The closely related peptides glucagon-like peptide (GLP-1) and glucagon have opposing effects on blood glucose. GLP-1 induces glucose-dependent insulin secretion in the pancreas, whereas glucagon stimulates gluconeogenesis and glycogenolysis in the liver. The identification of a hybrid peptide acting as both a GLP-1 agonist and a glucagon antagonist would provide a novel approach for the treatment of type 2 diabetes. Toward this end a series of hybrid peptides made up of glucagon and either GLP-1 or exendin-4, a GLP-1 agonist, was engineered. Several peptides that bind to both the GLP-1 and glucagon receptors were identified. The presence of glucagon sequence at the N terminus removed the dipeptidylpeptidase IV cleavage site and increased plasma stability compared with GLP-1. Targeted mutations were incorporated into the optimal dual-receptor binding peptide to identify a peptide with the highly novel property of functioning as both a GLP-1 receptor agonist and a glucagon receptor antagonist. To overcome the short half-life of this mutant peptide in vivo, while retaining dual GLP-1 agonist and glucagon antagonist activities, site-specific attachment of long chained polyethylene glycol (PEGylation) was pursued. PEGylation at the C terminus retained the in vitro activities of the peptide while dramatically prolonging the duration of action in vivo. Thus, we have generated a novel dual-acting peptide with potential for development as a therapeutic for type 2 diabetes.

Glucagon-like peptide-1 (GLP-1) and glucagon are members of a family of structurally related peptide hormones, the glucagon/secretin family. GLP-1 and glucagon originate from a common precursor, preproglucagon, which upon tissue-specific processing leads to production of GLP-1 in the intestine and glucagon in the pancreas (for review see Ref. 1). GLP-1 and glucagon constitute highly homologous peptides, displaying identity at 14 positions (45% identity) and similarity at 31 positions. The receptors for these two peptides are homologous (58% identity) and belong to the family of G-protein-coupled receptors (2, 3). However, despite the high degree of homology between the peptides and the homology between their receptors, GLP-1 and glucagon bind with a high degree of selectivity to their respective receptors (4). Exendin-4 is a structural homolog of GLP-1 (50% amino acid identity) isolated from saliva of the Gila monster that also acts as a potent agonist of the GLP-1 receptor (5).

Both GLP-1 and glucagon play major, but opposing, roles in overall glucose homeostasis. GLP-1 is synthesized in intestinal endocrine cells and induces glucose-dependent insulin secretion from the pancreas and thereby acts to lower plasma glucose concentrations without the risk of hypoglycemia (6, 7). This activity of GLP-1 has made it attractive as a therapeutic approach for the treatment of type 2 diabetes (for review see Ref. 8). Glucagon is secreted from α-cells in pancreatic islets in a glucose-dependent manner. Glucagon stimulates glycogenolysis and gluconeogenesis in the liver, resulting in elevation of plasma glucose. Glucagon has a critical role in maintaining serum glucose concentration, and glucagon receptor antagonists are being pursued as a potential therapeutic approach to inhibit hepatic glucose output (for review see Ref. 9). A molecule capable of both activation of the GLP-1 receptor and inhibition of the glucagon receptor has potential to be a highly effective and novel approach to control blood glucose in the treatment of type 2 diabetes.

Structure-activity studies have been performed to determine the role of individual amino acids within both GLP-1 and glucagon sequences. GLP-1 and glucagon have no defined structure in aqueous solution, but in the presence of micelles, adopt an α-helical structure in the midsection, with flexible N- and C-terminal regions (10, 11). This suggests that the helical structure is required for binding to their respective receptors. Mutations in the N-terminal region of both peptides result in receptor antagonists, suggesting the importance of the N terminus for receptor activation by both GLP-1 (12, 13) and glucagon (14–18).

In addition to the N-terminal region, many amino acids within the glucagon sequence have been shown to contribute to receptor binding and activation (for review see Ref. 19). For example, mutations at positions 11, 12, 16, 17, and 18 of glucagon appear to negatively affect receptor activation more than binding (20–23). Some possible increase in glucagon antagonism may also be provided by changes in amino acids 5, 7, 9, 11, 13, 21, and 29 (24).

For GLP-1, extensive mutational analysis has been reported. For example, alanine-scanning mutagenesis on GLP-1-(7–36)-amide implicates positions 1, 4, 6, 7, 9, 13, 15, 22, 23, and 26 to be critical for receptor binding (25, 26). On the other hand, combinatorial alanine substitutions at positions 8, 11, 12, and 16 of GLP-1 have a minimal effect on GLP-1 receptor binding and activation (27). In vivo, GLP-1 is rapidly degraded by dipeptidylpeptidase IV (DPP-IV), a protease responsible for cleaving peptides containing proline or alanine residues in the penultimate N-terminal position, resulting in the inactive GLP-1-(9–36)-amide metabolite (28). Removal of the DPP-IV site by mutagenesis greatly

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2 The abbreviations used are: GLP-1, glucagon-like peptide-1; IPGTT, intraperitoneal glucose tolerance test; DPP, dipeptidylpeptidase; PEG, polyethylene glycol; PEGylation, PEGylation of GLP-1; PEGylated, covalent attachment of long chained PEG to a target molecule; PBS, phosphate-buffered saline; TES, 2-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]aminoethanesulfonic acid; AUC, area under the curve; PEG-DAPD, PEGylated dual-acting peptide for diabetes; FA, fatty acid.
improves plasma stability (29). Exendin-4 has a prolonged half-life in vivo relative to GLP-1 likely due to the lack of the DPP-IV site (5).

The similarity between GLP-1 and glucagon, and between their respective receptors, raises the possibility of producing a hybrid peptide that can bind to both receptors. Indeed, Hjorth et al. (4) generated a chimeric peptide consisting of the N-terminal part of the glucagon molecule joined to the C-terminal part of the GLP-1 molecule that displayed high affinity for both receptors. However, the design of a peptide that binds both receptors and exhibits agonist activity on the GLP-1 receptor but antagonist activity on the glucagon receptor is more challenging.

A therapeutic drug based upon a hybrid peptide with dual GLP-1 agonist and glucagon antagonist action would still have to overcome the very short half-life (<5 min) inherent to its parent peptides, which makes them unsuitable for the treatment of type 2 diabetes. PEGylation, the covalent attachment of long chained polyethylene glycol (PEG) to a target molecule, has been successfully employed to increase the circulation half-life of proteins, allowing once-a-week dosing (for review see Ref. 30). PEGylation protects the protein from protease digestion and keeps the protein out of the kidney filtrate. However, the high molecular weight PEGs required for significant improvement of plasma half-life tend to have a negative effect on protein activity, due to steric hindrance. Given the small size of GLP-1 and glucagon peptides, and the fact that functionally important amino acids are spread throughout the peptide sequences, it is a further challenge to generate a PEGylated peptide that retains dual GLP-1 agonist and glucagon antagonist activities.

In the current study, we have engineered hybrid glucagon/GLP-1 peptides with targeted mutations that result in the identification of a peptide with both GLP-1 agonist and glucagon antagonist activity. We believe this is the first example of a single chimeric peptide that possesses opposing agonist and antagonist effects on related receptors. To enhance the duration of action, we have site-specifically modified the hybrid peptides with high molecular weight PEGs and succeeded in identifying a PEGylated peptide retaining both GLP-1 agonist and glucagon antagonist activities.

**EXPERIMENTAL PROCEDURES**

**Peptide Synthesis and PEGylation**—Peptides were supplied by Sigma Genosys (The Woodlands, TX) or SynPep (Dublin, CA). The peptides were characterized by high-performance liquid chromatography and mass spectrometry and were >90% pure (data not shown). Peptides were site-specifically PEGylated with 22- or 43-kDa PEG-maleimide purchased from Nektar Therapeutics (San Carlos, CA) and purified to >95% purity (data not shown).

**RINm5F Cell Culture**—RINm5F cells were maintained in RPMI 1640 medium containing 5% fetal bovine serum (IRI Biosciences) and 1% antibiotic-antimycotic solution (Invitrogen) at 37 °C in a humidified 5% CO₂ incubator.

**Preparation of RINm5F Cell Membranes**—RINm5F cells were washed with PBS, scraped in ice-cold HES buffer (20 mM Heps, 1 mM EDTA, 250 mM sucrose buffer containing protease inhibitors), and homogenized. Unbroken cells and nuclei were removed by centrifugation at 500 × g for 5 min at 4 °C. The supernatant was centrifuged at 40,000 × g for 20 min. Membranes were resuspended in HES.

**Preparation of Plasma Membranes from Rat Liver**—Male Sprague-Dawley rats were sacrificed, and livers were removed and placed into ice-cold TES buffer (20 mM Tris, pH 7.5, 1 mM EDTA, 255 mM sucrose containing protease inhibitors). The tissue was minced in 5 volumes of ice-cold TES, and a slurry was prepared using a Polytron. The slurry was further homogenized using a handheld Dounce homogenizer. The homogenate was passed through several layers of cheesecloth and spun at 25,000 × g for 10 min. The pellets were resuspended in TES, and plasma membranes were isolated from the interface of 1.2 M sucrose and TES cushion centrifugation at 180,000 × g. The resulting membranes were washed with TES using a 15-min centrifugation at 200,000 × g, resuspended in TES again, and purified on a second sucrose cushion at 200,000 × g. The final pellet was resuspended in TES.

**Competitive Binding of Peptide to the GLP-1 and Glucagon Receptors**—GF/C filtration plates (Millipore, Bedford, MA) were blocked with 0.3% or 0.1% polyethyleneimine for RINm5F membranes and rat liver plasma membranes, respectively. Filter plates were washed twice with binding buffer consisting of 20 mM Tris, 2 mM EDTA, pH 7.5, 1 mg/ml bovine serum albumin, and 1 mg/ml bacitracin. 5 μg of RINm5F cell plasma membranes or 3–5 μg of rat liver plasma membranes diluted in binding buffer were combined with 0.05 μCi of 125I-labeled GLP-1 or 125I-labeled glucagon and unlabeled test peptide at the indicated concentrations. Following a 1-h incubation at room temperature, the plates were washed three times with ice-cold PBS containing 1 mg/ml bovine serum albumin. The plates were dried, scintillant was added to each well, and plates were counted using a Wallac Microbeta counter.

**Measurement of Peptide Signaling through GLP-1 Receptor**—1.5 × 10⁵ RINm5F cells per well were seeded in 96-well plates and grown overnight. The cells were then washed twice with PBS and then incubated with peptide in Hepes-PBS containing 1% bovine serum albumin and 100 μM isobutylmethylxanthine for 15 min at 37 °C. The cells were lysed, and intracellular cAMP was determined using the cAMP Scintillation Proximity Assay direct screening assay system (Amersham Biosciences).

**Measurement of Peptide Signaling through Glucagon Receptor**—Hepatocytes were isolated according to a modified procedure of Berry (31). Freshly isolated rat hepatocytes were plated in 96-well plates at 7.5 × 10⁵ cells/well in Hepes-bicarbonate-PBS containing 1% bovine serum albumin and 100 μM isobutylmethylxanthine. Following equilibration at 37 °C in a 5% CO₂/95% O₂ environment for 10 min, peptide was added for an additional 15 min. The cells were lysed, and intracellular cAMP was determined as described above. The ability of the hybrid peptide to inhibit glucagon activity was measured as follows: Following equilibration at 37 °C in a 5% CO₂/95%O₂ environment for 10 min, 10 μM peptide was added to the cells followed immediately by either 2 or 10 nM glucagon for 15 min. The cells were lysed, and cAMP was determined.

**Measurement of Glucose Release from Rat Hepatocytes**—Hepatocytes were added into a flat bottom 96-well plate (2 × 10⁵/100 μl/well) and preincubated in a 37 °C incubator with constant shaking and under 95% O₂/5% CO₂ flow for 10 min. Hepatocytes were incubated for another 30 min after addition of glucagon with or without peptide. Cells were then lysed with 15% perchloric acid and plates were spun. The supernatant was neutralized with 1 M Tris-HCl (pH 8.0)/2.5 N KOH (45:55) and spun again. The resulting supernatant was analyzed for glucose with hexokinase and glucose-6-phosphate dehydrogenase and the A340 read on a fMAX plate reader (Molecular Devices, Sunnyvale, CA). Glucose output was calculated in the following manner: after subtracting the amount of glucose produced in the unstimulated hepatocytes from each data point, the percent inhibition was calculated.

**Insulin Release from Perifused Rat Islets**—The bi-phasic response of insulin release stimulated by peptides was tested in perifused rat islets. Fifty islets were loaded in a perfusion chamber and perfused with HEPES-Kreb’s-Ringer bicarbonate buffer containing 3 mM glucose at 37 °C. After 60 min, islets were exposed to buffer containing 8 mM glucose with or without peptide (50 nM) and perifused for another 30 min. Fractions of perfusate were collected at 1- or 5-min intervals for insulin determination. Insulin was measured using an enzyme-linked immunosorbent assay kit (Alpco Diagnostics, Windham, NH).
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Measurement of Peptide Stability in Rat Plasma—Fresh rat plasma was collected from male Wistar rats. The peptides were diluted in 100% rat plasma to 10-fold desired final concentration and incubated at 37 °C for 0, 3, 6, 18, or 24 h. Samples were then added to RINm5F cells, and cAMP production was measured after a 15-min exposure as outlined above. Percent peptide remaining was determined by comparing cAMP production at a given time point to cAMP production in a freshly diluted sample (zero time).

Intraportal Glucose Tolerance Test—Male Wistar rats (220–250 g) were purchased from Harlan (Indianapolis, IN). All procedures were approved by the Bayer Animal Care and Use Committee, and all experiments were performed in accordance with relevant guidelines and regulations. Male Wistar rats were either fasted overnight and then given peptide or vehicle by subcutaneous injection or they were given peptide or vehicle first and then fasted overnight, depending on the time interval between dosing and when the IPGTT was to be performed. At the appropriate time after dosing, the fasting blood glucose level was measured from tail-tip blood using a Glucometer (Bayer HealthCare, Mishawaka, IN), and the animals were given 2 g/kg glucose by intraperitoneal injection. Blood glucose was measured again after 15, 30, and 60 min. The area under the glucose curve (AUC) was calculated using the trapezoidal method, and the effect of the peptide on the AUC was expressed as a percentage of the AUC for the vehicle-treated group.

Statistical Analysis—In vitro results are means ± S.E. for the number of experiments (n) indicated in the figure legends. In vitro assay statistics were calculated by t test using GraphPad Prism (GraphPad Software, Inc., San Diego, CA). In vivo data are expressed ± S.E. Statistical analyses were performed using InStat (GraphPad). Treatment effects were analyzed by analysis of variance with post hoc analysis using Tukey-Kramer Multiple Comparisons Test (parametric methods) or the Kruskal-Wallis Test (non-parametric methods) when necessary. Differences are considered significant if p values of <0.05.

RESULTS

To engineer a long acting dual GLP-1 agonist and glucagon antagonist peptide, a three-stage mutagenesis strategy was employed. In the first stage, glucagon, GLP-1, and the related GLP-1 agonist, exendin-4, were combined to generate a series of chimeric peptides with the goal of identifying peptides with high affinity toward GLP-1 and glucagon receptors. In stage two, mutations known to antagonize the glucagon receptor but not known to antagonize the GLP-1 receptor were selectively introduced into the GLP-1/glucagon binding-optimized chimeric peptide identified in stage one, leading to the identification of a peptide with potential to act as both a GLP-1 agonist and glucagon antagonist. The GLP-1 agonist and glucagon antagonist activities of the stage two selected peptide was then tested in secondary cell assays to examine its ability to promote pancreatic islet insulin secretion and inhibit glucagon-induced hepatocyte glucose output. Finally, in stage three, cysteine and lysine mutagenesis procedures were performed to identify a position suitable for attachment of a high molecular weight PEG to prolong duration of action in vivo.

Stage 1: Optimization of Dual Binding Affinities to GLP-1 and Glucagon Receptors—As previously reported, both glucagon and GLP-1 displayed high affinity (IC50) and potency (EC50) for their own receptors but no significant cross-reactivity for the other receptor (Table 2). To identify novel peptides capable of binding to both receptors, 15 peptides (A1–A15) were synthesized (Table 1). Peptides A1 to A6 were designed to progressively introduce GLP-1-specific amino acids into the glucagon sequence from the C terminus. The N-terminal 18 amino acids of glucagon are critical for high affinity binding to its receptor, because C-terminal replacements with GLP-1 sequence beyond this point greatly reduce glucagon binding affinity (Table 2). The IC50 of A4–A6 at the glucagon receptor were at least 10-fold higher than that of peptides A1–A3. Exchange of only the three C-terminal amino acids of glucagon with the corresponding C-terminal GLP-1 amino acids, while only causing <3-fold decrease in glucagon receptor affinity, reduced glucagon agonist activity by >100-fold, as exhibited by peptide A1. Therefore, the precise amino acid sequence of the C-terminal portion of glucagon appears to be required for activation but not binding to the glucagon receptor. In contrast, binding to the GLP-1 receptor is much more tolerant to insertion of glucagon amino acids into the GLP-1 sequence. Even peptide A1, with 12 amino acid differences from GLP-1 in the N-terminal 26 positions, displayed relatively high affinity (IC50 = 6 nM) and potency (EC50 = 21.7 nM) for the GLP-1 receptor. Successive introduction of more GLP-1 amino acids into glucagon further enhanced activity at the GLP-1 receptor culminating in peptide A6, which has four amino acid differences from GLP-1 but almost an identical IC50 and EC50 as GLP-1. Peptide A3, a chimera sequence of glucagon–(1–19) and GLP-1–(20–30) possesses the most desired in vitro profile of a potent GLP-1 agonist (EC50 = 4.9 nM) and a strong glucagon receptor binder (IC50 = 7.7 nM). Moreover, peptide A3 is a weak glucagon agonist (EC50 = 361 nM) and could potentially act as a glucagon antagonist. However, consistent glucagon antago-
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TABLE 2
Hybrid peptide GLP-1/glucagon receptor binding and activity
GLP-1 and glucagon receptor binding (IC$_{50}$) and activation (EC$_{50}$) to receptors on RINm5F cells or rat liver membranes, respectively. Data are the mean ± S.E. of at least two experiments.

| Peptide | GLP-1 receptor | Glucagon receptor |
|---------|----------------|------------------|
|         | IC$_{50}$ (nM) | EC$_{50}$ (nM) | IC$_{50}$ (nM) | EC$_{50}$ (nM) | Maximum (%) |
| Glucagon | >1 µM | 1.9 ± 0.5 | 2.3 ± 0.3 | 4.1 ± 1.2 | 100 |
| GLP-1   | 0.4 ± 0.1 | 0.4 | >1 µM | >1 µM | 100 |
| Exendin-4 | 0.4 | 0.8 ± 0.2 | 6.6 ± 1.2 | 505 ± 142 | 103 ± 6.3 |
| A1      | 6.0 ± 1.7 | 21.7 ± 7.1 | 8.4 ± 1.3 | 567 ± 129 | 96.6 ± 7.4 |
| A2      | 3.5 ± 0.4 | 14.4 ± 4.0 | 7.7 ± 1.6 | 361 ± 124 | 92.9 ± 4.0 |
| A3      | 0.9 ± 0.2 | 4.9 ± 1.0 | 84 ± 14 | 365 | 100 |
| A4      | 0.18 ± 0.07 | 10.5 ± 6.8 | 190 ± 96 | 3021 ± 954 | 99.7 ± 9.5 |
| A5      | 2.1 ± 12 | 15.1 ± 0.7 | 243 ± 71 | 938 ± 200 | 106.5 ± 8.6 |
| A6      | 0.5 ± 0.1 | 2.0 ± 0.6 | 96 ± 8 | 75 ± 23 | 80.9 ± 8.4 |
| A7      | 12.3 ± 3.2 | 39 ± 15 | 9.7 ± 0.3 | 128.8 ± 5.3 | 80.2 ± 3.8 |
| A8      | 1.9 ± 0.7 | 5.6 ± 1.8 | 53 | 108 | 80.3 |
| A9      | 0.2 ± 0.1 | 9.2 ± 4.6 | — | — | — |
| A10     | 0.4 ± 0.1 | 6.6 ± 3.3 | — | — | — |
| A11     | 0.8 | 8.8 ± 6.9 | 21 ± 13 | 13 | 89.4 |
| A12     | 0.6 ± 0.4 | 6.1 ± 5.5 | 106 ± 10 | 92 | 92.3 |
| A13     | 0.2 ± 0.02 | 1.6 ± 0.4 | 452 ± 238 | 1458 ± 340 | 97.9 ± 16.7 |
| A14     | 0.27 ± 0.04 | 1.9 ± 0.3 | 216 ± 48 | 543 ± 177 | 78.4 ± 9.2 |
| A15     | 41 ± 12 | >1 µM | 101 ± 9 | >10 µM | 10.5 ± 10.5 |
| AN1     | 99 ± 4.7 | >1 µM | 264 ± 101 | >10 µM | 0.6 |
| AN2     | 28 ± 11 | 91 ± 19 | 13.1 ± 6.3 | 143 ± 25 | 81.3 ± 6.2 |
| AN3     | 64 ± 25 | 132 ± 19 | 372 ± 180 | >10 µM | 6.8 |
| AN4     | 5.9 ± 3.6 | 40 ± 17 | 116 ± 25 | 321 ± 102 | 46.8 ± 17.7 |
| AN5     | 1.5 ± 0.7 | 12.9 ± 3.3 | 28.8 ± 6.3 | 468 ± 182 | 72.1 ± 11.1 |
| AN6     | 4.5 ± 2.3 | 11.6 ± 0.6 | 31 ± 10 | 241 | 94.7 |
| AN7     | 0.6 ± 0.1 | 2.5 ± 0.5 | 16.4 ± 7.8 | 166 ± 80 | 103.1 ± 18.0 |
| AN8     | 10.2 ± 2.6 | 53 ± 14 | 42 ± 34 | >10 µM | 0.0 |
| AN9     | 3.0 ± 0.7 | 13.3 ± 4.9 | 69 ± 16 | 713 ± 140 | 43.6 ± 4.3 |
| AN10    | 9.7 ± 0.2 | >1 µM | 43 ± 26 | >10 µM | 0.0 |
| AN11    | 11.4 ± 4.3 | >1 µM | 26 ± 20 | >10 µM | 0.0 |
| AN12    | 3.0 ± 0.7 | >1 µM | NT | NT | NT |
| AN13    | 6.1 ± 29 | 463 ± 197 | 591 ± 176 | NT | NT |
| AN14    | 2.7 ± 16 | 31 ± 22 | 136 ± 100 | 705 ± 440 | 75.5 ± 16.0 |
| AN15    | 17.5 ± 6.3 | >1 µM | 163 ± 47 | 837 ± 656 | 58.2 ± 18.3 |
| AN16    | 40 ± 19 | 171 | 799 ± 245 | >10 µM | 2.7 ± 3.9 |
| AN18    | >1 µM | 1429 ± 364 | NT | NT | NT |

* NT, not tested.

Similar substitution of glucagon with exendin-4 amino acids (A7–A11) showed comparable results at the GLP-1 receptor, suggesting GLP-1- or exendin-4-specific amino acids in the N-terminal portion are not required for potent activities at the GLP-1 receptor. Unlike the GLP-1 amino acids, substitution of exendin-4 amino acids into glucagon had a more profound effect on glucagon receptor binding. Only peptide A8 displayed high affinity and potency at the glucagon receptor. Introduction of a combination of GLP-1 and exendin-4 amino acids into peptides (A12–A15) showed a similar trend and again did not produce any peptide with a better profile with respect to GLP-1 agonist activity and lack of glucagon agonist activity when compared with peptide A3. Thus, peptide A3 was selected as the lead peptide for this stage and the template sequence for the next stage of mutagenesis.

Stage 2: Optimization of GLP-1 Agonism and Glucagon Antagonism—To generate a peptide mutant that retains GLP-1 agonism but blocks glucagon from activating its receptor (i.e. glucagon antagonism) 18 peptides were designed that introduced known glucagon antagonist mutations into the template peptide A3 (Table 1). Mutations at positions 11, 12, and 16, which have been shown to affect glucagon receptor activation much more than glucagon receptor binding (21, 32), had a modest effect on GLP-1 receptor binding and activation as demonstrated by peptides AN5–AN10 and AN15 (Table 2). On the other hand, mutations in the N-terminal region, including residues 1, 3–5, and 9, which lead to strong glucagon antagonism (14–18), also greatly affected interactions at the GLP-1 receptor as illustrated by peptides AN1–AN4, AN11–AN14, and AN16–AN18. Furthermore, with the exception of peptides AN3, AN11, and AN12, these N-terminal mutations reduced glucagon receptor binding affinity by at least 10-fold as compared with the template peptide A3. Therefore, even though these mutations had little effect on glucagon receptor binding in the context of the glucagon sequence, they had a greater effect when combined with the C-terminal GLP-1 sequence. Unlike the parent peptide A3, the S11A/S16A double mutant, AN10, is only a partial agonist at the glucagon receptor, making it more likely to act as a glucagon antagonist.

**Peptide AN10:** A GLP-1 Agonist and Glucagon Antagonist—The hybrid peptide AN10 was selected from the panel of 33 peptides analyzed as having the optimal in vitro characteristics with respect to GLP-1 and glucagon receptor binding and activity. Peptide AN10 binds to the GLP-1 and glucagon receptors with IC$_{50}$ = 3 nM and 69 nM, respectively (Table 2 and Fig. 1, a and c). Peptide AN10 activates the GLP-1 receptor (EC$_{50}$ = 13 nM) and antagonizes the receptor to 98% the maximal level of receptor activation achieved by GLP-1 (Table 2 and Fig. 1d). Peptide AN10 is a weak agonist of the glucagon receptor (EC$_{50}$ = 713 nM) reaching only 44% the maximum activity of glucagon (Table 2 and Fig. 1d).

Peptide AN10 (10 µM) antagonizes glucagon (2 nM)-mediated cAMP production 22% in rat hepatocytes. The benchmark glucagon antagonist peptide DesHis(Glu)$^3$ glucagon (10 µM) antagonizes glucagon (2 nM)-
Design of GLP-1 Agonist/Glucagon Antagonist Peptides

mediated cAMP production 81% in rat hepatocytes (Fig. 2a). In addition, peptide AN10 (100 nM) antagonizes glucagon (1 nM)-mediated glucose output from rat hepatocytes 39% (Fig. 2b).

GLP-1 agonism was further assessed by examining the ability of peptide AN10 to increase insulin secretion from perfused rat islets. Islets were perfused with 8 mM glucose, 8 mM glucose plus AN10 (50 nM), or 8 mM glucose plus GLP-1 (50 nM), and samples were collected over 25 min. Insulin secretion increased over time in all conditions, but incubation with GLP-1 or AN10 increased the area under the curve 1.8-fold \( (p<0.05) \) as compared with 8 mM glucose alone. Therefore, AN10 increases insulin secretion from perfused rat islets in the presence of 8 mM glucose equivalent to that achieved by GLP-1 (Fig. 3).

The short in vivo duration of action of GLP-1 is accounted for in part by DPP-IV. Because AN10 does not contain the N-terminal sequence of GLP-1 (i.e. it does not contain an alanine in the second position), we tested whether it would be more stable than GLP-1 in plasma. Exendin-4, which has a glycine at position 2 instead of the alanine found in GLP-1, is resistant to DPP-IV cleavage and has prolonged activity in vivo (33). GLP-1, exendin-4, and AN10 were incubated in rat plasma at 37 °C for time periods ranging from 3 to 24 h. At various time points during this period the percentage of active peptide remaining was assessed in the GLP-1 receptor activation assay. For GLP-1, after 6-h incubation in rat plasma only 14% of active peptide remained. At this 6-h time point 40 and 95% of active exendin-4 and AN10 remained, respectively. Furthermore, after 18-h incubation 27 and 52% of active exendin-4 and AN10 remained, respectively (Fig. 4). Thus, AN10 has greatly improved stability in plasma compared with GLP-1.

Stage 3: Optimization of Site-specific PEGylation—Protein PEGylation has often been limited by the loss of potency due to blockage of the protein active site by the large unstructured PEG polymer, which may explain the very few examples of successful peptide, as opposed to protein, PEGylation reported to date. In an effort to maintain both GLP-1 agonism and glucagon antagonism of the hybrid peptide, it was neces-
Rat islets were perifused with a solution containing 8 mM glucose with or without 50 nM GLP-1 or AN10 as indicated. Insulin in the perifusate was measured using enzyme-linked immunosorbent assay. The results are the mean ± S.E. of 10 trials.

FIGURE 3. Insulin secretion from perifused rat islets. Rat islets were perifused with a solution containing 8 mM glucose with or without 50 nM GLP-1 or AN10 as indicated. Insulin in the perifusate was measured using enzyme-linked immunosorbent assay. The results are the mean ± S.E. of 10 trials.

FIGURE 4. Activity of peptides following incubation in rat plasma. Peptides were incubated in 100% rat plasma at the indicated concentrations, for the indicated time periods. The samples were then added to RINm5F cells, and CAMP production was measured after a 15-min exposure. Percent peptide remaining was determined by comparing CAMP production at a given time point to CAMP production in a freshly diluted sample (zero time).

MODEL OF AN10G. The "twisted helix" model of AN10G when in contact with the cell surface. The helical backbone of AN10G is represented by a thin ribbon. Receptor binding side of the peptide is predicted to be at the top, surrounded by the nine hydrophobic side chains (depicted as thick sticks) Phe⁷, Tyr⁹, Tyr¹¹, Leu¹³, Phe⁲⁵, Leu²⁶, and Val²⁷. Side chains for the remaining residues (all hydrophilic) are shown as thin sticks. The flexible N-terminal region is predicted to insert into the receptor during receptor activation. The C-terminal three residues are also expected to be flexible. Each Cα atom of the seven positions within GLP-1 sequence chosen for PEGylation is represented by a ball.

FIGURE 5. Model of AN10G. The "twisted helix" model of AN10G when in contact with the cell surface. The helical backbone of AN10G is represented by a thin ribbon. Receptor binding side of the peptide is predicted to be at the top, surrounded by the nine hydrophobic side chains (depicted as thick sticks) Phe⁷, Tyr⁹, Tyr¹¹, Leu¹³, Phe⁲⁵, Leu²⁶, and Val²⁷. Side chains for the remaining residues (all hydrophilic) are shown as thin sticks. The flexible N-terminal region is predicted to insert into the receptor during receptor activation. The C-terminal three residues are also expected to be flexible. Each Cα atom of the seven positions within GLP-1 sequence chosen for PEGylation is represented by a ball.

Design of GLP-1 Agonist/Glucagon Antagonist Peptides

TABLE 3

Cysteine and lysine mutant peptides

| Peptide | Sequence       |
|---------|----------------|
| AN10G   | HSQGKTSDVAKLDAKRAKEFIAWLKVGRG |
| ANC1    | HSQGKTSDVAKLDAKRAKEFIAWLKVGRG |
| ANC2    | HSQGKTSDVAKLDAKRAKEFIAWLKVGRG |
| ANC3    | HSQGKTSDVAKLDAKRAKEFIAWLKVGRG |
| ANC4    | HSQGKTSDVAKLDAKRAKEFIAWLKVGRG |
| ANC5    | HSQGKTSDVAKLDAKRAKEFIAWLKVGRG |
| ANC6    | HSQGKTSDVAKLDAKRAKEFIAWLKVGRG |
| ANC7    | HSQGKTSDVAKLDAKRAKEFIAWLKVGRG |
| ANC8    | HSQGKTSDVAKLDAKRAKEFIAWLKVGRG |
| ANC9    | HSQGKTSDVAKLDAKRAKEFIAWLKVGRG |
| ANC10   | HSQGKTSDVAKLDAKRAKEFIAWLKVGRG |
| ANC11   | HSQGKTSDVAKLDAKRAKEFIAWLKVGRG |
| ANK1    | HSQGKTSDVAKLDAKRAKEFIAWLKVGRG |
| ANK2    | HSQGKTSDVAKLDAKRAKEFIAWLKVGRG |
| ANK3    | HSQGKTSDVAKLDAKRAKEFIAWLKVGRG |
| ANK4    | HSQGKTSDVAKLDAKRAKEFIAWLKVGRG |
| ANC7K2  | HSQGKTSDVAKLDAKRAKEFIAWLKVGRG |

Bold font indicates mutated amino acids.

Peptide ANC1–ANC11 and ANK1–ANK4 were analyzed for GLP-1 receptor potency (EC₅₀ < 50 nM) (Table 4). Likewise, with the exception of ANC2, ANC10, and ANC11, these peptides exhibited reasonable glucagon...
Design of GLP-1 Agonist/Glucagon Antagonist Peptides

TABLE 4
Receptor binding and activity of the cysteine and lysine mutant peptides
GLP-1 and glucagon receptor binding (IC\text{50}) and activation (EC\text{50}) to receptors on RINm5F cells or rat liver membranes, respectively. Inhibition of glucagon activity is determined by assaying rat hepatocyte response to 10 nM glucagon in the presence or absence of 10 μM peptide. Data are the mean ± S.E. of at least two experiments.

| GLP-1 receptor | Glucagon receptor |
|----------------|-------------------|
| RIN IC\text{50} | Liver IC\text{50} | Maximum glucagon activity |
| µM            | µM               | %                         |
| ANC1          | 5.7 ± 1.4        | 25.6 ± 7.9               | 67.4 ± 12.5 |
| ANC2          | NT*              | 166 ± 44                 | 47.0 ± 7.1  |
| ANC3          | NT               | 74.5 ± 24                | 57.4 ± 13.8 |
| ANC4          | 4.4 ± 0.2        | 21.7 ± 7.8               | 70.4 ± 19.4 |
| ANC5          | 7.7 ± 2.2        | 54.0 ± 6.3               | 55.4 ± 15.9 |
| ANC6          | 10.2 ± 1.7       | 59.1 ± 15.9              | 49.9 ± 15.7 |
| ANC7          | 8.8 ± 4.2        | 65.5 ± 6.6               | 52.4 ± 14.7 |
| ANC8          | NT               | 63.2 ± 19                | 66.5 ± 18.3 |
| ANC9          | NT               | 87.6 ± 7.5               | 66.4 ± 9.9  |
| ANC10         | NT               | 21.4 ± 3.9               | 64.3 ± 7.6  |
| ANC11         | NT               | 18.1 ± 2.2               | 63.2 ± 15.9 |
| ANK1          | 8.1 ± 4.5        | 33.6 ± 7.5               | 50.9 ± 11.6 |
| ANK2          | 3.2 ± 1.6        | 32.3 ± 4.6               | 58.2 ± 14.3 |
| ANK3          | 1.4 ± 0.9        | 11.7 ± 2.5               | 44.1 ± 2.2  |
| ANK4          | 1.5 ± 1.0        | 16.1 ± 2.6               | 47.6 ± 8.7  |
| ANC7K2        | 3.5 ± 0.5        | 117 ± 17                 | 46.8 ± 5.7  |

* NT, not tested.

GLP-1 receptor activity of PEGylated peptides
Potency (EC\text{50}, nM) of PEGylated and non-PEGylated peptides toward the GLP-1 receptor was evaluated in the RINm5F cell cAMP assay as described under "Experimental Procedures." Data are mean ± S.E. of at least two trials.

| Peptide | No PEG | 22-kDa PEG | 43-kDa PEG |
|---------|--------|------------|------------|
| ANC1    | 35.0 ± 3.5 | 346.1 ± 79.0 | 358.6 ± 21.7 |
| ANC2    | 110 ± 15.3 | 476 ± 137.7  | 411.3 ± 16.1 |
| ANC3    | 415 ± 5.0  | 1445 ± 9.5   | 294 ± 50.8  |
| ANC4    | 364 ± 6.9  | 1147 ± 10.1  | 337 ± 22.3  |
| ANC5    | 442 ± 8.1  | 275.8 ± 37.8  | 388.4 ± 24.6 |
| ANC6    | 236 ± 2.1  | 106.6 ± 19.3  | 383.0 ± 7.0  |
| ANC7    | 25.2 ± 1.9  | 49.7 ± 17.6   | 236.1 ± 16.6 |
| ANC8    | 17.4 ± 2.2  | 65.9 ± 15.4   | 91.6 ± 17.4  |
| ANC9    | 16.1 ± 1.7  | 60.2 ± 16.7   | 66.9 ± 14.5  |
| ANC10   | 21.4 ± 3.9  | 98.1 ± 31.8   | 49.6 ± 11.5  |
| ANC11   | 18.1 ± 2.2  | 92.8 ± 1.7    | 49.6 ± 11.5  |
| ANC7K2  | 12.7 ± 1.7  | 16.5 ± 4.3    | 227 ± 19     |

P<0.01)

TABLE 5
GLP-1 receptor activity of PEGylated peptides

Peptide No PEG 22-kDa PEG 43-kDa PEG

GLP-1 receptor activity of PEGylated peptides

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receptor affinity (IC\text{50} < 100 nM). In the glucagon receptor activation assay, none of the peptides reached the maximum of glucagon-induced activity. Peptides ANC4–ANC7 and ANK1–ANK4 inhibited glucagon-induced cAMP activation (>15%). The lysine mutant peptides ANK1–ANK4 appear to possess a better in vitro profile compared with the cysteine mutant peptides ANC1–ANC11 in that the former displays more consistent glucagon receptor antagonism.

The eleven single cysteine mutants were PEGylated with a 22-kDa linear PEG or a 43-kDa branched PEG and tested in the GLP-1 receptor activation assay. PEG had a greater negative effect on activity when introduced on internal positions of the peptide (ANC1–ANC6) than at the C terminus (ANC7–ANC11) (Table 5). Therefore, the optimal site for PEGylation was determined to be at the C-terminal position 31 for 22-kDa PEG (i.e. ANC7). The branched 43-kDa PEG led to even greater activity loss, which can be recovered by inserting spacers between the PEG and the body of the peptide. For example, the 5-amino acid spacer allowed the 43-kDa PEGylated ANC11 to have the same in vitro activity as 22-kDa PEGylated ANC7.

To assess whether PEGylation improves in vivo duration of action ANC7-PEG, which has a cysteine added to the C terminus of AN10G followed by modification with a 22-kDa PEG, and the benchmark peptide, fatty acid-modified GLP-1 (FA-GLP-1), were examined for their ability to lower the blood glucose in an intraperitoneal glucose tolerance test (IPGTT) performed 3 or 17 h after subcutaneous administration of the peptides (100 μg/kg) in mice. FA-GLP-1 is equivalent to the long acting GLP-1 analogue, NN2211/liraglutide (37). When the IPGTT is performed 3 h after peptide administration, both ANC7-PEG (PEGylated with 22-kDa PEG) and FA-GLP-1 significantly lower the blood glucose area under the curve (AUC) by 38% (p < 0.01) and 41% (p < 0.01), respectively. When the IPGTT is performed 17 h after peptide administration FA-GLP-1 fails to significantly lower the glucose AUC, whereas ANC7-PEG decreases the glucose AUC 34% (p < 0.01) (Fig. 6).
We conclude that the PEGylated ANC7 peptide has prolonged activity in vivo.

Based on the overall in vitro profile of unPEGylated peptides (Table 4) and the GLP-1 receptor activity of PEGylated peptides (Table 5), the C-terminal position 31 (peptide ANC7) was selected as the optimal position for cysteine PEGylation. Because the lysine mutants ANK1–ANK4 showed more consistent glucagon receptor antagonism, compared with ANC7, cysteine substitution at position 31 was performed on one of the ANK series of peptides (ANK2) to generate the peptide ANC7K2 (Table 3). ANC7K2 is a cross between ANC7 and ANK2 and was selected as the optimal peptide for PEGylation. ANC7K2 is a potent GLP-1 receptor agonist (12.7 nM EC50) and consistently inhibited the glucagon receptor (Table 4). Modification of ANC7K2 with a 22-kDa PEG had minimal effects on GLP-1 receptor activation (Table 5).

**DISCUSSION**

In this report we describe the design of hybrid peptides with the unique property of activating the GLP-1 receptor but inhibiting the related glucagon receptor. This was achieved by first identifying peptides that bind to both receptors, followed by introduction of mutations that selectively reduce glucagon receptor activation without affecting glucagon receptor binding, while retaining GLP-1 receptor activation. Through detailed structure-activity and mutational analysis we then identified several potential sites for PEGylation. PEGylation at the C-terminal amino acid of the hybrid peptide was shown to retain in vitro activity and displayed prolonged duration of action in vivo.

In a previous study by Hjorth et al. (4), two hybrid peptides, identical to peptides A1 and A6, that bind to both the GLP-1 and glucagon receptors were described. However, the functional activity of these peptides was not described. We determined that peptide A1 is 11-fold less potent compared with GLP-1 in activating the GLP-1 receptor. Peptide A6, while binding to both the GLP-1 and glucagon receptors, has relatively low affinity for the glucagon receptor compared with glucagon itself. Thus these hybrid peptide backbones were not suitable as template sequences for optimization of GLP-1 agonism and glucagon antagonism. To identify a peptide that retains potent GLP-1 receptor activation and glucagon receptor affinity, we designed hybrid peptides A2 to A5 where the number of C-terminal GLP-1 amino acids are intermediate between that of peptides A1 and A6. Receptor binding and activation analysis of these novel peptides revealed A3 as the optimal peptide for GLP-1 receptor activation, with only 2.6-fold reduction in potency, yet retaining glucagon receptor binding affinity. Peptide A3 displays ~3-fold greater potency at the GLP-1 receptor compared with peptide A2, presumably because peptide A3 contains GLP-1 amino acids at positions 20 and 21, adjacent to positions 22 and 23, which are reported to be critical for GLP-1 receptor binding (25, 26). On the other hand, peptides A2 and A3 show comparable potency at the glucagon receptor, consistent with no significant effect on glucagon receptor interaction being detected upon replacement of the amino acid at position 21 of glucagon with that of GLP-1 (22). Likewise, peptide A2 shows ~2-fold greater potency at the GLP-1 receptor but comparable potency at the glucagon receptor compared with peptide A1, consistent with GLP-1 alanine-scanning mutagenesis data and the fact that positions 23 and 24 are not reported to be important for glucagon receptor interaction. Peptide A3 displays ~11-fold greater affinity for the glucagon receptor compared with peptide A4, because peptide A3 contains a glucagon amino acid at position 18, whereas peptide A4 contains a GLP-1 amino acid at this position. When this amino acid is mutated to that of GLP-1 in the context of glucagon, it results in an ~8-fold reduction in glucagon receptor affinity (20). Thus, our results lend further support to previous structure activity studies of GLP-1 and glucagon.

To engineer a selective glucagon antagonist peptide based on the A3 peptide backbone sequence, we introduced mutations known to antagonize the glucagon receptor. The literature was reviewed for glucagon peptide mutations that are reported to reduce receptor activation more than binding. [Tyr'Glu'2, des-His1, and [Glu'4]-glucagon bind to the glucagon receptor with comparable affinity to glucagon, but have at least 100-fold lower activity (17, 18). This is similar to what we observed for peptides AN1, AN2, and AN4, where we introduced the same mutations into the A3 peptide backbone. However, these mutations also greatly affected GLP-1 receptor activation, consistent with alanine-scanning mutagenesis of GLP-1 that suggest the importance of positions 1 and 9 for GLP-1 receptor activation (25, 26). The more conservative H1Y mutation of glucagon slightly improved affinity and reduced activity by 3-fold (17), but in the context of the A3 peptide (i.e. peptide AN3) this mutation did not reduce glucagon receptor activation. More importantly, AN3 is almost 20-fold less potent compared with the A3 peptide in activating the GLP-1 receptor. This indicates that GLP-1 receptor activation is more sensitive to mutations at position 1 compared with glucagon receptor activation.

In addition to positions 1 and 9 of peptide A3, we evaluated positions 3, 4, 5, 12, 17, and 18 based on glucagon antagonists [Asp'3, D-Phe'6, Ser'9, Tyr'17,18, Glu'21]-glucagon and [D-Phe'6, Tyr'3, 5-D1, Tyr'16, Arg'12, Lys'17, 18, Glu'21]-glucagon, which have 20-fold and 2-fold, respectively, lower affinity for the glucagon receptor compared with glucagon (38). In the context of the peptide A3 sequence, we detected a significant loss of glucagon receptor affinity due to the Q3D mutation, as demonstrated by the comparison of AN18 versus AN16. However, even AN16, with the G4H/T5Y/K12R triple mutation lost 21-fold affinity for glucagon receptor, compared with peptide A3. This suggests that the R17K/R18K mutations in [D-Phe'6, Tyr'3, 5-D1, Tyr'16, Arg'12, Lys'17, 18, Glu'21]-glucagon may contribute to improved glucagon receptor affinity. This is supported by the triple mutant R17K/R18K/D21E of glucagon that is five times more potent compared with glucagon in the glucagon receptor binding assay (39). Finally, single mutations at positions 4, 5, and 12 of peptide A3 as found in AN13, AN14, and AN15, respectively, greatly reduced glucagon receptor binding. Although we were able to confirm and further extend the understanding of glucagon receptor binding, mutations at positions 3, 4, 5, 12, 17, and 18 did not lead to a selective glucagon antagonist.

Positions 11 and 16 have not been implicated as being critical for GLP-1 activity by alanine scanning mutagenesis (25, 26). Moreover, S11A/S16A and S11A/S16N mutations of glucagon have minimal effect on glucagon receptor binding, but reduced receptor activation by at least 10-fold (21). Although these mutations did not result in glucagon antagonists in the context of glucagon backbone, double mutation S11A/S16A of peptide A3 resulted in the peptide AN10 that is a potent GLP-1 receptor agonist while at the same time inhibits glucagon receptor activation. Presumably this is because of its ability to bind to the glucagon receptor with relatively high affinity, yet it only partially activates the glucagon receptor with relatively weak potency. This large drop in glucagon, but not GLP-1, receptor activation may be caused by greater structure rigidity in this region when the native serine amino acids at positions 11 and 16 are replaced with alanine, which possess a greater helix-forming propensity (40). Glucagon receptor activation perhaps requires a more flexible midsection than GLP-1 receptor activation, as suggested by GLP-1 having a more defined structure than glucagon in this region. The midsection of GLP-1 covering positions 9 to 15 forms an α-helix (35), whereas positions 11 and 16 bracket the
glucagon “hinge region” that connects and orients the N-terminal activation and C-terminal binding regions (41). Alternatively, these serines may act as part of an active site triad together with His and Asp that has been suggested for glucagon receptor activation but not GLP-1 receptor activation (32). In conclusion, the combination of two sets of mutations, hybrid N-terminal glucagon and C-terminal GLP-1 sequence of the A3 peptide and double S11A/S16A mutations, that individually have some tendency for glucagon antagonism and minimal effect on GLP-1 activation, is sufficient to produce a selective glucagon antagonist.

GLP-1 has the potential to be a very effective therapeutic drug for the treatment of type 2 diabetes. Multiple approaches are being pursued in an effort to attain a duration of action that would allow for once daily, or more preferably, once weekly administration. Exendin-4 (Exenatide/Byetta®), which lacks the DPP-IV cleavage site, is the only GLP-1 analogue approved for treatment of type 2 diabetes, but it requires twice daily dosing. We eliminated the DPP-IV cleavage site by using glucagon amino acids at the N-terminus of the hybrid peptides. Lipidation, which is the attachment of a fatty acid moiety to a protein or peptide, has been used as an approach to improve plasma half-life. Fatty acid-modified GLP-1 (NN2211/liraglutide) is currently in Phase II clinical development and has demonstrated efficacious glycemic control in clinical studies (42, 43). To identify the optimal site for lipidation of GLP-1 the activity of GLP-1 lipidated at various positions throughout the peptide was examined. GLP-1 was lipidated at positions 2, 12, 17, 20, 21, 28, and 30 by introducing lysine mutations while mutating the existing lysines to arginines. Lipidation at all of these positions, except position 2, had no significant effect on peptide activity in vitro (44). In contrast, we observed that PEGylation with high molecular weight PEGs has a more significant effect on peptide activity in vitro compared with lipidation, especially when the PEG is attached to internal amino acids of the peptide, such as positions 20 and 21. The reduction in receptor activation appears to be mediated by a corresponding loss of receptor affinity, as PEGylated peptides also display weaker affinity for the receptor compared with the unPEGylated peptides. Thus, it is unlikely that the PEG makes any specific interaction with a particular region of the receptor, consistent with the nonspecific nature of PEG-protein interactions. Because PEG effects on binding and activation are similar, the high molecular weight PEGs conjugated to the C-terminus of the peptide may simply interfere with receptor binding through steric interference of the C-terminal region of the peptide, responsible for the initial contact with the receptor. This interference with the C-terminal interaction would result in a subsequent reduction of activation potency mediated by the N-terminal region of the peptide, consistent with the two-site model of activation of family B GPCRs (41). Nevertheless, in many cases the reduced activity of PEGylated proteins in vitro due to reduced receptor affinity is more than compensated for by the significantly increased stability and extended half-life of the protein in vivo, which results in prolonged exposure and receptor occupancy (45).

From the studies described, ANC7K2 was identified as the optimal GLP-1 agonist/glucagon antagonist peptide for PEGylation. This peptide has been PEGylated with 43-kDa PEG, and its in vivo activity has been studied. This PEGylated peptide, referred to as PEG-DAPD (PEGylated dual-acting peptide for diabetes), has prolonged glucose lowering activity following glucose tolerance tests in mice, rats, and dogs. This is evidence of insulin secretion through GLP-1 receptor agonism in vivo. In rats glucose-lowering efficacy is evident when a glucose tolerance test is performed up to 65 h after a single subcutaneous injection of the peptide, demonstrating the dramatically prolonged in vivo activity of the peptide compared with native GLP-1. Furthermore, PEG-DAPD lowers blood glucose following a glucagon challenge in mice, evidence of in vivo glucagon antagonism. PEG-DAPD also lowers fasting blood glucose in a diabetic animal model, db/db mice. In conclusion, the mutagenesis data described herein provide further mechanistic insights into GLP-1 and glucagon receptor activation and inhibition, respectively, and have led to the identification of novel dual-acting GLP-1 agonist/glucagon antagonist peptides, which could have potential for development as a therapy for the treatment of type 2 diabetes.

REFERENCES

1. Mayo, K. E., Miller, L. J., Bataille, D., Dalle, S., Goke, B., Thorens, B., and Druecker, D. J. (2003) Pharmacol. Rev. 55, 167–194
2. Jelinek, I. J., Lok, S., Rosenberg, G. B., Smith, R. A., Grant, F. J., Biggs, S., Bensch, P. A., Kuiper, J. L., Sheppard, P. O., and Sprecher, C. A. (1993) Science 259, 1614–1616
3. Thorens, B. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 8641–8645
4. Hjorth, S. A., Adelhorst, K., Pedersen, B. B., Kirk, O., and Schwartz, T. W. (1994) J. Biol. Chem. 269, 30121–30124
5. Goke, R., Fehmhan, H. C., Linn, T., Schmidt, H., Krause, M., Eng, J., and Goke, B. (1993) J. Biol. Chem. 268, 19650–19655
6. Mojsov, S., Kopcynski, M. G., and Habener, J. F. (1990) J. Biol. Chem. 265, 8001–8008
7. Weir, G. C., Mojsov, S., Hendrick, G. K., and Habener, J. F. (1989) Diabetes 38, 338–342
8. Rogers, O. A., Baron, M., and Philo-Tsimikas, A. (2005) Expert. Opin. Investig. Drugs 14, 705–727
9. Jiang, G., and Zhang, B. B. (2003) Am. J. Physiol. 284, E671–E678
10. Thornton, K., and Gorenstein, D. G. (1994) Biochemistry 33, 3532–3539
11. Ying, J., Ahn, J. M., Jacobsen, N. E., Brown, M. F., and Hruby, V. J. (2003) Biochemistry 42, 2825–2835
12. Knudsen, L. B., and Pridal, L. (1996) Eur. J. Pharmacol. 318, 429–435
13. Montroze-Rafizadeh, C., Yang, H., Rodgers, B. D., Beaday, A., Pritchett, L. A., and Eng, J. (1997) J. Biol. Chem. 272, 21201–21206
14. Azieh, B. Y., Ahn, J. M., Caspari, R., Shenderovich, M. D., Trivedi, D., and Hruby, V. J. (1997) J. Med. Chem. 40, 2555–2562
15. Gysin, B., Trivedi, D., Johnson, D. G., and Hruby, V. J. (1986) Biochemistry 25, 8281–8284
16. Unson, C. G., Andreu, D., Gurzenza, E. M., and Merrifield, R. B. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 4083–4087
17. Unson, C. G., Macdonald, D., and Merrifield, R. B. (1993) Arch. Biochem. Biophys. 300, 747–750
18. Unson, C. G., Macdonald, D., Ray, K., Durrah, T. L., and Merrifield, R. B. (1991) J. Biol. Chem. 266, 2763–2766
19. Hruby, V. J. (1997) in Principles of Medical Biology (Bittar, E. E., and Bittar, N., eds) pp. 387–403, A1 Press, Greenwich, CT
20. Unson, C. G., Wu, C. R., Cheung, C. P., and Merrifield, R. B. (1998) J. Biol. Chem. 273, 10308–10312
21. Unson, C. G., Wu, C. R., Fitzpatrick, K. J., and Merrifield, R. B. (1994) J. Biol. Chem. 269, 12548–12551
22. Unson, C. G., Wu, C. R., and Merrifield, R. B. (1994) Biochemistry 33, 6884–6887
23. Unson, C. G., Gurzenza, E. M., and Merrifield, R. B. (1989) Peptides 10, 1171–1177
24. Smith, R. A., Sink, B., Lockhart, P., Mathewes, S., Gilbert, T., Walker, T., and Piggot, J. (1993) Mol. Pharmacol. 43, 741–748
25. Adelhorst, K., Hedegaard, B. B., Knudsen, L. B., and Kirk, O. (1994) J. Biol. Chem. 269, 6275–6278
26. Gallwitz, B., Witt, M., Paetzold, G., Morys-Wortmann, C., Zimmermann, B., Eckart, K., Folsch, U. R., and Schmidt, W. E. (1994) Eur. J. Biochem. 225, 1151–1156
27. Xiao, Q., Giguere, J., Parisien, M., Jeng, W., St-Pierre, S. A., Brubaker, P. L., and Wheeler, M. B. (2001) Biochemistry 40, 2860–2869
28. Mentlein, R., Gallwitz, B., and Schmidt, W. E. (1993) Eur. J. Biochem. 214, 829–835

3 C. Q. Pan, J. M. Buxton, S. L. Yung, I. Tom, L. Yang, H. Chen, M. MacDougall, A. Bell, T. H. Claus, K. B. Clairmont, and J. P. Whelan, unpublished data.
29. Ritzel, U., Leonhardt, U., Ottleben, M., Ruhmann, A., Eckart, K., Spiess, J., and Ramadori, G. (1998) J. Endocrinol. 159, 93–102
30. Harris, J. M., and Chess, R. B. (2003) Nat. Rev. Drug. Discov. 2, 214–221
31. Berry, M. N., and Friend, D. S. (1969) J. Cell Biol. 43, 506–520
32. Unson, C. G., and Merrifield, R. B. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 454–458
33. Greig, N. H., Holloway, H. W., De Ore, K. A., Jani, D., Wang, Y., Zhou, J., Garant, M. J., and Egan, J. M. (1999) Diabetologia 42, 45–50
34. Sturm, N. S., Lin, Y., Burley, S. K., Krstenansky, J. L., Ahn, J. M., Azizeh, B. Y., Trivedi, D., and Hruby, V. J. (1998) J. Med. Chem. 41, 2693–2700
35. Parker, J. C., Andrews, K. M., Rescek, D. M., Massefski, W., Jr., Andrews, G. C., Contillo, L. G., Stevenson, D. H., and Suleske, R. T. (1998) J. Pept. Res. 52, 398–409
36. Unson, C. G. (2002) Biopolymers 66, 218–235
37. Ribel, U., Larsen, M. O., Rolin, B., Carr, R. D., Wilken, M., Sturis, J., Westergaard, L., Deacon, C. F., and Knudsen, L. B. (2002) Eur. J. Pharmacol. 451, 217–225
38. Gysin, B., Johnson, D. G., Trivedi, D., and Hruby, V. J. (1987) J. Med. Chem. 30, 1409–1415
39. Krstenansky, J. L., Trivedi, D., Johnson, D., and Hruby, V. J. (1986) J. Am. Chem. Soc. 109, 1696–1698
40. Pace, C. N., and Scholtz, J. M. (1998) Biophys. J. 75, 422–427
41. Hruby, V. J., Ahn, J. M., and Trivedi, D. (2001) Curr. Med. Chem.-Immun. Endo. & Metab. Agents 1, 199–215
42. Madsbad, S., Schmitz, O., Ranstam, J., Jakobsen, G., and Matthews, D. R. (2004) Diabetes Care 27, 1335–1342
43. Feinglos, M. N., Saad, M. F., Pi-Sunyer, F. X., An, B., and Santiago, O. (2005) Diabet. Med. 22, 1016–1023
44. Knudsen, L. B., Nielsen, P. F., Huusfeldt, P. O., Johansen, N. L., Madsen, K., Pedersen, F. Z., Thogersen, H., Wilken, M., and Agerso, H. (2000) J. Med. Chem. 43, 1664–1669
45. Bailon, P., and Berthold, W. (1998) Pharma. Sci. Tech. Today 1, 352–356