Differential Structural Properties of GLP-1 and Exendin-4 Determine Their Relative Affinity for the GLP-1 Receptor N-Terminal Extracellular Domain†

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ABSTRACT: Glucagon-like peptide-1 (GLP-1) and exendin-4 (Ex4) are homologous peptides with established potential for treatment of type 2 diabetes. They bind and activate the pancreatic GLP-1 receptor (GLP-1R) with similar affinity and potency and thereby promote insulin secretion in a glucose-dependent manner. GLP-1R belongs to family B of the seven transmembrane G-protein coupled receptors. The N-terminal extracellular domain (nGLP-1R) is a ligand binding domain with differential affinity for Ex4 and GLP-1: low affinity for GLP-1 and high affinity for exendin-4. The superior affinity of nGLP-1R for Ex4 was previously explained by an additional interaction between nGLP-1R and the C-terminal Trp-cage of Ex4. In this study we have combined biophysical and pharmacological approaches thus relating structural properties of the ligands in solution to their relative binding affinity for nGLP-1R. We used both a tracer competition assay and ligand-induced thermal stabilization of nGLP-1R to measure the relative affinity of full length, truncated, and chimeric ligands for soluble refolded nGLP-1R. The ligands in solution and the conformational consequences of ligand binding to nGLP-1R were characterized by circular dichroism and fluorescence spectroscopy. We found a correlation between the helical content of the free ligands and their relative binding affinity for nGLP-1R, supporting the hypothesis that the ligands are helical at least in the segment that binds to nGLP-1R. The Trp-cage of Ex4 was not necessary to maintain a superior helicity of Ex4 compared to GLP-1. The results suggest that the differential affinity of nGLP-1R is explained almost entirely by divergent residues in the central part of the ligands: Leu^{10}−Gly^{30} of Ex4 and Val^{16}−Arg^{36} of GLP-1. In view of our results it appears that the Trp-cage plays only a minor role for the interaction between Ex4 and nGLP-1R and for the differential affinity of nGLP-1R for GLP-1 and Ex4.

Glucagon-like peptide-1 is a peptide hormone of 30 amino acid residues with regulatory functions in glucose homeostasis. It is produced primarily by the intestinal L cells and released to the blood stream in response to nutrient ingestion (*1*). Recent results suggest that GLP-1[1] could also be expressed by the α-cells of pancreatic islets (*2*). Exendin-4 is a peptide of 39 amino acid residues that was originally isolated from the venom of the lizard *Heloderma suspectum*, and it is homologous to GLP-1 (*3*). Both peptides bind and activate the pancreatic GLP-1 receptor (GLP-1R) and thereby potentiate glucose-induced insulin secretion of the *β*-cells (*4*, *5*). This effect and other regulatory properties make GLP-1 and exendin-4 promising therapeutic agents for the treatment of type 2 diabetes (*6*, *7*). Native GLP-1 is however rapidly degraded in vivo by dipetidylpeptidase IV (DPP IV), and physiologically more stable GLP-1 analogues are in clinical trials (*8*, *9*). Liraglutide is a long acting GLP-1 derivative in phase 3 development as a once daily treatment of type 2 diabetes (*9*). Exendin-4 is more resistant to DPP IV digestion and was recently approved by the FDA as a twice daily treatment of type 2 diabetes (*10*).

The GLP-1 receptor was cloned in 1992 and it belongs to family B of the seven transmembrane (7TM) G-protein coupled receptors (GPCRs) (*11*). This GPCR subfamily also includes receptors for the homologous peptide hormones glucagon, glucagon-like peptide-2 (GLP-2), glucose-dependent insulinotropic polypeptide (GIP), secretin, VIP, PACAP and the nonhomologous parathyroid hormone (PTH), calcitonin and corticotropin releasing factor (CRF) (*12*). These

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[1] Abbreviations: GLP-1, glucagon-like peptide-1; Ex4, exendin-4; GLP-1/Ex4, chimeric peptide GLP-1(7-26)/Ex4(21-39); nGLP-1R, GLP-1 receptor N-terminal extracellular domain; 7TM, seven transmembrane; GPCR, G protein-coupled receptor; DPP IV, dipeptidylpeptidase IV; PTH, parathyroid hormone; CRF, corticotrophin releasing factor; VIP, vasoactive intestinal polypeptide; PACAP, pituitary adenylate cyclase-activating polypeptide; DSC, differential scanning calorimetry; CD, circular dichroism; cAMP, cyclic adenosine 3′,5′-monophosphate; GdnHCl, guanidine hydrochloride.

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receptors are characterized by an N-terminal extracellular domain (Nt-domain) of 100−150 amino acids which contains a conserved pattern of three disulfide bonds (*13*−*16*). The NMR structure of the type 2β CRF receptor Nt-domain showed a protein dominated by sheets and flexible loops connected by the three disulfide bridges and a small core region with central conserved residues (*17*). The Nt-domain is an important ligand binding domain, and the isolated recombinant Nt-domains of the GLP-1, PTH, and CRF receptors are able to bind their respective ligands (*13*, *15*, *16*). The apparent binding affinity of the Nt-domain is typically lower than that of the corresponding full length receptor, except in the case of exendin-4 (*18*). The prevailing ligand binding model of family B 7TM GPCRs suggests a two-domain interaction mechanism (*19*). First, the Nt-domain of the receptor interacts with the C-terminal segment of the ligand, and this interaction is a major determinant of selectivity between the different homologous peptide ligands (*18*, *20*−*22*). Second, the N-terminus of the ligand interacts with the extracellular surface of the receptor core-domain (the 7TM helices and connecting loops), which leads to receptor activation (*23*−*28*). In fact, an 11 residue mimetic of the GLP-1 N-terminus is sufficient to activate GLP-1R (*29*). Recent result suggest that the Nt-domain itself might also be involved in receptor activation, although this was not shown for GLP-1R (*30*).

GLP-1 and exendin-4 (Ex4) are 50% identical, and they have similar binding affinity and potency at the full length GLP-1R. The similarity of the two peptides is distinct in the first 11 N-terminal residues where the identity is 82%, whereas the next 20 residues share only 35% identity. The N-terminus of Ex4 is necessary for receptor activation but less important for binding, and thus the N-terminally truncated peptide Ex4(9−39) is a competitive antagonist of GLP-1R (*31*). In GLP-1, the affinity and potency generating components are more intimate and difficult to identify separately (*32*). N-Terminal truncation of GLP-1 by two residues reduced the affinity by 100-fold and transformed the peptide into a partial agonist even at 1 μM (*33*). Therefore the N-terminus of GLP-1 appears to be important for both binding and activation of GLP-1R. Specific divergent segments in the central part of GLP-1 and Ex4 were suggested to explain the ability of Ex4(9−39) to maintain high affinity for GLP-1R regardless of the N-terminal truncation (IC_{50} of 1 nM relative to 0.2 nM of Ex4) (*33*).

Intriguingly, the isolated Nt-domain of GLP-1R (nGLP-1R) exhibits differential affinity for the two homologous ligands (*18*). Compared to the full length receptor, nGLP-1R maintains high affinity for both Ex4 and Ex4(9−39) whereas it binds GLP-1 with reduced affinity (*18*). Thus the ability of Ex4(9−39) to maintain high affinity for the full length receptor results from its high affinity interaction with nGLP-1R. Exendin-4 contains a C-terminal extension of nine amino acid residues know as the "Trp-cage" which is not present in GLP-1 (*34*). The NMR structure of Ex4 in aqueous trifluoroethanol (TFE) showed that the Trp-cage folds back onto the central helical part of Ex4, forming the smallest known protein-like fold which surrounds Trp^{25} (*35*). A ligand binding model for GLP-1R was suggested where the differential affinity of nGLP-1R for GLP-1 and Ex4 was explained by an additional interaction between the putative Trp-cage and nGLP-1R (*36*). The GLP-1 N-terminus was suggested to form a superior interaction with the core-domain of the full length GLP-1R, thereby compensating for the low affinity between the GLP-1 C-terminus and nGLP-1R (*36*). Accordingly the full length GLP-1R would bind the two homologous ligands with similar overall affinity, in spite of the differential affinity of the receptor subdomains (nGLP-1R and receptor core-domain) for the N- and C-terminal segments of GLP-1 and Ex4.

In this study we performed a biophysical characterization of free and ligand-bound nGLP-1R using full length, truncated, and chimeric exendin-4 and GLP-1 peptides. The thermal stability of free and ligand-bound nGLP-1R was measured by differential scanning calorimetry (DSC) and far-UV circular dichroism (CD). The conformational changes resulting from ligand binding were analyzed by fluorescence spectroscopy and far-UV CD measurements. In parallel we used tracer competition analyses to characterize ligand binding of both soluble nGLP-1R and GLP-1R in baby hamster kidney (BHK) cells and membranes. The results demonstrated a strong correlation between the α-helical content of the free ligands in solution and their affinity for nGLP-1R. The Trp-cage was not necessary for the superior helicity of Ex4 or for the differential affinity of nGLP-1R. We conclude that the differential affinity of nGLP-1R was determined almost exclusively by divergent residues in the central part of GLP-1 and Ex4 (Leu^{10}−Gly^{30} of Ex4 and Val^{16}−Arg^{36} of GLP-1).

EXPERIMENTAL PROCEDURES

Cloning, Expression, and Refolding of the Human GLP-1 Receptor N-Terminal Extracellular Domain (nGLP-1R). A cDNA fragment encoding the human nGLP-1R (Arg24−Tyr145) was amplified by PCR, cloned in the pET15b vector (Novagen), and transformed into the *Escherichia coli* expression strain BL21(DE3). The resulting expression vector encoded an N-terminal 6xHis-tag followed by a thrombin cleavage site and nGLP-1R. Expression and refolding of nGLP-1R was carried out essentially as previously described (*16*).

Purification of Refolded nGLP-1R. Correctly folded nGLP-1R was separated from impurities and incorrectly folded species of nGLP-1R by hydrophobic interaction chromatography (HIC) using Butyl Sepharose 4 Fast Flow (Amersham Biosciences). Initially, 0.5 M ammonium sulfate was added slowly to the refolding solution and stirred for 1 h at 4 °C. The resulting precipitates were removed by filtration before loading the HIC column. The column was eluted over a linear gradient with 0.1 M Tris-HCl pH 7.5, 0.8 M L-Arg. The L-Arg concentration of the eluted protein solution was diluted to 0.4 M by slow addition of 0.1 M Tris-HCl pH 7.5, 0.2 M Na_2SO_4, 4% glycerol, and incubated overnight at room temperature with 1000 units of bovine thrombin (Sigma). Four amino acid residues, Gly-Ser-His-Met, of the linker were still attached to the N-terminus of nGLP-1R after thrombin cleavage. The solution was concentrated to approximately 1 mg/mL in a stirred Amicon cell 8400 (Millipore). Finally, refolded nGLP-1R was purified by size exclusion chromatography on a Superdex 75 prep grade column (Amersham Biosciences) in 10 mM Tris-HCl pH 7.5, 0.1 M Na_2SO_4, 2% glycerol at a flow rate of 2 mL/min. The concentration of the collected protein was approximately

0.1 mg/mL (6.8 μM) as determined by UV absorption at 280 nm and the Beer−Lambert Law using a molar extinction coefficient of 46950 M^{-1} cm^{-1}.

Peptides and Tracers. The peptide ligands used in this study were synthesized and purified as previously described (*21*). The peptides were dissolved in the assay buffer for the particular measurement, the absorption at 280 nm of each dissolved peptide was measured, and the concentration was calculated using the Beer−Lambert Law and a molar extinction coefficient of 6970 M^{-1} cm^{-1} for GLP-1 and GLP-1/Ex4 and 5690 M^{-1} cm^{-1} for Ex4, Ex4(1−30), Ex4(9−39), and Ex4(10−39). The ^{125}I-GLP-1 tracer was specifically labeled on Tyr19. The ^{125}I-Bolton−Hunter labeled exendin (*9−39*) tracer was purchased from Perkin-Elmer (NEX335).

nGLP1R SPA Binding Assay. The nGLP1R in 0.1 M $NaHCO_3$ was biotinylated using 75 μg of BNHS (Sigma H1759) to 1 mg of protein. The biotinylated nGLP1R was subsequently dialyzed against PBS. All reagents and compounds were diluted in PBS with 0.05% v/v Tween 20. The binding assay was carried out in 96 well OptiPlates (Perkin-Elmer 6005290) in a final volume of 200 μL. Each well contained 2 mg of streptavidin coated SPA beads (Perkin-Elmer RPNQ007), 0.1 pmol of biotinylated nGLP1R, 50 pCi of ^{125}I-exendin (*9−39*), and test peptide in final concentrations ranging from 1000 nM to 0.064 nM. The plates were incubated on a shaker at RT for 3 h. The SPA particles were spun down by centrifugation for 10 min at 1500 rpm, and the plates were counted in a TopCount-NXT (Perkin-Elmer).

Cells and Membrane Preparation. Baby hamster kidney (BHK) cells stably expressing the human GLP-1 receptor were maintained in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum albumin (Invitrogen), 100 U/mL penicillin, 100 μg/mL streptomycin, and 1 mg/mL Geneticin. The cells were grown in 175 cm^2 flasks to ∼90% confluence, washed once in 10 mL of PBS, harvested by addition of 1.5 mL of Versene, diluted in 12 mL of PBS, pelleted by centrifugation for 2 min at 1300*g*, and resuspended either in FlashPlate assay buffer (Perkin-Elmer) for functional cAMP experiments or in 20 mM Na-HEPES, 10 mM EDTA, pH 7.4 for membrane preparations. For preparation of membranes the cell pellet was homogenized using an Ultrathurax for 30 s, spun down by centrifugation for 15 min at 20.000 rpm, and resuspended in 20 mM Na-HEPES, 0.1 mM EDTA, pH 7.4. Homogenization, centrifugation, and resuspension were repeated once, and the resulting membrane preparation was stored at −80 °C.

Binding Analyses of the Full Length GLP-1R. The membrane binding assay was carried out in Optiplates (Perkin-Elmer 6005290) in a final volume of 200 μL. Membranes, test peptides, and tracers were diluted in assay buffer; 50 mM HEPES, 5 mM EGTA, 5 mM $MgCl_2$, 0.005% v/v Tween 20, pH 7.4. Each well contained 0.1 mg/mL membrane protein, 0.5 mg of WGA SPA beads (Perkin-Elmer RPNQ0001), 50 pM tracer (^{125}I-GLP-1 or ^{125}I-Ex4-(9−39)), and test peptide in final concentrations ranging from 1 pM to 1 μM. The plates were incubated for 2 h at 30 °C. The SPA particles were spun down by centrifugation for 10 min at 1500 rpm, and the plates were counted in a TopCount-NXT (Perkin-Elmer).

The functional cAMP assay was carried out in 96 well FlashPlates (Perkin-Elmer) in a total volume of 100 μL. Test peptides were diluted in PBS with 0.02% v/v Tween 20 (peptide buffer), and nGLP-1R was diluted in 10 mM Tris-HCl pH 7.5, 100 mM Na_2SO_4, 2% glycerol (protein buffer). All wells contained the same ratio of peptide buffer and protein buffer. The test peptides and nGLP-1R were added to the FlashPlates and incubated for 25 min at 4 °C before the addition of 50 μL of cells with a density of 2×10^6 cells mL^{-1}. The concentration of test peptides ranged from 0.01 pM to 0.1 μM, and the concentration of nGLP-1R ranged from 75 to 600 nM. The plates were mixed gently for 5 min and incubated for 30 min at room temperature before the addition of 100 μL of stop solution containing ^{125}I-cAMP tracer and Detection Mix (Perkin-Elmer) according to the supplier's manual. The plates were incubated for 3 h before counting in a Packard γ-counter. All binding and functional data were analyzed by nonlinear regression using Prism 3.0 software (GraphPad Software Inc.).

Differential Scanning Calorimetry (DSC). The thermal stability of free and ligand-bound nGLP-1R was analyzed by DSC using a VP-DSC calorimeter (MicroCal, Inc., Northampton, MA). The nGLP-1R was concentrated to 70 μM by centrifugation at 3.000*g* in an Amicon Ultra-15 filter unit with 10 kDa cutoff (Millipore). In the samples used for calorimetric measurements, the concentration of nGLP-1R was 25 μM in 10 mM Tris-HCl pH 7.5, 0.1 M Na_2SO_4, 2% glycerol, and the concentration of peptide was 75 μM. The reference cell contained 10 mM Tris-HCl pH 7.5, 0.1 M Na_2SO_4, 2% glycerol. The samples were scanned from 20 to 90 °C at a rate of 60 °C/h, and the reversibility of the thermal unfolding was verified by rescanning using the same parameters. The experiments were repeated at least three times using different batches of nGLP-1R. Buffer/buffer thermograms were collected under identical conditions and used as background. The midpoint of thermal transition (T_m), the calorimetric enthalpy, and the van't Hoff enthalpy values were calculated from the thermograms subsequent to subtraction of background and baseline using the Origin software provided by the manufacturer. The values reported represent the average of at least three independent experiments.

Fluorescence Spectroscopy. Fluorescence emission spectra were recorded on a Perkin-Elmer luminescence spectrometer LS50B at 20 °C in a 3 mm quartz cuvette. The excitation wavelength was 295 nm, the emission scanning range was 300−400 nm, the slit widths were 5 nm, and the scan rate was 50 nm/min. The concentration of nGLP-1R and peptides was 4 μM in 10 mM Tris-HCl pH 7.5, 0.1 M Na_2SO_4, 2% glycerol. In order to record the spectrum of unfolded protein, nGLP-1R was denatured by addition of 4 M GdnHCl. The spectra of receptor−ligand complexes were recorded on samples containing a 1:1 molar ratio of nGLP-1R and ligand (4 μM). The samples were incubated for 1 min at room temperature before measuring, and all the spectra were corrected for buffer contributions.

Circular Dichroism Spectroscopy. Far-UV CD spectra were measured on a Jasco J-810 spectropolarimeter (Jasco Inc., Easton, MD) at 20 °C in a 1 mm quartz cuvette. The scanning range was 200−260 nm at a rate of 20 nm/min, the bandwidth was 1 nm, and the spectra were accumulated 10 times with a response time of 2 s. The concentration of nGLP-1R and peptides was 14 μM in 10 mM Tris-HCl pH 7.5, 0.1 M Na_2SO_4, 2% glycerol, and nGLP-1R was denatured by adding 6 M GdnHCl. CD spectra of receptor−

ligand complexes were measured on samples containing 14 μM nGLP-1R and 14 μM peptide that were incubated for 60 min at 20 °C. The sum spectra were measured in tandem cuvettes containing nGLP-1R and the ligand, respectively. The spectra were corrected for buffer contributions and converted to mean residue ellipticity according to Schmid, 1997 (*37*).

The thermal stability of nGLP-1R (7 μM) in absence or presence of ligands (21 μM) was also analyzed by CD spectroscopy. The samples were heated from 10 to 90 °C at a rate of 120 °C/h. and the mean residue ellipticity was measured at 203 nm with intervals of 2 °C using a time constant of 2 s. The measured signals were normalized and analyzed by a two-state model of unfolding.

RESULTS

The peptide ligands used in this study were exendin-4 (Ex4), GLP-1(7−37), Ex4(9−39), Ex4(10−39), Ex4(1−30), and the chimeric ligand GLP-1(7−26)/Ex4(21−39) (referred to as GLP-1/Ex4) (Figure 1A). The nGLP-1R was expressed, refolded, and purified essentially as earlier described, although in this study only four linker amino acid residues were attached to the N-terminus of nGLP-1R after 6xHis-tag removal and purification (*16*).

Pharmacological Profile of Refolded nGLP-1R. Ligand binding to nGLP-1R was analyzed by competition analysis using ^{125}I-Ex4(9−39) as the tracer. Titration curves and IC_{50} values are shown in Figure 1B and Table 1. The results suggested that Ex4, Ex4(9−39), Ex4(10−39), and Ex4(1−30) bound nGLP-1R with similar affinity, GLP-1/Ex4 bound nGLP-1R with intermediate affinity, and GLP-1 bound nGLP-1R with the lowest affinity.

Thermal Unfolding Analyzed by Differential Scanning Calorimetry (DSC). Ligand-induced thermal stabilization of nGLP-1R was measured by DSC of free and ligand-bound nGLP-1R. The thermogram of free nGLP-1R showed an endotherm with transition midpoint (T_m) at 45 °C and calorimetric denaturation enthalpy (ΔH_{cal}) of 56 kcal/mol (Figure 1C). No measurable change in ΔC_p was observed by thermal induced unfolding of the free ligands within the given concentrations (data not shown). The thermal stability of nGLP-1R was increased by the ligands in a concentration-dependent manner. Biphasic endotherms were observed at low molar ratios of ligand and nGLP-1R, suggesting that nGLP-1R unfolded independently in its free and ligand-bound forms (data not shown). A 3-fold molar excess of Ex4 gave a monophasic endotherm with T_m at 66 °C, ΔH_{cal} of 98 kcal mol^{-1}, and a van't Hoff enthalpy (ΔH_v) of 82 kcal (Figure 1C). The results showed that binding of Ex4 increased the midpoint of unfolding by 21 °C and increased the denaturation enthalpy by 42 kcal mol^{-1}. The results suggested that nGLP-1R unfolded only as an Ex4-bound protein most likely by a two-state process. DSC measurements of the other receptor−ligand complexes were performed on samples containing a 3-fold molar excess of the ligand, in order to get directly comparable T_m values representing the relative binding affinity of the various ligands (*38*, *39*). Ex4(9−39) and Ex4(10−39) increased the T_m to 65 °C (ΔT_m = 20 °C), Ex4(1−30) increased the T_m to 63 °C (ΔT_m = 18 °C), GLP-1/Ex4 increased the T_m to 58 °C (ΔT_m = 13 °C), and GLP-1 increased the T_m to 50 °C

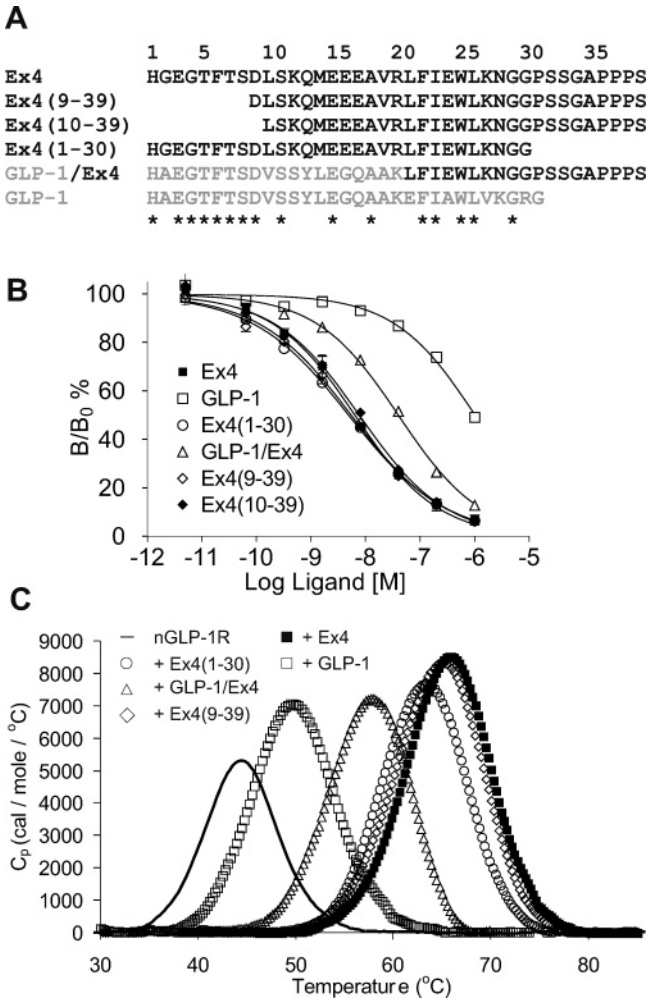

FIGURE 1: (A) Sequence alignment of the peptide ligands. From the top: Ex4(1−39), Ex4(9−39), Ex4(10−39), Ex4(1−30), the chimeric ligand GLP-1(7−26)/Ex4(21−39), and GLP-1(7−37). The N-terminal residue of Ex4 is His^1, which corresponds to His^7 of GLP-1, and the residues of the peptides are numbered accordingly. (B) Tracer competition assay with soluble refolded nGLP-1R using ^{125}I-Ex4(9−39) tracer. The graph shows a single representative experiment with all ligands included in the same experiment. (C) Differential scanning calorimetry of unbound and ligand-bound nGLP-1R. The concentration of nGLP-1R was 25 μM, and the concentration of the ligands was 75 μM. The data were collected at a scan rate of 60 °C/h from 20 to 90 °C.

(ΔT_m = 5 °C) (Figure 1C). The endotherms were all monophasic and gave reasonable fits to a two-state process, with typical $\Delta H_v/\Delta H_{cal}$ ratios of 0.8−1.2. Thermal unfolding of free and ligand-bound nGLP-1R was reversible and gave approximately the same T_m values after three scans (data not shown). High thermal stability of nGLP-1R was obtained with Ex4, Ex4(9−39), Ex4(10−39), and Ex4(1−30), intermediate thermal stability was obtained with GLP-1/Ex4, and low thermal stability was obtained with GLP-1. The ligand-induced thermal stabilization profile of nGLP-1R obtained by DSC corroborated the pharmacological profile of nGLP-1R obtained by tracer competition analyses.

Pharmacological Profile of the Full Length GLP-1R. Ligand binding to the human GLP-1R was analyzed by tracer competition analyses on membranes from BHK cells stably expressing GLP-1R using ^{125}I-Ex4(9−39) and ^{125}I-GLP-1 as tracers. Ex4, Ex4(1−30), GLP-1/Ex4, and GLP-1 gave similar IC_{50} values regardless of the choice of tracer,

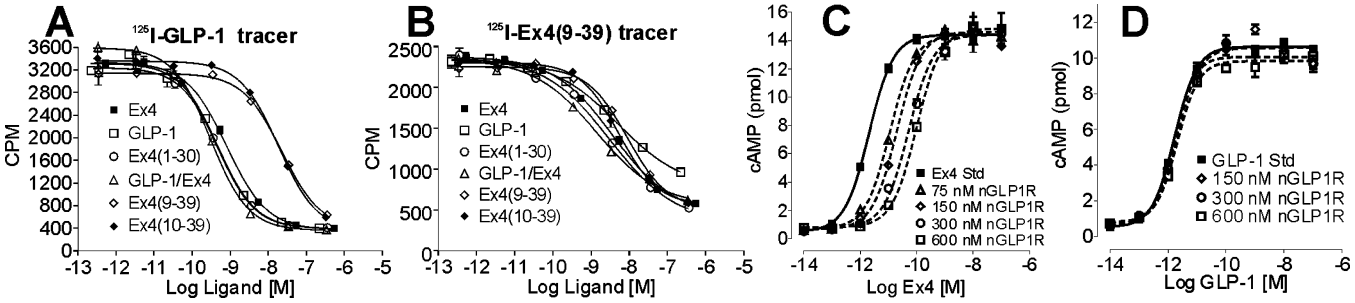

FIGURE 2: Pharamacological profile of the full length human GLP-1R. Tracer competion analysis using GLP-1R in membranes and (A) ^{125}I-GLP-1 as tracer or (B) ^{125}I-Ex4(9−39) as tracer. Competition between refolded nGLP-1R and full length GLP-1R for binding to (C) Ex4 or (D) GLP-1. Each graph shows a single representative experiment with all ligands included in the same experiment. In C and D the black curves show the cAMP response for the agonists in the absence of refolded nGLP-1R. The dark to light gray curves (broken lines) show the cAMP response in the presence of 75 nM, 150 nM, 300 nM, and 600 nM refolded nGLP-1R, using the same agonist concentrations.

Table 1: Pharmacological Profile of nGLP-1R and GLP-1R[a]

		GLP-1R		
		IC_{50} (nM) ± SD		
	nGLP-1R IC_{50} (nM) ± SD ^{125}I-Ex4(9−39)	^{125}I-GLP-1	^{125}I-Ex4(9−39)	EC_{50} (nM) ± SD
GLP-1	1120 ± 188	0.56 ± 0.15	4.5 ± 1.1	1.5 ± 0.2
Ex4	6.0 ± 0.83	0.73 ± 0.09	4.5 ± 0.72	1.5 ± 0.2
Ex4(9−39)	6.0 ± 0.50	24 ± 5.9	10 ± 1.4	
Ex4(10−39)	6.8 ± 1.3	20 ± 4.2	9.1 ± 3.5	
Ex4(1−30)	5.0 ± 0.45	0.43 ± 0.07	2.7 ± 0.59	1.4 ± 0.2
GLP−1/Ex4	41 ± 13	0.39 ± 0.07	3.0 ± 2.0	2.3 ± 0.5

[a] All values represent the mean ± SD of three or more independent experiments. IC_{50} values of nGLP-1R were measured on biotinylated nGLP-1R attached to streptavidin-coated SPA beads. IC_{50} and EC_{50} values of GLP-1R were measured on membrane preparations and whole cells, respectively.

suggesting that they bound GLP-1R with similar affinity (Figure 2A,B and Table 1). The N-terminally truncated ligands Ex4(9−39) and Ex4(10−39) showed differential effects with the two tracers. By comparison of Ex4 and Ex4-(9−39), N-terminal truncation reduced the ability to displace ^{125}I-GLP-1 by 33-fold but only reduced the ability to displace ^{125}I-Ex4(9−39) by 2-fold.

Ex4, GLP-1, Ex4(1−30), and GLP-1/Ex4 were full GLP-1R agonists with similar EC_{50} values in a functional cAMP assay using living BHK cells that stably expressed the full length GLP-1R (Figure 2C, 2D and Table 1). Competition for agonist binding between refolded nGLP-1R and full length GLP-1R was analyzed in the same functional assay. The addition of 600 nM nGLP-1R shifted the Ex4 dose−response curve by 49-fold to an EC_{50} value of 74 ± 19 pM (Figure 2C). The shift was dose dependent: lower concentrations of nGLP-1R (300 nM, 150 nM, and 75 nM) resulted in gradually reduced shifts of the Ex4 dose−response curve. The GLP-1 dose−response curve was shifted only 1.5-fold to an EC_{50} value of 2.2 ± 0.2 pM (1.5-fold) by the addition of 600 nM nGLP-1R (Figure 2D). The results demonstrated that the soluble refolded nGLP-1R was able to compete in a concentration-dependent manner with full length GLP-1R for Ex4 binding but essentially not for GLP-1 binding.

Fluorescence Spectroscopy. Fluorescence emission spectroscopy can provide information about the polarity of the local environment of Trp residues in proteins. Trp residues that are exposed to the aqueous solvent display an emission maximum around 355−360 nm, whereas the emission maximum of Trp residues buried in a nonpolar environment is shifted to shorter wavelength. The Nt-domain of GLP-1R contains seven Trp residues which allowed us to monitor denaturation and ligand binding by fluorescence spectroscopy. The emission maximum of refolded nGLP-1R occurred at 341 nm, suggesting significant but not complete solvent protection of all the Trp residues (Supporting Information, S1). Treatment of nGLP-1R with 4 M GdnHCl resulted in a red-shift of the fluorescence emission maximum to 355 nm as expected of a denatured protein.

The ligands have a conserved Trp residue in the C-terminus. Their fluorescence emission maximum ranged from 352 to 356 nm, and their fluorescence intensity was 5−10% compared to the intensity of equimolar free nGLP-1R (Figure 3B,D−F). The emission spectra of the ligands containing the C-terminal extension, Ex4, Ex4(9−39), Ex4(10−39), and GLP-1/Ex4, were blue-shifted by approximately 3 nm and had lower intensity compared to the spectra of the ligands without the extension: Ex4(1−30) and GLP-1 (Supporting Information, S2). The results suggested that the Trp residue of Ex4, Ex4(9−39), Ex4(10−39), and GLP-1/Ex4 was more protected from solvent exposure than the Trp residue of GLP-1 and Ex4(1−30). Apparently the C-terminal extension of Ex4 was necessary for the observed solvent protection of the Trp residue.

The fluorescence properties of ligand-bound nGLP-1R were analyzed on samples containing a 1:1 molar ratio of nGLP-1R and ligand. The concentration was 4 μM and thus above the expected dissociation constants. In order to evaluate the effect of ligand binding, the measured spectrum of a complex was compared to the sum spectrum of free nGLP-1R and the free ligand. By this comparison Ex4 induced a 2 nm blue-shift of the emission maximum and a 15% quench of the fluorescence intensity (Figure 3A,B). Titration of Ex4 showed that the maximum fluorescence quench and blue-shift were achieved at a 1:1 molar ratio, suggesting a 1:1 binding stoichiometry (data not shown). The results obtained with Ex4(9−39) and Ex4(10−39) (data not shown) were similar to those obtained with Ex4, suggesting that binding of these ligands directly or indirectly altered the solvent exposure of Trp residues in the complexes.

GLP-1 binding to nGLP-1R induced a small (0.5−1 nm) red-shift and induced an increase of the fluorescence intensity by 26% (Figure 3C,D). Binding of GLP-1 to nGLP-1R clearly had a different effect on the fluorescent properties compared to binding of Ex4. Binding of Ex4(1−30) and GLP-1/Ex4 to nGLP-1R induced a 1.5 nm blue-shift and a quench of 4 and 8%, respectively (Figure 3E,F). The results

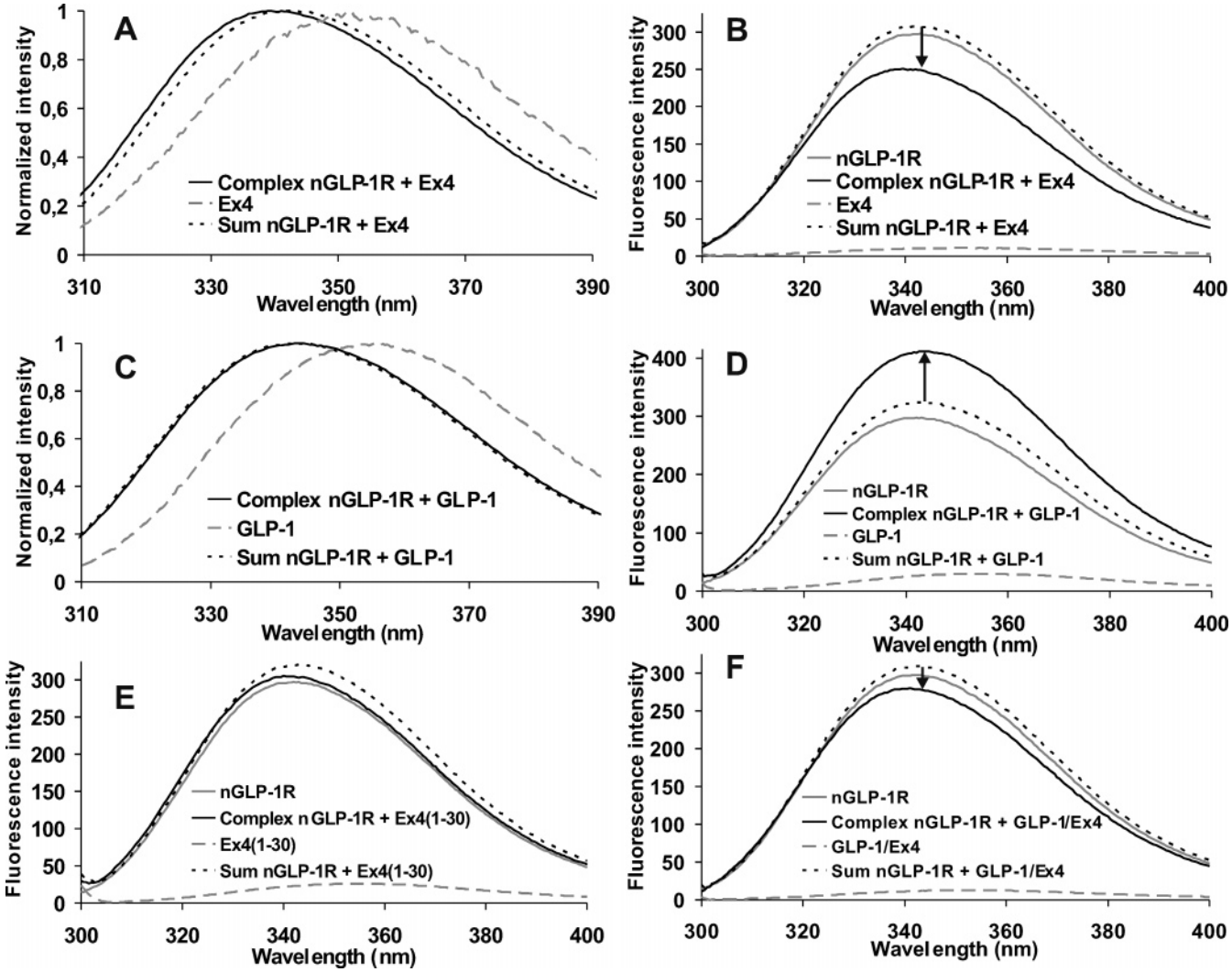

FIGURE 3: Fluorescence spectroscopy of unbound and ligand-bound nGLP-1R, unbound nGLP-1R: (gray —). Unbound ligands: (gray – – –). Complexes: (black —). Sum spectra of unbound nGLP-1R and ligand: (black – – –). The fluorescence spectrum of the complex with Ex4 is shown as normalized intensity (A) to illustrate the Ex4-induced blue-shift and as real intensity (B) to illustrate the Ex4-induced quench. The corresponding spectra of the complex with GLP-1 are shown in C and D. The spectra of the complexes with Ex4(1−30) and GLP-1/Ex4 in real intensity are shown in E and F. The concentration of nGLP-1R and ligands was 4 μM each.

showed that the Ex4 part of GLP-1/Ex4 maintained both the ability to blue-shift and the ability to quench the fluorescence signal. In addition, the ability to quench the fluorescence was specifically decreased by the C-terminal truncation of Ex4.

Addition of 3 M GdnHCl abolished the differential effects of the ligands on the fluorescence properties. The sum spectra of the individual denatured components were essentially identical to the spectra of the denatured complexes (data not shown).

Circular Dichroism (CD) Spectroscopy. Refolded nGLP-1R was analyzed by far-UV−CD spectroscopy (195−260 nm). The spectrum of refolded nGLP-1R was characteristic of a folded protein with secondary structure elements dominated by β-sheets and turns (Figure 4A). It showed a negative band with a minimum at 203 nm and a mean residue ellipticity of approximately 15.000 deg cm^2 dmol^{-1} at this wavelength. The CD spectrum of GdnHCl-treated nGLP-1R resembled that of a denatured protein. Previous studies of nGLP-1R and other Nt-domains in this receptor family gave similar results (*15*, *16*, *40*).

The free peptide ligands were also analyzed by CD spectroscopy (Figure 4B). The CD spectra of Ex4, Ex4(9−39), Ex4(10−39), and Ex4(1−30) in mean residue ellipticity were similar and had two negative bands at 206 and 224 nm, as expected for α-helical peptides. These ligands appeared to have a comparable overall α-helical content. The CD spectrum of GLP-1/Ex4 had two minima at 205 and 224 nm, and the overall intensity was lower than that of Ex4. Compared to Ex4, the ellipticity of the 224 nm band had decreased relative to the 205 nm band. The CD spectrum of GLP-1 showed the lowest overall negative mean residue ellipticity of the ligands. The short-wavelength negative band was shifted to 203 nm, and the relative ellipticity of the long-wavelength band was even lower than that of GLP-1/Ex4. The CD spectra of Ex4 and GLP-1 were similar in shape and relative intensity to previously published CD spectra measured in aqueous buffer (*41*).

Ligand binding of nGLP-1R may induce changes in the secondary structure of both the ligand and nGLP-1R. To analyze this, CD spectra were recorded on samples containing a 1:1 molar ratio of nGLP-1R and ligand, referred to as a

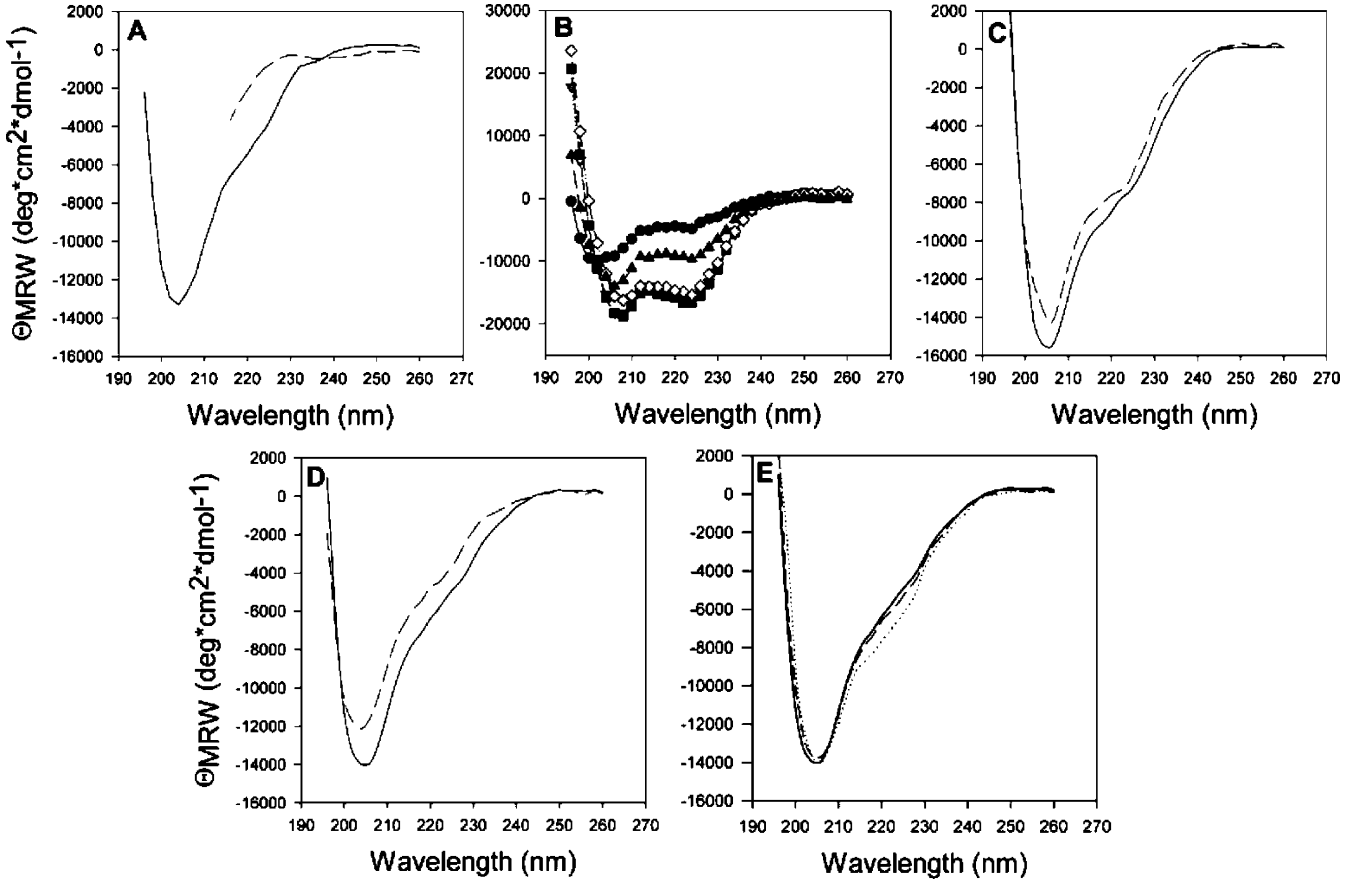

FIGURE 4: CD spectroscopy of unbound and ligand-bound nGLP-1R. (A) Refolded (—) and denatured (− − −) nGLP-1R. (B) The free peptide ligands: GLP-1 (black circle), GLP-1/Ex4 (dark triangles), Ex4(1−30) (light gray diamonds), Ex4 (dark squares), and Ex4(9−39) (gray triangles). (C) Ex4-bound nGLP-1R (—) and sum spectrum of unbound nGLP-1R and Ex4 (− − −). (D) GLP-1-bound nGLP-1R (—) and sum spectrum of unbound nGLP-1R and GLP-1 (− − −). (E) Comparison of the three complexes with GLP-1 (—), GLP-1/Ex4 (− − −), and Ex4 (•••). The concentration of ligand and nGLP-1R was 14 μM each.

complex spectrum. The complex spectrum was compared to the sum spectrum of the free ligand and free nGLP-1R, which represents the theoretical situation where nGLP-1R and the ligand are not interacting. A significant difference between the complex spectrum and the sum spectrum was considered as evidence for a change of secondary structure content induced by the ligand−receptor interaction.

By this comparison, Ex4 increased the overall negative ellipticity, suggesting that binding of Ex4 to nGLP-1R increased the total content of secondary structure in the complex compared to the free components, although the effect was relatively small (Figure 4C). By the same approach, we recorded CD spectra of complexes with the other ligands. The results obtained with Ex4(9−39), Ex4-(10−39), Ex4(1−30), and GLP-1/Ex4 were similar to the result obtained with Ex4 (data not shown). They all induced a small increase of the secondary structure content, but we were unable to identify significant differences between the different complexes by this comparison. The only ligand that gave a significantly different result was GLP-1, where the difference between the sum spectrum and the complex spectrum was significantly larger compared to the effect obtained with Ex4 (Figure 4D). Binding of GLP-1 to nGLP-1R shifted the short-wavelength band to a longer wavelength and gave a greater increase of the negative ellipticity than did Ex4. The results showed that the increase of secondary structure induced by the receptor−ligand interaction was more pronounced with GLP-1 than with Ex4.

Table 2: Mean Residue Ellipticity at 224 nm of Ligand-Bound nGLP-1R[a]

complex	$(\theta)_{224\ nm}$
nGLP-1R + Ex4	−7420 ± 155
nGLP-1R + Ex4(9−39)	−6820 ± 102
nGLP-1R + Ex4(1−30)	−6580 ± 126
nGLP-1R + GLP-1/Ex4	−5670 ± 100
nGLP-1R + GLP-1	−5260 ± 144

[a] Values represent the mean ± SD of three or more independent experiments.

In order to compare the helicity of the complexes, we listed their molar ellipticity at 224 nm and found that the signal of the complexes with Ex4, Ex4(9−39), Ex4(10−39), Ex4(1−30) was more negative than the signal of the complexes with GLP-1 and GLP-1/Ex4 (Table 2). The CD spectra of the complexes with GLP-1, GLP-1/Ex4, and Ex4 are shown in Figure 4E to illustrate this point. The results suggested that the complex with Ex4 had a higher helical content than the complexes with GLP-1 and GLP-1/Ex4.

Thermal Unfolding Analyzed by CD Spectroscopy. CD spectra of free and ligand-bound nGLP-1R were recorded at increased temperatures. A 3-fold molar excess of ligand was used for comparison with the DSC results. The degree of stabilization was ligand-dependent and correlated with the DSC results. The progress curve of the complex with Ex4 and GLP-1/Ex4 showed a T_m value of 62 °C and 59 °C, respectively (Supporting Information, S3).

DISCUSSION

The differential affinity of nGLP-1R for GLP-1 and exendin-4 is intriguing because the full length GLP-1R binds the two homologous agonists with similar affinity. The results presented in this study agree with the existing binding model where the N- and C-terminal parts of GLP-1 and Ex4 bind the receptor subdomains (nGLP-1R and core-domain) with differential affinity (*18*, *36*). However, the results appear to contradict previous results with regard to the suggested role of the Trp-cage in the differential affinity of nGLP-1R (*36*).

The effect of C-terminal truncation of Ex4 was previously studied by tracer competition analysis using nGLP-1R attached to the membrane via a single TM helix, and it was shown that Ex4, Ex4(1−30), and GLP-1 bound nGLP-1R with IC_{50} values of 12.6 nM, 316 nM, and 764 nM, respectively (*36*). It was suggested that the Trp-cage bound to nGLP-1R and provided an additional interaction that explained how Ex4 maintained high affinity for nGLP-1R (*36*). In our study, the relative binding affinity of the various ligands for nGLP-1R was established both by tracer competition analyses of nGLP-1R and by ligand-induced thermal stabilization of nGLP-1R. Our results confirm the differential affinity of nGLP-1R by showing that Ex4 and Ex4(9−39) maintain high affinity for nGLP-1R (IC_{50} of 7 and 6 nM respectively) whereas GLP-1 binds nGLP-1R with lower affinity (IC_{50} of 1120 nM). However, our results obtained with Ex4(1−30) apparently contradict previous results obtained with the same ligand (*36*). In our study, the removal of the C-terminal tail of Ex4 (residues Pro^{31}−Ser^{39}) only slightly decreased the ligand-induced thermal stabilization of nGLP-1R and had no apparent effect in the tracer competition assay with nGLP-1R compared to the results obtained with Ex4. Our results emphasize Leu^{10}−Gly^{30} of Ex4 (Val^{16}−Arg^{36} of GLP-1) as the most significant difference between GLP-1 and Ex4, in terms of affinity generating elements for the interaction with nGLP-1R. It appears that the differential affinity of nGLP-1R is explained in our study almost entirely by divergent residues in this segment of GLP-1 and Ex4. In view of these results we believe that the Trp-cage plays only a minor role for the interaction between Ex4 and nGLP-1R and for the differential affinity of nGLP-1R for GLP-1 and Ex4.

All the agonists in this study maintained similar affinity and potency on the full length GLP-1R. The results support our view that the Trp-cage is not necessary to maintain high affinity for GLP-1R or nGLP-1R, although previous results showed a reduced affinity of Ex4(1−30) compared to Ex4 (*36*, *42*). The GLP-1R binding results emphasized the important choice of tracer by showing that the N-terminally truncated ligands Ex4(9−39) and Ex4(10−39) were less competitive with the ^{125}I-GLP-1 tracer than with the ^{125}I-Ex4(9−39) tracer. The most likely explanation is that the affinity for the active state of GLP-1R is measured selectively by using the agonist tracer ^{125}I-GLP-1. Thus the agonists had higher affinity for the active conformation of GLP-1R than the antagonists. In contrast, the antagonist tracer ^{125}I-Ex4(9−39) probably binds nonselectively to both active and inactive states of GLP-1R. Accordingly the agonist competitors had a reduced Hill slope compared to the antagonist competitors using the ^{125}I-Ex4(9−39) tracer, suggesting two-site binding of the agonists. Nevertheless, the differential affinity of the receptor subdomains were not measurable with the agonists Ex4, Ex4(1−30), GLP-1/Ex4, and GLP-1, because these ligands obtain similar overall affinity on the full length receptor via differential affinity for the receptor subdomains. In the functional cAMP assay, the ability of the soluble nGLP-1R to compete for Ex4 binding with the full length GLP-1R is explained by the high affinity interaction between nGLP-1R and Ex4. Accordingly, the inability of soluble nGLP-1R to compete for GLP-1 binding is probably explained both by its low affinity for GLP-1 and by the superior interaction between the GLP-1 N-terminus and the GLP-1R core-domain.

Ex4(10−39) defined the minimal sequence that was sufficient to obtain maximum thermal stability of nGLP-1R, which suggests that Asp^9 of Ex4 is not important for binding to nGLP-1R. This is maybe not surprising because this Asp is conserved in GLP-1, glucagon, and Ex4 and has been implicated in receptor activation (*32*, *33*, *43*). The boundary defining the residues that interact with nGLP-1R and the residues that interact with the core-domain is not clear, and it remains to be shown if Ex4 can be further N-terminally truncated without loss of affinity for nGLP-1R. A previous study of chimeric ligands and receptors suggested that N-terminal residues until Leu^{20} of GLP-1 (Met^{14} of Ex4) interacted primarily with the GLP-1R core-domain (*21*). Accordingly, Glu^{21} of GLP-1 (Glu^{15} of Ex4) would define the boundary between the C-terminal part of GLP-1 which binds nGLP-1R and the N-terminal part of GLP-1 which binds the core-domain. In addition, Lys^{12} in the N-terminus of glucagon (Ser^{18} of GLP-1 and Lys^{12} of Ex4) has been implicated in interaction with a segment including the second extracellular loop of the glucagon receptor (*24*). The corresponding interaction could be relevant for GLP-1 binding to GLP-1R, given the cross-reactivity of glucagon on GLP-1R and the homology between glucagon, GLP-1, and their receptors (*21*). In this study, the DSC and tracer competition measurements showed that the chimeric ligand GLP-1/Ex4 bound nGLP-1R with reduced affinity relative to Ex4, suggesting that Leu^{10}−Arg^{20} of Ex4 (Val^{16}−Lys^{26} of GLP-1) play an important role for the differential affinity of nGLP-1R. This agrees with previous results which emphasized in particular the EEAVRL segments of Ex4 as being important for the ability of Ex4(9−39) to maintain high affinity for the full length GLP-1R (*33*). However, the EEAVRL segment cannot alone explain the differential affinity of nGLP-1R, and previous results showed that the specific substitution of this segment did not increase the affinity of GLP-1(9−30) for the full length rat GLP-1R (*36*). Collectively we believe that the residues Glu^{15}−Gly^{30} which include the EEAVRL segment of Ex4 form a superior interaction with nGLP-1R compared to the corresponding segment of GLP-1. Furthermore, depending on the intimacy between nGLP-1R and the core-domain of the full length receptor, the N-terminal half of GLP-1 including the VSSYL segment (LSKQM in Ex4) could be close to and/or interact with both nGLP-1R and the core-domain. Such intimacy between the subdomains of the secretin receptor was indicated by cross-linking, where photoactive probes in the N-terminus of secretin were able to cross-link specific residues in both receptor subdomains (*44*). Photoactive probes in the central and C-terminal part of secretin cross-link exclusively the Nt-domain of the secretin receptor (*45*).

GLP-1 and Ex4 have been studied by CD and NMR spectroscopy to understand their structure in aqueous solutions, DPC micelles, and organic solvents such as TFE (*34*, *41*). Ex4 is intrinsically more helical than GLP-1 in aqueous solutions, but it is believed that both ligands are primarily helical in their receptor-bound conformation, except for the conserved N-terminus and the Trp-cage of Ex4 (*34*, *46*). However, the Trp-cage is only partially populated in aqueous solutions, and a receptor-bound structure has not been solved of either GLP-1 or Ex4, so the extent to which the solution structures represent the receptor-bound conformation is not known. In this study we have combined biophysical and pharmacological approaches trying to relate structural properties of the free ligands to their relative binding affinity for nGLP-1R. We found a correlation between the helical content of the free ligands and their relative binding affinity for nGLP-1R, supporting the hypothesis that the ligands are helical at least in the segment that binds nGLP-1R. Accordingly, the loss of entropy by Ex4 binding to nGLP-1R is smaller than that of GLP-1 binding, because Ex4 is already more helical than GLP-1 in the unbound state, thus nGLP-1R binds Ex4 with higher affinity than GLP-1.

Ex4(1−30) which lacked the Trp-cage maintained a significantly higher helicity in solution than GLP-1 and maintained high affinity for nGLP-1R. The result suggests that the Trp-cage is not necessary for the superior helicity of Ex4 in aqueous solutions, supporting the idea that the Trp-cage folds onto a preformed helix as previously suggested (*35*). In this study, truncation of the Trp-cage altered the fluorescence properties, suggesting that even in aqueous buffers the C-terminal extension provides some degree of solvent protection of Trp^{25}. The solvent protection might also be explained by hydrophobic clustering of Trp^{25} and Pro^{31} which was suggested to C-cap the helix of Ex4 in a DPC micelle-associated state that lacked the Trp-cage (*34*, *35*). Nevertheless the similarity of the fluorescence spectra of GLP-1 and Ex4(1−30) is explained by the absence of the putative Trp-cage. The chimeric ligand GLP-1/Ex4 was designed on the basis of the NMR structure of Ex4 in order to allow formation of the Trp-cage. The results of fluorescence and CD spectroscopy suggested that the C-terminal conformation of GLP-1/Ex4 was similar to that of Ex4, although the overall helicity was decreased to a level that was intermediate between GLP-1 and Ex4 because of the GLP-1 segment. The GLP-1 segment included Gly^{22} (Glu^{16} of Ex4), which has been suggested to distort the helix of GLP-1 compared to the helix of Ex4 (*41*). The intermediate helicity of GLP1/Ex4 in solution correlated both with an intermediate affinity for nGLP-1R and an intermediate thermal stability of the complex with nGLP-1R.

The results of CD and fluorescence spectroscopy showed differential effects of GLP-1 and Ex4 binding to nGLP-1R. Binding of GLP-1 to nGLP-1R apparently induced a stronger increase of secondary structure in the resulting complex, compared to binding of Ex4. Given that Ex4 and GLP-1 are probably both helical in the segment that binds nGLP-1R and given that GLP-1 in solution is less helical than Ex4, the transition from the solution state to the nGLP-1R-bound state would be bigger with GLP-1 than with Ex4. We believe this is the most likely explanation for the differential interaction-induced effects with GLP-1 and Ex4, observed by CD spectroscopy. It has been suggested that peptide ligands for integral membrane receptors associate unspecifically with the membrane before they interact specifically with their receptor, and only minor conformational changes of PACAP(1−21) were observed upon transition from a micelle associated state to the receptor bound state (*47*). In this respect our binding assay with nGLP-1R would represent a transition from solution to nGLP-1R-bound without an intermediate membrane-association. The helicity of GLP-1 is increased by micelle association, and this could potentially increase the affinity of GLP-1 for nGLP-1R; however, Ex4 is still more helical than GLP-1 even in micelles (*34*). Furthermore the differential affinity of nGLP-1R for GLP-1 and Ex4 was originally described in parallel using both soluble and membrane-attached nGLP-1R (*18*).

We also used fluorescence spectroscopy to investigate ligand-induced structural changes although the presence of Trp residues in both nGLP-1R and the ligands limited the interpretations. Nevertheless, Ex4(10−39) defined the minimal sequence in our study which induced a maximum fluorescence blue-shift and quench of the nGLP-1R complex. The results suggested that one or more Trp residues of the complex were protected from solvent exposure directly by the interaction and/or indirectly by a conformational change induced by the interaction. Several of the Trp residues of GLP-1R have been implicated in ligand binding by site directed mutagenesis of GLP-1R, but it was not possible in this study to address them individually (*48*). The NMR study of the CRF2 receptor NT domain suggested ligand-induced folding of specific receptor segments and indeed Trp residues are present in the corresponding segment of nGLP-1R (*17*). Binding of GLP-1 had a significantly different effect on the fluorescence properties of nGLP-1R than all the other ligands including GLP-1/Ex4. The fluorescence signal was increased and slightly red-shifted by GLP-1, but quenched and blue-shifted by GLP-1/Ex4. In addition, GLP-1/Ex4 was more helical in solution and had higher affinity for nGLP-1R compared to GLP-1, but the CD spectra did not reveal any significant differences in the overall secondary structure of the complexes with GLP-1 and GLP-1/Ex4. Therefore it seemed that the GLP-1 induced fluorescence effects were related more to the local environment of the implicated Trp residues than to a differential effect of GLP-1 and GLP-1/Ex4 on the overall secondary structure. The Trp-cage is not necessary for the ligand-induced blue-shift as evident from the results obtained with Ex4(1−30). However, Ex4(1−30) had almost lost the ability to quench the fluorescence signal, whereas GLP-1/Ex4 despite lower affinity for nGLP-1R maintained the ability to quench the fluorescence. The differential fluorescence properties of the complexes suggested that the Trp-cage could be necessary for the quench effect, raising the possibility that the C-terminal tail of Ex4 induced solvent protection of one or more Trp residues of either the ligand itself or nGLP-1R. Given that the C-terminal truncation of Ex4 increased the fluorescence intensity both of Ex4(1−30) in solution and of the resulting complex with Ex4(1−30), it is tempting to speculate that the Trp-cage is present at least partially in the nGLP-1R-bound state and necessary for the quench of its own Trp residue. However with eight Trp residues in the complexes the effects are very difficult to decipher.

GLP-1 and Ex4(1−30) are comparable in the sense that they both lack the C-terminal tail of Ex4 and exhibit a similar

fluorescence spectrum in aqueous solution. However, Ex4-(1−30) had higher helicity and a correlating higher affinity for nGLP-1R. In addition, the pronounced long-wavelength CD band (224 nm) suggested that the complex with Ex4-(1−30) contained more α-helical structure than the complexes with GLP-1 and GLP-1/Ex4. Based on our CD experiments we could not address individually the structural changes of the ligands and nGLP-1R, so the effect could originate from nGLP-1R, the ligand, or both. But the results showed that the complexes with the more structured ligands (Ex4, Ex4(9−39), Ex4(10−39), and Ex4(1−30)) had a more pronounced $CD_{224\,nm}$ band. The results could suggest that the GLP-1 segment of the chimeric ligand is less helical than Ex4 also in the nGLP-1R bound state.

In summary, the full length GLP-1R binds Ex4 and GLP-1 with similar high affinity whereas the isolated nGLP-1R exhibits differential affinity for these two ligands as shown previously (*18*). The superior affinity of Ex4 for nGLP-1R is probably explained concomitantly by a superior helicity and by superior interactions due to specific divergent residues in the C-terminal part of the ligands (Glu^{15}−Gly^{30} in Ex4 and Glu^{21}−Arg^{36} in GLP-1). Conserved hydrophobic residues such as Phe^{28} and Leu^{32} of GLP-1 (Phe^{22} and Leu^{26} of Ex4) are probably on the same face of the helix providing conserved interactions with nGLP-1R (*32*, *36*). We believe that the Trp-cage plays only a minor role for the differential affinity of nGLP-1R for GLP-1 and Ex4.

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SUPPORTING INFORMATION AVAILABLE

Three figures showing fluorescence emission spectra of refolded and denatured nGLP1R (S1), fluorescence emission spectra of the free ligands (S2), and analysis of unfolding by CD spectroscopy (S3). This material is available free of charge via the Internet at http://pubs.acs.org.

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