Influence of Selective Fluorination on the Biological Activity and Proteolytic Stability of Glucagon-like Peptide-1

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The relative simplicity and high specificity of peptide therapeutics has fueled recent interest. However, peptide and protein drugs generally require injection and suffer from low metabolic stability. We report here the design, synthesis, and characterization of fluorinated analogues of the gut hormone peptide, GLP-1. Overall, fluorinated GLP-1 analogues displayed higher proteolytic stability with simultaneous retention of biological activity (efficacy). Fluorinated amino acids are useful for engineering peptide drug candidates and probing ligand−receptor interactions.

Introduction

Type 2 diabetes is a debilitating disease and has become a worldwide epidemic.[1] The unmet challenges in disease management are the lack of long-term efficacy in reducing hyperglycemia and the inability to stop progression. Glucagon-like peptide-1 (GLP-1[a]) is viewed as the basis for a new class of therapeutics to meet such needs.[2−4] GLP-1 has multifaceted actions: it stimulates insulin secretion in a glucose dependent manner, increases β-cell mass and function, and suppresses glucagon secretion and appetite.[3,4] These properties illuminate its pharmacological potential in regulating blood glucose homeostasis. The concept of using GLP-1 to treat type 2 diabetes has been validated in human subjects,[5] as administration of exogenous GLP-1 lowers blood sugar levels. However, the clinical utility of native GLP-1 is severely hampered by its rapid deactivation by the serine protease, dipeptidyl peptidase IV (DPP IV, EC 3.4.14.5).[3,4] GLP-1 has a half-life of less than 2 min after intravenous administration.[6] There is great interest in developing long-acting agents that emulate the functions of GLP-1.[3,7] Indeed, a 39-residue peptide that mimics the functional attributes of GLP-1, exenatide, with a half-life of 26 min after iv injection,[2] is currently on the market with a dosing frequency of twice a day.

Fluorine substitution has proven useful for improving the therapeutic index of medicinal agents. While the first examples may have been serendipitous, nevertheless, roughly one-fifth of the pharmaceuticals on the market contain fluorine.[8] Introduction of fluorine into small molecules often results in increased hydrophobicity and metabolic stability, eventually leading to improved bioactivity and bioavailability,[9] as evidenced in the case of the antihyperglycemic sitagliptin[10] and the antidepressant fluoxetine.[11] However, this strategy has rarely been explored in peptide/protein therapeutics, a class of promising agents with high specificity and low metabolic stability.[12]

In limited studies, myriad effects of fluorination on hormonal and antimicrobial peptides[13−19] have been documented. For example, replacement of Tyr4 and Phe8 with 4F-Phe resulted in two angiotensin II analogues with distinct functional profiles. The first of these analogues resulted in generating a competitive inhibitor of angiotensin receptors, while the latter is an equipotent agonist[13] in both rat blood pressure and oxytocic assays. Introduction of trifluoromethyl isoleucine and leucine into the cell lytic peptide melittin[14] and cell penetrating buforin[17] led to enhanced membrane binding affinity and increased bacteriostatic activity, respectively. In addition, introduction of hexafluorovaline[15] and hexafluoroleucine[17,18] into hormonal and antimicrobial peptides renders them more stable to certain hydrolytic enzymes.

We envisioned that incorporation of hexafluoroleucine at strategic sites in therapeutic peptides could modulate peptide−receptor interactions and provide improved resistance to proteolytic cleavage. We report here the synthesis of GLP-1 and selectively fluorinated analogues, their binding affinity to the cognate human receptor, (GLP-1R) and signal transduction efficacy, and their protease stability against DPP IV. A fluorinated analogue was also tested for its ability to regulate the blood glucose levels in vivo (see Supporting Information).

Experimental Procedures

Cell Culture and Receptor Transfection. COS-7 cells were cultured in DME supplemented with 10% FBS, penicillin G sodium (100 units/mL), streptomycin sulfate (100 μg/mL), 26 mM sodium bicarbonate, pH 7.4, at 37 °C in 5% CO_2 humidified air. Cells (0.8×10^6 cells/plate) were seeded in 10 cm dishes. The following day, cells were transiently transfected with 5 μg of pcDNA1 vector using diethylaminoethyl-dextran (DEAE-dextran). The vector contains cDNA encoding the human GLP-1 receptor (*h*GLP1-R)[20] and has been sequenced for identity.[21]

Receptor Binding Assay. Receptor binding of peptides was analyzed as previously described.[21] Briefly, COS-7 cells (10^4 cells/well) were subcultured onto 24-well tissue culture plates (Falcon, Primaria, BD Biosciences) a day after transfection. Competitive binding experiments were carried out the next day at 25 °C for 100 min using 17 pM [^{125}I]-exendin (9-39) amide as the radioligand. The peptides had a final concentration ranging from 3×10^{-6} to 3×10^{-11} M in 270 μL of binding buffer freshly prepared in HBSS containing 0.2% BSA, 0.15 mM phenylmethylsulfonyl fluoride (PMSF), 25 mM HEPES, pH 7.3. Nonspecific binding was determined in the presence of 1 μM unlabeled peptides. Cells were

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[a] Abbreviations: CD, circular dichroism; DPC, dodecylphosphocholine; DPP IV, dipeptidyl peptidase IV; GLP-1, glucagon-like peptide-1; *h*GLP-1R, human glucagon-like peptide-1 receptor; PMSF, phenylmethylsulfonyl fluoride; TFE, trifluoroethanol.

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Scheme 1. Sequences of Wild Type GLP-1(7-36) Amide, Fluorinated Analogues, Ex(9-39), and [^{125}I]-Ex(9-39)[a]

[a] The residues replaced with hexafluoroleucine in GLP-1 are underlined. The red arrow indicates the scissile bond that DPP IV cleaves. The conserved residues between GLP-1 and Ex(9-39) are colored blue. [^{125}I]-Ex(9-39) amide served as the radioligand for the competition binding assay, in which ^{125}I-labeled Bolton−Hunter reagent is conjugated to Lys12. **L**: 5,5,5,5′,5′,5′-2*S*-hexafluoroleucine. ORTEP drawing of the crystal structure of hexafluoroleucine methyl ester indicates the stereochemistry of α-carbon (*S*).

carefully washed before (1 × 1 mL) and after (3 × 1 mL) incubation with binding buffer. Cells were lysed in 1 N NaOH, washed with 1 N HCl, and transferred to polypropylene tubes (Sigma) for γ counting (Beckman Gamma Counter 5500B).

cAMP Assay. COS-7 (10^5 cells/well) cells were transferred 24 h after transfection to 24-well plates and cultured for another 24 h. Cells were stimulated with GLP-1 and analogues for 1 h at 25 °C in DME (without phenol red) supplemented with 1% BSA, 1 mM 3-isobutyl-1-methylxanthine (IBMX), 0.4 μM Pro-boroPro, and 25 mM HEPES, pH 7.4. Pro-boroPro ([1-(2-pyrrolidinylcarbonyl)-2-pyrrolidinyl] boronic acid) is a potent DPP IV inhibitor[22] (provided by Dr. W. W. Bachovchin, Tufts University). The final peptide concentrations ranged from 1×10^{-6} to 1×10^{-11} M in 10-fold increments in 270 μL of buffer. Upon removal of incubation buffer, cells were lysed by a freeze−thaw cycle in N_2 (*l*), followed by the addition of 200 μL M-Per to ensure the complete lysis of cells. The concentration of cAMP in its acetylated form was determined using a FlashPlate kit (PerkinElmer Life Sciences) with [^{125}I]-cAMP as the radioligand. Plate-bound radioactivity was measured using a Packard Topcount proximity scintillation counter.

Degradation of Peptides by DPP IV. The proteolytic stability of peptides toward DPP IV was determined by an analytical RP-HPLC assay. The chromogenic Gly-Pro-*p*-nitroanilide was used to calibrate the specific activity of DPP IV ($\Delta\varepsilon_{410nm}$ = 8800 $M^{-1}\cdot cm^{-1}$) in 100 mM Tris·HCl, pH 8.0. Peptides (10 μM) were separately incubated with DPP IV (20 units/L) in 50 mM Tris·HCl, 1 mM EDTA, pH 7.6 at 37 °C over 200 min.[23] Reactions were quenched with 600 μL of 0.2% TFA at time intervals and stored at −20 °C. An analytical column [J. T. Baker C_{18}, 5 μm, 4 mm × 250 mm] was used for separation and quantitation of intact and digested peptides with a binary solvent system of CH_3CN/H_2O/0.1%TFA (detection at 230 nm). First-order rate constants were obtained as the fitted value ± standard deviation using eq (1):

$$\ln[A] = -k\cdot t + \ln[A]_0 \quad (1)$$

where A is the concentration of peptides, k the first-order rate constant, t the reaction time in min, and $[A]_0$ the initial concentration of peptides. The fragments derived from the full-length peptides were manually collected and identified by ESI-MS.

Data Analysis. Data from radioligand binding and cAMP asssays were analyzed by nonlinear regression (GraphPad Prism v4.0, San Diego, CA) and are reported as mean ± SEM. Data were normalized relative to GLP-1 for both receptor binding and receptor activation assays.

Results

Peptide Design and Characterization. GLP-1 binds to its cognate seven transmembrane G protein-coupled receptor (GLP-1R) through noncovalent interactions.[24] GLP-1 consists of an *N*-terminal random coil segment[25] (7-13), two helical segments (13-20 and 24-36), and a less well-defined region (21-23). The *C*-terminal helix of GLP-1 is conformationally more stable than the *N*-terminal helix as judged by amide proton exchange experiments[25] and is essential for receptor binding.[24] Replacement of Phe28 and Ile29 with alanine in GLP-1 induces a dramatic loss of binding affinity to GLP-1R.[24] These two residues along with Trp31 and Leu32 are conserved between GLP-1 and exendin 4, a synthetic GLP-1R agonist with high binding affinity,[26] and are located on the C-terminal hydrophobic surface. In order to manipulate the binding affinity of GLP-1 to GLP-1R, Phe28, Ile29, and Leu32 were selectively substituted with hexafluoroleucine predicated by the notion that the superior hydrophobicity of hexafluoroleucine[27] would lead to enhanced binding affinity. Trp31 was retained because of its utility in concentration determination and the flat geometry and large volume of the aromatic side chain.

To confer resistance toward DPP IV, several *N*-terminal residues (at P1, P1′, and/or P2′ positions) were replaced with hexafluoroleucine, namely, Ala8, Glu9, Gly10, and both Ala8 and Glu9 to generate four fluorinated analogues. His7 was left unchanged due to its crucial role in receptor signaling.[28] In short, selective hexafluoroleucine substitution was performed to probe the effects of fluorinating GLP-1 at sites that are crucial for the proteolytic stability and ligand−receptor interactions. The sequences of the fluorinated analogues, GLP-1, and exendin (9-39) amide are shown in Scheme 1.

The crystal structure of hexafluoroleucine methyl ester hydrochloride salt (Scheme 1) validates the stereochemistry at α-carbon (*S* configuration). The secondary structures of all peptides were determined by circular dichroism (CD) in phosphate buffered saline (PBS), dodecylphosphocholine (DPC), and 35% (v/v) trifluoroethanol (TFE) in PBS. All fluorinated analogues were similar in structure to wt GLP-1 (representative spectra for GLP-1 and **F8**, see Figure 1; other data not shown), suggesting no significant structural perturbation arose from fluorination in buffered solutions despite the lower α-helical propensity of hexafluoroleucine compared to leucine and alanine.[29]

Binding Assay. The binding affinity of fluorinated analogues was measured by a competition-binding assay using [^{125}I]-Ex(9-39) amide as a radioligand. This Bolton−Hunter labeled peptide was assumed to have a similar affinity to *h*GLP-1R as Ex(9-39) amide because the modification at Lys12 side chain does not diminish receptor binding.[30] Homologous antagonist competitive binding experiments show that Ex(9-39) amide binds with a dissociation constant of 2.9 nM (three independent

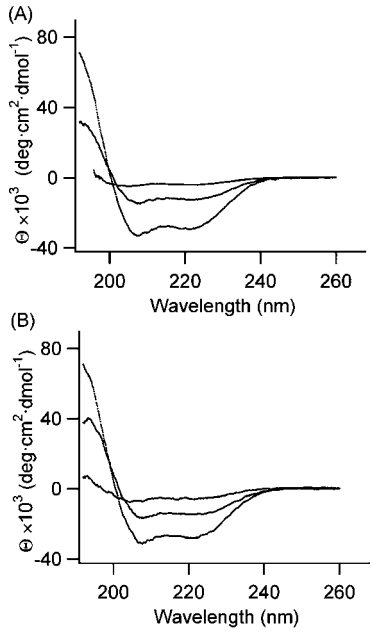

Figure 1. CD spectra of GLP-1 (A) and **F8** (B). Spectra in each panel correspond to peptides in 20 mM sodium phosphate (least helical), 40 mM dodecylphosphocholine, and 20 mM sodium phosphate containing 35% TFE (most helical) at 5 °C and pH 7.4. [Peptide] = 10 μM. Data represent the average of four scans.

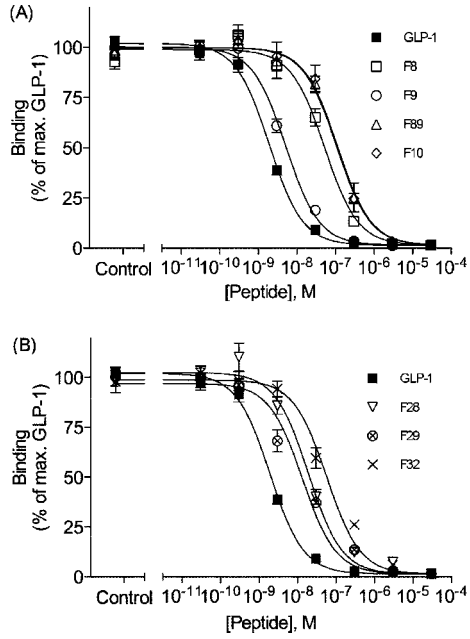

Figure 2. Binding of peptides to cloned human GLP-1R transiently expressed on COS-7 cells as examined by a competitive binding assay using [^{125}I]-Ex(9-39) as the radioligand. (A) *N*-Terminal analogues; (B) *C*-terminal analogues. Data represent five independent experiments in duplicate as mean ± SEM.

experiments in triplicate, see Supporting Information), in good agreement with previously reported values.[21,31] All seven fluorinated analogues bound to the *h*GLP-1R expressed on COS-7 cells that lack endogenous GLP-1R. **F9** had a binding affinity comparable to GLP-1 (IC_{50} 5.1 nM vs 1.9 nM, Figure 2 and Table 1), while **F29** and **F28** displayed 7-fold and 9.9-fold decreased affinity. **F8**, **F89**, **F10**, and **F32** were weaker binders with 27−60-fold increases in IC_{50} values (Figure 2A,B). The side chain carboxylate of Glu9 has been shown to be important for receptor binding, as substitution by Lys9 resulted in a significant loss in binding affinity.[32] Substitution with Ala9 also led to relatively poor receptor binding (30−80-fold higher IC_{50}),[24,33] while substitution by Asp9 did not result in a significant change.[33] These results, together with the similar binding affinity showed by **F9**, in which Glu9 is replaced by hexafluoroleucine, suggests that "polar hydrophobicity"[34] of hexafluoroisopropyl group is likely responsible for the apparent retention in binding ability. Ligand−receptor binding interfaces are frequently devoid of solvent water. Therefore, favorable polar interactions between CF_3 groups and possible electropositive regions (or dipoles) could in principle mimic the interaction between the carboxylate and its binding partner. Multipolar C−F···C═O, C−F···H−X (X = O, N, S), C−F···H−C_α, and C−F···side chain (of Arg, Gln, and Asn) interactions are commonly found in protein−ligand complexes in the PDB and CSD databases.[8] An alternative explanation is that bulky hydrophobic side chains at this position are well tolerated. The latter argument is supported by the prior observation that substitution of Glu9 with Leu9 resulted in only a 1.5-fold decrease in binding affinity.[35] The *N*-terminal modifications, except for **F9**, resulted in decreased binding affinity, while *C*-terminal modifications were well tolerated as evidenced by only slightly diminished binding to GLP-1R.

Formation of cAMP. In a radioimmunoassay, COS-7 cells expressing *h*GPL-1R were stimulated by peptides and the accumulation of intracellular secondary messenger cAMP was measured. All fluorinated peptides functioned as full agonists with the lone exception of **F89** (Figure 3). **F9**, **F32**, **F29**, and **F28** had a 2- to 5-fold decreased potency while retaining the same efficacy as GLP-1 (Figure 3 and Table 1). **F8** and **F10** displayed a moderate decrease in potency, but efficacies were judged to be equivalent by *p*-tests. A previous report[24] found that substitution of Gly10 with Ala completely abolished receptor activation ability, highlighting the crucial role of Gly 10. Our study indicates that bulky side chains can be accommodated at position 10. Replacement of Phe28 with Ala resulted in a 1000-fold decrease in receptor activation.[24] In contrast, substitution with hexafluoroleucine was well tolerated in terms of both receptor binding and activation, suggesting hydrophobicity is a required element while aromaticity is dispensable at this position. Interestingly, **F89** was a partial agonist with a maximal response being ~30% of that elicited by natural GLP-1. This is different from the binding ability of **F89** to GLP-1R, which is similar to **F10** in the range of tested concentrations. This result suggests that the correct positioning of His7 into the receptor-binding pocket is critical for receptor activation. Comparing the potency of **F89** to that of **F9**, it appears that the two adjacent side chains of hexafluoroleucine at positions 8 and 9 have enough steric bulk to perturb the spatial arrangement of His7. Overall, analogues with a lower receptor affinity, by and large, exhibited a higher EC_{50} value with respect to activation of adenylyl cyclase.

Proteolytic Stability. The major obstacle of using natural GLP-1 as a therapeutic is its rapid inactivation by the ubiquitous protease DPP IV ($t_{1/2} \approx 2$ min, iv in humans).[4] DPP IV is highly discriminating of the identity of residues at positions proximal to the scissile amide bond.[36] In particular, Pro and Ala are highly favored at the P1 position.[36] Other amino acids at this position enhanced the stability of peptides, as reported in the cases of GLP-1 derivatives with the substitutions Gly8, Aib8, Ser8, Thr8, Leu8.[37,38] However, other positions such as P1′ and P2′ have received only limited scrutiny. We found that substitution by hexafluoroleucine confers DPP IV resistance not only at position 8 but also at positions 9 and 10 (Figure 4). **F8** and **F89**

Table 1. Summary of the Receptor Binding, cAMP Production, and Proteolytic Stability of GLP-1 and Fluorinated Analogues

	binding to *h*GLP-1R			cAMP production			cleavage by DPP IV	
	IC_{50} (nM)[a]	max ($CI_{95\%}$)[b]	ratio	EC_{50} (nM)[c]	max ($CI_{95\%}$)	ratio	k (min^{-1})	ratio
GLP-1	1.9	99.1–104.9	1.0	1.0	95.6–103.8	1.0	0.0061	1
F8	52.2	94.6–103.2	27.3	73.0	82.6–94.9	73.8	0[d]	
F9	5.1	96.5–103.9	2.7	2.0	95.9–106.0	2.1	0.0050	1.5
F89	107.3	95.9–103.9	56.2	374.7	25.5–33.2	378.5	0[d]	
F10	113.1	95.8–104.2	59.2	67.3	82.0–98.1	68.0	0.0021	2.9
F28	18.9	98.1–106.9	9.9	5.4	99.0–108.5	5.5	0.0046	1.3
F29	13.4	92.4–101.7	7.0	3.6	91.0–111.0	3.6	0.0056	1.1
F32	57.1	94.6–103.0	29.9	2.4	88.1–106.8	2.4		

[a] IC_{50}: concentration required for 50% inhibition of the maximal binding. [b] $CI_{95\%}$: 95% confidence intervals. [c] EC_{50}: concentration required for producing 50% of the maximal response. [d] No detectable hydrolysis after 24 h.

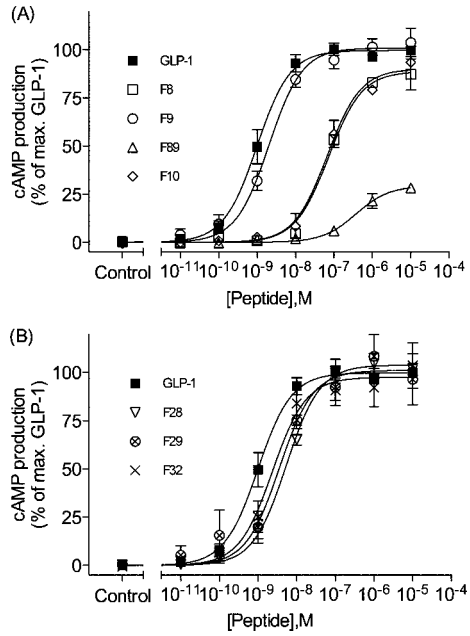

Figure 3. cAMP production stimulated by GLP-1 and fluorinated analogues using a radioimmunoassay with [^{125}I]-cAMP as the tracer. All values are normalized to the maximal cAMP level induced by GLP-1. (A) *N*-Terminal analogues; (B) *C*-terminal analogues. Data represent five independent experiments in duplicate as mean ± SEM.

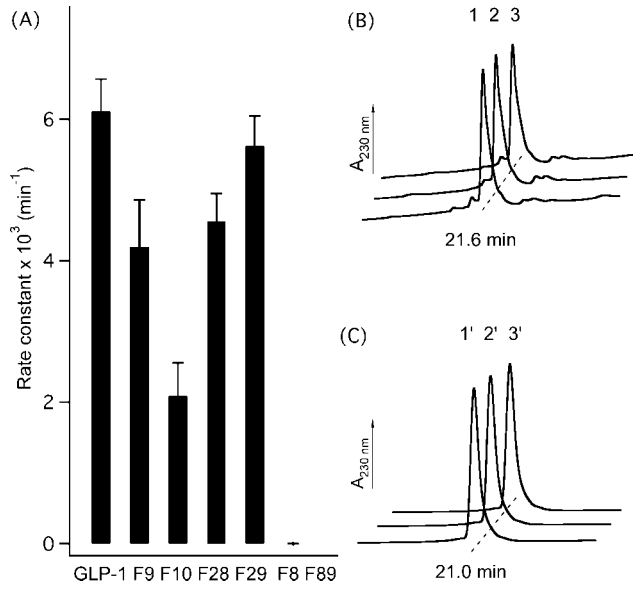

Figure 4. (A) Rate constants of peptide degradation by DPP IV. [Peptide] = 10 μM. [DPP IV] = 20 U/L. Error bars represent one standard deviation. (B) RP-HPLC traces of digestive mixtures of **F8**. Peaks 1, 2, and 3 represent **F8** at 0, 48 h at [DPP IV] = 20 U/L, and 1 h at [DPP IV] = 200 U/L. (C) RP-HPLC traces of digestive mixtures of **F89**. Peaks 1′, 2′, and 3′ denote **F89** at 0, 1, and 24 h at [DPP IV] = 20 U/L. No digestive products for either **F8** or **F89** by DPP IV were detectable. HPLC traces are offset along the time axis for clarity.

completely resisted enzymatic hydrolysis, as no fragments were detected after 24 h incubation with DPP IV (Figure 4B,C). **F8** was further incubated with DPP IV at a 10-fold higher concentration; no digested fragments were detected after 1 h. **F9** and **F10** exhibited 1.5-fold and 2.9-fold increased stability relative to GLP-1. RP-HPLC analysis showed the formation of only one other major peak, which was identified by ESI-MS to be the peptide fragments GLP-1(9-36), or fluorinated GLP-1(9-36). The kinetic data reported here for the fluorinated GLP-1 analogues could be correlated to prolonged metabolic stability in vivo, which has been established by Deacon and co-workers.[38] Green et al.[39] have reported that intraperitoneal injection of Val8-GLP-1 into (*ob*/*ob*) mice resulted in increased insulin levels and reduced plasma glucose presumably due to its protracted lifetime. Indeed, Val8-GLP-1 is 220-fold and 3.5-fold less potent than GLP-1 with respect to in vitro receptor binding and cAMP stimulation.[40] McIntosh and colleagues[41] have recently demonstrated that subcutaneous administration of complete DPP IV resistant Ser8(P)2-GLP-1 into Wistar rats resulted in a more pronounced reduction in the glycemic profile, although it was deemed not to be statistically significant. Therefore, **F8**, **F9**, and **F10** have the potential as candidates for further animal glucose tolerance study (see Supporting Information).

Conclusion and Outlook

In our continuing efforts to probe effects of fluorination on peptides/proteins, the results here are informative in that this is the first study of hexafluoroleucine containing hormonal ligand−receptor interaction investigated to date. Introduction of hexafluoroleucine into GLP-1 at strategic sites conferred resistance against its regulatory protease, DPP IV. Although the in vitro binding affinity and signal transduction activity decreased slightly, the all-important efficacy was retained in 6 out of 7 fluorinated analogues. Other interesting findings were that (1) the P2′ site had a significant impact on protease stability, (2) double substitutions at 8 and 9 positions turned GLP-1 into a partial agonist with retention of maximal binding ability, (3) **F9** was similar to natural GLP-1 in every aspect, suggesting a large hydrophobic side chain is well tolerated at position 9 or that the CF_3 group is able to make multipolar contacts in the binding interface. Considering the successful introduction of fluorine into small molecule drugs, incorporation of fluorinated amino acids into naturally bioactive peptides demands further investigation. We are currently conducting further in vivo studies

on fluorinated GLP-1s so that the integrated metabolic effects can be evaluated. We are also exploring suitable systems for further application of fluorinated amino acids in modulating biological functions.

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Supporting Information Available: Experimental procedures and analytical data. This material is available free of charge via the Internet at http://pubs.acs.org.

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