The Isolated N-terminal Domain of the Glucagon-like Peptide-1 (GLP-1) Receptor Binds Exendin Peptides with Much Higher Affinity than GLP-1*

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Two fragments of the receptor for glucagon-like peptide-1 (GLP-1), each containing the N-terminal domain, were expressed and characterized in either bacterial or mammalian cells. The first fragment, rNT-TM1, included the N-terminal domain and first transmembrane helix and was stably expressed in the membrane of human embryonic kidney 293 cells. The second, 6H-rNT, consisted of only the N-terminal domain of the receptor fused with a polyhistidine tag at its N terminus. The latter fragment was expressed in Escherichia coli in the form of inclusion bodies from which the protein was subsequently purified and refolded in vitro. Although both receptor fragments displayed negligible 125I-labeled GLP-1(7–36)amide-specific binding, they both displayed high affinity for the radiolabeled peptide antagonist 125I-exendin-4(9–39). Competition binding studies demonstrated that the N-terminal domain of the GLP-1 receptor maintains high affinity for the agonist exendin-4 as well as the antagonists exendin-4(3–39) and exendin-4(9–39) whereas, in contrast, GLP-1 affinity was greatly reduced. This study shows that although the exendin antagonists are not dependent upon the extracellular loops and transmembrane helices for maintaining their normal high affinity binding, the endogenous agonist GLP-1 requires regions outside of the N-terminal domain. Hence, distinct structural features in exendin-4, between residues 9 and 39, provide additional affinity for the N-terminal domain of the receptor. These data are consistent with a model for the binding of peptide ligands to the GLP-1 receptor in which the central and C-terminal regions of the peptides bind to the N terminus of the receptor, whereas the N-terminal residues of peptide agonists interact with the extracellular loops and transmembrane helices.

It is well known that the action of glucose on pancreatic islets results in increased plasma insulin levels. Nevertheless, high blood glucose levels are not solely responsible for increased insulin secretion (for review, see Ref. 1). For example, in 1964 McIntyre et al. (2) demonstrated that intravenous injection of glucose resulted in a smaller insulin release than that resulting from intrajeunal glucose injection, even though the latter produced lower blood glucose levels compared with the former. Hence, glucose-dependent insulin secretion requires a nutrient-dependent component, which was believed to be an endocrine termter termed an “incretin” (3). It has since been demonstrated that two hormones, glucagon-like peptide-1 and glucose-dependent insulinotropic polypeptide, are responsible for the incretin effect (1).

The predominant active form of GLP-1 is actually glucagon-like peptide-1(7–36)amide (termed GLP-11 throughout this paper), a 30-residue peptide hormone derived from the post-translational modification of proglucagon in intestinal L cells (1). GLP-1 not only increases glucose-dependent insulin secretion (4–6), but it also decreases glucose-dependent glucagon secretion (7, 8) and decelerates gastric emptying (9). In addition, GLP-1 has been shown to reduce appetite in rats (10) and to stimulate proinsulin gene transcription and biosynthesis in pancreatic β-cells (11, 12). The physiological roles of GLP-1 in maintaining blood sugar levels, via a glucose-dependent mechanism, have heightened interest in the GLP-1 receptor (GLP-1R) as a target for glucose-dependent therapeutic agents designed to treat hyperglycemia resulting from diabetes (13, 14). Unfortunately, the half-life of GLP-1 itself after subcutaneous injection is very short because of dipeptidyl peptidase IV cleavage of the first 2 N-terminal residues (15), and so future research requires the design of physicochemically stable GLP-1R agonists.

The venom of the Gila monster Heloderma suspectum contains a mixture of compounds that includes several peptides related in sequence to GLP-1. Two of these, exendin-3 and exendin-4, are 39-amino acid peptides that share ~50% sequence identity to GLP-1 itself and are indeed potent GLP-1R agonists (Fig. 1) (16, 17). Interestingly, although GLP-1 affinity is highly sensitive to N-terminal cleavage, exendin-4 can be truncated by up to 8 residues at its N terminus without significant loss of affinity, suggesting that relative to GLP-1, the central and/or C-terminal residues form additional stabilizing contacts with the receptor (15, 18). Nevertheless, the first two amino acids are also essential for the efficacy of exendin peptides because, once removed, the truncated exendin peptides function as antagonists or inverse agonists (16–19).

Although the N termini of GLP-1 and exendin-4 are almost identical, exendin-4 contains 9 extra amino acids at the C terminus, which have been shown by NMR analysis to form a
Fig. 1. Ligands and receptor constructs used in this work. A, aligned amino acid sequences of several GLP-1R peptide ligands. B, schematic representation of the wild-type rat GLP-1R (rGLP-1R) and the two truncated rat receptors used in this study, rNT-TM1 and 6H-rNT. The N-terminal domain is represented by gray ovals, and transmembrane helices are shown as gray cylinders. The expression of rGLP-1R and rNT-TM1 in a mammalian system is indicated by the putative glycosylation shown with the two Y-shaped symbols. The 6H-rNT protein was expressed in a bacterial system and lacks glycosylation but contains a His$_6$ tag (HHHHHH) close to the N terminus.

The GLP-1 Receptor N-terminal Domain

GLP-1(7–36)amide, exendin-4, exendin-4(3–39), and exendin-4(9–39) were from Bachem (Saffron Walden, UK). $^{125}$I-GLP-1(7–36)amide was a gift from Novo Nordisk A/S (Copenhagen, Denmark). $^{125}$I-Exendin-4(9–39) was purchased from PerkinElmer Life Sciences. The expression vectors pCDNA3 and pQE-30 were from Invitrogen and Qiagen, respectively. The M15(pREP4) E. coli strain and nickel-nitri-locetic acid resin were purchased from Qiagen. Oligonucleotides were obtained from Sigma. Restriction and modifying enzymes were from MBI Fermentas-Helena Biosciences (Sunderland, UK), Promega (Southampton, UK), Invitrogen (Paisley, Scotland, UK), and New England Biolabs. Cell culture reagents were obtained from Invitrogen and Sigma. General chemicals were from Merck and Sigma.

Methods

Plasmid Construction—The cDNA encoding amino acids Met$^1$-Leu$^{171}$ (N-terminal domain, including the putative signal sequence, and first transmembrane α-helix) of the GLP-1R was synthesized by PCR using as a template the pcDNA3 vector containing the full-length rat GLP-1R gene originally provided in pcdNA1 by Dr. B. Thorens (21, 22). The forward and reverse oligonucleotides incorporated the Hin$\text{III}$ and Xho$\text{I}$ recognition sites, respectively, to facilitate insertion into the pcDNA3 expression vector. The expressed receptor fragment was named rNT-TM1. For the second construct, the cDNA sequence encoding amino acids Ala$^{14}$-Leu$^{396}$ (N-terminal domain without the signal sequence) of the GLP-1R was amplified by PCR using the same template as above. The forward and reverse oligonucleotides incorporated the Hint$\text{III}$ and Hind$\text{III}$ recognition sites, respectively, for subcloning into homologous sites in the pQE-30 expression vector. The resultant construct was identical to that of Wilmen et al. (28), taking into account a misprint in the original paper concerning the restriction enzymes used. The expressed protein was named 6H-rNT. The integrity of both constructs was verified by automated nucleotide sequencing.

Expression of rNT-TM1 in HEK 293 Cells—HEK 293 cells were stably transfected with pCDNA3/rNT-TM1 and membrane preparations from these cells prepared as described previously (21).

Expression of 6H-rNT in E. coli Cells and in Vitro Refolding from Inclusion Bodies—Insertion of the cDNA encoding the N-terminal domain of the GLP-1R into the pQE-30 vector led to the addition of 12 extra amino acids at the N terminus of the GLP-1R fragment, including a His$_6$ tag (MRGSHHHHHHHGS). Bacterial expression and protein refolding were carried out according to Bazarsuren et al. (26) except that

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2 R. López de Maturana, S. Al-Sabah, and D. Donnelly, unpublished results.

3 A. Wilmen, personal communication.
the pulse renaturation stage was carried out manually, and functional characterization of the protein in radioligand binding assays was carried out after the refolding stage but prior to purification via ion exchange chromatography.

**Binding to HEK-293 Cell Membranes**—75 μl of membranes thawed slowly on ice was diluted appropriately in MBS (20 mM HEPES pH 7.4, 2.5 mM CaCl2, 1 mM MgCl2, 50 mg/liter bacitracin) and mixed with 75 μl of 0.2 nM 125I-exendin-4(9–39) (50 pm final concentration), 75 μl of MBS and 75 μl of unlabeled peptides at a final concentration ranging from 10^{-6} to 10^{-12} M, diluted in MBS. Nonspecific binding was estimated in the presence of 1 μM unlabeled ligand. Reactions were incubated for 1 h at room temperature. Unbound ligand was washed by rapid vacuum filtration (Brandel cell harvester; Gaithersburg, MD) through glass-fiber paper (Whatman G/F grade filter paper) pre-soaked in 5% non-fat powdered milk. Filters were rinsed three times with 5 ml of ice-cold phosphate-buffered saline. Then they were cut, allowed to dry, and filter-bound radioactivity was measured in a gamma counter.

**Competition binding assays**—Triplicate values with S.E. displayed as error bars. Counts were estimated in the presence of 10^{-6} M unlabeled ligand. Reactions were carried out after the refolding stage but prior to purification via ion exchange chromatography.

**RESULTS**

Although the wild type GLP-1R displayed high affinity for 125I-GLP-1, membranes prepared from HEK-293 cells containing rNT-TM1 showed barely detectable binding of the radiolabeled agonist used at 50 pM (data not shown). However, using the agonist 125I-exendin-4(9–39) as the tracer, again at 50 pM, resulted in high specific binding, which allowed the determination of the IC_{50} values for various peptides via competition binding. From such binding assays, the IC_{50} values for the antagonists exendin-4(3–39) and exendin-4(9–39) (IC_{50} = 7.9 and 15.8 nM, respectively) were found to be similar to those obtained with HEK-293 cell membranes expressing the wild type rat GLP-1R, termed rGLP-1R (IC_{50} = 10.0 and 5.0 nM, respectively; Tables I and II and Figs. 2–4). The agonist exendin-4 also displayed a similar affinity at both receptors (IC_{50} = 1.6 nM wild type versus 6.3 nM rNT-TM1). However, in marked contrast, the affinity of rNT-TM1 for GLP-1 was estimated to be decreased >50-fold (IC_{50} = 316 nM versus 6.3 nM at the full-length receptor; Table II and Figs. 3 and 4). The B_{max} value for rNT-TM1 determined from antagonist binding studies suggested that expression levels of this construct were reduced to 10% of rGLP-1R levels (6.8 ± 1 pmol/mg versus 67.7 ± 12.1 pmol/mg).

**Radiolabeled exendin-4(9–39)**—was then used in competition binding studies to determine the binding parameters of the truncated receptor that had been refolded from inclusion bodies. For this part of the study, we used the isolated N-terminal domain from both the rat and human GLP-1Rs. The human N-terminal domain 6H-hNT had already been characterized extensively (26), whereas the rat N-terminal domain 6H-rNT provides a more consistent comparison with the data from rGLP-1R and rNT-TM1. High affinity for exendin-4(9–39) at the isolated N-terminal receptor domain from the human receptor was observed with both the highly purified 6H-hNT preparation and the partially purified 6H-rNT preparation (Table I and Fig. 2). Further analysis demonstrated that 6H-rNT also maintained very high affinity for exendin-4, exendin-4(3–39), and exendin-4(9–39) (IC_{50} = 1.0, 1.3, and 0.6 nM, respectively). However, in contrast to these exendin peptides, GLP-1 affinity was >60-fold lower than at rGLP-1R (IC_{50} = 398 nM versus 6.3 nM respectively; Tables I and II and Figs. 2, 3, and 5).

**DISCUSSION**

**Hypothesis**—Because we have shown previously that the binding site for the N-terminal region of the GLP-1 hormone involves the extracellular loop regions (21), it is perhaps not surprising that it has been reported that GLP-1 binds to the isolated N-terminal domain of the GLP-1R with much lower affinity than it does to the wild type receptor (26, 28, 29). However, because the high affinity of exendin-4 for GLP-1R is neither dependent upon its own N-terminal residues (18) nor the loop regions of the receptor (21), we hypothesized that it may bind with high affinity to the isolated N terminus of GLP-1R. Such knowledge would provide important information in understanding ligand binding to this receptor, and it may also suggest means by which GLP-1 affinity may be improved by identifying the unique properties of exendin-4.

**Constructs and Receptor Fragments**—To test this hypothesis,
we made two constructs designed to express N-terminal fragments of the rat GLP-1R. The first truncated receptor (rNT-TM1), which included the N-terminal domain and first transmembrane helix of the receptor, was expressed in HEK-293 cell membranes. Because it is membrane-anchored via the single transmembrane helix and is processed by the eukaryotic expression machinery, the resultant binding data can be easily compared with the rat wild type receptor expressed in the same system (rGLP-1R). The second truncated receptor (6H-rNT), consisting of the N-terminal receptor domain fused with a His6 tag, was expressed in E. coli and refolded from inclusion bodies. If correctly refolded in vitro, the data obtained from the analysis of this receptor fragment should be comparable with that obtained for rNT-TM1 because this was refolded by the eukaryotic processing machinery in vivo. However, the soluble protein obtained from E. coli holds additional potential for further structural studies that require greater expression levels and solubility than can easily be obtained for membrane proteins, and hence its characterization is of great interest.

The construct and expression system used to synthesize 6H-rNT were identical to those used previously by Wilmen et al. (28). However, they obtained their protein from the soluble fraction, whereas we found that the vast majority of the protein expressed in this system was in the form of insoluble inclusion bodies. Although we could increase the yield of soluble protein by lowering the induction temperature, most of the protein was still expressed in the insoluble fraction (data not shown). We therefore denatured and refolded the inclusion body protein according to a method based closely upon that of Bazarsuren et al. (26) and analyzed the resultant protein for ligand binding.

As a positive control, we analyzed a sample of the refolded human GLP-1R N-terminal domain, which previously had been extensively characterized by CD, cross-linking, affinity chromatography, surface plasmon resonance, and isothermal titration calorimetry (26). In addition to acting as a positive control for the refolding of the rat analog, 6H-rNT, our data (Table I and Fig. 2) further confirm the correct refolding of 6H-hNT itself because we have demonstrated for the first time that it binds exendin-4(9–39) with very high affinity by using a radio-labeled ligand known to be highly selective for the GLP-1R.

rNT-TM1—In competition binding studies using the radiolabeled antagonist peptide exendin-4(9–39), the affinities of the exendin peptides at the truncated rNT-TM1 were determined to be similar to those at rGLP-1R. Hence, there are clearly critical interactions between regions in these peptides and the receptor N terminus. However, GLP-1 displayed an IC50 at the truncated receptor which was more than 50-fold higher than at rGLP-1R (Table II and Fig. 5). Hence there is clearly a difference between the agonists GLP-1 and exendin-4 because the latter is much less dependent either on regions on the receptor outside of the N-terminal domain of the receptor (Table II and Figs. 3–5) or on its own N-terminal residues (15, 18).

The observation of a >50-fold reduction in affinity at rNT-TM1 highlights the reason why we initially failed to detect GLP-1 affinity using the radiolabeled agonist 125I-GLP-1. Because we used the radiolabeled agonist at 50 pM, the receptor occupancy at this ligand concentration would have been extremely low and, coupled with the reduced Bmax, the binding would have become undetectable. Nevertheless, it is clear from the competition studies that GLP-1 maintains reasonable affinity for rNT-TM1, albeit 50-fold reduced compared with the wild type receptor.

6H-rNT—The isolated N-terminal domain of the human GLP-1R has been expressed, refolded, and extensively characterized (26). The correct refolding of this domain (6H-hNT) was shown by the binding of GLP-1 using either surface plasmon resonance or isothermal titration calorimetry. However, because the affinity for GLP-1 was found to be significantly lower than that seen at rGLP-1R expressed in mammalian cells, there was the possibility that the domain possessed a structure altered from that of the whole receptor, possibly because of the lack of glycosylation in the bacterial expression system or the in vitro refolding methodology. However, the very high affinity binding for the antagonist exendin-4(9–39) strongly suggests that this domain is correctly refolded and that the loss of GLP-1 affinity is because of the absence of interactions between the GLP-1 and other regions of the receptor. Hence, the determination of the disulfide bonding pattern for 6H-hNT (26) can be viewed with very high confidence.

To compare rNT-TM1, our membrane-anchored N-terminal domain for the rat GLP-1R, properly with the refolded N-terminal domain from E. coli, we decided to avoid potential species differences and hence generated a soluble version of the N-terminal domain for the rat GLP-1R. The analysis of this refolded protein confirmed that is was correctly folded because
it bound exendin-4(9–39) with very high affinity (<1 nM). It is not clear why this affinity is higher than that of GLP-1R itself; this may result from the different nature of binding assays used or perhaps from an absence of the juxtaposed membrane bilayer in the 6H-rNT preparation. Further analysis also revealed that this protein bound exendin-4 and exendin-4(9–39) with affinity comparable with that observed at rGLP-1R. However, as with the membrane-anchored rNT-TM1, the natural agonist GLP-1 showed very reduced affinity (>60-fold) at the isolated N-terminal domain compared with the full-length receptor. Overall, this part of the study confirmed the results using rNT-TM1 and further reveals the potential for detailed structural studies of this important domain.

Comparison with Other Studies—The isolated N-terminal domain of GLP-1R and other Family B GPCRs has been documented previously to have a reduced affinity for their endogenous ligands. In competition binding studies with agonist tracer, the IC50 for GLP-1 at the soluble rat GLP-1R N-terminal domain purified from COS-7 cells and immobilized on Ni2+ beads was 450 nM (29). The refolded N-terminal domain of the human GLP-1R was reported to have a Kd of 47 nM, as determined by surface plasmon resonance, and 144 nM from isothermal titration calorimetry (26). Hence our data using GLP-1 are consistent with these previous observations in showing that normal GLP-1 affinity requires regions outside of the N-terminal domain.

The results obtained with rNT-TM1 are somewhat similar to those obtained using the membrane-anchored N-terminal fragment of the rat receptor for putitary adenylate cyclase-activating peptide subtype 1 expressed in COS-7 cells, where a 19-fold reduction in agonist affinity was observed at this similarly truncated receptor (32). The results are also very similar to the analogous corticotropin-releasing factor receptor fragment expressed in COSM6 cells, which showed no detectable binding in homologous competition binding assays when the agonist uro-cortin was used as tracer but bound with low nM affinity to the antagonist astressin when this peptide was used as the tracer in competition binding studies (33).

Comparison of our 6H-rNT data with a study on the N-terminal domain of corticotropin-releasing factor receptor 1 may also be useful because several similarities can be observed (25). This domain was produced as a soluble protein in E. coli and characterized using a radiolabeled peptide antagonist for corticotropin-releasing factor receptor 1, astressin. The Kd value for astressin at this receptor fragment was 50 nM compared with 1.8 nM for the full-length receptor in mammalian cells. Interestingly, one agonist (urocortin) was able to displace 125I-astressin binding, although with a slightly reduced affinity, whereas two other agonists (corticotropin-releasing factor and sauavagine) were not able to compete for 125I-astressin binding. This example illustrates how agonists with a relatively high degree of sequence identity may have different binding modes on the same receptor.

In general, studies focused on the N terminus of Family B GPCRs report very variable decreases in hormone affinity ranging between 20- and 1,000-fold (23, 25, 32); others do not mention affinity values specifically, suggesting that affinity was too low to be quantified properly in the particular systems (27, 28). Reports on similar rNT-TM1 constructs showing membrane presence but no detectable binding to their cognate hormones also exist (34, 35).

Conclusions and Model—In contrast to the GLP-1 binding requirements, the present analysis shows that the exendin antagonists do not require binding epitopes outside the N-terminal domain. This was first indicated after the analysis of site-directed mutations in the extracellular loops of GLP-1R, which reduced GLP-1 affinity but did not markedly reduce exendin-4(9–39) affinity, indicating that this peptide does not need intact loops for high affinity binding to GLP-1R (21).2 Because the normal affinity of exendin antagonists only requires interactions with the N-terminal domain of the receptor, it strongly suggests that the region between residues 9 and 39 interacts with the N terminus. The equivalent region in GLP-1 (residues 15–30) most likely also binds to this region because both peptides share a conserved face on the putative helix formed by this region (21). However, there are clearly additional interactions made by residues between positions 9 and 39 of exendins, compared with residues 15–30 of GLP-1, which result in the increased affinity of exendins for the N-terminal domain of the receptor and also reduce the sensitivity of exendin ligands to N-terminal truncation. Hence, the fact that exendin-4 binding does not depend greatly on the extracellular loops does not necessitate that it requires an activation pocket different from that of GLP-1. The increased affinity of exendin-4 and the GLP-1R N terminus may be provided by the C-terminal Trp-cage motif (20) or perhaps by the increased helicity of the middle region of this peptide compared with GLP-1 (36, 37). Increasing peptide helicity has been proposed to improve binding to the N-terminal domain of the calcitonin receptor (38).

We can view the interaction of these peptides and GLP-1 as being mediated by up to three types of interaction, defined below as N, H, and Ex (respectively for N-terminal, Helix, and Exendin or Extra; Fig. 6). There are 9 residues conserved on a common face of the putative central helix in GLP-1 and exendin-4(9–39) (21) which are likely to form a conserved re-
ceptor-peptide interaction site termed H. However, in addition to this shared property, the two peptides also reveal three distinguishing characteristics. First, GLP-1 has an extra 8 residues at its N terminus, which are known to be critical for its efficacy (18) and which interact with the receptor core (21) (the N interaction). Because exendin-4(9–39) lacks this interaction, it cannot activate the receptor and is therefore an antagonist. Second, exendin-4(9–39) possesses an extra 9 residues at its C terminus which form a putative Trp-cage (20). Third, the central regions of the two peptides contain 13 different residues, mainly clustered on one face of an ideal α-helix (21). Although the first difference explains the different intrinsic activities of the two peptides, one or both of the latter two differences are likely to account for the increased affinity of exendin-4(9–39) for the isolated N-terminal domain of the receptor (the Ex interaction). Exendin-4 can be viewed as a peptide that retains the agonistic properties of GLP-1 because it shares a very similar N terminus and also the high affinity for the isolated N-terminal domain of the receptor typical of the antagonist exendin-4(9–39).

Because exendin-4 can interact with the receptor via all three interaction sites H, N, and Ex (Fig. 6Bi), whereas GLP-1 only interacts via H and N (Fig. 6Bii). Hence the removal of the N interaction between GLP-1 and its receptor (e.g. either by truncation of the peptide or by mutation of the receptor (21)) has a much more drastic effect on affinity (>50-fold reduction) than removal of the N interaction between exendin-4 and GLP-1R (4-fold reduction). The combination of the H and Ex interactions is sufficient to maintain high affinity of exendin-4(9–39) for GLP-1R. Because this peptide cannot make the N interaction, the removal of the entire receptor core has a negligible effect upon its binding affinity (Fig. 6Biii). The proposed model further highlights the potential of 125I-exendin-4(9–39) as a useful radiolabel for studying mutant receptors in which the strength of the receptor-peptide interaction site termed

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