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Research Article

Functional Characterization of N-Terminally GFP-Tagged GLP-1 Receptor

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The glucagon-like peptide-1 receptor (GLP-1 receptor) mediates important effects on peripheral tissues and the central nervous system. It seems one of the most promising therapeutic targets for treatment of diabetes mellitus type 2. Surprisingly, very little is known about the cellular mechanisms that regulate its function in vivo. One of the approaches to study receptor dynamics, expression, or signaling is using GFP-tagged fluorescent proteins. In this study, we synthesized and characterized N-terminally GFP-tagged GLP-1 (GFP-GLP-1) receptor in CHO cells. We demonstrated that GFP-GLP-1 receptor is weakly expressed in the plasma membranes and is functionally coupled to adenylyl cyclase via heterotrimeric G-proteins, similarly as its wild type.

1. Introduction

The glucagon-like peptide-1 (GLP-1) receptor mediates the effects of GLP-1(7-36)amide or GLP-1(7-37) on peripheral tissues, such as the stimulation of glucose-induced insulin secretion by pancreatic β-cells [1], arterial blood pressure, heart rate [2], and lung surfactant synthesis [3], and in the central nervous system it exerts effects on food and water intake [4] and enhances associative and spatial learning [5]. GLP-1 also induces β-cells proliferation and is protective against apoptosis in both β-cells and neurons [6]. GLP-1 has therapeutic potential; it stimulates insulin secretion and lowers plasma glucose in patients with noninsulin-dependent diabetes mellitus type 2 [7, 8]. Moreover, exendin-4, an exogenous GLP-1 receptor agonist isolated from the Gila monster lizard, has very similar effects [9, 10] and has recently been approved for the treatment of type 2 diabetes.

GLP-1 receptor is a member of the G-protein coupled receptors (GPCRs) and is classified within secretin/vasointestinal peptide (VIP) receptor family B [11, 12]. GLP-1 receptor-mediated signaling involves activation of at least two signaling pathways: (i) adenylyl cyclase/cAMP/protein kinase A pathway which increases the level of cAMP [13–15], inhibits the K_{ATP} channels in β-cells [16, 17], enhances currents through voltage-dependent Ca^{2+} channels [16, 18], and increases the level of free cytosolic calcium in cAMP dependent manner [19] is primarily involved in glucose-stimulated insulin secretion, and (ii) phosphatydilinositol 3-kinase/extracellular signal-related kinase/protein kinase Cξ pathway, which affects β-cell insulin gene transcription and proliferation [20, 21]. In addition, some potential cross talk between these two pathways has been linked to phosphodiesterase [22] and cAMP-GEFII-Rim2 complex [23]. GLP-1 might also stimulate release of inositol trisphosphate (IP_3)-sensitive Ca^{2+} stores because it increases inositol phosphate production in COS-7 cells transfected with the recombinant GLP-1 receptor [14]. Overexpressed GLP-1 receptor couples to different types of α subunits of G-proteins in Chinese hamster ovary cells [24] and sf9 cells [25]. Point and block deletion mutations of the receptor and peptide-based interaction mapping of the receptor revealed the importance of the third intracellular (IC_3) loop in activation of G-proteins and adenylyl cyclase [25–29].

Surprisingly, very little is known about the regulation of membrane expression and trafficking of the GLP-1 receptor in vivo. According to our knowledge only one paper discusses that GFP-tagged GLP-1 receptor interacts with caveolin-1 in association that is necessary for receptor trafficking

to the cell membrane in HEK 293 and MIN6 cells [30]. Works on cellular localization are done with C-terminally GFP-tagged GLP-1 (GLP-1-GFP) receptor [30, 31]. This GLP-1 receptor-GFP neither is displaying the same kinetic data as its wild type nor is fully characterized regarding receptor binding data, G-protein activation and coupling to its downstream effectors such as adenylyl cyclase or phospholipase C. However, the physiological relevance of these studies obtained with C-terminally GFP-tagged GLP-1 receptor is questionable; therefore, further studies are needed to clarify expression and trafficking of the GLP-1 receptor in vivo.

In this study, we used CHO cells to characterize N-terminally GFP-tagged GLP-1 (GFP-GLP-1) receptor. We demonstrated that GFP-GLP-1 receptor is localized in plasma membranes and is functionally coupled to adenylyl cyclase via heterotrimeric G-proteins, similarly as its wild type.

2. Materials and Methods

2.1. Cell Cultures. CHO cells were grown as monolayer culture in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Sigma F2442), 8,9 mg/L L-alanine, 15,0 mg/L L-asparagine, 13,3 mg/L L-aspartatic acid, 14,7 mg/L L-glutamatic acid, 7,5 mg/L glicine, 11,5 mg/L L-proline, 10,5 mg/L L-serine, and 2 mM L-glutamine at 37°C in a 5% CO_2 atmosphere.

Rin m5F cells were grown as monolayer culture in RPMI-1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin at 37°C in a 5% CO_2 atmosphere.

2.2. Constructing GFP-GLP-1 Receptor. The cDNA clone encoding the human GLP-1 receptor in pBluescript SK with the size ≈1.6 kb was subcloned into EcoRI site of either pEGFP-C3 or pcDNA3. CHO cells were transiently transfected with GFP-GLP-1 receptor or wild type GLP-1 receptor. Fluorescent derivative was analyzed by using confocal microscopy and immunoblotting. All cDNA clones were verified by sequencing.

2.3. Overexpression of GFP-GLP-1 Receptor and GLP-1 Receptor in CHO Cells. CHO cells were plated in 24-well plates at 5×10^4 cells/well and incubated 24 hours in growth medium supplemented with 10% fetal bovine serum. Cells were washed with HBSS for 2 times and incubated in 0.4 mL growth medium supplemented with 0.1 mL of OPTIMEM-1 medium with 1.0 μg plasmid DNA/well, 2.5 μL LipofectAMINE2000/1.0 μg of DNA for 24 or 48 hours at 37°C [32]. The same procedure was used to express GFP-GLP-1 receptor in Rin m5F cells.

Cells were plated in 6-well plates at 50×10^4 cells/well and incubated 24 hours in growth medium supplemented with 10% fetal bovine serum. Cells were washed with HBSS for 2 times and incubated in 1.5 mL growth medium supplemented with 0.5 mL of OPTIMEM-1 medium with 4.0 μg plasmid DNA/well, 12.5 μL LipofectAMINE2000/4.0 μg of DNA for 24 or 48 hours at 37°C.

2.4. Whole Cell Competition Binding Assay. After expressing GLP-1 receptor in 24-well plates, CHO cells were rinsed 3 times with HBSS. Cells were incubated together with 100 μL of 200 pM [^{125}I]GLP-1(7-36)amide (100 pM/well) and 100 μL of binding buffer (0.2% BSA, 10 mM NaHepes, pH = 7.4 in HBSS) without or with GLP-1(7-36)amide ranging from 10^{-11} to 10^{-6} M for 30 minutes at 37°C. After incubation, the cells were transferred on ice and washed 3 times with a binding buffer. The total cellular radioactivity was determined by scraping the cells in 0.5 mL of lysis buffer (1% SDS (w/v), 0.2 M NaOH) and counted in a gamma counter. The specific binding was determined by subtracting the nonspecific binding obtained in the presence of 10^{-6} M unlabelled GLP-1(7-36)amide from the total cell associated radioactivity. The same procedure was used to determine the binding of GLP-1(7-36)amide to endogenous receptor in Rin m5F cells (plated at 3.25×10^5 cells/well for 48 hours). Meanwhile, competition binding assay for GFP-GLP-1 receptor was carried out in 6-well plates with 200 μL of [^{125}I]GLP-1(7-36)amide and 200 μL of binding buffer with or without GLP-1. The rest remains the same.

2.5. Membrane Preparation. After expressing a recombinant GLP-1 receptor and GFP-GLP-1 receptor in 6-well plates, CHO cells were harvested by scraping and processed for plasma membranes according to the protocol (Bavec 2004a), but with minor modifications. Cells (for approximately 7.5×10^6) were centrifuged at 500× g for 10 minutes and suspended with 0.75 mL of ice-cold lysis buffer (50 mM NaHepes, pH = 8.0, 0.1 mM EDTA, 3 mM $MgCl_2$, 100 mM NaCl, 10 mM β-mercaptoetanol, and protease inhibitors 2 μM pepstatin and 1 mM phenylmethylsulphonyl fluoride). Cells were lysed by Teflon/glass Potter homogenizer for 10 times at 1500 rpm. Cell lysates were centrifuged at 750× g for 10 minutes. The supernatant was centrifuged at 100.000× g for 60 minutes. The resultant pellet was then suspended in TE buffer (10 mM Tris-HCl, 0.1 mM EDTA; pH = 7.5) and stored at −80°C. All operations were carried out at 4°C. The protein concentration in membrane preparations was between 2.0 and 4.0 mg/mL as determined by Biorad Protein Assay at 595 nm.

2.6. GTPγS Binding Studies. The rate of [^{35}S]GTPγS binding to G-proteins from plasma membranes was followed as previously described [33] with minor modification. Briefly, the membranes (final protein concentration in the assay mixture in recombinant GLP-1 receptor and GFP-GLP-1 receptor enriched CHO plasma membranes preparation was 500 μg/mL) were incubated with 5 mM $MgCl_2$, 1 mM dithiothreitol, 150 mM NaCl, 1 μM GDP, and 1–5 nM [^{35}S]GTPγS (approximately 150.000–200.000 cpm per assay) at 25°C in TE buffer (pH 7.5), for 5 minutes with or without 100 nM GLP-1(7-36)amide. The unbound [^{35}S]GTPγS was washed out by rapid filtration of the reaction mixture through Millipore GF/C glass-fiber filters under vacuum three times with 5 mL of cold TE buffer. After extraction of the radioactive material overnight in 20 mL of Emulsifier-Safe (Packard, USA) scintillation liquid, radioactivity was determined with

LKB 1214 Rackbeta liquid scintillation counter. Blank values were determined by the same procedure in samples in which the membranes were replaced with buffer.

2.7. Cyclic AMP Measurements. After expressing (24 hours posttransfection) GFP-GLP-1 receptor or GLP-1 receptor in 6-well plates, CHO cells were rinsed once with HBSS. Cells were incubated with 500 μL of GLP-1(7-36)amide (100 nM/well) or 10 μM forskolin in stimulating buffer (0.2% BSA, 10 mM NaHepes, pH = 7.4 in HBSS) for 15 minutes at 37°C. After stimulation, stimulating buffer was removed and replaced with 150 μL of TE buffer (50 mM Tris, 5 mM EDTA, pH = 7.4). Cells were lysed by scraping, followed by boiling at 100°C for 5 minutes and centrifuged at 16000× g for 10 minutes. The supernatant was collected and the total content of cAMP was measured by a specific radioimmunoassay (Amersham). The same procedure was used to determine the accumulation of cAMP in Rin m5F cells and CHO wild type cells.

2.8. Immunoblotting Analysis. After expressing GFP-GLP-1 receptor in 6-well plates, CHO cells were rinsed once with HBSS and lysed with 0.2% Triton X-100. Cell lysate were centrifuged at 750× g for 10 minutes. Pellet was resuspended in TE buffer and used for further manipulation. Immunoblotting analysis was performed as described previously [34, 35] with minor modifications. After the run on SDS-PAGE (Hoefer SE600, 16 cm long) with a current of 30 mA (60 mA for two gels) for stacking (2.5 mL 0.5 M Tris-HCl, pH = 6.8, 1.5 mL 40% acrylamide mix 37.5 : 1, 100 μL 10% SDS, 100 μL 10% ammonium persulphate, 10 μL TEMED, 5.8 mL destillated water) and 45 mA (80 mA for two gels) for running gel (7.5 mL 1.5 M Tris-HCl, pH = 8.8, 8.25 mL 40% acrylamide mix 37.5 : 1, 300 μL 10% SDS, 300 μL 10% ammonium persulphate, 20 μL TEMED, 14.25 mL destillated water) for 2-3 hours (EPS 601, Amersham Biosciences) in running buffer (3.0 g Tris base, 14.4 g glycine, 1.0 g SDS in 1l destillated water), proteins were transferred from the gel onto nitrocellulose membranes with a constant current of 400 mA for 5 hours (TE 62 Transphor II) in transfer buffer (1l methanol plus 3,5 l destillated water plus 500 mL TB10x: 60 g Tris base, 288 g glycine). The run and transfer of proteins were cooled at 9°C (MultiTemp III). The proteins on the blot were visualized with 0.2% Ponceou S in 5% CH_3COOH. After staining, the proteins were incubated for 1 hour in blocking buffer (2% bovine serum albumin in TBS; 20 mM Tris-HCl, 500 mM NaCl; pH = 7.5). The blocking buffer was then replaced by a buffer TTBS (0.05% Tween 20 in TBS) and incubated with the primary monoclonal mouse IgG antiGFP (JL-8, Clontech, 1 : 8000) at room temperature for 2 hours. The primary antibodies were washed three-times in TTBS buffer for 10 minutes and incubated with secondary peroxidase-conjugated goat antimouse IgG antibodies (1 : 1000, Calbiochem) at room temperature for 1 hour. After the wash in TTBS buffer three-times for 10 minutes and once in TBS for 10 minutes, the immunoreactive proteins caught on the blot were exposed for 15–60 seconds to Kodak X-Omat film using ECL chemiluminescence detection kit (Amersham Pharmacia Biotech, RPN 2106).

The same procedure was used to determine the expression of GLP-1 receptor in CHO cells, except 5% low fatty acid (0.6 g/100 g milk) milk in TBS for blocking; primary policlonal rabbit IgG anti-GLP-1R (Bernard Thorens, Lausanne, Switzerland, 1 : 1000) and secondary peroxidase-conjugated goat antirabbit IgG antibodies (1 : 1000, Calbiochem) were used in immunoblotting analysis. The rest remains the same.

2.9. Confocal Microscopy. Coverslips with CHO cells expressing GFP-GLP-1 receptor were examined with a Zeiss LSM 510 confocal microscope (Jena, Germany). The fluorescent images were collected by a plan-apochromatic oil immersion objective (63 magnification and the numerical aperture 1.4) using 488-nm Ar-Ion and 543-nm He-Ne laser excitation. GFP-GLP-1 receptor and plasma membrane FM4-64 dye fluorescence were collected through the BP 505–530-nm and LP 560-nm emission filters, respectively.

2.10. Insulin Release. After expressing GFP-GLP-1 receptor in 6-well plates (50×10^4 cells/well), Rin m5F cells were rinsed 3 times with HBSS. Cells were then incubated with 500 μL of buffer (0.2% BSA, 10 mM NaHepes, pH = 7.4 in Krebs-Ringer bicarbonate buffer) without or with 10 nM GLP-1(7-36)amide for 60 minutes at 37°C. Aliquots of the incubation medium were taken for radioimmunoassay of insulin [36]. Insulin secretion was expressed as μU insulin/50×10^4 cells/h.

2.11. Calculations. Displacement binding data were analysed using a nonlinear curve fitting program (Graph Pad Prism 4) and fitted to dose response curve. The bottom parameter was fixed and constant was equal to 0, and the top parameter was put to a constraint to 100. Data are shown as mean ± SEM.

2.12. Statistics. Statistical analysis (one-way ANOVA) was carried out by Prism 4 computer program (GraphPad Software, USA), which was used also for graphical presentation of the results. In ANOVA analysis Tukey-Kramer multiple comparison test was used to reveal significant differences between groups of interest; $P > .05$ was not considered significant.

2.13. Materials. CHO and Rin m5F cells were obtained from ECACC, UK. Acrylamide/bis-acrylamide, SDS, and Tris electrophoresis reagents were from Eurobio (France). OPTIMEM-1 was from Gibco (UK). LipofectAMIN 2000 reagent was from Invitrogen Life Technologies (New Zaland). ECL kit, low molecular protein markers, cAMP radioimmunoassay kit, [^{125}I]GLP-1(7-36)amide, and [^{35}S]GTPγS were obtained from Amersham Pharmacia Biotech, UK. *Tert*-butyloxycarbonyl amino acids were from Bachem, Switzerland, and Chemimpex, USA. Fetal bovine serum, Dulbecco's modified Eagle's medium (DMEM), Hank's Balanced Salt Solution (HBSS), GLP-1(7-36)amide, forskolin, TEMED, and ammonium persulphate were supplied from Sigma Aldrich Co, USA. Nitrocellulose transfer membranes were from Schleicher and Schuell, Germany. Mouse monoclonal anti-GFP (JL-8) was from Clontech, USA. Goat

MVSKGEELFT GVVPILVELD GDVNGHKFSV SGEGEGDATY

GKLTLKFICT TGKLPVPWPT LVTTLTYGVQ CFSRYPDHMK

QHDFFKSAMP EGYVQERTIF FKDDGNYKTR AEVKFEGDTL

VNRIELKGID FKEDGNILGH KLEYNYNSHN VYIMADKQKN

GIKVNFKIRH NIEDGSVQLA DHYQQNTPIG DGPVLLPDNH

YLSTQSALSK DPNEKRDHMV LLEFVTAAGI TLGMDELYKY

SDLELKLRI**R GRRFRRWQRW PSPELPA**MAG APGPLRLALL

LLGMVGRAGP RPQGATVSLW ETVQKWREYR RQCQRSLTED

PPPATDLFCN RTFDEYACWP DGEPGSFVNV SCPWYLPWAS

SVPQGHVYRF CTAEGLWLQK DNSSLPWRDL SECEESKRGE

RSSPEEQLLF LYIIYTVGYA LSFSALVIAS AILLGFRHLH

CTRNYIHLNL FASFILRALS VFIKDAALKW MYSTAAQQHQ

WDGLLSYQDS LSCRLVFLLM QYCVAANYYW LLVEGVYLYT

LLAFSVFSEQ WIFRLYVSIG WGVPLLFVVP WGIVKYLYED

EGCWTRNSNM NYWLIIRLPI LFAIGVNFLI FVRVICIVVS

KLKANLMCKT DIKCRLAKST LTLIPLLGTH EVIFAFVMDE

HARGTLRFIK LFTELSFTSF QGLMVAILYC FVNNEVQLEF

RKSWERWRLE HLHIQRDSSM KPLKCPTSSL SSGATAGSSM

YTATCQASCS

Figure 1: Deduced amino acid sequence of the GFP-GLP-1 receptor. GFP (shaded box); MCS (underlined); 5′-untranslated region of the clone hGLPR-6(20) (bold); human GLP-1 receptor (normal).

antirabbit IgG and goat antimouse IgG horseradish peroxidase conjugates were from Calbiochem, USA. Emulsifier-Safe scintillation liquid was from Packard, USA. Insulin radioimmunoassay kit was from Milipore USA. Tris-HCl and methanol were from Merck, Germany. $MgCl_2$, NaCl, and EDTA were from Kemika (Croatia). Biorad Protein Assay was from BioRad, USA. Restriction and ligation enzymes were from Fermentas; the cDNA clone encoding the human GLP-1 receptor and anti-GLP-1R primary polyclonal rabbit IgG antibodies were gift from Bernard Thorens (Lausanne, Switzerland).

3. Results

3.1. Preparation, Expression, and Localization of the GFP-GLP-1 Receptor. CHO cells were transiently transfected with the N-terminal tagged enhanced green fluorescent protein to GLP-1 receptor called GFP-GLP-1 receptor. The deduced amino acid sequence of the GFP-GLP-1 receptor (non-glycosylated) with the theoretical M_w of 83.5 kDa is represented in Figure 1. Cells expressing fluorescent GFP-GLP-1 receptor are shown in Figure 2(a). In addition, the expression of the GFP-GLP-1 receptor in total membranes from CHO cells was analyzed by SDS-PAGE and followed by protein transfer from the gel onto nitrocellulose membrane. In contrast to the control nontransfected cells (Figure 2(b), lane 3) and EGFP-C3 transfected cells (Figure 2(b), lane 2) protein immunostaining showed protein band with the M_w of 90 kDa (Figure 2(b), lane 1), which might correspond to the glycosylated form (Göke et al. 1994) of GFP-GLP-1 receptor. Much more apparent protein band with M_w of 70–75 kDa (Figure 2(b), lane 1) might correspond to the non-glycosylated form of GFP-GLP-1 receptor. Other two protein bands with M_w 180 kDa and 276 kDa were unidentified.

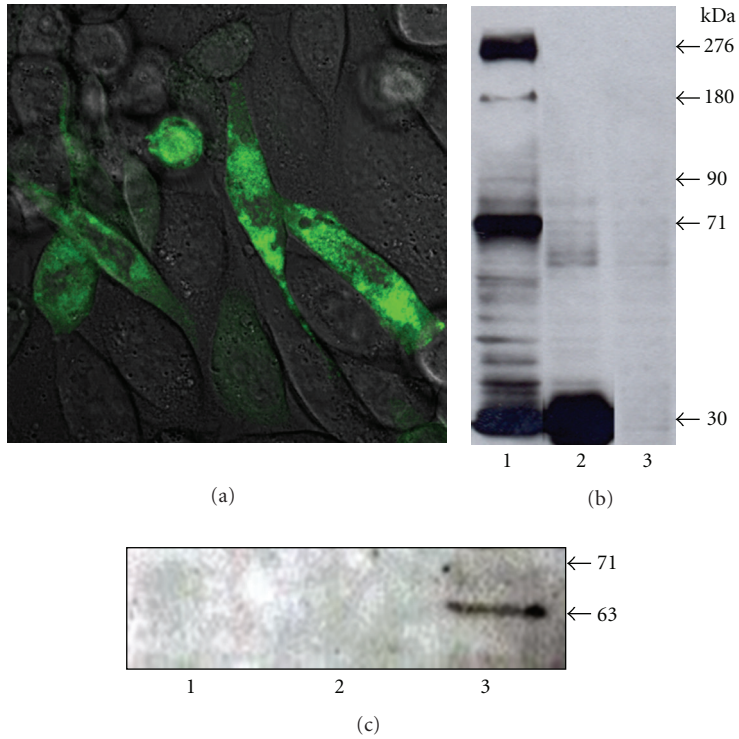

Figure 2: Expression analysis of recombinant GFP-GLP-1 receptor from CHO cells. (a) CHO cells expressing GFP-GLP-1 receptor are viewed under standard light conditions or under argon-ion laser light source (488 nm) 24-hour posttransfection (merge pictures). (b) Western blot analysis with the anti-GFP primary polyclonal mouse IgG antibodies. Lane 1: membranes with the GFP-GLP-1 receptor; lane 2: membranes with the GFP; lane 3: membranes CHO. (c) Western blot analysis with the anti-GLP-1R primary polyclonal rabbit IgG antibodies. Lane 1: membranes CHO; lane 2: membranes with the GFP-GLP-1 receptor; lane 3: membranes with the GLP-1 receptor. The amount of protein loaded in each well in SDS-PAGE was 50 μg.

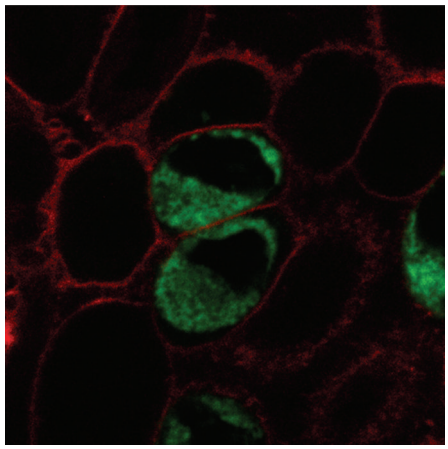

Figure 3: Localization of the GFP-GLP-1 receptor. CHO cells expressing GFP-GLP-1 receptor are viewed under argon-ion laser light source (488 nm) and helium-neon laser light source (543 nm) 24-hour posttransfection. Most of the protein is found outside of the nucleolus, in internal membranes (GFP, green dye), but very small amount in plasma membrane (FM4-64, red dye). Imaging is performed 24–48 hours posttransfection.

We speculate that these high molecular weight protein structures may result due to overexpression or/and high temperature dependant protein aggregation. This promotes formation of GFP-GLP-1 receptor oligomers, such as dimers or tetramers. Protein band with 30 kDa probably represents GFP protein. Unfortunately, we were unable to detect GFP-GLP-1 receptor, both 70–75 kDa and 90 kDa form, with anti-GLP-1R antibodies (Figure 2(c)). It is most likely that GFP tagging hinders epitopes on molecule of GLP-1 receptor [30]. To examine the level of expression and localization of GFP-GLP-1 receptor in CHO cells, confocal imaging was performed. Approximately 25%–40% of CHO cells were efficiently transfected with GFP-GLP receptor. Most of the GFP-GLP-1 receptor was found outside the nucleolus, in internal membranes (Figure 2(a) and Figure 3; protein is dyed in green); meanwhile, its signal from plasma membranes was very weak (Figure 3; plasma membranes are dyed in red).

3.2. Characterization of GFP-GLP-1 Receptor Binding Properties. CHO cells transfected with the wild type receptor construct prepared either in the pcDNA3 (GLP-1 receptor) or in the pEGFP-C3 (GFP-GLP-1 receptor) vectors displayed affinity binding of GLP-1(7-36)amide with IC_{50} of 18.2 ± 0.8 nM and 8.9 ± 3.8 nM, respectively (Table 1 and Figure 4) at 25°C. The number of binding sites for GLP-1 receptor and GFP-GLP-1 receptor on surface of each CHO cell was 102 500 ± 1700 and 1830 ± 220, respectively. The binding GLP-1(7-36)amide to endogenous GLP-1 receptor from RIN

Table 1: Effect of GLP-1(7-36)amide on GLP-1 receptor binding sites from Rin m5F cells and CHO cells transiently expressing human GLP-1 receptor and GFP-GLP-1 receptor.

	IC_{50} (nM)	B_{max} (receptor/cell)
GLP-1 receptor from CHO cells	18.2 ± 0.8	102500 ± 1700
GFP-GLP-1 receptor from CHO cells	8.9 ± 3.8	1830 ± 220
Endogenous GLP-1 receptor from Rin m5F cells	0.56 ± 0.02	1107 ± 121

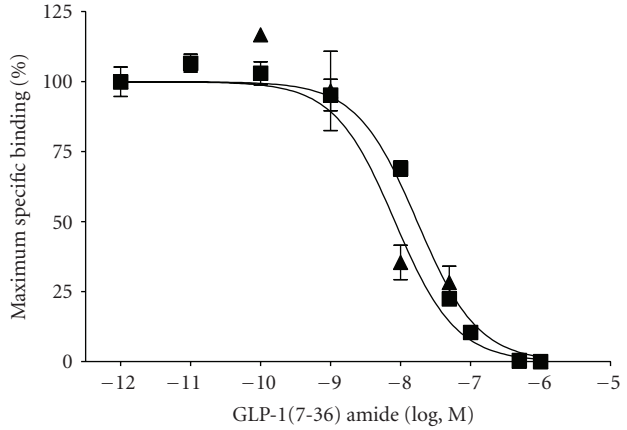

Figure 4: Displacement of [^{125}I] GLP-1(7-36)amide binding to its receptor by GLP-1 from CHO cells. GLP-1 receptor (squares); GFP-GLP-1 receptor (triangles). Each dot with the indicated standard error represents the mean value of three independent experiments done in triplicate (for GLP-1 receptor) and in duplicates (for GFP-GLP-1 receptor). See Results for the details.

m5F cells showed lower values of IC_{50} = 0.56 ± 0.02 nM and lower receptor expression with 1107 ± 121 binding sites per cell (Table 1).

3.3. Functional Coupling of GFP-GLP-1 Receptor with G-Protein. The binding of [^{35}S]GTPγS to G-proteins in plasma membranes from CHO cells is time dependent, and one phase exponential association curve was fitted to the experimental data giving maximal [^{35}S]GTPγS binding of 467 fmol/mg (data not shown). The apparently linear part of the curve (0–5 minutes) was used to assess the initial rate of [^{35}S]GTPγS binding, and the following values were obtained: 13.4 fmol/mg/min for GFP-GLP-1 receptor, 9.8 fmol/mg/min for GLP-1 receptor in CHO overexpressed plasma membranes, and 11.1 fmol/mg/min in the membranes from nontransfected CHO cells. GLP-1(7-36)amide increased the initial rate of [^{35}S]GTPγS binding as compared to the basal activity of CHO transfected cells. Maximal increase of the initial rate of [^{35}S]GTPγS binding was 72% ($P < .01$) over the basal for GFP-GLP-1 receptor and 67% ($P < .05$) over the basal for GLP-1 receptor transfected CHO cells (Figure 5).

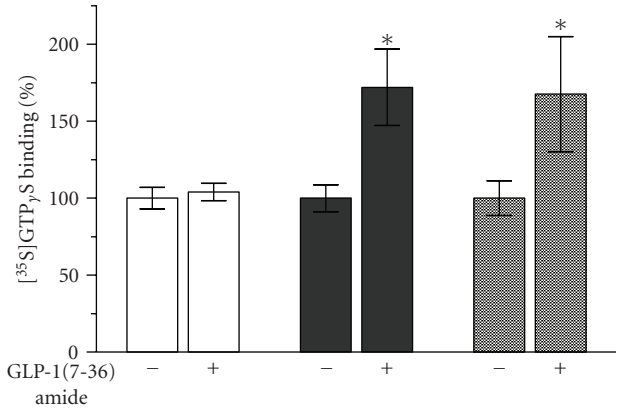

Figure 5: Effect of GLP-1(7-36)amide on the initial rate of [^{35}S]GTPγS binding to the plasma membranes from CHO cells transiently expressing GLP-1 receptor and GFP-GLP-1 receptor. Mock (empty bar); GFP-GLP-1 receptor (filled bar); GLP-1 receptor (patterned bar). 100% = 11.1 fmol/mg/min for CHO, 13.4 fmol/mg/min for GFP-GLP-1 receptor, and 9.8 fmol/mg/min for GLP-1 receptor (basal values, no GLP-1(7-36)amide added). Each bar with the indicated standard deviation represents the mean value of three independent experiments done in duplicate. ANOVA: F(5, 12) = 9.95; P <.001. Tukey-Kramer multiple comparison test was used to reveal significant differences between groups of interest. See Results for the details.

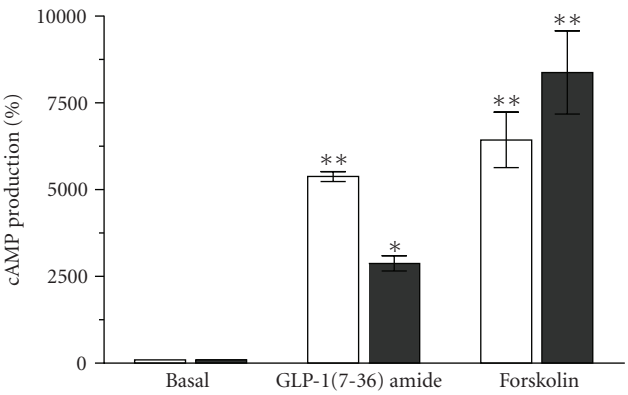

Figure 6: Effect of GLP-1(7-36)amide and forskolin on the production of cAMP from CHO cells transiently expressing GLP-1 receptor and GFP-GLP-1 receptor. GLP-1 receptor (empty bar); GFP-GLP-1 receptor (filled bar). 100% = 56.8 fmol/mg and 37.7 fmol/mg for GLP-1 receptor and GFP-GLP-1 receptor, respectively (basal value, no ligand added). Each bar with the indicated standard deviation represents the mean value of three independent experiments done in duplicate. ANOVA: F(5, 12) = 98.14; P <.0001. Tukey-Kramer multiple comparison test was used to reveal significant differences between groups of interest. See Results for the details.

3.4. The Effect of GFP-GLP-1 Receptor on cAMP Production. We further demonstrated the coupling of the GFP-GLP-1 receptor to adenylyl cyclase. GLP-1(7-36)amide induced cAMP production from GFP-GLP-1 receptor and GLP-1 receptor-transfected CHO cells revealed 2900% (P <.001) and 5400% (P <.001) increase over the basal, respectively (Figure 6). The observed effect in both types of transfected

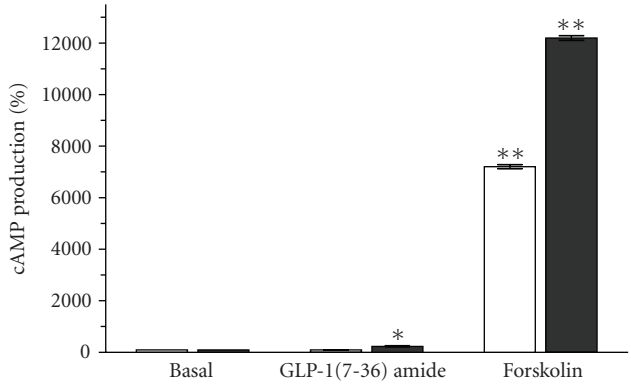

Figure 7: Effect of GLP-1(7-36)amide and forskolin on the production of cAMP from CHO and Rin m5F cells. Wild type CHO cells (empty bar); Rin m5F cells (filled bar). 100 % = 46.7 fmol/mg and 40.9 fmol/mg for CHO cells and Rin m5F, respectively (basal value, no ligand added). Each bar with the indicated standard deviation represents the mean value of three independent experiments done in duplicate. ANOVA: $F(5, 12) = 31250$; $P < .0001$. Tukey-Kramer multiple comparison test was used to reveal significant differences between groups of interest. See Results for the details.

cells was increased 12- and 22-fold, respectively, in comparison with the effect on Rin m5F cells that naturally express GLP-1 receptor. This means that the production of cAMP in Rin m5F cells was increased up to 240% ($P < .05$) of the basal in the response to maximally stimulating concentration of GLP-1(7-36)amide (Figure 7). Wild type CHO cells without GLP-1 receptor show no effect upon stimulation by a hormone (Figure 7). The stimulation of cAMP production in response to forskolin was significantly increased by both cell types ($P < .001$). GFP-GLP-1 receptor cells are not more sensitive to forskolin in comparison to GLP-1 receptor cells, because ANOVA analysis Tukey-Kramer multiple comparison test has not revealed significant differences between these groups of interest.

3.5. The Effect of GFP-GLP-1 Receptor on Insulin Release. In Rin m5F cells, GLP-1 stimulated insulin release more than 3-fold as compared to basal release ($P < .05$), (Table 2). Cells transfected with the GFP-GLP-1 receptor vectors significantly displayed 14.5-fold GLP-1 dependent increase in insulin release ($P < .001$) (Table 2).

4. Discussion

In the present report we localized N-terminally GFP-tagged GLP-1 receptor in CHO cells and characterized its binding properties and the coupling to the G-proteins and adenylyl cyclase. Our findings show that (1) the fusion protein is primarily localized on the internal membranes near nucleous, while its concentration on the plasma membrane is low; (2) two types of the fusion proteins might be localized on the membranes, nonglycosylated on the internal membranes and glycosylated type on the plasma membrane; (3) the fusion protein which specifically binds the GLP-1 on the cell surface

Table 2: Effect of GLP-1(7-36)amide on insulin release from Rin m5F cells transiently expressing human GFP-GLP-1 receptor. Each bar with the indicated standard deviation represents the mean value of five independent experiments done in duplicate. ANOVA: $F(3, 16) = \mathbf{291.6}$; $P < .0001$. Tukey-Kramer multiple comparison test was used to reveal significant differences between groups of interest.

Condition	Insulin release (μU/50 $\times$ 10^4 cells/h)
Basal (no addition of GLP-1(7-36)amide)	21.5 ± 1.7
+ GLP-1(7-36)amide	69.9 ± 5.4*
+ GFP-GLP-1 receptor	26.9 ± 3.2
+ GFP-GLP-1 receptor + GLP-1(7-36)amide	388.5 ± 45.6**

*$P < .05$ versus basal (no addition).
**$P < .001$ versus + GFP-GLP-1 receptor.

and when activated is able to transfer the signal to adenylyl cyclase via heterotrimeric G-proteins and induces insulin release.

It is evident that, depending on the site of GFP insertion, the tagging can produce substantial changes in the properties of the receptors. Whereas GFP tagging does not seem to alter the function of some receptors [37–39], it is known that for some receptors GFP alters their function [40, 41], membrane localization [42], or rate of membrane incorporation [43]. There is no general rule which site of GFP insertion presents better GFP tagging. In some cases N-terminus is better choice than C-terminus [39] and vice-versa [37]. C-terminally GFP-tagged GLP-1 receptor neither is displaying the same membrane localization as its wild type [31] nor is fully characterized regarding receptor binding data, G-protein activation and coupling to its downstream effectors such as adelylyl cyclase or PLC [30]. With this question in mind, we synthesized and analyzed the properties of N-terminally GFP-tagged GLP-1 receptor expressed in CHO cells.

Regarding the spatial distribution of GFP-GLP-1 receptor, the overall fluorescence was strongly polarized to the region near the nucleolus. Contrary, fluorescence in plasma membranes was very weak. Immunolabeling of GFP-GLP-1 receptor on nitrocellulose membrane showed more than one protein band. Intensity of protein bands with the molecular weight of 90 kDa and 71 kDa, which might correspond to glycosylated and nonglycosylated forms of receptor, respectively, perfectly correlates with the amount of fluorescence in plasma membrane and region near nucleolus, respectively. Glycosylation of GLP-1 receptor is a precondition for regular receptor translocation to plasma membrane [44], but not for the receptor binding. We do not have direct biochemical proof for glycosylation state of the receptor, but Figure 2(b) shows that most of the receptor might be nonglycosylated. This raises the possibility that either the tag interferes with glycosylation of the receptor and blocks its translocation from endoplasmatic reticulum and Golgi apparatus to plasma membrane or it is misfolded, and most receptors do not reach the compartments, where glycosylation occurs. In addition, only a minority of GFP-GLP-1 receptor is properly glycosylated and translocates to plasma membrane (Figure 2 and Table 1).

The functionality of the GFP-GLP-1 receptor was assessed by measuring its binding properties and the coupling to the G-proteins and adenylyl cyclase, followed by insulin release. Tagging GFP to the amino terminus led to receptor that showed very similar binding affinity and G-protein coupling but accumulated lower concentration of cAMP in response to agonist application (Figures 4, 5, and 6). In addition, reduced accumulation of cAMP was due to reduced number of receptors in plasma membrane. Reduced number of receptors (Table 1) was correlated with decreases in both amount of protein (Figure 2(b)) and fluorescence (Figure 3) in plasma membrane. Reduced number of receptors should also reduce the effect on G-protein coupling. But it did not. Why the effect on G-protein coupling was not reduced? In this experiment we have used the total membrane preparation instead of the whole cell system. The number of the tag and wild type receptors was equal and resulted in the same effect on G-protein coupling in both membrane preparations. This means that both types of GFP-GLP-1 receptors localized, in plasma membrane and in internal membranes, are functional and able to transfer the signal to G-proteins (Figure 5). Additionally, after GLP-1 stimulation GFP-GLP-1 receptor significantly induced insulin release from Rin m5F cells (Table 2).

Our results show that GFP tagging to the amino terminus can alter the properties of GLP-1 receptor. In spite of these minor alternations in functionality of the receptor we believe that N-terminally GFP-tagged GLP-1 receptor is useful tool for further studies such as receptor dynamics, expression, signaling, and oligomerization. Combining different modern in vivo and in silico biology methods [45, 46] will help in investigation of new synthetic and natural ligands of receptor for the treatment of diabetes mellitus type 2.

Abbreviations

GPCR:	G-protein coupled receptor
GLP-1:	Glucagon-like peptide-1
GFP- GLP-1:	GFP-tagged GLP-1
GFP:	Green fluorescent protein.

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