The isolated N-terminal extracellular domain of the glucagon-like peptide-1 (GLP)-1 receptor has intrinsic binding activity

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Abstract The glucagon-like peptide 1 (7–37)/(7–36) amide (GLP-1) receptor belongs to a new subclass of seven transmembrane domain, G-protein coupled receptors comprising several receptors for peptide hormones. The receptors of this family share many common motifs including a relatively large N-terminal extracellular domain. The GLP-1 receptor is presently attracting much attention, since it is the target protein of the antidiabetic gut hormone GLP-1. To establish the functional significance of the N-terminal part of the GLP-1 receptor for ligand binding, the extracellular domain was isolated and purified. Utilizing CHL cells expressing the cloned GLP-1 receptor, we demonstrate that the isolated, solubilized N-terminal part of the receptor protein competes for GLP-1 binding with the intact wild-type receptor. Moreover, in cross-linking experiments radiolabeled GLP-1 was covalently attached to the isolated N-terminus, thereby demonstrating direct physical interaction of both components. By Western blot analysis two specific bands were detectable, representing the N-terminal receptor protein in the presence or absence of bound ligand. These data underline the significance of the N-terminal domain of the GLP-1 receptor for ligand binding.

Key words: GLP-1 receptor; N-terminal domain; Intrinsic binding activity

1 Introduction

Glucagon-like peptide 1 (GLP-1 (7–37)/(7–36) amide) arises as a post-translation product of the intestinal proglucagon gene [1]. GPL-1 potently stimulates glucose-induced insulin secretion and proinsulin gene expression [2]. It has therapeutic potential for diabetes mellitus treatment [3]. Recent data have demonstrated that injection of GLP-1 into the brain inhibits feeding in rats [4]. GLP-1 binding sites are widespread [3] and have been characterized in pancreatic B cells [5], gastric glands [6], lung [7,8] and brain [9].

Together with the receptors for vasoactive intestinal polypeptide, pituitary adenylate cyclase activating polypeptide (PACAP), parathormone, glucagon, gastric inhibitory polypeptide (GIP), and calcitonin, the GLP-1 receptor (GLP-1R) belongs to a subclass of seven transmembrane, G-protein coupled receptors (GPCRs) [10]. All members of this family of GPCRs share a similar basic structure and membrane topology. In contrast to the superfamily of adrenergic receptors, this polypeptide receptor subclass contains a relatively large N-terminal extracellular domain containing highly conserved amino acids including 6 cysteine residues. In meticulous studies, binding sites for agonists and antagonists were defined for the adrenergic receptor family, which facilitate the construction of a prototypical seven transmembrane receptor model [11,12]. In contrast, only a few structure-function analyses have been reported for peptide hormone receptors, however, a detailed understanding of the molecular mechanisms of the ligand-receptor interaction is of outstanding pharmacological interest.

Recent studies revealed evidence for the importance of the extracellular N-terminal domain for ligand binding in this subclass of GPCRs [13–19]. Point mutation experiments within the N-terminus of the rat GLP-1 receptor suggested a dominant biological function for binding [14]. Utilizing the purified, solubilized N-terminal domain of the rat GLP-1 receptor (N-T GPL-1R), it could now be demonstrated that this part of the receptor exhibits intrinsic ligand binding affinity.

2. Materials and methods

2.1. Materials

Chemicals, all of highest grade, were purchased from Sigma (Deisenhofen, Germany) or Merck (Darmstadt, Germany). Sera, media, and other tissue culture reagents were from Gibco Laboratories and enzymes were from Boehringer Mannheim (Germany). The cross-linking reagent disuccinimidyl suberate (DSS) was obtained from Pierce Europe (Oud-Bijerland, The Netherlands).

^{125}I-GPL-1 and ^{125}I-glucagon were prepared as described previously [20]; the specific activity of the radiolabel amounted to 2000 Ci/mmol.

2.2. Cell culture

CHL (Chinese hamster lymphoblast) cells stably transfected with the rat wild-type GLP-1 receptor were grown in RPMI 1640 medium supplemented with 10% bovine calf serum, 1 mM sodium pyruvate and 1% non-essential amino acids at 37°C in 95% air and 5% CO_2.

2.3. Purification of the N-terminal extracellular domain of the GLP-1 receptor

The extracellular domain of the rat GLP-1 receptor without the putative leader sequence (amino acids 20–144) was purified by using the 6×His tag system (QIAGEN, Germany). The N-terminus was amplified by PCR with specific oligonucleotides generating *Eco*RI and *Bam*HI restriction sites at the 5′ and 3′ ends of the amplified fragment to facilitate subcloning in pQE30 vector (QIAGEN, Germany). The sequence of the construct (pQE30 N-T GLP-1R) was verified by sequencing. This plasmid was transformed into the *E. coli* strain M15 [21]. Positive expression clones were checked by screening of small-scale expression cultures. For the purification of large amounts of NT GLP-1R, 500 ml LB broth liquid cultures were incubated at 37°C until the OD_{600nm} reached 0.7–0.9 IPTG (isopropyl-β-D-thiogalactopyranoside) was added to a final concentration of 0.5 mM and the culture was allowed to continue growth for 3 h at 37°C. Cells were harvested by centrifugation. The pellet was resuspended in 1 M urea, 50 mM NaH_2PO_4, 10 mM Tris, pH 7.9 at 3 vols. per g wet weight. Cells were disrupted by lysozyme treatment (1 mg/ml final concentration, incubated on ice for 30 min) followed by sonification (10×30 s bursts/1 min cooling/200 W, using a Labsonic sonifier, B. Braun, Germany). Samples were centrifuged for 20 min at 12000×*g*. 8 ml of a 50% slurry of Ni-NTA resin was added to 8 ml of the supernatant and stirred on ice for 60 min. The resin was loaded onto a column and washed twice with 80 ml solution buffer (1 M

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urea, 50 mM NaH_2PO_4, 10 mM Tris, pH 7.9). The N-T GLP-1R protein was eluted with a 20 ml of a pH 7.5–5.0 gradient in solution buffer and 1 ml fractions were analyzed on SDS-PAGE. Purity of N-T GLP-1R was checked by silver staining of SDS gels. The majority of the protein eluted at pH 5.9.

Immunization of rabbits was performed by Eurogentec (Belgium). The antisera raised yielding polyclonal antibodies were tested according to standard protocols.

The N-terminal domain of the GIP receptor (N-T GIPR) was purified using the pMAL system (New England Biolabs, USA). Briefly, the DNA encoding the N-terminal domain of GIP receptor was amplified by PCR, subcloned downstream of the maltose-binding protein (MBP) into the pMAL-c2 vector. The sequence of the construct was verified by sequencing. The plasmid was transformed into the *E. coli* strain TB1. Cells were inoculated in LB-broth+2% glucose. At an OD_{600nm} of 0.7, IPTG was added to a final concentration of 0.5 mM. Proteins were isolated in the same way as described for N-T GLP-1R (see above) with the exception that amylase resin was used instead of Ni-NTA resin. The fusion protein MBP-N-T GIPR was eluted with 5 ml of elution buffer (1 M urea, 50 mM NaH_2PO_4, 10 mM Tris, pH 6.5+10 mM maltose). To remove the MBP fusion protein, eluted samples were treated with factor Xa for 2 days at 4°C. Proteins were separated on a native polyacrylamide gel and N-T GIPR was isolated by electroelution using a GE200 Sixpac Gel Eluter (Hoefer Scientific Instruments, San Francisco, USA).

2.4. Binding assay of the purified N-terminal domain of the GLP-1 receptor

Transfected cells carrying the rat wild-type GLP-1 receptor were detached from culture plates using phosphate-buffered saline (pH 7.3) containing 1.5 mM EDTA. Cells were centrifuged and resuspended in buffer (2.5 mM Tris-HCl, 120 mM NaCl, 1.2 mM $MgSO_4$, 5 mM KCl, 15 mM CH_3COONa; pH 7.4) supplemented with 1% human serum albumin, 0.1% bacitracin, and 1 mM EDTA. Cells were incubated for 30 min at 37°C with ^{125}I-GLP-1 (20000 cpm) in the absence (controls) and presence of 6.5×10^{-7}, 2.6×10^{-7}, or 1.3×10^{-7} M of the purified extracellular domain. Cells were centrifuged for 5 min at $10000\times g$ (4°C), washed in ice-cold incubation buffer and centrifuged again. The cell pellet was counted using a γ-counter. As additional control, instead of the extracellular domain of the GLP-1 receptor the purified N-terminal part of the related GIP receptor as well as bacterial lysozyme were added. To examine whether formation of disulfide bridges within the extracellular domain is important for hormone binding, 6.5×10^{-7}, 2.6×10^{-7}, or 1.3×10^{-7} M of the purified protein and 10^{-4} M of unlabeled GLP-1, respectively, were incubated for 10 min at room temperature in the presence of 10 mM β-mercaptoethanol before addition to the binding assay.

2.5. Cross-linking experiments with the purified extracellular domain

Cross-linking experiments were performed as described [22]. Briefly, purified protein (1 mg/ml) was resuspended in cross-linking buffer (10 mM HEPES, 118 mM NaCl, 4.7 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, 40 μM PMSF) containing 0.01% (w/v) bacitracin, and 0.01% (w/v) human serum albumin, (pH 7.4). The protein was incubated with ^{125}I-GLP-1 ($\sim2\times10^6$ cpm) in the absence and presence of unlabeled GLP-1(7–36) amide (final concentration 1 μM) at 20°C for 30 min. DSS (final concentration 0.1 mM) dissolved in DMSO (final concentration 1% (v/v)) was added, and the samples incubated for 10 min at 20°C. The reaction was stopped by addition of Tris-HCl buffer (50 mM Tris-HCl, pH 7.0) containing 1 mM EDTA and 1% SDS. Samples were further analyzed by gel electrophoresis (SDS/12% PAGE) followed by autoradiography. Dried gels were exposed to Kodak type X-Omat AR films at −80°C for up to 5 days with the use of a light-intensifying screen.

3. Results

3.1. Construction of the solubilized N-terminal domain of the GLP-1 receptor

The N-terminal domain of the GLP-1 receptor consists of 144 amino acids. Using the corresponding cDNA this part of the receptor DNA without the putative leader sequence was amplified by PCR and subcloned into a bacterial expression vector. The protein termed N-T GLP-1R was overexpressed, purified to homogeneity and used for all subsequent experiments (Fig. 1).

3.2. Binding activity of the N-terminal domain

To examine whether the N-terminal domain of the GLP-1 receptor has intrinsic binding activity this purified solubilized protein was used as specific competitor in binding assays. CHL cells carrying the wild-type GLP-1 receptor were incubated with radiolabeled GLP-1 and a range of concentrations of the purified N-terminal domain was added. 90% of labeled GLP-1 was competitively removed from the wild-type receptor in the presence of 6.5×10^{-7} M of solubilized extracellular receptor domain. Decreasing the concentration to 2.6×10^{-7} and 1.3×10^{-7} M led to a competition activity of 87 and 43%, respectively (Fig. 2).

In order to exclude the possibility that not the solution

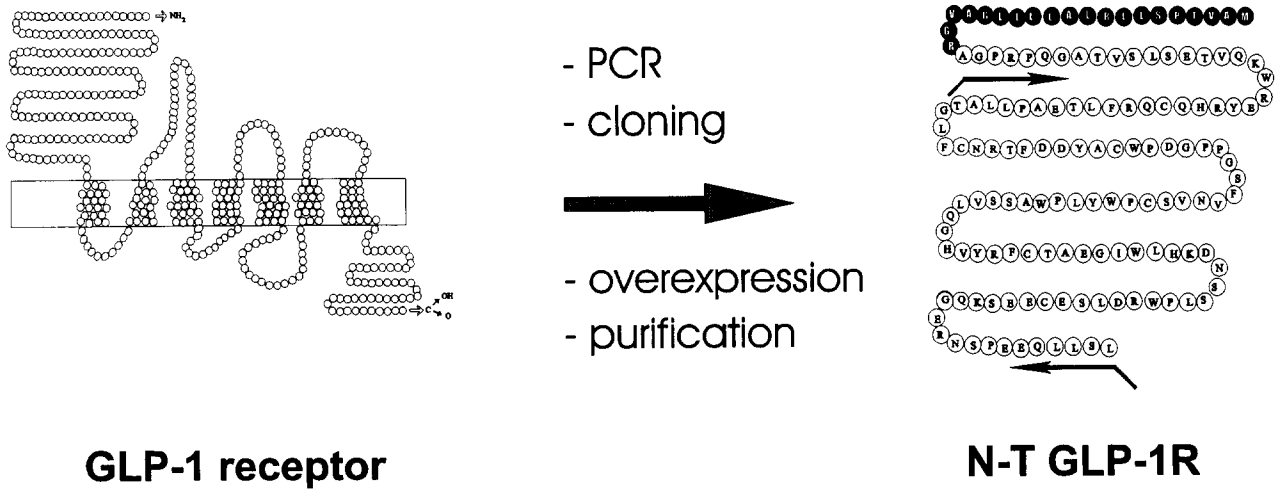

Fig. 1. Schematic representation of the construction of N-T GLP-1R. The corresponding cDNA of the rat wild-type GLP-1 receptor was used to amplify the N-terminal domain by PCR. The resulting DNA fragment was cloned into a bacterial expression vector and the overexpressed N-T GLP-1R protein was purified. The oligonucleotides used are indicated by arrows. Closed circles indicate the putative secretory signal peptide.

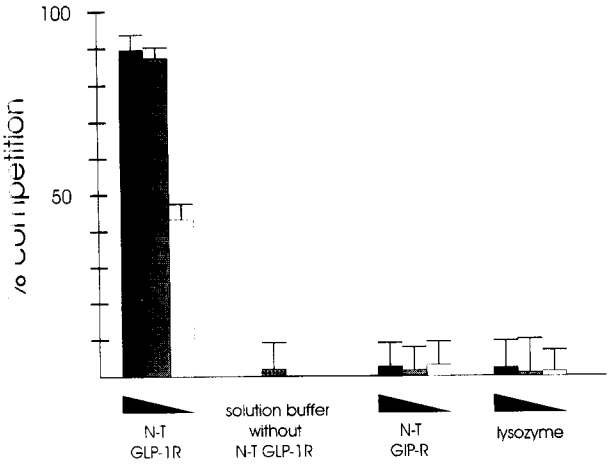

Fig. 2. Competition ability of the isolated extracellular domain of the GLP-1 receptor. Increasing amounts of the isolated N-terminal part of the GLP-1 receptor (N-T GLP-1R) were added as competitor in a binding assay using CHL cells expressing the entire wild-type GLP-1 receptor and ^{125}I-GLP-1(7–36) amide as radiolabeled tracer. As controls plain solution buffer without N-T GLP-1R, or the N-terminal domain of the GIP receptor (N-T GIPR), or bacterial lysozyme were employed. Shown is percent competition vs. concentration (M) of competitor proteins. ▶ indicates 6.5×10^{-7}, 2.6×10^{-7}, and 1.3×10^{-7} M protein . Each bar shows the mean (± S.E.M.) of three or four experiments, each performed in duplicate or triplicate.

buffer but rather N-T GLP-1R exhibits competition activity, the experiment was repeated using solution buffer without the solubilized protein. The amount of buffer was the same as in the experiments with N-T GLP-1R. As shown in Fig. 2 the solution buffer does not lead to any detectable competition of ^{125}I-GLP-1. Therefore, the shift of binding of peptide label suggests attachment to the isolated N-terminus of the receptor.

The N-terminal domain of the GIP (gastric inhibitory polypeptide) receptor (N-T GIPR), a close relative of the GLP-1 receptor (41% homology), was tested as a control for its ability to compete within the same assay system. No specific binding of GLP-1 to the GIP receptor was detectable [23]. As further controls bacterial lysozyme was used. N-T GIPR and lysozyme were added to the binding assay in the same concentrations as the solubilized extracellular domain of the GLP-1 receptor. None of these two proteins showed any competition activity. This suggested specificity for the ligand binding ability of the N-terminal domain of the GLP-1 receptor (Fig. 2).

3.3. *The tertiary structure of the extracellular domain is important for its competition activity*

Similar to other receptors of the subfamily, the GLP-1 receptor contains 6 highly conserved cysteines in the amino-terminal domain. Analysis of the related VIP receptor has recently shown the importance of these amino acids for ligand binding [15]. For the GLP-1 receptor this was studied by incubation of N-T GLP-1R with the reducing agent β-mercaptoethanol which induces the complete loss of the disulfide-bridge dependent tertiary structure. In order to determine whether β-mercaptoethanol interferes with the binding assay, unlabeled ligand (GLP-1) was incubated with the same concentration of reducing agent as the purified N-terminal domain. β-Mercaptoethanol treated GLP-1 showed concentration-dependent inhibition of ^{125}I-GLP-1 binding to the receptor similar to that of the untreated hormone. This indicated no interference of the reducing agent with the peptide or the assay system in general. In contrast, incubation of the

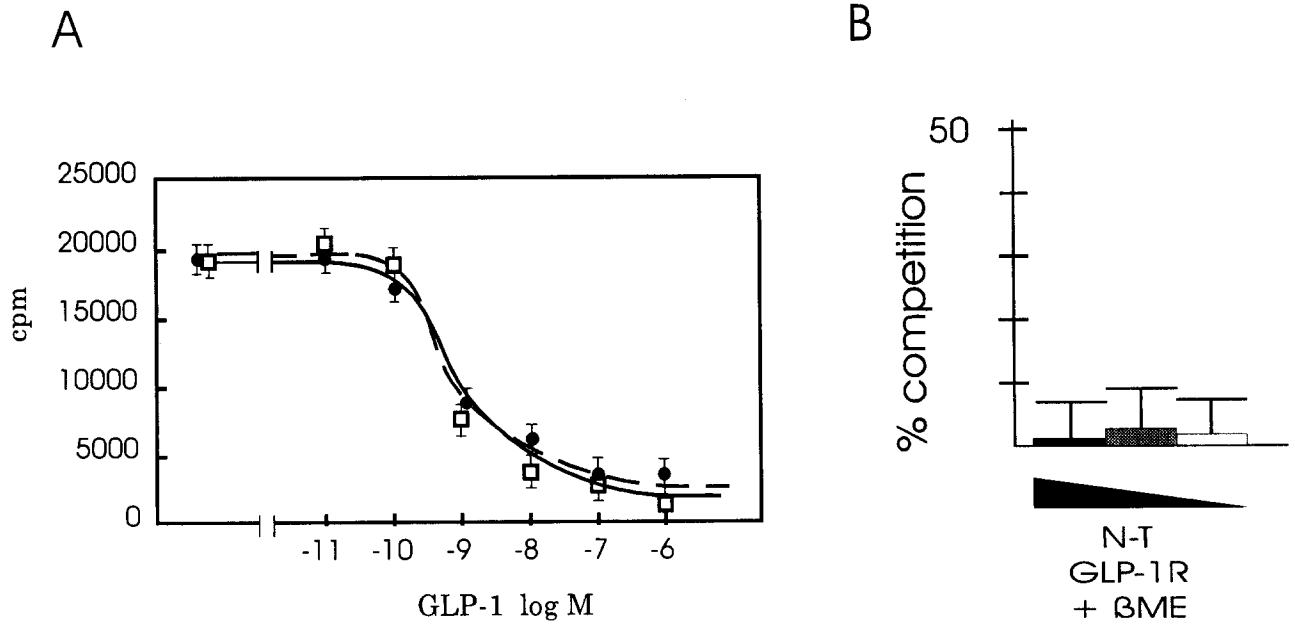

Fig. 3. (A) Displacement of ^{125}I-GLP-1(7–36) amide binding to stably transfected CHL cells by unlabeled GLP-1 treated (● – – ●) or not treated (□——□) with β-mercaptoethanol. Analysis of the binding data was performed using the PC program Prism (GraphPad, San Diego, USA). (Data are means ± S.E.M., (n = 2)). (B) N-T GLP-1R was treated with 10 mM β-mercaptoethanol before being added as competitor in a binding assay using CHL cells expressing the entire wild-type GLP-1 receptor and ^{125}I-GLP-1(7–36) amide as radiolabeled tracer. ▶ indicates 6.5×10^{-7}, 2.6×10^{-7} and 1.3×10^{-7} M protein.

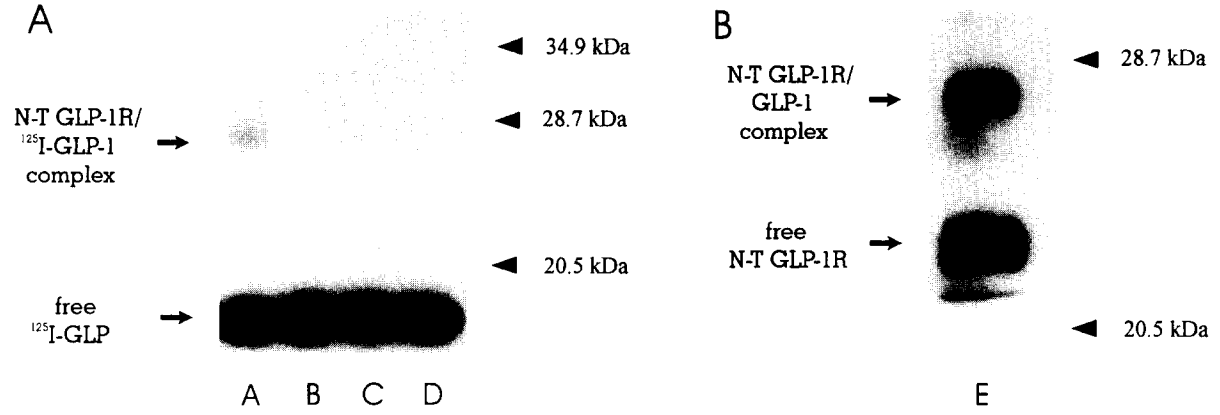

Fig. 4. (A) Cross-linking of ^{125}I-GLP-1(7–36) amide to the isolated, solubilized domain of the GLP-1 receptor. The isolated amino terminal domain was incubated in the absence (lane A) and presence (lane B) of 1 μM unlabeled GLP-1(7–36) amide. The complex was crosslinked using 0.1 mM DSS. In lane C and D N-T GIPR or lysozyme instead of the purified N-terminal part of the GLP-1 receptor was used. Samples were separated on SDS/12% PAGE. An autoradiogram of the dried gel is shown. (B) Western blot analysis of the cross-linked N-T GLP-1R/GLP-1(7–36) amide complex (lane E). The isolated N-terminal domain of the GLP-1 receptor was cross-linked to unlabeled GLP-1(7–36) amide. Proteins were separated on SDS/12% PAGE, the gel being blotted onto a nitrocellulose membrane and hybridized with an anti-GLP-1R antibody. Two bands are detectable representing the unbound (free N-T GLP-1R, 22 kDa) and the bound (N-T GLP-1R/GLP-1 complex, 28 kDa) N-terminal domain of the GLP-1 receptor. The arrows indicate the crosslinked complex and the arrow heads the mobilities of a low range protein standard (34.9 kDa = carbonic anhydrase, 28.7 kDa = soybean trypsin inhibitor, 20.5 kDa = lysozyme).

isolated N-terminal domain with β-mercaptoethanol led to a complete loss of its competition activity (Fig. 3B).

3.4. Specific interaction between the amino-terminal domain and GLP-1

In order to study the direct physical interaction between GLP-1 and the N-terminal domain of the GLP-1 receptor the latter was covalently cross-linked to ^{125}I-GLP-1 using DSS. After separation of the proteins by SDS PAGE, a single ligand-binding protein complex with an apparent molecular mass of 28 kDa was detected (Fig. 4A). In control experiments, a specific band was not found using either the extracellular part of the GIP receptor or lysozyme. In Western analysis employing anti-GLP-1 receptor antibody two bands with apparent molecular masses of 22 and 28 kDa, respectively, were detected representing the N-terminal domain in the presence and absence of bound ligand (Fig. 4B).

4. Discussion

Our data support the concept that the extracellular domain of peptide hormone receptors contains, at least in part, elements for recognizing and binding of corresponding ligands. Analysis of chimeras of various receptors supported this hypothesis [16–19]. For example, replacement of the amino-terminal domain of the VIP receptor by the homologous region from the secretin receptor and vice versa abolished high affinity binding [19]. With an alternative experimental approach we studied the putative binding affinity of isolated, solubilized N-terminal domain of the GLP-1 receptor.

The present results demonstrate for the first time not only competitive activity of the receptor N-terminus (Fig. 2) but also hint at a direct physical interaction between GLP-1 and the extracellular domain of the GLP-1 receptor. It seems that this part of the receptor is a major ligand recognition site. In support of our data, it has been reported that a truncated form of the PACAP receptor consisting only of the membrane-anchored extracellular domain showed some binding activity [24]. Both approaches suggest that the N-terminal receptor domain alone is already capable of ligand binding in this receptor subfamily, although with lower affinity than the entire receptor protein.

Incubation of N-T GLP-1R with β-mercaptoethanol led to complete loss of competition activity. This indicates that the correct formation of the tertiary structure of the N-terminal domain is a prerequisite for its ligand binding. This is supported by a recently published study using mutational analysis of the VIP receptor. Here, Gaudin et al. demonstrated that cysteine residues within the N-terminal domain are likely to be involved in the formation of intramolecular disulfide bonds maintaining the structural topology necessary for ligand binding [15].

The ligand binding ability of solubilized N-terminal receptor domains may be a physiologically important phenomenon. This has been shown earlier for members of the glycoprotein receptor family. Isolated receptor N-termini are here naturally generated by tissue-specific alternative splicing processes. An mRNA encoding the N-terminal domain of the TSH receptor was isolated by Graves et al. [25]. Tsai-Morris et al. [26] demonstrated that the solubilized form of the extracellular domain of the gonadotropin receptor exhibits binding activity similar to that of the entire receptor.

The extracellular domain of the GLP-1 receptor has a lower affinity for GLP-1 binding as the full length receptor. This indicates that additional elements of the receptor are involved to achieve high affinity ligand binding. In this context, it is of interest that for the PTH receptor Lee et al. [27] have shown that in addition to the N-terminal domain the third extracellular loop is also involved in PTH(1–34) binding.

In summary, the data presented here demonstrate that the isolated N-terminal domain of the GLP-1 receptor has the ability to interact directly with its ligand GLP-1.

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