# Biological Activities of Glucagon-Like Peptide-1 Analogues in Vitro and in Vivo

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ABSTRACT: Studies support a role for glucagon-like peptide 1 (GLP-1) as a potential treatment for diabetes. However, since GLP-1 is rapidly degraded in the circulation by cleavage at $Ala_2$, its clinical application is limited. Hence, understanding the structure−activity of GLP-1 may lead to the development of more stable and potent analogues. In this study, we investigated GLP-1 analogues including those with N-, C-, and midchain modifications and a series of secretin-class chimeric peptides. Peptides were analyzed in CHO cells expressing the hGLP-1 receptor (R7 cells), and in vivo oral glucose tolerance tests (OGTTs) were performed after injection of the peptides in normal and diabetic (*db*/*db*) mice. [D-$Ala_2$]GLP-1 and [$Gly_2$]GLP-1 showed normal or relatively lower receptor binding and cAMP activation but exerted markedly enhanced abilities to reduce the glycemic response to an OGTT in vivo. Improved biological effectiveness of [D-$Ala_2$]GLP-1 was also observed in diabetic *db*/*db* mice. Similarly, improved biological activity of acetyl- and hexenoic-$His_1$-GLP-1, glucagon$_{(1-5)}$-, glucagon$_{(1-10)}$-, PACAP$_{(1-5)}$-, VIP$_{(1-5)}$-, and secretin$_{(1-10)}$-GLP-1 was observed, despite normal or lower receptor binding and activation in vitro. [$Ala_{8/11/12/16}$] substitutions also increased biological activity in vivo over wtGLP-1, while C-terminal truncation of 4−12 amino acids abolished receptor binding and biological activity. All other modified peptides examined showed normal or decreased activity in vitro and in vivo. These results indicate that specific N- and midchain modifications to GLP-1 can increase its potency in vivo. Specifically, linkage of acyl-chains to the α-amino group of $His_1$ and replacement of $Ala_2$ result in significantly increased biological effects of GLP-1 in vivo, likely due to decreased degradation rather than enhanced receptor interactions. Replacement of certain residues in the midchain of GLP-1 also augment biological activity.

Glucagon-like peptide 1 (GLP-1) is an important glucoincretin hormone that is secreted from intestinal L cells in response to nutrient ingestion. The biologically active forms of GLP-1, [GLP-1$_{(7-36)\ amide}$ and GLP-1$_{(7-37)}$], have been demonstrated to possess multiple functions including enhancement of glucose-dependent insulin secretion, stimulation of proinsulin gene expression, and suppression of glucagon secretion and gastric emptying (reviewed in refs *1* and *2*). Additional actions of GLP-1 may include enhancement of insulin sensitivity, induction of β cell differentiation and proliferation, and the suppression of feeding (*3*, *4*). Several reports indicate that GLP-1 not only enhances insulin secretion in normal subjects but also in those with Type 2 diabetes mellitus (DM). These studies suggest that GLP-1 receptor agonists may have great clinical potential for the treatment of patients with Type 2 DM (*5*, *6*).

GLP-1 signal transduction has been studied in β cells and cell lines extensively (*2*). The β cell has specific, high affinity receptors for GLP-1 and upon interaction with its receptor this peptide increases cAMP levels in several β cell models (reviewed in ref *1*). There is a general consensus that through the second messenger cAMP, GLP-1 can also increase intracellular $Ca^{2+}$ to ultimately trigger insulin secretion (reviewed in refs *1*, *7*, and *8*). However, the intracellular mechanism of GLP-1 leading to increases in $Ca^{2+}$ and insulin exocytosis are likely multifaceted and are not completely understood. Several reports suggest that GLP-1, through PKA, acts at the level of the ATP-sensitive $K^+$ channel ($K_{ATP}$) facilitating channel inactivation, cell depolarization, and the activation of voltage-dependent $Ca^{2+}$ channels promoting $Ca^{2+}$ influx (*9*−*11*; reviewed in ref *1*). We and others have also demonstrated potential GLP-1 targets in the β cell distal to $K_{ATP}$, including the L-type voltage-dependent $Ca^{2+}$ channel (*12*), nonselective cation channels (NSCC) (*13*), intracellular $Ca^{2+}$ stores (*14*), chloride channels (*15*), and the exocytotic machinery itself (*16*). It is likely that the insulinotropic activity of GLP-1 is translated through actions on several of these targets, with a common linkage to PKA.

Although multiple immunoreactive forms of GLP-1 are present in vivo, the major form of circulating and biologically active GLP-1 is GLP-1$_{(7-36)amide}$. To allow for a more direct structural comparison with GLP-2, glucagon, and other secretin family peptides and to provide clarity, we have adopted a modified 30-amino acid numbering system for GLP-1 throughout this paper [e.g., GLP-1$_{(1-30)amide}$] (Table 1). In vivo, GLP-1 is rapidly inactivated by degradation to

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[1] Abbreviations: glucagon-like peptide 1 (GLP-1), dipeptidyl-peptidase IV (DP IV), neutral endopeptidases (NEPs).

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Table 1: Structures (aa 1−18) of GLP-1 and Analogues

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| WT GLP-1 | H | A | E | G | T | F | T | S | D | V | S | S | Y | L | E | G | Q | A |
| [Phe1]GLP-1 | F | A | E | G | T | F | T | S | D | V | S | S | Y | L | E | G | Q | A |
| [Tyr1]GLP-1 | Y | A | E | G | T | F | T | S | D | V | S | S | Y | L | E | G | Q | A |
| Acetyl-GLP-1 | ac H | A | E | G | T | F | T | S | D | V | S | S | Y | L | E | G | Q | A |
| Hexenoic-GLP-1 | he H | A | E | G | T | F | T | S | D | V | S | S | Y | L | E | G | Q | A |
| [dAla2]GLP-1 | H | dA | E | G | T | F | T | S | D | V | S | S | Y | L | E | G | Q | A |
| [Leu2]GLP-1 | H | L | E | G | T | F | T | S | D | V | S | S | Y | L | E | G | Q | A |
| [Gly2]GLP-1 | H | G | E | G | T | F | T | S | D | V | S | S | Y | L | E | G | Q | A |
| [Asp3]GLP-1 | H | A | D | G | T | F | T | S | D | V | S | S | Y | L | E | G | Q | A |
| [Ala3]GLP-1 | H | A | A | G | T | F | T | S | D | V | S | S | Y | L | E | G | Q | A |
| [Ala4]GLP-1 | H | A | E | A | T | F | T | S | D | V | S | S | Y | L | E | G | Q | A |
| Hexenoic-[dAla2]GLP-1 | he H | dA | E | G | T | F | T | S | D | V | S | S | Y | L | E | G | Q | A |
| Glucagon(1-5)GLP-1 | H | S | Q | G | T | F | T | S | D | V | S | S | Y | L | E | G | Q | A |
| Glucagon(1-10)GLP-1 | H | S | Q | G | T | F | T | S | D | Y | S | S | Y | L | E | G | Q | A |
| GIP(1-10)GLP-1 | Y | A | E | G | T | F | I | S | D | Y | S | S | Y | L | E | G | Q | A |
| PACAP(1-5)GLP-1 | H | S | D | G | I | F | T | S | D | V | S | S | Y | L | E | G | Q | A |
| PACAP(1-10)GLP-1 | H | S | D | G | I | F | T | D | S | Y | S | S | Y | L | E | G | Q | A |
| PHI(1-5)GLP-1 | H | A | D | G | V | F | T | S | D | V | S | S | Y | L | E | G | Q | A |
| PHI(1-10)GLP-1 | H | A | D | G | V | F | T | S | D | F | S | S | Y | L | E | G | Q | A |
| Secretin(1-10)GLP-1 | H | S | D | G | T | F | T | S | E | L | S | S | Y | L | E | G | Q | A |
| VIP(1-5)GLP-1 | H | S | D | A | V | F | T | S | D | V | S | S | Y | L | E | G | Q | A |
| VIP(1-10)GLP-1 | H | S | D | A | V | F | T | D | N | Y | S | S | Y | L | E | G | Q | A |
| [Ala12/16]GLP-1 | H | A | E | G | T | F | T | S | D | V | S | A | Y | L | E | A | Q | A |
| [Ala8/11/16]GLP-1 | H | A | E | G | T | F | T | A | D | V | A | S | Y | L | E | A | Q | A |
| [Ala8/12/16]GLP-1 | H | A | E | G | T | F | T | A | D | V | S | A | Y | L | E | A | Q | A |
| [Ala11/12/16]GLP-1 | H | A | E | G | T | F | T | S | D | V | A | A | Y | L | E | A | Q | A |
| [Ala8/11/12/16]GLP-1 | H | A | E | G | T | F | T | A | D | V | A | A | Y | L | E | A | Q | A |

$GLP\text{-}1_{(3-30)amide}$ (reviewed in ref *17*). A number of experiments have shown that dipeptidyl-peptidase IV (DP IV), widely present in the brush border epithelium and the capillaries of the *lamina propria*, as well as in the circulation, is the predominant enzyme that cleaves N-terminal GLP-1 between $Ala_2$ and $Glu_3$ (*18*, *19*). The GLP-1 molecule is also potentially susceptible to degradation by neutral endopeptidases, including NEP 24.11 (*20*), which has been shown to render biologically inactive cleavage products. The feasibility of designing protease-resistant, and thus, more stable GLP-1 analogues, has been realized, as a D-$Ala_2$-GLP-1 analogue is resistant to DP IV cleavage and has improved metabolic stability in vitro (*21*). A similar modification ($Gly_2$) to the structurally related intestinotropic peptide, GLP-2, not only renders that molecule resistant to DP IV cleavage but also improves biological activity in vivo (*22*, *23*). These studies suggest that targeted modifications of the GLP-1 molecule may produce a more efficient peptide that can be used to treat patients with Type 2 DM.

In the present study, we have designed a series of GLP-1 analogues, including several novel chimeric peptides, and evaluated their ability to bind and activate the human GLP-1 receptor in vitro and to reduce the glycemic response to an oral glucose challenge in vivo. Results indicate that several modifications to GLP-1 can improve its potency in vivo without improving in vitro receptor binding or activation. Specifically, modifications to $His_1$ and $Ala_2$ of GLP-1 resulted in significantly increased bioactivity, likely due to decreased degradation by DP IV. These analogues were more effective in both normal and diabetic mice (*db*/*db*). Replacement of certain residues in the middle chain of GLP-1 also enhanced its biological activity, while truncation to the C-terminus resulted in dramatically reduced receptor binding and biological activity.

## EXPERIMENTAL PROCEDURES

*Peptide Synthesis.* Wild-type (wt) GLP-1 and peptide analogues were synthesized using the solid-phase method (*24*) on a Rink amide resin (*25*), as previously described (*26*, *27*). In brief, couplings were effected with benzotriazole-1-yl-oxy-tris-(dimethylamnio)-phosphonium hexafluorophosphate (BOP) in the presence of diisopropylethylamine in dimethylflormamide (DMF). The completed peptides were simultaneously cleaved from the resin and fully deprotected with trifluoroacetic acid (TFA) in a mixture of triisopropylsilane, ethanedithiol, and thioanisole as scavenger at room temperature for 60 min. The crude peptides were purified by reversed-phase high performance liquid chromatography and subsequently analyzed by FAB mass spectrometry and amino acid analysis. Peptide purity was routinely >95%, as previously described (*26*). All peptides were lyophilized and stored at −20 °C until use.

*Cell Culture.* Chinese hamster ovary (CHO) cells stably expressing the recombinant human GLP-1 receptor (termed R7 cells) (*28*) were generated via selection with G418. Cells cultured in DMEM containing 10% FBS were used for less than 10 passages and examined frequently for functional expression via ligand binding using wt GLP-1.

*Iodination of GLP-1 and Ligand Binding Assay.* Synthetic wt human GLP-1 (Bachem California Inc, Torrance, CA) was radiolabeled with carrier-free $^{125}I$ using the Chloramine-T method, as described previously (*29*). The iodination reaction mixture was purified by reversed-phase adsorption to a C-18 Sep-Pak cartridge (Waters Associates, Milford, MA). Binding assays were performed as previously described (*30*). Briefly, on the day of assay, CHO/GLP-1R (R7) cells were washed twice in PBS and recovered from the plates with 2 mM EDTA in PBS. Cells ($1 \times 10^6$) were incubated for 60 min at 37 °C in binding assay buffer [DMEM containing 1% BSA

and 0.1% Trasylol (Bayer, Toronto, ON)] with $^{125}$I-GLP-1 and unlabeled GLP-1 or peptide analogues at concentration of $10^{-12}$ to $10^{-6}$ M, in a final volume of 200 $\mu$L. Cell suspensions were centrifuged at 14000*g*, and then cell pellets were washed with cold PBS. The radioactivity contained in the cell pellets was counted on a $\gamma$-counter, and binding analyses were performed as described by Irwin et al (*29*).

*cAMP Radioimmunoassay (RIA).* cAMP assays were performed as previously described (*30*). Briefly, cells were passaged into 12-well plates 1 day before the assay and then washed with PBS, followed by assay buffer (DMEM containing 1% BSA), and preincubated at 37 °C for 30 min. Cells were stimulated with GLP-1 or peptide analogues in the presence of 3-isobutyl-1-methylxanthine (IBMX, 1 $\mu$M) for 30 min. The cAMP was extracted with 80% ethanol and stored at −20 °C until RIA. Aliquot of the extracts were dried under vacuum and reconstituted with cAMP buffer for RIA (Biomedical Technologies, Stoughton, MA) (*29*).

*In Vivo Experiments.* The in vivo biological activity of wt GLP-1 and analogues was evaluated during an oral glucose tolerance test (OGTT) in normal female CD1 mice (6 wk of age; Charles River Canada, Montreal, QC, Canada) or obese, diabetic female *db*/*db* mice (8−10 wks of age; C57BLKS/J-M +/+Lepr$^{db}$; Jackson Laboratories, Bar Harbor, ME). Mice fasted for 16−17 h and given a s.c. (CD1 mice) or i.p. (*db*/*db* mice) injection of either PBS (vehicle) or peptide (at doses ranging from 0.1 to 100 $\mu$g) at $t = -5$ min. At $t = 0$ min, mice were administered 1.5 mg/g glucose by gastric gavage, and blood glucose levels were determined at $t = 0$, 10, 20, 30, 60, 90, and 120 min using tail vein blood on a One Touch Glucose Meter (Lifescan Canada Ltd, Burnaby, BC, Canada). Mice were normally tested on two separate occasions separated by a minimum period of 3 days. All animal protocols were approved by the University of Toronto Animal Care Committee.

*Data Analysis.* All values are expressed as mean ± SEM of at least three independent experiments unless stated otherwise. Ligand binding displacement curves and cAMP dose−response curves were plotted using PRISM GraphPad (GraphPad Software, San Diego, CA). The glycemic area-under-the curve (AUC) was determined using the trapezoidal rule. Statistical differences between responses were determined by ANOVA using n-1 post-hoc custom hypotheses tests, for each peptide analogue as compared to the same dose of WT GLP-1, while all doses of WT GLP-1 and doses of the analogues below 1 $\mu$g (the lowest dose of WT GLP-1 tested) were compared to the response observed in mice receiving PBS (vehicle) alone.

## RESULTS

GLP-1 analogues were categorized into four major groups: N-terminally modified, chimeric, midchain modified and C-terminally truncated peptides (Table 1). All analogues were compared to wt GLP-1 for their ability to bind and activate the GLP-1R in vitro and to reduce the glycemic AUC during an OGTT in vivo.

*N-Terminally Modified GLP-1 Analogues.* To examine specific amino acids in the N-terminus of GLP-1 for their empirical importance in GLP-1 receptor binding and activation, a series of analogues was created, by replacement of $His_1$ with aromatic amino acids Phe or Tyr, or by addition of an acetyl- or hexenoyl-acyl chain to the α-amino group of $His_1$. All of these analogues bound to the hGLP-1R expressed in CHO-K1 (R7) cells but with lower affinity than that of native GLP-1 ($IC_{50}$'s =15.2−58.0 nM vs 4.8 ± 1.9 nM for wt GLP-1; Figure 1, panel A, and Table 2). As expected from the binding experiments, these peptide analogues also increased the accumulation of cAMP in R7 cells with higher $EC_{50}$ values than wt GLP-1 (20−182 nM vs 2.6 ± 0.5 nM for wt GLP-1; Figure 1, panel B, and Table 2). Furthermore, addition of an acetyl- or hexenoyl-acyl chain to $His_1$ had a more dramatic effect on receptor activation than on binding, resulting in ∼10-fold greater increments in their $EC_{50}$'s than in their $IC_{50}$'s. In oral glucose tolerance tests in vivo, wt GLP-1 was found to significantly lower the glycemic response at the 25 and 100 $\mu$g doses, as compared to mice receiving vehicle alone (Figure 1, panel C). When compared to the wt peptide, substitution of $His_1$ with Phe or Tyr did not improve the ability of GLP-1 to lower the glycemic response to oral glucose in vivo (Figure 1, panel C). Unexpectedly, however, acetyl-, but not hexenoic-GLP-1, was found to exhibit increased effectiveness in vivo, markedly reducing the glycemic response to an OGTT as compared to wt GLP-1 ($P < 0.01$ at the 25 $\mu$g dose).

Several additional N-terminal modifications to GLP-1 were also tested based upon the known specificity of DP IV for cleavage of peptide/proteins with Ala or Pro at position 2 (*18*). These included D-$Ala_2$, $Gly_2$, $Leu_2$, $Ala_3$, $Asp_3$, $Ala_4$, and hexenoic-D-$Ala_2$. [D-$Ala_2$]-, [$Gly_2$]-, [$Asp_3$]-, and [hexenoyl-D-$Ala_2$]-GLP-1 did not exhibit remarkable changes in receptor binding ($IC_{50}$'s = 1.8−14.5 nM, respectively). However, all of these analogues exhibited rightward shifts in the cAMP dose−response curves (Figure 2, panels A and B, and Table 3). Despite their decreased ability to activate the GLP-1R in vitro, [D-$Ala_2$]-, [$Gly_2$]-, and [hexenoic-D-$Ala_2$]-GLP-1 were all found to exhibit increased biological activity in vivo, as compared to the WT peptide ($P < 0.05$−0.01 at the 1 $\mu$g dose for [D-$Ala_2$]- and [$Gly_2$]-GLP-1, and $P < 0.05$−0.01 at the 5 $\mu$g dose for all three peptides; Figure 2, panel C). In contrast, [$Ala_3$]- and [$Ala_4$]-GLP-1 were all found to be relatively poor analogues with respect to receptor binding ($IC_{50}$'s = 155−255 nM) and activation ($EC_{50}$'s = 125 to >1000 nM), and their in vivo biological activity was not enhanced as compared to wt GLP-1.

*Chimeric GLP-1 Analogues.* Although GLP-1 shares a relatively high homology with other peptides in the glucagon/secretin family, none of these peptides can efficiently bind or activate the recombinant GLP-1 receptor. The receptor binding affinity of glucagon for the GLP-1 receptor, for example, is ∼1000-fold lower than that of GLP-1 (*31*). In the present study, a series of chimeric peptides was created in which the N-terminus of GLP-1 was replaced by the corresponding region of several related peptides (Table 1). $Glucagon_{(1-5)}$GLP-1 (equivalent to [$Ser_2$,$Gln_3$]-GLP-1) and $glucagon_{(1-10)}$GLP-1 (= [$Ser_2$,$Gln_3$,$Tyr_{10}$]-GLP-1) peptide analogues showed relatively small effects on receptor binding and activation, although $glucagon_{(1-5)}$GLP-1 had markedly enhanced in vivo biological activity ($P < 0.05$ vs WT peptide at the 1 and 5 $\mu$g doses, and $P < 0.01$ vs PBS alone at the 0.5 $\mu$g dose; Figures 3−4 and Table 4). Conversely, $VIP_{(1-5)}$-GLP-1(= [$Ser_2$,$Asp_3$,$Ala_4$,$Val_5$]-GLP-1) and $VIP_{(1-10)}$GLP-1 (= [$Ser_2$,$Asp_3$,$Ala_4$,$Val_5$,$Asp_8$,$Asn_9$]-GLP-1), which shared only 1 of 5 and 3 of 10 amino acids, respectively, were poor

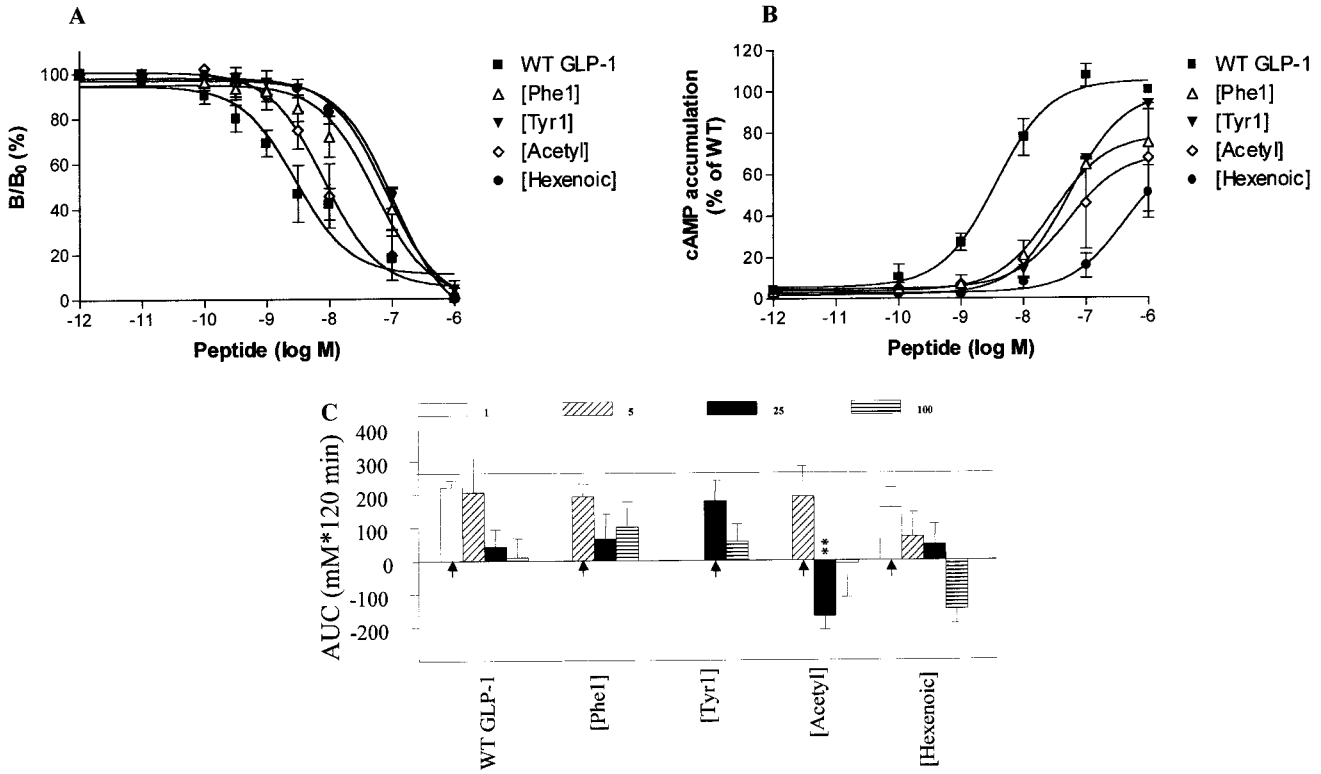


FIGURE 1: Analysis of receptor binding, cAMP production, and in vivo biological activity of wt GLP-1 and GLP-1 analogues with N-terminal $His_1$ modifications. (*A*) Binding of peptides to the GLP-1R was examined by $^{125}$I-GLP-1 competitive binding assay in CHO (R7) cells ($n = 3$). (B) Analysis of cAMP production stimulated by wt GLP-1 and GLP-1 analogues in CHO (R7) cells ($n = 3$). (C) Effects of wt GLP-1 and GLP-1 analogues (1, 5, 25, or 100 $\mu$g/mouse; some peptides were not tested at all doses) on the delta-glycemic response to an oral glucose challenge in CD1 mice ($n = 50$ for PBS-treated animals, $n = 3-4$ for all other treated groups). The cumulative response over a 2 h period is represented as the area under the curve (AUC). The horizontal line indicates the AUC for PBS-treated mice ($= 268 \pm 21$ mM·120min), and the arrowhead indicates the lowest dose of each peptide tested. $^{**}P < 0.01$ vs same dose of WT GLP-1.

Table 2: Receptor Binding and cAMP Production in R7 Cells Induced by WT GLP-1 and Analogues with N-Terminal His1 Modification (Mean ± SEM, $n = 3$)

| | receptor binding ($IC_{50}$, nM) | cAMP production ($EC_{50}$, nM) | cAMP max (% WT max) |
|---|---|---|---|
| WT GLP-1 | 4.8 ± 1.9 | 2.6 ± 0.5 | 100 |
| [$Phe_1$]GLP-1 | 58.0 ± 15.3 | 19.9 ± 2.5 | 75.0 ± 2.3 |
| [$Tyr_1$]GLP-1 | 49.3 ± 20.7 | 33.7 ± 2.7 | 93.8 ± 2.4 |
| acetyl-GLP-1 | 15.2 ± 3.3 | 161.0 ± 65.0 | 45.7 ± 0.5 |
| hexenoic-GLP-1 | 24.3 ± 5.8 | 182.0 ± 43.0 | 52.8 ± 0.8 |

activators of the receptor. Interestingly, both $VIP_{(1-10)}$- and $PACAP_{(1-10)}$GLP-1 demonstrated only limited binding to the GLP-1R. Nonetheless, both of these peptides exhibited relatively normal biological activity in vivo, indicating the importance of factors in addition to receptor interactions in mediating biologic effectiveness. Finally, $GIP_{(1-10)}$GLP-1 ($=$ [$Tyr_1$,$Ile_7$,$Tyr_{10}$]-GLP-1) was considerably less efficient in its ability to activate than bind the GLP-1R and was largely inactive in vivo, supporting an essential role for $His_1$ in activation of the GLP-1R.

*Midchain GLP-1 Modifications and C-Terminally Truncated Analogues.* Multiple substitutions of Ala for $Ser_8$, $Ser_{11}$, $Ser_{12}$, and/or $Gly_{16}$ in midchain of GLP-1 gave rise to five analogues, of which only [$Ala_{8/11/16}$] and [$Ala_{8/11/12/16}$] displayed comparable receptor binding and activation characteristics to those of wt GLP-1 (Figure 5 and Table 5). Unexpectedly, however, several of these peptides exhibited enhanced biologic activity in vivo, with $Ala_{12/16}$ being markedly more potent than wt GLP-1 ($P < 0.05-0.01$ at the 5, 25, and 100 $\mu$g doses). These data indicate that modifications to the so-called *hinge* region of GLP-1 are well tolerated.

To determine if the C-terminus of GLP-1 is critical for receptor binding and/or activation, three C-terminally truncated peptides were designed (GLP-$1_{1-18}$, GLP-$1_{1-22}$, and GLP-$1_{1-26}$). The deletion of as few as four C-terminal amino acids greatly reduced receptor binding, signaling ($IC_{50}$ and $EC_{50}$: >1000 nM; Table 5) and biological activity (data not shown). Further truncations completely abolished all receptor interactions and biological activity.

*Effects of Selected GLP-1 Analogues in Diabetic db/db Mice.* As GLP-1 is under consideration as a therapeutic agent for the treatment of Type 2 DM, additional studies were conducted to establish the effectiveness of selected peptides in diabetic animals. The *db*/d*b* mouse is a model of Type 2 DM, due to a homozygous mutation in the leptin receptor that leads to hyperphagic obesity, hyperglycemia, and hyperinsulinemia (reviewed in ref *32*). Basal glycemia was markedly elevated in these mice (11.3 ± 0.8 mM in *db*/*db* mice vs. 5.1 ± 0.2 mM in control, CD1 mice), as was the glycemic response to an oral glucose challenge (1674 ± 232 mM·120 min in *db*/*db* mice vs 268 ± 21 mM·120 min in control, CD1 mice). Administration of [$dAla_2$]GLP-1 to diabetic mice was associated with normalization of glycemia for the duration of the 2 h period studied, and consistent with the findings made in normal mice, the response to this analogue was markedly greater than observed for the wt peptide ($P < 0.05-0.001$ for all doses tested; Figure 6). In contrast, and also consistent with the data from normal mice,

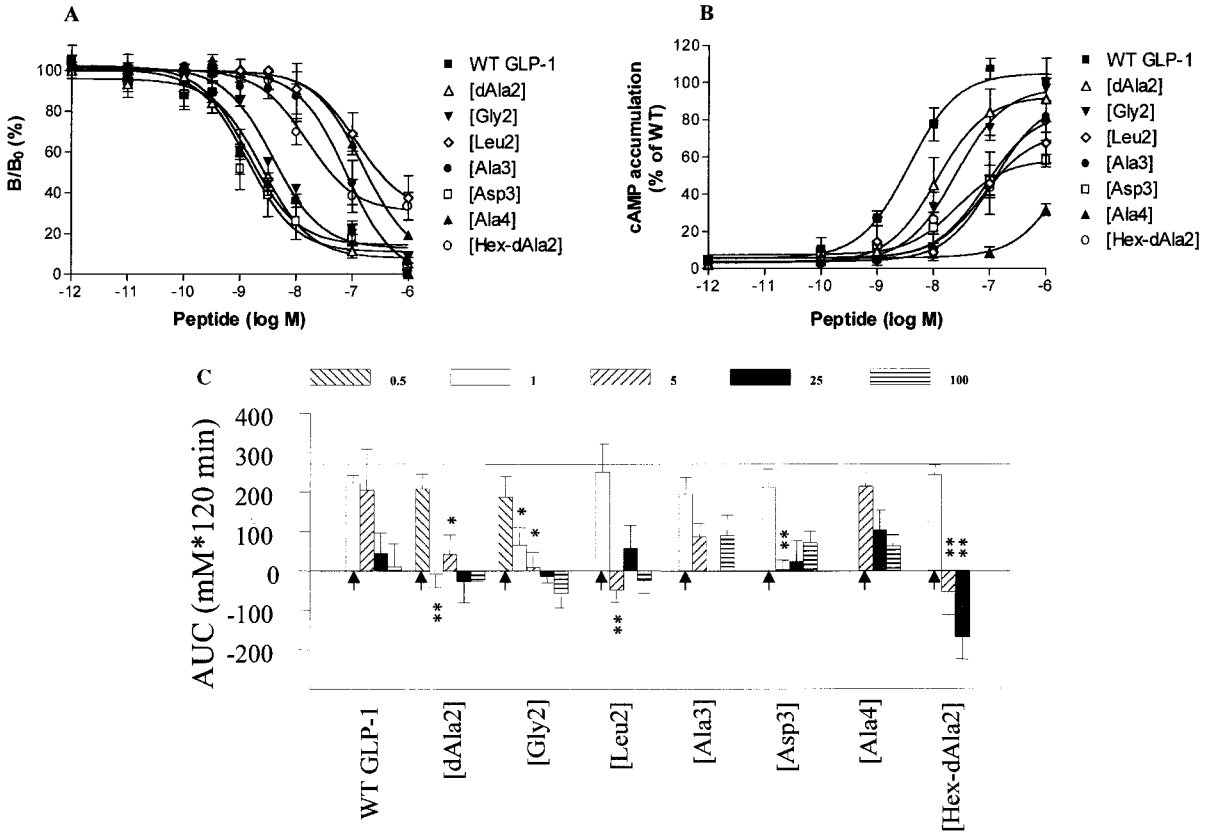


FIGURE 2: Analysis of receptor binding, cAMP production, and in vivo biological activity of wt GLP-1 and GLP-1 analogues with N-terminal amino acid substitutions. (A) Binding of peptides to the GLP-1R was examined by $^{125}$I-GLP-1 competitive binding assay in CHO (R7) cells ($n = 3$). (B) Analysis of cAMP production stimulated by wt GLP-1 and GLP-1 analogues in CHO (R7) cells ($n = 3$). (C) Effects of wt GLP-1 and GLP-1 analogues (0.5, 1, 5, 25, or 100 $\mu$g/mouse; some peptides were not tested at all doses) on the delta-glycemic response to an oral glucose challenge in CD1 mice ($n = 50$ for PBS-treated animals, $n = 3-4$ for all other treatment groups). The cumulative response over a 2 h period is represented as the area under the curve (AUC). The horizontal line indicates the AUC for PBS-treated mice ($= 268 \pm 21$ mM·120min), and the arrowhead indicates the lowest dose of each peptide tested. *$P < 0.05$, **$P < 0.01$ vs same dose of WT GLP-1.

Table 3: Receptor Binding and cAMP Production in R7 Cells Induced by WT GLP-1 and Analogues with N-Terminal Amino Acids (2−4) Substitution (Mean ± SEM, $n = 3$)

| | receptor binding ($IC_{50}$, nM) | cAMP production ($EC_{50}$, nM) | cAMP max (% WT max) |
|---|---|---|---|
| WT GLP-1 | 2.4 ± 1.5 | 4.1 ± 1.8 | 100 |
| [D-Ala$_2$]GLP-1 | 1.8 ± 0.2 | 41.0 ± 3.1 | 93.4 ± 1.3 |
| [Gly$_2$]GLP-1 | 4.9 ± 0.9 | 29.6 ± 6.4 | 96.8 ± 7.5 |
| [Leu$_2$]GLP-1 | 154.5 ± 58.6 | >1000 | 81.2 ± 17.5 |
| [Ala$_3$]GLP-1 | 194.6 ± 78.3 | 124.6 ± 29.6 | 81.7 ± 8.5 |
| [Asp$_3$]GLP-1 | 2.7 ± 1.3 | 118.5 ± 4.3 | 58.1 ± 3.4 |
| [Ala4]GLP-1 | 255.4 ± 54.6 | >1000 | 30.9 ± 3.7 |
| hexenoic-[D-Ala2]GLP-1 | 14.5 ± 3.1 | >1000 | 86.7 ± 26.4 |

the biological effectiveness of Ala$_{8/11/12/16}$-GLP-1 was not different from that of wt GLP-1.

## DISCUSSION

The considerable interest in GLP-1 as a treatment for Type 2 DM has sparked interest in the design of more potent GLP-1R agonists. Of great importance to such studies is the fact that GLP-1 is very rapidly degraded by DP IV, limiting its biological half-life and potential clinical application. Although DP IV inhibitors may prevent the degradation of GLP-1 and improve glucose tolerance (*33*), these inhibitors may also affect the biological activities of other important polypeptides, such as GIP and GLP-2, due to the presence of similar substrate cleavage sites (*22*, *23*, *34*). Therefore, one of the more effective and promising approaches is to develop DP IV-resistant and potentially more potent GLP-1 analogues by modification of the primary structure of GLP-1. Of particular importance in the present study was the ability to correlate the capacity of each peptide for receptor binding and activation to its biological activity in the whole animal, an approach that has only been used to a limited extent in other studies of GLP-1 analogues (*37*, *39*) (see Table 6). The results indicate that these functions may be dissociated, most likely due to additional determinants of in vivo activity, such as changes in peptide degradation rates. One additional factor that must be considered in the interpretation of the results of the in vivo studies relates to the pluripotential actions of GLP-1 on glycemia in the whole animal, which not only include stimulation of glucose-dependent insulin secretion, but also inhibition of glucagon release and gastric emptying (reviewed in ref *2*). Nonetheless, administration of GLP-1 and its analogues to the whole animal in association with an oral glucose tolerance test is the most representative test of GLP-1 actions in vivo, as related to its potential use in humans with Type 2 DM. Furthermore, use of the oral glucose tolerance test allows assessment of GLP-1 bioeffectiveness under physiological conditions, as compared to an IV glucose tolerance test, for example.

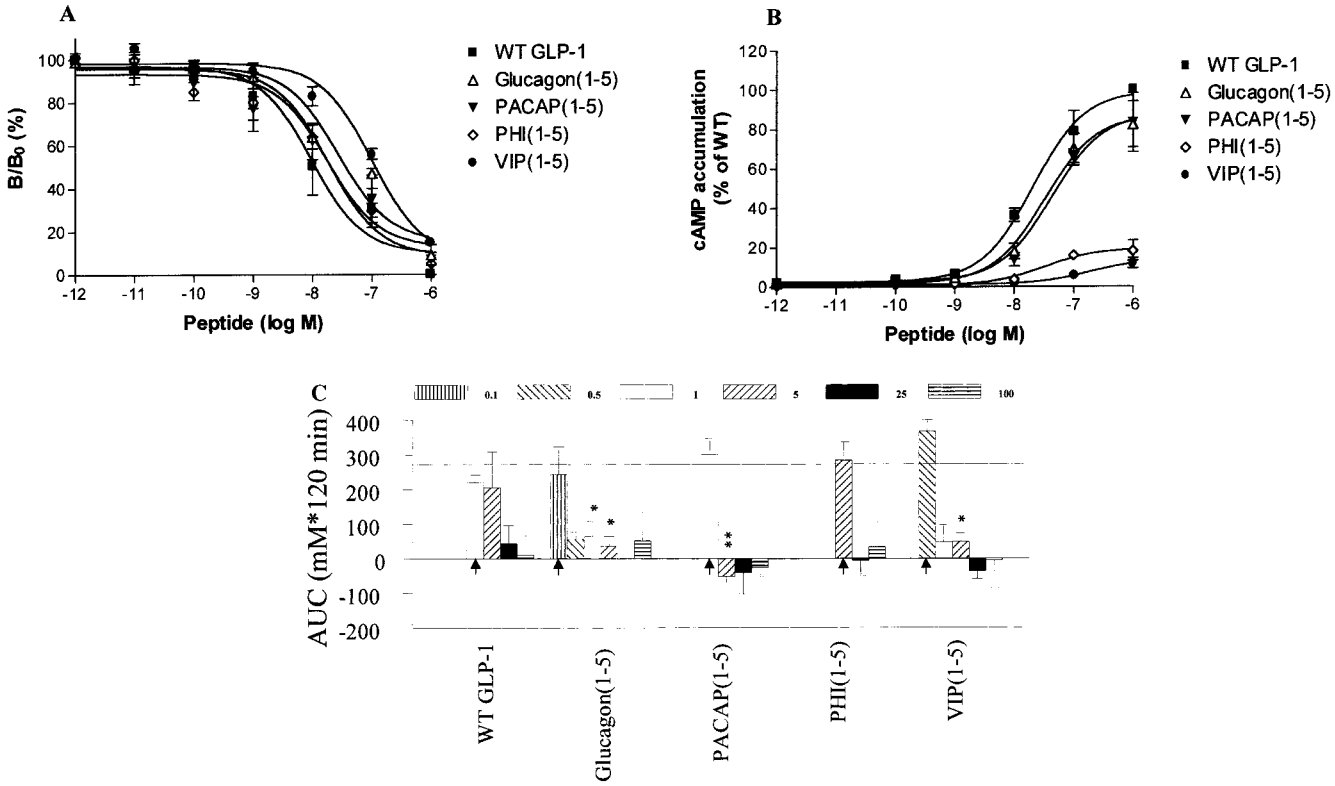


FIGURE 3: Analysis of receptor binding, cAMP production, and in vivo biological activity of wt GLP-1 and chimeric$_{(1-5)}$-GLP-1 analogues. (A) Binding of peptides to the GLP-1R cells was examined by $^{125}$I-GLP-1 competitive binding assay in CHO (R7) cells ($n = 3$). (B) Analysis of cAMP production stimulated by wt GLP-1 and GLP-1 analogues in CHO (R7) cells ($n = 3$). (C) Effects of wt GLP-1 and GLP-1 analogues (0.1, 0.5, 1, 5, 25, 100 $\mu$g/mouse; some peptides were not tested at all doses) on the delta-glycemic response to an oral glucose challenge in CD1 mice ($n = 50$ for PBS-treated animals, $n = 3-4$ for all other treatment groups). The cumulative response over a 2 h period is represented as the area under the curve (AUC). The horizontal line indicates the AUC for PBS-treated mice ($= 268 \pm 21$ mM·120 min), and the arrowhead indicates the lowest dose of each peptide tested. *$P < 0.05$, **$P < 0.01$ vs same dose of WT GLP-1.

In the present study, replacement of $His_1$ in GLP-1 by the aromatic amino acids Tyr or Phe resulted in decreased GLP-1R binding and activation, consistent with a report that the positive charge on the imidazole ring is the essential component of $His_1$ for GLP-1 action (*35*) (Table 6). Similarly, modification of $His_1$ by covalent attachment of acyl chains (e.g., acetyl, hexenoyl) to the α-amino group of GLP-1 also decreased receptor binding and activation efficiency; however, these analogues exhibited insulinotropic activity that was equal to or greater than that of wt GLP-1 in vivo, likely due to the prevention of DP IV degradation. This is supported by the results of others showing that *N*-methylated-GLP-1, desamino-GLP-1, and imidazol-lactic-acid-GLP-1 are poorly degraded by DP IV (*36*, *37*). In keeping with the crucial role of DP IV in regulating GLP-1 action, GLP-1 analogues in which $Ala_2$ was substituted by D-$Ala_2$ or $Gly_2$ were markedly more potent in vivo as compared to wt GLP-1, despite in vitro receptor binding and activation characteristics that were comparable or slightly decreased. These two analogues have recently been shown, by us and others, to be DP IV-resistant in vitro and in vivo (*21*, *37*, *38*, *47*). Since these amino acid substitutions at position 2 of the GLP-1 molecule reduce enzymatic degradation by DP IV and did not markedly affect receptor recognition, it appears to be a promising approach to produce highly biologically active long-term GLP-1 analogues. In agreement with this, other analogues with amino acid changes in position 2, including $Ser_2$-GLP-1, $Thr_2$-GLP-1, $Aib_2$-GLP-1 (*21*, *39*) also have been shown to exhibit improved stability. Finally, consistent with the results of previous studies (*40*), replacement of $Glu_3$ with either Asp or Ala and of $Gly_4$ with Ala decreased receptor activation. These findings suggest the importance of the carboxyl side chain at position 3 in GLP-1 function, while $Gly_4$ may be important in the conformational flexibility of GLP-1 because of its lack of a bulky side chain. However, these analogues all had near normal biological effects in vivo, suggesting that exchange of the side chain at positions 3 and 4 may influence the recognition by DP IV, leading to a longer half-life and stronger insulinotropic effects.

The N-terminal structure of GLP-1 was further analyzed using a series of novel chimeric peptides in which the N-terminus of GLP-1 was replaced by that of a structurally related peptide. In our hands, glucagon is a very weak agonist of the GLP-1R, and GIP, VIP, PACAP, PHI, secretin, etc., are not capable of binding to the GLP-1R (data not shown). The activities of chimeric GLP-1 peptides have thus revealed insight into the structural characteristics of the N-terminal 10 amino acids. Glucagon$_{(1-5)}$-, glucagon$_{(1-10)}$-, PACAP$_{(1-5)}$, PACAP$_{(1-10)}$-, secretin$_{(1-10)}$-, VIP$_{(1-5)}$-, and VIP$_{(1-10)}$GLP-1 all displayed normal or reduced receptor binding and signaling, while their biological effects in vivo were normal-enhanced. These findings may be explained by the $Ser_2$ vs $Ala_2$ transversion that occurs in each of these chimeric peptides. Previous studies have shown that $Ser_2$-GLP-1 is more stable in plasma and possesses a more potent insulin secretory response as compared to wt GLP-1 (*38*, *39*) (Table 6). Many of these peptides have Asp at position 3 rather

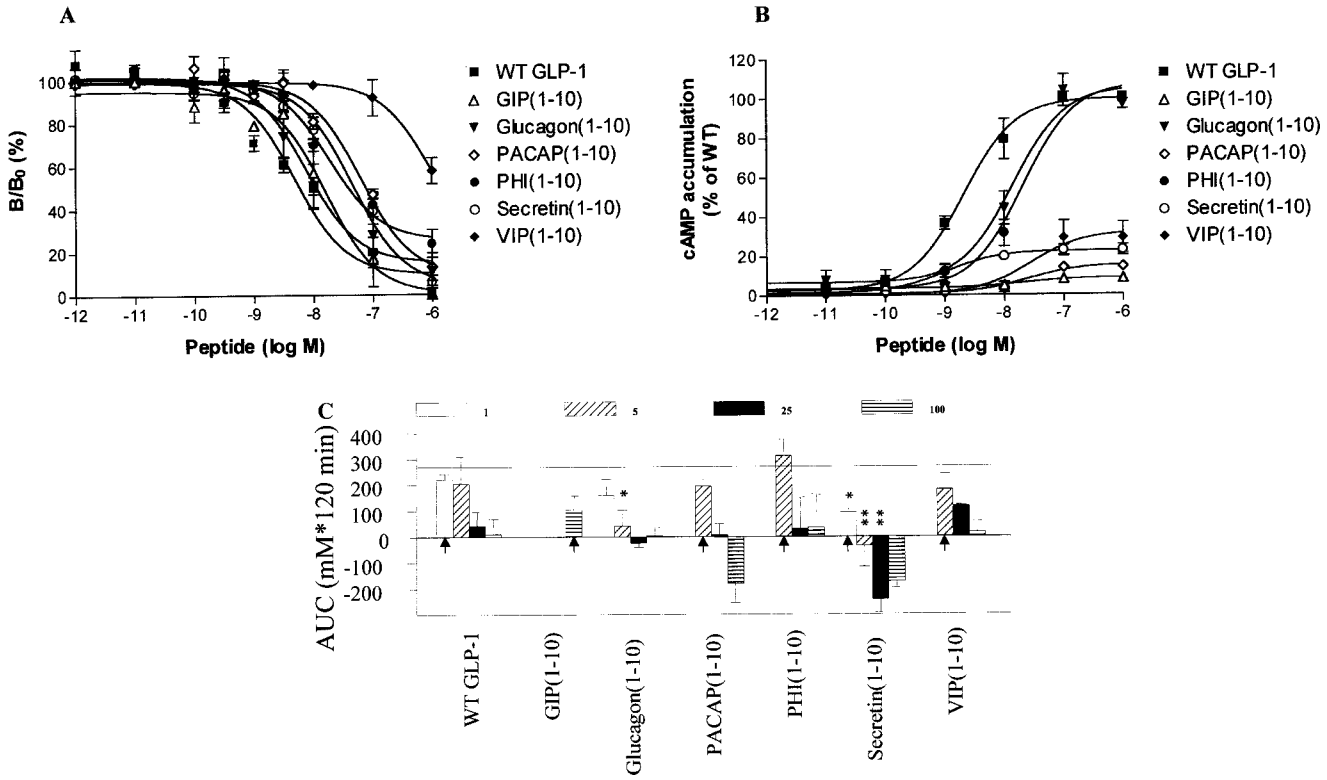


FIGURE 4: Analysis of receptor binding, cAMP production, and in vivo biological activity of wt GLP-1 and chimeric$_{(1-10)}$-GLP-1 analogues. (A) Binding of peptides to the GLP-1R was examined by $^{125}$I-GLP-1 competitive binding assay in CHO (R7) cells ($n = 3$). (B) Analysis of cAMP production stimulated by wt GLP-1 and GLP-1 analogues in CHO (R7) cells ($n = 3$). (C) Effects of wt GLP-1 and GLP-1 analogues (1, 5, 25, or 100 $\mu$g/mouse; some peptides were not tested at all doses) on the delta-glycemic response to an oral glucose challenge in CD1 mice ($n = 50$ for PBS-treated animals, $n = 3-4$ for all other treatment groups). The cumulative response over a 2 h period is represented as the area under the curve (AUC). The horizontal line indicates the AUC for PBS-treated mice ($= 268 \pm 21$ mM·120min), and the arrowhead indicates the lowest dose of each peptide tested. *$P < 0.05$, **$P < 0.01$ vs same dose of WT GLP-1.

Table 4: Receptor Binding and cAMP Production in R7 Cells by WT GLP-1 and Chimeric Peptides (Mean ± SEM, $n = 3$)

| | receptor binding ($IC_{50}$, nM) | cAMP production ($EC_{50}$, nM) | cAMP max (% WT max) |
|---|---|---|---|
| WT GLP-1 | 2.8 ± 1.2 | 3.4 ± 0.9 | 100 |
| glucagon$_{(1-5)}$GLP-1 | 8.2 ± 2.3 | 5.1 ± 0.9 | 82.4 ± 11.5 |
| glucagon$_{(1-10)}$GLP-1 | 10.1 ± 4.5 | 16.1 ± 0.7 | 109.7 ± 5.4 |
| PACAP$_{(1-5)}$GLP-1 | 3.4 ± 1.5 | 7.4 ± 1.2 | 83.2 ± 14.8 |
| PACAP$_{(1-10)}$GLP-1 | 39.3 ± 2.9 | 154.7 ± 18.7 | 13.9 ± 1.5 |
| PHI$_{(1-5)}$GLP-1 | 11.6 ± 2.2 | 4.9 ± 1.8 | 18.4 ± 5.1 |
| PHI$_{(1-10)}$GLP-1 | 21.8 ± 10.0 | 30.9 ± 12.1 | 107.1 ± 0.3 |
| VIP$_{(1-5)}$GLP-1 | 58.5 ± 12.8 | 94.2 ± 27.6 | 12.1 ± 2.5 |
| VIP$_{(1-10)}$GLP-1 | 157.7 ± 65.8 | >1000 | 29.3 ± 10.8 |
| GIP$_{(1-10)}$GLP-1 | 53.2 ± 2.5 | >1000 | 8.1 ± 1.8 |
| secretin$_{(1-10)}$GLP-1 | 29.5 ± 13.3 | 0.19 ± 0.1 | 22.4 ± 2.7 |

than Glu, suggesting that substitutions at residue 3 are well tolerated in terms of receptor activation. These findings are consistent with the observation that replacement of $Glu_3$ with Ala did not change peptide activity. Furthermore, as the analogues glucagon$_{(1-5)}$GLP-1 and glucagon$_{(1-10)}$GLP-1 have $Gln_3$ and $Gln_3$, $Tyr_{10}$ substitutions, respectively, but exhibit comparable binding properties, these data further suggest that positions 3 and 10 of GLP-1 are not critical. However, in contrast to the similar chimerics with replacement of only amino acids 1−5, PACAP$_{(1-10)}$- and VIP$_{(1-10)}$GLP1 exhibited remarkably decreased receptor binding and activation; secretin$_{(1-10)}$GLP-1 also exhibited a very limited capacity for receptor interaction. Although all of these chimerics have Ser at position 2, and may therefore possess a longer half-life in vivo (*21*, *39*), the exchange of $Asp_9$ by Ser in PACAP, by Asn in VIP, and by Glu in secretin appears to dramatically alter their ability to interact with the receptor, possibly due to a conformation change. Consistent with this possibility, an interaction of the negatively charged $Asp_9$ with the positively charged $His_1$ of glucagon has been reported, and these amino acids, together with $Ser_{16}$, form the catalytic triad of glucagon (*41*). Previous Ala scanning studies have also indicated that $Asp_9$ is a critical residue for GLP-1 receptor activation (*42*). Finally, substitution of amino acids 1−10 in GLP-1 with those of GIP (= [$Tyr_1$,$Ile_7$,$Tyr_{10}$]GLP-1) confirms that $His_1$ is critical for receptor activation. Taken together, these chimeric studies indicate that $His_1$ and $Asp_9$ are critical for GLP-1 receptor binding and activation. However, as these residues are conserved among most of the members of this subfamily of peptides, it is conceivable that they are more important for receptor activation than for selective receptor recognition.

Combined replacement of amino acids at positions 8, 11, 12, and/or 16 with Ala in GLP-1 (e.g., $Ala_{12/16}$, $Ala_{11/12/16}$, and $Ala_{8/11/12/16}$) appeared to increase biological effectiveness in vivo despite receptor binding and activation that were comparable to or less efficient than those of wt GLP-1. This could possibly have resulted from a conformation change of the peptide, which potentially decreased degradation. Previous study showed that NEP 24.11 cleaves GLP-1 at multiple positions of the midchain in GLP-1 (*43*, *44*). Therefore, modification of mid GLP-1 may improve stability in vivo; this remains to be determined. These modifications

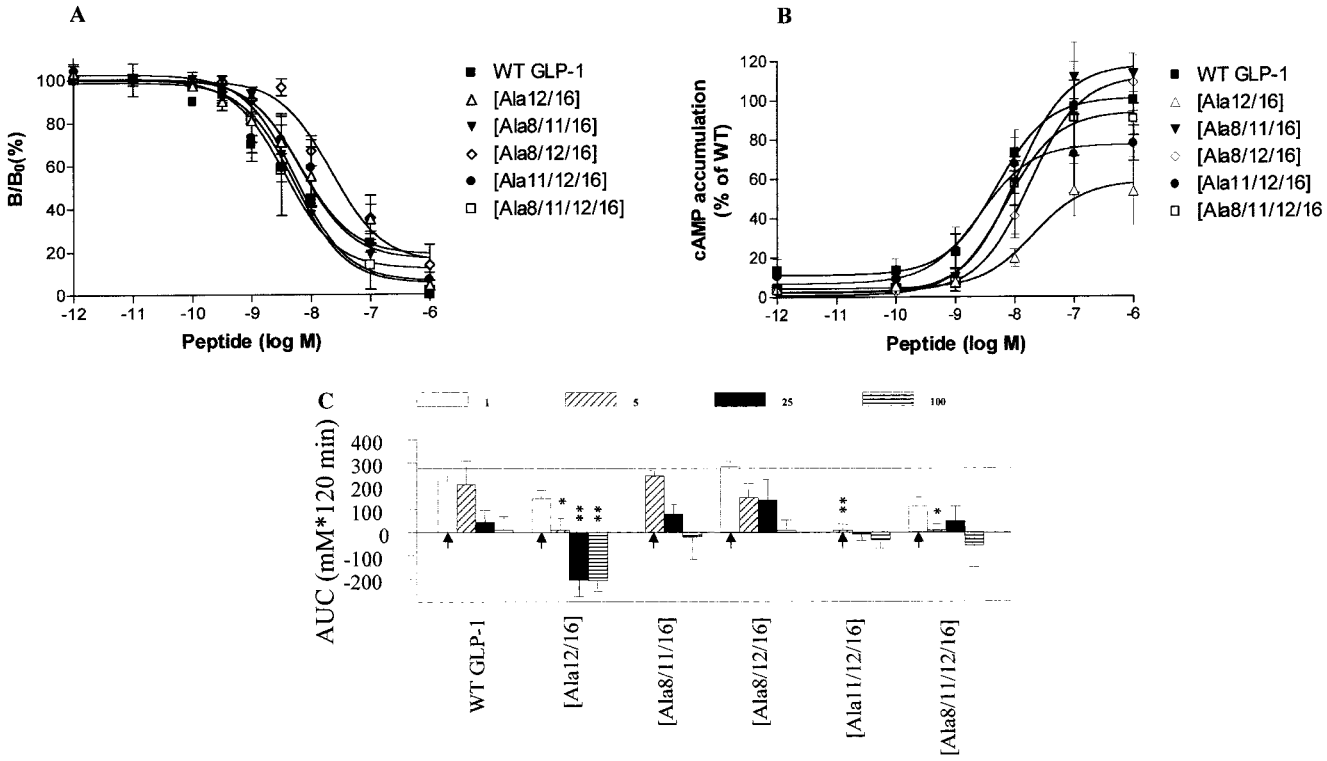


FIGURE 5: Analysis of receptor binding, cAMP production, and in vivo biological activity of wt GLP-1 and GLP-1 analogues with mid-sequence amino acid substitutions. (A) Binding of peptides to the GLP-1R was examined by $^{125}$I-GLP-1 competitive binding assay in CHO (R7) cells ($n = 3$). (B) Analysis of cAMP production stimulated by wt GLP-1 and GLP-1 analogues in CHO (R7) cells ($n = 3$). (C) Effects of wt GLP-1 and GLP-1 analogues (1, 5, 25, or 100 $\mu$g/mouse; some peptides were not tested at all doses) on the delta-glycemic response to an oral glucose challenge in CD1 mice ($n = 50$ for PBS-treated animals, $n = 3-4$ for all other treatment groups). The cumulative response over a 2 h period is represented as the area under the curve (AUC). The horizontal line indicates the AUC for PBS-treated mice ($= 268 \pm 21$ mM·120min), and the arrowhead indicates the lowest dose of each peptide tested. *$P < 0.05$, **$P < 0.01$ vs same dose of WT GLP-1.

Table 5: Receptor Binding and cAMP Production in R7 Cells by WT GLP-1 and Analogues with Midportion Modification and C-Terminal Truncation (Mean ± SEM, $n = 3$)

| | receptor binding ($IC_{50}$, nM) | cAMP production ($EC_{50}$, nM) | cAMP max (% WT max) |
|---|---|---|---|
| WT GLP-1 | 4.0 ± 1.1 | 4.2 ± 1.5 | 100 |
| [$Ala_{12/16}$]GLP-1 | 15.6 ± 9.7 | 29.3 ± 0.7 | 86.2 ± 7.9 |
| [$Ala_{8/11/16}$]GLP-1 | 4.6 ± 1.1 | 15.2 ± 6.5 | 120.8 ± 12.9 |
| [$Ala_{8/12/16}$]GLP-1 | 40.7 ± 12.8 | 18.4 ± 5.8 | 108.3 ± 12.9 |
| [$Ala_{11/12/16}$]GLP-1 | 14.4 ± 5.2 | 3.9 ± 1.6 | 76.6 ± 22.3 |
| [$Ala_{8/11/12/16}$]GLP-1 | 5.3 ± 0.8 | 7.9 ± 1.3 | 95.8 ± 12.0 |
| GLP-1(1−26) | >1000 | >1000 | 52.8+13.0 |
| GLP-1(1−22) | ND | ND | ND |
| GLP-1(1−18) | ND | ND | ND |

however, provide important novel structural information demonstrating that the midportion of GLP-1 could possibly be minimized while preserving activity. This observation opens the way to short GLP-1 analogues or mimetics.

The C-terminal sequences of the glucagon-GLP subfamily are highly heterogeneous in relation to the N-terminus, suggesting this region determines binding selectivity. C-terminally truncated GLP-$1_{(1-26)}$ displayed minimal binding and signaling, while GLP-$1_{(1-22)}$ and GLP-$1_{(1-18)}$ lost all function in vitro and in vivo, implying that the C-terminal region or absolute peptide length are essential for interaction of GLP-1 with its receptor. In agreement with our results, replacement of the last four residues of GLP-1 with the last three residues of glucagon also results in a 475-fold decrease

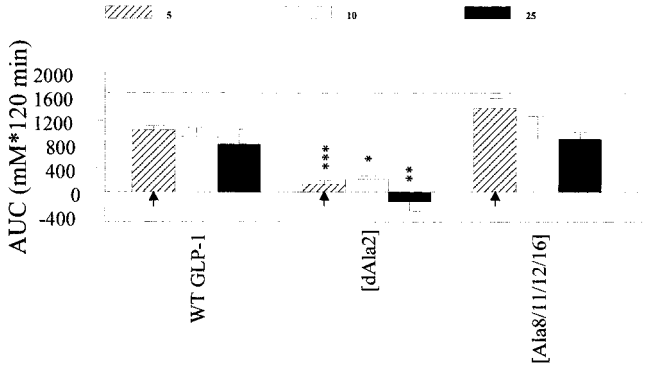


FIGURE 6: Analysis of in vivo biological activity of wt GLP-1 and GLP-1 analogues in diabetic *db*/*db* mice. wt GLP-1 or selected GLP-1 analogues (5, 25, or 100 $\mu$g/mouse) were tested for their ability to reduce the glycemic response to an oral glucose challenge in diabetic *db*/*db* mice ($n = 3-4$ for all treatment groups). The cumulative response over a 2 h period is represented as the area under the curve (AUC). The horizontal line indicates the AUC for PBS-treated mice ($= 1674 \pm 232$ mM·120min), and the arrowhead indicates the lowest dose of each peptide tested. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs same dose of WT GLP-1.

in GLP-1R binding affinity (*31*). Since the amino acid sequence at the C-terminus of these peptides is not conserved, it was proposed that this region might be important for specific ligand−receptor interaction, while the N-terminus may be primarily involved in receptor activation. This is supported by the fact that the N-terminally truncated peptides,

Table 6: Summary of the Studies in GLP-1 Analogues as Compared to WT GLP-1[a]

| | In Vitro | | | | In Vivo | | | Reference |
|---|---|---|---|---|---|---|---|---|
| | Degradation | Binding | cAMP | Insulin | Insulin | Glucose | Animal model | |
| [Phe1]GLP-1 | | D (12) | D (7.7) | | | D | mouse | |
| [Tyr1]GLP-1 | | D (10) | D (13) | | | D | mouse | |
| Acetyl-GLP-1 | | D (3.2) | D (62) | | | I | mouse | |
| Acetyl-GLP-1 | D | NC | NC | | I | I | minipig | Siegel E. G., et al, 37 |
| Hexenoic-GLP-1 | | D (5.1) | D (70) | | | I | mouse | |
| [dAla2]GLP-1 | | NC (0.8) | D (10) | | | I/I | mouse/db/db mouse | |
| [dAla2]GLP-1 | D | NC | NC | | NC | NC | minipig | Siegel E. G., et al, 37 |
| [Leu2]GLP-1 | | D (64) | D (>200) | | | D | mouse | |
| [Gly2]GLP-1 | | NC (2.0) | D (7.2) | | | I | mouse | |
| [Gly2]GLP-1 | D | NC | NC | | NC | NC | minipig | Siegel E. G., et al, 37 |
| [Gly2]GLP-1 | D | NC | | NC (islet) | | | | Deacon, C. F., et al, 21 |
| [Asp3]GLP-1 | | NC (0.8) | D (29) | | | NC | mouse | |
| [Ala3]GLP-1 | | D (80) | D (30) | | | D | mouse | |
| [Ala3]GLP-1 | | D | D | | | | | Gallwitz, B., et al, 42 |
| [Ala4]GLP-1 | | D (106) | (>200) | | | NC | mouse | |
| [Ala4]GLP-1 | | D (>200) | D (>200) | | | | | Gallwitz, B., et al, 42 |
| [Ala4]GLP-1 | | D | D | | | | | Ritzel, U., et al, 39 |
| Hexenoic-[dAla2]GLP-1 | | D (6.0) | (>200) | | | I | mouse | |
| Glucagon(1-5)GLP-1 | | NC (2.9) | NC (1.5) | | | I | mouse | |
| Glucagon(1-5)GLP-1 | | D(13) | | | | | | Hjorth, S. A., et al, 46 |
| Glucagon(1-10)GLP-1 | | NC (3.6) | NC (4.7) | | | NC | mouse | |
| GIP(1-10)GLP-1 | | D (19) | D (>200) | | | D | mouse | |
| PACAP(1-5)GLP-1 | | NC (1.2) | NC (2.2) | | | I | mouse | |
| PACAP(1-10)GLP-1 | | D (14) | D (46) | | | NC | mouse | |
| PHI(1-5)GLP-1 | | NC (4.1) | NC (1.4) | | | NC | mouse | |
| PHI(1-10)GLP-1 | | D (7.8) | D (46) | | | NC | mouse | |
| Secretin(1-10)GLP-1 | | D (11) | I (0.05) | | | I | mouse | |
| VIP(1-5)GLP-1 | | D (21) | D (28) | | | NC | mouse | |
| VIP(1-10)GLP-1 | | D (56) | D (>200) | | | NC | mouse | |
| [Ala12/16]GLP-1 | | NC (3.9) | D (7.0) | | | I | mouse | |
| [Ala8/11/16]GLP-1 | | NC (1.2) | NC (3.6) | | | NC | mouse | |
| [Ala8/12/16]GLP-1 | | D (10) | NC (3.2) | | | NC | mouse | |
| [Ala11/12/16]GLP-1 | | NC (3.6) | NC (0.9) | | | D | mouse | |
| [Ala8/11/12/16]GLP-1 | | NC (1.3) | NC (1.9) | | | D/NC | mouse/ db/db mouse | |
| [dHis1] GLP-1 | None | D (9.1) | D | | NC | NC | minipig | Siegel, E.G., et al, 37 |
| desaminoHis1 GLP-1 | None | | | | I | I | minipig | Siegel, E.G., et al, 37 |
| desaminoHis1 GLP-1 | None | D (15) | | | | | | Gallwitz, B., et al, 36 |
| N-me-His1-GLP-1 | None | NC | | | | | | Gallwitz, B., et al, 36 |
| alpha-me-His1-GLP-1 | D | NC | | | | | | Gallwitz, B., et al, 36 |
| imi-His1-GLP-1 | None | NC | | | | | | Gallwitz, B., et al, 36 |
| [Ser2]GLP1 | None | | | I (HIT cell) | I | D | rat | Ritzel, U., et al, 39 |

[a] D: decrease; I: increase; NC: no change; values in parentheses represent fold increase in $IC_{50}$ [binding] or $EC_{50}$ [cAMP] as compared to WT GLP-1.

$GLP\text{-}1_{(3-30)}$ and $exendin_{(9-39)}$ bind the GLP-1R, but do not activate signaling, while $exendin_{(1-39)}$(*45*) and xenGLP-1B (*29*) have identical or even higher binding affinity to the GLP-1R as compared to wt GLP-1 despite divergent C-termini. Of note, $Gly_{29}$ is the only conserved residue among these peptides, and this amino acid is not shared outside GLP-1 and exendin. It is therefore conceivable that this residue may play a critical role in recognition of the GLP-1R.

In summary, GLP-1 analogues were tested for both their receptor interactions in vitro and their physiological activity in vivo. Results indicated that both the N-terminus and C-terminus of GLP-1 are important for biological function, such that the N-terminus carries common but important consensus residues for receptor activation, while the C-terminus appears critical for the specific receptor recognition. Although few GLP-1 analogues possessed superior binding or activation of the receptor in vitro, several analogues possessed superior biological effects in vivo in both normal and diabetic mice. Future studies on these peptides will require analysis of both DP IV and NEP 24.11 degradation rates, as well as the mechanism of action in vivo, including effects on insulin and glucagon secretion, as well as gastric emptying.

## ACKNOWLEDGMENT

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