-1 (1–37), GLP-1(7–37)), glicentin-related pancreatic peptide and the carboxy-terminal extension peptide. The L-cells of the intestine process proglucagon to GLP-1(1–37), GLP-1(7–37), glicentin and an amidated form of intervening peptide II (Holst, 1997). GLP-1 (7–37) and GLP-1(7–37) amide are the biologically active forms of GLP-1. GLP-1 not only makes  $\beta$ -cell competent for glucose-stimulated insulin secretion (Holz et al., 1993) but also inhibits glucagons secretion, slows gastric emptying, and reduces energy intake (Verdich et al., 2001). It also has trophic effects on  $\beta$ -cell (Buteau et al., 1999).

In the islets, the  $\beta$ -cell-aggregate is surrounded by  $\alpha$ -cells which secrete glucagon and some GLP-1 but these locally produced hormones do not act on  $\beta$ -cells (Moens et al., 2002). GLP-1 is rapidly degraded in the circulation by dipeptidyl peptidase IV (DPP-IV). It has been demonstrated that GLP-1 level in some type 2 diabetic patients is low (Mannucci et al., 2000) and it has been suggested that this could be due to increased degradation by DPP-IV (Pala et al., 2003). A synthetic glycine-substituted GLP-1 called GLP-1-Gly8 is resistant to degradation by DPP-IV and is as effective as GLP-1 (Burcelin et al., 1999a). Such GLP-1 derivatives are candidates for treatment of type 2 diabetes (Burcelin et al., 1999a). Under normal circumstances  $\beta$ -cell produces neither glucagon nor GLP-1 (Mojsov et al., 1986). We hypothesized that if insulin-secreting cells could be made to secrete GLP-1 then the hormone could act on the  $\beta$ -cells in an autocrine or paracrine manner and exert beneficial effects. In this paper we demonstrate that insulin-secreting cell lines can be made to secrete a DPP-IV-resistant form of GLP-1 and that such cells become more responsive to the glucose-stimulus in terms of insulin secretion.

## Methods

### *Reagents*

Recombinant human GLP-1 (7–37) and polyclonal rabbit anti-GLP-1 (7–37) (catalogue number IHC7123) were from Peninsula Laboratories, Inc, California, U.S.A. pCRE-Luc plasmid was from

Stratagene Corp (La Jolla, CA, USA). The tissue culture reagents and G418 sulphate were from Invitrogen (Lidingö, Sweden). Rat insulin ELISA kits were from Mercodia AB (Uppsala, Sweden). Sources of other reagents are indicated in the text.

### *Cells culture*

INS-1E cells were a gift from Drs. Claes Wollheim and Pierre Maechler (Geneva). These cells and the new clones derived from them were cultured as described before (Asfari et al., 1992).

### *The Plasmid DNA construct*

The plasmid (pDM2) consisted of the GLP-1-(7–37) coding sequence where the alanine residue at position 8 was replaced by a glycine (GLP-1-Gly8) to make GLP-1 resistant to degradation by DPP-IV (Burcelin et al., 1999b). Upstream of the GLP-1-Gly8 coding sequence, a 30 amino acid prosequence (glicentin-related pancreatic polypeptide) was placed to increase the stability of the newly synthesized polypeptides. A furin cleavage site was added at the GLP-1-Gly8 and the prosequence junction. This would allow secretion of the bioactive peptide by cleavage of the prosequence by the endopeptidase furin. To direct the newly synthesized polypeptide to the secretory pathway, a signal peptide was placed upstream of the prosequence. A signal peptidase removes the signal peptide at the time of translocation of the newly synthesized peptide into the endoplasmic reticulum. Expression of the plasmid pDM2 was driven by the cytomegalovirus promoter.

### *Generation of stable cell lines*

We chose a clonal cell line (S4 cells) derived from the rat insulinoma INS-1E cells. S4 cells secrete insulin in response to stimulation by physiologically relevant concentrations of glucose only modestly. The cells were grown in a humidified incubator (95% air and 5% CO<sub>2</sub>) at 37 °C in RPMI 1640 culture medium containing 5% heat-inactivated fetal bovine serum, 50 µM 2-mercaptoethanol, 11 mM Glucose, 10 mM Hepes, 1 mM sodium pyruvate, 1 mM L-glutamine and 50 µg per ml gentamycin. In preliminary experiments, we evaluated the optimal dose of the selective antibiotic G418. The optimal concentration for selection was 400 µg per ml. Cells grown to ~70% confluence in 10 cm Petri-dishes were transfected by using the plasmids pDM2 (40 µg), and pEGFP-Luc (4 µg) by 25 kDa polyethylenimine (PEI)-based transfections reagent (5 µg of plasmid/2.4 µl of 0.1 M PEI solution). pEGFP-Luc is a hybrid of the EGFP and the luciferase genes. Transfection with pDM2 and pEGFP-Luc allowed double selection by G418 and also by identifying green cells with a fluorescent microscope. The transfections efficiency was ~30–40% as monitored by GFP expression under fluorescence microscopy. Stable cell lines were made following standard procedures (Sambrook and Russel, 2001). The expression of pDM2 was evaluated also by RT-PCR. Seven of the selected clones were expanded.

### *Immunocytochemistry*

We used polyclonal rabbit anti-GLP-1 (7–37) (Peninsula Laboratories, Inc., Catalog. Nr. IHC 7123). This antibody reacts with GLP-1 (7–37) and does not react with GLP-1 (7–36) or glucagon. The secondary antibody was swine anti-rabbit immunoglobulin (1:200) (Dako, code number Z

0196) and the tertiary antibody was polyclonal rabbit anti-swine immunoglobulin conjugated with horse-radish peroxidase (1:200) (Dako, code number P 0164). All anti-sera were diluted in 0.1% BSA-c (Aurion) in TBS consisting of 50 mM Tris, 150 mM NaCl, pH 7.6. Cells cultured on glass slides were washed with 0.1% Tween-20 in PBS (pH-7.4) for 5 minutes, fixed with 100% acetone for 10 minutes and washed three times. Samples were then blocked by 0.1% BSA-c in TBS for 1 hour. Slides were incubated overnight with the primary antibody (1:500), washed and incubated with the secondary antibody for 30 minutes. After washing again, incubation was done with the peroxidase-conjugated tertiary antibody for 30 minutes. 3,3'-Diaminobenzidine (Sigma) was used as a substrate system for peroxidase. Slides were mounted in an aqueous mounting medium (Dako).

#### *Assay for bioactivity of secreted GLP-1-Gly8*

For bioassay of GLP-1 activity, we used a Chinese hamster lung fibroblast cell line (clone 5 CHL cells) that was transformed to express the cloned GLP-1 receptor (Widmann et al., 1994). These cells were cultured in DMEM containing G418 (200 µg/ml) and FCS (10%). The cells were grown to ~70% confluence and then transfected with the pGTC50CMVLucN4 plasmid (in this plasmid the luciferase gene is driven by the CMV promoter) and the luciferase activity was analyzed 40 hrs later. There was strong luciferase activity in these experiments (about 3445-fold higher than cells that had not been transfected with this plasmid) suggesting that the clone 5 CHL cells can be transfected efficiently under our experimental conditions and that the luciferase gene can be used as a read out system.

We used the PathDetect CRE *cis*-Reporting system (Stratagene) to estimate the cAMP concentration in the clone 5 CHL cells as a measure of the amount of GLP-1 or GLP-1-Gly8 secreted by the S4 cells or the transfected cells (Chepurny and Holz, 2002). Clone 5 CHL cells were transfected with pCRE-Luc by the PEI method. In this plasmid, expression of the luciferase gene is directed by a multimerised synthetic cAMP response element. CRE-Luc consists of 4 CREs with the sequence 5'-(AGCC[TGACGTCA]-GAG)-3'. Non-transfected S4 cells and the two clones of the transfected cells (clone 1 and clone 3) were grown for one week and the supernatants collected and frozen at -20 °C. On the day of the experiments the conditioned media were thawed. Two µl of the conditioned media or recombinant GLP-1 was added to the transfected clone 5 CHL cells in 24 well plates (100,000 cells per well). After overnight incubation clone 5 CHL cells were washed, lysed and luciferase activity was measured using a luciferase assay kit and a TD-20/20 luminometer (Turner Designs).

#### *Insulin secretion*

Cells (200,000/well) were seeded in 24-well plates and cultured for 6–7 days before using them for insulin release assay. On the day of experiment, cells were incubated in RPMI medium without glucose for 2 hours. Cells attached to the wells were then washed three times with warm (37°C) medium containing (in mM): NaCl 140, NaHCO<sub>3</sub> 2, KCl 3.6, NaH<sub>2</sub>PO<sub>4</sub> 0.5, MgSO<sub>4</sub> 0.5, Hepes 10, CaCl<sub>2</sub> 1.5, BSA 0.1% and incubated for 30 minutes at 37°. Cells were then incubated with 500 µl of the test solutions, by adding solution to one well at a time, every 20 sec. After one hour of incubation, 200 µl of supernatant was transferred to Eppendorf tubes, again one well at a time, every 20 sec. The aliquots were placed on ice. The collected materials were then immediately centrifuged to guard

against measuring content of insulin in any whole cells that might have detached from the well and contaminated the buffer. Supernatants were kept on ice used for insulin ELISA.

### Statistical analyses

Numerical data are presented as means  $\pm$  S.E. In insulin release assays, statistical significance were judged by Student's *t*-test for unpaired data. Mann-Whitney U test was used for judging statistical significance in luciferase assays.

## Results

For the purpose of this study, we chose a clone of insulin-secreting cells derived from the INS-1E cells. This clone of cells (S4 cells) secreted insulin when medium glucose concentration was increased from 3 mM to 11.2 mM. However, the increase in insulin secretion was only about two fold. We tested whether engineering of these cells to secrete GLP-1-gly8, in addition to insulin, could enhance the glucose-induced insulin secretion from these cells. S4 cells were transformed to express the pDM2 plasmid. Twelve different clones were identified and seven clones were expanded. Two of the clonal cell lines (Glipsulin cells, clone 1 and clone 3) were chosen for further evaluation.

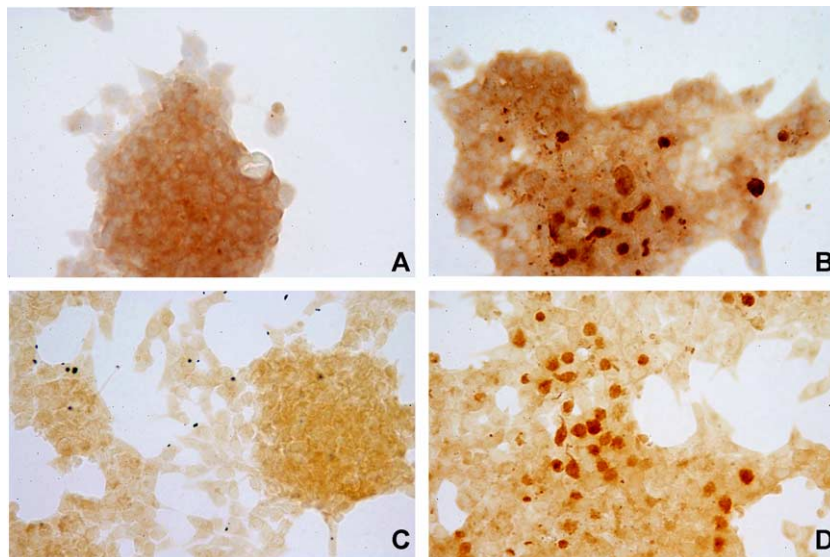


Fig. 1. Detection of GLP-1 immunoreactivity in the cells. Figure shows GLP-1 immunoreactivity in non-transfected S4 cells (control cells) and one clone of Glipsulin cells (clone 3). Slides were stained with polyclonal rabbit anti-GLP-1 (7–37) antibody as described in the methods section. (A). Control S4 cells without primary antibody; (B). Control S4 cells with primary antibody; (C). Glipsulin cells (clone 3) without primary antibody; and (D). Glipsulin cells (clone 3) with the primary antibody. The figures are representative of experiments that have been repeated at least three times.

In Fig. 1, cells were stained with an antibody against GLP-1. This antibody does not cross react with glucagon and it cannot distinguish between GLP-1 and GLP-1-Gly8. GLP-1-like immunoreactivity was present in both the non-transfected S4 cells (Fig. 1B) and in the Glipsulin cells (clone 3) (Fig. 1D). Although these cells are clonal, the level of GLP-1-like immunoreactivity was not equal in all of the cells. However, since immunocytochemistry as used in our study is not a quantitative method, it was not possible to ascertain whether Glipsulin cells had more GLP-1-like immunoreactivity compared to the control cells. For a quantitative analysis of GLP-1-Gly8 secretion we used the clone 5 CHL cells that express the cloned GLP-1 receptor. These CHL cells were transiently transfected with a luciferase-based reporter pCRE-Luc. CRE-Luc consists of a promoter

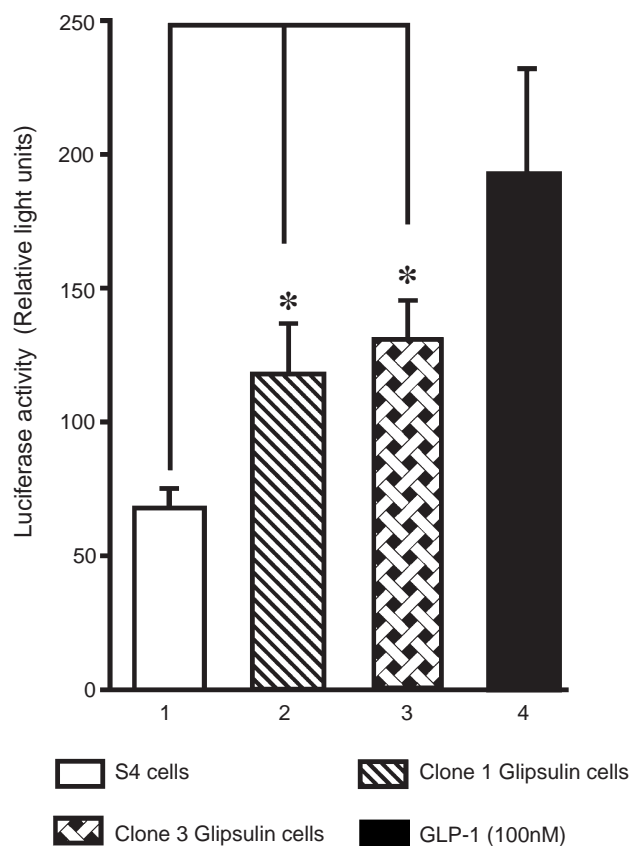


Fig. 2. Stimulation of luciferase activity in clone 5 CHL cells expressing GLP-1 receptor by the conditioned media. Effect of secreted GLP-1 Gly8 was estimated from stimulation of luciferase gene expression under the transcriptional control of a CRE-promoter. Clone 5 CHL cells expressing GLP-1 receptor were transfected with the pCRE-Luc *cis*-reporter plasmid and treated with the conditioned media obtained from cultures of S4 cells or Glipsulin cells (clone 1 and clone 3). Clone 5 CHL cell lysates were measured for luciferase activity. Luciferase activity is expressed as relative light units. In column 1 conditioned medium from non-transfected S4 cells was added. In column 2 and 3, conditioned media from cultures of clone 1 Glipsulin cells and clone 3 Glipsulin cells, respectively, were added. Conditioned media obtained from both clone 1 and clone 3 cells increased luciferase activity significantly (\*  $p < 0.05$ ) as compared to the luciferase activity stimulated by the conditioned medium obtained from the non-transfected S4 cells. Column 4 shows luciferase activity stimulated by recombinant GLP-1 (100 nM).

incorporating multimerized cAMP response elements (CREs) and is sensitive to increases in cAMP levels. We collected the conditioned media from the culture flasks after culturing the S4 cells and the Glipsulin cells for one week. Under such conditions native GLP-1 secreted into the medium is degraded by the DPP-IV present in the serum. The conditioned medium obtained from the non-transfected S4 cells did not stimulate CRE-Luc activity in clone 5 CHL cells as compared to the activity in control experiments where no addition was made. Recombinant GLP-1 increased CRE-Luc activity indicating that the method is suitable for bioassay of GLP-1 activity (Fig. 2). Conditioned medium obtained from both the clone 1 and clone 3 Glipsulin cells increased CRE-Luc activity significantly ( $p < 0.05$ ) compared to that obtained from the non-transfected S4 cells (Fig. 2). These results suggest that GLP-1-Gly8 expressed in the Glipsulin cells is processed normally and that the secreted GLP-1-Gly8 is biologically active even in the presence of DPP-IV in the culture medium.

We next tested insulin secretion in response to glucose stimulation in the control S4 cells and the Glipsulin cells. As shown in Fig. 3, control S4 cells responded to glucose stimulation with a  $2.18 \pm 0.12$

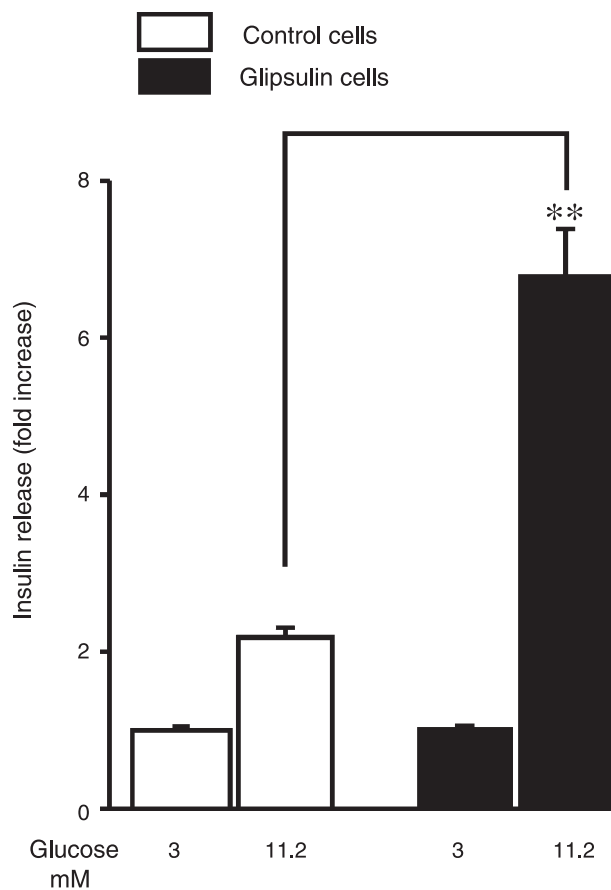


Fig. 3. Glucose-stimulated insulin secretion in the Glipsulin cells. Cells were incubated for 1 hour in the presence of low (3 mM) or high (11.2 mM) glucose. Glucose-stimulated insulin secretion was  $2.18 \pm 0.12$  fold in the non-transfected S4 cells (open bar) and  $6.77 \pm 0.6$  fold in the Glipsulin cells (clone 3) (solid bars) (\*\*  $p < 0.001$ ). The results represent mean  $\pm$  S.E.M of four experiments.

fold increase in insulin secretion. Under similar conditions, Glipsulin cells (clone 3) responded to glucose stimulation with a  $6.77 \pm 0.6$  fold increase in insulin secretion ( $p < 0.001$ ).

## Discussion

There is currently considerable interest about the prospective use of GLP-1 in the treatment of type 2 diabetes. However, this hormone has a very short half-life and has to be administered by continuous infusion. Normal  $\beta$ -cells and insulinoma cells possess GLP-1 receptors (Kang et al., 2001). We reasoned that it would be advantageous if  $\beta$ -cells could synthesize and secrete their own bioactive GLP-1. In this study, we started with a rat insulinoma cell line (S4 cells) that responded to glucose stimulation with only a two-fold increase in insulin secretion. We found that these insulinoma cell lines contain GLP-1, which probably results from aberrant expression of the proglucagon gene. However the level of secretion of GLP-1 from these cells is probably low and the secreted GLP-1 will be readily degraded by the DPP-IV present in the serum in the culture medium. Thus, there was no detectable GLP-1-activity in the conditioned medium collected from the non-transfected S4 cells. We transfected S4 cells and obtained the stable Glipsulin cells that express GLP-1-Gly8, which is resistant to degradation by DPP-IV. Our results suggest that the GLP-1-Gly8 expressed in the Glipsulin cells is normally cleaved at the furin cleavage site. This was expected since the normal  $\beta$ -cells and insulinoma cells express endogenous furin (Kayo et al., 1996; Sawada et al., 2001). The GLP-1-Gly8 secreted by Glipsulin cells is biologically active. Thus conditioned media obtained from the Glipsulin cell clones stimulated CRE-Luc activity in clone 5 CHL cells expressing GLP-1 receptor. Consistent with this, the glucose-stimulated insulin secretion in the Glipsulin cells was significantly higher than that in the non-transfected S4 cells. The increased glucose-responsiveness of the Glipsulin cells is consistent with the fact that GLP-1 makes  $\beta$ -cells competent (Holz et al., 1993) and that it promotes differentiation of insulinoma cells (de la et al., 2001). Multiple mechanisms are involved in mediating stimulation of insulin secretion by GLP-1. These include regulation of ATP-sensitive  $K^+$  channels, voltage-gated  $Ca^{2+}$  channels, voltage-dependent  $K^+$  channels, non-selective cation channels, the ryanodine receptors, regulation of cellular energy homeostasis and exocytosis and regulation of insulin gene transcription (Skoglund et al., 2000; MacDonald et al., 2002).

Our results confirm those reported in an excellent recent study by Hui et al who demonstrate that transfections of MIN6 cells with gene fragment encoding for GLP-1 increase insulin secretion in response to glucose (Hui et al., 2002). In that study, increased insulin secretion was not observed in cells where GLP-1 gene was under the control of CMV promoter. In our study where the GLP-1 gene was under the control of CMV promoter, insulin secretion in response to glucose was dramatically increased. The reason for such differences is unclear. Furthermore, our cells secrete a mutant GLP-1, which is resistant to DPP-IV. The rationale for this approach derives from the consideration that DPP-IV activity in type 2 diabetes may be elevated (Pala et al., 2003). Our approach differs from that of Chepurny et al who over-expressed GLP-1 receptors in insulinoma cells to allow the autocrine action of the small amount of readily degradable GLP-1 that is secreted by the insulinoma cells (Chepurny and Holz, 2002). To what extent our results obtained from a tumour cell line, can be extrapolated to normal and diabetic  $\beta$ -cells remains unclear. However, it is noteworthy that the level of expression of GLP-1 receptor in diabetic  $\beta$ -cells appears to be normal (Gutniak et al., 1992) and that normal  $\beta$ -cells do not secrete any GLP-1.



## Conclusions

We report the establishment of stable Glipsulin cells that synthesize and secrete both insulin and a bioactive DPP-IV-resistant form of GLP-1. Prolonged exposure to GLP-1-Gly8 secreted by these cells improves their glucose-responsiveness. These observations suggest that GLP-1-Gly8-based gene therapy may be a possible approach for type 2 diabetes, a condition where glucose-responsiveness of  $\beta$ -cells is diminished.

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