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REGULAR ARTICLE

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# Over-expression of the glucagon-like peptide-1 receptor on INS-1 cells confers autocrine stimulation of insulin gene promoter activity: a strategy for production of pancreatic β-cell lines for use in transplantation

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**Abstract** To develop transplantable β-cell lines for the treatment of diabetes mellitus, we have taken advantage of the property of INS-1 cells to synthesize and secrete not only insulin, but also small quantities of the insulinotropic hormone glucagon-like peptide-1 (GLP-1). In INS-1 cells over-expressing the β-cell GLP-1 receptor (GLP-1-R), we have shown, by radioimmune assay and bioassay of conditioned medium, that an autocrine signaling mechanism of hormone action exists whereby self-secreted GLP-1 acts as a competence factor in support of insulin gene transcription. INS-1 cells also exhibit insulin gene promoter activity, as assayed in cells transfected with a rat insulin gene I promoter-luciferase construct (RIP1-Luc). The GLP-1-R agonist exendin-4 stimulates RIP1-Luc activity in a glucose-dependent manner, an effect mediated by endogenous GLP-1-Rs, and is blocked by the serine/threonine protein kinase inhibitor Ro 31-8220. Over-expression of GLP-1-R in transfected INS-1 cells reduces the threshold for exendin-4 agonist action, whereas basal RIP1-Luc activity increases 2.5-fold in the absence of added agonist. The increase of basal RIP1-Luc activity is a consequence of autocrine stimulation by self-secreted GLP-1 and is blocked by introduction of (1) an inactivating W39A mutation in the N-terminus ligand-binding domain of GLP-1-R or (2) mutations in the third cytoplasmic loop that prevent G protein coupling. No evidence for constitutive ligand-independent signaling properties of the GLP-1-R has been obtained. Over-expression of GLP-1-R increases the potency and efficacy of D-glucose as a stimulator of RIP1-Luc. Thus, INS-1 cells over-expressing the GLP-1-R recapitulate the incretin hormone effect of circulating GLP-1, thereby providing a possible strategy by which β-cell lines may be engineered for efficient glucose-dependent insulin biosynthesis and secretion.

**Keywords** Glucagon-like peptide-1 · Insulin gene expression · β-Cell lines · Transplantation · Diabetes mellitus · INS-1 cells

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## Introduction

One therapeutic strategy now under investigation for use in the treatment of diabetes mellitus centers on the utilization of islet transplantation, whereby insulin-secreting β-cells are introduced into individuals with compromised endocrine pancreas function (Robertson et al. 2000). Although demonstrated to be efficacious, a major limitation of islet transplantation is the uncertain availability of a ready source of donor pancreatic islets. This dilemma has prompted interest in the development of stem cell or pancreatic ductal cell lines capable of β-cell neogenesis (Serup et al. 2001; Bonner-Weir et al. 2000). Efforts have also been directed at achieving "molecular engineering" of endocrine cell lines, so that cells are optimized for efficient glucose-dependent insulin secretion (Clark et al. 1997). The INS-1 cell line developed by Asfari and colleagues (1992) is a radiation-induced rat insulinoma and is one of the best characterized cell lines suitable for this latter purpose. It is amenable to the molecular engineering approach, and INS-1 cells have indeed been engineered to secrete human insulin in a glucose-dependent manner (Hohmeier et al. 2000).

Ideally, engineering of insulinoma cell lines should achieve a stable phenotype characteristic of fully differentiated β-cells. In particular, these cell lines must exhibit not only robust insulin gene transcription, but also preproinsulin biosynthesis, proinsulin processing, and regulated insulin exocytosis. All of these insulinotropic functions are known to be stimulated by metabolism of D-glucose in mature β-cells (Newgard and McGarry 1995). However, a major draw back to the use of insulinoma cell lines for purposes of transplantation is that such cells exhibit diminished responsiveness to glucose (Clark et al. 1997; Hohmeier et al. 2000; Ferber et al. 1994). One approach to resolving this problem is the randomized subcloning of insulinoma cell variants, which, for reasons that have yet to be elucidated, respond to glucose over the appropriate physiological range of blood glucose concentrations (Hohmeier et al. 2000).

An alternative approach to the engineering of cell lines for use in transplantation has been suggested by our observation that the glucose responsiveness of β-cells is profoundly stimulated by the insulinotropic hormone glucagon-like peptide-1-(7–36)-amide (GLP-1; Holz and Habener 1992; Holz et al. 1993). GLP-1 acts as a physiological incretin hormone in humans, amplifying glucose-dependent insulin gene transcription, insulin biosynthesis, and secretion (Drucker 1998; Kieffer and Habener 1999; Skoglund et al. 2000). The effects of GLP-1 are mediated by GLP-1 receptors (GLP-1-R; Thorens 1992), viz., G-protein-coupled receptors expressed not only on β-cells, but also on most insulinoma cell lines previously investigated. Surprisingly, insulinoma cell lines such as INS-1 and HIT-T15 have a propensity to synthesize not only insulin, but also small quantities of proglucagon, glucagon, and GLP-1 (Philippe et al. 1986; Shennan et al. 1989; Diem et al. 1990; Tateishi et al. 1994; Wang et al. 2001). This propensity may be a consequence of the limited tendency of the insulinoma cell to revert to a precursor multipotent endocrine cell phenotype in which proinsulin and proglucagon biosynthesis coexist (Wang et al. 2001). With this information in mind, we reasoned that self-secreted GLP-1 might act in INS-1 cells as an autocrine factor to amplify glucose-dependent insulin biosynthesis and/or secretion. Furthermore, the over-expression of GLP-1-Rs on these cells might achieve an autocrine induction of glucose responsiveness over the full physiological range of blood glucose concentrations.

Here, we have tested for such an autocrine stimulatory effect of GLP-1 at the level of insulin gene promoter activity, as assessed in INS-1 cells expressing endogenous GLP-1-Rs or in cells over-expressing recombinant GLP-1-Rs. We report that the autocrine stimulatory effect of self-secreted GLP-1 at the rat insulin I gene promoter (RIP1) is only marginal in INS-1 cells expressing endogenous GLP-1-Rs but is fully apparent in transfected cells over-expressing GLP-1-Rs. In these transfected cells, autocrine stimulation by GLP-1 amplifies the glucose-responsiveness of insulin gene promoter activity, increasing the potency and efficacy of glucose as an insulinotropic stimulus.

## Materials and methods

### Cell culture

INS-1 cells (passages 70–79) were cultured in RPMI 1640 containing 10 mM HEPES, 11.1 mM glucose, 10% fetal bovine serum (FBS), 100 µU/ml penicillin G, 100 µg/ml streptomycin, 2.0 mM L-glutamine, 1.0 mM sodium pyruvate, and 50 µM 2-mercaptoethanol (Asfari et al. 1992). Cells were maintained at 37°C in a humidified incubator gassed with 5% $CO_2$, passaged by trypsinization, and subcultured once a week. HIT-T15, HEK-293, and CHO cells were obtained from the American Type Culture Collection (Rockville, Md.). HIT-T15 cells (passages 60–65) were cultured in Ham's F-12 medium containing 10 mM glucose and supplemented with 10% heat-inactivated horse serum and 2.5% FBS. CHO cells were cultured in Ham's F-12 containing 10% FBS, whereas HEK-293 cells were cultured in DMEM medium containing 10% FBS. Culture media and additives were from GIBCO BRL (Rockville, Md.).

### Plasmid DNA constructs

A –410 bp fragment of RIP1 was fused to the coding sequence of luciferase in pGL3-basic vector (Promega, Madison, Wis.) to generate RIP1-Luc (Fig. 1A; numbering of RIP1 as described in Skoglund et al. 2000). CRE-Luc (Fig. 1B) was from Stratagene (La Jolla, Calif.) and consisted of four CREs with the sequence 5′-(AGCC[TGACGTCA]GAG)-3′ driving expression of luciferase. Wild-type rat GLP-1-R cDNA in pcDNA1 was subcloned into pcDNA3.1Zeo (Invitrogen, Carlsbad, Calif.). Site-directed mutagenesis of the GLP-1-R cDNA was achieved by using the QuickChange Mutagenesis Kit (Stratagene). Trp residue 39 codon 5′-TGG-3′ was converted to Ala by nucleotide substitution to generate codon 5′-GCG-3′. The W39A receptor mutation confers diminished ligand-binding activity to the receptor (Van Eyll et al. 1996) as verified by nucleotide sequencing. The IC3-1 and DM-1 rat GLP-1-R mutants are defective in G protein coupling (Salapatek et al. 1999) and are pcDNA3-based expression constructs in which nucleotide residues 334–336 and 331–333 are deleted, respectively. DNA for all transfections was obtained from transformed JM-109 cells and purified by using the Wizard DNA Purification System (Promega).

### Transfection protocol and luciferase assay for INS-1 cells

Adherent INS-1 cells grown to 40%–60% confluence in Falcon 60-mm tissue culture dishes (Becton Dickenson, Franklin Lakes, N.J.) were transfected by using commercially available reagents consisting of Lipofectamine Plus (GIBCO BRL). Transfection efficiency was 10%–15% as determined by use of a plasmid constructed by our laboratory and in which expression of enhanced green fluorescent protein (pEGFP; Clonetech, Palo Alto, Calif.) was placed under the control of the rat insulin II gene promoter (G.G. Holz, O.G. Chepurny, unpublished). Cells to be transfected with RIP1-Luc were rinsed twice in phosphate-buffered saline, lifted by trypsinization, and suspended in normal cell culture medium containing plasmid DNA and transfection reagents. The cells were plated onto 96-well cell culture plates (Costar 3610) at a volume of 100 µl cell suspension per well containing approximately $5\times10^4$ cells/well. INS-1 cells were exposed to this transfection cocktail for 16 h. The transfection cocktail was then removed and replaced with normal cell culture medium. After an 8-h equilibration in culture medium, the solution was replaced with RPMI 1640 containing 2.8 mM glucose and 0.1% human serum albumin (HSA, fraction V; Sigma, St. Louis, Mo.). After an over-night incubation, the cells were then exposed to RPMI 1640 containing 11.1 mM glucose, 0.1% HSA, and indicated concentrations of exendin-4 (Ex-4) or exendin-(9–39) from Sigma. After a 4-h exposure to test substances, cells were lysed and assayed for luciferase-catalyzed photoemissions by using a luciferase assay kit (Tropix,

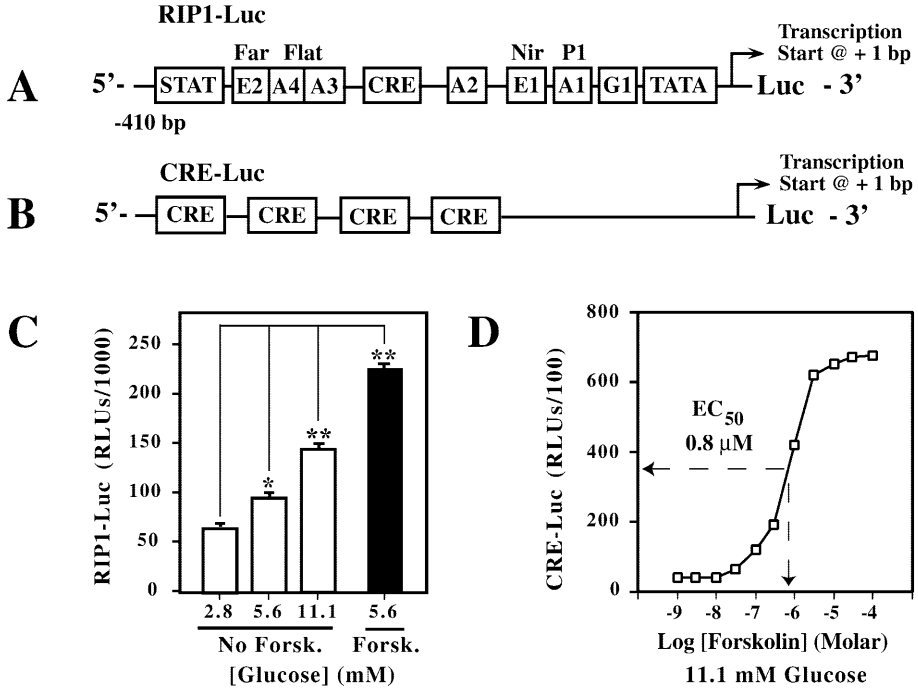


**Fig. 1** **A** Design of the RIP1-Luc construct incorporating regulatory elements (*boxes*) found within –410 bp of the RIP1 sequence. **B** Design of the CRE-Luc synthetic construct incorporating four multimerized CREs. **C** Stimulation by D-glucose and forskolin of luciferase expression in INS-1 cells transfected with RIP1-Luc. Cells were equilibrated in RPMI 1640 medium containing the indicated concentrations of glucose for 24 h prior to the experiment. Exposure to forskolin (2 μM) was for 4 h. Luciferase activity of INS-1 cell lysates is expressed as Relative Light Units (*RLUs*). Statistical significance is indicated as $*P \leq 0.005$ or $**P \leq 0.001$ and is expressed relative to values of RIP1-Luc activity obtained with 2.8 mM glucose. **D** Dose-response relationship describing the stimulatory effect of forskolin (4-h exposure) on luciferase activity measured in INS-1 cells equilibrated in RPMI 1640 medium containing 11.1 mM glucose and transfected with CRE-Luc

Bedford, Mass.) and a dual injection-port luminometer allowing automated application of ATP and luciferin solutions (Model TR-717, Perkin Elmer Applied Biosystems, Foster City, Calof.). Experiments with HEK-293 or CHO cells were performed as described for INS-1 cells, except that the duration of exposure to CRE-Luc during the transfection procedure was 4 h. In addition, these cells did not undergo glucose and serum deprivation, as was the case for INS-1 cells. Experiments were carried out in triplicate. Statistical analysis was performed with the ANOVA test combined with Fisher's PLSD test.

### Radioimmune assay for GLP-1

GLP-1-like immunoreactivity in alcohol extracts of INS-1-cell-conditioned medium was determined by radioimmune assay (RIA) with an immunoassay kit (Linco Research, St. Charles, Mo.). The guinea pig anti-GLP-1-(7–36)-amide antibody is directed at the N-terminus of GLP-1 and recognizes only the active forms of the hormone. $^{125}$I-GLP-1-(7–36)-amide was purified by high-pressure liquid chromatogaphy, and the assay was run under disequilibrium binding conditions at 4°C. A standard curve was constructed by logit-log transformation of the binding data. The $EC_{50}$ for displacement of $^{125}$I-GLP-1-(7–36)-amide by non-radioactive GLP-1-(7–36)-amide synthetic standard was 70 pM, and the lower limit of detection was 5 pM. Assays were performed in triplicate. The intra- and inter-assay co-efficients of variation were 5% and 8%, respectively.

## Results

### Luciferase reporters for analysis of GLP-1-R-mediated stimulus-transcription coupling

Signal transduction properties of the GLP-1-R were initially evaluated in INS-1 cells transiently transfected with either of two types of luciferase-based reporters, viz., RIP1-Luc or CRE-Luc. RIP1-Luc (Fig. 1A) incorporates –410 bp of RIP1 fused to the coding sequence of firefly luciferase and contains regulatory elements that confer sensitivity to glucose and GLP-1. CRE-Luc (Fig. 1B) consists of a synthetic promoter incorporating multimerized cAMP response elements (CREs) and is sensitive to cAMP-elevating agents such as forskolin, in addition to GLP-1. The CRE of RIP1-Luc confers novel signaling properties to the insulin gene promoter because its nucleotide sequence (5′-TGACGTCC-3′) deviates from the consensus palindromic CRE found within CRE-Luc (5′-TGACGTCA-3′) by a single nucleotide substitution. When transfected into INS-1 cells, RIP1-Luc activity is stimulated by a 24-h exposure to glucose over a concentration range of 2.8–11.1 mM of the sugar (Fig. 1C). Furthermore, the action of glucose is potentiated by a 4-h treatment with forskolin (Fig. 1C). In contrast, CRE-Luc is relatively insensitive to glucose but is stimulated by treatment with forskolin over a concentration range of 0.1–10 μM (Fig. 1D).

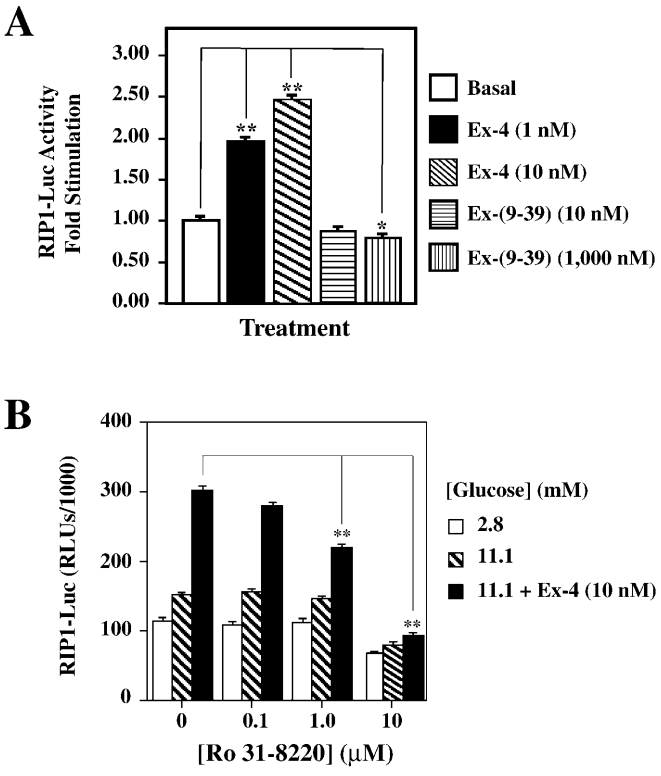


**Fig. 2** **A** Pharmacological properties of endogenous GLP-1 receptors expressed on INS-1 cells. Cells were transfected with RIP1-Luc and equilibrated in RPMI 1640 medium containing 11.1 mM glucose. Cells were then exposed for 4 h to the indicated concentrations of Ex-4 or exendin-(9–39) (*Ex-(9–39)*). Luciferase activities are expressed as Fold Stimulation relative to a basal value of 1.0 determined in the absence of added peptides. **P*≤0.05 or ***P*≤0.001 indicates statistical significance relative to basal values. **B** Dose-response relationship describing the inhibitory action of protein kinase inhibitor Ro 31–8220. INS-1 cells transfected with RIP1-Luc were pretreated with the indicated concentrations of Ro 31–8220 for 30 min at 37°C in RPMI 1640 medium containing 2.8 or 11.1 mM glucose. Cells were then exposed for 4 h to RPMI 1640 containing Ro 31–8220, glucose, and Ex-4. ***P*≤0.001 indicates statistical significance relative to RIP1-Luc activity measured in the absence of Ro 31–8220

## Pharmacological properties of endogenous GLP-1-Rs expressed on INS-1 cells

Endogenous receptors for GLP-1 on INS-1 cells mediate stimulatory actions of Ex-4, a GLP-1-R agonist (Goke et al. 1993). To demonstrate that the stimulatory action of Ex-4 at RIP1-Luc was indeed mediated by GLP-1-Rs, transfected INS-1 cells were subjected to an over-night exposure to RPMI 1640 containing 2.8 mM glucose, 0.1% HSA, and no added FBS. Cells were then transferred to medium containing 11.1 mM glucose and 0.1% HSA for treatment with various pharmacological agents. Under these conditions, a 4-h exposure to 10 nM Ex-4 produced a 2.5-fold increase of RIP1-Luc activity (Fig. 2A). In contrast, the GLP-1-R antagonist exendin-(9–39) produced a small but statistically significant suppression of basal RIP1-Luc activity when tested at a concentration of 1000 nM, but not 10 nM (Fig. 2A). These pharmacological properties of GLP-1-Rs expressed on INS-1 cells match closely the properties of endogenous GLP-1-Rs expressed on rat pancreatic β-cells (Goke et al. 1993).

## Kinase inhibitor Ro 31–8220 blocks stimulatory effects of Ex-4 at RIP1

A series of preliminary experiments (data not shown) demonstrated that the stimulatory action of Ex-4 at RIP1-Luc was not blocked by a 30-min pretreatment and subsequent 4-h exposure to protein kinase inhibitors that exhibited selectivity for protein kinase A (H-89 or KT572C, 10 μM each), calcium-calmodulin-regulated kinase-II (KN-93, 10 μM), mitogen-activated protein kinases (SB 203580 or PD 98059, 40 μM each), tyrosine kinases (genistein at 10 μM; lavendustin A at 1 μM; PP2 at 120 nM), or phosphatidylinositol 3-kinase (wortmanin at 1 μM or LY 294002 at 100 μM). However, the action of Ex-4 was blocked in a concentration-dependent manner by Ro 31–8220 (Fig. 2B), a serine-threonine protein kinase inhibitor that exhibits selectivity for protein kinase C (Alessi 1997), the RSK ribosomal S6 kinases (Alessi 1997; Xing et al. 1996), and the MSK mitogen/stress-activated protein kinases (Deak et al. 1998). K-252c (1 μM), an inhibitor of protein kinases A and C, was ineffective in this assay (data not shown).

## Autocrine stimulation of RIP1 as a consequence of GLP-1-R over-expression

As discussed above, the GLP-1-R antagonist exendin-(9–39) exhibited a small, statistically significant, inhibitory effect on basal RIP1-Luc activity when evaluated by using INS-1 cells expressing endogenous GLP-1-Rs. This effect is interpretable in terms of the known antagonist properties of exendin-(9–39) at the GLP-1-R (Goke et al. 1993), or possibly via its action as an inverse agonist to counter the ligand-independent constitutive signaling properties of GLP-1-Rs (Serre et al. 1998). An antagonist action of exendin-(9–39) at the GLP-1-R would be expected to reduce basal RIP1-Luc activity in INS-1 cells only if an autocrine effect of self-secreted GLP-1 is present. If this were the case, it might be predicted that over-expression of GLP-1-Rs would amplify the autocrine signaling pathway and increase the effectiveness of exendin-(9–39) by rendering INS-1 cells highly sensitive to self-secreted GLP-1. As a test of this line of reasoning, INS-1 cells were transfected not only with RIP1-Luc, but also the GLP-1-R cDNA, and assays of luciferase activity were performed 48 h post-transfection.

INS-1 cells over-expressing wild-type (WT) GLP-1-Rs exhibited a marked increase of basal RIP1-Luc activity, as measured after a 4-h incubation in RPMI 1640 medium containing 11.1 mM glucose, but to which no GLP-1 was added (Fig. 3A). No such effect was observed after transfection with empty vector (EV) not

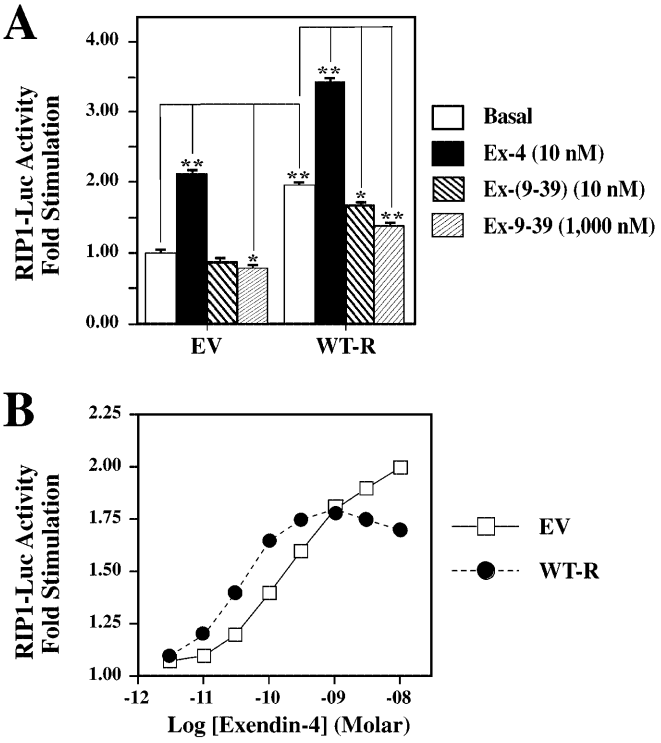


**Fig. 3** **A** Over-expression of GLP-1 receptors on INS-1 cells stimulates basal activity of RIP1-Luc. Cells were transfected with 200 ng/well luciferase reporter, and 10 ng/well wild-type GLP-1-R (*WT-R*) or pcDNA3.1 empty vector (*EV*). Effects of Ex-4 or exendin-(9–39) (*Ex-(9–39)*) were then assessed 48 h post-transfection under conditions in which cells were equilibrated for 4 h in fresh RPMI 1640 medium containing 11.1 mM glucose. Note that, in cells transfected with WT-R, the antagonist action of exendin-(9–39) was enhanced. Values of significance are expressed relative to basal luciferase activities measured in cells transfected with EV or WT-R. *$P \leq 0.05$; **$P \leq 0.001$. **B** Dose-response relationship describing the stimulatory action of Ex-4 measured in INS-1 cells equilibrated in RPMI 1640 medium containing 11.1 mM glucose. Cells were transfected with 200 ng/well RIP1-Luc and 10 ng/well wild-type (*WT*) GLP-1-R or empty vector (*EV*)

containing GLP-1-R cDNA (Fig. 3A). Initially, this finding was interpreted as evidence favoring constitutive ligand-independent stimulatory effects of the GLP-1-R at RIP1. However, it should be noted that the half-life for firefly luciferase in mammalian cells is 3–5 h, so that any stimulatory effect of self-secreted GLP-1 present in the transfection medium might be expected to persist under conditions in which cells are transferred to fresh medium. Therefore, we sought to examine in greater detail the way in which GLP-1-R over-expression influences RIP1 activity.

An autocrine signaling hypothesis is supported by our observation that INS-1 cells secrete GLP-1 (see below), and by our finding that the inhibition of basal RIP1-Luc activity by exendin-(9–39) is magnified in cells over-expressing the GLP-1-R (Fig. 3A; compare EV vs WT GLP-1-R transfectants). This is as expected if cells over-expressing GLP-1-Rs exhibit an increased sensitivity to self-secreted GLP-1, thereby allowing for a stronger antagonist action of exendin-(9–39). Such a conclusion is also supported by our dose-response analysis. We demonstrated that the threshold and $EC_{50}$ values for stimulation of RIP1-Luc by Ex-4 were approximately 30 pM and 200 pM, respectively, for cells expressing endogenous GLP-1-Rs (Fig. 3B, EV transfectants). In contrast, over-expression of the GLP-1-R in the presence of endogenous GLP-1-Rs led to a left-ward shift of the dose-response relationship, such that the threshold and $EC_{50}$ values for stimulation by Ex-4 decreased to 7 pM and 40 pM, respectively (Fig. 3B, WT transfectants).

## Effects of GLP-1-R over-expression require ligand binding and G protein coupling

As an additional, more rigorous test of the autocrine signaling hypothesis, we next determined whether the stimulatory effect of GLP-1-R over-expression required binding of GLP-1 to its receptor. To test this possibility, a W39A substitution was introduced into the extracellular N-terminus of the receptor. This mutation was previously reported to inhibit binding of GLP-1 to its receptor (Van Eyll et al. 1996). INS-1 or HIT-T15 insulinoma cells transfected with wild-type GLP-1-Rs exhibited an increase of basal RIP1-Luc activity and of CRE-Luc activity (Fig. 4A–D). These assays were performed by using cells harvested directly from the transfection medium, thereby demonstrating that the effect of receptor over-expression did not require the transfer of cells to fresh medium (compare Figs. 3A and 4A). Notably, no such increase of RIP1-Luc or CRE-Luc activity was observed after transfection with the W39A mutant receptor (Fig. 4A–D). To confirm that the mutation did indeed influence the binding of GLP-1 to its receptor, wild-type and W39A mutant receptors were expressed in CHO cells, and the effects of Ex-4 at CRE-Luc were evaluated. In cells over-expressing wild-type receptors, the threshold concentration for Ex-4 was determined to be 3 pM, and the $EC_{50}$ was estimated to be 20 pM (Fig. 5A). For cells expressing the W39A mutant, the threshold and $EC_{50}$ values increased to 20 pM and 100 pM, respectively (Fig. 5A).

Autocrine effects of self-secreted GLP-1 would also be expected to require the coupling of GLP-1-Rs to downstream effector G proteins. As a test of this prediction, DM-1 and IC3-1 mutant receptors were prepared in which deletions were introduced into the third intracellular cytoplasmic loop, modifications previously reported to inhibit G protein coupling (Salapatek et al. 1999). When transfected into INS-1 cells, the DM-1 and IC3-1 variants failed to reproduce fully the stimulation of basal RIP1-Luc activity normally observed in cells transfected with wild-type receptors (Fig. 5B; note that, in these cells, the stimulatory action of Ex-4 was mediated by endogenous GLP-1-Rs). These observations indicated that the increase of basal RIP1-Luc activity observed in INS-1 cells over-expressing wild-type receptors probably resulted from autocrine effects of self-secreted GLP-1 act-

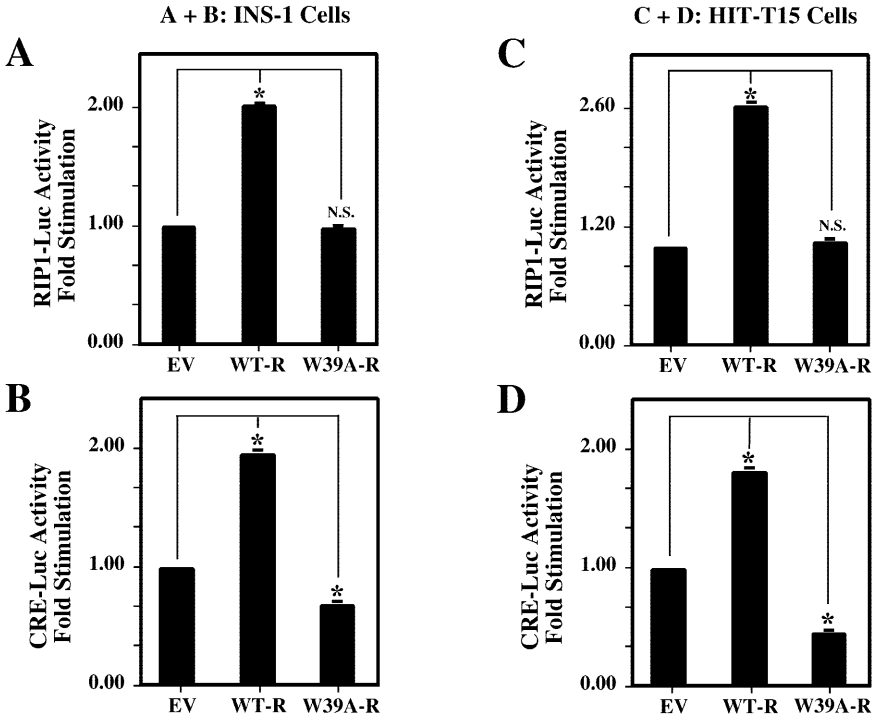


**Fig. 4A–D** Introduction of a W39A mutation into the N-terminus of the GLP-1-R abrogates the stimulatory effects of receptor over-expression at RIP1-Luc or CRE-Luc. INS-1 cells (**A**) or HIT-T15 cells (**C**) were transfected with 200 ng/well RIP1-Luc and 10 ng/well of empty vector (*EV*), wild-type GLP-1-R (*WT-R*), or mutant receptor (*W39A-R*). Basal luciferase activities were then assessed 48 h post-transfection without transfer of cells to fresh medium. Note that the activity of RIP1-Luc was elevated in cells transfected with WT-R but not W39A-R. Similar findings were obtained for INS-1 cells (**B**) or HIT-T15 cells (**D**) transfected with CRE-Luc. **P*≤0.005, *N.S.* Not significant

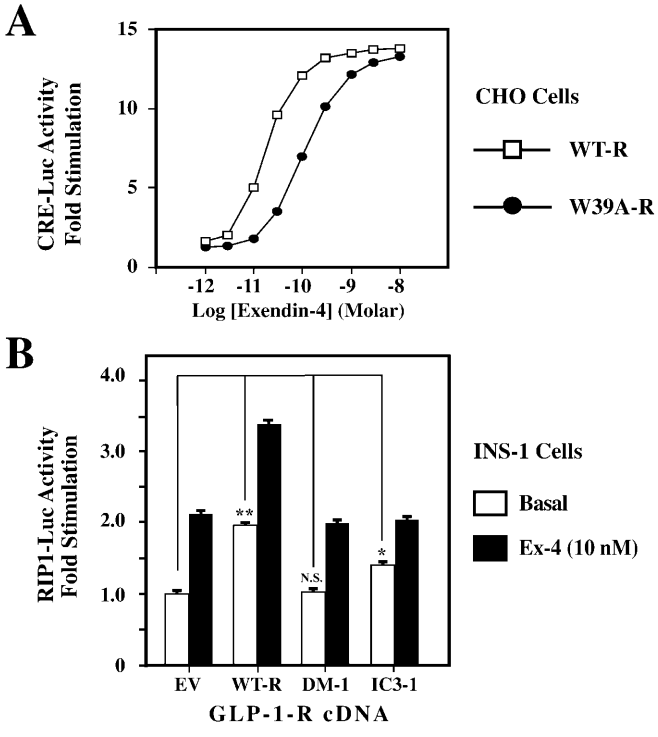


**Fig. 5** **A** Dose-response relationship describing the action of Ex-4 in CHO cells transfected with 200 ng/well CRE-Luc, and 50 ng/well of either wild-type GLP-1-R (*WT-R*) or the W39A mutant receptor (*W39A-R*). **B** Mutations introduced into the third intracellular loop of the GLP-1-R abrogate or diminish stimulatory effects of receptor over-expression, as evaluated in INS-1 cells transfected with RIP1-Luc. Cells were transfected with 200 ng/well RIP1-Luc and 10 ng/well of either WT-R or one of two types of receptor variants incorporating mutations of the third intracellular loop (*DM-1*, *IC3-1*). Note that over-expression of DM-1 or IC3-1 failed to reproduce the stimulatory effect of WT-R when evaluating effects on basal RIP1-Luc activity. The stimulatory effect of Ex-4 measured in INS-1 cells transfected with DM-1 or IC3-1 represents effects of the peptide mediated by endogenous GLP-1 receptors. **P*≤0.01, ***P*≤0.001, *N.S.* Not significant

ing via ligand binding and conventional receptor-mediated G protein coupling.

## INS-1 cells secrete biologically active GLP-1

The ability of INS-1 cells to secrete small quantities of GLP-1 was demonstrated more directly by RIA of conditioned medium. RPMI 1640 medium was exposed to 80% confluent INS-1 cell cultures for 48 h, and extracts of the medium were found to contain detectable quantities of GLP-1-like immunoreactivity (Fig. 6A). Based on this analysis, the concentration of GLP-1 in the conditioned medium was estimated to be 10–15 pM. No such GLP-1-like immunoreactivity was measured in conditioned medium obtained from CHO cells (negative control, data not shown). To demonstrate that the GLP-1-like immunoreactivity found in conditioned medium was indeed biologically active GLP-1, a bioassay was conducted with CHO cells co-transfected with GLP-1-R cDNA and CRE-Luc (Fig. 6B). One set of CHO cells was exposed for 4 h to RPMI culture medium (RPMI-CM) that had previously been used to culture INS-1 cells. A second set of cells was exposed under identical conditions to normal RPMI that was not conditioned.

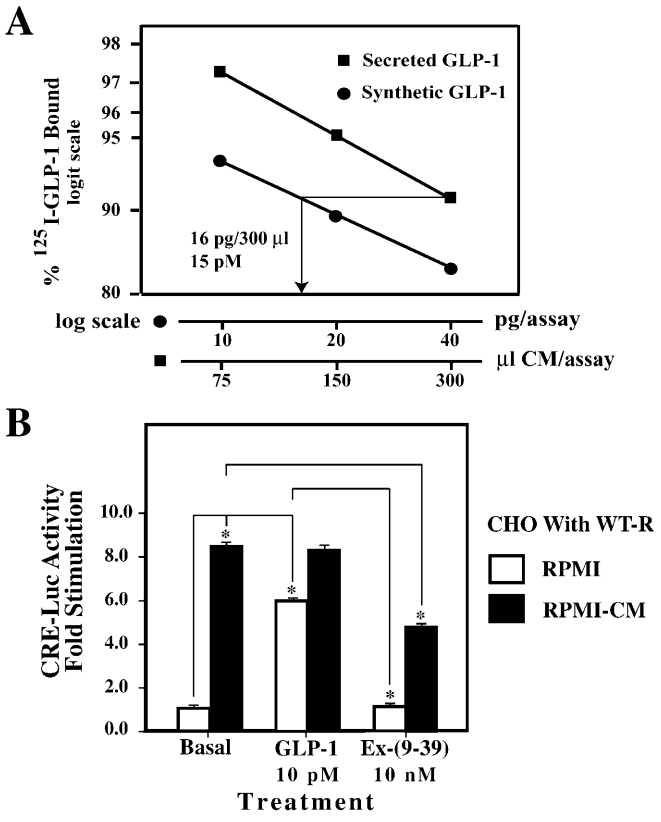


**Fig. 6** **A** INS-1 cells secrete GLP-1, as demonstrated by RIA of culture medium with an antiserum specific for GLP-1-(7–36)-amide and GLP-1-(7–37). Illustrated are parallel displacement curves generated for synthetic GLP-1-(7–36)-amide (*circles*) and GLP-1-like immunoreactivity (*squares*) found in INS-1 cell conditioned medium. Displacement curves were linearized by logit-log transformation of the binding data. A standard curve was generated by assaying serial dilutions of synthetic GLP-1-(7–36)-amide (*upper abscissa*). The *lower abscissa* indicates the volume in microliters of conditioned medium (*CM*) subjected to RIA. **B** INS-1 cells secrete GLP-1, as demonstrated by the use of conditioned media in combination with CHO cells expressing the recombinant GLP-1-R. CHO cells were transfected with 200 ng/well CRE-Luc and 50 ng/well wild-type GLP-1-R (*WT-R*). CHO cells were then exposed to normal culture medium (*RPMI*) or culture medium that was conditioned by a prior 48-h exposure to INS-1 cells (*RPMI-CM*). Note that the basal activity of CRE-Luc was stimulated by RPMI-CM, an effect mimicked by 10 pM GLP-1, but not by RPMI. Note also that the action of RPMI-CM was inhibited by 10 nM of the specific GLP-1-R antagonist exendin-(9–39) (*Ex-(9–39)*). *$P \leq 0.001$

When compared with normal RPMI, the conditioned medium derived from INS-1 cells stimulated an 8.5-fold increase of basal CRE-Luc activity, and this effect was inhibited (Fig. 6B) by pretreatment of CHO cells with exendin-(9–39). No effect of conditioned medium was observed in CHO cells transfected with empty expression vector (data not shown).

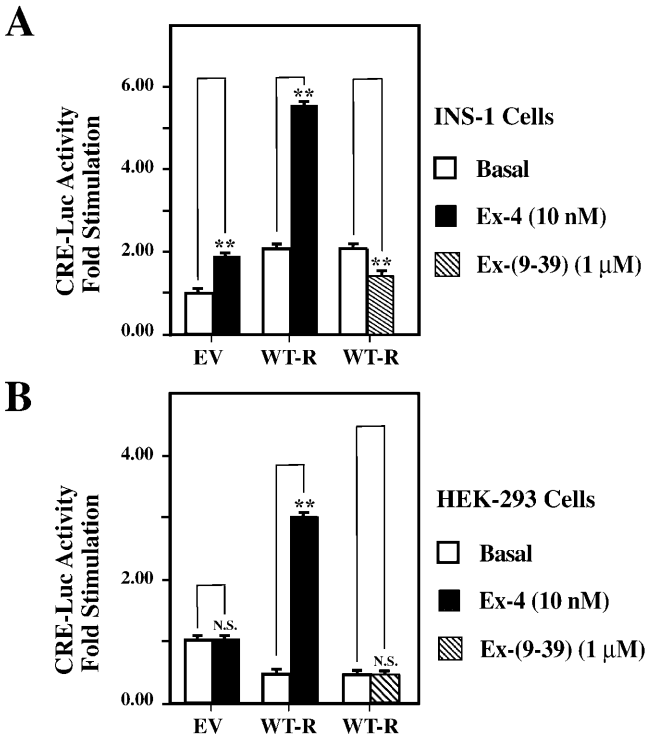


**Fig. 7A, B** A test for ligand-independent constitutive signaling properties of the GLP-1-R. INS-1 cells (**A**) or HEK 293 cells (**B**) were transfected with 200 ng/well CRE-Luc, and either empty vector (*EV*) or the wild-type GLP-1-R (*WT-R*; 10 ng/well for INS-1; 50 ng/well for HEK 293). **A** INS-1 cells equilibrated for 4 h in RPMI 1640 medium containing 11.1 mM glucose exhibited increased basal RIP1-Luc activity when transfected with WT-R. **B** Over-expression of the WT-R on HEK 293 cells produced inhibition of basal CRE-Luc activity. The stimulatory action of Ex-4 was only observed when HEK 293 cells were transfected with WT-R. Under these conditions, no inverse agonist action of exendin-(9–39) (*Ex-(9–39)*) was measured (**B**). **$P \leq 0.001$, *N.S.* Not significant

## A test for constitutive ligand-independent signaling properties of the GLP-1-R

GLP-1-Rs may exhibit ligand-independent constitutive signaling properties, measurable as increased basal cAMP production and increased basal insulin secretion (Serre et al. 1998; Montrose-Rafizadeh et al. 1997). Furthermore, constitutive signaling of the receptor might be reversed by exendin-(9–39) acting as an inverse agonist (Serre et al. 1998). If this were the case, constitutive signaling properties of GLP-1-Rs might also contribute to increased basal RIP1-Luc activity measured in our assay of INS-1 cells over-expressing the GLP-1-R (Fig. 7A). A direct test for constitutive signaling properties of the GLP-1-R expressed on INS-1 cells is not possible since these cells express not only endogenous GLP-1-Rs, but also secrete small quantities of GLP-1. However, a test for constitutive activity is possible by using heterologous expression systems where endogenous GLP-1-Rs and GLP-1 secretion are not present. Therefore, we evaluated the effect of GLP-1-R over-expression by using

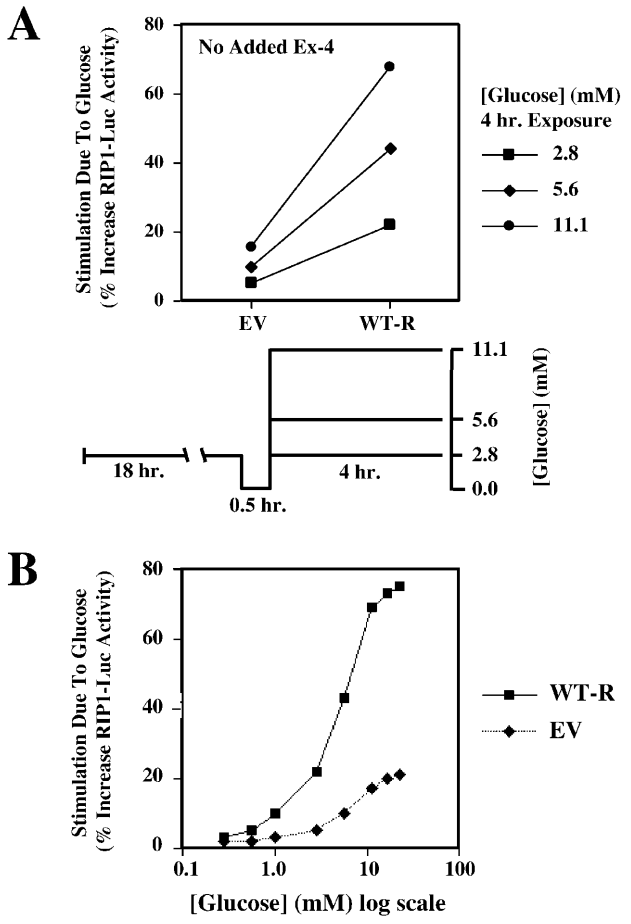


**Fig. 8A, B** Over-expression of the GLP-1-R on INS-1 cells confers increased sensitivity of RIP1-Luc to glucose. **A** INS-1 cells equilibrated in normal RPMI 1640 culture medium containing 11.1 mM glucose were co-transfected with RIP1-Luc (200 ng/well) and 10 ng/well of empty expression vector (*EV*) or wild-type GLP-1-R (*WT-R*). At 30 h post-transfection, cells were transferred to RPMI 1640 medium containing 2.8 mM glucose. Following an 18-h equilibration in medium containing 2.8 mM glucose, the cells were re-equilibrated for 0.5 h in fresh medium containing no added glucose (*bottom panel*). This glucose-free medium was then replaced with fresh medium containing 2.8, 5.6, or 11.1 mM glucose, and cells were allowed to equilibrate for an additional 4 h. Such step-wise increases of glucose concentration produced a dose-dependent increase of basal RIP1-Luc activity that was facilitated in cells over-expressing the GLP-1-R (*top panel*). **B** Dose-response relationship illustrating the short-term (4 h) stimulatory effect of glucose to increase basal RIP1-Luc activity in INS-1 cells transfected with empty expression vector (*EV*) or wild-type GLP-1-R (*WT-R*). Receptor over-expression produced a shift to the left of the glucose dose-response relationship (increased potency) and increased the maximal effect of glucose (increased efficacy)

HEK-293 or CHO cells co-transfected with CRE-Luc. Notably, in these systems, GLP-1-R over-expression produced a marked inhibition of basal CRE-Luc activity, rather than a stimulation (Fig. 7B; data for CHO cells not shown). Furthermore, in these cells, no inverse agonist action of exendin-(9–39) was observed (Fig. 7B).

### Facilitation of glucose-dependent RIP1 activity by GLP-1-R over-expression

Based on the findings summarized above, a strategy for the engineering of insulin-producing cell lines for use in transplantation was suggested. We observed that over-expression of the GLP-1-R facilitated short-term stimulatory effects of glucose on insulin gene promoter activity. Normally, INS-1 cells exhibited glucose-dependent stimulation of RIP1-Luc activity, and this effect was manifest when cells were equilibrated for 24 h at concentrations of glucose between 2.8 and 11.1 mM (see Fig. 1B). However, we observed that glucose was a less effective stimulus when its short-term effects were measured by using an experimental design in which INS-1 cells were initially deprived of glucose and then exposed to a step-wise increase of glucose concentration (Fig. 8A). Notably, the short-term stimulatory action of glucose was potentiated by as much as 4-fold in INS-1 cells over-expressing GLP-1-Rs (Fig. 8A). Dose-response analysis demonstrated that this increased sensitivity to glucose was manifest as an increased potency (left-ward shift of the dose-response relationship) and an increased efficacy (increased effect) when evaluating stimulatory effects of glucose at RIP1-Luc (Fig. 8B).

## Discussion

### Molecular engineering of β-cell lines for use in transplantation

Previous attempts to engineer cell lines that secrete insulin in a glucose-dependent manner have focused on the introduction of the insulin gene into endocrine cell lines of extra-pancreatic origin (Hughes et al. 1992). Attention has also been focused on the optimization of the proximal steps of glucose sensing by the introduction of isoforms of the glucose transporter and the glucokinase characteristic of β-cells (Hughes et al. 1992, 1993). Here, we have explored an alternative approach, viz., the use of insulinoma cell lines that synthesize and secrete insulin and that express the full complement of signal-transducing components found in β-cells. Our approach was prompted by previous studies suggesting that it might be possible to take advantage of the unusual ability of insulinoma cells to secrete small quantities of GLP-1. This secretory capacity has long been recognized (Philippe et al. 1986; Shennan et al. 1989; Diem et al. 1990; Tateishi et al. 1994; Wang et al. 2001), although not fully studied. It is most likely the consequence of the aberrant expression of proglucagon (Wang et al. 2001), a prohormone normally restricted to the α-cells of the islets, some neurons of the central nervous system, and L-cells of the distal intestine. Given that insulinoma cells express GLP-1-Rs, we reasoned that there might exist an autocrine signaling mechanism of hormone action in these cells whereby self-secreted GLP-1 facilitates glucose-dependent insulin biosynthesis and secretion. GLP-

1 is an established competence factor for efficient β-cell glucose signaling (Holz and Habener 1992; Holz et al. 1993), and the innate ability of insulinoma cells to secrete and to respond to GLP-1 might be of considerable significance when developing a strategy for the engineering of cell lines for use in transplantation.

### Demonstration of an autocrine GLP-1 signaling mechanism in INS-1 cells

To explore these possibilities, we demonstrated that INS-1 cells do indeed secrete GLP-1, as demonstrated by RIA of culture medium or by use of conditioned medium in conjunction with a bioassay of CRE-Luc activity in CHO cells expressing GLP-1-Rs. We then demonstrated an autocrine action of self-secreted GLP-1 by assessing the functional consequences of GLP-1-R over-expression in INS-1 cells transfected with RIP1-Luc. Our findings lead us to conclude that GLP-1 is an autocrine stimulator of RIP1, an effect most clearly evident under conditions in which high level expression of GLP-1-Rs is achieved. Indeed, over-expression of GLP-1-R produces a leftward shift of the Ex-4 dose-response relationship and lowers the threshold for agonist stimulation from 30 pM to 7 pM. We conclude that increased sensitivity of transfected INS-1 cells to GLP-1 most likely allows these cells to respond to the 10–15 pM concentrations of GLP-1 present in culture medium after over-night equilibration. This autocrine effect is also observed in HIT-T15 cells and is not restricted to RIP1, since stimulation of basal CRE-Luc activity is also observed after over-expression of GLP-1-R.

It is notable that the autocrine effect of self-secreted GLP-1 was observed under two non-identical experimental conditions. In one set of experiments, cells were placed in fresh RPMI 1640 to which no GLP-1 was added and then assayed for RIP1-Luc activity after a 4-h equilibration. Since the RPMI contained 11.1 mM glucose, the increase of basal RIP1-Luc activity measured in cells over-expressing GLP-1-Rs was probably secondary to glucose-dependent exocytosis of GLP-1. In the second set of experiments, RIP1-Luc activity was measured immediately after removal of the cells from culture medium. Under these conditions, the increase of basal RIP1-Luc activity measured in cells over-expressing the GLP-1-R was the consequence of receptor stimulation resulting from GLP-1 present in conditioned medium. In both types of experiments, basal RIP1-Luc activity increased two-fold, and the sensitivity of RIP1 to glucose was markedly facilitated. Such findings are indicative of the induction of glucose competence by GLP-1 (Holz and Habener 1992; Holz et al. 1993), a unique insulinotropic effect conferred by an autocrine signaling pathway present in INS-1 cells.

### Autocrine stimulation by GLP-1 requires ligand binding and G protein coupling

The mechanism by which GLP-1-R over-expression upregulates RIP1-Luc activity was demonstrated to require ligand binding and receptor/G protein interactions. The effects of GLP-1-R over-expression were markedly reduced after the introduction of the DM-1 or IC3-1 deletions into the receptor's third intracellular loop, a region of the receptor previously demonstrated to play a critical role as a mediator of G protein interactions (Salapatek et al. 1999). It should be noted, however, that previous studies have suggested a possible ligand-independent constitutive activity of the receptor (Serre et al. 1998; Montrose-Rafizadeh et al. 1997), an effect that might be G-protein-mediated and that could contribute to the increased basal RIP1-Luc activity observed in INS-1 cells over-expressing GLP-1-Rs. In this scenario, exendin-(9–39) is proposed to act not simply as an antagonist of GLP-1 binding, but as an inverse agonist with negative intrinsic activity (Serre et al. 1998). Evidence arguing against this interpretation is provided by our demonstration that the stimulatory effect of receptor over-expression was abrogated by introduction of a W39A substitution into the N-terminus of GLP-1-R. This mutation was previously shown to inhibit the binding of GLP-1 to its receptor (Van Eyll et al. 1996), an observation confirmed by our analysis of CHO cells in which the mutant receptor was shown to possess little signaling activity at concentrations of Ex-4 in the 1–20 pM range. Evidently, the W39A mutant receptor is unable to respond to low concentrations of GLP-1, as are present in INS-1-conditioned medium.

In the light of these observations, the increased effectiveness of exendin-(9–39) observed in INS-1 cells over-expressing the GLP-1-R appears to result from its ability to act as a conventional receptor antagonist, thereby blocking stimulatory effects of self-secreted GLP-1. These 4-h studies were performed with fresh medium containing 11.1 mM glucose, a concentration sufficient to support stimulus-secretion coupling and exocytosis of GLP-1 in INS-1 cells. Therefore, it is apparent that self-secreted GLP-1 probably acts in an autocrine manner, accumulating near the plasma membrane at concentrations near-threshold for signaling mediated by endogenous GLP-1-Rs, and at concentrations clearly supra-threshold for signaling mediated by recombinant GLP-1-Rs expressed at high levels.

It should also be noted that our studies of CHO and HEK 293 cells have demonstrated that over-expression of the GLP-1-R leads to an inhibition of basal CRE-Luc activity, not a stimulation. Therefore, our findings with heterologous expression systems confirm a previous study in which no constitutive activity of the GLP-1-R was observed (Chen et al. 1999). The simplest interpretation of the findings presented here is that over-expression of GLP-1-Rs allows INS-1 cells to respond to GLP-1 present in the culture medium. Under these conditions, exendin-(9–39) exerts an antagonist action, whereby it prevents the binding of GLP-1 to its receptor.

### Signal transduction properties of the GLP-1-R that confer insulinotropic action

We have previously reported that the stimulation of RIP1 by GLP-1 results from activation of a protein-kinase-A-independent signaling pathway (Skoglund et al. 2000). In the present study, we extend upon these observations by demonstrating that the GLP-1-R is coupled to a pathway insensitive not only to inhibitors of protein kinase A, but also to calcium-calmodulin-regulated kinase-II, mitogen-activated protein kinases, phosphatidylinositol 3-kinase, and tyrosine kinases. In contrast, we demonstrate that signaling mediated by the GLP-1-R is blocked by Ro 31–8220. This serine-threonine protein kinase inhibitor exhibits selectivity for protein kinase C, RSK, and MSK. Given that the protein kinase C inhibitor K-252c failed to exert an inhibitory effect in our assay, the weight of the current evidence suggests a possible role for RSK- or MSK-like kinases as down-stream effectors of GLP-R signaling.

## Conclusion

It has been reported that over-expression of the GLP-1-R on RIN 1046–38 rat insulinoma cells has a major facilitatory effect on cellular glucose responsiveness (Montrose-Rafizadeh et al. 1997). Receptor over-expression has been shown to augment glucose-induced insulin secretion, even in the absence of exogenously added GLP-1. The effect of receptor over-expression is accompanied by increased basal [cAMP] and is associated with a potentiation of glucose-dependent $Ca^{2+}$ signaling. We suggest that such effects of receptor over-expression are a consequence of the autocrine effect of self-secreted GLP-1, as reported here. Of additional significance is our finding that GLP-1-R over-expression amplifies the acute stimulatory effect of glucose at the insulin gene promoter. This augmentation of glucose-dependent stimulus-transcription coupling might be of significant therapeutic importance given that it would be expected to up-regulate the content of preproinsulin mRNA and insulin, itself. Such an insulinotropic effect might compensate for molecular defects of glucose-dependent insulin biosynthesis, as are known to occur in type-2 diabetes mellitus. In conclusion, the findings presented here suggest a strategy by which insulinoma cell lines used for purposes of transplantation might be optimized for efficient glucose-dependent insulin gene expression.

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## References

Alessi DR (1997) The protein kinase C inhibitors Ro 31–8220 and GF 109203X are equally potent inhibitors of MAPKAP kinase-1beta (Rsk-2) and p70 S6 kinase. FEBS Lett 402:121–123

Asfari M, Janjic D, Meda P, Li G, Halban PA, Wollheim CB (1992) Establishment of 2-mercaptoethanol-dependent differentiated insulin-secreting cell lines. Endocrinology 130:167–178

Bonner-Weir S, Taneja M, Weir GC, Tatarkiewicz K, Song KH, Sharma A, O'Neil, JJ (2000) In vitro cultivation of human islets from expanded ductal tissue. Proc Natl Acad Sci USA 97:7999–8004

Clark SA, Quaade C, Constandy H, Hansen P, Halban P, Ferber S, Newgard CB, Normington K (1997) Novel insulinoma cell lines produced by iterative engineering of GLUT2, glucokinase, and human insulin expression. Diabetes 46:958–967

Chen G, Jayawickreme C, Way J, Armour S, Queen K,Watson C, Ignar D, Chen WJ, Kenakin T (1999) Constitutive receptor systems for drug discovery. J Pharmacol Toxicol Methods 42:199–206

Deak M, Clifton AD, Lucocq JM, Alessi DR (1998) Mitogen- and stress-activated protein kinase-1 (MSK1) is directly activated by MAPK and SAPK2/p38, and may mediate activation of CREB. EMBO J 17:4426–4441

Diem P, Walseth TF, Zhang HJ, Robertson RP (1990) Secretion and degradation of glucagon by HIT cells. Endocrinology 127:1609–1612

Drucker DJ (1998) Glucagon-like peptides. Diabetes 47:159–169

Ferber S, BeltrandelRio H, Johnson JH, Noel RJ, Cassidy LE, Clark S, Becker TC, Hughes SD, Newgard CB (1994) GLUT-2 gene transfer into insulinoma cells confers both low and high affinity glucose-stimulated insulin release. Relationship to glucokinase activity. J Biol Chem 269:11523–11529

Goke R, Fehmann HC, Linn T, Schmidt H, Krause M, Eng J, Goke B (1993) Exendin-4 is a high potency agonist and truncated exendin-(9–39)-amide an antagonist at the glucagon-like peptide 1-(7–36)-amide receptor of insulin-secreting β-cells. J Biol Chem 268:19650–19655

Hohmeier HE, Mulder H, Chen G, Henkel-Rieger R, Prentki M, Newgard CB (2000) Isolation of INS-1-derived cell lines with robust ATP-sensitive $K^+$ channel-dependent and -independent glucose-stimulated insulin secretion. Diabetes 49:424–430

Holz GG, Habener JF (1992) Signal transduction crosstalk in the endocrine system: pancreatic β-cells and the glucose competence concept. Trends Biochem Sci 17:388–393

Holz GG, Kuhtreiber WM, Habener JF (1993) Pancreatic β-cells are rendered glucose competent by the insulinotropic hormone glucagon-like peptide-1(7–37). Nature 361:362–365

Hughes SD, Johnson JH, Quaade C, Newgard CB (1992) Engineering of glucose-stimulated insulin secretion and biosynthesis in non-islet cells. Proc Natl Acad Sci USA 89:688–692

Hughes SD, Quaade C, Johnson JH, Ferber S, Newgard CB (1993) Transfection of AtT-20ins cells with GLUT-2 but not GLUT-1 confers glucose-stimulated insulin secretion: relationship to glucose metabolism. J Biol Chem 268:15205–15212

Kieffer TJ, Habener JF (1999) The glucagon-like peptides. Endocr Rev 20:876–913

Montrose-Rafizadeh C, Wang Y, Janczewski AM, Henderson TE, Egan JM (1997) Over expression of glucagon-like peptide-1 receptor in an insulin-secreting cell line enhances glucose responsiveness. Mol Cell Endocrinol 130:109–117

Newgard CB, McGarry JD (1995) Metabolic coupling factors in pancreatic β-cell signal transduction. Annu Rev Biochem 64:689–719

Philippe J, Mojsov S, Drucker DJ, Habener JF (1986) Proglucagon processing in a rat islet cell line resembles phenotype of intestine rather than pancreas. Endocrinology 119:2833–2839

Robertson RP, Davis C, Larsen J, Stratta R, Sutherland, DE (2000) Pancreas and islet transplantation for patients with diabetes. Diabetes Care 23:112–116

Salapatek AM, MacDonald PE, Gaisano HY, Wheeler MB (1999) Mutations to the third cytoplasmic domain of the glucagon-like peptide-1 receptor can functionally uncouple GLP-1-stimulated insulin secretion in HIT-T15 cells. Mol Endocrinol 13:1305–1317

Serre V, Dolci W, Schaerer E, Scrocchi L, Drucker D, Efrat S, Thorens B (1998) Exendin-(9–39) is an inverse agonist of the murine glucagon-like peptide-1 receptor: implications for basal intracellular cyclic adenosine 3′,5′-monophosphate levels and β-cell glucose competence. Endocrinology 139:4448–4454

Serup P, Madsen OD, Mandrup-Poulsen T (2001) Islet and stem cell transplantation for treating diabetes. BMJ 322:29–32

Shennan KI, Holst JJ, Docherty K (1989) Proglucagon expression, post-translational processing and secretion in SV40-transformed islet cells. Mol Cell Endocrinol 67**:**93–99

Skoglund G, Hussain MA, Holz GG (2000) Glucagon-like peptide-1 stimulates insulin gene promoter activity by protein kinase A-independent activation of the rat insulin I gene cAMP response element. Diabetes 49:1156–1164

Tateishi K, Shima K, Funakoshi A, Yasunami Y, Iwamoto N, Matsuoka Y (1994) Glucagon-like peptide-1 (GLP-1) molecular forms in human pancreatic endocrine tumors resemble those in intestine rather than pancreas. Diabetes Res Clin Pract 25:43–49

Thorens B (1992) Expression cloning of the pancreatic β-cell receptor for the gluco-incretin hormone glucagon-like peptide-1. Proc Natl Acad Sci USA 89:8641–8645

Van Eyll B, Goke B, Wilmen A, Goke R (1996) Exchange of W39 by A within the N-terminal extracellular domain of the GLP-1 receptor results in a loss of receptor function. Peptides 17: 565–570

Wang H, Maechler P, Ritz-Laser B, Hagenfeldt KA, Ishihara H, Philippe J, Wollheim CB (2001) PDX-1 level defines pancreatic gene expression pattern and cell lineage differentiation. J Biol Chem 276:25279–25286

Xing J, Ginty DD, Greenberg M (1996) Coupling of the RAS-MAPK pathway to gene activation by RSK-2, a growth factor-regulated CREB kinase. Science 273:959–963