Agonist-induced internalization and recycling of the glucagon-like peptide-1 receptor in transfected fibroblasts and in insulinomas

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Glucagon-like peptide-1 (GLP-1) is the most potent stimulator of glucose-induced insulin secretion and its pancreatic β-cell receptor is a member of a new subfamily of G-protein-coupled receptors which includes the receptors for vasoactive intestinal polypeptide, secretin and glucagon. Here we studied agonist-induced GLP-1 receptor internalization in receptor-transfected Chinese hamster lung fibroblasts using three different approaches. First, iodinated GLP-1 bound at 4 °C to transfected cells was internalized with a $t_\frac{1}{2}$ of 2–3 min following warming up of the cells to 37 °C. Secondly, exposure to GLP-1 induced a shift in the distribution of the receptors from plasma membrane-enriched to endosomes-enriched membrane fractions, as assessed by Western blot detection of the receptors using specific antibodies. Thirdly, continuous exposure of GLP-1 receptor-expressing cells to iodinated GLP-1 led to a linear accumulation of peptide degradation products in the medium following a lag time of 20–30 min, indicating a continuous cycling of the receptor between the plasma membrane and endosomal compartments.

Potassium depletion and hypertonicity inhibited transferrin endocytosis, a process known to occur via coated pit formation, as well as GLP-1 receptor endocytosis. In contrast to GLP-1, the antagonist exendin-(9–39) did not lead to receptor endocytosis. Surface re-expression following one round of GLP-1 receptor endocytosis occurred with a half-time of about 15 min. The difference in internalization and surface re-expression rates led to a progressive redistribution of the receptor in intracellular compartments upon continuous exposure to GLP-1. Finally, endogenous GLP-1 receptors expressed by insulinoma cells were also found to be internalized upon agonist binding. Together our data demonstrate that the GLP-1 receptor is internalized upon agonist binding by a route similar to that taken by single transmembrane segment receptors. The characterization of the pathway and kinetics of GLP-1-induced receptor endocytosis will be helpful towards understanding the role of internalization and recycling in the control of signal transduction by this receptor.

INTRODUCTION

Glucagon-like peptide-1 (GLP-1) is a hormone produced in intestinal L cells by a specific proteolytic processing of the preproglucagon molecule [1–3]. Following ingestion of a glucose-containing meal, this peptide is released into the circulation and binds to a specific receptor on the surface of pancreatic islet β cells, thereby stimulating glucose-induced insulin secretion through the activation of adenylate cyclase [4]. This receptor is a G-protein-coupled receptor [5,6] of the glucagon, vasoactive intestinal polypeptide, calcitonin and secretin receptor subfamily [7]. These receptors are 25–50 % identical to each other, but share less than 10 % sequence identity with the other G-protein-coupled receptors. So far only limited information is available regarding the biology of this subfamily of receptors. Here we report on the pathway and kinetics of endocytosis and recycling to the plasma membrane of the GLP-1 receptor following agonist binding.

Internalization and surface re-expression is a process that operates on many membrane receptors. Generally, nutrient receptors such as those for transferrin or low-density lipoproteins internalize constitutively. For hormone receptors such as the epidermal growth factor or insulin receptors, internalization is induced by ligand binding [8]. These two types of receptors, however, follow the same route of endocytosis and surface re-expression. They are first concentrated in clathrin-coated pits

which pinch off from the plasma membrane and form clathrin-coated vesicles [9]. These vesicles rapidly uncoat and fuse together or with pre-existing endosomal structures called early endosomes or CURL (compartment for uncoupling of receptor and ligand) [10]. These tubulo-vesicular structures have an acidic content (pH ~ 5–6) and are the site where ligands dissociate from their receptors. While the ligands are targeted to late endosomes and lysosomes, where they are eventually degraded, the receptors return to the plasma membrane, probably via carrier vesicles formed from the tubular part of the early endosomes [11].

While most of these data have been obtained with single transmembrane segment receptors [12], less is known of the internalization pathway of multiple transmembrane segments proteins such as G-protein-coupled receptors. Exposure of G-protein-coupled receptors (i.e. the β_2-adrenergic receptor) to agonists has been shown to lead to a rapid decrease in the number of cell-surface binding sites which could, however, be recovered in a low-density membrane fraction [13,14]. This phenomenon has been referred to as the 'sequestration' of receptors. Recently, however, it has been demonstrated that endocytosis of the mating factor receptor was prevented in yeast strains deficient in clathrin expression [15]. Also it was shown, by immunofluorescence microscopy using specific antibodies, that the β_2-adrenergic as well as the substance P receptors were internalized in a compartment in which transferrin receptors also accumulated [16,17], and from which recycling to the plasma

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membrane could occur. These data therefore suggest that G-protein-coupled receptors are internalized and recycled to the plasma membrane by a similar pathway to that described for receptors containing single transmembrane domains.

A detailed knowledge of receptor internalization, surface re-expression and change in the steady-state surface expression upon ligand binding is important to obtain. Indeed, these processes may modulate the coupling of receptors to their effectors. In particular, it has been suggested that receptor internalization may promote or participate in fast receptor desensitization [18,19], although it now appears that rapid desensitization and endocytosis are separate processes [20–23]. Internalization may, however, be required for receptor resensitization. Indeed, fast desensitization of the β_2-adrenergic receptor results from its phosphorylation by protein kinase A or β-adrenergic receptor kinase [24–27] and resensitization may require exposing the phosphorylated receptor to phosphatase-containing intracellular vesicles, an event which takes place during passage of the receptor along the endocytic/exocytic pathway [24,28,29]. Also, it is not yet clear whether receptor sequestration and long-term down-regulation are initiated by the same internalization pathways, even though genetic analysis and study of receptor mutants have suggested that both phenomena can be dissociated [22,30,31]. In addition, it is particularly important to understand how agonist binding may affect GLP-1 receptor expression, since GLP-1 or agonists of the GLP-1 receptor are now being tested as new drugs for the stimulation of insulin secretion in patients with non-insulin-dependent diabetes mellitus in replacement of the currently used hypoglycaemic agents.

In the present study, we used the rat GLP-1 receptor stably expressed in fibroblasts as a model for this subfamily of receptors. We studied the pathways and kinetics of agonist-dependent internalization and recycling to the plasma membrane of the receptor and we extended these data by showing that the endogenous receptors of insulinoma cells were also endocytosed after agonist binding. These data are required for further structural studies on receptor internalization and desensitization.

MATERIALS AND METHODS

Cells and cell culture

The 1056A insulinoma cell line [32] was provided by Dr. Jacques Philippe, University of Geneva, Switzerland and cultured in RPMI-c [RPMI 1640 medium, 10% fetal-calf serum (FCS), 2 mM glutamine, 1 mM sodium pyruvate, 50 μM 2-mercaptoethanol, 50 units/ml streptomycin/penicillin, 10 mM Hepes, 2 g/l NaHCO$_3$, pH 7.2] as described previously [33]. Clone 5 is a Chinese hamster lung (CHL) fibroblast (CCL39, American Type Culture Collection, Rockville, MD, U.S.A.) cell line stably transfected with the rat GLP-1 receptor cDNA. It was established [33] by co-transfection with plasmid pGLPR-1 containing the rat GLP-1 receptor cDNA [5] and with plasmid pWL-neo (Stratagene GmbH, Zurich, Switzerland) carrying the gene for resistance to neomycin. This cell line was routinely cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% FCS, 2 mM glutamine, 50 units/ml streptomycin/penicillin (DMEM-c). For all experiments, cells were either seeded at 1.5×10^4 cells/ml in 2 ml of DMEM-c in 12-well clusters (COSTAR no. 3512, Technomara AG, Wallisellen, Switzerland) and incubated for 2 days at 37 °C in 5% CO$_2$, or plated in 100-mm-diam. culture dishes and used at > 50% confluency. For metabolic inhibitor treatment, cells were washed twice with 2 ml of glucose-free HBS buffer (130 mM NaCl, 20 mM Hepes, pH 7.4, 0.9 mM NaH$_2$PO$_4$, 0.8 mM MgSO$_4$, 5.4 mM KCl,

1.8 mM CaCl$_2$, 25 μM Phenol Red) and incubated for 30 min in the same buffer containing 20 mM 2-deoxyglucose and 10 mM sodium azide (MI medium). Control cells were incubated in glucose-free HBS buffer. Potassium depletion and hypertonic treatment were performed exactly as described by Hansen and collaborators [34]. Briefly, cells were incubated for 30 min at 37 °C in DME-H (DMEM, 20 mM Hepes, 2 mM glutamine). For potassium depletion, cells were rinsed twice in K$^+$-free buffer (140 mM NaCl, 20 mM Hepes, 1 mM CaCl$_2$, 1 mM MgCl$_2$, 1 g/l glucose, pH 7.4), hypotonically shocked for 5 min at 37 °C in K$^+$-free buffer/water (1:1, v/v), washed three times in K$^+$-free buffer and incubated in the same buffer (treated cells) or DME-H (control cells) for 30 min at 37 °C. For hypertonic treatment, cells were washed twice in DME-H containing 0.45 M sucrose and incubated in the same buffer (treated cells) or DME-H (control cells) for 45 min at 37 °C. COS-G cells were transfected with plasmid pGLPR-1 as described previously [33] and were analysed 2 days after transfection. A pool of about 25 pGLPR-1-transfected CHL fibroblast clones was established as described for clone 5.

Binding studies

Iodination of GLP-1 was carried out as described previously [5,6]. Specific radioactivity was 500–800 c.p.m./fmol. Binding at 4 °C was performed in Hanks' balanced salt solution (HBSS) (GIBCO BRL no. 042-04065; Life Technologies A. G., Basel, Switzerland), containing 0.5% BSA and 20 mM Hepes, pH 7.4 (binding buffer), for at least 5 h, at which time equilibrium was reached (results not shown). For saturation binding, 50–200 pM iodinated GLP-1 was mixed with 3 nM unlabelled GLP-1. Following incubation with ^{125}I-GLP-1, cells were washed three to five times with 1 ml of HBSS/20 mM Hepes, pH 7.4, and lysed in 400–1000 μl of 0.2 M NaOH/1% SDS. Non-specific binding was defined as binding in the presence of 1 μM unlabelled GLP-1 and was subtracted in every case from the total binding value. Measurements were performed in triplicate unless otherwise stated. Radioactivity was determined using a gamma counter.

Ferri-transferrin

Human transferrin (10 mg in 0.5 ml of 0.15 M NaCl) was mixed with 5 μl of KHCO$_3$ and then with 24 μl of 5 mg/ml FeSO$_4$. The solution was incubated on ice for 30 min, centrifuged and the supernatant containing ferri-transferrin was stored at 4 °C. Iodination of ferri-transferrin (20 mg) was carried out as described for GLP-1. The iodinated protein was separated from free iodine on a G-50 Sephadex column (Pharmacia LKB, Uppsala, Sweden). In some experiments iodinated human transferrin from Dupont NEN (NEX 212) was used.

Measurement of cell-surface GLP-1 receptor expression

Following incubation in DMEM/20 mM Hepes, pH 7.4, with the indicated substances, cells were chilled on ice, washed three times with HBSS/20 mM Hepes, pH 7.4, incubated for 2–3 min in pH 3 buffer (50 mM glycine/150 mM NaCl, pH 3), washed once with the same buffer and once again with HBSS/20 mM Hepes, pH 7.4. Cells were then subjected to saturation binding for 16–18 h at 4 °C as described above.

Determination of association and dissociation constants

Association constant (k_{on})

Cells were treated with metabolic inhibitors (see above) and incubated for various periods of times at 37 °C in binding buffer

containing 3.2 nM ^{125}I-GLP-1, 20 mM 2-deoxyglucose and 10 mM azide. Cell-associated radioactivity was then determined. With a concentration of 3.2 nM GLP-1, 95% of receptors are occupied at equilibrium. A 60 min incubation at 37 °C was sufficient to reach binding equilibrium (data not shown). Maximal binding was thus set as (c.p.m. bound after 60 min incubation) divided by 0.95. The k_{on} value is derived from the slope of the straight line obtained by plotting c.p.m. bound versus time for early time points. The slope is equal to $k_{on} \times$ [free ligand] $\times$ maximal binding [35].

Dissociation constant (k_{off})

Cells were treated with metabolic inhibitors (see above) and incubated in the presence of 200 pM of ^{125}I-GLP-1 for 5 h at 4 °C in MI medium containing 0.5% BSA. After two washes with glucose-free HBS buffer, 400 μl of prewarmed MI medium containing 1 μM unlabelled GLP-1 was added for the indicated times. Cell-associated radioactivity was then determined. The k_{off} values are equal to the slopes of the straight lines obtained by plotting $\ln(B^{\circ}/B)$ as a function of time, where B° is the initial binding and B the binding at time t.

GLP-1 internalization measurement

Internalization of ^{125}I-GLP-1 was assessed as the increase in radioiodinated peptide remaining cell-associated following acid washes. Cells were incubated with ^{125}I-GLP-1 at 4 °C for 4–6 h, washed and incubated for various times at 37 °C in DMEM-c. They were then washed once with ice-cold HBSS/20 mM Hepes and incubated for 2 min at 4 °C with pH 3 buffer (50 mM glycine/150 mM NaCl, pH 3) (acid-resistant radioactivity) or with ice-cold HBSS/20 mM Hepes, pH 7.4 (total cell-associated radioactivity). Cells were then washed once with the same buffer, once with ice-cold HBSS/20 mM Hepes, pH 7.4, and lysed with 400–500 μl of 0.2 M NaOH/1% SDS. Measurements were performed in triplicate. Radioactivity was determined as described above. There was a fraction of surface-bound ^{125}I-GLP-1 that could not be removed at pH 3 prior to incubation at 37 °C (20–40%).

Preparation of antibodies

Antigens consisted of fusion proteins made of the glutathione-S-transferase (GST) and either the N-terminal or C-terminal tail of the receptor. For the N-terminal region, a segment of the GLP-1 receptor cDNA corresponding to amino acids 19–145 was amplified by PCR with specific oligonucleotides so that SmaI and EcoRI restriction sites were added to the 5′ and 3′ termini of the amplified fragment to facilitate subcloning in the pGEX-2T vector (Pharmacia). For the C-terminal tail, the pGLPR-87 cDNA clone [5] was cut with HincII (position 1235, amino acid 407) and XbaI (poly-linker of pcDNA-1). After filling in the ends with the Klenow fragment of DNA polymerase I, the 749 bp fragment was subcloned in pGEX-2T. Fusion proteins consisting of the C-terminal tail of the receptor were expressed in bacteria, purified on glutathione–agarose beads and eluted with reduced glutathione [36]. For purification of the N-terminal fusion protein, the bacterial lysate was run on preparative SDS/PAGE and the band corresponding to the fusion protein extracted by electroelution. Immunization of rabbits was performed by a first subcutaneous injection of the antigen emulsified in complete Freud's adjuvant followed by repeated injections of the antigen emulsified in incomplete adjuvant. The C-terminus-specific antibody [antibody 9(3)] was affinity-purified from an ammonium sulphate γ-globulin fraction in two steps: first, the

GST-specific antibodies were removed by absorption on an affinity column consisting of Affi-gels 15 and 10 (1:1, v/v) (Bio-Rad Laboratories AG, Glattbrug, Switzerland) to which the GST protein was covalently coupled. The receptor-specific antibodies were then purified from the effluent of the first column by absorption on a second column containing the antigen coupled to Affi-gel beads. After elution with 0.58% acetic acid and dialysis against phosphate-buffered saline (PBS: 2.7 mM KCl/1.5 mM KH_2PO_4/137 mM NaCl/8 mM Na_2HPO_4) the antibodies were stored in small aliquots at -20 °C. IgG from the sera of rabbits injected with the fusion protein containing the N-terminal tail of the GLP-1 receptor [antibody 20(2)] were prepared by absorption of the serum on a Protein A–Sepharose (Pharmacia) column, followed by elution with 0.58% acetic acid, dialysis and storage as described above.

Sucrose gradients and Western blotting analysis

High- and low-density membranes were separated on sucrose step gradients according to a slightly modified version of the method of Kurz and Perkins [37]. Briefly, confluent cells in 10-cm-diam. culture dishes were treated with 500 μg/ml concanavalin A (no. C-7275; Sigma, Buchs, Switzerland) in HBSS/20 mM Hepes for 5 min at 4 °C. They were then rinsed in 1 mM Tris (pH 7.4)/2 mM EDTA (lysis buffer), lysed for 20 min on ice in the same buffer and scraped into a final volume of 1 ml of lysis buffer. These broken cells were loaded on a sucrose step gradient consisting of 4 ml of 60% sucrose, 4 ml of 38% sucrose and 4 ml of 15% sucrose, all buffered with 20 mM Tris, pH 7.4, and centrifuged at 112000 g for 1 h at 2 °C in a Beckman SW40 TI rotor. Membranes at the 15–38% sucrose interface (low-density fraction) and 38–60% sucrose interface (high-density fraction) were collected, diluted with lysis buffer and centrifuged at 212000 g for 30 min at 2 °C. In the case of insulinoma cells, lysis was performed for 10 min in the absence of EDTA and the broken cells loaded on to a sucrose step gradient consisting of 2 ml each of the following sucrose concentrations: 65%, 55%, 45%, 35%, 25% and 15%. After centrifugation, each interface was collected and pelleted as described above. Pellets were resuspended in 50 μl of solubilization buffer (PBS containing 1% SDS, 2 mM N-ethylmaleimide, 1 mM PMSF, 5 mM EDTA). Samples (10 μl) of the resuspended pellets were loaded on an SDS/7.5%-PAGE gel. The amount of protein loaded was not affected by GLP-1 pretreatment (see Table 1). After migration, proteins were transferred to nitrocellulose filters (Schleicher and Schuell, BA83 0.2 μm, no. 401380). Filters were incubated at room temperature with TBS (10 mM Tris, pH 7.4, 150 mM NaCl)/0.2% Nonidet P-40 (TBS/NP40) for 20 min, followed by a 20 min incubation with TBS/0.1% Tween 20 (TBS/Tween). They were then incubated at 37 °C for 30 min with TBS/5% powdered milk (TBS/milk). Filters were blotted with 5 μg of antibody 20(2)/ml for 1 h at room temperature or with 2 μg of antibody 9(3)/ml. Alternatively, the blots were incubated with a 1/400–1/800 dilution of an anti-BiP serum (a kind gift from Professor Kathy Geering, University of Lausanne, Lausanne, Switzerland) in the same conditions. Filters were again washed with TBS/NP-40, TBS/Tween and TBS/milk as described above, followed by five incubations at room temperature with TBS (5 min each). Rabbit immunoglobulins were detected with the Enhanced Chemiluminescence detection system (no. RPN 2106; Amersham International, Little Chalfont, Bucks., U.K.) according to the manufacturer's protocol. When indicated, the bands corresponding to GLP-1 receptors or to BiP were analysed by densitometry scanning using a video imaging device (Bio-

Print, Vilber Lourmat) and the ImageQuant software, version 3.22 (Molecular Dynamics).

Endoglycosidase treatment

Endoglycosidase treatments were performed on detergent-solubilized membranes prepared from cell lysates. Confluent cells were hypotonically lysed as described above and a membrane fraction was prepared by layering the lysate on a sucrose step gradient consisting of 5.5 ml of 55 % sucrose and 5.5 ml of 5 % sucrose buffered with 20 mM Tris, pH 7.4. After centrifugation, the material at the 5–55 % sucrose interface was collected and pelleted.

Peptide: N-glycosidase F (PNGase F) treatment

Membrane pellets were resuspended in 150–250 μl of denaturing buffer (0.5 % SDS/1 % 2-mercaptoethanol/2 mM PMSF) containing 50 mM sodium phosphate, pH 7.5. Aliquots (50 μl) were then prepared to which (1:10, v/v) 10 % Triton X-100, (1:100, v/v) 200 mM PMSF and 1000 units of PNGase F (New England Biolabs GmbH, Schwalbach, Germany) were added.

Endo-β-glycosaminidase H (Endo H) treatment

Membrane pellets were resuspended in 150–250 μl of denaturing buffer and (1:10, v/v) 0.5 M sodium citrate (pH 5.5) was added. Aliquots (50 μl) were then prepared to which (1:100, v/v) 200 mM PMSF and 1000 units of recombinant Endo H (Endo H_r; New England Biolabs) were added.

PNGase F and Endo H digestions were performed at 37 °C for 1–2 h. Control reactions did not receive any enzyme. The samples were then mixed with 50 μl of solubilization buffer and loaded on an SDS/7.5 %-PAGE gel. Detection of the GLP-1 receptor was performed by Western blotting analysis as described above using the 20(2) antibodies for PNGase F-treated samples and the 9(3) antibodies for the Endo H-treated samples.

Cross-linking of ^{125}I-GLP-1 to its receptor

Confluent clone 5 cells cultured in 100-mm-diam. culture dishes were washed twice with 4 ml of HBSS/20 mM Hepes, pH 7.4, and incubated for 40 min at room temperature with 10^6 c.p.m. of ^{125}I-GLP-1 (about 1 nM) in 2 ml of binding buffer in the presence or absence of 1 μM unlabelled GLP-1. After four washes with HBSS/Hepes, the cells were incubated with 100 μM bifunctional cross-linker dithiobis(succinimidyl)propionate (no. 22585; Pierce Europe B. V., Oud Beijerland, The Netherlands) prepared in HBSS/Hepes for 15 min on ice. The cells were then washed twice and incubated for 5 min with 10 ml of 150 mM NaCl/20 mM Tris, pH 7.4, on ice to block cross-linker molecules that did not react with amine groups. After four more washes, the cells were scraped into 2 ml of PBS containing 167 μM aprotinin and centrifuged at 200 g for 5 min. The pellet was resuspended in 1 ml of 20 mM Hepes, pH 7.4, containing 250 mM sucrose and 167 μM aprotinin, and incubated on ice for 15 min. The cells were then homogenized with the A pestle of a Dounce homogenizer (Kontes Glass Co., Vineland, NJ, U.S.A.). The number of strokes was adjusted so that about 80–90 % of the cells were broken, as assessed by Trypan Blue exclusion. Cellular debris were pelleted by a 10 min centrifugation at 200 g at 4 °C and the supernatant was centrifuged at 170000 g for 15 min at 4 °C. The membrane pellet was solubilized in 100 μl of PBS/1 % SDS/1 mM PMSF/2 mM N-ethylmaleimide/5 mM EDTA, 50 μl of which were mixed with 50 μl of 160 mM Tris, pH 6.8, containing

5 mM EDTA, 20 % glycerol, 36.8 mg/ml iodoacetamide, 10 % SDS and a trace amount of Bromophenol Blue. Samples were then loaded on an SDS/7.5 %-PAGE gel. After migration, the gel was dried and autoradiography was performed at −70 °C using Kodak X-OMAT film (no. F-5388; Sigma).

Measurement of GLP-1 degradation products

Following incubation with cells, 1 vol. of ^{125}I-GLP-1-containing culture supernatant (400 μl) was mixed with 1 vol. of ice-cold 20 % trichloroacetic acid (TCA) solution, incubated on ice for 1 h in an Eppendorf tube and centrifuged at 4 °C for 15 min at 18000 g. The supernatants were transferred to new tubes and 500 μl of 10 % TCA was used to wash the pellets and was added to the first supernatants. There was a certain amount of radioactivity in stock solutions of ^{125}I-GLP-1 that was not precipitable by 10 % TCA (about 15 %). This radioactivity mostly corresponded to free iodine, as determined by chloroform extraction [38] (data not shown), and was always subtracted from the radioactivity that remained in the supernatants. Iodinated GLP-1 incubated with untransfected CCL39 or incubated with clone 5 in the presence of an excess of unlabelled GLP-1 was degraded in a time-dependent manner (data not shown). The rate of this receptor-independent degradation was, however, much lower compared with the receptor-dependent degradation (about 15 %). The receptor-independent degradation was always subtracted from total degradation.

Measurements of cell surface-bound and internalized ^{125}I-diferric transferrin in clone 5 cells

Cells were incubated with 1–2 nM ^{125}I-transferrin for 2 h at 4 °C to allow binding to surface-expressed receptors or for 15 min at 37 °C to allow binding and internalization of the transferrin-receptor complexes. High- and low-density membrane fractions were prepared as described above and their radioactivities were measured in a gamma counter. Non-specific binding was measured in the presence of 0.5–1 μM unlabelled ferri-transferrin and was subtracted from the total binding values.

RESULTS AND DISCUSSION

Clone 5 as a cellular model with which to study GLP-1 receptor

Clone 5 is a CHL fibroblast cell line stably transfected with the rat GLP-1 receptor cDNA. The affinity constant for GLP-1 binding measured at 4 °C was 0.2 nM with about 80000 binding sites per cell. This affinity constant was almost identical to that measured for GLP-1 binding to the endogenous receptor of insulinoma cell lines. In addition, cyclic AMP production following GLP-1 stimulation of clone 5 and insulinoma cells displayed indistinguishable dose–response curves (EC_{50} values of about 0.5 nM) [33]. Neither clone 5 cells nor insulinomas were coupled to the phospholipase C pathway [33]. Studies on the kinetic parameters for GLP-1 binding to its receptor and for ligand-induced internalization and surface re-expression were performed on transfected fibroblasts for two main reasons. First, the high expression level of the receptor in fibroblasts allows for more accurate binding and internalization studies, as assessed by techniques used in the present work. Secondly, the use of cells devoid of endogenous GLP-1 receptor will permit us, in the future, to perform structure–function studies to determine which parts of the receptor are required for internalization and desensitization.
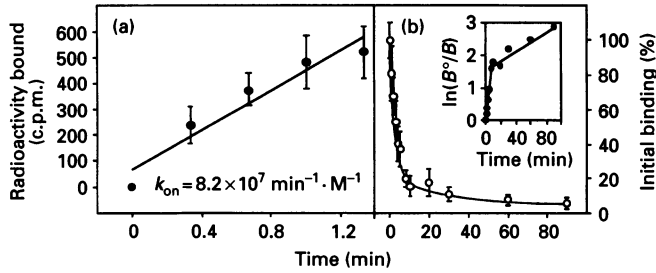
Figure 1   GLP-1 association and dissociation rates

(a) ^{125}I-GLP-1 association to its receptor. Cells were exposed to ^{125}I-GLP-1 at 37 °C in the presence of metabolic inhibitors and the amount of ^{125}I-GLP-1 associated with the receptor was determined after different times as described in the Materials and methods section. Results are expressed as the mean ± S.D. of sextuplet determinations. Binding at equilibrium (60 min incubation) was 1461 ± 208 c.p.m. (b) ^{125}I-GLP-1 dissociation at 37 °C in the presence of energy inhibitors. Cells were incubated with 200 pM ^{125}I-GLP-1 in the presence of metabolic inhibitors for 5 h at 4 °C. After washings, the cells were incubated at 37 °C in the presence of an excess of unlabelled peptide and the radioactivity remaining bound at the indicated times was determined. Results are expressed as the mean ± S.D. of sextuplet determinations. Binding at $t = 0$ min, 1234 ± 65 c.p.m. Inset: the data of the main Figure were replotted as $\ln(B^0/B)$ as a function of time where B^0 is the initial binding and B the binding at time t. Two first-order rates of dissociation were measured: 0.21 min^{-1} and 0.015 min^{-1}. The plots in (a) and (b) are representative of three separate experiments.

Association and dissociation constants

The rate constant for GLP-1 association to the receptor was measured at 37 °C in the presence of metabolic inhibitors (20 mM 2-deoxyglucose and 10 mM sodium azide in glucose-free buffer) to prevent internalization of the receptor in the course of the experiment (see Figure 6, left-hand panel, and Table 2). The k_{on} value derived from the kinetics of GLP-1 association shown in Figure 1(a) and from two other experiments was $(8.0 \pm 1.2) \times 10^7$ min$^{-1} \cdot$M^{-1} (mean ± S.D.), a value close to that found for most agonists binding to their receptor [39]. The rate of association of GLP-1 to its receptor was temperature-dependent, since the k_{on} decreased to $(3.6 \pm 1.1) \times 10^6$ min$^{-1} \cdot$M^{-1} at 4 °C (two experiments; mean ± S.E.M.). For measurement of the GLP-1 dissociation rate, GLP-1 was first bound at 4 °C in the presence of energy inhibitors, the cells were washed and transferred back to 37 °C to measure the rate of GLP-1 dissociation. Two components were measured: a fast component with a k_{off} of 0.21 min^{-1} ($t_\frac{1}{2} = 3.3$ min) and a slow component with a k_{off} of 0.015 min^{-1} ($t_\frac{1}{2} = 46$ min) (Figure 1b). The proportion of receptors with fast k_{off} was 64 ± 16 % (mean ± S.D.; five experiments).The presence of two rate constants for dissociation indicates that the GLP-1 receptor can be present in two affinity states. A high-affinity state ($K_d = k_{off}/k_{on} = 0.015$ min$^{-1}/8.0 \times 10^7$ min$^{-1} \cdot$M$^{-1} = 1.9 \times 10^{-10}$ M) which corresponds to the affinity derived from Scatchard analysis performed at 4 °C on transfected fibroblasts [33], or performed by others at 37 °C on membranes of RINm5F insulinoma cells [40], and a low- affinity state ($K_d = 0.21$ min$^{-1}/8.0 \times 10^7$ min$^{-1} \cdot$M$^{-1} = 2.6 \times 10^{-9}$ M). Two affinities are commonly found for members of the G-protein-coupled receptors and the high-affinity form is thought to be due to interaction of the receptor with G-proteins [18,19,41].

Kinetics of ^{125}I-GLP-1 internalization

To measure the rate of GLP-1 endocytosis, ^{125}I-GLP-1 was prebound to clone 5 cells at 4 °C, unbound ligand was washed off and the cells warmed up to 37 °C. After different periods of time, total cell-associated radioactivity and acid-resistant radioactivity
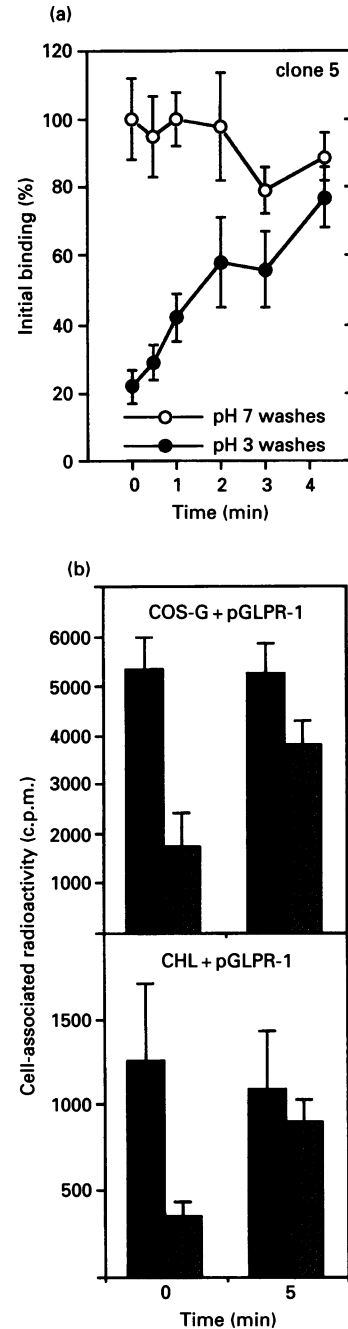

Figure 2 ^{125}I-GLP-1 internalization at 37 °C by cells expressing the transfected GLP-1 receptor

Clone 5 cells (a) and COS-G and pools of CHL fibroblasts transfected with pGLPR-1 (b) were incubated with 200 pM ^{125}I-GLP-1 for 5 h at 4 °C. After washing, prewarmed medium was added for the indicated times and the amount of cell-associated and internalized ^{125}I-GLP-1 was determined. The Figure shows the mean (± S.D. of triplicate determinations) of total cell-associated (○ and solid bars) and acid wash-resistant radioactivity (● and striped bars) as a function of time. GLP-1 was internalized with a $t_\frac{1}{2}$ of about 2–3 min. These experiments have been performed five times, twice and once for clone 5 cells, transfected COS cells and pools of transfected CHL fibroblasts respectively.

were determined following washings at pH 7.4 and pH 3.0 respectively. Figure 2(a) shows that, in the case of clone 5 cells, acid-resistant radioactivity increased rapidly upon rewarming. After 5 min, virtually all the cell-associated radioactivity could
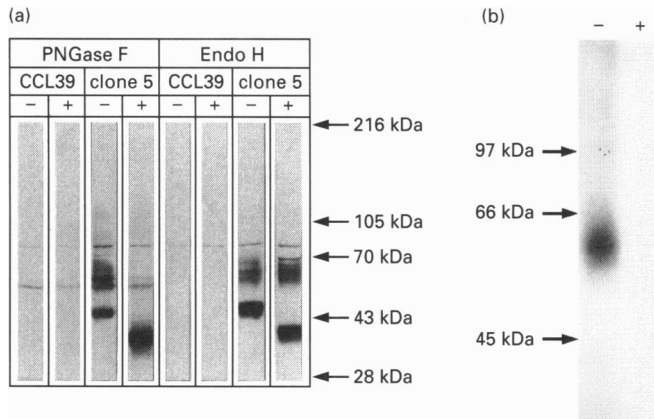
(a)

(b)

Figure 3   Characterization of the GLP-1 receptor expressed in transfected fibroblasts

(**a**) Endoglycosidase treatments. Membranes from clone 5 cells and from untransfected CCL39 fibroblasts were solubilized in detergents and incubated in the presence (+) or in the absence (−) of PNGase F or Endo H. GLP-1 receptors were then detected by Western blot analysis using either the anti-N-terminal antibody (PNGase F panel) or the anti-C-terminal antibody (Endo H panel). Both antibodies recognize ~ 64 kDa and 44 kDa bands in transfected cells. Upon PNGase F treatment both forms are converted into a 35 kDa band, while only the 44 kDa band is converted into the 35 kDa form by Endo H. This gel is representative of two separate experiments. (**b**) Cross-linking of ^{125}I-GLP-1 to its receptor. Clone 5 cells were incubated with iodinated GLP-1 in the absence (−) or in the presence (+) of 1 μM unlabelled GLP-1, followed by the addition of the cross-linker dithiobis(succinimidyl)propionate. Cross-linked receptors were then detected by SDS/PAGE and autoradiography. Only the 64 kDa band was detected, indicating that only the high-molecular-mass form of the GLP-1 receptor can bind the agonist. This gel is representative of five separate experiments.

not be displaced by acid washes anymore, suggesting that the majority of ^{125}I-GLP-1 molecules had been internalized. The half-time of internalization was estimated to be about 2–3 min. The decrease in total cell-associated radioactivity observed in Figure 2(a) is unlikely to be due to peptide degradation, because during the first 20–30 min of incubation at 37 °C, no ^{125}I-GLP-1 degradation products are detected in the extracellular medium (see Figure 4). Decrease in cell-surface radioactivity thus represents dissociation of GLP-1 molecules from receptors still present at the cell surface.

Importantly, internalization of GLP-1 by clone 5 is not specific to this cell line since internalization was also observed in transiently transfected COS-G cells and in a pool of CHL

fibroblasts stably expressing the wild-type GLP-1 receptors (Figure 2b).

Characterization of the GLP-1 receptors in transfected fibroblasts

Both the N- and C-terminal GLP-1 receptor antibodies were used to characterize the receptors expressed in transfected fibroblasts. For determination of the receptor-specific bands, non-transfected fibroblasts were analysed in parallel. The relationship between the different bands was assessed by glycosidase treatments. Figure 3 shows in the PNGase F panel that the N-terminal antibody recognized two major bands present only in transfected cells: an upper band centred around 64 kDa and a lower band migrating with an apparent molecular mass of 44 kDa. The presence of the same two bands was found in insulinoma cells and in β cells of the pancreas (B. Thorens, unpublished work). This indicates that they are not due to inappropriate or unregulated expression of the GLP-1 receptor in transfected cells. Treatment with PNGase F converted both bands into a faster migrating band with an apparent molecular mass of 35 kDa, suggesting that both the 64 kDa and 44 kDa bands were the same polypeptide with different oligosaccharide side chains. In the right-hand panel, the C-terminal antibody recognized the same two bands in the transfected cells. Endo H treatment converted only the 44 kDa form into the 35 kDa polypeptide. This therefore indicates that the 44 kDa band is a core-glycosylated form of the receptor probably still present in the endoplasmic reticulum or in a site not distal from the cis-mid Golgi [42,43]. The 64 kDa form is a fully glycosylated form present at the plasma membrane. Cross-linking experiments indeed indicated that only the higher-molecular-mass form could bind iodinated GLP-1 (Figure 3b).

Internalization of the GLP-1 receptor

It is possible to prepare cellular fractions enriched in plasma membranes and endosomal compartments by centrifugation of cell homogenates on sucrose density gradients [20,37,44]. Here we have separated low (endosomal)- and high (plasma membrane)-density membrane fractions on sucrose step gradients consisting of 60 %, 38 % and 15 % sucrose. The enrichment of the different fractions in endosomes and plasma membrane was assessed by the presence of ^{125}I-transferrin either bound at 4 °C or bound and internalized at 37 °C [45,46]. Presence of endoplasmic reticulum membranes was assessed by the detection of the marker protein BiP [43,47]. As summarized in Table 1, the low-density fraction contains 15 % of the transferrin bound at

Table 1   Characterization of low- and high-density membrane fractions of clone 5 cells

Abbreviation: N.D., not determined.

GLP-1 preincubation[a]	Fraction[b]	Protein content (μg/fraction)[c]	BiP (% of total)[d]	^{125}I-Transferrin (% of total)		GLP-1 receptors (% of total)	
				4 °C[e]	37 °C[e]	Core-gylcosylated[f]	Mature[f]
No	Light	17.1 ± 1.7	24 ± 6	15.4 ± 5.2	59.0 ± 9.6	39.6 ± 5.7	17.2 ± 5.2
	Heavy	53.1 ± 3.1	76 ± 6	84.6 ± 5.2	41.0 ± 9.6	60.4 ± 5.7	82.8 ± 5.2
Yes	Light	17.6 ± 2.4	16 ± 8	N.D.	N.D.	40.6 ± 4.7	49.2 ± 4.8
	Heavy	54.0 ± 7.6	84 ± 8	N.D.	N.D.	59.4 ± 4.7	50.8 ± 4.8

[a] Preincubation was performed at 37 °C for 15 min with 10 nM GLP-1. [b] Light and heavy fractions correspond to the 15–38% sucrose interface and 38–60% sucrose interface respectively. [c] Mean ± S.D. ($n = 3$); representative of two independent determinations. [d] Mean ± S.E. M. of 6–7 separate experiments. [e] Mean ± S.E.M. of 3–4 separate experiments. [f] Mean ± S.E.M. of five separate experiments.
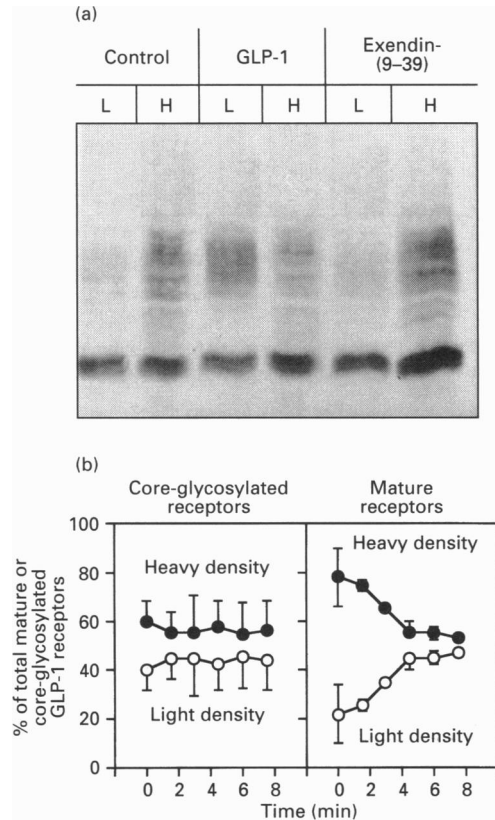
Figure 4   GLP-1 receptor internalization as detected by Western blot analysis

(a) Clone 5 cells were incubated for 15 min at 37 °C in control medium or in medium containing 10 nM GLP-1 or 10 nM exendin-(9–39). High- and low-density membranes were separated on a sucrose gradient and the receptor present in each fraction detected by Western blot analysis. Exposure of the cells to GLP-1, but not to exendin-(9–39), induced redistribution of the mature form of the receptor to low-density membranes whereas no change in core-glycosylated receptor distribution was observed. This gel is representative of two separate experiments. (b) Clone 5 cells were incubated with 100 nM GLP-1 at 4 °C in binding buffer for 0.5–3 h to saturate the GLP-1 receptors present at the cell surface. Cells were then incubated for the indicated periods of time with 100 nM GLP-1 at 37 °C in DMEM. Receptors present in high- and low-density membranes were detected as described above. The intensity of the bands corresponding to mature and core-glycosylated GLP-1 receptors were measured by densitometry scanning. Only the results of the densitometry scanning are shown and are expressed as percentages of the total core-glycosylated or mature receptor as a function of time. The amount of total mature or core-glycosylated receptors did not change significantly over the time course of the experiment. Results are expressed as the mean ± S.E.M. of two separate experiments.

4 °C while 85 % was in the high-density fraction. In contrast, when transferrin was bound and internalized at 37 °C, 60 % of the transferrin was shifted to the low-density fraction and 40 % was recovered in the high-density fraction. This indicated that the low-density fraction was enriched in endosomes and the high-density fraction enriched in plasma membranes. The endoplasmic reticulum marker protein BiP was mostly present in the heavy membrane fraction ($\sim$ 80 %) but was also present in the low-density fraction ($\sim$ 20 % of the total). The distribution of this marker was unaffected by incubation of the cells with GLP-1.

To obtain a direct assessment of GLP-1 receptor internalization, we have thus fractionated total cell membranes into heavy and light membrane fractions and detected GLP-1 receptors by Western blot analysis. Figure 4(a) shows that in control conditions, the majority of mature GLP-1 receptors

(83 %) was in the high-density membrane fraction. In contrast, after GLP-1 incubation there was a shift in the distribution of the mature GLP-1 receptor from the high- to the low-density membrane fraction. Quantification of this redistribution showed that the amount of mature receptor increased from 17 % to almost 50 % upon agonist incubation (Table 1). This strongly indicated that GLP-1 induced redistribution of its receptor to an endosomal compartment. The amount of core-glycosylated GLP-1 receptor present in the light and heavy membrane fractions did not change upon exposure of the cells to GLP-1 (see Table 1), consistent with the inability of this form to bind GLP-1 and with its proposed intracellular localization.

The proportion of core-glycosylated GLP-1 receptors in the low-density fraction was not identical to that of BiP (40 % versus 24 %; Table 1). This could indicate that not all of the core-glycosylated GLP-1 receptors were localized to the endoplasmic reticulum.

To measure the rate of GLP-1 receptor endocytosis directly, distribution of mature GLP-1 receptors in light and heavy membrane fractions was monitored as a function of time of incubation following saturation of the receptors with their agonist. Figure 4(b) shows that mature GLP-1 receptors progressively redistributed to the low-density membrane fraction and this was accompanied by a parallel loss of receptors from the high-density membrane fraction. The $t_{\frac{1}{2}}$ of GLP-1 appearance in the endosomes-enriched fraction was about 2–3 min which was identical to the $t_{\frac{1}{2}}$ for iodinated GLP-1 internalization, further suggesting that the receptor and its agonist are endocytosed as a complex. After about 5 min, the percentage of mature GLP-1 receptors in the light and heavy membrane fractions remained stable with about 40 % of the receptors being in the low-density fraction and 60 % in the high-density fraction. This apparent steady-state distribution suggests that, in the presence of GLP-1, receptors cycle between the plasma membrane and endosomal compartments.

Cycling of the GLP-1 receptor

For a number of receptors, such as those for asialoglycoproteins [48], insulin [49] or low-density lipoproteins [38], accumulation of degraded peptides in the medium has been shown to result from receptor-dependent internalization of the ligand followed by its targeting towards, and degradation within, the lysosomes; while, at the same time, receptors were continuously recycled between the cell surface and the endosomal compartment. To assess whether GLP-1 receptors were subjected to such a cycling in the presence of the ligand, clone 5 cells were exposed to iodinated GLP-1 for up to 4 h and both the cell-associated radioactivity and appearance of ^{125}I-GLP-1 degradation products in the culture medium were measured. Figure 5 shows that cell-associated radioactivity increased during the first hour of incubation and then plateaued or even decreased during the subsequent hours. In contrast, after a lag time of 20–30 min, ^{125}I-GLP-1 degradation products continuously accumulated in the medium. Values of ^{125}I-GLP-1 degradation in Figure 5 were corrected for degradation by untransfected CHL fibroblasts or by clone 5 cells incubated in the presence of an excess of unlabelled GLP-1. In both circumstances this receptor-independent degradation amounted to about 15 % of the total degradation measured in the experiment presented in Figure 5. Taken together, these results indicate that GLP-1 induces a continuous cycling of the receptor between the cell surface and an intracellular compartment and that the internalized ligand is continuously degraded, probably as a result of targeting to lysosomes.
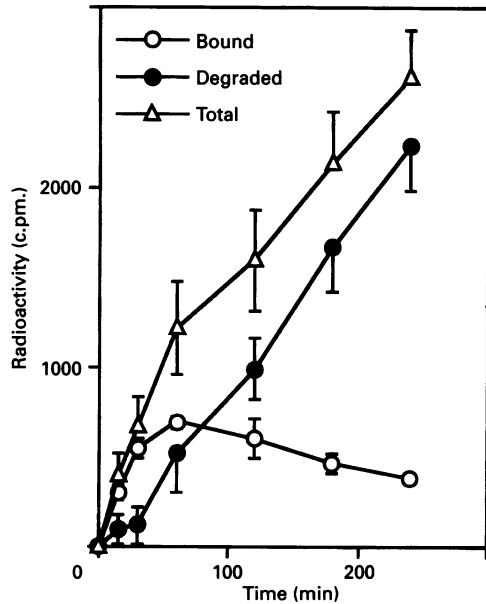
Figure 5   Uptake and degradation of ^{125}I-GLP-1 by clone 5 cells at 37 °C

Cells were incubated with 3.05 nM ^{125}I-GLP-1 at 37 °C. At the indicated times, medium was removed and analysed for ^{125}I-degradation product. In addition, ^{125}I-GLP-1 specifically bound to the cells was determined. The Figure represents the mean $\pm$ S.D. of sextuplet determinations of cell-associated radioactivity, amount of ^{125}I-degradation products present in the medium, and the sum of the two previous values. This Figure is representative of three separate experiments.

Ligand-induced GLP-1 receptor endocytosis occurs via coated pits

Potassium depletion and incubation in hypertonic medium inhibit endocytosis via coated pits by preventing clathrin and HA2 adaptors from interacting [34]. To verify that these treatments were effective blockers of coated pit-dependent internalization in clone 5 cells, their effect on diferric transferrin internalization, a process known to occur via coated pits [50], was assessed. Table 2 shows that metabolic inhibitors (2-deoxyglucose and azide in glucose-free buffer) completely blocked internalization of transferrin as only 12.9 % of the total radioactivity was found in the low-density membrane fraction, a percentage corresponding to contaminating plasma membranes in this fraction (see Table 1). Hypertonicity also totally inhibited internalization of transferrin (Table 2). By comparison with metabolic inhibitor treatment and hypertonicity, potassium depletion inhibited internalization of transferrin by only about 30%. Thus hypertonicity is a strong inhibitor of coated pit-dependent internalization in clone 5 cells,
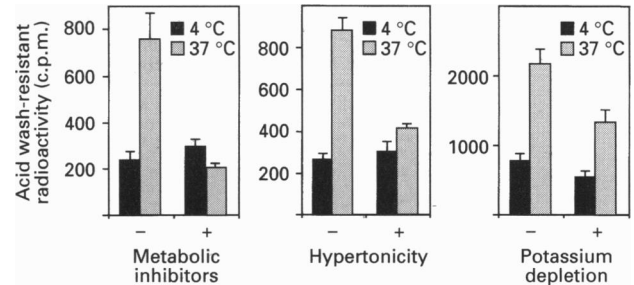

Figure 6 ^{125}I-GLP-1 endocytosis by clone 5 cells in the presence of energy inhibitors, or in hypertonic or potassium-depleted media

Cells were incubated with 200 pM ^{125}I-GLP-1 for 5 h at 4 °C in control medium (−), in (a) the presence of metabolic inhibitors (2-deoxyglucose and sodium azide), in (b) hypertonic or in (c) potassium-depleted media. After washing, the different prewarmed media were added for 8 min (left-hand panel) or 4 min (middle and right-hand panels) at 37 °C (stippled bars) or kept at 4 °C (solid bars). Radioactivity resistant to acid washes was then measured in triplicate. Internalization was completely blocked by metabolic inhibitors, while hypertonicity and potassium depletion blocked internalization by about 80% and 40% respectively, suggesting that endocytosis took place, at least in part, via coated pits. These plots are representative of two separate experiments.

while potassium depletion is less potent. We then assessed the ability of these treatments to inhibit internalization of iodinated GLP-1 in clone 5 cells. As shown in Figure 6, 60–75 % of the GLP-1 initially bound to clone 5 cells at 4 °C in the absence of any treatment rapidly became acid wash-resistant upon rewarming, indicating that it was internalized. Hypertonicity and, to a lesser extent, potassium depletion inhibited endocytosis (Figure 6, middle and right-hand panels). Since the hypertonic treatment did not completely inhibit endocytosis of iodinated GLP-1 in contrast to what was found for internalization of transferrin, it is possible that a fraction of the GLP-1 receptors was internalized via a non-clathrin-dependent endocytic route. Nevertheless, our results indicate that endocytosis of the GLP-1–GLP-1-receptor complex occurs mainly via clathrin-coated pits. This route of receptor endocytosis is therefore very similar to that described for β_2 adrenergic receptors [16] or the substance P receptor [17], as well as for single transmembrane receptors such as those for epidermal growth factor [51] or transferrin [52] (see also Table 1).

Antagonist binding does not trigger endocytosis

Exendin-(9–39) is an antagonist of the GLP-1 receptor [6,53]. This compound binds to the receptor with the same affinity as GLP-1 but does not induce cyclic AMP formation and can actually inhibit the production of cyclic AMP induced by GLP-

Table 2   Effect of metabolic inhibitors, hypertonicity and potassium depletion on the endocytosis of transferrin

Cells were treated with 2-deoxyglucose and azide, incubated in hypertonic medium or in medium deprived of potassium (+) or incubated in control medium (−), as described in the Materials and methods section. Iodinated transferrin was then added for 15 min at 37 °C and light and heavy membrane fractions were prepared by centrifugation on sucrose step gradients. Radioactivity in each fraction was determined in a gamma counter. Results are expressed as the percentage of the total radioactivity (light + heavy fractions) (mean $\pm$ S.E.M. of two independent experiments).

Fractions	Metabolic inhibitors		Hypertonicity		Potassium depletion	
	−	+	−	+	−	+
Light	59.3 ± 1.9	12.9 ± 1.1	60.1 ± 5.7	12.8 ± 5.5	52.4 ± 1.6	39.6 ± 3.8
Heavy	40.7 ± 1.9	87.1 ± 1.1	39.9 ± 5.7	87.2 ± 5.5	47.6 ± 1.6	60.4 ± 3.8

Table 3   The antagonist exendin-(9–39) does not induce internalization of GLP-1 receptor

Cells were incubated for 40 min at 37 °C in control medium or medium containing 10 nM GLP-1 or 10 nM exendin-(9–39). The cells were then washed and cell-surface-receptor expression was measured by saturation binding. Results are expressed as specific c.p.m. bound ± S.D. ($n = 6$). Values in parentheses are percentages of initial binding.

	GLP-1 receptor cell surface expression (c.p.m.)		
	Control	10 nM GLP-1	10 nM Exendin-(9–39)
Experiment 1	537 ± 64 (100)	157 ± 39 (29)	545 ± 72 (101)
Experiment 2	1458 ± 420 (100)	145 ± 329 (10)	1394 ± 281 (96)
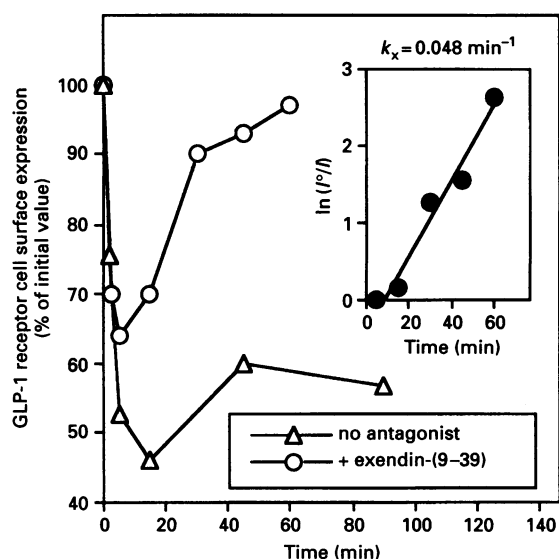
Figure 7   Surface re-expression of GLP-1 receptor binding sites after one round of endocytosis

Cells were incubated for 5 h at 4 °C with 10 nM GLP-1. After washing, prewarmed medium with or without 50 nM of the antagonist exendin-(9–39) was added for the indicated periods of time and cell-surface receptor expression was measured by saturation binding analysis. Results are expressed as the mean ± S.D. [triplicate determinations when exendin-(9–39) was not used and sextuplet determinations otherwise]. Error bars have been omitted for clarity. Relative errors on specific bound radioactivity varied from 10–40% (mean of 19%) and 3–11% (mean of 6%) for the experiments performed in the absence and in the presence of exendin-(9–39), respectively. Inset: the data in the main Figure corresponding to the incubation in the presence of exendin-(9–39) were replotted as $\ln(I^0/I)$ as a function of time, where I corresponds to intracellular GLP-1 receptors (calculated as 100 — values of the main Figure) and I^0 the corresponding value at $t = 5$ min (when GLP-1 receptor internalization is maximum). The externalization constant (k_x) was 0.048 min⁻¹, which corresponds to a $t_\frac{1}{2}$ of 14.4 min. Initial ¹²⁵I-GLP-1 binding: 2058 ± 237 c.p.m. and 4234 ± 476 c.p.m. for the experiments performed in the absence and in the presence of exendin-(9–39) respectively. This plot is representative of three separate experiments.

1 on clone 5 (data not shown). To determine whether binding of this antagonist to the GLP-1 receptor could trigger endocytosis, two different sets of experiments were performed. First, we incubated clone 5 cells with 10 nM GLP-1 or 10 nM exendin-(9–39) for 40 min. Following washings with an acidic buffer, the total number of cell-surface receptors was measured by saturating binding analysis. Table 3 shows that GLP-1 induced a 70–90 % decrease in cell-surface receptors while cells incubated in the presence of exendin-(9–39) had the same number of surface receptors compared with untreated cells. Secondly, distribution of mature GLP-1 receptors into light and heavy membrane

fractions was determined following clone 5 incubation with 10 nM GLP-1 or 10 nM exendin-(9–39). Figure 4(a) shows that, while GLP-1 induced redistribution of the receptors to endosomal compartments, exendin-(9–39) was unable to do so. The results of these two experiments indicate that despite an affinity for the receptor similar to GLP-1 itself, the antagonist exendin-(9–39) is unable to trigger rapid receptor endocytosis. This indicates that binding of the ligand to its receptor is not sufficient to induce internalization but that a change in conformation of the receptor by the ligand is probably required for clustering in coated pits to be initiated. Whether the same change in conformation as that leading to activation of G-protein is responsible for internalization is not known. However, it has been shown that receptors unable to activate G-proteins can internalize following agonist binding [54]. It seems therefore that activation of two different domains of the receptor are required for G-protein activation and for receptor internalization.

Kinetics of GLP-1 receptor re-expression at the cell surface

GLP-1 receptor re-expression to the cell surface following agonist-induced internalization was assessed by inducing one round of receptor endocytosis and measuring the kinetics of re-expression of GLP-1-binding sites at the cell surface. Clone 5 cells were incubated at 4 °C with 10 nM unlabelled GLP-1 for 5 h and washed free of unbound ligand. The cells were then warmed up to 37 °C and rapidly chilled after different periods of time. The cells were then washed at acidic pH and the number of surface receptors was determined by ¹²⁵I-GLP-1 saturation binding. Figure 7 shows that after rewarming the cells to 37 °C, there was a rapid decrease in the number of cell-surface receptors, as expected from the kinetics of internalization measured previously (Figures 2a and 4b). However, in these conditions, no clear reappearance of GLP-1 binding sites to the cell surface could be detected. Since the experiment of Figure 5 suggested a continuous cycling of the receptors to the cell surface, we suspected that GLP-1, which binds to non-specific sites from which it cannot be completely removed by the washings performed at the end of the incubation at 4 °C, could bind to the receptor emerging from intracellular compartments, thereby inducing rapid re-endocytosis. To test this hypothesis, we performed the same experiment but with the addition of an excess of exendin-(9–39) in the warming medium, expecting that binding of the antagonist would prevent receptor re-endocytosis. In these conditions, we measured the same rapid rate of receptor endocytosis during the first 5 min of rewarming. However, in contrast to the previous experiment, we were now able to measure surface re-expression of the receptor to control levels (Figure 7). This suggests that the endocytosed receptor is quantitatively cycled

Table 4   Surface re-expression of internalized GLP-1 receptor as measured by Western blot analysis

Cells were incubated for 5 h at 4 °C with 10 nM GLP-1. After washing, prewarmed medium containing 50 nM of the antagonist exendin-(9–39) was added for the indicated periods of time. Cells were then lysed and high- and low-density membranes were separated on sucrose step gradients. GLP-1 receptor in each fraction was detected by Western blot analysis as described in Figure 4(b). Results are expressed as the percentage of total core-glycosylated or mature receptors present in the low- and high-density fractions (mean ± S.E.M. of two independent experiments).

Incubation time	Fraction...	Core-glycosylated receptors (%)		Mature receptors (%)	
		Low-density	High-density	Low-density	High-density
0 min		35.0 ± 2.7	65.0 ± 2.7	11.6 ± 6.3	88.4 ± 2.7
5 min		33.8 ± 1.6	66.2 ± 1.6	30.0 ± 1.0	70.0 ± 1.0
60 min		37.5 ± 1.8	62.5 ± 1.8	19.0 ± 0.3	81.0 ± 0.3

back to the plasma membrane and that, in the absence of antagonist, binding of remaining GLP-1 induces rapid receptor endocytosis. The rate constant k_x of GLP-1 receptor re-expression was derived after logarithmic transformation of the binding data obtained in the presence of exendin-(9–39) (Figure 7 inset). The value of k_x obtained from Figure 7 and two other experiments was 0.047 ± 0.001 min^{-1} (mean ± S.D.) which corresponds to a $t_\frac{1}{2}$ of 15 min. In an additional experiment, clone 5 cells were incubated for 30 min with 400 μM cycloheximide and thereafter treated as described in Figure 7, but in the continuous presence of 400 μM cycloheximide. The result of this experiment was essentially identical to that of Figure 7, indicating that GLP-1 receptor cell-surface re-expression did not involve, in a significant manner, newly synthesized proteins.

It could be argued that the decrease and reappearance of GLP-1 binding sites at the cell surface shown in Figure 7 could have resulted from a change of affinity rather than from recycling of the receptors. Western blot analysis of low- and high-density membrane fractions was thus used to directly follow the redistribution of the receptors in the experimental conditions described in Figure 7. Clone 5 cells were preincubated for 5 h at 4 °C with 10 nM GLP-1 to saturate the receptors with the agonist. After washing, the cells were incubated for different periods of times at 37 °C in the presence of exendin-(9–39). As shown in Table 4, there was a 2.6-fold increase in mature GLP-1 receptors in the low-density fraction after 5 min incubation at 37 °C, reaching 30% of the total mature pool, and a corresponding decrease in the high-density fraction. After 1 h at 37 °C, the amount of mature GLP-1 receptors decreased to 19 % in the low-density fraction and increased to 81 % in the high-density fraction. In contrast, no change in the amount of core-glycosylated GLP-1 receptor present in the low- and high-density fractions was observed. These data are consistent with a receptor internalization/surface re-expression cycle induced by agonist binding. We cannot, however, exclude the possibility that the receptors which reappeared at the cell surface came, at least in part, from the intracellular, core-glycosylated receptors pool.

Redistribution of GLP-1 receptor in the continuous presence of the ligand

The difference in the rates of internalization ($t_\frac{1}{2}$ of 2–3 min) and recycling to the plasma membrane ($t_\frac{1}{2}$ of 15 min) should lead to a progressive decrease in the number of cell-surface-expressed receptors. Figure 8 shows that continuous incubation of clone 5 cells with GLP-1 indeed led to a time-dependent decrease in cell-surface receptor expression which was dependent on the GLP-1 concentration used. At 100 pM GLP-1, no significant change in cell-surface receptor number could be detected. At 3.2 nM GLP-
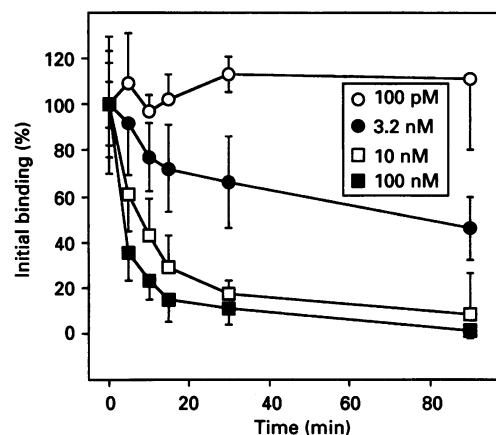
Figure 8   Redistribution of GLP-1 receptors in clone 5 cells in the continuous presence of the ligand

Cells were incubated with the indicated GLP-1 concentrations. At the indicated times, the cells were chilled on ice, washed and cell-surface GLP-1 receptor expression was measured by saturation binding analysis. Results are expressed as the mean ± S.D. of triplicate determinations. Increasing concentrations of peptides led to a time- and dose-dependent decrease in the number of receptors present on the plasma membrane of clone 5 cells. This plot is representative of two separate experiments.

1, the cell-surface receptor expression rapidly decreased by about 30 % during the first 15–20 min. At 10 nM and 100 nM GLP-1, the initial decrease occurred more rapidly and was more pronounced. The estimated fraction of internalized receptors calculated from Western blot analysis of sucrose gradient fractions was less than when calculated from saturation binding experiments (compare Figures 4b and 8). This probably results from an imperfect separation of endocytosed and surface receptors using cellular fractionation. Further experiments will determine whether the decreased number of cell-surface receptors modifies signal transduction.

Internalization of GLP-1 receptors in insulinoma cells

Since the above data were obtained with transfected cells, it was of particular interest to see whether GLP-1 receptor internalization could be demonstrated in a more physiological situation. We thus used 1056A insulinoma cells that endogenously express these receptors. Cells were incubated in control medium or with 10 nM GLP-1 for 15 min, lysed, and the cell homogenate layered over a step sucrose gradient and centrifuged
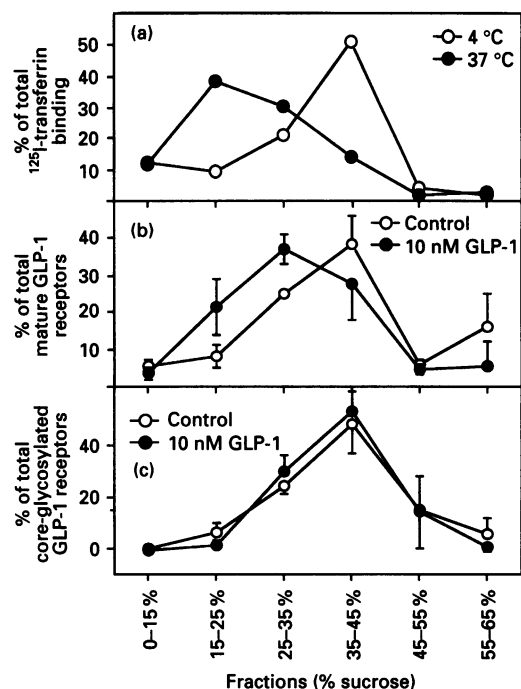
Figure 9   Agonist-induced GLP-1 receptor internalization in insulinoma cells

(a) 1056A cells were incubated in RPMI-c with 2 nM ^{125}I-transferrin for 2 h at 4 °C or for 15 min at 37 °C. The cells were then lysed and fractionated by centrifugation in a step sucrose gradient. Radioactivity of each fraction was counted in a gamma counter. Non-specific binding was determined in the presence of 1 μM unlabelled transferrin and was subtracted in each case. (b) and (c) 1056A cells were incubated in control medium or with 10 nM GLP-1 for 15 min at 37 °C, lysed and fractionated. GLP-1 receptors in each fraction were detected following gel electrophoresis and Western blotting. The amount of receptors in each fraction was determined by densitometry scanning of the bands corresponding to mature receptors (b) or to the core-glycosylated form (c). Results are expressed as the mean ± S.E.M. of two separate experiments. While the distribution of core-glycosylated receptors in the sucrose gradient was unaffected by GLP-1, the agonist induced a redistribution of mature receptors to lower-density membrane fractions, where internalized iodinated transferrin molecules were found.

to equilibrium. In control conditions, the majority of mature GLP-1 receptors migrated between 25 % and 45 % sucrose with a peak at the 35–45 % sucrose interface (Figure 9, middle panel). This is where most of the plasma membrane fraction was found as defined by the localization of transferrin molecules that were bound to cells at 4 °C (Figure 9, upper panel). When the cells were preincubated with the agonist at 37 °C, there was a shift in the distribution of mature receptors to lower-density fractions. These fractions also contained transferrin internalized at 37 °C (compare upper and middle panels of Figure 9), suggesting that internalized receptors may be co-localized in transferrin-containing endosomes as has been directly demonstrated to occur for the β-adrenergic receptor [16]. In contrast the distribution of core-glycosylated receptors was unaffected by GLP-1 pre-incubation (Figure 9, lower panel). These results indicate that GLP-1 receptors expressed at the surface of β cells are also internalized following agonist binding.

Conclusions

Altogether, our data indicate that the GLP-1 receptor, a member of a new subfamily of G-protein-coupled receptors, is internalized and recycled to the plasma membrane as a consequence of agonist binding and that this diacytosis pathway is similar to the

classical route followed by single-transmembrane segment receptors. In addition, these data, by precisely characterizing the pathway and kinetics of GLP-1 receptor internalization and recycling, will permit studies of the receptor structures required for endocytosis. Also, since experiments under way indicate that the receptor can be desensitized under certain circumstances, the role of internalization and surface recycling in these processes will be facilitated by the present study.

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