Structural Determinants for Activity of Glucagon-like Peptide-2[†]

Mark P. DaCambra,[‡] Bernardo Yusta,[§,||] Martin Sumner-Smith,[⊥] Anna Crivici,[⊥] Daniel J. Drucker,[§,||,#] and Patricia L. Brubaker*[,‡,§,#]

Departments of Physiology and Medicine, University of Toronto, Toronto, Canada, M5S 1A8, NPS Allelix Inc., Mississauga, Ontario, Canada, L4V 1V7, and The Banting and Best Diabetes Centre, The Toronto General Hospital, University of Toronto, Toronto, Canada, M5G 2C4

Received March 3, 2000; Revised Manuscript Received May 16, 2000

ABSTRACT: Glucagon-like peptide-2 (GLP-2) is a 33 amino acid gastrointestinal hormone that regulates epithelial growth in the intestine. Dipeptidylpeptidase IV cleaves GLP-2 at the position 2 alanine, resulting in the inactivation of peptide activity. To understand the structural basis for GLP-2 action, we studied receptor binding and activation for 56 GLP-2 analogues with either position 2 substitutions or alanine replacements along the length of the peptide. The majority of position 2 substitutions exhibited normal to enhanced GLP-2 receptor (GLP-2R) binding; in contrast, position 2 substitutions were less well tolerated in studies of receptor activation as only Gly, Ile, Pro, α-aminobutyric acid, D-Ala, or nor-Val substitutions exhibited enhanced GLP-2R activation. In contrast, alanine replacement at positions 5,6,17, 20, 22, 23, 25, 26, 30, and 31 led to diminished GLP-2R binding. Position 2 substitutions containing Asp, Leu, Lys, Met, Phe, Trp, and Tyr, and Ala substitutions at positions 12 and 21 exhibited normal to enhanced GLP-2R binding but greater than 75% reduction in receptor activation. D-Ala^2, Pro^2 and Gly^2, Ala^{16} exhibited significantly lower EC_{50}s for receptor activation than the parent peptide ($p < 0.01-0.001$). Circular dichroism analysis indicated that the enhanced activity of these GLP-2 analogues was independent of the α-helical content of the peptide. These results indicate that single amino acid substitutions within GLP-2 can confer structural changes to the ligand−receptor interface, allowing the identification of residues important for GLP-2R binding and receptor activation.

Glucagon-like peptide-2 (GLP-2) is a 33 amino acid proglucagon-derived peptide (PGDP) secreted by the L cell of the intestinal epithelium (*1, 2*). GLP-2 augments intestinal hexose transport and reduces gastric emptying in rats and pigs, respectively, within 30−60 min following peptide infusion (*3, 4*). GLP-2 administration to rodents for several days produces expansion of the small bowel epithelium via stimulation of crypt cell proliferation and inhibition of villus enterocyte apoptosis (*5*−*7*). GLP-2 also prevents parenteral nutrition-associated intestinal hypoplasia (*8*), and augments the endogenous adaptive response to intestinal resection in rats following major small bowel resection (*9*). The reparative actions of GLP-2 have also been observed in models of intestinal inflammation in that a GLP-2 analogue, h[Gly2]-GLP-2, ameliorated epithelial injury in the small and large intestine of mice following induction of experimental enteritis in vivo (*10, 11*).

The diverse number of GLP-2 actions in the gastrointestinal tract remains poorly understood. A GLP-2 receptor (GLP-2R) has recently been cloned from hypothalamic and intestinal cDNA libraries and appears to be a new member of the glucagon/secretin 7-transmembrane, G-protein-coupled receptor (GPCR) superfamily (*12*). The GLP-2R is expressed in a highly tissue-specific manner, suggesting that transcriptional regulation of GLP-2R expression represents an important control mechanism for regulating the specificity of GLP-2 action. Consistent with the structure of the related glucagon, GLP-1, and secretin receptors, the GLP-2R contains a large extracellular amino terminus thought to be involved in ligand binding. Analysis of GLP-2R signaling in fibroblasts demonstrates coupling of the GLP-2R to activation of adenylyl cyclase and production of cAMP (*12, 13*). Although GLP-2 also activates AP-1-dependent signaling pathways, no stimulation of intracellular calcium influx was observed following activation of the rat GLP-2R in transfected BHK cells in vitro (*13*).

The amino acid sequence of GLP-1 is identical in mouse, rat, and human species (*14*−*16*). Although not as highly conserved as GLP-1, the GLP-2 amino acid sequence is also conserved throughout vertebrate evolution [(*17*) and Figure 1], with rat and human GLP-2 differing by only one amino acid (Thr^{19} in the rat versus Ala^{19} in human). Despite the emerging biological importance of GLP-2, little information

[†] This work was supported in part by grants from the Medical Research Council of Canada (to P.L.B. and D.J.D.) and NPS Allelix Inc. (to D.J.D.). D.J.D. is a Senior Scientist of the Medical Research Council of Canada, and a consultant to NPS Allelix Inc.

* Correspondence should be addressed to this author at Room 3366, Medical Sciences Building, University of Toronto, Toronto, Ontario, M5S 1A8, Canada. Tel. and Fax: (416) 978-2593. E-mail: p.brubaker@utoronto.ca.

[‡] Department of Physiology, University of Toronto.

[§] Department of Medicine, University of Toronto.

[||] The Banting and Best Diabetes Centre, The Toronto General Hospital.

[⊥] NPS Allelix Inc.

[#] These authors were equal contributors to this study.

DOCKET ALARM Find authenticated court documents without watermarks at docketalarm.com.

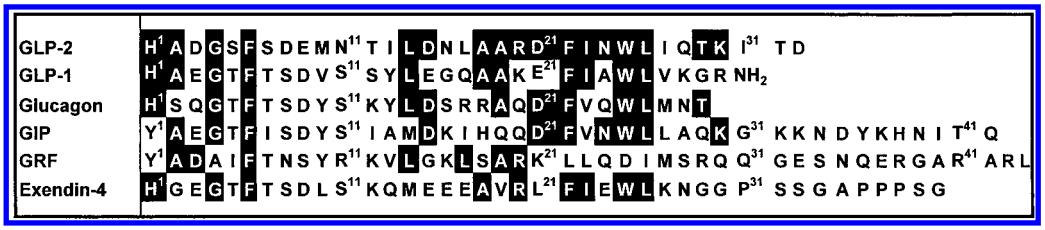

FIGURE 1: Sequences of hGLP-2^{1-33}, GLP-$1^{7-36NH2}$, glucagon, hGIP, and *Heloderma suspectum* exendin-4^{1-39}. Amino acids conserved between GLP-2 and the other glucagon-related peptides are shaded.

is available correlating GLP-2 structure with determinants of GLP-2 action. In studies analyzing the metabolism of circulating GLP-2, the first two amino acids (His^1-Ala^2) were found to be cleaved by the enzyme dipeptidylpeptidase IV (DP-IV), rendering the peptide biologically inactive (*18*, *19*). Consistent with these findings, an Ala^2Gly substitution in GLP-2 renders the peptide DP IV-resistant, and enhances the biological effectiveness of GLP-2 in vivo (*18*).

Structure−function analyses of glucagon-like peptide action have identified specific residues critical for receptor binding and signal transduction. For example, His^1, Asp^9, and Ser^{16} have been defined as a catalytic triad in glucagon (*20*−*23*), while His^1, Phe^6, and Phe^{22}-Ile^{23} appear to be important for the biological action of GLP-1 (*24*−*26*) (Figure 1). To understand the specific structural determinants important for GLP-2 binding and receptor activation, we have now carried out an analysis of position 2 analogues and an alanine substitution scan of the GLP-2 molecule.

EXPERIMENTAL PROCEDURES

Peptides. Wild-type rat GLP-2^{1-33} (rGLP-2) and human (h) [Gly^2]GLP-2 (American Peptide Co. Inc., Sunnyvale, CA) were used as control peptides. Exendin-4, glucagon, GLP-1, and GIP were from Bachem California Inc. (Torrance, CA); 26 hGLP-2 analogues, each with a different amino acid or amino acid derivative substituted for Ala^2, and 31 h[Gly^2]-GLP-2 analogues, each with an $Xxx^n \rightarrow Ala^n$ mutation, were synthesized by Chiron Technologies Inc. (Raleigh, NC). The peptide synthesis was performed on Chiron Mimitopes proprietary Multiple Peptide Synthesis grafted HEMA crowns. Free Fmoc-protected α-amino acids were in situ activated with DIC/HOBt using DMF as the solvent. Fmoc deprotection was completed after each coupling cycle using 20% v/v piperidine in DMF. Side chain deprotection and cleavage were carried out using a TFA/scavenger mixture. Side chain deprotection byproducts were removed using an ether/petroleum ether/methanol solvent mixture.

Synthetic peptides from Chiron were purified using RP-HPLC and a C-18 preparative HPLC column with an acetonitrile gradient in 1% TFA/distilled water. Quality control analysis was carried out for each peptide using both analytical HPLC and mass spectrometric analysis. Peptide purity ranged from 97 to 100%, with a mean purity of 99%. A repeat quantitative peptide amino acid analysis was also carried out using an aliquot from the actual peptide stock used in each experiment, and predicted peptide concentrations were corrected for actual peptide content for all full dose−response assays. The stability and demonstrated DP-IV resistance of [Gly^2]GLP-2 led to its selection as the base

^{125}I-Labeled GLP-2. ^{125}I (2×10^8 Bq in NaOH; Amersham Life Science, Oakville, ON, Canada) was used to iodinate rGLP-2 on His^1 (*27*). Tracer was purified by passage through a SepPak cartridge of C18 silica (Waters Associates, Milford, MA) to a specific activity of approximately 21 TBq/mmol.

GLP-2R Binding Assay. Analysis of receptor binding was carried out using a membrane fraction prepared from BHK cells stably transfected with the rat GLP-2 receptor (rGLP-2R-BHK) (*13*). The cells were washed with PBS, and harvested in the same buffer used for membrane preparation. Ten milliliters of incubation buffer (25 mM Hepes, 140 mM NaCl, 0.9 mM $MgCl_2$, 5 mM KCl, 1.8 mM $CaCl_2$, pH 7.4, 17 mg/L Diprotin A, and 100 μM phenanthroline) was added to approximately 10×10^7 cells and homogenized with a Polytron tissue homogenizer for 15 s. The homogenate was centrifuged at 1000*g* for 10 min at 4 °C, and the supernatant (membrane-enriched fraction) was aliquoted and stored at −70 °C until required. The protein concentration was determined by the method of Lowry (*28*).

Polypropylene tubes (1.5 mL) were used for the receptor binding assay, each containing peptides dissolved in 50 μL of incubation buffer. Tracer (101 kBq/50 μL of buffer) and 25 μg of freshly thawed membrane protein were gently mixed in 400 μL of incubation buffer, and the tubes were incubated with shaking for 2 h at 4 °C. The reaction was terminated by centrifugation at 13000*g* at 4 °C for 15 min. The membrane pellets were washed 3 times with 1.5 mL of cold 50 mM Tris buffer, pH 7.4, and the amount of bound radioactivity was ascertained in the pellet fraction. Total binding was defined as the amount of ^{125}I-GLP-2 bound to the membranes in the absence of nonradiolabeled GLP-2 and was approximately 1.3% of total ^{125}I-GLP-2 added per tube. Nonspecific binding (NSB) was defined as the amount of ^{125}I-GLP-2 binding to the membranes in the presence of 1 μM nonradiolabeled GLP-2 and was consistently 0.1% of total binding. The percent ^{125}I-GLP-2 specific binding for any given dose of unlabeled GLP-2 or GLP-2 analogue was therefore determined as: $100 \times$ [cpm bound in the presence of unlabeled peptide − NSB cpm]/[total cpm − NSB cpm].

cAMP Determination. Levels of cAMP were determined using rGLP-2R-BHK cells in 24 well plates (*13*). Peptides were diluted in 300 μL of DMEM containing 100 μM 3-isobutyl-1-methylxanthine (IBMX; Sigma Chemical Co., St. Louis, MO), and incubated with the cells for 10 min at 37 °C. Control incubations (DMEM with IBMX in the absence of peptides) were carried out for each experiment. The reaction was stopped by the addition of 1 mL of absolute ethanol at −20 °C. Cells were then homogenized, and cAMP levels determined in dried aliquots using a cAMP radioim-

DOCKET ALARM Find authenticated court documents without watermarks at docketalarm.com.

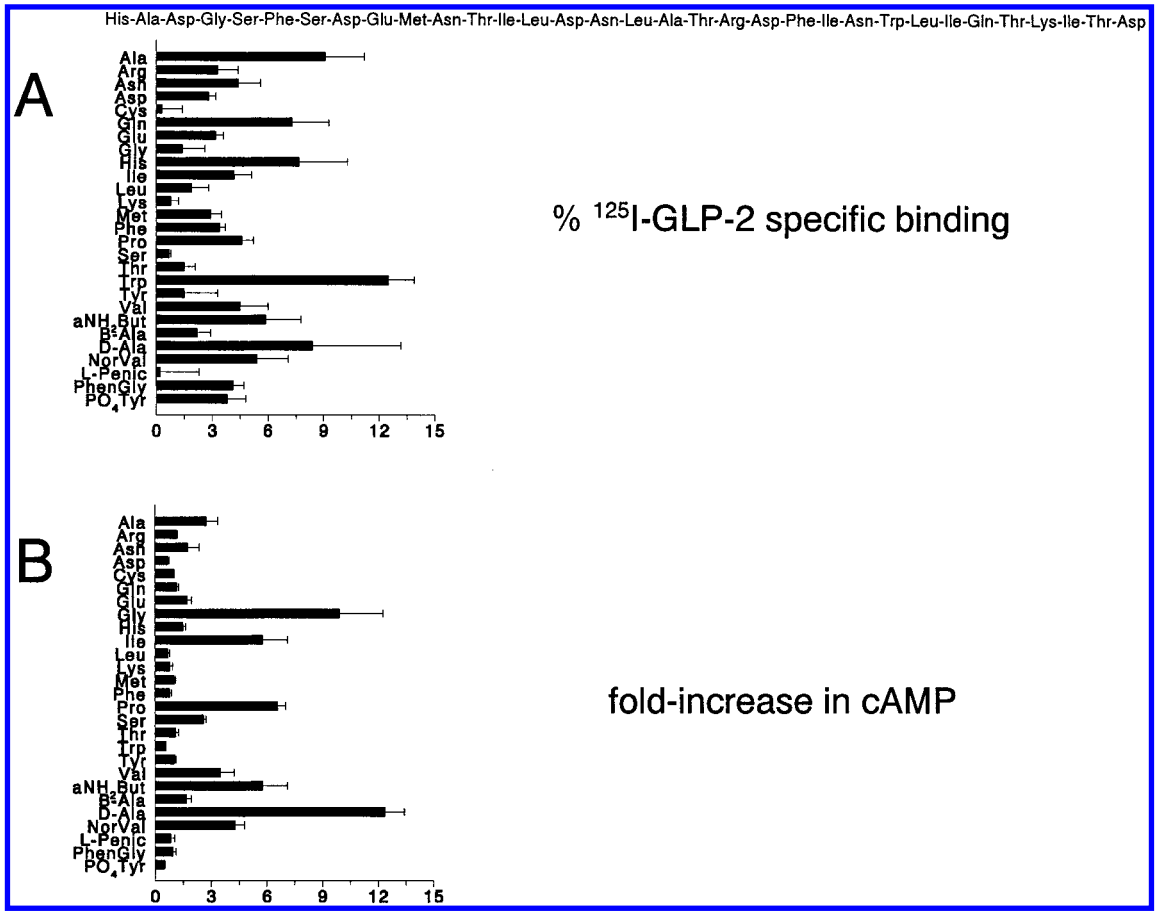

FIGURE 2: GLP-2R binding (A: as a percent of total specific binding) and activation (B: as the fold increase over basal cAMP production) by position 2 analogues of GLP-2. Each analogue was tested in triplicate at 100, 500, and 1000 pM, and the data for the 1000 pM dose are shown. The rGLP-2 sequence is shown at the top of the figure.

Circular Dichroism Spectroscopy. Peptides were dissolved in water to a final concentration of 35 μM, and the UV spectra between 180 and 260 nm were determined in a Molecular Dichroism Spectrometer (Aviv, Lakewood, NJ) at room temperature using a 0.1 cm cell path length. Each peptide was scanned 5 times, and the absorption spectra were averaged. The molar ellipticity was determined as [the absorption at 222 nm $\times$ 100]/[nM peptide $\times$ the number of amino acids in the peptide]. The percent helicity was then calculated as 100 $\times$ (the molar ellipticity/34 000), where 34 000 represents the molar ellipticity of a peptide with 100% α-helicity.

Data Analysis. Screening assays were performed in triplicate, while full dose−response curves were carried out in duplicate or triplicate in each of 3−4 different experiments. Data are expressed as mean ± SEM. Half-maximal inhibition of GLP-2 binding (inhibitory concentration or IC_{50}), and the half-maximal effective concentration (EC_{50}) and the maximum effect (E_{max}) for stimulation of cAMP were calculated using GraphPad Prism 2.00 Software (GraphPad Software Inc., San Diego, CA). All peptides tested exhibited two-site competition binding, and it was assumed for the purposes of the IC_{50} calculations that both sites express equal probability of ligand binding. Because of difficulties in accurately assessing the high-affinity IC_{50} for individual curves, only the mean for the averaged data is reported. Statistical differences between groups were assessed by

RESULTS

For analysis of the properties of the position 2 and alanine scanning mutants, we utilized a previously described stable BHK fibroblast cell line transfected with the rat GLP-2 receptor (*13*). Characterization of the specificity of GLP-2R binding and receptor activation demonstrated that the cloned rat GLP-2R responds specifically to nanomolar concentrations of GLP-2, but not to equivalent concentrations of related members of the glucagon peptide superfamily (*12, 13*), including GLP-$1^{7-36NH2}$, glucagon, GIP, and exendin (data not shown). Following observations that the amino acid at position 2 was a key determinant of GLP-2 degradation and bioactivity in vivo (*18, 19*), we initially analyzed the receptor binding and signaling properties of a series of position 2-substituted GLP-2 molecules. The 26 analogues with Ala^2 substitutions included all physiologic amino acids as well as L-pencillamine, β-alanine, D-alanine, phosphotyrosine, α-aminobutyric acid, norvaline, and phenylglycine. The majority of position 2 substitutions exhibited normal to enhanced GLP-2 receptor binding, as illustrated by their ability to displace specific binding of ^{125}I-GLP-2 (Figure 2A). In contrast, position 2 substitutions were less well tolerated in studies of receptor activation. Only the natural amino acids Gly, Ile, or Pro or the synthetic amino acids α-aminobutyric acid, D-Ala, or nor-Val at position 2 exhibited enhanced GLP-2R activation in BHK-GLP-2R cells (Figure 2B). Hence,

DOCKET ALARM Find authenticated court documents without watermarks at docketalarm.com.

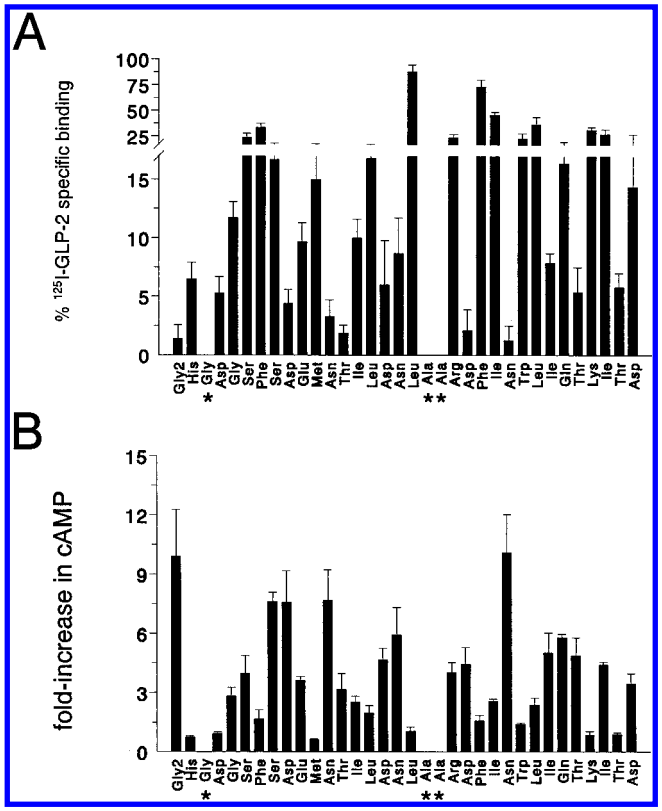

FIGURE 3: rGLP-2R binding (A: as a percent of total specific binding) and activation (B: as the fold increase over basal cAMP production) by alanine-substituted analogues of GLP-2. Each analogue was tested in triplicate at 100, 500, and 1000 pM, and the data for the 1000 pM dose are shown. An asterisk indicates that Gly^2 and the native alanine at positions 18 and 19 were neither substituted nor tested.

cAMP generation is clearly sensitive to the specific residue present at this position of the GLP-2 molecule.

For the alanine scan, Ala was substituted for the native amino acid at all positions in h[Gly^2]GLP-2 except for Gly^2 and the Ala residues already at positions 18 and 19. In contrast to the modest effects of varying position 2 amino acids on GLP-2 binding, alanine replacement at positions 5,6,17, 20, 22, 23, 25, 26, 30, and 31 led to diminished GLP-2R binding (Figure 3A). Several alanine-substituted molecules, notably Thr^{12}Ala and Asp^{21}Ala, exhibited normal GLP-2R binding yet markedly reduced generation of cAMP (Figure 3B). Furthermore, His^1Ala and Asp^3Ala exhibited markedly reduced receptor activation despite only modest changes in receptor binding, further establishing the importance of the GLP-2 amino terminus for coupling of the GLP-2R to adenylyl cyclase-dependent signal transduction.

Several analogues were observed to exhibit normal to enhanced receptor binding and/or cAMP generating ability, including h[Gly^2]GLP-2, h[D-Ala^2], and h[Pro^2]-GLP-2 (Figure 2). A full analysis of GLP-2R binding and cAMP generation for these peptides over a broad range of concentrations is shown in Figure 4 and Table 1. rGLP-2 displayed a two-site binding curve, with 50% inhibition of binding at 2.2 pM (high affinity; IC_{50}-1) and 49.2 ± 10.5 nM (low affinity; IC_{50}-2). Receptor activation by rGLP-2, as assessed by cAMP generation, exhibited an EC_{50} of 14.0 ± 2.9 nM and an E_{max} of 28.4 ± 2.2 pmol of cAMP/mg of protein. values of 4.8 pM and 42.9 ± 5.6 nM, respectively, and an EC_{50} and E_{max} of 9.2 ± 0.6 nM and 27.2 ± 2.8 pmol of cAMP/mg of protein, respectively. While the high- and low-affinity binding of h[D-Ala^2]GLP-2 was not different from that of rGLP-2, the IC_{50}-2 for h[Pro^2]GLP-2 was 5-fold higher than that of both rGLP-2. In contrast, h[Pro^2]GLP-2 values to activate the rGLP-2R were 2−3-fold-enhanced compared to rGLP-2 ($p < 0.01$−0.001; Figure 4 and Table 1).

In comparison with h[Gly^2]GLP-2, the base peptide used for the Ala scanning studies, both h[Gly^2,Ala^5]GLP-2 and h[Gly^2,Ala^{16}]GLP-2 were found to exhibit similar binding characteristics (Figure 4 and Table 1). Although the E_{max} was slightly reduced for h[Gly^2,Ala^5]GLP-2 in comparison with the base peptide ($P < 0.01$), the EC_{50} for h[Gly^2,Ala^{16}]-GLP-2 was more than 5-fold lower than that of h[Gly^2]GLP-2 ($P < 0.001$; Figure 4 and Table 1).

To establish whether the biological activities of the various GLP-2 analogues were affected by the α-helicity of the peptides, circular dichroism (CD) scanning was performed on rGLP-2 as well as the five analogues studied in detail. Two additional peptides which exhibited markedly reduced abilities to activate the rGLP-2R in the scanning assays, h[Thr^2]GLP-2 and h[Gly^2,Ala^6]GLP-2, were also analyzed for comparative purposes (Figure 5). rGLP-2 exhibited a far-UV CD spectrum consistent with a helical content of approximately 11%. Although similar degrees of α-helicity were observed for h[D-Ala^2]GLP-2, h[Pro^2]GLP-2, h[Gly^2,-Ala^5]GLP-2, h[Gly^2,Ala^6]GLP-2, and h[Gly^2,Ala^{16}]GLP-2 (13−15%), h[Gly^2]GLP-2 and h[Thr^2]GLP-2 exhibited marked increases in α-helicity (18 and 24%, respectively). Thus, the α-helical content of GLP-2 and its analogues did not appear to correlate with their ability to bind and/or activate the rGLP-2R.

DISCUSSION

GLP-2 has been shown to increase intestinal mass and enhance intestinal adaptation in normal rodents as well as in a number of pathophysiological models of intestinal resection and/or inflammation (*29*). Understanding the structural determinants of GLP-2 function may provide useful information for the design of more potent GLP-2 analogues or GLP-2 antagonists. The recent cloning of the GLP-2R (*12*) has therefore provided an opportunity to determine the structure/function relationships of GLP-2 molecules in vitro.

Initial analyses of the rGLP-2R in stably expressed BHK cells demonstrated a high degree of specificity for GLP-2 compared to other structurally related peptides, consistent with the findings of GLP-2R specificity using transfected COS or EBNA cells (*12*). The related peptides glucagon and GIP bound the rGLP-2R very poorly and failed to activate cAMP biosynthesis, while no physiologically significant binding of GLP-1 or the lizard GLP-1 analogue exendin-4 was detected [(*12*) and our study]. Furthermore, carboxy-terminal extensions to the GLP-2 molecule generally retained the ability to bind and activate the GLP-2R, whereas amino-terminal truncations exhibited significant reductions in binding and GLP-2R activation (*12*).

In studies utilizing the rat GLP-2R in transfected 293-EBNA cells, Munroe et al. found IC_{50}-1 (60 pM) and -2 (259

DOCKET ALARM Find authenticated court documents without watermarks at docketalarm.com.

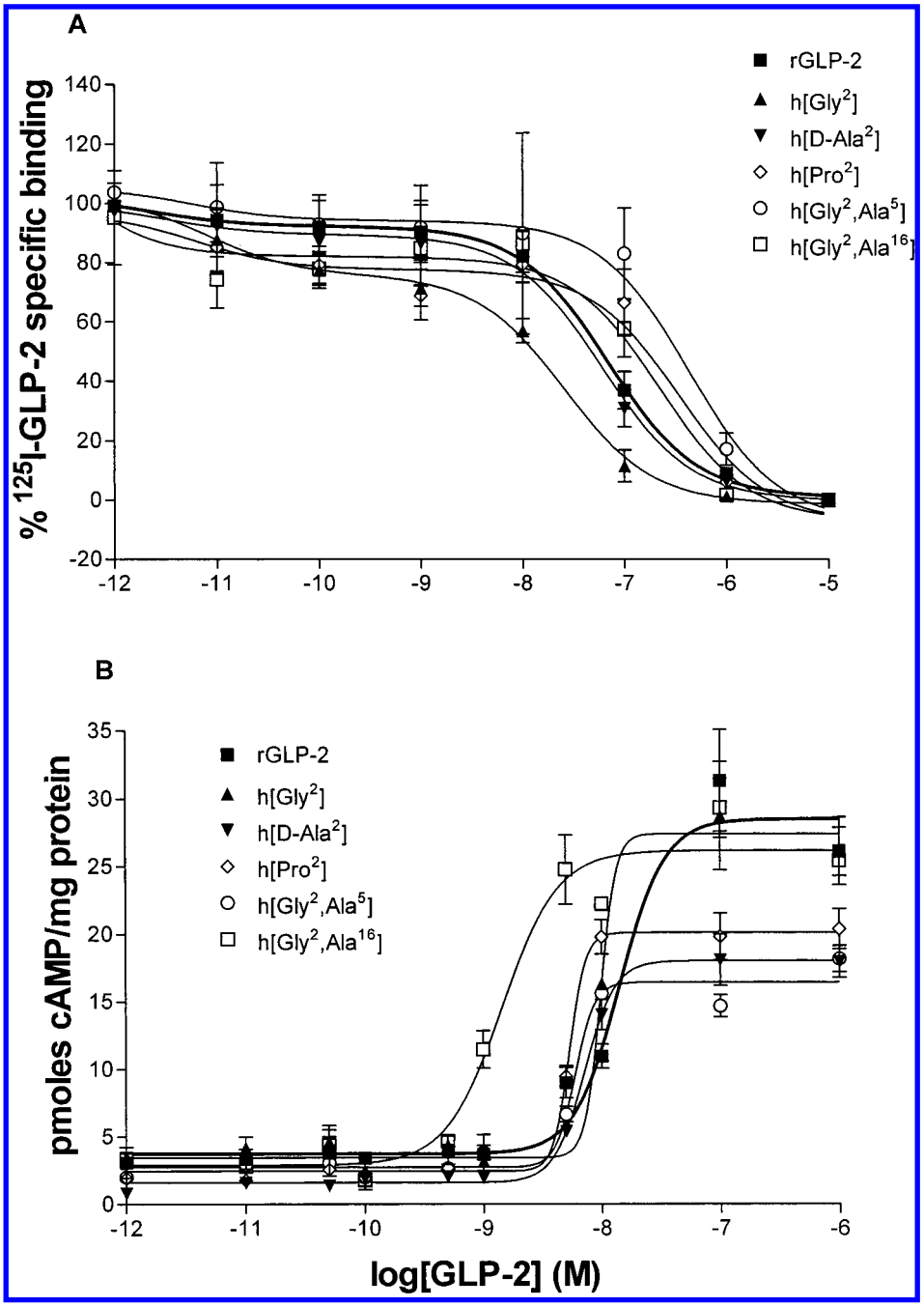

FIGURE 4: rGLP-2R binding (A) and activation (B) by rGLP-2, h[Gly^2]GLP-2, h[D-Ala^2]GLP-2, h[Pro^2]GLP-2, h[Gly^2,Ala^5]GLP-2, and h[Gly^2,Ala^{16}]GLP-2 ($n = 4-5$). Membranes for binding studies were prepared from BHK cells stably transfected with the rGLP-2R, while cAMP were levels were determined in rGLP-2-BHK cells in culture.

cells (*12*). Similarly, rGLP-2 was ~40-fold less potent in stimulating cAMP in COS cells. In contrast, the EC_{50} for rGLP-2 was ~0.6 nM in stably transfected 3G2R cells (*12*) and ~0.06 nM for both rGLP-2R and h[Gly2]-GLP-2 in studies with BHK-GLP-2R cells (*13*). Consistent with the results of our previous studies using BHK-GLP-2R cells (*13*), we did not observe significant differences between h[Gly^2]-GLP-2 and rGLP-2 in their abilities to bind and activate the rGLP-2R. Hence, the minor differences in receptor binding and activation that have been reported to date may be attributable to potential differences in the different cell lines utilized for studies of GLP-2R signaling.

determinant of GLP-2 biological activity in vivo. Substitution of the position 2 alanine with a glycine conferred resistance to degradation and enhanced biological potency in vivo (*18*, *19*, *30*). The related peptides GIP and GLP-1 are also substrates for DP-IV-mediated cleavage, and modifications to the amino terminus of these peptides confer enhanced resistance to inactivation and substantially greater bioactivity in vivo (*31*−*33*). Analysis of GLP-2 molecules with position 2 substitutions identified several amino acid substitutions that resulted in enhanced biological activity compared to the native peptide, including proline and D-alanine. Given the short incubation time period involved in assessment of

DOCKET ALARM Find authenticated court documents without watermarks at docketalarm.com.

DOCKET ALARM

Explore Litigation Insights

Docket Alarm provides insights to develop a more informed litigation strategy and the peace of mind of knowing you're on top of things.

Real-Time Litigation Alerts

Keep your litigation team up-to-date with **real-time alerts** and advanced team management tools built for the enterprise, all while greatly reducing PACER spend.

Our comprehensive service means we can handle Federal, State, and Administrative courts across the country.

Advanced Docket Research

With over 230 million records, Docket Alarm's cloud-native docket research platform finds what other services can't. Coverage includes Federal, State, plus PTAB, TTAB, ITC and NLRB decisions, all in one place.

Identify arguments that have been successful in the past with full text, pinpoint searching. Link to case law cited within any court document via Fastcase.

Analytics At Your Fingertips

Learn what happened the last time a particular judge, opposing counsel or company faced cases similar to yours.

Advanced out-of-the-box PTAB and TTAB analytics are always at your fingertips.

API

Docket Alarm offers a powerful API (application programming interface) to developers that want to integrate case filings into their apps.

LAW FIRMS
Build custom dashboards for your attorneys and clients with live data direct from the court.

Automate many repetitive legal tasks like conflict checks, document management, and marketing.

FINANCIAL INSTITUTIONS
Litigation and bankruptcy checks for companies and debtors.

E-DISCOVERY AND LEGAL VENDORS
Sync your system to PACER to automate legal marketing.