Characterization of glucagon-like peptide-1 receptor-binding determinants

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ABSTRACT

Glucagon-like peptide 1 (GLP-1) is a potent insulinotropic hormone currently under study as a therapeutic agent for type 2 diabetes. Since an understanding of the molecular mechanisms leading to high-affinity receptor (R) binding and activation may facilitate the development of more potent GLP-1R agonists, we have localized specific regions of GLP-1R required for binding. The purified N-terminal fragment (hereafter referred to as NT) of the GLP-1R produced in either insect (Sf9) or mammalian (COS-7) cells was shown to bind GLP-1. The physical interaction of NT with GLP-1 was first demonstrated by cross-linking (^{125}I -GLP-1/NT complex band at ~ 28 kDa) and secondly by attachment to Ni^{2+} -NTA beads. The GLP-1R NT protein attached to beads bound GLP-1, but with lower affinity (inhibitory concentration (IC_{50}): 4.5×10^{-7} M) than wild-type (WT) GLP-1R (IC_{50} : 5.2×10^{-9} M). The low affinity of GLP-1R NT suggested that other receptor domains may contribute to GLP-1 binding. This was

supported by studies using chimeric glucose-dependent insulinotropic polypeptide (GIP)/GLP-1 receptors. GIP(1–151)/GLP-1R, but not GIP(1–222)/GLP-1R, exhibited specific GLP-1 binding and GLP-1-induced cAMP production, suggesting that the region encompassing transmembrane (TM) domain 1 through to TM3 was required for binding. Since it was hypothesized that certain charged or polar amino acids in this region might be involved in binding, these residues (TM2–TM3) were analyzed by substitution mutagenesis. Five mutants (K197A, D198A, K202A, D215A, R227A) displayed remarkably reduced binding affinity. These studies indicate that the NT domain of the GLP-1R is able to bind GLP-1, but charged residues concentrated at the distal TM2/extracellular loop-1 (EC1) interface (K197, D198, K202) and in EC1 (D215 and R227) probably contribute to the binding determinants of the GLP-1R.

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INTRODUCTION

Glucagon-like peptide-1 (GLP-1) is an important glucoregulatory hormone that is secreted from intestinal L cells in response to nutrient ingestion. The biologically active forms of GLP-1, (GLP-1(7–36) amide and GLP-1(7–37)), have been demonstrated to possess multiple functions, including enhancement of glucose-dependent insulin secretion, stimulation of proinsulin biosynthesis and inhibition of food intake (reviewed by Fehmann *et al.* 1995, Drucker 1998). Additional actions of GLP-1 may include enhancement of insulin sensitivity and the induction of β -cell protection, proliferation and differentiation (Buteau *et al.* 1999, Sandhu *et al.* 1999, Wang *et al.* 1999, Xu *et al.* 1999). Several

reports indicate that GLP-1 enhances insulin secretion not only in normal subjects, but also in those with type 2 diabetes mellitus. These studies suggest that GLP-1-receptor agonists have great clinical potential for the treatment of patients with type 2 diabetes mellitus (Gutniak *et al.* 1992, 1996, Nathan *et al.* 1992, Nauck *et al.* 1993).

The biological functions of GLP-1 are mediated through high-affinity GLP-1 receptors (GLP-1Rs) (reviewed by Thorens 1993). This receptor, cloned initially from rat pancreatic islet cells (Thorens 1992) and subsequently from a human pancreatic insulinoma (Dillon *et al.* 1993), predicts a seven-transmembrane G-protein-coupled receptor (GPCR) which couples with G_s and the protein kinase A (PKA) pathway to stimulate insulin

secretion. Recent studies demonstrate that GLP-1R can also couple with other G proteins, however, including G_q11, G_i1 and G_i2 (Montrose-Rafizadeh *et al.* 1999), and activates the mitogen-activated protein kinase pathway in CHO/GLP-1R and β -cell lines (Buteau *et al.* 1999, Montrose-Rafizadeh *et al.* 1999). This strongly suggests the possible involvement of GLP-1R in the regulation of β -cell mass via PKA-dependent and PKA-independent mechanisms.

Given the therapeutic potential of GLP-1R agonists, it is important to understand how this large peptide hormone (30 amino acids) interacts with its receptor. The GLP-1R belongs to the B-class receptor family that includes receptors for glucagon, GLP-2, glucose-dependent insulinotropic polypeptide (GIP) and secretin. A key structural feature of this receptor family is a large extracellular N-terminal domain (hereafter referred to as NT) containing consensus sites for asparagine-linked glycosylation and six conserved cysteine residues that form putative disulfide bonds which probably contribute to the development of a highly folded structure (EMBL 1997). Pertinent to the present study are several lines of evidence that suggest that, in receptors within the B-class family, the NT domain contains putative binding sites (Holtman *et al.* 1995, Bergwitz *et al.* 1996, Gourlet *et al.* 1996, Graziano *et al.* 1996, Van Eyll *et al.* 1996, Wilmen *et al.* 1996, 1997). Our work, using a series of chimeric GLP-1 and GIP receptors, supports a role for the GLP-1R and GIP receptor (GIP R) NT domains in this process (Gelling *et al.* 1997). However, this work and that of others also suggest that the NT domain of the B-class receptors is insufficient for high-affinity binding or receptor activation (Buggy *et al.* 1995, Hjorth & Schwartz 1996, Holtmann *et al.* 1995, 1996).

In the present study, we provide evidence supporting a role for the GLP-1R NT domain in ligand binding, by using a purified recombinant NT-domain protein. Moreover, using a series of chimeric receptors and a site-directed mutagenic approach, we have analyzed other domains and specific charged and polar amino acids that potentially associate with the ligand. These data demonstrate that GLP-1R binding may be complex, involving the NT domain and certain charged residues in the distal transmembrane domain 2 (TM2) and first extracellular loop 1 (EC1).

MATERIALS AND METHODS

Preparation of rat GLP-1 receptor constructs

The wild-type (WT) rat GLP-1 receptor DNA was introduced into HindIII and XhoI sites of vector

pcDNA 3 (Invitrogen, CA, USA) for mutagenesis. Mutations were introduced into the GLP-1R coding sequence by oligonucleotide-directed mutagenesis using a double-stranded template (Stratagene, CA, USA). Mutant sites were verified by dideoxy sequencing with T7 polymerase (Pharmacia, Uppsala, Sweden). WT and mutant receptor cDNAs were analyzed in COS-7 cells for binding assays and cAMP-accumulation determinations, as described below. Receptor mutants that were found to show little or no function (binding and cAMP accumulation) were cloned into the pEGFP vector (EGFP, enhanced green fluorescent protein) (Clontech, CA, USA) to produce a receptor-GFP fusion protein for further analysis, as we have previously described (Salapatek *et al.* 1999).

NT (GLP-1R, 1–143 amino acids) and full-length GLP-1 constructions were obtained for baculoviral expression via cloning into pFastBac1 at ApaI/NotI sites. The NT or WT GLP-1R baculovirus constructs contained *c-myc* and (His)₆ tags for immunodetection and purification. Cloning was verified by dideoxy sequencing. A mammalian GLP-1R NT construction was produced by cloning the NT domain (1–143 amino acids) into the pSecTag2 vector (Invitrogen) at HindIII/XhoI sites. The protein expressed from this vector was fused at the N-terminus to the murine immunoglobulin kappa (Igk) chain leader sequence, for protein secretion, and at the C-terminus to a peptide containing the *c-myc* epitope and (His)₆, for detection and subsequent purification (see below).

Cell culture and transfection

COS-7 cells cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and grown in 10 cm tissue-culture dishes were transfected with plasmid DNA encoding WT, or mutant, GLP-1Rs (5 or 10 μ g for binding assays and cAMP assays respectively) using the diethylaminoethyl-dextran method as described previously (Wheeler *et al.* 1999). For baculoviral expression, the GLP-1R NT constructs were transformed into DH10 Bac-competent cells (Gibco BRL, Burlington, ON, Canada) containing a baculovirus shuttle vector (bacmid). Colonies indicative of the presence of the recombinant bacmid were selected for isolation of recombinant bacmid DNA. Recombinant bacmid DNA was isolated and purified according to the manufacturer's instructions (Gibco BRL). Sf9 cells at 60–80% confluence were infected and then harvested 4 days post-infection; this was

followed by Western blotting to determine protein expression.

Ligand binding and cAMP assays

Synthetic human GLP-1(7–36) amide was radio-labeled with carrier-free I^{125} by using the chloramine-T method as previously described (Gelling *et al.* 1997). The iodination reaction mixture was purified by reverse-phase adsorption to a C-18 Sep-pak cartridge (Waters Associates, Milford, MA, USA). COS-7 cells expressing WT and mutant receptors, 72 h post-transfection, were washed twice in PBS and recovered from plates with 2 mM EDTA in PBS. Cells (5×10^5 /tube) were incubated for 45 min at 37 °C in binding assay buffer with I^{125} -GLP-1 and unlabelled GLP-1 at concentrations of 10^{-12} to 10^{-6} M, in a final volume of 200 μ l. The cell suspension was centrifuged at 12 000 *g* and the cell pellet was washed with cold PBS prior to counting, as previously described (Salapatek *et al.* 1999). Binding assays were also performed on semi-purified plasma membranes prepared from SF-9 cells. Briefly, 100 μ g plasma membrane expressing NT was incubated with I^{125} -GLP-1 tracer and increasing concentrations of non-radiolabelled GLP-1. After 45 min incubation at 37 °C, the mixture solution was centrifuged and the pellets counted. The protein concentration was determined with the BCA Protein Assay Reagent Kit (Pierce, Rockford, IL, USA).

For cAMP measurements, COS-7 cells expressing the WT and mutant receptors were passaged into 6- or 12-well plates 24 h post-transfection. After culture for an additional 48 h, cells were washed in PBS then assay buffer (DMEM containing 1% BSA) and were then preincubated for 30 min at 37 °C. Cells were stimulated with GLP-1 in the presence of 3-isobutyl-1-methylxanthine (IBMX, 1 μ M) for 30 min. The cAMP was extracted using 80% ethanol and stored (–20 °C) until radioimmunoassay. The cAMP concentrations were measured as described previously (Salapatek *et al.* 1999).

NT-protein purification and NT- Ni^{2+} -NTA domain binding assay

Ni^{2+} -NTA Superflow resin was used for purification of the (His)₆-tagged NT GLP-1R fusion protein. The purification procedure was modified according to the manufacturer's instructions (Qiagen Inc., Mississauga, ON, Canada). Briefly, an aliquot of slurry of Ni^{2+} -NTA resin was washed

with sonication buffer (50 mM sodium phosphate, pH 7.8, 300 mM NaCl) and incubated with cell media containing secreted NT protein or the control protein (prostate-specific antigen, PSA) for 1 h at room temperature. The media mixture was loaded onto a column and then washed with sonication buffer and wash buffer (50 mM sodium phosphate, 300 mM NaCl, 10% glycerol, pH 6.0). The recombinant protein was eluted with 50, 200 and 500 mM imidazole. The fractions were collected and analyzed by Western blotting as described below. For binding assays, the cell media containing GLP-1R NT or PSA were incubated with Ni^{2+} -NTA resin for 1 h at room temperature followed by three washes with wash buffer. The resin beads containing NT or PSA protein (100 μ g protein) were then incubated with I^{125} -GLP-1 for 45 min in the presence or absence of unlabelled GLP-1. After a centrifugation step, the pellet was washed with cold PBS and the level of specific radioactivity determined.

Western blotting and cross-linking

Purified samples (10 μ g protein) were separated by 10% SDS-PAGE and transferred to a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA, USA). After an overnight incubation with blocking buffer, the membrane was washed three times with TBS-T buffer (0.05 M Tris, 0.15 M NaCl, 0.1% (v/v) Tween) before incubation with 1:5000 primary antibody (anti-*myc* or anti-His; Invitrogen) followed by an anti-mouse or an anti-rabbit horse-radish peroxidase-conjugated secondary antibody (1:5000) respectively. Blots were then detected with ECL reagents according to the manufacturer's instructions (Amersham Pharmacia Biotech UK Ltd, Amersham, Bucks, UK). For cross-linking, plasma membranes (100 μ g) were incubated with I^{125} -GLP-1 (50 000 c.p.m.) in Hepes buffer (50 mM, pH 7.5) containing 0.02% BSA for 45 min at 37 °C in the presence or absence of 1–10 μ M GLP-1. The membrane mixtures were centrifuged at 12 000 *g* for 5 min at 4 °C and pellets were resuspended in 10 mM Hepes (pH 7.5) containing 0.02% BSA. The cross-linker disuccinimidyl suberate (DSS) in dimethylsulfoxide was added to the solution at a final concentration of 1 mM. After an incubation period (10 min) at 4 °C, an ammonium acetate solution was added (final concentration 10 mM) to quench the reaction. Membranes were spun at 12 000 *g* for 5 min at 4 °C and the pellets were resuspended in Hepes buffer (10 mM, pH 7.5) containing 0.02% BSA. The mixture was incubated for an additional 30 min, centrifuged and then resuspended in 10 mM sodium phosphate buffer

containing 2% SDS (pH 7.5). Samples were prepared for SDS-PAGE. Gels were dried and exposed to film for 3–14 days using one intensifying screen.

Confocal microscopy

On glass cover-slips in six-well plates, COS-7 cells were grown and transfected with plasmids encoding EGFP-tagged WT or selected mutant GLP-1R constructions; 72 h post-transfection, the cells were analyzed by confocal microscopy as described previously (Salapatek *et al.* 1999).

Data analysis

All values are expressed as the means $\pm$ S.E.M. of at least three independent experiments. Statistical analyses were performed using the Student's paired *t*-test (INSTAT; GraphPad Software, San Diego, CA, USA). $P < 0.05$ was taken as the level of significant difference.

RESULTS

Sf9-cell NT-domain expression

The NT domain of the GLP-1R cloned into pFastBacHTb and pFastBac1 was transformed into DH10 Bac-competent cells containing a baculoviral shuttle vector. Recombinant bacmids were isolated and used to infect Sf9 cells as described in the Materials and Methods. Two days post-infection, the NT-domain protein was characterized by Western blot analysis (Fig. 1). The NT domain expressed without the putative GLP-1R leader sequence (–LS; see Fig. 2) appeared as a ~ 17 kDa product (Fig. 1B), whereas the NT domain expressed with the leader sequence (+LS; Fig. 1C) appeared as two larger isoforms, (~ 21 and ~ 24 kDa; see lanes 2 and 3). The NT-domain protein with or without the LS was treated with PNGase F (to cleave N-linked oligosaccharides): NT(–LS) was not affected, but NT(+LS) collapsed to a single 21 kDa product (lane 4). To clarify the location of the expressed non-glycosylated and glycosylated NT-domain proteins in Sf9 cells, the infected cells were fractionated into cytosolic and plasma-membrane fractions by using sucrose-gradient centrifugation followed by Western blot analysis. As shown in Fig. 1D, the glycosylated (+LS) NT GLP-1R was mainly associated with the plasma-membrane fraction, there being minimal expression in the cytosol (left

panel, lanes 1 and 2). However, the non-glycosylated NT domain was found in both the plasma-membrane and the cytosolic fractions (right panel, lanes 1 and 2). The fractions obtained from untransfected cells contained no specific signal (lanes 3 and 4).

Sf9 NT-domain binding and cross-linking

In order to determine the potential physical interaction between GLP-1 and the NT domain of GLP-1R, the NT was covalently cross-linked to 125 I-GLP-1 by using DSS (see the Materials and Methods). The complex solution was subsequently separated by SDS-PAGE. A ligand-binding protein with a molecular mass of 28 kDa was observed with glycosylated NT GLP-1R (Fig. 3, panel 2). This band was competed with excess GLP-1 (10 μ M), demonstrating the specificity of the peptide for the NT. Interestingly, larger specific bands were observed, suggesting that the NT domain may complex as a dimer, or perhaps as larger complexes (Fig. 3, panel 2). Cells transfected with full-length WT GLP-1R exhibited a strong interaction, as demonstrated by an intense signal of the predicted size (~ 65 kDa). As expected, the intensity of the ligand-binding complex was dramatically reduced in the presence of GLP-1 (10 μ M) (Fig. 3, panel 3). Non-glycosylated NT-domain protein and protein obtained from control cells (Fig. 3, panels 1 and 4 respectively) did not form detectable complexes. These data suggest that the GLP-1R NT domain is capable of interacting with GLP-1, but that the interaction is probably weaker than that of the intact GLP-1R (given that approximately equal amounts of protein were used in these studies). These data also suggest that the NT domain may, in part, contribute to receptor dimerization, which has been described for members of the A-class receptor family (Ng *et al.* 1996, Han & Hampson 1999, Jordan & Devi 1999).

In order to test the hypothesis that the NT domain binds GLP-1, but with lower affinity, the plasma-membrane fraction of Sf9 cells expressing the NT was used in a competitive displacement binding assay. WT GLP-1R membranes (100 μ g) showed typical GLP-1 binding ($IC_{50} = 12$ nM). The glycosylated NT domain of GLP-1R showed a lower binding affinity compared with the full-length GLP-1R, specific displacement being observed at GLP-1 concentrations between 0.1 and 1 μ M (data not shown). The non-glycosylated NT domain showed no detectable displacement at 1 μ M GLP-1 (data not shown), in agreement with cross-linking studies that suggest that the NT(–LS) protein is non-functional.

FIGURE 1. Expression of the GLP-1R NT domain generated in Sf9 cells. (A) Schematic diagram of the constructs (Tn7, transposon 7; pPolh, polyhedrin promoter) encoding the NT cDNA with (top) and without (bottom) the putative leader sequence (LS). (B) Expression of the GLP-1R NT domain in infected Sf9 cells was analysed by Western blotting: lane 1, uninfected Sf9 cells (control); lane 2, the NT domain expressed without the putative LS appears as a ~17 kDa band; lane 3, the NT domain (with the LS), which appears as two isoforms (~21 kDa and ~24 kDa). (C) Deglycosylation of protein samples obtained from infected Sf9 cells was performed by treatment with peptide-N-glycosylase (PNGase) F followed by Western blot analysis. GLP-1R NT without the LS (lanes 1 and 2) and with the LS (lanes 3 and 4) were untreated (lanes 1 and 3) or treated (lanes 2 and 4) with PNGase F. The glycosylated form and the non-glycosylated form are indicated by the upper and lower arrows respectively. (D) The locations of the expressed non-glycosylated and glycosylated NTs were determined by analyzing the cytosolic and plasma-membrane fractions obtained by sucrose-gradient centrifugation. Left panel: glycosylated NT was associated with the plasma membrane (lane 1) and showed minimal expression in the cytosol (lane 2). Right panel: non-glycosylated NT was present in both the plasma membrane (lane 1) and cytosolic fractions (lane 2). Plasma-membrane and cytosolic fractions of untransfected Sf9 cells are shown in lanes 3 and 4 respectively, in both panels. Molecular mass markers are indicated beside each panel.

FIGURE 2. Schematic representation of the predicted structure of the rat GLP-1R. The GLP-1 receptor is predicted to contain an NT, three EC and IC loops, seven TM domains and a carboxy-terminal (CT) tail. Amino acids are identified by the one-letter code. ●, represent the amino acids that were substituted by A in this study. ● represent the leader sequence.

GLP-1R NT purification and binding

To isolate a GLP-1R NT domain that is produced in mammalian cells and does not require a detergent solubilization step, COS-7 cells were transfected with the construct pSec NT GLP-1R (Fig. 4A). Since this construct is designed to produce a secretable protein, in theory the NT domain should accumulate in culture media. Three days post-transfection, soluble protein containing the *myc*-(His)₆ tag was purified using an Ni²⁺-NTA Superflow column and eluted with increasing concentrations of imidazole (50–500 mM). The elution fractions were analysed by Western blotting using anti-*myc* antibody. As shown in Fig. 4B, a strong protein band of molecular mass 30 kDa (lanes 2 and 3) was observed in the eluate fractions (50 and 200 mM imidazole), but was not present in control cell media, unpurified media or washing

buffer. Since the deduced molecular masses of the NT domain and the vector fusion protein were approximately 22 kDa and 5 kDa respectively, the larger apparent size of the protein could be attributed to post-translational glycosylation. Additionally, the appearance of the 80 kDa band may represent non-specific binding with components present in the culture medium (since it was also present in the control fraction).

To determine if the purified NT protein obtained from a mammalian expression system was able to bind GLP-1, the NT-domain protein was absorbed onto Ni²⁺-NTA beads by using the procedure described in the Materials and Methods (see Fig. 4C) to perform a competitive displacement assay. When compared with the non-specific binding (2668 ± 276 c.p.m./tube, *n*=3), represented by the counts obtained from the control beads attached to an equivalent amount of expressed PSA control

FIGURE 3. GLP-1R NT cross-linking and binding to GLP-1. Chemical cross-linking of ^{125}I -GLP-1 to NT, without the LS (panel 1) and with the LS (panel 2), and full-length GLP-1R (lane 3) expressed in Sf9 plasma membrane in the presence or absence of GLP-1 (10 μM). Panel 2: a major ^{125}I -GLP-1/NT complex (28 kDa) was revealed (lower arrow) which was diminished with excess GLP-1. Panel 3: an ~ 65 kDa band indicative of the ^{125}I -GLP-1/GLP-1R complex (upper arrow) was also diminished with 10 μM GLP-1. Non-glycosylated NT, GIP R or uninfected Sf9 plasma membranes did not bind ^{125}I -GLP-1 (panels 2 and 4). Molecular mass markers are shown to the right of the panels.

protein (~ 100 μg), the NT protein was found to be able to bind ^{125}I -GLP-1 (3690 ± 627 c.p.m./tube, $n=3$). The value for specific binding was 1021 ± 218 c.p.m./tube. Since the radioactivity associated with beads with attached PSA could not be displaced with GLP-1 at concentrations as high as 10 μM (2668 ± 276 vs 2517 ± 171 , $n=3$), it was considered to be background radioactivity. In contrast, with NT protein, specific binding was inhibited in the presence of graded increases in unlabelled GLP-1 with an IC_{50} of 450 ± 35 nM (Fig. 4D). This value was remarkably high relative to that of the full-length GLP-1R expressed in COS-7 cells (5.3 ± 1.9 nM).

Chimeric GIP/GLP-1 receptor

The relatively low affinity of the GLP-1R NT for GLP-1 suggests that other domains within the GLP-1R contribute to high-affinity binding. In order to test this hypothesis, a chimeric-receptor strategy was employed. Although the GIP receptor shares approximately 40% homology with GLP-1R, we have shown that neither binds or is activated by the other's ligand, at concentrations as high as 1 μM . Two chimeric receptors (see Fig. 5A), GIP(1–151)/GLP-1R (CH3) and GIP(1–222)/GLP-1R (CH4), were produced and expressed in

COS-7 cells. Previous studies showed that both of these receptor chimeras efficiently bound, and were activated by, GIP (Gelling *et al.* 1997). Interestingly, we found that the GIP(1–151)/GLP-1R construct (CH3) also bound GLP-1 with a high affinity relative to that of WT GLP-1R (1.4 ± 1.0 nM vs 3.8 ± 0.8 nM; Fig. 5B), but the maximum binding (B_{max}) for ^{125}I -GLP-1 was reduced approximately 12-fold (376 ± 43 vs 3932 ± 1234 c.p.m./tube). Of further interest was the observation that this chimeric receptor was still activated by GLP-1 (10 nM), with a ~ 2.5 -fold increase over basal cAMP production (Fig. 5D). In contrast, GIP(1–222)/GLP-1R (CH4) lost the ability to bind GLP-1 or to lead to any measurable increase in cAMP accumulation, despite the observation that both CH3 and CH4 were bound and activated efficiently by GIP (Fig. 5C and E; CH3: $B_{\text{max}} = 5150 \pm 1424$ c.p.m./tube, $\text{IC}_{50} = 18.3 \pm 1.5$ nM; CH4: $B_{\text{max}} = 1636 \pm 466$ c.p.m./tube, $\text{IC}_{50} = 2.8 \pm 0.9$ nM). Results obtained from the CH3 chimeric receptor suggested that specific domains and amino acid residues distal to the NT contribute to GLP-1 binding and receptor activation. The fact that CH4 apparently did not interact with GLP-1 suggested that the TM1–TM3 region of the receptor contains these important moieties. The studies described below address this hypothesis.

FIGURE 4. Expression and functional analysis of the GLP-1R NT domain secreted from COS-7 cells. (A) Schematic representation of the NT-pSecTag construct. Expressed NT was fused at the N-terminus to the murine Igk chain LS, for protein secretion, and at the C-terminus to a peptide containing *c-myc* and (His)₆ epitopes, for purification and detection. (B) Secreted NT protein was purified on an Ni²⁺-NTA column and sequentially eluted with imidazole after a complete wash, followed by Western blot analysis. Lanes: 1, unpurified cell media; 2, wash fraction (after loading of media onto the column); 3–5, the fractions sequentially eluted by 50, 200 and 500 mM imidazole; 6, control (fresh DMEM media). Molecular mass markers are shown to the right of the panel. (C) The diagram represents the competitive binding assay of purified NT protein. NT protein with the (His)₆ tag associated with Ni²⁺-NTA beads bound ¹²⁵I-GLP-1 and cold GLP-1. (D) Radioligand binding analysis of purified NT. The specific binding was determined by subtraction of non-specific counts (determined using the PSA control; see the Materials and Methods) from the total counts. Data are means ± S.E.M. of three independent experiments.

FIGURE 5. Analysis of binding and signaling of WT and chimeric GIP/GLP-1 R expressed in COS-7 cells. (A) Schematic representations of GIP R, GLP-1R, and two chimeric receptors GIP(1–151)/GLP-1R (CH3) and GIP(1–222)/GLP-1R (CH4). (B) Binding of the chimeric receptors was examined for ^{125}I -GLP-1 binding in COS-7 cells compared with GLP-1R. (C) Binding of the chimeric receptors was examined for ^{125}I -GIP binding in COS-7 cells compared with GLP-1R. (D) Analysis of GLP-1 (100 nM) stimulated cAMP production in cells expressing GIP R, GLP-1R and chimeric receptors. (E) Analysis of GIP (100 nM) stimulated cAMP production in cells expressing GIP R and chimeric receptors. Values are the means $\pm$ S.E.M. of three different transfection experiments (* $P < 0.05$).

TABLE 1. Receptor binding and activation in transfected COS-7 cells

Region		Receptor	IC ₅₀ (nM)	cAMP (% of WT)	B _{max} (% of WT)
TM2		WT GLP-1R	5.3 ± 1.9	100	100
		N182A	2.3 ± 0.8	35.8 ± 14.6	23.7 ± 6.7
		R190A	>100	26.9 ± 8.2	20.5 ± 5.4
		K197A	26.9 ± 8.2	25.0 ± 23.5	30.4 ± 8.0
EC1		D198A	52.4 ± 18.4	19.9 ± 3.3	16.2 ± 1.7
		K202A	20.3 ± 10.0	71.4 ± 8.7	44.7 ± 8.6
		H212A	2.5 ± 0.8	102.4 ± 19.7	92.9 ± 4.1
		D215A	22.7 ± 8.4	56.7 ± 16.3	50.9 ± 12.1
		D222A	6.5 ± 3.9	81.9 ± 41.3	94.5 ± 12.6
		R227A	>100	90.2 ± 32.0	31.1 ± 6.5
TM3		N240A	>100	8.4 ± 2.7	14.4 ± 4.6
		E247A	6.0 ± 1.7	63.6 ± 16.8	39.0 ± 5.5

Ligand binding of GLP-1R substitution mutants

Chimeric receptor studies (Fig. 5) suggested that TM1–TM3 contain important residues for binding. Since it is predicted that receptor–ligand interaction within the B-class family and its respective peptides may involve interactions between charged residues (salt bridge formation), a series of alanine (A) substitutions targeting these charged and polar residues was performed (see Fig. 2). The effects of receptor mutations on GLP-1 binding properties and on receptor activation were analyzed. Mutants N182A, H212A, D222A and E247A did not significantly change receptor binding affinity (IC₅₀=2.3 ± 0.8, 2.5 ± 0.8, 6.5 ± 3.0 and 6.0 ± 1.7 nM respectively; see Table 1, Fig. 6A and B) relative to that of the WT receptor (5.2 ± 1.9 nM). N182A and E247A did display comparable decreases in both B_{max} and cAMP accumulation, which are indicative of less efficient cell-surface expression (Table 1). In contrast, K197A, D198A, K202A, D215A and R227A displayed remarkable reductions in binding affinity (IC₅₀ values 4- to 20-fold higher than that of the WT GLP-1R; Table 1, Fig. 6A and B), and this was accompanied by a reduced B_{max} value (Table 1). These data suggest that K197, D198, K202, D215 and R227 directly (by disrupting the specific interaction between the peptide and receptor) or indirectly (by changing the general conformation of the receptor) contribute to high-affinity GLP-1R binding. Mutant receptors R190A and N240A had extremely low values for B_{max} as well as cAMP accumulation (<27% of that of the WT receptor), suggesting that these residues are essential for ligand binding and/or cell-surface expression. To determine if they, along with the

WT GLP-1R, were appropriately targeted to the plasma membrane, pGLP-1R–EGFP constructions were generated and transfected into COS-7 cells for observation using confocal microscopy (Fig. 7). COS-7 cells expressing the WT GLP-1R displayed typical high-density fluorescence at the plasma membrane. In contrast, the expression of R190A or N240A was similar to that of pEGFP alone, showing a cytosolic pattern of expression, which suggests that these mutants were not targeted to the plasma membrane. As such, it was not possible to assess their contribution to surface-receptor binding. In contrast, N182A, which exhibited normal affinity but reduced expression, was appropriately targeted to the plasma membrane, but the fluorescence intensity was reduced relative to that of the WT GLP-1R (Fig. 7C).

DISCUSSION

Precise and efficient signal transduction through a GPCR requires highly specific interactions with its ligand. Varied methodological approaches have shown that each of the receptor classes displays a unique set of binding characteristics. The receptors for the glycoprotein hormones contain large NT domains that provide sites for high-affinity binding (reviewed by Ji *et al.* 1998). In contrast, the receptors for biogenic amines contain very small extracellular NT domains, so their binding elements are concentrated deep within the hydrophobic transmembrane regions. The members of the secretin B-class family of receptors have been less well studied, but, nevertheless, there is increasing evidence to suggest that several domains within the receptor interact with its ligand and that the binding characteristics may be quite complex and receptor-type specific. Given the great interest in designing small molecule-based GLP-1R agonists that are more suitable for the treatment of type 2 diabetes, it is important to understand the key structural features of the receptor that are required for receptor binding and activation. The present studies support the hypothesis that high-level binding and specificity require multiple contacts, between the receptor and GLP-1, that involve the receptor NT, TM2 and EC1. The GLP-1R, like other members of the secretin B-receptor family, contains a moderately large NT domain (predicted size 146 amino acids) that probably attains a highly folded structure. Several reports have now provided evidence that the NT domain does, indeed, play some role in the binding process, but it is unclear if the NT alone is sufficient to promote high-affinity binding. Those studies

A

B

FIGURE 6. Binding analysis of mutant GLP-1Rs in COS-7 cells. (A) Binding ability of the TM2 and (B) EC1 loop GLP-1R mutants examined for ^{125}I -GLP-1 binding. Data are the means $\pm$ S.E.M. of three independent experiments.

using site-directed mutagenesis demonstrate that residues within the GLP-1R NT, when mutated, cause loss-of-function mutants, or, in the case of K38 or R40, a significant decrease in the K_d value (Van Eyll *et al.* 1996, Wilmen *et al.* 1997). One of the most convincing indications that the NT does interact with GLP-1, however, came from Wilmen *et al.* (1996), who used a non-glycosylated recombinant GLP-1R NT fragment. After chemical refolding of the NT protein, it was capable of being cross-linked to GLP-1, but the affinity of the peptide for the receptor fragment was not

determined. In the present study, the GLP-1R NT was examined using two approaches. First, the NT domain, with or without the putative LS, was generated via baculoviral expression (Sf9 cells). Only the NT with the LS was glycosylated (presumably correctly folded) and was capable of being cross-linked to GLP-1 (Fig. 3). Secondly, a GLP-1R NT secreted from COS-7 cells and attached to Ni^{2+} -NTA beads could also be shown to bind GLP-1 (Fig. 4D). In both cases, however, binding was sufficiently weak to suggest that the NT domain alone was perhaps incapable of the full

FIGURE 7. Localization of GLP-1R expression in COS-7 cells. Transfected cells expressing EGFP-tagged receptor mutants were analysed by confocal microscopy 3 days post-transfection. (A) GFP vector control, (B) R190A mutant, (C) N182A mutant, (D) WT GLP-1R mutant and (E) N240A mutant are shown. These micrographs are representative of two experiments.

binding ability that was displayed by the intact receptor. In the purified NT binding model, the IC_{50} value for GLP-1R NT was 450 ± 35 nM, as opposed to 5.3 ± 1.9 nM for the intact receptor expressed in COS-7 cells. The precise residues within the NT that interact with the GLP-1 have not been identified, but GLP-1R mutagenic studies (Van Eyll *et al.* 1996, Wilmen *et al.* 1997) and studies with other B-class receptors may serve as a basis for speculation as to their location. For the glucagon receptor, a 12 amino acid area near TM1 (aa 126–137) has been implicated. Hydrophobic residues F184 and L187 in the distal NT portion of the parathyroid hormone (PTH) receptor were found to interact directly with the NT portion of PTH (Carter *et al.* 1999), and Dong and colleagues showed that the secretin receptor NT could interact specifically with Val4 of secretin (Dong *et al.*

1999a,b). Additionally, a naturally occurring mutation of the GH-releasing factor (GHRF) receptor (D60G) leading to a phenotype (*little mice*) caused by the loss of the receptor's ability to bind its ligand (Lin *et al.* 1993).

Despite the evidence of an interaction between the NT domain and GLP-1, the significant shift in binding affinity of the GLP-1R NT fragment suggested that the NT requires anchoring elements so that it can be appropriately presented to its ligand. Alternatively, additional binding elements may be found distal to the GLP-1R NT. The latter scenario is supported by several lines of evidence. For other B-class receptors, the EC loops and TM domains can contribute to binding. Glucagon/GLP-1R chimera studies identified several non-contiguous domains, in the receptor, that are important for high-affinity glucagon binding, specifically at EC1, EC3, TM3, TM4 and TM6 (Buggy *et al.* 1995). For the secretin receptor, critical domains have been localized to EC1, EC2 and TM2 (Holtmann *et al.* 1996, Di Paolo *et al.* 1998). In addition, TM1 and TM2 for the VIP receptor have been implicated (Hashimoto *et al.* 1997). Conversely, GIP/GLP-1 receptor chimeric studies (Gelling *et al.* 1997) clearly show that the binding selectivity of GIP for the GIP receptor requires the GIP R NT domain. Interestingly, these chimerics have also been useful for examining GLP-1 binding, since the GIP(1–151)/GLP-1 R construct (CH3) also binds GLP-1 with a high affinity relative to that of the WT receptor (Fig. 5B). In comparison, GIP(1–222)/GLP-1 R (CH4) lost the ability to bind GLP-1, suggesting that the TM1–TM3 region of the receptor contains these important moieties. The advantage of these chimeric experiments over conventional loss-of-function mutations lies with the fact that these chimeric proteins retained, or gained additional, GIP binding and signaling activity (Fig. 5C and E), ensuring that they were appropriately expressed on the cell surface.

Although it is conceivable that any residues within TM1–TM3 may be involved in ligand–receptor interactions, the strong possibility that stable receptor–peptide complexes involve ionic interactions (intramolecular ion pairing) supports the need to examine charged residues within this region. Thus, Holtmann *et al.* (1996) demonstrated that two basic residues, H189 and K190, at the first EC loop of the secretin receptor may be involved in ligand–receptor interactions. Although, in the present study, several charged residues were found not to affect GLP-1R affinity, K197A, D198A, K202A, and D215A and R227A displayed remarkably reduced binding affinity (IC_{50} values 4- to

FIGURE 8. Analysis of GLP-1-stimulated cAMP accumulation in COS-7 cells expressing GLP-1R mutants. GLP-1 (10 nM) induced cAMP production in cells expressing mutant GLP-1R. Open bars represent the cAMP contents without GLP-1 treatment, whereas solid bars represent the cAMP contents with GLP-1 treatment. The values are the means $\pm$ S.E.M. of three independent experiments.

20-fold higher than that of WT GLP-1 receptor; Table 1, Figs 6 and 8), and this is accompanied by a reduced value for $B_{\max}$ (Table 1). Upon B-class receptor alignment, K197, and D198 and R227, are conserved in the majority of the receptors of this family. In fact, a single substitution with alanine in place of R166, K173 or D174 of the secretin receptor (equivalent to R190, K197 and D198 of GLP-1R) or in place of D196 of the vasoactive intestinal peptide (VIP)-1 receptor (equivalent to D198 of GLP-1R) affects binding (Du *et al.* 1997, Di Paolo *et al.* 1998, 1999). These data suggest that those residues are important for ligand-receptor interactions across the receptor class. In contrast, K202 and D215 are unique to the GLP-1R and are therefore more likely to contribute to binding specificity. Hence, it is conceivable that in this study, the replacement of positively charged K and R or negatively charged D with A would block the potential association with the oppositely charged residues of GLP-1, reducing affinity. Alternatively, these residues may stabilize the receptor, but this is less likely than with the residues found in the TM helices. For example, in this study, mutants with extremely low $B_{\max}$ values and poor receptor activation were found in TM1 (data not shown), TM2 and TM3, and, when tagged with GFP, were incapable of being efficiently translocated and inserted into the plasma membrane (Fig. 7).

Investigations detailing the molecular structures of peptide hormones and the interactions between a receptor and its ligand may facilitate the design

of structurally smaller, potent receptor agonists or antagonists. For example, a 4-amino-acid VIP derivative has been found to possess more neurotrophic activity than the native VIP (Gozes *et al.* 1999) and a small non-peptidyl growth hormone secretagogue has been produced recently (DeVita 2000). On the basis of these and other advances, future studies on GLP-1 and its receptor may uncover interactions that might provide important information for the rational design of novel small GLP-1R agonists. The present study may suggest, however, that it is not possible to produce a small molecule based on GLP-1 structure, because of the complex interactions between the peptide and its receptor.

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