

114
111

CAVELIER
ABOGADOS

WEB SITE: www.caveller-abogados.com
E-MAIL: caveller@colomsat.net.co

EDIFICIO SISKI
CARRERA 4 No. 72 - 35
BOGOTÁ 8, COLOMBIA

SUPERINDUSTRIA Y COMERCIO

Radicación : 00073865 00000001

Folios: 141

Fecha (REC): 2000-12-08 17:25:13

Trámite : 002 PATENTES D 1 REGISTRO/ 329 COSPAREN

Dependencia: 2620 DIVISION DE NUEVAS CREACIONES

Señora Jefe de la
DIVISION DE NUEVAS CREACIONES
SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
E. S. D.

Referencia : Expediente No. 00.073.865
Solicitante : BAYER CORPORATION.
Asunto : Solicitud de patente para:
"AGONISTAS DEL RECEPTOR 3 (R3) DEL PEPTIDO
ACTIVADOR DE LA ADENILATO CICLASA DE LA
PITUITARIA Y SUS METODOS DE USO FARMACOLOGICO"
Fecha de solicitud : 28 de septiembre de 2000

TRAMITE 002 EVENTO 001 ACTUACION 329

1. Yo, Emilio FERRERO, obrando como apoderado de la solicitante en el asunto de la referencia, atentamente, presento con este escrito las copias certificadas de las solicitudes de patente estadounidenses No. 09/407,832 del 28 de septiembre de 1999 y No.09/595,280 del 15 de junio de 2000 junto con las respectiva traducciones de sus carátulas y reivindicaciones de las cuales se reclama prioridad en esta solicitud de patente.

2. Ruego a la Señora Jefe se sirva aceptar este escrito con sus anexos, y se continúe con el trámite de la solicitud.

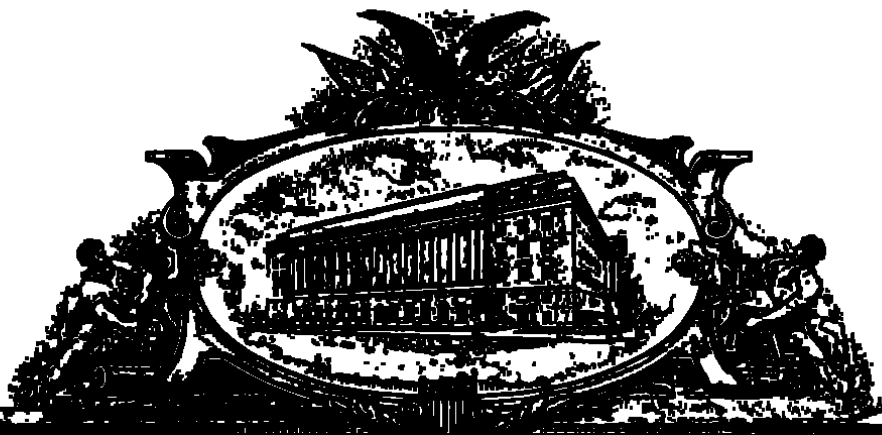
Bogotá, 29 DIC. 2000
Señora Jefe,


EMILIO FERRERO
T.P.A. No. 31.929

COLO 09597-801-017-1
JJC-ID-173

#5
112

PA 330121



THE UNITED STATES OF AMERICA

TO ALL TO WHOM THESE PRESENTS SHALL COME:

UNITED STATES DEPARTMENT OF COMMERCE

United States Patent and Trademark Office

November 28, 2000

THIS IS TO CERTIFY THAT ANNEXED HERETO IS A TRUE COPY FROM THE RECORDS OF THE UNITED STATES PATENT AND TRADEMARK OFFICE OF THOSE PAPERS OF THE BELOW IDENTIFIED PATENT APPLICATION THAT MET THE REQUIREMENTS TO BE GRANTED A FILING DATE UNDER 35 USC 111.

APPLICATION NUMBER: 09/407,832

FILING DATE: September 28, 1999



By Authority of the
COMMISSIONER OF PATENTS AND TRADEMARKS

L. Edelen

L. EDELEN
Certifying Officer

46
113

Attorney Docket No. 5053

UNITED STATES PATENT AND TRADEMARK OFFICE

Box Patent Application
Assistant Commissioner for Patents
Washington, DC 20231

A
Boxseq

CERTIFICATION UNDER 37 C.F.R. 1.19

I hereby certify that this New Application Transmittal and the documents referred to as enclosed therein are being deposited with the United States Postal Service on this date September 28, 1999, in an envelope as "Express Mail Post Office to Addressee" Mailing Label Number EI791841345US, addressed to the: Assistant Commissioner for Patents, Box Patent Application, Washington, D.C. 20231.

Amir Z. P.
Signature of person certifying / Boatrix Alvir

NEW UTILITY PATENT APPLICATION TRANSMITTAL
UNDER 37 CFR §1.51(b) AND § 1.53(b)

☒ Inventors / ☐ Application Identifier:

Clark Pan

Manami Tsubumi

Express Mail Label No.: EI791841345US

Title of Application: Glucose-dependent Insulin Secretagogue Polypeptides and Their Methods of Use

Type of application: ☒ Original (nonprovisional)

☐ Provisional

☐ Continuation

☐ Continuation-in-part (CIP)

☐ Divisional

Benefit of the prior US provisional applications indicated below is claimed under 35 USC § 119(e). Please amend the specification by inserting, before the first line, the following sentence: "This application claims the benefit of U.S. Provisional Application(s) No(s):

Application Number

Day/Month/Year Filed

1.

2.

117
114

Papers accompanying this transmittal that are required for filing under 37 CFR § 1.53(b):

- ☒ Fee transmittal form, in duplicate
- ☐ Provisional Application Cover Sheet
- ☒ Specification, 30 pages
- ☒ Claims, 3 pages
- ☒ Abstract, 1 page
- ☒ Drawings, 7 pages:
 - ☐ formal,
 - ☒ informal,
- ☐ Declaration, _____ pages
 - ☐ Executed by inventors
 - ☐ Executed by other: _____

Additional papers accompanying this transmittal:

- ☒ Return Receipt Postcard
- ☐ Power of Attorney
- ☐ Assignment to Bayer Corporation
 - ☐ Cover Sheet
 - ☐ Assignment Document(s)
- ☒ Nucleotide and/or Amino Acid Sequence Submission
 - ☒ Computer Readable Copy
 - ☒ Paper Copy (identical to computer readable copy) 22 pages
 - ☐ Statement verifying identity of above copies
- ☐ Information Disclosure Statement
- ☐ Form PTO-1449
- ☐ Copies of References
- ☐ Verified English Translation of the Application
- ☐ Certified Copy of Priority Document(s)
 - Country _____ Application No. _____ Date filed _____
- ☐ Preliminary Amendment
- ☐ Other: _____

Applicants request that the patent application accompanying this transmittal be duly filed with the United States Patent and Trademark Office as of the date indicated in the Certificate of Mailing below.

09407832.0928.9

115
115

Address all written correspondence to:

Mr. Jeffrey M. Greenman
Vice President, Patents & Licensing
Bayer Corporation
400 Morgan Lane
West Haven, Connecticut 06518

Direct all telephone calls to Huw R. Jones at (203) 812-2317.

Respectfully submitted by:



Huw R. Jones
Reg. No. 33,918

09407832-092899

419
116

TITLE OF THE INVENTION

Glucose-dependent Insulin Secretagogue Polypeptides and their Methods of Use

BACKGROUND OF THE INVENTION

5 1. Field of the Invention

This invention relates to newly identified polypeptides and the use of such polypeptides for therapeutic purposes. More particularly, the polypeptides of the present invention are useful in stimulating the release of insulin from pancreatic beta cells in a glucose-dependent manner, thereby providing a treatment option for those
10 individuals afflicted with a metabolic disorder such as Diabetes or impaired glucose tolerance, a prediabetic state.

2. Background of the Related Art

Diabetes is characterized by impaired glucose metabolism manifesting itself
15 among other things by an elevated blood glucose level in the diabetic patient. Underlying defects lead to a classification of diabetes into two major groups: type 1 diabetes, or insulin dependent diabetes mellitus (IDDM), which arises when patients lack beta-cells producing insulin in their pancreatic glands, and type 2 diabetes, or non-insulin dependent diabetes mellitus (NIDDM), which occurs in patients with an
20 impaired beta-cell function and alterations in insulin action.

Type 1 diabetic patients are currently treated with insulin, while the majority of type 2 diabetic patients are treated with agents that stimulate beta-cell function or with agents that enhance the tissue sensitivity of the patients towards insulin. Over time almost one-half of type II diabetic subjects lose their response to these agents
25 and then must be placed on insulin therapy. The drugs presently used to treat Type II Diabetes include:

Alpha-glucosidase inhibitors (PRECOSE®, VOGLIBOSE™, and
MIGLITOL®) reduce the excursion of postprandial glucose by delaying the
30 absorption of glucose from the gut. These drugs are safe and provide treatment for mild to moderately affected diabetic subjects. However, gastrointestinal side effects have been reported in the literature.

Insulin sensitizers. Insulin sensitizers are drugs that enhance the body's response to insulin. REZULIN™ (troglitazone) activates the PPAR gamma receptor

and modulates the activity of a set of genes that have not been well described. Although effective, this drug can produce liver toxicity. A black box warning on its label requiring monthly liver function tests for the first eight months of use and periodic tests thereafter has adversely affected sales. The FDA recently amended the product labeling requiring Warner-Lambert to withdraw its monotherapy indication, and recommending that initial use should be limited to patients who are not adequately controlled by other therapy. The new labeling also recommends more extensive liver function monitoring.

Insulin secretagogues (sulfonylureas and other agents that act by the ATP-dependent K⁺ channel). SFUs are standard therapy for Type II diabetics that have mild to moderate fasting glycemia. The SFUs have limitations that include a potential for inducing hypoglycemia, weight gain, and high primary and secondary failure rates. 10 to 20% of initially treated patients fail to show a significant treatment effect (primary failure). Secondary failure is demonstrated by an additional 20-30% loss of treatment effect after six months on an SFU. Insulin treatment is required in 50% of the SFU responders after 5-7 years of therapy (Scheen, A.J., et al., Diabetes Res. Clin. Pract. 6:533-543 (1989)).

GLUCOPHAGE™ (metformin HCl) is a biguanide that lowers blood glucose through an unknown mechanism. The drug is effective at lowering blood glucose in mildly and moderately affected subjects and does not have the side effects of weight gain or the potential to induce hypoglycemia. However, GLUCOPHAGE has a number of side effects including gastrointestinal disturbances and lactic acidosis. GLUCOPHAGE is contraindicated in diabetics over the age of 70 and in subjects with impairment in renal or liver function. Finally, GLUCOPHAGE has the same primary and secondary failure rates as the SFUs.

Insulin treatment is instituted after diet, exercise and oral medications have failed to adequately control blood glucose. This treatment has the drawbacks that it is an injectable, that it can produce hypoglycemia, and that it causes weight gain.

Because of the problems with current treatments, new therapies to treat Type II diabetes are needed. In particular, new treatments to retain normal (glucose-dependent) insulin secretion are needed. Such new drugs should have the following characteristics: dependent on glucose for promoting insulin secretion, i.e. produce insulin secretion only in the presence of elevated blood glucose; low primary and

secondary failure rates; and preserve islet cell function. The strategy to develop the new therapy disclosed herein is based on the cyclic adenosine monophosphate (cAMP) signaling mechanism and its effects on insulin secretion.

Cyclic AMP is a major regulator of the insulin secretion process. Elevation of this signaling molecule promotes the closure of the K⁺ channels following the activation of protein kinase A pathway. Closure of the K⁺ channels causes cell depolarization and subsequent opening of Ca⁺⁺ channels, which in turn leads to exocytosis of insulin granules. Little if any effects on insulin secretion occurs in the absence of low glucose concentrations (Weinham, A., et. al., Diabetes 47: 1426-1435 (1998)). Secretagogues like pituitary adenylyl cyclase activating peptide ("PACAP") and GLP-1 use the cAMP system to regulate insulin secretion in a glucose-dependent fashion (Komatsu, M., et al., Diabetes 46: 1928-1938, (1997)).

PACAP is a potent stimulator of glucose-dependent insulin secretion from pancreatic beta-cells. Three different PACAP receptor types (R1, R2, and R3) have been described (Harmar, A. et. al., Pharmacol. Reviews 50: 265-270 (1998)). R1 is located predominately in the CNS, whereas R2 and R3 are more widely distributed. Also, R2 and R3 bind VIP with high affinity. R2 is located in the CNS as well as in liver, lungs and intestine. R3 is located in the CNS, pancreas, skeletal muscle, heart, kidney, adipose tissue, testis and stomach. Recent work argues that R3 is responsible for the insulin secretion from beta cells (Inagaki, N. et. al., PNAS 91: 2679-2683, (1994)). This insulinotropic action of PACAP is mediated by the GTP binding protein Gs. Accumulation of intracellular cAMP in turn activates the nonselective cation channels in beta cells increasing [Ca⁺⁺]_i, and promotes exocytosis of insulin-containing secretory granules.

PACAP is the newest member of the superfamily of metabolic, neuroendocrine and neurotransmitter peptide hormones that exert their action through the cAMP-mediated signal transduction pathway (Arimura, Regul. Peptides 37:287-303 (1992)). The biologically active peptides are released from the biosynthetic precursor in two molecular forms, either as a 38-amino acid peptide (PACAP-38) and/or as a 27-amino acid peptide (PACAP-27) with an amidated carboxyl termini (Arimura, *supra*).

The highest concentrations of the two forms of the peptide are found in the brain and testis (reviewed in Arimura, *supra*). The shorter form of the peptide,

PACAP-27, shows 68% structural homology to vasoactive intestinal polypeptide (VIP). However, the distribution of PACAP and VIP in the central nervous system suggests that these structurally related peptides have distinct neurotransmitter functions (Koves et al., Neuroendocrinology 54:159-169, (1991)).

Recent studies have demonstrated diverse biological effects of PACAP-38, from a role in reproduction (McArdle, Endocrinology 135:815-817 (1994)) to ability to stimulate insulin secretion (Yada et al., J. Biol. Chem. 269:1290-1293 (1994)).

Vasoactive intestinal peptide (VIP) is a 28 amino acid peptide that was first isolated from hog upper small intestine (Said and Mutt, Science 169: 1217-1218, 1970; U.S. Patent No. 3,879,371). This peptide belongs to a family of structurally-related, small polypeptides that includes helodermin, secretin, the somatostatins, and glucagon. The biological effects of VIP are mediated by the activation of membrane-bound receptor proteins that are coupled to the intracellular cAMP signaling system. These receptors were originally known as VIP-R1 and VIP-R2, however, they were later found to be the same receptors as PACAP-R2 and PACAP-R3.

To improve the stability of VIP in human lung fluid, Bolin et al (Biopolymers 37: 57-66, (1995)) made a series of VIP analogs designed to enhance the helical propensity of this peptide and reduce proteolytic degradation. Substitutions were focused on positions 8, 12, 17, and 25-28 which were implicated to be unimportant for receptor binding. Moreover, the "GGT" sequence was tagged onto the C-terminus of VIP mutants with the hope of more effectively capping the helix. Finally, to further stabilize the helix, several cyclic analogs were synthesized (US Patent No. 5,677,419). Although these efforts were not directed toward receptor selectivity, they yielded two analogs that have greater than 100-fold PACAP-R3 selectivity (Gourlet et al., Peptides 18: 403-408, (1997); Xia et al., J. Pharmacol. Exp. Ther., 281: 629-633, (1997)).

GLP-1 is released from the intestinal L-cell after a meal and functions as an incretin hormone (i.e. it potentiates glucose-induced insulin release from the pancreatic beta-cell). It is a 37-amino acid peptide that is differentially expressed by the Glucagon gene, depending upon tissue type. The clinical data that support the beneficial effect of raising cAMP levels in β -cells have been collected with GLP-1.

Infusions of GLP-1 in poorly controlled Type II diabetics normalized their fasting blood glucose levels (Gutniak, M., et al., New Eng. J. Med 326:1316-1322, (1992)) and with longer infusions improved the beta cell function to those of normal subjects (Rachman, J. et al., Diabetes 45: 1524-1530, (1996)). A recent report has shown that GLP-1 improves the beta cells' ability to respond to glucose in subjects with impaired glucose tolerance (Byrne M., et al., Diabetes 47: 1259-1265 (1998)). All of these effects, however, are short-lived because of the short half-life of the peptide. Recently Novo Nordisk has discontinued clinical trials with GLP-1. This failure reportedly was due to a very short plasma half-life of the peptide of a few minutes.

EXENDIN 4™. Amylin Pharmaceuticals is conducting Phase I trials with EXENDIN 4 (AC2993), a 39 amino acid peptide originally identified in Gila Monster. Phase II trials have recently begun. Amylin claims preclinical results showing a 4 hour duration of efficacy and efficacy in animal models when AC2993 is administered subcutaneously, orally, and nasally. However, at doses of 0.2 and 0.3ug/kg, the incidence of headaches, postural hypotension, nausea and vomiting was significant.

There exists a need for an improved peptide that has the glucose-dependent insulin secretagogue activity of PACAP, GLP-1, or EXENDIN 4, and yet has less side-effects.

SUMMARY OF THE INVENTION

This invention provides novel polypeptides that function *in vivo* to stimulate insulin release from pancreatic beta cells in a glucose-dependent fashion. These insulin secretagogue polypeptides are shown to stimulate insulin release in rat islet cells *in vitro*, and *in vivo*. The polypeptides of the present invention provide a new therapy for patients with metabolic disorders such as decreased endogenous insulin production, in particular type 2 diabetics, or in patients with impaired glucose tolerance, a prediabetic state that has a mild alteration in insulin secretion. In particular, the invention is a polypeptide selected from the group consisting of SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:18 and SEQ ID NO:32 or a functional equivalent thereof. The invention is also directed to a method of treating diabetes in a mammal comprising administering a therapeutically effective amount of the insulin secretagogue

09407832.09289

124
121

polypeptides to said mammal. Also disclosed are methods of making the polypeptides, both recombinant and synthetic.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 depicts the amino acid sequences (SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:18 and SEQ ID NO:32) of the claimed insulin secretagogue polypeptides.

FIG. 2 is a sequence alignment of VIP mutants and native polypeptides VIP, PACAP38, GLP-1, and EXENDIN-4. Conserved residues are bolded and shaded in dark gray while conservative changes are shaded in light gray.

FIG. 3 is a restriction map of a typical plasmid containing the GST-peptide fusion.

FIGS. 4A-4B are graphs showing the effects of GLP-1 or R3P3 on insulin release from rat islets *in vitro*.

FIG. 5 is a graph showing the R3P3 peptide effect on glucose disposal.

FIG. 6 is a bar chart showing the effect of PACAP and related polypeptides on intestinal water content of Balb/C mice.

FIG. 7 is a bar chart showing that 1 nmole/kg dose of R3P3, R3P12, R3P13, or GLP-1 enhance glucose disposal in the rat by subcutaneous route of administration.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

This invention provides novel polypeptides that function *in vivo* to stimulate insulin release from pancreatic beta cells in a glucose-dependent fashion. The polypeptides are shown to stimulate insulin release in rat islet cells *in vitro*, and *in vivo*. The polypeptides of the present invention provide a new therapy for patients with decreased endogenous insulin production, in particular type 2 diabetics.

A. Discussion

PACAP, VIP, GLP-1 and Exendin-4 are polypeptides capable of stimulating insulin release in a glucose-dependent fashion. However, this fact alone does not guarantee glucose reduction *in vivo*. Since PACAP is known to bind to PACAP-R1, -R2 and -R3 receptors, and VIP is known to bind to PACAP-R2 and -R3, it was

09407832-092899
668260-23870460

thought that they may have similar conserved structural features. The stacking alignment below shows the primary structural relationships:

5	VIP NO:1)	1	HSDAVFTDNY	TRLRQDAVK	KYLNSILN-NH ₂	28	(SEQ ID
	PACAP38 ID NO:2)	1	HSDGIFIDSY	SKYRQDAVK	KYLAAVLGKR	YKQEVKIK-NH ₂	38 (SEQ
	GLP-1 ID NO:3)	1	HAGTFTSDV	SSYLEGQAQK	KFLANLVKGR-NH ₂	30 (SEQ	
10	Exendin 4 NO:4)	1	HGGTFTSDL	SKQMEKAVR	LFIEMLKNG	PSSGAPPPS-NH ₂	39 (SEQ ID

(where single-letter abbreviations for amino acids can be found in Zubay, Biochemistry 2d ed., 1988, MacMillan Publishing, New York, p. 33). The

polypeptides of the present invention are most closely related to VIP in terms of their primary structure.

The inventors herein set out to create a new polypeptide that could lead to a selective glucose-dependent insulin secretagogue in the hope that selective activation would indeed lead to a glucose dependant pathway to insulin secretion by pancreatic beta cells, with concomitant glucose reduction *in vivo*. In that light, they first studied the structures of PACAP27 and VIP in an effort to determine the residues most likely responsible for receptor selectivity. It is known that PACAP and VIP do not reduce glucose *in vivo*, but due to non-specific interactions, glucose release is triggered.

Previously, both PACAP and VIP have been mutagenized extensively for various reasons. For instance, serial deletions of PACAP27 and PACAP38 from both ends confirmed the importance of both termini for receptor binding (Gourlet et al., Eur. J. Pharm. 287: 7-11, (1995); Gourlet et al., Regul. Peptides 62: 125-130, (1996)). Rat brain membrane binding and adenylate cyclase activities of PACAP27/VIP hybrid mutants implicated the importance of the N-terminal residues of PACAP for PACAP-R1 recognition (Ando et al., Biomed. Rept. Proteins Nucleic Acids 2:41-46, (1996)). Increasing the basicity of Leu¹⁷-PACAP27 and Leu¹⁷-VIP by making K15R, K20R, and K21R mutations and extension of the C-terminus with "GKR" sequence led to an increase in duration of guinea pig tracheal relaxant activity proposed to be due to protection from heparin binding (Kashimoto et al., Ann. NY Acad. Sci. 805: 505-510, (1996)). Gourlet et al. (Biochim. Biophys. Acta 1314: 267-273, (1996)) demonstrated that the Q16R mutant of VIP and PACAP possessed greater affinities than their respective native polypeptides for PACAP-R2

and R1, respectively. Gourlet et al. (Peptides 18: 1539-1545, (1997)) developed a high affinity R2-selective agonist by making the chimeric substituted peptide [K15, R16, L27] VIP(1-7)/GRF(8-27). N-terminal acylation and D-Phe2 substitution of this selective agonist led to a potent R2 selective antagonist (Gourlet et al., Peptides 18 1555-1560, (1997)). VIP mutants Y22L and Y22A, but not Y22F, display lower affinity for PACAP-R3, suggesting the importance of an aromatic group at position 22 for receptor R3 binding but not for receptor R2 binding (Gourlet, Eur. J. Biochem. 348: 95-99, (1998)). Helodermin and helospectin, VIP-like peptides isolated from the salivary gland venom of lizards exhibit ~100-fold PACAP-R3 selectivity (Gourlet, Ann. NY Acad. Sci. 865: 247-252, (1998)). Photoaffinity labeling of PACAP27 by replacing F6 and Y22 with *p*-benzoyl-L-phenylalanine (pBz) or K15, K20, and K21 with pBz, suggested that K15 and F22 are closer to PACAP-R1 than F6, K20, and K21 (Cao et al., Eur. J. Biochem. 244: 400-406, (1997); Cao et al., Ann. NY Acad. Sci. 865: 82-91, (1998)).

The inventors herein have found several polypeptides that cause the stimulation of release of insulin in a glucose-dependent manner and cause glucose reduction in vivo. Those polypeptides bear some similarity to VIP and PACAP. In particular, a stacked alignment shows the following:

20	VIP NO:1)	1 HSDAVFTDNY TLRLRQMAVK KYLSILN-NH ₂	28 (SEQ ID
	PACAP38 ID NO:2)	1 HSDGIFTDSY SRKRQMAVK KYLAAVLGR YKQVKNK-NH ₂	36 (SEQ
	R3P1 ID NO:6)	Ac-HSDAVFTENY TLRLRQLAAK KYLNDLKKGG T-NH ₂	31 (SEQ
25	R3P3 ID NO:8)	1 HSDAVFTENY TLRLRQLAAK KYLNDLKKGG T	31 (SEQ
	R3P9 ID NO:12)	1 HSDAVFTDNY TLRLRQMAVK KYLSIKKGG T	31 (SEQ
30	R3P10 ID NO:13)	1 HSDAVFTENY TLRLRQLAAK KYLNDLLKGG T	31 (SEQ
	R3P12 ID NO:15)	1 HSDAVFTDNY TLRLRQLAAK KYLNDIKKGG T	31 (SEQ
	R3P13 ID NO:16)	1 HSDAVFTDNY TLRLRQLAAK KYLNDIKK-NH ₂	28 (SEQ
35	R3P19 ID NO:18)	1 HSDAVFTENY TLRLRQLAAK KYLNDLKKGG TSWCEPGWCR	40 (SEQ
	R3P36 ID NO:32)	1 HSDAVFTDNY TLRLRQLAAK KYLNDIKKR Y	31 (SEQ
40			

However, there is no teaching in the scientific or patent literature that suggest the surprising result taught herein, namely, that select modifications to the VIP and

09407832.092899

PACAP sequences might lead to a polypeptide with the ability to stimulate insulin secretion in a glucose-dependent fashion, and reduce glucose concentration.

Certain terms used throughout this specification will now be defined, and others will be defined as introduced.

5 The term "polynucleotide encoding a polypeptide" encompasses a polynucleotide which includes only coding sequence for the polypeptide as well as a polynucleotide which includes additional coding and/or non-coding sequence.

10 The present invention further relates to polynucleotides which hybridize to the hereinabove-described sequences if there is at least 70%, preferably at least 90%, and more preferably at least 95% identity between the sequences. The present invention particularly relates to polynucleotides encoding polypeptides which hybridize under stringent conditions to the hereinabove-described polynucleotides. As herein used, the term "stringent conditions" means hybridization will occur only if there is at least 95% and preferably at least 97% identity between the sequences.
15 The polynucleotides which hybridize to the hereinabove described polynucleotides in a preferred embodiment encode polypeptides which retain substantially the same biological function or activity as the mature polypeptide encoded by the cDNAs.

20 "Stringent hybridization conditions" refers to an overnight incubation at 42° C in a solution comprising 50% formamide, 5x SSC (750 mM NaCl, 75 mM sodium citrate), 50 mM sodium phosphate (pH 7.6), 5x Denhardt's solution, 10% dextran sulfate, and 20 µg/ml denatured, sheared salmon sperm DNA, followed by washing the filters in 0.1x SSC at about 65°C.

25 The terms "fragment," "derivative" and "analog" when referring to the polypeptides of FIG. 1, means polypeptides which retain essentially the same biological function or activity as such polypeptides. Thus, an analog includes an allelic variant or a proprotein which can be activated by cleavage of the proprotein portion to produce an active mature polypeptide. A fragment is a portion of the polypeptide which retains substantially similar functional activity, as shown in the *in vivo* models disclosed herein. A derivative includes all modifications to the
30 polypeptide which preserve the functions disclosed herein and include additional structure and attendant function, e.g., PEGylated polypeptides which have greater half-life, fusion proteins which confer targeting specificity or an additional activity such as toxicity to an intended target.

The polypeptides of the present invention may be recombinant polypeptides, natural polypeptides or synthetic polypeptides, preferably recombinant polypeptides. The fragment, derivative or analog of the polypeptides of FIG. 1 may be (i) one in which one or more of the amino acid residues are substituted with a conserved or non-conserved amino acid residue (preferably a conserved amino acid residue) and such substituted amino acid residue may or may not be one encoded by the genetic code, or (ii) one in which one or more of the amino acid residues includes a substituent group, or (iii) one in which the mature polypeptide is fused with another compound, such as a compound to increase the half-life of the polypeptide (for example, polyethyleneglycol), or (iv) one in which the additional amino acids are fused to the mature polypeptide, such as a leader or secretory sequence or a sequence which is employed for purification of the mature polypeptide or a proprotein sequence, or v) one in which the polypeptide sequence is fused with a larger protein, i.e. human albumin, an antibody or Fc, for increased duration of effect. Such fragments, derivatives and analogs are deemed to be within the scope of those skilled in the art from the teachings herein.

The polypeptides of the present invention can be composed of amino acids joined to each other by peptide bonds or modified peptide bonds, i.e., peptide isosteres, and may contain amino acids other than the 20 gene-encoded amino acids. The polypeptides may be modified by either natural processes, such as posttranslational processing, or by chemical modification techniques which are well known in the art. Such modifications are well described in basic texts and in more detailed monographs, as well as in a voluminous research literature. Modifications can occur anywhere in a polypeptide, including the peptide backbone, the amino acid side-chains and the amino or carboxyl termini. It will be appreciated that the same type of modification may be present in the same or varying degrees at several sites in a given polypeptide. Also, a given polypeptide may contain many types of modifications. Polypeptides may be branched, for example, as a result of ubiquitination, and they may be cyclic, with or without branching. Cyclic, branched, and branched cyclic polypeptides may result from posttranslation natural processes or may be made by synthetic methods. Modifications include acetylation, acylation, ADP-ribosylation, amidation, covalent attachment of flavin, covalent attachment of a heme moiety, covalent attachment of a nucleotide or nucleotide derivative, covalent

attachment of a lipid or lipid derivative, covalent attachment of phosphatidylinositol, cross-linking, cyclization, disulfide bond formation, demethylation, formation of covalent cross-links, formation of cysteine, formation of pyroglutamate, formylation, gamma-carboxylation, glycosylation, GPI anchor formation, hydroxylation, iodination, methylation, myristoylation, oxidation, pegylation, proteolytic processing, phosphorylation, prenylation, racemization, selenoylation, sulfation, t-transfer-RNA mediated addition of amino acids to proteins such as arginylation, and ubiquitination. (See, for instance, Proteins. Structure and Molecular Properties, 2nd ed., T. E. Creighton, W.H. Freeman and Company, New York (1993); Posttranslational Covalent Modification of Proteins, B. C. Johnson, ed., Academic Press, New York, pgs. 1-12 (1983); Seifter et al., Meth. Enzymol 182:626-646 (1990); Rattan et al., Ann. N.Y. Acad. Sci. 663:48-62 (1992)).

The polypeptides of the present invention include the polypeptides of Fig. 1, including SEQ ID NOs: 6,8,12,13,15,16,18 and 32, as well as those sequences having insubstantial variations in sequence from them. An "insubstantial variation" would include any sequence, substitution, or deletion variant that maintains the insulin secreting activity demonstrated herein. These functional equivalents may include polypeptides which have at least a 90% identity to the polypeptides of Fig. 1, and more preferably at least a 95% identity to the polypeptides of Fig. 1, and still more preferably at least a 97% identity to the polypeptides of Fig. 1, and also include portions of such polypeptides having the same biological activity. As known in the art "similarity" between two polypeptides is determined by comparing the amino acid sequence and its conserved amino acid substitutes of one polypeptide to the sequence of a second polypeptide. Such conservative substitutions include those described by Dayhoff in The Atlas of Protein Sequence and Structure 2 (1978), and by Argos in EMBO J., 8:779-785 (1989). For example, amino acids belonging to one of the following groups represent conservative changes:

- ala, pro, gly, gln, asn, ser, thr;
- cys, ser, tyr, thr;
- val, ile, leu, met, ala, phe;
- lys, arg, his;
- phe, tyr, trp, his; and
- asp, glu.

5 The present invention also relates to vectors which include polynucleotides of the present invention, host cells which are genetically engineered with vectors of the invention and the production of polypeptides of the invention by recombinant techniques. Host cells may be genetically engineered (transduced or transformed or transfected) with the vectors of this invention which may be, for example, a cloning vector or an expression vector. The vector may be, for example, in the form of a plasmid, a viral particle, a phage, etc. The engineered host cells can be cultured in conventional nutrient media modified as appropriate for activating promoters, or selecting transformants. The culture conditions, such as temperature, pH and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan. The polynucleotide of the present invention may be employed for producing a polypeptide by recombinant techniques. Thus, for example, the polynucleotide sequence may be included in any one of a variety of expression vehicles, in particular vectors or plasmids for expressing a polypeptide. Such vectors include chromosomal, non-chromosomal and synthetic DNA sequences, e.g., derivatives of SV40; bacterial plasmids; phage DNA; yeast plasmids; vectors derived from combinations of plasmids and phage DNA, viral DNA such as vaccinia, adenovirus, fowl pox virus, and pseudorabies. However, any other vector or plasmid may be used as long as they are replicable and viable in the host.

25 The appropriate DNA sequence may be inserted into the vector by a variety of procedures. In general, the DNA sequence is inserted into an appropriate restriction endonuclease site by procedures known in the art. Such procedures and others are deemed to be within the scope of those skilled in the art. The DNA sequence in the expression vector is operatively linked to an appropriate expression control sequence(s) (promoter) to direct mRNA synthesis. As representative examples of such promoters, there may be mentioned: LTR or SV40 promoter, the *E. coli*. lac or trp, the phage lambda P_L promoter and other promoters known to control expression of genes in prokaryotic or eukaryotic cells or their viruses. The expression vector also contains a ribosome binding site for translation initiation and a transcription terminator. The vector may also include appropriate sequences for amplifying expression. In addition, the expression vectors preferably contain a gene to provide a phenotypic trait for selection of transformed host cells such as

0 407832 . 092899

dihydrofolate reductase or neomycin resistance for eukaryotic cell culture, or such as tetracycline or ampicillin resistance in *E. coli*. The vector containing the appropriate DNA sequence as herein above described, as well as an appropriate promoter or control sequence, may be employed to transform an appropriate host to permit the host to express the protein. As representative examples of appropriate hosts, there may be mentioned: bacterial cells, such as *E. coli*, *Salmonella typhimurium*, *Streptomyces*; fungal cells, such as yeast; insect cells, such as *Drosophila* S2 and *Spodoptera* Sf9; animal cells such as CHO, COS or Bowes melanoma; adenoviruses; plant cells, etc. The selection of an appropriate host is deemed to be within the scope of those skilled in the art from the teachings herein.

The present invention also includes recombinant constructs comprising one or more of the sequences as broadly described above. The constructs comprise a vector, such as a plasmid or viral vector, into which a sequence of the invention has been inserted, in a forward or reverse orientation. In a preferred aspect of this embodiment, the construct further comprises regulatory sequences, including, for example, a promoter, operably linked to the sequence. Large numbers of suitable vectors and promoters are known to those of skill in the art, and are commercially available. The following vectors are provided by way of example. Bacterial: pQE70, pQE60, pQE-9 (Qiagen), pBS, phagescript, psiX174, pBluescript SK, pBaKS, pNH8a, pNH16a, pNH18a, pNH46a (Stratagene); pTRC99A, pKK223-3, pKK233-3, pDR540, PRITS (Pharmacia). Eukaryotic: pWLneo, pSV2cat, pOG44, pXT1, pSG (Stratagene) pSVK3, pBPV, pMSG, PSVL (Pharmacia). However, any other plasmid or vector may be used as long as they are replicable and viable in the host. Promoter regions can be selected from any desired gene using CAT(chloramphenicol transferase) vectors or other vectors with selectable markers. Two appropriate vectors are pKK232-8 and pCM7. Particular named bacterial promoters include *lacI*, *lacZ*, T3, T7, *gpt*, lambda P_R, P_L and *trp*. Eukaryotic promoters include CMV immediate early, HSV thymidine kinase, early and late SV40, LTRs from retrovirus, and mouse metallothionein-I. Selection of the appropriate vector and promoter is well within the level of ordinary skill in the art.

The present invention also relates to host cells containing the above-described construct. The host cell can be a higher eukaryotic cell, such as a mammalian cell, or a lower eukaryotic cell, such as a yeast cell, or the host cell can be a prokaryotic cell,

such as a bacterial cell. Introduction of the construct into the host cell can be effected by calcium phosphate transfection, DEAE-Dextran mediated transfection, or electroporation (Davis, L., Dibner, M., Battey, I., Basic Methods in Molecular Biology, (1986)). The constructs in host cells can be used in a conventional manner to produce the gene product encoded by the recombinant sequence. Alternatively, the polypeptides of the invention can be synthetically produced by conventional peptide synthesizers.

Mature proteins can be expressed in mammalian cells, yeast, bacteria, or other cells under the control of appropriate promoters. Cell-free translation systems can also be employed to produce such proteins using RNAs derived from the DNA constructs of the present invention. Appropriate cloning and expression vectors for use with prokaryotic and eukaryotic hosts are described by Sambrook, et al., Molecular Cloning: A Laboratory Manual, Second Edition, (Cold Spring Harbor, N.Y., 1989), the disclosure of which is hereby incorporated by reference.

Transcription of a DNA encoding the polypeptides of the present invention by higher eukaryotes is increased by inserting an enhancer sequence into the vector. Enhancers are cis-acting elements of DNA, usually from about 10 to 300 bp, that act on a promoter to increase its transcription. Examples include the SV40 enhancer on the late side of the replication origin (bp 100 to 270), a cytomegalovirus early promoter enhancer, a polyoma enhancer on the late side of the replication origin, and adenovirus enhancers. Generally, recombinant expression vectors will include origins of replication and selectable markers permitting transformation of the host cell, e.g., the ampicillin resistance gene of *E. coli* and *S. cerevisiae* TRP1 gene, and a promoter derived from a highly-expressed gene to direct transcription of a downstream structural sequence. Such promoters can be derived from operons encoding glycolytic enzymes such as 3-phosphoglycerate kinase (PGK), α factor, acid phosphatase, or heat shock proteins, among others. The heterologous structural sequence is assembled in appropriate phase with translation, initiation and termination sequences, and preferably, a leader sequence capable of directing secretion of translated protein into the periplasmic space or extracellular medium. Optionally, the heterologous sequence can encode a fusion protein including an N-terminal identification peptide imparting desired characteristics, e.g., stabilization or simplified purification of expressed recombinant product.

Useful expression vectors for bacterial use are constructed by inserting a structural DNA sequence encoding a desired protein together with suitable translation, initiation and termination signals in operable reading phase with a functional promoter. The vector will comprise one or more phenotypic selectable markers and an origin of replication to ensure maintenance of the vector and to, if desirable, provide amplification within the host. Suitable prokaryotic hosts for transformation include *E.coli*, *Bacillus subtilis*, *Salmonella typhimurium* and various species within the genera *Pseudomonas*, *Streptomyces*, and *Staphylococcus*, although others may also be employed as a matter of choice. Useful expression vectors for bacterial use can comprise a selectable marker and bacterial origin of replication derived from commercially available plasmids comprising genetic elements of the well known cloning vector pBR322 (ATCC 37017). Such commercial vectors include, for example, pKK223-3 (Pharmacia Fine Chemicals, Uppsala, Sweden) and GEM1 (Promega Biotech, Madison, Wis., USA). These pBR322 "backbone" sections are combined with an appropriate promoter and the structural sequence to be expressed.

After transformation of a suitable host strain and growth of the host strain to an appropriate cell density, the selected promoter is derepressed by appropriate means (e.g., temperature shift or chemical induction) and cells are cultured for an additional period. Cells are typically harvested by centrifugation, disrupted by physical or chemical means, and the resulting crude extract retained for further purification. Microbial cells employed in expression of proteins can be disrupted by any convenient method, including freeze-thaw cycling, sonication, mechanical disruption, or use of cell lysing agents.

Various mammalian cell culture systems can also be employed to express recombinant protein. Examples of mammalian expression systems include the COS-7 lines of monkey kidney fibroblasts, described by Gluzman, *Cell* 23:175 (1981), and other cell lines capable of expressing a compatible vector, for example, the C127, 3T3, CHO, HeLa and BHK cell lines. Mammalian expression vectors will comprise an origin of replication, a suitable promoter and enhancer, and also any necessary ribosome binding sites, polyadenylation site, splice donor and acceptor sites, transcriptional termination sequences, and 5' flanking nontranscribed sequences. DNA sequences derived from the SV40 viral genome, for example, SV40 origin,

early promoter, enhancer, splice, and polyadenylation sites may be used to provide the required non-transcribed genetic elements.

5 The polypeptides of the present invention may be recovered and purified from recombinant cell cultures by methods used heretofore, including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxyapatite chromatography and lectin chromatography. Protein refolding steps can be used, as necessary, in completing configuration of the mature protein. Finally, high performance liquid chromatography (HPLC) can be employed for final purification steps.

10 The polypeptides of the present invention may be a product of chemical synthetic-procedures, or produced by recombinant techniques from a prokaryotic or eukaryotic host (for example, by bacterial, yeast, higher plant, insect and mammalian cells in culture). Depending upon the host employed in a recombinant production procedure, the polypeptides of the present invention may be glycosylated with mammalian or other eukaryotic carbohydrates or may be non-glycosylated. Polypeptides of the invention may also include an initial methionine amino acid residue.

20 The polypeptides of the present invention, as a result of the ability to stimulate insulin secretion from pancreatic islet cells *in vitro*, and by causing a decrease in blood glucose *in vivo*, may be employed in treatment of Type 2 Diabetes (non-insulin dependent diabetes mellitus). Also, the polypeptides may be used to prevent subjects with impaired glucose tolerance from proceeding to develop Type II diabetes. In addition, the polypeptides of the invention may be used for treatment of asthma (Bolin et al Biopolymer 37:57-66 (1995); US Patent No. 5,677,419)(showing that polypeptide R3P0 is active in relaxing guinea pig tracheal smooth muscle); hypotension induction (VIP induces hypotension, tachycardia, and facial flushing in asthmatic patients (Morice, A.H., and Sever, P.S., Peptides 7:279-280 (1986); Morice, A., et al., The Lancet II, 1225-1227 (1983)), male reproduction problems (Siow, Y., et al., Effects of vasoactive intestinal peptide on human sperm motility, Arch. Androl. 1999 Jul-Aug; 43(1):67-71); as a anti-apoptosis/neuroprotective agent (Brenneman D.R., et al., VIP neurotrophism in the central nervous system: multiple effectors and identification of a femtomolar-acting neuroprotective peptide, Ann. N.

25
30

Y. Acad. Sci. 1998 Dec. 11;865:207-12); cardioprotection during ischemic events (Kalfin R., et al., Protective role of intracoronary vasoactive intestinal peptide in ischemic and reperfused myocardium, J. Pharmacol. Exp. Ther. 1994 Feb;268(2):952-8; Das, D.K., et al., Coordinated role of vasoactive intestinal peptide and nitric oxide in cardioprotection, Ann. N. Y. Acad. Sci. 1998 Dec 11;865:297-308), and finally as an anti-ulcer agent (Tuncel, et al., The protective effect of vasoactive intestinal peptide (VIP) on stress-induced gastric ulceration in rats, Ann. N. Y. Acad. Sci. 1998 Dec 11;865:309-22.

The polypeptides of the present invention may be employed in combination with a suitable pharmaceutical carrier to comprise a pharmaceutical composition for parenteral administration. Such compositions comprise a therapeutically effective amount of the polypeptide and a pharmaceutically acceptable carrier or excipient. Such a carrier includes but is not limited to saline, buffered saline, dextrose, water, glycerol, ethanol, and combinations thereof. The formulation should suit the mode of administration. The invention also provides a pharmaceutical pack or kit comprising one or more containers filled with one or more of the ingredients of the pharmaceutical compositions of the invention. Associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration. In addition, the polypeptides of the present invention may be employed in conjunction with other therapeutic compounds.

The pharmaceutical compositions may be administered in a convenient manner such as by the oral, topical, intravenous, intraperitoneal, intramuscular, subcutaneous, intranasal or intradermal routes. The pharmaceutical compositions are administered in an amount which is effective for treating and/or prophylaxis of the specific indication. In general, they are administered in an amount of at least about 350ng (0.1nmol)/kg body weight and in most cases they will be administered in an amount not in excess of about 35ug (10nmol)/kg body weight per day. In most cases, the dosage is from about 0.1 µg/kg to about 100 mg/kg body weight daily, taking into account the routes of administration, symptoms, etc. These numbers do not take into account the bioavailability of the peptide in vivo, in which case more or less may be required to attain the effective dose required. One of ordinary skill in the

art is able to determine through dosing experiments or other conventional means the gross amount required to produce an effective dosage.

A polypeptide of the invention may also be employed in accordance with the present invention by expression of such polypeptide *in vivo*, which is often referred to as "gene therapy." Thus, for example, cells may be engineered with a polynucleotide (DNA or RNA) encoding for the polypeptide *ex vivo*, the engineered cells are then provided to a patient to be treated with the polypeptide. Such methods are well-known in the art. For example, cells may be engineered by procedures known in the art by use of a retroviral particle containing RNA encoding for the polypeptide of the present invention.

Local delivery of the insulin secretagogues using gene therapy may provide the therapeutic agent to the target area, i.e., the pancreas. For instance a pancreas-specific promoter was used to create a beta-cell pancreatic tumor mouse model (Hanahan, D., Heritable formation of pancreatic beta-cell tumours in transgenic mice expressing recombinant insulin/simian virus 40 oncogenes, *Nature* 315(6015):115-22 (1985)).

Both *in vitro* and *in vivo* gene therapy methodologies are contemplated. Several methods for transferring potentially therapeutic genes to defined cell populations are known. See, e.g., Mulligan, "The Basic Science Of Gene Therapy", *Science*, 260: 926-31 (1993). These methods include:

1) Direct gene transfer. See, e.g., Wolff *et al.*, "Direct Gene transfer Into Mouse Muscle In Vivo", *Science*, 247:1465-68 (1990);

2) Liposome-mediated DNA transfer. See, e.g., Caplen *et al.*, "Liposome-mediated CFTR Gene Transfer To The Nasal Epithelium Of Patients With Cystic Fibrosis", *Nature Med.* 3: 39-46 (1995); Crystal, "The Gene As A Drug", *Nature Med.* 1:15-17 (1995); Gao and Huang, "A Novel Cationic Liposome Reagent For Efficient Transfection Of Mammalian Cells", *Biochem. Biophys. Res. Comm.*, 179:280-85 (1991);

3) Retrovirus-mediated DNA transfer. See, e.g., Kay *et al.*, "In Vivo Gene Therapy Of Hemophilia B: Sustained Partial Correction In Factor IX-Deficient Dogs", *Science*, 262:117-19 (1993); Anderson, "Human Gene Therapy", *Science*, 256:808-13 (1992).

09407832-092899

4) DNA Virus-mediated DNA transfer. Such DNA viruses include adenoviruses (preferably Ad-2 or Ad-5 based vectors), herpes viruses (preferably herpes simplex virus based vectors), and parvoviruses (preferably "defective" or non-autonomous parvovirus based vectors, more preferably adeno-associated virus based vectors, most preferably AAV-2 based vectors). See, e.g., *Ali et al.*, "The Use Of DNA Viruses As Vectors For Gene Therapy", *Gene Therapy*, 1:367-84 (1994); United States Patent 4,797,368, incorporated herein by reference, and United States Patent 5,139,941, incorporated herein by reference.

The choice of a particular vector system for transferring the gene of interest will depend on a variety of factors. One important factor is the nature of the target cell population. Although retroviral vectors have been extensively studied and used in a number of gene therapy applications, these vectors are generally unsuited for infecting non-dividing cells. In addition, retroviruses have the potential for oncogenicity. However, recent developments in the field of lentiviral vectors may circumvent some of these limitations. See *Naldini et al.*, In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector, *Science* 272:263-7 (1996).

Retroviruses from which the retroviral plasmid vectors hereinabove mentioned may be derived include, but are not limited to, Moloney Murine Leukemia Virus, spleen necrosis virus, retroviruses such as Rous Sarcoma Virus, Harvey Sarcoma Virus, avian leukosis virus, gibbon ape leukemia virus, human immunodeficiency virus, adenovirus, Myeloproliferative Sarcoma Virus, and mammary tumor virus. In one embodiment, the retroviral plasmid vector is derived from Moloney Murine Leukemia Virus.

Adenoviruses have the advantage that they have a broad host range, can infect quiescent or terminally differentiated cells, such as neurons or hepatocytes, and appear essentially non-oncogenic. See, e.g., *Ali et al.*, *supra*, p. 367. Adenoviruses do not appear to integrate into the host genome. Because they exist extrachromosomally, the risk of insertional mutagenesis is greatly reduced. *Ali et al.*, *supra*, p. 373.

Adeno-associated viruses exhibit similar advantages as adenoviral-based vectors. However, AAVs exhibit site-specific integration on human chromosome 19 (*Ali et al.*, *supra*, p. 377).

In a preferred embodiment, the DNA encoding the polypeptide insulin secretagogues of this invention is used in gene therapy for disorders such as diabetes.

According to this embodiment, gene therapy with DNA encoding polypeptide insulin secretagogues or muteins of this invention is provided to a patient in need thereof, concurrent with, or immediately after diagnosis.

The skilled artisan will appreciate that any suitable gene therapy vector containing polypeptide insulin secretagogues, DNA or DNA of fragment, derivative or analog of polypeptide insulin secretagogues may be used in accordance with this embodiment. The techniques for constructing such a vector are known. See, e.g., Anderson, W.F., "Human Gene Therapy," *Nature*, 392 25-30 (1998); Verma, I.M., and Somia, N., "Gene Therapy - Promises, Problems, and Prospects," *Nature*, 389 239-242 (1998). Introduction of the polypeptide insulin secretagogues DNA-containing vector to the target site may be accomplished using known techniques.

The vector includes one or more promoters. Suitable promoters which may be employed include, but are not limited to, the retroviral LTR; the SV40 promoter; and the human cytomegalovirus (CMV) promoter described in Miller, et al., *Biotechniques*, 7(9): 980-990 (1989), or any other promoter (e.g., cellular promoters such as eukaryotic cellular promoters including, but not limited to, the histone, pol III, and β -actin promoters). Other viral promoters which may be employed include, but are not limited to, adenovirus promoters, thymidine kinase (TK) promoters, and B19 parvovirus promoters. The selection of a suitable promoter will be apparent to those skilled in the art from the teachings contained herein.

The nucleic acid sequence encoding the polypeptide of the present invention is under the control of a suitable promoter. Suitable promoters which may be employed include, but are not limited to, adenoviral promoters, such as the adenoviral major late promoter; or heterologous promoters, such as the cytomegalovirus (CMV) promoter; the respiratory syncytial virus (RSV) promoter; inducible promoters, such as the MMT promoter, the metallothionein promoter; heat shock promoters; the albumin promoter; the ApoAI promoter; human globin promoters; viral thymidinekinase promoters, such as the Herpes Simplex thymidine kinase promoter; retroviral LTRs (including the modified retroviral LTRs hereinabove described); the β -actin promoter; and human growth hormones

promoters. The promoter also may be the native promoter which controls the gene encoding the polypeptide.

The retroviral plasmid vector is employed to transduce packaging cell lines to form producer cell lines. Examples of packaging cells which maybe transfected include, but are not limited to, the PES01, PA317, ψ -2, ψ -AM, PA12, T19-14X, VT-19-17-H2, ψ CRE, ψ CRIP, GP+E-86, GP+envAm12, and DAN cell lines as described in Miller, *Human Gene Therapy*, 1: 5-14 (1990), which is incorporated herein by reference in its entirety. The vector may transduce the packaging cells through any means known in the art. Such means include, but are not limited to, electroporation, the use of liposomes, and CaPO_4 precipitation. In one alternative, the retroviral plasmid vector may be encapsulated into a liposome, or coupled to a lipid, and then administered to a host. The producer cell line generates infectious retroviral vector particles which include the nucleic acid sequence(s) encoding the polypeptides. Such retroviral vector particles then may be employed, to transduce eukaryotic cells, either *in vitro* or *in vivo*. The transduced eukaryotic cells will express the nucleic acid sequence(s) encoding the polypeptide. Eukaryotic cells which may be transduced include, but are not limited to, embryonic stem cells, embryonic carcinoma cells, as well as hematopoietic stem cells, hepatocytes, fibroblasts, myoblasts, keratinocytes, endothelial cells, and bronchial epithelial cells.

A different approach to gene therapy is "transkaryotic therapy" wherein the patient's cells are treated *ex vivo* to induce the dormant chromosomal genes to produce the protein of interest after reintroduction to the patient. Transkaryotic therapy assumes the individual has a normal complement of genes necessary for activation. Transkaryotic therapy involves introducing a promoter or other exogenous regulatory sequence capable of activating the nascent genes, into the chromosomal DNA of the patients' cells *ex vivo*, culturing and selecting for active protein-producing cells, and then reintroducing the activated cells into the patient with the intent that they then become fully established. The "gene activated" cells then manufacture the protein of interest for some significant amount of time, perhaps for as long as the life of the patient. U.S. Patent Nos. 5,641,670 and 5,733,761 disclose in detail this concept, and are hereby incorporated by reference in their entirety.

09407832-0928 6

In order that this invention may be better understood, the following examples are set forth. These examples are for the purpose of illustration only, and are not to be construed as limiting the scope of the invention in any manner. All publications mentioned herein are incorporated by reference in their entirety.

5

B. Examples.

Protocols and Procedures

1. Protocol for Rat Islet Isolation

10 Sprague Dawley rats (275-320 g) were used as the source of donor islets. Briefly, the pancreas was filled with 10 ml of cold reconstituted Liberase RI (Boehringer Mannheim), harvested and incubated with additional 5 ml enzyme solution in water bath for 30 minutes. Tissue suspension was washed twice with cold 10% FBS/Hanks buffer (Gibco), resuspended in 8 ml 25% ficoll (Sigma) and then layered with 5 ml
15 each of 23%, 20% and 11% ficoll. The islets in the 20% layer after centrifugation were removed, washed twice with cold 10% FBS/Hank buffer and resuspended in 10% FBS/RPMI 1640 media (Sigma).

2. Peptide-Induced Elevation of Insulin Levels in Rats.

20 Wistar rats are fasted overnight (17 hrs) and then anesthetized with pentobarbital (0.1 ml/100 g BW). Glucose (0.4 g/kg dissolved in 1% human albumin-saline) +/- peptide (dissolved in 1% human albumin-saline) is injected intravenously into the tail vein.

The rats are eye-bled 1 minute after the injection and 50-100ul of the plasma are
25 assayed for insulin level with the Linco RIA kit (Linco Research, Inc., St. Charles, MO).

3. Effect of Peptides on Intraperitoneal Glucose Tolerance in Rats.

Wistar rats are fasted overnight and then anesthetized with pentobarbital.

30 The rats were eye-bled (zero time) and the peptide (in 1% human albumin) was injected into the tail vein. Five minutes later, 1 g/kg of glucose (in saline) was injected intraperitoneally, and the rats were eye-bled after 15, 30 and 60 minutes. Plasma glucose levels were determined using the Technicon Axon autoanalyzer,

Bayer Diagnostics division of Bayer Corporation, Tarrytown NY, operated using Method No. SM4-2143F90 "Glucose".

4. Effect of Peptides on Intestinal Water Retention in Mice.

Male mice were fasted for 24 hours, and their water bottles were taken away for 2 - 3 hours before the start of the experiment. Peptide or saline was injected subcutaneously into conscious mice. The mice were euthanized with CO₂ 10 minutes after dosing, and the small intestine dissected out and weighed (1). The intestine was cut open, the water in the lumen absorbed with filter paper, and the intestine re-weighed (2). The amount of intestinal water (g) = weight (1) - weight (2).

5. Peptide Synthesis Methodology.

The following general procedure was followed to synthesize some of the polypeptides of the invention. Peptide synthesis was carried out by the Fmoc/t-Butyl strategy (Peptide Synthesis Protocols (1994), Volume 35 by Michael W. Pennington & Ben M. Dunn) under continuous flow conditions using Rapp-Polymers PEG-Polystyrene resins (Rapp-Polymers, Tübingen, Germany). At the completion of synthesis, peptides are cleaved from the resin and de-protected using TFA/DTT/H₂O/Triisopropyl silane (88/5/5/2). Peptides were precipitated from the cleavage cocktail using cold diethyl ether. The precipitate was washed three times with the cold ether and then dissolved in 5% acetic acid prior to lyophilization. Peptides were checked by reversed phase chromatography on a YMC-Pack ODS-AQ column (YMC, Inc., Wilmington, NC) on a Waters ALLIANCE® system (Waters Corporation, Milford, MA) using water/acetonitrile with 3% TFA as a gradient from 0% to 100% acetonitrile, and by MALDI mass spectrometry on a VOYAGER DE™ MALDI Mass Spectrometer, (model 5-2386-00, PerSeptive BioSystems, Framingham, MA). Matrix buffer (50/50 dH₂O/acetonitrile with 3% TFA) peptide sample added to Matrix buffer 1/1. Those peptides not meeting the purity criteria of >95% are purified by reversed phase chromatography on a Waters Delta Prep 4000 HPLC system (Waters Corporation, Milford, MA).

2. Examples

Example 1. Peptide Cloning

The recombinant expression of VIP has been attempted previously with mixed results. Simoncsits et al (*Eur. J. Biochem.* 178: 343-350, (1988)) expressed Leu¹⁷, G²⁹-VIP or Leu¹⁷Gly²⁹ Lys³⁰ Arg³¹-VIP as a C-terminal fusion to the N-terminal part of the *E. coli* β -galactosidase gene in *E. coli*. The removal of the methionine at position 17 eliminates a CNBR cleavage site. C-terminal addition of Gly or Gly-Lys-Arg was designed for potential *in vivo* C-terminal amidation by mammalian PAMase. Upon CNBR cleavage of the fusion proteins at the methionine introduced N-terminal to the VIP mutants, free VIP mutants were purified and shown to possess similar activities as the native VIP, although the activities were measured only at saturating concentrations of the peptides. Raingeaud et al (*Biochimie* 78: 14-25 (1996)) expressed VIP as polymeric C-terminal fusions to glutathione S-transferase (GST) in *E. coli*. The free polymeric or monomeric VIP peptides were released upon sequential cleavages by Factor Xa and hydroxylamine. The requirement for the two-step cleavage led to inefficiency and a mixture of products. Polymeric or monomeric VIP peptides produced by this method were less active than the native VIP. An improved version of the construct using only Factor Xa cleavage yielded a VIP mutant with a seven residue C-terminal extension that was also less active than the native VIP (Ottavi et al, *Biochimie* 80: 289-293 (1998)). No expression of PACAP has been reported to date. To establish a robust method for expressing PACAP, VIP, and their mutants, their genetic codes were cloned C-terminal to GST with a single Factor Xa recognition site separating the monomeric peptide and GST. The gene encoding Factor Xa recognition site fused to PACAP38, VIP, and R3P3 has been synthesized by hybridizing two overlapping single-stranded DNA fragments (70-90mers) containing a Bam HI or Xho I restriction enzyme site immediately 5' to the DNA sequence of the gene to be cloned, followed by DNA synthesis of the opposite strands via the large fragment of DNA polymerase I (Life Technologies, Inc., Gaithersburg, MD). The double-stranded product is then digested by Bam HI and Xho I and ligated into pGEX-6P-1 (Pharmacia) which has also been cleaved by Bam HI and Xho I. The DNA sequences of the cloned peptide genes are listed below.

PACAP38:
ATCGAAGGTCGTCACTCCGATGGTATCTTCACCGACTCCTACTCTCGGTA
CCGCAAGCAGATGGCTGTAAAGAAATATCTGGCTGCAGTCCTAGGCAAA
CGTTACAAGCAACGCGTTAAAAACAAGTAATGA (SEQ ID NO:54)

5

VIP:
ATCGAAGGTCGTCACTCCGACGCTGTTTTACCGACAACCTACACGCGTCT
GCGTAAACAGATGGCTGTAAAGAAATACCTGAATTCCATCCTGAACTAAT
GA (SEQ ID NO:55)

10

R3P3:
ATCGAAGGTCGTCACTCCGATGCTGTTTTACCGAAAACTACACCAAGCT
TCGTAAACAGCTGGCAGCTAAGAAATACCTCAACGACCTGAAAAAGGGC
GGTACCTAATGA (SEQ ID NO: 56)

15

The first 12 nucleotides (underlined) encode the "IEGR" Factor Xa recognition site while
the last 6 (also underlined) represent the two stop codons. When cloned into pGEX-6P-1,
the following protein sequences were expressed as fusions with glutathione S-transferase
(GST):

20

PACAP38: IEGRHSDGIFTDSYSRYRKQMAVKKYLA AVL GKRYKQ RVKNK
(SEQ ID NO:2)

VIP: IEGRHSDAVFTDNYTRLRKQMAVKKYLN SILN (SEQ ID NO:1)

25

R3P3: IEGRHSDAVFTENYTKLRKQLAAKKYLNDLKKGGT (SEQ ID
NO:3)

A restriction map of a typical plasmid containing the GST-peptide fusion is shown as
Figure 3.

30

Example 2. Peptide Recombinant Expression and Purification

BL21 cells (Stratagene) transformed with the GST-peptide fusion containing
plasmids were grown at 37°C until OD₆₀₀ reached 0.6 to 1.0 and induced by 1 mM
IPTG (Life Technologies) for 2 hours at 37°C. 2L of cells were spun at 7,700 g for
15 minutes, weighed, and stored at -20°C for at least 3 hours. The frozen cell pellet
was resuspended in 100 mL ice-cold PBS with 250 ul of protease inhibitor cocktail
(Cat No. P-8465, Sigma Chemical) per gram of cells, sonicated at 3x for 1 minute
with 15 second breaks. Cellular debris were spun down at 10,000 g for 20 minutes.
The supernatant was mixed with 2 mL of 50% Glutathione Sepharose 4B resin

35

40

09407832.092899

144
141

(Pharmacia) on a shaker overnight at 4 °C. The resins were spun down at 1,500 g for 15 minutes, packed into empty Poly-Prep Chromatography Columns (Bio-Rad), washed with 30 mL PBS followed by 10mL of Factor Xa buffer (1 mM CaCl₂, 100 mM NaCl, and 50 mM Tris-HCl, pH 8.0). The peptides were cleaved off the column by 60 units of Factor Xa (Pharmacia) in 1 mL of Factor Xa buffer for overnight at 4 °C and run on C18 HPLC (Beckman System Gold), using a 2 mL loop and flow rate of 2 mL/min with the following program: 10 minutes of Buffer A (0.1% TFA/H₂O), 30 minutes of gradient to Buffer B (0.1% TFA/ACN), 10 minutes of Buffer A, 10 minutes of gradient, and 10 minutes of Buffer A. Peak fractions (1 mL each) were collected and screened by 10-20% Tricine-SDS gel electrophoresis. Fractions containing the peptides of Table 1 were pooled and dried down. Typical yields are several hundred micrograms of free peptides per liter of *E. coli* culture. Recombinant R3P3 has been tested to have the same activities as its synthetic version *in vivo*.

The following table contains some selected polypeptides made according to the Peptide Synthesis protocol discussed above, or recombinantly as described in Example 2. Recombinant peptides are denoted with a small letter "r" in front of the peptide designator.

Table 1

Peptide No.	Sequence	SEQ ID NO:
R3P0	Ac-HSDAVFTENYTKLRKQNIeAAKKYLNDLKKGGT-NH ₂	5
R3P1	Ac-HSDAVFTENYTKLRKQLAAKKYLNDLKKGGT-NH ₂	6
R3P2	Ac-HSDAVFTENYTKLRKQLAAKKYLNDLKKGGT	7
R3P3	HSDAVFTENYTKLRKQLAAKKYLNDLKKGGT	8
R3P4	Ac-HSDAVFTENOCH ₃ -YTKLRKQNIeAAKKYLNDLKK-NH ₂	9
R3P5	HSDAVFTENYTKLRKQLAAKKYLNDLKK	10
R3P8	HSDAVFTDNYTRLRKQMAVKKYNSIKK-NH ₂	11
R3P9	HSDAVFTDNYTRLRKQMAVKKYLSIKKGGT	12
R3P10	HSDAVFTENYTKLRKQLAAKKYLNDLLNGGT	13
R3P11	HSDAVFTDNYTKLRKQLAAKKYLNDILNGGT	14
R3P12	HSDAVFTDNYTRLRKQLAAKKYLNDIKKGGT	15
R3P13	HSDAVFTDNYTRLRKQLAAKKYLNDIKK-NH ₂	16
R3P14	HSDAVFTDNYTRLRKQMAVKKYLNDLKKGGT	17
R3P19	HSDAVFTENYTKLRKQLAAKKYLNDLKKGGTSWCEPGWCR	18
R3P20	HSDAVFTDNYTRLRKQMAAKKYLNDIKKGGT	19
R3P21	HSDAVFTDNYTRLRKQLAVKKYLNDIKKGGT	20
R3P22	HSDAVFTDNYTRLRKQLAAKKYLSIKKGGT	21
R3P24	HSDAVFTDNYTRLRKQLAAKKYLNDIKKGGT	22
R3P25	HSDAVFTDNYTRLRKQLAVKKYLSIKKGGT	23
R3P26	HSDAVFTDNYTRLRKQMAAKKYLSIKKGGT	24

09407832 - 92899

146
142

0 407832.092899

R3P29	HSDAVFTDNYTRLRKQLAVKKYLNDIKKGGT	25
R3P30	HSDAVFTDNYTRLRKQLAAKKYLNSIKKGGT	26
R3P31	HSDAVFTDNYTRLRKQLAAKKYLNDIKKGG	27
R3P32	HSDAVFTDNYTRLRKQLAAKKYLNDIKKG	28
R3P33	HSDAVFTDNYTRLRKQLAAKKYLNDIKK	29
R3P34	HSDAVFTDNYTRLRKQLAAKKYLNDIKKQ	30
R3P35	HSDAVFTDNYTRLRKQLAAKKYLNDIKKQ	31
R3P36	HSDAVFTDNYTRLRKQLAAKKYLNDIKKKRY	32
rR3P41	HSDAVFTDNYTRLRKQMAVKKYLNSIKK	33
rR3P42	HSDAVFTDNYTRLRKQMAVKKYLNSIKK	34
rR3P43	HSDAVFTDNYTRLRKQMAVKKYLNSILK	35
rR3P44	HSDAVFTDNYTRLRKQMAVKKYLNSILN	36
rR3P45	HSDAVFTDNYTRLRKQMAVKKYLNSILN	37
rR3P46	HSDAVFTDNYTRLRKQLAVKKYLNSILN	38
rR3P47	HSDAVFTDNYTRLRKQMAAKKYLNSILN	39
rR3P48	HSDAVFTDNYTRLRKQMAVKKYLNDILN	40
rR3P49	HSDAVFTDNYTRLRKQMAAKKYLNSIKK	41
rR3P50	HSDAVFTDNYTRLRKQMAAKKYLNSILK	42
rR3P51	HSDAVFTDNYTRLRKQMAAKKYLNSIKK	43
rR3P52	HSDAVFTDNYTRLRKQMAAKKYLNSIKKORY	44
rR3P53	HSDAVFTDNYTRLRKQMAAKKYLNSIKKOR	45
rR3P54	HSDAVFTDNYTRLRKQMAAKKYLNSIKKK	46
rR3P55	HSDAVFTDNYTRLRKQMAAKKYLNSIKKKRY	47
rR3P56	HSDAVFTDNYTRLRKQMAVKKYLNSIKKKRY	48
rR3P57	HSDAVFTDNYTRLRKQMAVKKYLNSIKKKR	49
rR3P58	HSDAVFTDNYTRLRKQMAVKKYLNSIKKK	50
rR3P59	HSDAVFTDNYTRLRKQMAVKKYLNSIKKKRY	51
rR3P60	HSDAVFTDNYTRLRKQVAAKKYLQSIKK	52
rR3P61	HSDAVFTDNYTRLRKQLAAKKYLQTIKK	53

Example 3. Insulin secretion by rat islets

Peptide R3P3 (0.1-100 nM) stimulates insulin secretion from isolated rat islets in a glucose-dependent fashion. These studies compare the effects on islet cells of R3P3 peptide and GLP-1.

Rat islets were isolated and treated with GLP-1 or R3P3 at either 3 or 8 mM glucose in the medium in accordance with the rat islet protocol described above, under "Protocols and Procedures." As shown by Figures 4A-4B, peptide R3P3 significantly enhances the release of insulin from islets in a concentration-dependent fashion and this effect is similar to that of GLP-1, a known insulin secretagogue.

Example 4. In vivo insulin and glucose response. As shown in Table 2 below, peptide R3P3 increases plasma insulin one minute after IVGTT in fasted rats with an Emax of 1nmol/kg i.v.

In accordance with Standard Protocol #2 above, overnight fasted Wistar rats were anesthetized with pentobarital; eye-bled for basal insulin; i.v. injected with glucose $\pm$ peptide and tail bled after 1 minute. N=12 rats/group. The graph in Figure 5 shows that the polypeptide R3P3 causes an increase in glucose disposal that accompanies the increase in insulin secretion. The peptide or vehicle was given i.v. followed by a glucose load given i.p. The plasma glucose was followed over the time period indicated. As shown in the graph, R3P3 markedly accelerated the disposal of blood glucose.

Example 5. Diarrhea side effects.

As described in standard Protocol #5 above, fasted mice were injected s.c. with the indicated peptide (5 nmol/kg or 22-24 nmol/kg). Five minutes after the injection, 0.3 ml of water was given p.o. Five min after the water dose, the animals were euthanized and the water content in the small intestine determined. As shown in Figure 6, VIP injections at the two doses caused a marked increase in the water content of the small intestine lumen over the vehicle (saline) control. Importantly at the highest dose, the R3 peptides caused only a very small increase (i.e. roughly 10%) in comparison to VIP. At the 5 nmol/kg dose, the peptides did not produce any change in water content in the small gut.

Example 6. Effect of Peptides on Intraperitoneal Glucose Tolerance in Rats

As described in standard protocol # 4, Wistar rats were fasted overnight and then anesthetized with Pentobarbital. The rats were eye-bled (zero time) and the peptide (in 1% human albumin) was injected subcutaneously. Five minutes later, 1 g/kg of glucose (in saline) was injected intraperitoneally, and the rats were eye-bled after 30 minutes. Plasma glucose levels were determined using the Axon autoanalyzer, and are shown in Figure 7.

Figure 7 shows that plasma glucose elevated to 160 mg/dl above basal (100mg/dl) in rats treated with vehicle 30 minutes after IPGTT (IP Glucose Tolerance Test). In rats injected with peptides R3P3, R3P12 and R3P13, this elevation of plasma glucose was significantly reduced, verifying the insulin-producing effect. At 1 nmole/kg s.c., the glucose-lowering effect of each peptide was similar to that observed with an equivalent dose of GLP-1.

Example 7. Glucose-dependent insulin secreting polypeptides.

Table 2 contains a list of the peptides that stimulated release of insulin *in vivo* in the IPGTT assay, or *in vitro* through the rat islet assay. As shown by the data, the peptides enhance glucose-mediated insulin release *in vivo* and *in vitro*.

Plasma insulin: Data are expressed as % of plasma insulin one minute after IVGTT (0.4g/kg glucose) with either saline or 1 nmole/kg peptide in Wistar rats. Blood was drawn from the eye and insulin was measured with the rat insulin RIA kit (Linco Research, Inc., St. Charles, MO).

Islet insulin release: Rat islets were isolated from Sprague-Dawley rats as described in "Protocols and Procedures" and treated with either vehicle or specified peptide (10nM) for 2hrs. Insulin concentration in the medium was measured with the Linco rat insulin RIA kit. Glucose concentration in the medium was 8mM. The data are expressed as % of [insulin] in 8mM glucose alone.

Table 2

Peptide	Plasma insulin (% of basal)	Islet insulin release (% of basal)
PACAP 27	320	186
R3P0	ND	264
R3P1	480	250
R3P3	361	275
rR3P3	450	212
R3P9	ND	221
R3P10	ND	174
R3P12	302	324
R3P13	465	170
R3P19	285	ND
R3P36	255	ND

ND: not determined

All publications and patents mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described compositions and methods of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the above-described modes

09407832-0928999

148
145

for carrying out the invention which are obvious to those skilled in the field of molecular biology or related fields are intended to be within the scope of the following claims.

09407832.0 2899

CLAIMS

We claim:

1. A polypeptide selected from the group consisting of SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:18 and SEQ ID NO:32 or a functional equivalent thereof.
2. A polynucleotide encoding a polypeptide sequence of claim 1, or a degenerate variant thereof.
3. A vector comprising a polynucleotide of claim 2.
4. A host cell comprising a vector of claim 3.
5. A method for producing a polypeptide comprising:
 - a) culturing the host cell of claim 4 under conditions suitable for the expression of said polypeptide; and
 - b) recovering the polypeptide from the host cell culture.
6. A pharmaceutical composition comprising a polypeptide of claim 1 in combination with a pharmaceutically acceptable carrier.
7. A gene therapy composition comprising a polynucleotide of claim 2 in combination with a therapeutically effective gene therapy vector.
8. The polypeptide of claim 1 wherein said polypeptide is represented by SEQ ID NO: 6.
9. The polypeptide of claim 1 wherein said polypeptide is represented by SEQ ID NO: 8.
10. The polypeptide of claim 1 wherein said polypeptide is represented by SEQ ID NO: 12.

150
147

11. The polypeptide of claim 1 wherein said polypeptide is represented by SEQ ID NO: 13.

5 12. The polypeptide of claim 1 wherein said polypeptide is represented by SEQ ID NO: 15.

13. The polypeptide of claim 1 wherein said polypeptide is represented by SEQ ID NO: 16.

10 14. The polypeptide of claim 1 wherein said polypeptide is represented by SEQ ID NO: 18.

15 15. The polypeptide of claim 1 wherein said polypeptide is represented by SEQ ID NO: 32.

16. A method of treating a metabolic disorder in a mammal comprising administering a therapeutically effective amount of a peptide selected from the group consisting of SEQ ID NO: 5, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:18 and SEQ ID NO:32 to said mammal.

17. The method of claim 16 wherein said metabolic disorder is type 2 diabetes.

25 18. The method of claim 16 wherein said therapeutically effective amount ranges from about 0.1ug/kg to about 1mg/kg

19. The method of claim 16 wherein said metabolic disorder is the pre-diabetic state of impaired glucose tolerance.

30 20. A vasoactive intestinal peptide variant having one of the structures of Figure 1, and the functional equivalents thereof.

09407832-092899

151
148

21. A method of stimulating insulin release in a glucose-dependent manner in a mammal in need thereof by administering to said mammal a polypeptide selected from the group consisting of the polypeptides of Figure 1.

09407832.092899

Abstract

This invention provides novel peptides that function *in vivo* to stimulate insulin release from pancreatic beta cells in a glucose-dependent fashion. These insulin secretagogue peptides are shown to stimulate insulin release in rat islet cells *in vitro*, and *in vivo*. The peptides of the present invention provide a new therapy for patients with decreased endogenous insulin production, in particular type 2 diabetics. In particular, the invention is a polypeptide selected from a specific group of VIP/PACAP-related polypeptides, or functional equivalents thereof. The invention is also directed to a method of treating a metabolic disease in a mammal comprising administering a therapeutically effective amount of the insulin secretagogue peptides to said mammal. Also disclosed are methods of making the peptides, both recombinant and synthetic.

9407832-092899

159
150

SEQUENCE LISTING

<110> Pan, Clark
Tsutsumi, Manami

<120> Glucose-dependant Insulin Secretagogue Polypeptides and
Their Methods of Use

<130> 5053

<140> NOT ASSIGNED

<141> 1999-09-28

<160> 56

<170> PatentIn Ver. 2.0

<210> 1

<211> 28

<212> PRT

<213> Homo sapiens

<220>

<221> PEPTIDE

<222> (1)..(28)

<220>

<221> MOD_RES

<222> (28)

<223> AMIDATION

<400> 1

His	Ser	Asp	Ala	Val	Phe	Thr	Asp	Asn	Tyr	Thr	Arg	Leu	Arg	Lys	Gln		
1				5				10						15			
Met Ala Val Lys Lys Tyr Leu Asn Ser Ile Leu Asn																	
								20									25

<210> 2

<211> 38

<212> PRT

<213> Homo sapiens

<220>

<221> PEPTIDE

<222> (1)..(38)

09407832-092899

174
157

<220>
<221> MOD_RES
<222> (38)
<223> AMIDATION

<400> 2

His Ser Asp Gly Ile Phe Thr Asp Ser Tyr Ser Arg Tyr Arg Lys Gln
1 5 10 15

Met Ala Val Lys Lys Tyr Leu Ala Ala Val Leu Gly Lys Arg Tyr Lys
20 25 30

Gln Arg Val Lys Asn Lys
35

<210> 3
<211> 30
<212> PRT
<213> Homo sapiens

<220>
<221> PEPTIDE
<222> (1)..(30)

<220>
<221> MOD_RES
<222> (30)
<223> AMIDATION

<400> 3

His Ala Glu Gly Thr Phe Thr Ser Asp Val Ser Ser Tyr Leu Glu Gly
1 5 10 15

Gln Ala Ala Lys Glu Phe Ile Ala Trp Leu Val Lys Gly Arg
20 25 30

<210> 4
<211> 39
<212> PRT
<213> Homo sapiens

<220>
<221> PEPTIDE
<222> (1)..(39)

<220>

09407832.092899
668260.2E870460

<221> MOD_RES
<222> (39)
<223> AMIDATION

<400> 4

His Gly Glu Gly Thr Phe Thr Ser Asp Leu Ser Lys Gln Met Glu Glu
1 5 10 15

Glu Ala Val Arg Leu Phe Ile Glu Trp Leu Lys Asn Gly Gly Pro Ser
20 25 30

Ser Gly Ala Pro Pro Pro Ser
35

<210> 5
<211> 31
<212> PRT
<213> Homo sapiens

<220>
<221> PEPTIDE
<222> (1)..(31)

<220>
<221> PEPTIDE
<222> (17)
<223> Xaa=Nle

<220>
<221> MOD_RES
<222> (1)
<223> ACETYLATION

<220>
<221> MOD_RES
<222> (31)
<223> AMIDATION

<400> 5

His Ser Asp Ala Val Phe Thr Glu Asn Tyr Thr Lys Leu Arg Lys Gln
1 5 10 15

Xaa Ala Ala Lys Lys Tyr Leu Asn Asp Leu Lys Lys Gly Gly Thr
20 25 30

<210> 6

156
153

<211> 31
<212> PRT
<213> Homo sapiens

<220>
<221> PEPTIDE
<222> (1)..(31)

<220>
<221> MOD_RES
<222> (1)
<223> ACETYLATION

<220>
<221> MOD_RES
<222> (31)
<223> AMIDATION

<400> 6
His Ser Asp Ala Val Phe Thr Glu Asn Tyr Thr Lys Leu Arg Lys Gln
1 5 10 15

Leu Ala Ala Lys Lys Tyr Leu Asn Asp Leu Lys Lys Gly Gly Thr
20 25 30

<210> 7
<211> 31
<212> PRT
<213> Homo sapiens

<220>
<221> PEPTIDE
<222> (1)..(31)

<220>
<221> MOD_RES
<222> (1)
<223> ACETYLATION

<400> 7
His Ser Asp Ala Val Phe Thr Glu Asn Tyr Thr Lys Leu Arg Lys Gln
1 5 10 15

Leu Ala Ala Lys Lys Tyr Leu Asn Asp Leu Lys Lys Gly Gly Thr
20 25 30

09407832-223/0460

157
154

<210> 8
<211> 31
<212> PRT
<213> Homo sapiens

<220>
<221> PEPTIDE
<222> (1)..(31)

<400> 8
His Ser Asp Ala Val Phe Thr Glu Asn Tyr Thr Lys Leu Arg Lys Gln
1 5 10 15

Leu Ala Ala Lys Lys Tyr Leu Asn Asp Leu Lys Lys Gly Gly Thr
20 25 30

<210> 9
<211> 28
<212> PRT
<213> Homo sapiens

<220>
<221> PEPTIDE
<222> (1)..(28)

<220>
<221> PEPTIDE
<222> (9)
<223> Xaa-N-OCH3

<220>
<221> PEPTIDE
<222> (17)
<223> Xaa-Nle

<220>
<221> MOD_RES
<222> (1)
<223> ACETYLATION

<220>
<221> MOD_RES
<222> (28)
<223> AMIDATION

<400> 9
His Ser Asp Ala Val Phe Thr Glu Xaa Tyr Thr Lys Leu Arg Lys Gln

09407832.092899

158
155

1 5 10 15
Xaa Ala Ala Lys Lys Tyr Leu Asn Asp Leu Lys Lys
 20 25

<210> 10
<211> 28
<212> PRT
<213> Homo sapiens

<220>
<221> PEPTIDE
<222> (1)..(28)

<400> 10
His Ser Asp Ala Val Phe Thr Glu Asn Tyr Thr Lys Leu Arg Lys Gln
1 5 10 15

Leu Ala Ala Lys Lys Tyr Leu Asn Asp Leu Lys Lys
 20 25

<210> 11
<211> 27
<212> PRT
<213> Homo sapiens

<220>
<221> PEPTIDE
<222> (1)..(27)

<220>
<221> MOD_RES
<222> (27)
<223> AMIDATION

<400> 11
His Ser Asp Ala Val Phe Thr Asp Asn Tyr Thr Arg Leu Arg Lys Gln
1 5 10 15

Met Ala Val Lys Lys Tyr Asn Ser Ile Lys Lys
 20 25

<210> 12
<211> 31
<212> PRT

09407832.092899

<213> Homo sapiens

<220>

<221> PEPTIDE

<222> (1)..(31)

<400> 12

His Ser Asp Ala Val Phe Thr Asp Asn Tyr Thr Arg Leu Arg Lys Gln
1 5 10 15

Met Ala Val Lys Lys Tyr Leu Asn Ser Ile Lys Lys Gly Gly Thr
20 25 30

<210> 13

<211> 31

<212> PRT

<213> Homo sapiens

<220>

<221> PEPTIDE

<222> (1)..(31)

<400> 13

His Ser Asp Ala Val Phe Thr Glu Asn Tyr Thr Lys Leu Arg Lys Gln
1 5 10 15

Leu Ala Ala Lys Lys Tyr Leu Asn Asp Leu Leu Asn Gly Gly Thr
20 25 30

<210> 14

<211> 31

<212> PRT

<213> Homo sapiens

<220>

<221> PEPTIDE

<222> (1)..(31)

<400> 14

His Ser Asp Ala Val Phe Thr Asp Asn Tyr Thr Lys Leu Arg Lys Gln
1 5 10 15

Leu Ala Ala Lys Lys Tyr Leu Asn Asp Ile Leu Asn Gly Gly Thr
20 25 30

160
157

<210> 15
<211> 31
<212> PRT
<213> Homo sapiens

<220>
<221> PEPTIDE
<222> (1)..(31)

<400> 15
His Ser Asp Ala Val Phe Thr Asp Asn Tyr Thr Arg Leu Arg Lys Gln
1 5 10 15
Leu Ala Ala Lys Lys Tyr Leu Asn Asp Ile Lys Lys Gly Gly Thr
20 25 30

<210> 16
<211> 28
<212> PRT
<213> Homo sapiens

<220>
<221> PEPTIDE
<222> (1)..(28)

<220>
<221> MOD_RES
<222> (28)
<223> AMIDATION

<400> 16
His Ser Asp Ala Val Phe Thr Asp Asn Tyr Thr Arg Leu Arg Lys Gln
1 5 10 15
Leu Ala Ala Lys Lys Tyr Leu Asn Asp Ile Lys Lys
20 25

<210> 17
<211> 31
<212> PRT
<213> Homo sapiens

<220>
<221> PEPTIDE
<222> (1)..(31)

00407832-25870760

<400> 17

His Ser Asp Ala Val Phe Thr Asp Asn Tyr Thr Arg Leu Arg Lys Gln
1 5 10 15

Met Ala Val Lys Lys Tyr Leu Asn Asp Leu Lys Lys Gly Gly Thr
20 25 30

<210> 18

<211> 40

<212> PRT

<213> Homo sapiens

<220>

<221> PEPTIDE

<222> (1)..(40)

<400> 18

His Ser Asp Ala Val Phe Thr Glu Asn Tyr Thr Lys Leu Arg Lys Gln
1 5 10 15

Leu Ala Ala Lys Lys Tyr Leu Asn Asp Leu Lys Lys Gly Gly Thr Ser
20 25 30

Trp Cys Glu Pro Gly Trp Cys Arg
35 40

<210> 19

<211> 31

<212> PRT

<213> Homo sapiens

<220>

<221> PEPTIDE

<222> (1)..(31)

<400> 19

His Ser Asp Ala Val Phe Thr Asp Asn Tyr Thr Arg Leu Arg Lys Gln
1 5 10 15

Met Ala Ala Lys Lys Tyr Leu Asn Asp Ile Lys Lys Gly Gly Thr
20 25 30

<210> 20

<211> 31

<212> PRT

162
159



<213> Homo sapiens

<220>

<221> PEPTIDE

<222> (1)..(31)

<400> 20

His Ser Asp Ala Val Phe Thr Asp Asn Tyr Thr Arg Leu Arg Lys Gln
1 5 10 15

Leu Ala Val Lys Lys Tyr Leu Asn Asp Ile Lys Lys Gly Gly Thr
20 25 30

<210> 21

<211> 31

<212> PRT

<213> Homo sapiens

<220>

<221> PEPTIDE

<222> (1)..(31)

<400> 21

His Ser Asp Ala Val Phe Thr Asp Asn Tyr Thr Arg Leu Arg Lys Gln
1 5 10 15

Leu Ala Ala Lys Lys Tyr Leu Asn Ser Ile Lys Lys Gly Gly Thr
20 25 30

<210> 22

<211> 31

<212> PRT

<213> Homo sapiens

<220>

<221> PEPTIDE

<222> (1)..(31)

<400> 22

His Ser Asp Ala Val Phe Thr Asp Asn Tyr Thr Arg Leu Arg Lys Gln
1 5 10 15

Leu Ala Ala Lys Lys Tyr Leu Asn Asp Ile Lys Asn Gly Gly Thr
20 25 30

09407832.092899

163
160

<210> 23
<211> 31
<212> PRT
<213> Homo sapiens

<220>
<221> PEPTIDE
<222> (1)..(31)

<400> 23
His Ser Asp Ala Val Phe Thr Asp Asn Tyr Thr Arg Leu Arg Lys Gln
1 5 10 15

Leu Ala Val Lys Lys Tyr Leu Asn Ser Ile Lys Lys Gly Gly Thr
20 25 30

<210> 24
<211> 31
<212> PRT
<213> Homo sapiens

<220>
<221> PEPTIDE
<222> (1)..(31)

<400> 24
His Ser Asp Ala Val Phe Thr Asp Asn Tyr Thr Arg Leu Arg Lys Gln
1 5 10 15

Met Ala Ala Lys Lys Tyr Leu Asn Ser Ile Lys Lys Gly Gly Thr
20 25 30

<210> 25
<211> 31
<212> PRT
<213> Homo sapiens

<400> 25
His Ser Asp Ala Val Phe Thr Asp Asn Tyr Thr Arg Leu Arg Lys Gln
1 5 10 15

Leu Ala Val Lys Lys Tyr Leu Asn Asp Ile Lys Asn Gly Gly Thr
20 25 30

<210> 26

09407832.092899

164
161

<211> 31
<212> PRT
<213> Homo sapiens

<220>
<221> PEPTIDE
<222> (1)..(31)

<400> 26
His Ser Asp Ala Val Phe Thr Asp Asn Tyr Thr Arg Leu Arg Lys Gln
1 5 10 15

Leu Ala Ala Lys Lys Tyr Leu Asn Ser Ile Lys Asn Gly Gly Thr
20 25 30

<210> 27
<211> 30
<212> PRT
<213> Homo sapiens

<220>
<221> PEPTIDE
<222> (1)..(30)

<400> 27
His Ser Asp Ala Val Phe Thr Asp Asn Tyr Thr Arg Leu Arg Lys Gln
1 5 10 15

Leu Ala Ala Lys Lys Tyr Leu Asn Asp Ile Lys Lys Gly Gly
20 25 30

<210> 28
<211> 29
<212> PRT
<213> Homo sapiens

<220>
<221> PEPTIDE
<222> (1)..(29)

<400> 28
His Ser Asp Ala Val Phe Thr Asp Asn Tyr Thr Arg Leu Arg Lys Gln
1 5 10 15

Leu Ala Ala Lys Lys Tyr Leu Asn Asp Ile Lys Lys Gly
20 25

09407832.092899

163
162

<210> 29
<211> 28
<212> PRT
<213> Homo sapiens

<220>
<221> PEPTIDE
<222> (1)..(28)

<400> 29
His Ser Asp Ala Val Phe Thr Asp Asn Tyr Thr Arg Leu Arg Lys Gln
1 5 10 15

Leu Ala Ala Lys Lys Tyr Leu Asn Asp Ile Lys Lys
20 25

<210> 30
<211> 29
<212> PRT
<213> Homo sapiens

<220>
<221> PEPTIDE
<222> (1)..(29)

<400> 30
His Ser Asp Ala Val Phe Thr Asp Asn Tyr Thr Arg Leu Arg Lys Gln
1 5 10 15

Leu Ala Ala Lys Lys Tyr Leu Asn Asp Ile Lys Lys Gln
20 25

<210> 31
<211> 30
<212> PRT
<213> Homo sapiens

<220>
<221> PEPTIDE
<222> (1)..(30)

<400> 31
His Ser Asp Ala Val Phe Thr Asp Asn Tyr Thr Arg Leu Arg Lys Gln
1 5 10 15

09407832-092899

166
163

Leu Ala Ala Lys Lys Tyr Leu Asn Asp Ile Lys Lys Asn Gln
20 25 30

<210> 32
<211> 31
<212> PRT
<213> Homo sapiens

<220>
<221> PEPTIDE
<222> (1)..(31)

<400> 32
His Ser Asp Ala Val Phe Thr Asp Asn Tyr Thr Arg Leu Arg Lys Gln
1 5 10 15

Leu Ala Ala Lys Lys Tyr Leu Asn Asp Ile Lys Lys Lys Arg Tyr
20 25 30

<210> 33
<211> 28
<212> PRT
<213> Homo sapiens

<220>
<221> PEPTIDE
<222> (1)..(28)

<400> 33
His Ser Asp Ala Val Phe Thr Asp Asn Tyr Thr Arg Leu Arg Lys Gln
1 5 10 15

Met Ala Val Lys Lys Tyr Leu Asn Ser Ile Lys Lys
20 25

<210> 34
<211> 28
<212> PRT
<213> Homo sapiens

<220>
<221> PEPTIDE
<222> (1)..(28)

09407832-092899

167
164

<400> 34

His Ser Asp Ala Val Phe Thr Asp Asn Tyr Thr Arg Leu Arg Lys Gln
1 5 10 15

Met Ala Val Lys Lys Tyr Leu Asn Ser Ile Lys Asn
20 25

<210> 35

<211> 28

<212> PRT

<213> Homo sapiens

<220>

<221> PEPTIDE

<222> (1)..(28)

<400> 35

His Ser Asp Ala Val Phe Thr Asp Asn Tyr Thr Arg Leu Arg Lys Gln
1 5 10 15

Met Ala Val Lys Lys Tyr Leu Asn Ser Ile Leu Lys
20 25

<210> 36

<211> 28

<212> PRT

<213> Homo sapiens

<220>

<221> PEPTIDE

<222> (1)..(28)

<400> 36

His Ser Asp Ala Val Phe Thr Asp Asn Tyr Thr Glu Leu Arg Lys Gln
1 5 10 15

Met Ala Val Lys Lys Tyr Leu Asn Ser Ile Leu Asn
20 25

<210> 37

<211> 28

<212> PRT

<213> Homo sapiens

<220>

15

09407832-092899

165
165

<221> PEPTIDE
<222> (1)..(28)

<400> 37

His Ser Asp Ala Val Phe Thr Asp Asn Tyr Thr Arg Leu Arg Glu Gln
1 5 10 15

Met Ala Val Lys Lys Tyr Leu Asn Ser Ile Leu Asn
20 25

<210> 38
<211> 28
<212> PRT
<213> Homo sapiens

<220>
<221> PEPTIDE
<222> (1)..(28)

<400> 38

His Ser Asp Ala Val Phe Thr Asp Asn Tyr Thr Arg Leu Arg Lys Gln
1 5 10 15

Leu Ala Val Lys Lys Tyr Leu Asn Ser Ile Leu Asn
20 25

<210> 39
<211> 28
<212> PRT
<213> Homo sapiens

<220>
<221> PEPTIDE
<222> (1)..(28)

<400> 39

His Ser Asp Ala Val Phe Thr Asp Asn Tyr Thr Arg Leu Arg Lys Gln
1 5 10 15

Met Ala Ala Lys Lys Tyr Leu Asn Ser Ile Leu Asn
20 25

<210> 40
<211> 28
<212> PRT

09407832.092899

169
166

<213> Homo sapiens

<220>

<221> PEPTIDE

<222> (1)..(28)

<400> 40

His Ser Asp Ala Val Phe Thr Asp Asn Tyr Thr Arg Leu Arg Lys Gln
1 5 10 15

Met Ala Val Lys Lys Tyr Leu Asn Asp Ile Leu Asn
20 25

<210> 41

<211> 28

<212> PRT

<213> Homo sapiens

<220>

<221> PEPTIDE

<222> (1)..(28)

<400> 41

His Ser Asp Ala Val Phe Thr Asp Asn Tyr Thr Arg Leu Arg Lys Gln
1 5 10 15

Met Ala Ala Lys Lys Tyr Leu Asn Ser Ile Lys Asn
20 25

<210> 42

<211> 28

<212> PRT

<213> Homo sapiens

<220>

<221> PEPTIDE

<222> (1)..(28)

<400> 42

His Ser Asp Ala Val Phe Thr Asp Asn Tyr Thr Arg Leu Arg Lys Gln
1 5 10 15

Met Ala Ala Lys Lys Tyr Leu Asn Ser Ile Leu Lys
20 25

09407887-092899

170
167

<210> 43
<211> 28
<212> PRT
<213> Homo sapiens

<220>
<221> PEPTIDE
<222> (1)..(28)

<400> 43
His Ser Asp Ala Val Phe Thr Asp Asn Tyr Thr Arg Leu Arg Lys Gln
1 5 10 15
Met Ala Ala Lys Lys Tyr Leu Asn Ser Ile Lys Lys
20 25

<210> 44
<211> 31
<212> PRT
<213> Homo sapiens

<220>
<221> PEPTIDE
<222> (1)..(31)

<400> 44
His Ser Asp Ala Val Phe Thr Asp Asn Tyr Thr Arg Leu Arg Lys Gln
1 5 10 15
Met Ala Ala Lys Lys Tyr Leu Asn Ser Ile Lys Lys Lys Arg Tyr
20 25 30

<210> 45
<211> 30
<212> PRT
<213> Homo sapiens

<220>
<221> PEPTIDE
<222> (1)..(30)

<400> 45
His Ser Asp Ala Val Phe Thr Asp Asn Tyr Thr Arg Leu Arg Lys Gln
1 5 10 15
Met Ala Ala Lys Lys Tyr Leu Asn Ser Ile Lys Lys Lys Arg

09407832-068260-22870460

171
168

20

25

30

<210> 46
<211> 29
<212> PRT
<213> Homo sapiens

<220>
<221> PEPTIDE
<222> (1)..(29)

<400> 46
His Ser Asp Ala Val Phe Thr Asp Asn Tyr Thr Arg Leu Arg Lys Gln
1 5 10 15

Met Ala Ala Lys Lys Tyr Leu Asn Ser Ile Lys Lys Lys
20 25

<210> 47
<211> 31
<212> PRT
<213> Homo sapiens

<220>
<221> PEPTIDE
<222> (1)..(31)

<400> 47
His Ser Asp Ala Val Phe Thr Asp Asn Tyr Thr Arg Leu Arg Lys Gln
1 5 10 15

Met Ala Ala Lys Lys Tyr Leu Asn Ser Ile Lys Asn Lys Arg Tyr
20 25 30

<210> 48
<211> 31
<212> PRT
<213> Homo sapiens

<220>
<221> PEPTIDE
<222> (1)..(31)

<400> 48
His Ser Asp Ala Val Phe Thr Asp Asn Tyr Thr Arg Leu Arg Lys Gln

09407832.092899

172
169

1 5 10 15
Met Ala Val Lys Lys Tyr Leu Asn Ser Ile Lys Lys Lys Arg Tyr
 20 25 30

<210> 49
<211> 30
<212> PRT
<213> Homo sapiens

<220>
<221> PEPTIDE
<222> (1)..(30)

<400> 49
His Ser Asp Ala Val Phe Thr Asp Asn Tyr Thr Arg Leu Arg Lys Gln
1 5 10 15

Met Ala Val Lys Lys Tyr Leu Asn Ser Ile Lys Lys Lys Arg
 20 25 30

<210> 50
<211> 29
<212> PRT
<213> Homo sapiens

<220>
<221> PEPTIDE
<222> (1)..(29)

<400> 50
His Ser Asp Ala Val Phe Thr Asp Asn Tyr Thr Arg Leu Arg Lys Gln
1 5 10 15

Met Ala Val Lys Lys Tyr Leu Asn Ser Ile Lys Lys Lys
 20 25

<210> 51
<211> 31
<212> PRT
<213> Homo sapiens

<220>
<221> PEPTIDE
<222> (1)..(31)

09407832.0 2899

173
170

<400> 51

His Ser Asp Ala Val Phe Thr Asp Asn Tyr Thr Arg Leu Arg Lys Gln
1 5 10 15

Met Ala Val Lys Lys Tyr Leu Asn Ser Ile Lys Asn Lys Arg Tyr
20 25 30

<210> 52

<211> 28

<212> PRT

<213> Homo sapiens

<220>

<221> PEPTIDE

<222> (1)..(28)

<400> 52

His Ser Asp Ala Val Phe Thr Asp Asn Tyr Thr Arg Leu Arg Lys Gln
1 5 10 15

Val Ala Ala Lys Lys Tyr Leu Gln Ser Ile Lys Lys
20 25

<210> 53

<211> 28

<212> PRT

<213> Homo sapiens

<220>

<221> PEPTIDE

<222> (1)..(28)

<400> 53

His Ser Asp Ala Val Phe Thr Asp Asn Tyr Thr Arg Leu Arg Lys Gln
1 5 10 15

Ile Ala Ala Lys Lys Tyr Leu Gln Thr Ile Lys Lys
20 25

<210> 54

<211> 132

<212> DNA

<213> Artificial Sequence

09407832.092899

174
A1

<220>

<223> Description of Artificial Sequence: Expression
Oligomer

<400> 54

atcgaagggtc gtcactccga tggatatctc accgactcct actctcggta ccgcaagcag 60
atggctgtaa agaaatatct ggctgcagtc ctaggcaaac gttacaagca acgcgttaaa 120
aacaagtaat ga 132

<210> 55

<211> 102

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Expression
Oligomer

<400> 55

atcgaagggtc gtcactccga cgctgttttc accgacaact acacgcgtct gcgtaaacag 60
atggctgta agaaatacct gaattccatc ctgaactaat ga 102

<210> 56

<211> 111

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Expression
Oligomer

<400> 56

atcgaagggtc gtcactccga tgetgttttc accgaanact acaccaagct tcgtaaacag 60
ctggcagcta agaaatacct caacgacctg aaaaagggcg gtacctaata a 111

09407832.092899
668260.25870460

475
172

R3P1	Ac-HSDAVFTENY TKLRKQLAAK KYLNDLKGG T-NH ₂	31 (SEQ ID NO:6)
R3P3	1 HSDAVFTENY TKLRKQLAAK KYLNDLKGG T	31 (SEQ ID NO:8)
R3P9	1 HSDAVFTENY TKLRKQLAAK KYLNDLKGG T	31 (SEQ ID NO:12)
R3P10	1 HSDAVFTENY TKLRKQLAAK KYLNDLKGG T	31 (SEQ ID NO:13)
R3P12	1 HSDAVFTENY TKLRKQLAAK KYLNDLKGG T	31 (SEQ ID NO:15)
R3P13	1 HSDAVFTENY TKLRKQLAAK KYLNDLKGG T	28 (SEQ ID NO:16)
R3P19	1 HSDAVFTENY TKLRKQLAAK KYLNDLKGG TSWCEPWCR	40 (SEQ ID NO:18)
R3P36	1 HSDAVFTENY TKLRKQLAAK KYLNDLKGG Y	31 (SEQ ID NO:32)

0 407832.092899

Figure 1

176
173

09407832-092899

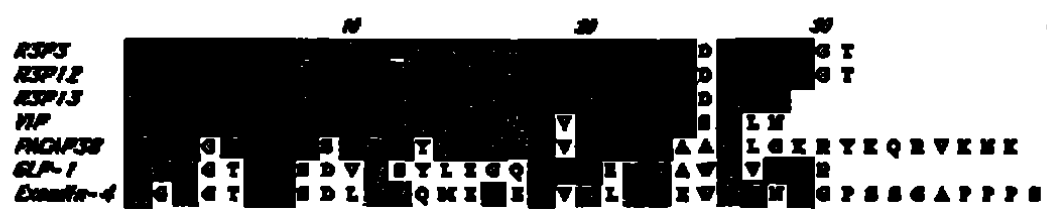


Figure 2

177
174

pGex6P-1/rR3P3

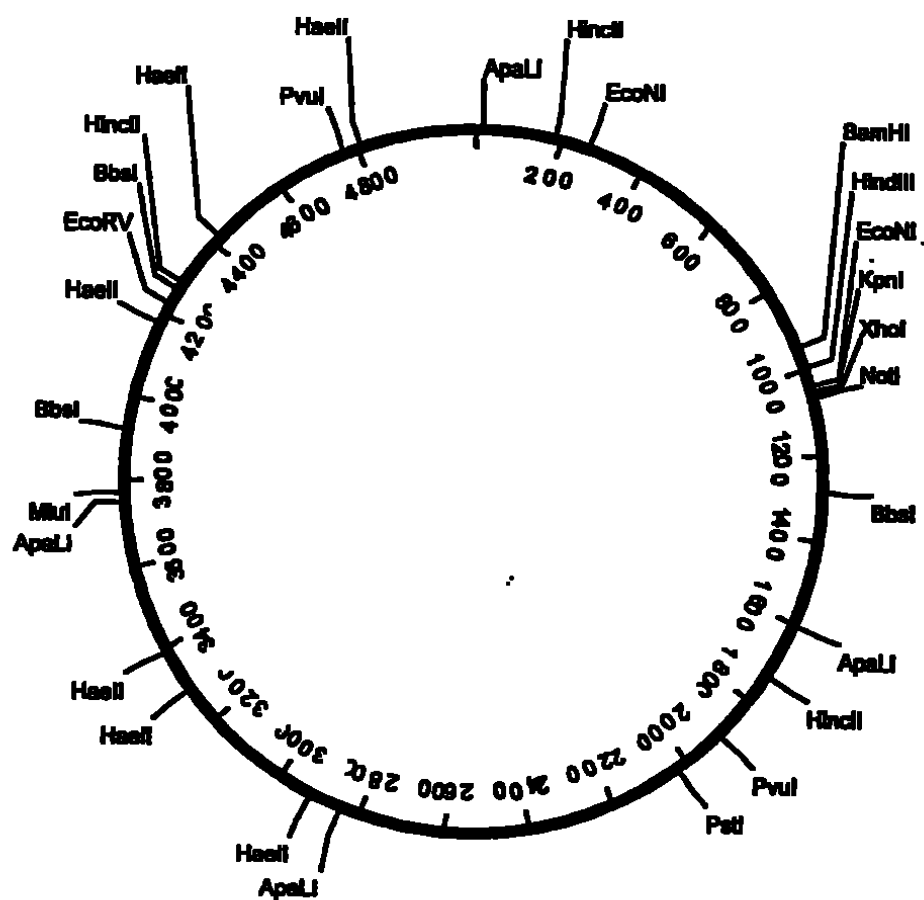


Figure 3

09407832.092899

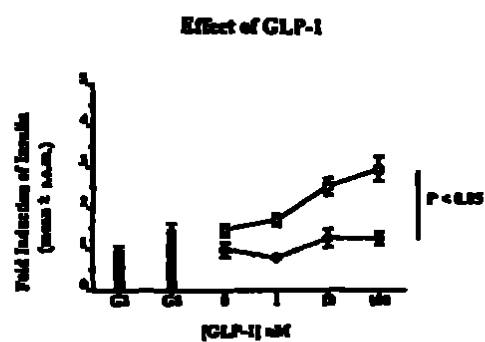


Figure 4A

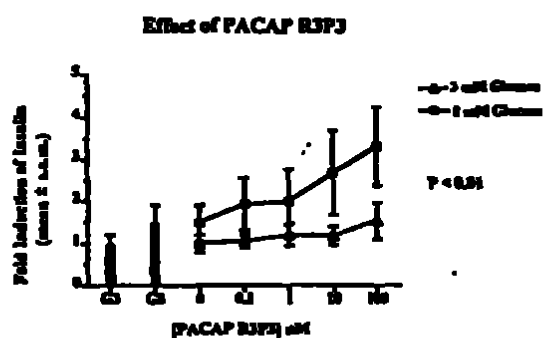


Figure 4B

09407832-092899

179
176

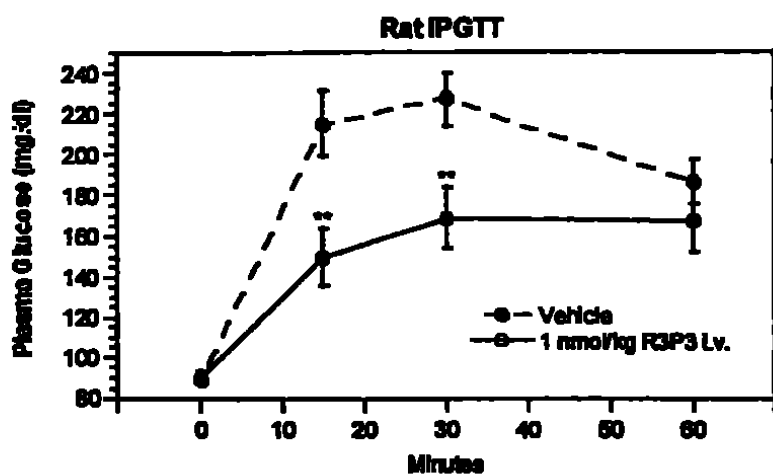


Figure 5

09407832.092889
668260.28870460

80
177

Effect of PACAP and Related Peptides on Intestinal Water Content of Balb/C Mice

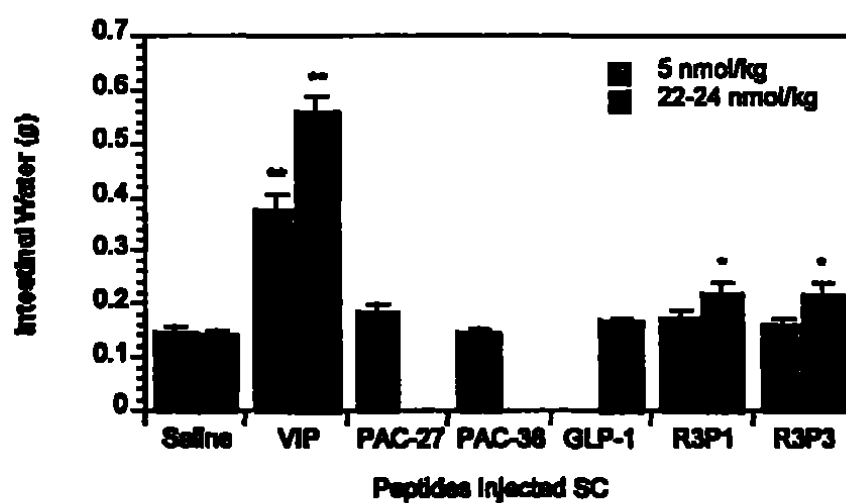


Figure 6

09407832-092899

178

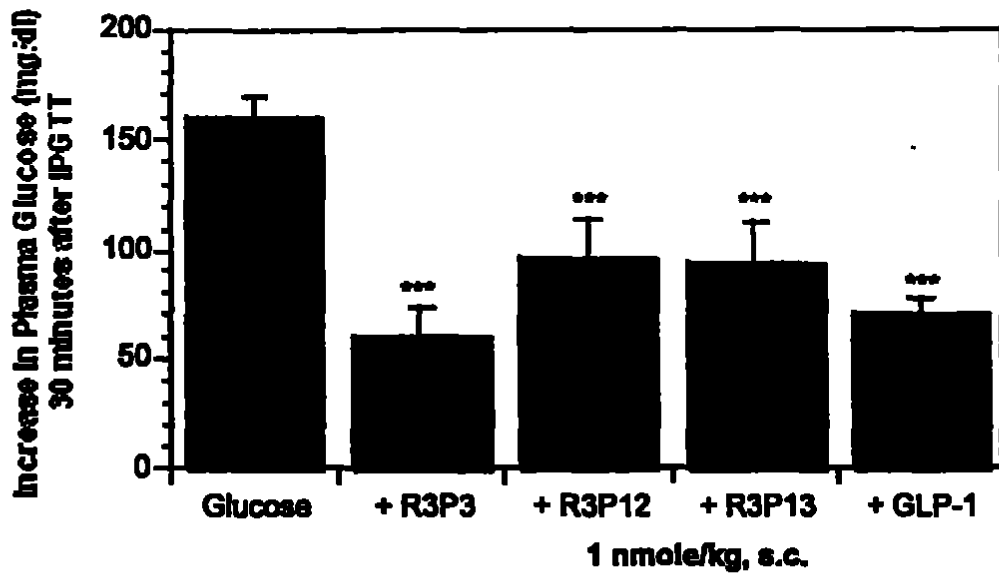


Figure 7

0407832.09880.28260.68260

183
179



LOS ESTADOS UNIDOS DE AMÉRICA

A TODO AQUEL A QUIEN CONCIERNA LO PRESENTE

DEPARTAMENTO DE COMERCIO DE LOS ESTADOS UNIDOS

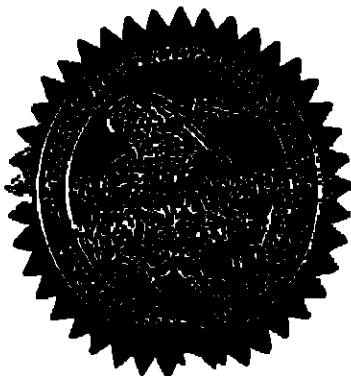
Oficina de Patentes y Marcas de los Estados Unidos

28 de Noviembre de 2000

LA PRESENTE ES PARA CERTIFICAR QUE ADJUNTA A LA MISMA HAY UNA COPIA FIEL DE LOS REGISTROS DE LA OFICINA DE PATENTES Y MARCAS DE LOS ESTADOS UNIDOS DE LOS DOCUMENTOS DE LA SOLICITUD DE PATENTE IDENTIFICADA EN LO QUE SIGUE, QUE CUMPLEN LOS REQUISITOS PARA OTORGARLE UNA FECHA DE PRESENTACIÓN BAJO 35 USC 111.

NÚMERO DE SOLICITUD: 09/407.832

FECHA DE PRESENTACIÓN: 28 de Septiembre de 1999



**Por la Autoridad del
COMISARIO DE PATENTES Y MARCAS**

L. EDELEN

Oficial certificador

REIVINDICACIONES

1. Un polipéptido seleccionado del grupo que consiste de SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:18 y SEQ ID NO:32 o un equivalente funcional del mismo.
2. Un polinucleótido que codifica una secuencia de polipéptidos de la reivindicación 1 o una variante degenerada del mismo.
3. Un vector que comprende un polinucleótido de la reivindicación 2.
4. Una célula huésped que comprende un vector de la reivindicación 3.
5. Un método para producir un polipéptido que comprende:
 - a) Cultivar la célula huésped de la reivindicación 4 bajo condiciones ideales para la expresión de dicho polipéptido; y
 - b) Recuperar el polipéptido del cultivo de la célula huésped.
6. Una composición farmacéutica que comprende el polipéptido de la reivindicación 1 en combinación con un portador farmacéuticamente aceptable.
7. Una composición de terapia genética que comprende un polipéptido de la reivindicación 2 en combinación con un vector terapéuticamente efectivo de la terapia genética.

8. El polipéptido de la reivindicación 1 donde dicho polipéptido esta representado por SEQ ID NO:6.
9. El polipéptido de la reivindicación 1 donde dicho polipéptido esta representado por SEQ ID NO:8.
10. El polipéptido de la reivindicación 1 donde dicho polipéptido esta representado por SEQ ID NO:12.
11. El polipéptido de la reivindicación 1 donde dicho polipéptido esta representado por SEQ ID NO:13.
12. El polipéptido de la reivindicación 1 donde dicho polipéptido esta representado por SEQ ID NO:15.
13. El polipéptido de la reivindicación 1 donde dicho polipéptido esta representado por SEQ ID NO:16.
14. El polipéptido de la reivindicación 1 donde dicho polipéptido esta representado por SEQ ID NO:18.
15. El polipéptido de la reivindicación 1 donde dicho polipéptido esta representado por SEQ ID NO:32.
16. Un método para tratar un desorden metabólico en un mamífero que comprende la administración de una cantidad terapéuticamente

efectiva de un péptido seleccionado del grupo que consiste de SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:18 y SEQ ID NO:32 a dicho mamífero.

17.El método de la reivindicación 16 donde dicho desorden metabólico es la diabetes tipo 2.

18.El método de la reivindicación 16 donde dicha cantidad terapéuticamente efectiva varía entre 0.1µg/kg hasta 1 mg/kg.

19.El método de la reivindicación 16 donde dicho desorden metabólico es el estado prediabético de la tolerancia a la glucosa deteriorada.

20.Una variante del péptido vasoactivo intestinal que tiene una de las estructuras de la figura 1, y los equivalentes funcionales del mismo.

21.Un método para estimular la liberación de insulina en una forma dependiente de glucosa en un mamífero con necesidad de la misma administrando a dicho mamífero un polipéptido seleccionado del grupo que consiste de polipéptidos de la figura 1.

COLO 09597-801-017-1

JJC-ID-176

787
183

PA 220229



THE UNITED STATES OF AMERICA

TO ALL TO WHOM THESE PRESENTS SHALL COME:

UNITED STATES DEPARTMENT OF COMMERCE

United States Patent and Trademark Office

December 01, 2000

THIS IS TO CERTIFY THAT ANNEXED HERETO IS A TRUE COPY FROM THE RECORDS OF THE UNITED STATES PATENT AND TRADEMARK OFFICE OF THOSE PAPERS OF THE BELOW IDENTIFIED PATENT APPLICATION THAT MET THE REQUIREMENTS TO BE GRANTED A FILING DATE UNDER 35 USC 111.

APPLICATION NUMBER: 09/595,280

FILING DATE: June 15, 2000



By Authority of the
COMMISSIONER OF PATENTS AND TRADEMARKS

T. Wallace
T. WALLACE

Certifying Officer

UNITED STATES PATENT AND TRADEMARK OFFICE

Box Patent Application
Assistant Commissioner for Patents
Washington, DC 20231

CERTIFICATION UNDER 37 C.F.R. 1.10

I hereby certify that this New Application Transmittal and the documents referred to as enclosed therein are being deposited with the United States Postal Service on this date June 15, 2000, in an envelope as "Express Mail Post Office to Addressee" Mailing Label Number EF292875128US, addressed to the Assistant Commissioner for Patents, Box Patent Application, Washington, D.C. 20231.

Lauren Fitzgerald
Signature of person certifying

NEW UTILITY PATENT APPLICATION TRANSMITTAL
UNDER 37 CFR §1.81(b) AND § 1.53(b)

☒ Inventors / ☐ Application Identifier:

Clark Pan

Manami Tautsumi

Express Mail Label No.: EF292875128US

Armen B. Shanafelt

Title of Application: PITUITARY ADENYLATE CYCLASE ACTIVATING PEPTIDE (PACAP) RECEPTOR 3 (R3) AGONISTS AND THEIR PHARMACOLOGICAL METHODS OF USE

Type of application: ☐ Original (nonprovisional)
☐ Provisional
☐ Continuation
☒ Continuation-in-part (CIP)
☐ Divisional

Benefit of the prior US provisional applications indicated below is claimed under 35 USC § 119(e). Please amend the specification by inserting, before the first line, the following sentence: "This application claims the benefit of U.S. Provisional Application(s) No(s):

Application Number	Day/Month/Year Filed
1.	
2.	

259
185

Papers accompanying this transmittal that are required for filing under 37 CFR § 1.53(b):

- ☒ Fee transmittal form, in duplicate
- ☐ Provisional Application Cover Sheet
- ☒ Specification, 38 pages
- ☒ Claims, 1 pages
- ☒ Abstract, 1 page
- ☒ Drawings, 18 pages:
 - ☐ formal,
 - ☒ Informal,
- ☒ Declaration, 3 pages
 - ☒ Executed by inventors
 - ☐ Executed by other: _____

Additional papers accompanying this transmittal:

- ☒ Return Receipt Postcard
- ☐ Power of Attorney
- ☐ Assignment to Bayer Corporation
 - ☐ Cover Sheet
 - ☐ Assignment Document(s)
- ☐ Nucleotide and/or Amino Acid Sequence Submission
 - ☐ Computer Readable Copy
 - ☐ Paper Copy (identical to computer readable copy) _____ pages
 - ☐ Statement verifying identity of above copies
- ☐ Information Disclosure Statement
- ☐ Form PTO-1449
- ☐ Copies of References
- ☐ Verified English Translation of the Application
- ☐ Certified Copy of Priority Document(s)
 - Country _____ Application No. _____ Date filed _____
- ☐ Preliminary Amendment
- ☐ Other: _____

Applicants request that the patent application accompanying this transmittal be duly filed with the United States Patent and Trademark Office as of the date indicated in the Certificate of Mailing above.

03575280-051504

190
186

Address all written correspondence to:

Mr. Jeffrey M. Greenman
Vice President, Patents & Licensing
Bayer Corporation
400 Morgan Lane
West Haven, Connecticut 06516

Direct all telephone calls to Alice A. Brewer at (203) 812-2705.

Respectfully submitted by:

Alice A. Brewer
Alice A. Brewer
Reg. No. 32,888

005730-0000000000

၆၆
 ၆၇
 ၆၈
 ၆၉
 ၇၀
 ၇၁
 ၇၂
 ၇၃
 ၇၄
 ၇၅
 ၇၆
 ၇၇
 ၇၈
 ၇၉
 ၈၀
 ၈၁
 ၈၂
 ၈၃
 ၈၄
 ၈၅
 ၈၆
 ၈၇
 ၈၈
 ၈၉
 ၉၀
 ၉၁
 ၉၂
 ၉၃
 ၉၄
 ၉၅
 ၉၆
 ၉၇
 ၉၈
 ၉၉
 ၁၀၀

5

10

15

20

25

30

35

40

Secondary failure is demonstrated by an additional 20-30% loss of treatment effect after six months on an SFU. Insulin treatment is required in 50% of the SFU responders after 5-7 years of therapy (Scheen, A.J., et al., Diabetes Res. Clin. Pract. 6:533-543 (1989)).

GLUCOPHAGE™ (metformin HCl) is a biguanide that lowers blood glucose by decreasing hepatic glucose output and increasing peripheral glucose uptake and utilization. The drug is effective at lowering blood glucose in mildly and moderately affected subjects and does not have the side effects of weight gain or the potential to induce hypoglycemia. However, GLUCOPHAGE has a number of side effects including gastrointestinal disturbances and lactic acidosis. GLUCOPHAGE is contraindicated in diabetics over the age of 70 and in subjects with impairment in renal or liver function. Finally, GLUCOPHAGE has the same primary and secondary failure rates as the SFUs.

Insulin treatment is instituted after diet, exercise, and oral medications have failed to adequately control blood glucose. This treatment has the drawbacks that it is an injectable, that it can produce hypoglycemia, and that it causes weight gain.

Because of the problems with current treatments, new therapies to treat type 2 diabetes are needed. In particular, new treatments to retain normal (glucose-dependent) insulin secretion are needed. Such new drugs should have the following characteristics: dependent on glucose for promoting insulin secretion, i.e. produce insulin secretion only in the presence of elevated blood glucose; low primary and secondary failure rates; and preserve islet cell function. The strategy to develop the new therapy disclosed herein is based on the cyclic adenosine monophosphate (cAMP) signaling mechanism and its effects on insulin secretion.

Cyclic AMP is a major regulator of the insulin secretion process. Elevation of this signaling molecule promotes the closure of the K⁺ channels following the activation of protein kinase A pathway. Closure of the K⁺ channels causes cell depolarization and subsequent opening of Ca⁺⁺ channels, which in turn leads to exocytosis of insulin granules. Little if any effects on insulin secretion occurs in the absence of low glucose concentrations (Weinhaus, A., et al., Diabetes 47: 1426-1435 (1998)). Secretagogues like pituitary adenylylate cyclase activating peptide ("PACAP") and GLP-1 use the cAMP system to regulate insulin secretion in a glucose-dependent fashion (Komatsu, M., et al., Diabetes 46: 1928-1938, (1997)).

PACAP is a potent stimulator of glucose-dependent insulin secretion from pancreatic beta-cells. Three different PACAP receptor types (R1, R2, and R3) have been described (Harner, A. et al., Pharmacol. Reviews 50: 265-270 (1998)). PACAP displays no receptor selectivities, having comparable activities and potencies at all three receptors. R1 is located predominately in the CNS, whereas R2 and R3 are more widely distributed. R2 is located in the CNS as well as in liver, lungs and intestine. R3 is located in the CNS, pancreas, skeletal muscle, heart, kidney, adipose tissue, testis and stomach. Recent work argues that R3 is responsible for the insulin secretion from beta cells (Inagaki, N. et al., PNAS 91: 2679-2683, (1994)). This insulinotropic action of PACAP is mediated by the GTP binding protein Gs.

Accumulation of intracellular cAMP in turn activates the nonselective cation channels in beta cells increasing $[Ca^{++}]$, and promotes exocytosis of insulin-containing secretory granules.

5 PACAP is the newest member of the superfamily of metabolic, neuroendocrine and neurotransmitter peptide hormones that exert their action through the cAMP-mediated signal transduction pathway (Arimura, Regul. Peptides 37:287-303 (1992)). The biologically active peptides are released from the biosynthetic precursor in two molecular forms, either as a 38-amino acid peptide (PACAP-38) and/or as a 27-amino acid peptide (PACAP-27) with an amidated carboxyl termini (Arimura, *supra*).

10 The highest concentrations of the two forms of the peptide are found in the brain and testis (reviewed in Arimura, *supra*). The shorter form of the peptide, PACAP-27, shows 88% structural homology to vasoactive intestinal polypeptide (VIP). However, the distribution of PACAP and VIP in the central nervous system suggests that these structurally related peptides have distinct neurotransmitter functions (Koves et al., Neuroendocrinology 54:159-169, (1991)).

15 Recent studies have demonstrated diverse biological effects of PACAP-38, from a role in reproduction (McArdle, Endocrinology 135:815-817 (1994)) to ability to stimulate insulin secretion (Yada et al., J. Biol. Chem. 269:1290-1293 (1994)).

20 Vasoactive intestinal peptide (VIP) is a 28 amino acid peptide that was first isolated from hog upper small intestine (Said and Mutt, Science 169: 1217-1218, 1970; U.S. Patent No. 3,879,371). This peptide belongs to a family of structurally-related, small polypeptides that includes helodermin, secretin, the somatostatins, and glucagon. The biological effects of VIP are mediated by the activation of membrane-bound receptor proteins that are coupled to the intracellular cAMP signaling system. These receptors were originally known as VIP-R1 and VIP-R2, however, they were later found to be the same receptors as PACAP-R2 and PACAP-R3. VIP displays comparable activities and potencies at PACAP-R2 and PACAP-R3.

25 To improve the stability of VIP in human lung fluid, Bolin et al (Biopolymers 37: 57-68, (1995)) made a series of VIP variants designed to enhance the helical propensity of this peptide and reduce proteolytic degradation. Substitutions were focused on positions 8, 12, 17, and 25-28, which were implicated to be unimportant for receptor binding. Moreover, the "GGT" sequence was tagged onto the C-terminus of VIP mutants with the hope of more effectively capping the helix. Finally, to further stabilize the helix, several cyclic variants were synthesized (US. Patent No. 5,677,419). Although these efforts were not directed toward receptor selectivity, they yielded two analogs (designated herein as R3P0 and R3P4) 30 that have greater than 100-fold PACAP-R3 selectivity (Gourlet et al., Peptides 18: 403-408, (1997); Xia et al., J. Pharmacol. Exp. Ther., 281: 628-633, (1997)).

35 GLP-1 is released from the intestinal L-cell after a meal and functions as an incretin hormone (i.e. it potentiates glucose-induced insulin release from the pancreatic beta-cell). It is a 37-amino acid peptide that is differentially expressed by the Glucagon gene, depending upon tissue type. The clinical data that support the beneficial effect of raising cAMP levels in 40

β -cells have been collected with GLP-1. Infusions of GLP-1 in poorly controlled type 2 diabetics normalized their fasting blood glucose levels (Gutniak, M., et al., New Eng. J. Med. 328:1318-1322, (1992)) and with longer infusions improved the beta cell function to those of normal subjects (Rachman, J. et al., Diabetes 45: 1524-1530, (1996)). A recent report has shown that GLP-1 improves the β -cells' ability to respond to glucose in subjects with impaired glucose tolerance (Byrne M., et al., Diabetes 47: 1259-1265 (1998)). All of these effects, however, are short-lived because of the short half-life of the peptide. Recently Novo Nordisk has discontinued clinical trials with GLP-1. This failure reportedly was due to a very short plasma half-life of the peptide of a few minutes.

EXENDIN 4™, Amylin Pharmaceuticals is conducting Phase I trials with EXENDIN 4 (AC2993), a 39 amino acid peptide originally identified in Gila Monster. Phase II trials have recently begun. Amylin claims preclinical results showing a 4 hour duration of efficacy and efficacy in animal models when AC2993 is administered subcutaneously, orally, and nasally. However, at doses of 0.2 and 0.3ug/kg, the incidence of headaches, postural hypotension, nausea and vomiting was significant.

There exists a need for an improved peptide that has the glucose-dependent insulin secretagogue activity of PACAP, GLP-1, or EXENDIN 4, and yet has fewer side-effects.

SUMMARY OF THE INVENTION

This invention provides novel polypeptides that function *in vivo* as agonists of the PACAP R3 receptor (hereafter, R3) and are effective in the treatment of diseases and conditions that can be ameliorated by agents having R3 agonist activity. Preferably, the polypeptides of this invention are selective R3 agonists, having greater potency at R3 than at R2 and R1. For example, but not by way of limitation, these polypeptides stimulate insulin release from pancreatic beta cells in a glucose-dependent fashion and subsequent plasma glucose reduction. These insulin secretagogue polypeptides are shown to stimulate insulin release in rat and human islet cells *in vitro* and *in vivo*. Unlike PACAP-27, these secretagogue polypeptides also lower blood glucose *in vivo* more than vehicle control upon glucose challenge.

The polypeptides of the present invention provide a new therapy for patients with, for example, metabolic disorders such as those resulting from decreased endogenous insulin secretion, in particular type 2 diabetics, or for patients with impaired glucose tolerance, a prediabetic state that has a mild alteration in insulin secretion.

In particular, one aspect of the invention is a polypeptide selected from the group consisting of SEQ ID NOs: 11 through 14, SEQ ID NO: 18, SEQ ID NOs: 21 through 28, SEQ ID NOs: 32 through 36, SEQ ID NOs: 40 through 53, SEQ ID NOs: 57 through 61, SEQ ID NOs: 63 through 99, SEQ ID NOs: 102 through 119, SEQ ID NOs: 121 through 137, SEQ ID NOs: 139 through 177, SEQ ID NOs: 179, 180, SEQ ID NOs: 183 through 202 and fragments, derivatives and variants thereof that demonstrate at least one biological function that is substantially the same as the polypeptides of the listed SEQ ID NOs. (collectively,

196
191

"polypeptides of this invention"), including functional equivalents thereof. A preferred embodiment of this invention is a polypeptide selected from the group consisting of SEQ ID NOs. 12, 18, 21-28, 32-35, 41, 43-53, 63, 66, 70-92, 94-99, 102-104, 107, 109, 112-119, 121-137, 139, 140, 142-153, 156-174, and 187, and fragments, derivatives, and variants thereof that demonstrate at least one biological function that is substantially the same as the polypeptides of the listed SEQ ID NOs. A more preferred embodiment of this invention is a polypeptide selected from the group consisting of SEQ ID NOs 18, 24, 25, 32, 33, 43-50, 52, 53, 70-87, 82, 98, 99, 104, 107, 112-114, 129-131, 137, 140, 144, 147-151, 156-159, and 161-173, and fragments, derivatives and variants thereof that demonstrate at least one biological function that is substantially the same as the polypeptides of the listed SEQ ID NOs. A most preferred embodiment of this invention is a polypeptide selected from the group consisting of SEQ ID NOs 18, 32, 43, 47, 50, 52, 72, 83, 86, and 87, and fragments, derivatives and variants thereof that demonstrate at least one biological function that is substantially the same as the polypeptides of the listed SEQ ID NOs.

Another embodiment of the invention is a polynucleotide that encodes for the polypeptides of this invention, and the attendant vectors and host cells necessary to recombinantly express the polypeptides of this invention. These polynucleotide sequences include those identified as SEQ ID NOs: 204, 207-211, 214-230, and 232-301. Preferred polynucleotides include those identified as SEQ. ID. NOs 204, 207-208, 215, 217-230, 232-234, 237, 239, 242-268, 270-281, and 284-301. More preferred polynucleotides include those identified as SEQ ID NOs 207, 218-224, 228-230, 234, 237, 242-244, 258-260, 266, 268, 272, 275-279, 284-287, and 289-301. Most preferred polynucleotides include those identified as SEQ ID NO 217, 221, and 226.

Antibodies and antibody fragments that selectively bind the polypeptides of this invention are also provided. Such antibodies are useful in detecting the polypeptides of this invention, and can be identified and made by procedures well known in the art, including those analogous to that described in Example 17 below.

The invention is also directed to a method of treating diabetes and/or other diseases or conditions affected by the polypeptides of this invention, preferably effected by the R3 agonist function of the polypeptides of this invention, in a mammal, comprising administering a therapeutically effective amount of any of the polypeptides of the present invention or any polypeptide active at R3 such as SEQ ID NO 5 and 9 to said mammal.

Also disclosed are methods of making the polypeptides of this invention, both recombinant and synthetic.

BRIEF DESCRIPTION OF THE DRAWING

FIG. 1 depicts amino acid sequences of polypeptides of SEQ ID NO: 11 through 14, SEQ ID NO: 18, SEQ ID NOs: 21 through 28, SEQ ID NOs: 32 through 36, SEQ ID NOs: 40 through 53, SEQ ID NOs: 57 through 61, SEQ ID NOs: 63 through 99, SEQ ID NOs: 102

196
192

through 118, SEQ ID NOs: 121 through 137, SEQ ID NOs: 139 through 177, SEQ ID NOs: 179, 180, SEQ ID NOs: 183 through 202 which are claimed polypeptides.

FIG. 2 is a sequence alignment of VIP mutants and native polypeptides VIP, PACAP38, GLP-1, EXENDIN-4 and examples of R3 selective polypeptides. Conserved residues are bolded and shaded in dark gray while conservative changes are shaded in light gray.

FIG. 3 is a restriction map of a typical plasmid containing the GST-peptide fusion.

FIGS. 4A-4B are graphs showing the effects of GLP-1 or R3P3 on insulin release from rat islets *in vitro*.

FIG. 5 is a graph showing the R3P3 peptide effect on glucose disposal.

FIG. 6 is a bar chart showing the effect of PACAP and related polypeptides on intestinal water content of Balb/C mice.

FIG. 7 is a bar chart showing that 1 nmole/kg dose of R3P3, R3P12, R3P13, or GLP-1 enhance glucose disposal in the rat by subcutaneous route of administration.

FIG 8 depicts polynucleotide sequences SEQ ID NOs 54 through 56 and 203 through 301 that encode for the polypeptides of this invention.

The first 6 nucleotides represent the Bam HI restriction enzyme recognition site followed by the next 12 nucleotides which encode the "IEGR" Factor Xa recognition site. The last 6 nucleotides represent the Xho I or Eco RI restriction enzyme recognition site preceded by the 6 nucleotides that encode the two stop codons. The nucleotides between the Factor Xa site and stop codons encode the amino acid sequence of the corresponding polypeptide. The nucleotides between the two restriction sites are cloned into the corresponding restriction sites on the pGEX-6P-1 vector (Amersham Pharmacia Biotech). The SEQ ID NOs are denoted in parentheses.

FIG 9 shows the effect of PACAP-27, VIP and receptor selective agonists on the heart rate in conscious dogs (see example 15).

FIG. 10 shows detection of R3P66 by polyclonal antibodies produced in rabbits immunized with R3P66 C-terminus sequence (Ac-CRKQVAAKKYLQSIKNKRY-COOH), using ELISA.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

This invention provides novel polypeptides, and fragments, derivatives and variants thereof that demonstrate at least one biological function that is substantially the same as the polypeptides of Fig. 1 (collectively, polypeptides of this invention). The polypeptides of this invention function *in vivo* as R3 agonists or otherwise in the prevention and/or treatment of such diseases or conditions as diabetes, asthma, hypertension, male reproduction problems including human sperm motility, cardiovascular diseases, ulcers, and other conditions identified herein, or function otherwise as described later herein. Preferably, the

[illegible]

10

15

20

25

30

35

40

118
194

known that PACAP and VIP do not reduce glucose *in vivo*, but, rather, they stimulate glucose release from the liver. It has been shown that activation of R2 increases plasma glucose levels *in vivo*. Previously, both PACAP and VIP have been mutagenized extensively for various reasons. For instance, serial deletions of PACAP27 and PACAP38 from both ends confirmed the importance of both termini for receptor binding (Gourlet et al., Eur. J. Pharm. 287: 7-11, (1995); Gourlet et al., Regul. Peptides 62: 125-130, (1996)). Rat brain membrane binding and adenylate cyclase activities of PACAP27/VIP hybrid mutants implicated the importance of the N-terminal residues of PACAP for PACAP-R1 recognition (Ando et al., Biomed. Pept. Proteins Nucleic Acids 2:41-46, (1996)). Increasing the basicity of Leu¹⁷-PACAP27 and Leu¹⁷-VIP by making K15R, K20R, and K21R mutations and extension of the C-terminus with "GKR" sequence led to an increase in duration of guinea pig tracheal relaxant activity proposed to be due to protection from heparin binding (Kashimoto et al., Ann. NY Acad. Sci. 805: 505-510, (1996)). Gourlet et al. (Biochim. Biophys. Acta 1314: 267-273, (1996)) demonstrated that the Q16R mutant of VIP and PACAP possessed greater affinities than their respective native polypeptides for PACAP-R2 and R1, respectively. Gourlet et al. (Peptides 18: 1539-1545, (1997)) developed a high affinity R2-selective agonist by making the chimeric substituted peptide [K15, R16, L27] VIP(1-7)GRF(8-27). N-terminal acylation and D-Phe2 substitution of this selective agonist led to a potent R2 selective antagonist (Gourlet et al., Peptides 18 1555-1560, (1997)). VIP mutants Y22L and Y22A, but not Y22F, display lower affinity for PACAP-R3, suggesting the importance of an aromatic group at position 22 for receptor R3 binding but not for receptor R2 binding (Gourlet, Eur. J. Biochem. 348: 95-99, (1996)). Helodermin and helospectin, VIP-like peptides isolated from the salivary gland venom of lizards exhibit ~100-fold PACAP-R3 selectivity (Gourlet, Ann. NY Acad. Sci. 885: 247-252, (1998)). Photoaffinity labeling of PACAP27 by replacing F8 and Y22 with p-benzoyl-L-phenylalanine (pBz) or K15, K20, and K21 with pBz₂ suggested that K15 and F22 are closer to PACAP-R1 than F8, K20, and K21 (Cao et al., Eur. J. Biochem. 244: 400-406, (1997); Cao et al., Ann. NY Acad. Sci., 885: 82-91, (1998)).

The inventors herein have found several polypeptides that cause the stimulation of release of insulin in a glucose-dependent manner and cause glucose reduction *in vivo*. Those polypeptides bear some similarity to VIP and PACAP. In particular, a stacked alignment shows the following:

			SEQ.	
			ID NO.	
35	VIP	1 HSDAVFTDNY TRLRKQMAVK KYLNSILN-NH ₂	28	1
	PACAP38	1 HSDGIFTDSY SRYRKQMAVK KYLA AVL GKR YKQRVKNK-NH ₂	38	2
	R3P1	Ac-HSDAVFTENY TKLRKQLAAK KYLNDLKGG T-NH ₂	31	6
	R3P3	1 HSDAVFTENY TKLRKQLAAK KYLNDLKGG T	31	8
	R3P12	1 HSDAVFTDNY TRLRKQLAAK KYLNDIKGG T	31	15
40	R3P13	1 HSDAVFTDNY TRLRKQLAAK KYLNDIKK-NH ₂	28	16
	R3P36	1 HSDAVFTDNY TRLRKQLAAK KYLNDIKGR Y	31	32
	R3P66	1 HSDAVFTDNY TRLRKQVAAK KYLQSIKNR Y	31	72

005730-005730

179
195

However, there is no teaching in the scientific or patent literature that suggests that select modifications to the VIP and PACAP sequences lead to a polypeptide with the ability to stimulate insulin secretion in a glucose-dependent fashion, and reduce plasma glucose concentration.

5 Certain terms used throughout this specification will now be defined, and others will be defined as introduced. The single letter abbreviation for a particular amino acid, its corresponding amino acid, and three letter abbreviation are as follows: A, alanine (ala); C, cysteine (cys); D, aspartic acid (asp); E, glutamic acid (glu); F, phenylalanine (phe); G, glycine (gly); H, histidine (his); I, isoleucine (ile); K, lysine (lys); L, leucine (leu); M, methionine (met); N, asparagine (asn); P, proline (pro); Q, glutamine (gln); R, arginine (arg);
10 S, serine (ser); T, threonine (thr); V, valine (val); W, tryptophan (trp); Y, tyrosine (tyr).

The term "polynucleotide encoding a polypeptide" encompasses a polynucleotide which includes only coding sequence for the polypeptide as well as a polynucleotide which includes additional coding and/or non-coding sequence. The present invention further relates to polynucleotides which hybridize to the hereinabove-described sequences if there is at least about 70%, preferably at least about 80%, and more preferably at least about 95% identity between the sequences. The present invention particularly relates to polynucleotides encoding polypeptides which hybridize under stringent conditions to the hereinabove-described polynucleotides. As herein used, the term "stringent conditions" means "stringent hybridization conditions" Preferably, hybridization will occur only if there is at least about 90% and preferably about 95% through 97% identity between the sequences. The polynucleotides which hybridize to the hereinabove described polynucleotides in a preferred embodiment encode polypeptides which retain substantially the same biological function or activity as the mature polypeptide encoded by the cDNAs.

25 "Functional equivalent" and "substantially the same biological function or activity" each means that degree of biological activity that is within about 30% to 100% or more of that biological activity demonstrated by the polypeptide to which it is being compared when the biological activity of each polypeptide is determined by the same procedure. For example, a polypeptide that is functionally equivalent to a polypeptide of Fig. 1 is one that, when tested in the Cyclic AMP stimulation proximity assay of Specific Example 16, demonstrates accumulation of cAMP in CHO cell line expressing the human PACAP/VIP R2 (PACAP R3) receptor.

A polypeptide of this invention that is an R3 agonist is one that demonstrates about 30% -100% or more of maximal PACAP-27 R3 agonist activity when tested in the protocol of Example 16. The preferred polypeptides of this invention that are selective agonists for R3 over PACAP R2 and R1 receptors are those polypeptides that demonstrate the ratio of R3 agonist activity to R2 activity of about 10:1 or greater, and more preferably, about 100:1 or greater, and/or demonstrate the ratio of R3 agonist activity to R1 receptor activity of about 10:1 or greater, and more preferably, about 100:1 or greater when the polypeptide is tested
40 in the protocol of Example 16, using cells that express the appropriate receptors.

"Stringent hybridization conditions" refers to an overnight incubation of the two pieces of polynucleotides to be hybridized at 42° C in a solution comprising 50% formamide, 5x SSC (750 mM NaCl, 75 mM sodium citrate), 50 mM sodium phosphate (pH 7.8), 5x Denhardt's solution, 10% dextran sulfate, and 20 µg/ml denatured, sheared salmon sperm DNA, followed by washing the filters in 0.1x SSC at about 65°C.

The terms "fragment," "derivative", and "variant", when referring to the polypeptides of FIG. 1, means fragments, derivatives, and variants of the polypeptides which retain substantially the same biological function or activity as such polypeptides, as described further below.

An analog includes a propolypeptide which includes within it, the amino acid sequence of the polypeptide of this invention. The active polypeptide of this invention can be cleaved from the additional amino acids that complete the propolypeptide molecule by natural, *in vivo* processes or by procedures well known in the art such as by enzymatic or chemical cleavage. For example, the 28-amino acid native peptide VIP is naturally expressed as a much larger polypeptide which is then processed *in vivo* to release the 28-amino acid active mature peptide.

A fragment is a portion of the polypeptide which retains substantially similar functional activity, as shown in the *in vivo* models disclosed herein as described further below.

A derivative includes all modifications to the polypeptide which substantially preserve the functions disclosed herein and include additional structure and attendant function, e.g., PEGylated polypeptides which have greater half-life, fusion polypeptides which confer targeting specificity or an additional activity such as toxicity to an intended target, as described further below.

The polypeptides of the present invention may be recombinant polypeptides, natural purified polypeptides or synthetic polypeptides.

The fragment, derivative, or variant of the polypeptides of the present invention may be (i) one in which one or more of the amino acid residues are substituted with a conserved or non-conserved amino acid residue (preferably a conserved amino acid residue) and such substituted amino acid residue may or may not be one encoded by the genetic code, or (ii) one in which one or more of the amino acid residues includes a substituent group, or (iii) one in which the mature polypeptide is fused with another compound, such as a compound to increase the half-life of the polypeptide (for example, polyethyleneglycol), or (iv) one in which the additional amino acids are fused to the mature polypeptide, such as a leader or secretory sequence or a sequence which is employed for purification of the mature polypeptide or a propolypeptide sequence, or v) one in which the polypeptide sequence is fused with a larger polypeptide, i.e. human albumin, a antibody or Fc, for increased duration of effect. Such fragments, derivatives, and variants and analogs are deemed to be within the scope of those skilled in the art from the teachings herein.

Preferably, the derivatives of the present invention will contain conservative amino acid substitutions (defined further below) made at one or more predicted, preferably nonessential amino acid residues. A "nonessential" amino acid residue is a residue that can be altered from the wild-type sequence of a protein without altering the biological activity, whereas an "essential" amino acid residue is required for biological activity. A "conservative amino acid substitution" is one in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art. These families include amino acids with basic side chains (e.g., lysine, arginine, histidine), acidic side chains (e.g., aspartic acid, glutamic acid), uncharged polar side chains (e.g., glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (e.g., alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (e.g., threonine, valine, isoleucine) and aromatic side chains (e.g., tyrosine, phenylalanine, tryptophan, histidine). Non conservative substitutions would not be made for conserved amino acid residues or for amino acid residues residing within a conserved protein domain, such as residues 19 and 27 where such residues are essential for protein activity such as R3 activity and/or R3 selectivity. Fragments, or biologically active portions include polypeptide fragments suitable for use as a medicament, to generate antibodies, as a research reagent, and the like. Fragments include peptides comprising amino acid sequences sufficiently similar to or derived from the amino acid sequences of a polypeptide of this invention and exhibiting at least one activity of that polypeptide, but which include fewer amino acids than the full-length polypeptides disclosed herein. Typically, biologically active portions comprise a domain or motif with at least one activity of the polypeptide. A biologically active portion of a polypeptide can be a peptide which is, for example, 5 or more amino acids in length. Such biologically active portions can be prepared synthetically or by recombinant techniques and can be evaluated for one or more of the functional activities of a polypeptide of this invention by means disclosed herein and/or well known in the art.

Variants of the polypeptides of this invention include polypeptides having an amino acid sequence sufficiently similar to the amino acid sequence of the SEQ ID NOs of Fig. 1 or a domain thereof. The term "sufficiently similar" means a first amino acid sequence that contains a sufficient or minimum number of identical or equivalent amino acid residues relative to a second amino acid sequence such that the first and second amino acid sequences have a common structural domain and/or common functional activity. For example, amino acid sequences that contain a common structural domain that is at least about 45%, preferably about 75% through 98%, identical are defined herein as sufficiently similar. Preferably, variants will be sufficiently similar to the amino acid sequence of the preferred polypeptides of this invention. Variants include variants of polypeptides encoded by a polynucleotide that hybridizes to a polynucleotide of this invention or a complement thereof under stringent conditions. Such variants generally retain the functional activity of the polypeptides of this invention. Libraries of fragments of the polynucleotides can be used

to generate a variegated population of fragments for screening and subsequent selection. For example, a library of fragments can be generated by treating a double-stranded PCR fragment of a polynucleotide with a nuclease under conditions wherein nicking occurs only about once per molecule, denaturing the double-stranded DNA, renaturing the DNA to form double-stranded DNA which can include sense/antisense pairs from different nicked products, removing single-stranded portions from reformed duplexes by treatment with S1 nuclease, and ligating the resulting fragment library into an expression vector. By this method, one can derive an expression library that encodes N-terminal and internal fragments of various sizes of the polypeptide of this invention.

10 Variants include polypeptides that differ in amino acid sequence due to mutagenesis. Variants that function as R3 agonists can be identified by screening combinatorial libraries of mutants, for example truncation mutants, of the polypeptides of this invention for R3 agonist activity.

15 In one embodiment, a variegated library of analogs is generated by combinatorial mutagenesis at the nucleic acid level and is encoded by a variegated gene library. A variegated library of variants can be produced by, for example, enzymatically ligating a mixture of synthetic oligonucleotides into gene sequences such that a degenerate set of potential variant amino acid sequences is expressible as individual polypeptides, or, alternatively, as a set of larger fusion proteins (for example, for phage display) containing the set of sequences therein. There are a variety of methods that can be used to produce 20 libraries of potential variants from a degenerate oligonucleotide sequence. Chemical synthesis of a degenerate gene sequence can be performed in an automatic DNA synthesizer, and the synthetic gene then ligated into an appropriate expression vector. Use of a degenerate set of genes allows for the provision, in one mixture, of all of the sequences encoding the desired set of potential variant sequences. Methods for synthesizing degenerate oligonucleotides are known in the art (see, e.g., Narang (1983) *Tetrahedron* 39:3; Itakura et al (1984) *Annu. Rev. Biochem.* 53:323; Itakura et al (1984) *Science* 198:1058; Ike et al (1983) *Nucleic Acid Res.* 11:477).

25 Several techniques are known in the art for screening gene products of combinatorial libraries made by point mutations or truncation and for screening cDNA libraries for gene products having a selected property. Such techniques are adaptable for rapid screening of the gene libraries generated by the combinatorial mutagenesis of R-agonist polypeptides. The most widely used techniques, which are amenable to high through-put analysis for screening large gene libraries typically include cloning the gene library into replicable expression vectors, transforming appropriate cells with the resulting library of vectors and expressing the combinatorial genes under conditions in which 35 detection of a desired activity facilitates isolation of the vector encoding the gene whose product was detected. Recursive ensemble mutagenesis (REM), a technique that enhances the frequency of functional mutants in the libraries, can be used in combination with the screening assays to identify the desired variants.

40

263
199

The invention also provides chimeric or fusion polypeptides. Examples include those polypeptides of this invention described in SEQ ID NOs 18 and 172 which are fusions of the pancreatic targeting sequence "SWCEPGWCR" (Rajotte D., et al (1998) *J Clin Invest* 102:430-437) with SEQ ID NOs 8 and 32, respectively. The targeting sequence is designed to localize the delivery of the polypeptide to the pancreas to minimize potential side effects. The polypeptides of this invention can be composed of amino acids joined to each other by peptide bonds or modified peptide bonds, i.e., peptide isosteres, and may contain amino acids other than the 20 gene-encoded amino acids. The polypeptides may be modified by either natural processes, such as posttranslational processing, or by chemical modification techniques which are well known in the art. Such modifications are well described in basic texts and in more detailed monographs, as well as in a voluminous research literature. Modifications can occur anywhere in a polypeptide, including the peptide backbone, the amino acid side-chains and the amino or carboxyl termini. It will be appreciated that the same type of modification may be present in the same or varying degree at several sites in a given polypeptide. Also, a given polypeptide may contain many types of modifications. Polypeptides may be branched, for example, as a result of ubiquitination, and they may be cyclic, with or without branching. Cyclic, branched, and branched cyclic polypeptides may result from posttranslation natural processes or may be made by synthetic methods. Modifications include acetylation, acylation, ADP-ribosylation, amidation, covalent attachment of flavin, covalent attachment of a heme moiety, covalent attachment of a nucleotide or nucleotide derivative, covalent attachment of a lipid or lipid derivative, covalent attachment of phosphatidylinositol, cross-linking, cyclization, disulfide bond formation, demethylation, formation of covalent cross-links, formation of cysteine, formation of pyroglutamate, formylation, gamma-carboxylation, glycosylation, GPI anchor formation, hydroxylation, iodination, methylation, myristoylation, oxidation, pegylation, proteolytic processing, phosphorylation, prenylation, racemization, selenoylation, sulfation, transfer-RNA mediated addition of amino acids to proteins such as arginylation, and ubiquitination. (See, for instance, Proteins, Structure and Molecular Properties, 2nd ed., T. E. Creighton, W.H. Freeman and Company, New York (1983); Posttranslational Covalent Modification of Proteins, B. C. Johnson, ed., Academic Press, New York, pgs. 1-12 (1983); Seifter et al., Meth. Enzymol 182:626-648 (1990); Rattan et al., Ann. N.Y. Acad. Sci. 663:48-62 (1992)).

The polypeptides of the present invention include the polypeptides of Fig. 1, that are SEQ ID NOs: 11 through 14, SEQ ID NO: 18, SEQ ID NOs: 21 through 28, SEQ ID NOs: 32 through 38, SEQ ID NOs: 40 through 53, SEQ ID NOs: 57 through 61, SEQ ID NOs: 63 through 99, SEQ ID NOs: 102 through 119, SEQ ID NOs: 121 through 137, SEQ ID NOs: 139 through 177, SEQ ID NOs: 179, 180, SEQ ID NOs: 183 through 202, as well as those sequences having insubstantial variations in sequence from them. An "insubstantial variation" would include any sequence, substitution, or deletion variant that maintains substantially at least one biological function of the polypeptides of this invention, preferably R3 agonist activity, and more preferably selective R3 agonist activity, and most preferably,

204
200

the insulin secreting activity demonstrated herein. These functional equivalents may preferably include polypeptides which have at least about a 80% identity to the polypeptides of Fig. 1, and more preferably at least a 95% identity to the polypeptides of Fig. 1, and still more preferably at least a 97% identity to the polypeptides of Fig.1, and also include portions of such polypeptides having substantially the same biological activity. However, any polypeptide having insubstantial variation in amino acid sequence from the polypeptides of Fig. 1 that demonstrates functional equivalency as described further herein is included in the description of the present invention.

As known in the art "similarity" between two polypeptides is determined by comparing the amino acid sequence and its conserved amino acid substitutes of one polypeptide to the sequence of a second polypeptide. Such conservative substitutions include those described above and by Dayhoff in The Atlas of Protein Sequence and Structure 5 (1978), and by Argos in EMBO J., 8:779-785 (1989). For example, amino acids belonging to one of the following groups represent conservative changes:

- ala, pro, gly, gln, asn, ser, thr;
- cys, ser, tyr, thr;
- val, ile, leu, met, ala, phe;
- lys, arg, his;
- phe, tyr, trp, his; and
- asp, glu.

The present invention also relates to vectors which include polynucleotides of the present invention, host cells which are genetically engineered with vectors of the invention and the production of polypeptides of the invention by recombinant techniques. Host cells may be genetically engineered (transduced or transformed or transfected) with the vectors of this invention which may be, for example, a cloning vector or an expression vector. The vector may be, for example, in the form of a plasmid, a viral particle, a phage, etc. The engineered host cells can be cultured in conventional nutrient media modified as appropriate for activating promoters, or selecting transformants. The culture conditions, such as temperature, pH and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan. The polynucleotide of the present invention may be employed for producing a polypeptide by recombinant techniques. Thus, for example, the polynucleotide sequence may be included in any one of a variety of expression vehicles, in particular vectors or plasmids for expressing a polypeptide. Such vectors include chromosomal, non-chromosomal and synthetic DNA sequences, e.g., derivatives of SV40; bacterial plasmids; phage DNA; yeast plasmids; vectors derived from combinations of plasmids and phage DNA, viral DNA such as vaccinia, adenovirus, fowl pox virus, and pseudorabies. However, any other vector or plasmid may be used as long as they are replicable and viable in the host.

The appropriate DNA sequence may be inserted into the vector by a variety of procedures. In general, the DNA sequence is inserted into an appropriate restriction

265
201

endonuclease site by procedures known in the art. Such procedures and others are deemed to be within the scope of those skilled in the art. The DNA sequence in the expression vector is operatively linked to an appropriate expression control sequence(s) (promoter) to direct mRNA synthesis. As representative examples of such promoters, there may be mentioned: LTR or SV40 promoter, the *E. coli* *lac* or *trp*, the phage lambda P_L promoter and other promoters known to control expression of genes in prokaryotic or eukaryotic cells or their viruses. The expression vector also contains a ribosome binding site for translation initiation and a transcription terminator. The vector may also include appropriate sequences for amplifying expression. In addition, the expression vectors preferably contain a gene to provide a phenotypic trait for selection of transformed host cells such as dihydrofolate reductase or neomycin resistance for eukaryotic cell culture, or such as tetracycline or ampicillin resistance in *E. coli*. The vector containing the appropriate DNA sequence as herein above described, as well as an appropriate promoter or control sequence, may be employed to transform an appropriate host to permit the host to express the protein. As representative examples of appropriate hosts, there may be mentioned: bacterial cells, such as *E. coli*, *Salmonella typhimurium*, *Streptomyces*; fungal cells, such as yeast; insect cells, such as *Drosophila* S2 and *Spodoptera* Sf9; animal cells such as CHO, COS or Bowes melanoma; adenoviruses; plant cells, etc. The selection of an appropriate host is deemed to be within the scope of those skilled in the art from the teachings herein.

The present invention also includes recombinant constructs comprising one or more of the sequences as broadly described above. The constructs comprise a vector, such as a plasmid or viral vector, into which a sequence of the invention has been inserted, in a forward or reverse orientation. In a preferred aspect of this embodiment, the construct further comprises regulatory sequences, including, for example, a promoter, operably linked to the sequence. Large numbers of suitable vectors and promoters are known to those of skill in the art, and are commercially available. The following vectors are provided by way of example. Bacterial: pQE70, pQE60, pQE-9 (Qiagen), pBS, phagescript, psiX174, pBluescript SK, pBakS, pNH8a, pNH16a, pNH18a, pNH48a (Stratagene); pTRC99A, pKK223-3, pKK233-3, pDR540, PRIT5 (Pharmacia). Eukaryotic: pWLneo, pSV2cat, pOG44, pXT1, pSG (Stratagene) pSVK3, pBPV, pMSG, PSVL (Pharmacia). However, any other plasmid or vector may be used as long as they are replicable and viable in the host. Promoter regions can be selected from any desired gene using CAT(chloramphenicol transferase) vectors or other vectors with selectable markers. Two appropriate vectors are pKK232-8 and pCM7. Particular named bacterial promoters include *lacI*, *lacZ*, T3, T7, *gpt*, lambda P_R , P_L and *trp*. Eukaryotic promoters include CMV immediate early, HSV thymidine kinase, early and late SV40, LTRs from retrovirus, and mouse metallothionein-I. Selection of the appropriate vector and promoter is well within the level of ordinary skill in the art.

The present invention also relates to host cells containing the above-described construct. The host cell can be a higher eukaryotic cell, such as a mammalian cell, or a lower eukaryotic cell, such as a yeast cell, or the host cell can be a prokaryotic cell, such as

a bacterial cell. Introduction of the construct into the host cell can be effected by calcium phosphate transfection, DEAE-Dextran mediated transfection, or electroporation (Davis, L., Digner, M., Batley, I., Basic Methods in Molecular Biology, (1986)). The constructs in host cells can be used in a conventional manner to produce the gene product encoded by the recombinant sequence. Alternatively, the polypeptides of the invention can be synthetically produced by conventional peptide synthesizers.

Mature proteins can be expressed in mammalian cells, yeast, bacteria, or other cells under the control of appropriate promoters. Cell-free translation systems can also be employed to produce such proteins using RNAs derived from the DNA constructs of the present invention. Appropriate cloning and expression vectors for use with prokaryotic and eukaryotic hosts are described by Sambrook, et al., Molecular Cloning: A Laboratory Manual, Second Edition, (Cold Spring Harbor, N.Y., 1989), the disclosure of which is hereby incorporated by reference.

Transcription of a DNA encoding the polypeptides of the present invention by higher eukaryotes is increased by inserting an enhancer sequence into the vector. Enhancers are cis-acting elements of DNA, usually from about 10 to 300 bp, that act on a promoter to increase its transcription. Examples include the SV40 enhancer on the late side of the replication origin (bp 100 to 270), a cytomegalovirus early promoter enhancer, a polyoma enhancer on the late side of the replication origin, and adenovirus enhancers. Generally, recombinant expression vectors will include origins of replication and selectable markers permitting transformation of the host cell, e.g., the ampicillin resistance gene of *E. coli* and *S. cerevisiae* TRP1 gene, and a promoter derived from a highly-expressed gene to direct transcription of a downstream structural sequence. Such promoters can be derived from operons encoding glycolytic enzymes such as 3-phosphoglycerate kinase (PGK), α factor, acid phosphatase, or heat shock proteins, among others. The heterologous structural sequence is assembled in appropriate phase with translation, initiation and termination sequences, and preferably, a leader sequence capable of directing secretion of translated protein into the periplasmic space or extracellular medium. Optionally, the heterologous sequence can encode a fusion protein including an N-terminal identification peptide imparting desired characteristics, e.g., stabilization or simplified purification of expressed recombinant product.

Useful expression vectors for bacterial use are constructed by inserting a structural DNA sequence encoding a desired protein together with suitable translation, initiation and termination signals in operable reading phase with a functional promoter. The vector will comprise one or more phenotypic selectable markers and an origin of replication to ensure maintenance of the vector and to, if desirable, provide amplification within the host. Suitable prokaryotic hosts for transformation include *E. coli*, *Bacillus subtilis*, *Salmonella typhimurium* and various species within the genera *Pseudomonas*, *Streptomyces*, and *Staphylococcus*, although others may also be employed as a matter of choice. Useful expression vectors for bacterial use can comprise a selectable marker and bacterial origin of replication derived

207
203

from commercially available plasmids comprising genetic elements of the well known cloning vector pBR322 (ATCC 37017). Such commercial vectors include, for example, pK223-3 (Pharmacia Fine Chemicals, Uppsala, Sweden) and GEM1 (Promega Biotec, Madison, Wis., USA). These pBR322 "backbone" sections are combined with an appropriate promoter and the structural sequence to be expressed.

After transformation of a suitable host strain and growth of the host strain to an appropriate cell density, the selected promoter is derepressed by appropriate means (e.g., temperature shift or chemical induction) and cells are cultured for an additional period. Cells are typically harvested by centrifugation, disrupted by physical or chemical means, and the resulting crude extract retained for further purification. Microbial cells employed in expression of proteins can be disrupted by any convenient method, including freeze-thaw cycling, sonication, mechanical disruption, or use of cell lysing agents.

Various mammalian cell culture systems can also be employed to express recombinant protein. Examples of mammalian expression systems include the COS-7 lines of monkey kidney fibroblasts, described by Gluzman, Cell 23:175 (1981), and other cell lines capable of expressing a compatible vector, for example, the C127, 3T3, CHO, HeLa and BHK cell lines. Mammalian expression vectors will comprise an origin of replication, a suitable promoter and enhancer, and also any necessary ribosome binding sites, polyadenylation sites, splice donor and acceptor sites, transcriptional termination sequences, and 5' flanking nontranscribed sequences. DNA sequences derived from the SV40 viral genome, for example, SV40 origin, early promoter, enhancer, splice, and polyadenylation sites may be used to provide the required non-transcribed genetic elements.

The polypeptides of the present invention may be recovered and purified from recombinant cell cultures by methods used heretofore, including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxyapatite chromatography and lectin chromatography. Protein refolding steps can be used, as necessary, in completing configuration of the mature protein. Finally, high performance liquid chromatography (HPLC) can be employed for final purification steps.

The polypeptides of this invention may be a product of chemical synthetic procedures, or produced by recombinant techniques from a prokaryotic or eukaryotic host (for example, by bacterial, yeast, higher plant, insect and mammalian cells in culture). Depending upon the host employed in a recombinant production procedure, the polypeptides of this invention may be glycosylated with mammalian or other eukaryotic carbohydrates or may be non-glycosylated. Polypeptides of this invention may also include an initial methionine amino acid residue. An isolated or purified polypeptide of this invention, or biologically active portion thereof, is substantially free of other cellular material, or culture medium when produced by recombinant techniques, or substantially free of chemical precursors or other chemicals when chemically synthesized. Preferably, an isolated

258
204

polypeptide of this invention is substantially free of cellular material and has less than about 30% (by dry weight) of non-polypeptide, or contaminating, material. When the polypeptide of this invention or a biologically active portion thereof is recombinantly produced, preferably culture medium represents less than about 30% of the volume of the polypeptide preparation. When this invention is produced by chemical synthesis, preferably the preparations contain less than about 30% by dry weight of chemical precursors or non-invention chemicals.

The polypeptides of this invention can be conveniently isolated as described in the specific examples below. A preparation of purified polypeptide is at least about 70% pure; preferably, the preparations are 85% through 99% pure. Purity of the preparations can be assessed by any means known in the art, such as SDS-polyacrylamide gel electrophoresis and Mass Spec/Liquid Chromatography.

Polynucleotide sequences encoding a polypeptide of this invention can be synthesized, in whole or in part, using chemical methods well known in the art (see, for example, Caruthers *et al*, *Nucl. Acids Res. Symp. Ser.* 215 - 223, 1980; Horn *et al*, *Nucl. Acids Res. Symp. Ser.* 225-232, 1980). The polynucleotide that encodes the polypeptide can then be cloned into an expression vector to express the polypeptide.

As will be understood by those of skill in the art, it may be advantageous to produce the polypeptide-encoding nucleotide sequences possessing non-naturally occurring codons. For example, codons preferred by a particular prokaryotic or eukaryotic host can be selected to increase the rate of polypeptide expression or to produce an RNA transcript having desirable properties, such as a half-life which is longer than that of a transcript generated from the naturally occurring sequence.

The nucleotide sequences disclosed herein can be engineered using methods generally known in the art to alter the polypeptide-encoding sequences for a variety of reasons, including but not limited to, alterations which modify the cloning, processing, and/or expression of the polypeptide or mRNA product. DNA shuffling by random fragmentation and PCR reassembly of gene fragments and synthetic oligonucleotides can be used to engineer the nucleotide sequences. For example, site-directed mutagenesis can be used to insert new restriction sites, alter glycosylation patterns, change codon preference, produce splice variants, introduce mutations, and so forth.

Alternatively, the polypeptides of this invention can be produced using chemical methods to synthesize its amino acid sequence, such as by direct peptide synthesis using solid-phase techniques (see, for example, Merrifield, *J. Am. Chem. Soc.* 85, 2149 - 2154, 1963; Roberge *et al*, *Science*, 269, 202 - 204, 1995). Polypeptide synthesis can be performed using manual techniques or by automation. Automated synthesis can be achieved, for example, using Applied Biosystems 431A Peptide Synthesizer (Perkin Elmer). Optionally, fragments of the polypeptide can be separately synthesized and combined using chemical methods to produce a full-length molecule.

209
205

The newly synthesized polypeptide can be substantially purified by preparative high performance liquid chromatography (see, for example, Creighton, **PROTEINS: STRUCTURES AND MOLECULAR PRINCIPLES**, WH Freeman and Co., New York, N.Y., 1983). The composition of a synthetic polypeptide of the present invention can be confirmed by amino acid analysis or sequencing by, for example, the Edman degradation procedure (see, Creighton, *supra*). Additionally, any portion of the amino acid sequence of the polypeptide can be altered during direct synthesis and/or combined using chemical methods with sequences from other proteins to produce a variant polypeptide or a fusion polypeptide.

The polypeptides of the present invention, as a result of the ability to stimulate insulin secretion from pancreatic islet cells *in vitro*, and by causing a decrease in blood glucose *in vivo*, may be employed in treatment of type 2 Diabetes (non-insulin dependent diabetes mellitus). Also, the polypeptides may be used to prevent subjects with impaired glucose tolerance from proceeding to develop type 2 diabetes. In addition, the polypeptides of the invention may be used for treatment of asthma (Bolin et al *Biopolymer* 37:57-66 (1995); US Patent No. 5,677,419) (showing that polypeptide R3P0 is active in relaxing guinea pig tracheal smooth muscle); hypotension induction (VIP induces hypotension, tachycardia, and facial flushing in asthmatic patients (Morice, A.H., and Sever, P.S., *Peptides* 7:279-280 (1986); Morice, A., et al., *The Lancet* II, 1225-1227 (1983)), male reproduction problems (Slow, Y., et al., *Effects of vasoactive intestinal peptide on human sperm motility*, *Arch. Androl.* 1999 Jul-Aug; 43(1):67-71); as a anti-apoptosis/neuroprotective agent (Brenneman D.E., et al., *VIP neurotrophism in the central nervous system: multiple effectors and identification of a femtomolar-acting neuroprotective peptide*, *Ann. N. Y. Acad. Sci.* 1998 Dec 11;865:207-12); cardioprotection during ischemic events (Kaifin R., et al., *Protective role of intracoronary vasoactive intestinal peptide in ischemic and reperfused myocardium*, *J. Pharmacol. Exp. Ther.* 1994 Feb;268(2):852-8; Das, D.K., et al., *Coordinated role of vasoactive intestinal peptide and nitric oxide in cardioprotection*, *Ann. N. Y. Acad. Sci.* 1998 Dec 11;865:297-308), and finally as an anti-ulcer agent (Tuncel, et al., *The protective effect of vasoactive intestinal peptide (VIP) on stress-induced gastric ulceration in rats*, *Ann. N. Y. Acad. Sci.* 1998 Dec 11;865:309-22).

The polypeptides of the present invention may be employed in combination with a suitable pharmaceutical carrier to comprise a pharmaceutical composition for parenteral administration. Such compositions comprise a therapeutically effective amount of the polypeptide and a pharmaceutically acceptable carrier or excipient. Such a carrier includes but is not limited to saline, buffered saline, dextrose, water, glycerol, ethanol, and combinations thereof. The formulation should suit the mode of administration. The invention also provides a pharmaceutical pack or kit comprising one or more containers filled with one or more of the ingredients of the pharmaceutical compositions of the invention. Associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration.

2A
206

in addition, the polypeptides of the present invention may be employed in conjunction with other therapeutic compounds.

The pharmaceutical compositions may be administered in a convenient manner such as by the oral, topical, intravenous, intraperitoneal, intramuscular, subcutaneous, intranasal or intradermal routes. The pharmaceutical compositions are administered in an amount which is effective for treating and/or prophylaxis of the specific indication. In general, they are administered in an amount of at least about 350ng (0.1nmol)/kg body weight and in most cases they will be administered in an amount not in excess of about 35ug (10nmol)/kg body weight per day. In most cases, the dosage is from about 0.1 µg/kg to about 100 mg/kg body weight daily, taking into account the routes of administration, symptoms, etc. These numbers do not take into account the bioavailability of the peptide in vivo, in which case more or less may be used to attain the effective dose desired. One of ordinary skill in the art is able to determine through dosing experiments or other conventional means the gross amount to be used to produce an effective dosage.

A polypeptide of the invention may also be employed in accordance with the present invention by expression of such polypeptide in vivo, which is often referred to as "gene therapy." Thus, for example, cells may be engineered with a polynucleotide (DNA or RNA) encoding for the polypeptide ex vivo, the engineered cells are then provided to a patient to be treated with the polypeptide. Such methods are well-known in the art. For example, cells may be engineered by procedures known in the art by use of a retroviral particle containing RNA encoding for the polypeptide of the present invention.

Local delivery of the insulin secretagogues using gene therapy may provide the therapeutic agent to the target area, i.e., the pancreas. For instance a pancreas-specific promoter was used to create a beta-cell pancreatic tumor mouse model (Hanahan, D., Heritable formation of pancreatic beta-cell tumors in transgenic mice expressing recombinant insulin/simian virus 40 oncogenes, Nature 315(6015):115-22 (1985)).

Both in vitro and in vivo gene therapy methodologies are contemplated. Several methods for transferring potentially therapeutic genes to defined cell populations are known. See, e.g., Mulligan, "The Basic Science Of Gene Therapy", Science, 260: 826-31 (1993). These methods include:

1) Direct gene transfer. See, e.g., Wolff et al., "Direct Gene transfer Into Mouse Muscle In Vivo", Science, 247:1465-68 (1990);

2) Liposome-mediated DNA transfer. See, e.g., Caplan et al., "Liposome-mediated CFTR Gene Transfer To The Nasal Epithelium Of Patients With Cystic Fibrosis", Nature Med. 3: 39-46 (1995); Crystal, "The Gene As A Drug", Nature Med. 1:15-17 (1995); Gao and Huang, "A Novel Cationic Liposome Reagent For Efficient Transfection Of Mammalian Cells", Biochem. Biophys. Res. Comm., 178:280-85 (1991);

3) Retrovirus-mediated DNA transfer. See, e.g., Kay et al., "In Vivo Gene Therapy Of Hemophilia B: Sustained Partial Correction In Factor IX-Deficient Dogs", Science, 262:117-19 (1993); Anderson, "Human Gene Therapy", Science, 258:808-13 (1992).

00506280-051500

207

4) DNA Virus-mediated DNA transfer. Such DNA viruses include adenoviruses (preferably Ad-2 or Ad-5 based vectors), herpes viruses (preferably herpes simplex virus based vectors), and parvoviruses (preferably "defective" or non-autonomous parvovirus based vectors, more preferably adeno-associated virus based vectors, most preferably AAV-2 based vectors). See, e.g., Ali et al., "The Use Of DNA Viruses As Vectors For Gene Therapy", Gene Therapy, 1:367-84 (1994); United States Patent 4,797,368, incorporated herein by reference, and United States Patent 5,138,941, incorporated herein by reference.

The choice of a particular vector system for transferring the gene of interest will depend on a variety of factors. One important factor is the nature of the target cell population. Although retroviral vectors have been extensively studied and used in a number of gene therapy applications, these vectors are generally unsuited for infecting non-dividing cells. In addition, retroviruses have the potential for oncogenicity. However, recent developments in the field of lentiviral vectors may circumvent some of these limitations. See Naldini et al., In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector, Science 272:263-7 (1998).

Retroviruses from which the retroviral plasmid vectors hereinabove mentioned may be derived include, but are not limited to, Moloney Murine Leukemia Virus, spleen necrosis virus, retroviruses such as Rous Sarcoma Virus, Harvey Sarcoma Virus, avian leukosis virus, gibbon ape leukemia virus, human immunodeficiency virus, adenovirus, Myeloproliferative Sarcoma Virus, and mammary tumor virus. In one embodiment, the retroviral plasmid vector is derived from Moloney Murine Leukemia Virus.

Adenoviruses have the advantage that they have a broad host range, can infect quiescent or terminally differentiated cells, such as neurons or hepatocytes, and appear essentially non-oncogenic. See, e.g., Ali et al., *supra*, p. 367. Adenoviruses do not appear to integrate into the host genome. Because they exist extrachromosomally, the risk of insertional mutagenesis is greatly reduced. Ali et al., *supra*, p. 373.

Adeno-associated viruses exhibit similar advantages as adenoviral-based vectors. However, AAVs exhibit site-specific integration on human chromosome 19 (Ali et al., *supra*, p. 377).

In a preferred embodiment, the DNA encoding the polypeptide insulin secretagogues of this invention is used in gene therapy for disorders such as diabetes.

According to this embodiment, gene therapy with DNA encoding polypeptide insulin secretagogues or mutants of this invention is provided to a patient in need thereof, concurrent with, or immediately after diagnosis.

The skilled artisan will appreciate that any suitable gene therapy vector containing polypeptide insulin secretagogues, DNA or DNA of fragment, derivative or variant of polypeptide insulin secretagogues may be used in accordance with this embodiment. The techniques for constructing such a vector are known. See, e.g., Anderson, W.F., "Human Gene Therapy," Nature, 392 25-30 (1998); Verma, I.M., and Somia, N., "Gene Therapy - Promises, Problems, and Prospects," Nature, 389 239-242 (1998). Introduction of the

polypeptide insulin secretagogues DNA-containing vector to the target site may be accomplished using known techniques.

5 The vector includes one or more promoters. Suitable promoters which may be employed include, but are not limited to, the retroviral LTR; the SV40 promoter; and the human cytomegalovirus (CMV) promoter described in Miller, et al., *Biotechniques*, 7(9): 980-990 (1989), or any other promoter (e.g., cellular promoters such as eukaryotic cellular promoters including, but not limited to, the histone, pol III, and β -actin promoters). Other viral promoters which may be employed include, but are not limited to, adenovirus promoters, thymidine kinase (TK) promoters, and B19 parvovirus promoters. The selection of a suitable promoter will be apparent to those skilled in the art from the teachings contained herein.

15 The nucleic acid sequence encoding the polypeptide of the present invention is under the control of a suitable promoter. Suitable promoters which may be employed include, but are not limited to, adenoviral promoters, such as the adenoviral major late promoter; or heterologous promoters, such as the cytomegalovirus (CMV) promoter; the respiratory syncytial virus (RSV) promoter; inducible promoters, such as the MMT promoter, the metallothionein promoter; heat shock promoters; the albumin promoter; the ApoA1 promoter; human globin promoters; viral thymidinekinase promoters, such as the Herpes Simplex thymidine kinase promoter; retroviral LTRs (including the modified retroviral LTRs hereinabove described); the β -actin promoter; and human growth hormone promoters. The promoter also may be the native promoter which controls the gene encoding the polypeptide.

20 The retroviral plasmid vector is employed to transduce packaging cell lines to form producer cell lines. Examples of packaging cells which maybe transfected include, but are not limited to, the PE501, PA317, ψ -2, ψ -AM, PA12, T19-14X, VT-19-17-H2, ψ CRE, ψ CRIP, GP+E-86, GP+envAm12, and DAN cell lines as described in Miller, *Human Gene Therapy*, 1: 5-14 (1990), which is incorporated herein by reference in its entirety. The vector may transduce the packaging cells through any means known in the art. Such means include, but are not limited to, electroporation, the use of liposomes, and CaPO_4 precipitation. In one alternative, the retroviral plasmid vector may be encapsulated into a liposome, or coupled to a lipid, and then administered to a host. The producer cell line generates infectious retroviral vector particles which include the nucleic acid sequence(s) encoding the polypeptides. Such retroviral vector particles then may be employed, to transduce eukaryotic cells, either in vitro or in vivo. The transduced eukaryotic cells will express the nucleic acid sequence(s) encoding the polypeptide. Eukaryotic cells which may be transduced include, but are not limited to, embryonic stem cells, embryonic carcinoma cells, as well as hematopoietic stem cells, hepatocytes, fibroblasts, myoblasts, keratinocytes, endothelial cells, and bronchial epithelial cells.

35 A different approach to gene therapy is "transkaryotic therapy" wherein the patient's cells are treated ex vivo to induce the dormant chromosomal genes to produce the protein of interest after reintroduction to the patient. Transkaryotic therapy assumes the individual has

a normal complement of genes necessary for activation. Transkaryotic therapy involves introducing a promoter or other exogenous regulatory sequence capable of activating the nascent genes, into the chromosomal DNA of the patients' cells ex vivo, culturing and selecting for active protein-producing cells, and then reintroducing the activated cells into the patient with the intent that they then become fully established. The "gene activated" cells then manufacture the protein of interest for some significant amount of time, perhaps for as long as the life of the patient. U.S. Patent Nos. 5,841,870 and 5,733,781 disclose in detail this concept, and are hereby incorporated by reference in their entirety.

In order that this invention may be better understood, the following examples are set forth. These examples are for the purpose of illustration only, and are not to be construed as limiting the scope of the invention in any manner. All publications mentioned herein are incorporated by reference in their entirety.

Example 1. Protocol for Rat Islet Isolation

Sprague Dawley rats (275-320 g) were used as the source of donor islets. Briefly, the pancreas was filled with 10 ml of cold reconstituted Liberase RI (Boehringer Mannheim), harvested and incubated with additional 5 ml enzyme solution in water bath for 30 minutes. Tissue suspension was washed twice with cold 10% FBS/Hanks buffer (Gibco), resuspended in 8 ml 25% ficoll (Sigma) and then layered with 5 ml each of 23%, 20% and 11% ficoll. The islets in the 20% layer after centrifugation were removed, washed twice with cold 10% FBS/Hank buffer and resuspended in 10% FBS/RPMI 1640 media (Sigma).

Example 2. Protocol for Assaying Peptide-Induced Elevation of Insulin Levels in Rats.

Wistar rats are fasted overnight (17 hrs) and then anesthetized with pentobarbital (0.1 ml/100 g BW). Glucose (0.4 g/kg dissolved in 1% human albumin-saline) +/- peptide (dissolved in 1% human albumin-saline) is injected intravenously into the tail vein. The rats are eye-bled 1 minute after the injection and 50-100ul of the plasma are assayed for insulin level with the Linco RIA kit (Linco Research, Inc., St. Charles, MO).

Example 3. Protocol for Determining the Effect of Peptides on Intraperitoneal Glucose Tolerance in Rats.

Wistar rats are fasted overnight and then anesthetized with pentobarbital. The rats were eye-bled (zero time) and the peptide (in 1% human albumin) was injected into the tail vein. Five minutes later, 1 g/kg of glucose (in saline) was injected intraperitoneally, and the rats were eye-bled after 15, 30 and 60 minutes. Plasma glucose levels were determined using the Technicon Axon autoanalyzer, Bayer Diagnostics division of Bayer Corporation, Tarrytown NY, operated using Method No. SM4-2143F90 "Glucose".

714
210

Example 4. Protocol for Determining the Effect of Peptides on Intestinal Water Retention in Rats.

Male rats were fasted for 24 hours, and their water bottles were taken away for 2 - 3 hours before the start of the experiment. Peptide or saline was injected subcutaneously into conscious rats. The rats were euthanized with CO₂ 10 minutes after dosing, and the small intestine dissected out and weighed (1). The intestine was cut open, the water in the lumen absorbed with filter paper, and the intestine re-weighed (2). The amount of intestinal water (g) = weight (1) - weight (2). -

Example 5. Peptide Synthesis Methodology.

The following general procedure was followed to synthesize some of the polypeptides of the invention. Peptide synthesis was carried out by the FMOCA-Butyl strategy (Peptide Synthesis Protocols (1994), Volume 35 by Michael W. Pennington & Ben M. Dunn) under continuous flow conditions using Rapp-Polymers PEG-Polystyrene resins (Rapp-Polymers, Tübingen, Germany). At the completion of synthesis, peptides are cleaved from the resin and de-protected using TFA/DTT/H₂O/Trisopropyl silane (88/5/5/2). Peptides were precipitated from the cleavage cocktail using cold diethyl ether. The precipitate was washed three times with the cold ether and then dissolved in 5% acetic acid prior to lyophilization. Peptides were checked by reversed phase chromatography on a YMC-Pack ODS-AQ column (YMC, Inc., Wilmington, NC) on a Waters ALLIANCE® system (Waters Corporation, Milford, MA) using water/acetonitrile with 3% TFA as a gradient from 0% to 100% acetonitrile, and by MALDI mass spectrometry on a VOYAGER DE™ MALDI Mass Spectrometer, (model 5-2386-00, PerSeptive BioSystems, Framingham, MA). Matrix buffer (50/50 dH₂O/acetonitrile with 3% TFA) peptide sample added to Matrix buffer 1/1. Those peptides not meeting the purity criteria of >95% are purified by reversed phase chromatography on a Waters Delta Prep 4000 HPLC system (Waters Corporation, Milford, MA).

Example 6. Peptide Cloning

The recombinant expression of VIP has been attempted previously with mixed results. Simoncits et al (Eur. J. Biochem. 178: 343-350, (1988)) expressed Leu¹⁷, Gly²⁸-VIP or Leu¹⁷Gly²⁸Lys³⁰Arg³¹-VIP as a C-terminal fusion to the N-terminal part of the E. coli β -galactosidase gene in E. coli. The removal of the methionine at position 17 eliminates a CNBR cleavage site. C-terminal addition of Gly or Gly-Lys-Arg was designed for potential in vivo C-terminal amidation by mammalian PAMase. Upon CNBR cleavage of the fusion proteins at the methionine introduced N-terminal to the VIP mutants, free VIP mutants were purified and shown to possess similar activities as the native VIP, although the activities were measured only at saturating concentrations of the peptides. Raingeaud et al (Biochimie 76: 14-25 (1996)) expressed VIP as polymeric C-terminal fusions to glutathione

216
211

S-transferase (GST) in *E. coli*. The free polymeric or monomeric VIP peptides were released upon sequential cleavages by Factor Xa and hydroxylamine. The requirement for the two-step cleavage led to inefficiency and a mixture of products. Polymeric or monomeric VIP peptides produced by this method were less active than the native VIP. An improved version of the construct using only Factor Xa cleavage yielded a VIP mutant with a seven residue C-terminal extension that was also less active than the native VIP (Ottavi et al, *Biochimie* 80: 289-293 (1998)). No expression of PACAP has been reported to date. To establish a robust method for expressing PACAP, VIP, and their mutants, their genetic codes were cloned C-terminal to GST with a single Factor Xa recognition site separating the monomeric peptide and GST. The gene encoding Factor Xa recognition site fused to DNA sequence of the peptide to be produced has been synthesized by hybridizing two overlapping single-stranded DNA fragments (70-90mers) containing a Bam HI or Xho I restriction enzyme site immediately 5' to the DNA sequence of the gene to be cloned, followed by DNA synthesis of the opposite strands via the large fragment of DNA polymerase I (Life Technologies, Inc., Gaithersburg, MD). The DNA sequences chosen for each gene was based on the reverse translation of the designed amino acid sequence of each peptide. In some cases, the gene encoding the peptide is generated by PCR mutagenesis (Picard, V, et al, *Nucleic Acids Res* 22: 2587-91 (1994); Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, New York) of a gene already made by the method described above. The double-stranded product is then digested by Bam HI and Xho I and ligated into pGEX-6P-1 (Amersham Pharmacia Biotech) which has also been cleaved by Bam HI and Xho I. The DNA sequences of the cloned peptide genes are listed in Figure 8.

For example, when DNA sequences of SEQ ID NOs: 54, 55, and 56 are cloned into pGEX-6P-1, the following polypeptide sequences were expressed as fusions with glutathione S-transferase (GST):

PACAP38: IEGRHSDGIFTDSYSRYRKQMAVKKYLA AVL GKRYKQRVKIK (SEQ ID NO:2)

VIP: IEGRHSDAVFTDNYTRLRKQMAVKKYLNSILN (SEQ ID NO:1)

R3P3: IEGRHSDAVFTENYTKLRQLAAKKYLN DLKKG GT (SEQ ID NO:8)

A restriction map of a typical plasmid containing the GST-peptide fusion is shown as Figure 3.

Example 7. Peptide Recombinant Expression and Purification

BL21 cells (Stratagene) transformed with the GST-peptide fusion containing plasmids were grown at 37°C until OD₆₀₀ reached 0.6 to 1.0 and induced by 1 mM IPTG (Life Technologies) for 2 hours at 37°C. 2L of cells were spun at 7,700 g for 15 minutes, weighed, and stored at -20°C for at least 3 hours. The frozen cell pellet was resuspended in 100 mL ice-cold PBS with 250 µl of protease inhibitor cocktail (Cat No. P-8485, Sigma Chemical) per gram of cells, sonicated at 3x for 1 minute with 15 second breaks. Cellular debris were

R3P34	HSDAVFTDNYTRLRKQLAAKKYLNDIKKQ	30
R3P35	HSDAVFTDNYTRLRKQLAAKKYLNDIKGNQ	31
R3P36	HSDAVFTDNYTRLRKQLAAKKYLNDIKKKRY	32
rR3P41	HSDAVFTDNYTRLRKQMAVKKYLSIKK	33
rR3P42	HSDAVFTDNYTRLRKQMAVKKYLSIKN	34
rR3P43	HSDAVFTDNYTRLRKQMAVKKYLSILK	35
rR3P44	HSDAVFTDNYTRLRKQMAVKKYLSILN	36
rR3P45	HSDAVFTDNYTRLRKQMAVKKYLSILN	37
rR3P48	HSDAVFTDNYTRLRKQLAVKKYLSILN	38
rR3P47	HSDAVFTDNYTRLRKQMAAKKYLSILN	39
rR3P48	HSDAVFTDNYTRLRKQMAVKKYLNDILN	40
rR3P49	HSDAVFTDNYTRLRKQMAAKKYLSIKN	41
rR3P50	HSDAVFTDNYTRLRKQMAAKKYLSILK	42
rR3P51*	HSDAVFTDNYTRLRKQMAAKKYLSIKK	43
rR3P52	HSDAVFTDNYTRLRKQMAAKKYLSIKKKRY	44
rR3P53*	HSDAVFTDNYTRLRKQMAAKKYLSIKKKR	45
rR3P54	HSDAVFTDNYTRLRKQMAAKKYLSIKKK	46
rR3P55*	HSDAVFTDNYTRLRKQMAAKKYLSIKNKRY	47
rR3P58	HSDAVFTDNYTRLRKQMAVKKYLSIKKKRY	48
rR3P57	HSDAVFTDNYTRLRKQMAVKKYLSIKKKR	49
rR3P58	HSDAVFTDNYTRLRKQMAVKKYLSIKKK	50
rR3P59	HSDAVFTDNYTRLRKQMAVKKYLSIKNKRY	51
rR3P60	HSDAVFTDNYTRLRKQVAKKYLSIKK	52
rR3P61	HSDAVFTDNYTRLRKQLAAKKYLQTIKK	53
R3P6	HSDGIFTESYSRYRKQMAVKKYLAALKKKRYKQRVKNK	57
R3P7	HSDAVFTENYTRLRKQMAVKKYLSILKK-NH ₂	58
R3P15	HSDGIFTDSYSRYRKQMAVKKYLSAVRHGGT-NH ₂	59
R3P16	HSDGIFTDSYSRYRKQMAVKKYLAAVKQGGT-NH ₂	60
R3P17	HSDGIFTDSYSRYRKQMAVKKYLAAVKKYLAAVRHG-NH ₂	61
R3P18	SWCEPGWCRHSDAVFTENYTKLRKQLAAKKYLNDLKKGGT	62
R3P23	HSDAVFTDNYTRLRKQLAAKKYLNDILKGGT	63
R3P27	HSDAVFTDNYTRLRKQLAAKKYLNDILNGGT	64
R3P28	HSDAVFTDNYTRLRKQLAVKKYLNDILKGGT	65
R3P37	HSDGIFTDSYSRYRKQLAAKKYLADVKKGGT	66
R3P38	HSDGIFTDSYSRYRKQLAAKKYLADVKK	67
R3P39	HSDGIFTDSYSRYRKQLAVKKYLAAVKK	68
R3P40	HSDGIFTDSYSRYRKQMAVKKYLAAVKK	69
R3P62	HSDAVFTDNYTRLRKQVAAKKYLSIKK	70
R3P65	HSDAVFTDNYTRLRKQMAAKKYLSIKNKR	71
R3P66	HSDAVFTDNYTRLRKQVAAKKYLSIKNKRY	72
R3P67	HSDAVFTDNYTRLRKQLAAKKYLNTIKNKRY	73
R3P68	HSDAVFTDNYTRLRKQVAAKKYLSIKNKRY	74
R3P69	HSDAVFTDNYTRLRKQMAAKKYLSIKNKRY	75
R3P70	HSDAVFTDNYTRLRKQMAAKKYLNTIKNKRY	76
R3P71	HSDAVFTDQYTRLRKQMAAKKYLSIKNKRY	77
R3P72	HSDAVFTDQYTRLRKQLAAKKYLNTIKNKRY	78
R3P73	HSDAVFTDNYTRLRKQMAAHKYLSIKNKRY	79
R3P74	HSDAVFTDNYTRLRKQMAAKHYLSIKNKRY	80
R3P75	HSDAVFTDQYTRLRKQLAAHKYLNTIKNKRY	81
R3P76	HSDAVFTDQYTRLRKQLAAKHLYLNTIKNKRY	82
R3P77	HSDAVFTDNYTRLRKQVAAKKYLSIKKKR	83
R3P78	HSDAVFTDNYTRLRKQVAAKKYLSIKKKR	84
R3P79	HSDAVFTDNYTRLRKQVAAKKYLSIKNKRY	85
R3P80	HSDAVFTDNYTRLRKQVAVKKYLSIKKKR	86
R3P81	HSDAVFTDNYTRLRKQVAVKKYLSIKKK	87
R3P82	HSDAVFTDNYTRLRKQVAVKKYLSIKNKRY	88
R3P83	HSDAVFTDNYTRLRKQVAAKKYLSILKKRY	89

218
214

R3P84	HSDAVFTDNYTRLRKQVAAKKYLSILKKR	90
R3P85	HSDAVFTDNYTRLRKQVAAKKYLSILKK	91
R3P86	HSDAVFTDNYTRLRKQVAAKKYLSIKNK	92
R3P87	HSDAVFTDNYTRLRKQVAVKKYLSILKKRY	93
R3P88	HSDAVFTDNYTRLRKQVAVKKYLSILKKR	94
R3P89	HSDAVFTDNYTRLRKQVAVKKYLSILKK	95
R3P92	HSDAVFTDNYTRLRKQVAAKKYLSILNKRY	97
R3P93	HSDAVFTDNYTRLRKQVAAKKYLSILNKR	98
R3P94	HSDAVFTDNYTRLRKQVAAKKYLSILNK	99
rR3P97	HSDAVFTDNYTRLRKQMACKKYLSIKNKR	100
rR3P98	HSDAVFTDNYTRLRKQMAKKYLSIKNKR	101
rR3P99	HSDAVFTDNYTRLRKQMAEKKYLSIKNKR	102
rR3P100	HSDAVFTDNYTRLRKQMAFKKYLSIKNKR	103
rR3P101	HSDAVFTDNYTRLRKQMAKKYLSIKNKR	104
rR3P102	HSDAVFTDNYTRLRKQMAHKYLSIKNKR	105
rR3P103	HSDAVFTDNYTRLRKQMAIKKYLSIKNKR	106
rR3P104	HSDAVFTDNYTRLRKQMAKKKYLSIKNKR	107
rR3P105	HSDAVFTDNYTRLRKQMALKKYLSIKNKR	108
rR3P106	HSDAVFTDNYTRLRKQMAKKYLSIKNKR	109
rR3P107	HSDAVFTDNYTRLRKQMANKKYLSIKNKR	110
rR3P108	HSDAVFTDNYTRLRKQMAPKKYLSIKNKR	111
rR3P109	HSDAVFTDNYTRLRKQMAQKKYLSIKNKR	112
rR3P110	HSDAVFTDNYTRLRKQMASKKYLSIKNKR	113
rR3P111	HSDAVFTDNYTRLRKQMASKKYLSIKNKR	114
rR3P112	HSDAVFTDNYTRLRKQMATKKYLSIKNKR	115
rR3P113	HSDAVFTDNYTRLRKQMAVKKYLSIKNKR	116
rR3P114	HSDAVFTDNYTRLRKQMAWKYLSIKNKR	117
rR3P115	HSDAVFTDNYTRLRKQMAVKKYLSIKNKR	118
rR3P116	HSDAVFTDNYTRLRKQMAAKKYLSIANKR	119
rR3P117	HSDAVFTDNYTRLRKQMAAKKYLSIANKR	120
rR3P118	HSDAVFTDNYTRLRKQMAAKKYLSIDNKR	121
rR3P119	HSDAVFTDNYTRLRKQMAAKKYLSIENKR	122
rR3P120	HSDAVFTDNYTRLRKQMAAKKYLSIFNKR	123
rR3P121	HSDAVFTDNYTRLRKQMAAKKYLSIGNKR	124
rR3P122	HSDAVFTDNYTRLRKQMAAKKYLSIHNR	125
rR3P123	HSDAVFTDNYTRLRKQMAAKKYLSIINKR	126
rR3P124	HSDAVFTDNYTRLRKQMAAKKYLSIMNKR	127
rR3P125	HSDAVFTDNYTRLRKQMAAKKYLSINNKR	128
rR3P126	HSDAVFTDNYTRLRKQMAAKKYLSIPNKR	129
rR3P127	HSDAVFTDNYTRLRKQMAAKKYLSIQNKR	130
rR3P128	HSDAVFTDNYTRLRKQMAAKKYLSIRNKR	131
rR3P129	HSDAVFTDNYTRLRKQMAAKKYLSISNKR	132
rR3P130	HSDAVFTDNYTRLRKQMAAKKYLSITNKR	133
rR3P131	HSDAVFTDNYTRLRKQMAAKKYLSIVNKR	134
rR3P132	HSDAVFTDNYTRLRKQMAAKKYLSIWNKR	135
rR3P133	HSDAVFTDNYTRLRKQMAAKKYLSIYNKR	136
rR3P134	HSDAVFTDNYTRLRKQMAAKKYLSIKNAR	137
rR3P135	HSDAVFTDNYTRLRKQMAAKKYLSIKNCR	138
rR3P136	HSDAVFTDNYTRLRKQMAAKKYLSIKNDR	139
rR3P137	HSDAVFTDNYTRLRKQMAAKKYLSIKNER	140
rR3P138	HSDAVFTDNYTRLRKQMAAKKYLSIKNFR	141
rR3P139	HSDAVFTDNYTRLRKQMAAKKYLSIKNGR	142
rR3P140	HSDAVFTDNYTRLRKQMAAKKYLSIKNHR	143
rR3P141	HSDAVFTDNYTRLRKQMAAKKYLSIKNIR	144
rR3P142	HSDAVFTDNYTRLRKQMAAKKYLSIKNLR	145
rR3P143	HSDAVFTDNYTRLRKQMAAKKYLSIKNMR	146
rR3P144	HSDAVFTDNYTRLRKQMAAKKYLSIKNNR	147
rR3P145	HSDAVFTDNYTRLRKQMAAKKYLSIKNPR	148

5053P1-001-001-001

219
215

rR3P146	HSDAVFTDNYTRLRKQMAAKKYLNSIKNQR	149
rR3P147	HSDAVFTDNYTRLRKQMAAKKYLNSIKNRR	150
rR3P148	HSDAVFTDNYTRLRKQMAAKKYLNSIKNSR	151
rR3P149	HSDAVFTDNYTRLRKQMAAKKYLNSIKNTR	152
rR3P150	HSDAVFTDNYTRLRKQMAAKKYLNSIKNVR	153
rR3P151	HSDAVFTDNYTRLRKQMAAKKYLNSIKNWR	154
rR3P152	HSDAVFTDNYTRLRKQMAAKKYLNSIKNYR	155
rR3P153	HSDAVFTDNYTRLRKQMAAKKYLNSIKNKA	156
rR3P155	HSDAVFTDNYTRLRKQMAAKKYLNSIKNKD	157
rR3P156	HSDAVFTDNYTRLRKQMAAKKYLNSIKNKE	158
rR3P157	HSDAVFTDNYTRLRKQMAAKKYLNSIKNKF	159
rR3P158	HSDAVFTDNYTRLRKQMAAKKYLNSIKNKG	160
rR3P159	HSDAVFTDNYTRLRKQMAAKKYLNSIKNKH	161
rR3P160	HSDAVFTDNYTRLRKQMAAKKYLNSIKNKI	162
rR3P161	HSDAVFTDNYTRLRKQMAAKKYLNSIKNKK	163
rR3P162	HSDAVFTDNYTRLRKQMAAKKYLNSIKNKL	164
rR3P163	HSDAVFTDNYTRLRKQMAAKKYLNSIKNKM	165
rR3P164	HSDAVFTDNYTRLRKQMAAKKYLNSIKNKN	166
rR3P165	HSDAVFTDNYTRLRKQMAAKKYLNSIKNKP	167
rR3P166	HSDAVFTDNYTRLRKQMAAKKYLNSIKNKQ	168
rR3P167	HSDAVFTDNYTRLRKQMAAKKYLNSIKNKS	169
rR3P168	HSDAVFTDNYTRLRKQMAAKKYLNSIKNKT	170
rR3P169	HSDAVFTDNYTRLRKQMAAKKYLNSIKNKV	171
rR3P170	HSDAVFTDNYTRLRKQMAAKKYLNSIKNKW	172
rR3P171	HSDAVFTDNYTRLRKQMAAKKYLNSIKNKY	173
R3P172	HSDAVFTDNYTRLRKQVAACKYLSIKNKRYSWCEPGWCR	174
R3P173	HSDAVFTDDYTRLRKEVAACKYLESIKDKRY	175
PAC1:	ESDGIFTDSYSRYRKQMAVKKYLA AVL-NH ₂	176
PAC2:	HKGIFTDSYSRYRKQMAVKKYLA AVL-NH ₂	177
PAC3:	HSKGIFTDSYSRYRKQMAVKKYLA AVL-NH ₂	178
PAC4:	HSDKIFTDSYSRYRKQMAVKKYLA AVL-NH ₂	179
PAC5:	HSDGKFTDSYSRYRKQMAVKKYLA AVL-NH ₂	180
PAC6:	HSDGIKFTDSYSRYRKQMAVKKYLA AVL-NH ₂	181
PAC7:	HSDGIFKDSYSRYRKQMAVKKYLA AVL-NH ₂	182
PAC8:	HSDGIFTKSYSRYRKQMAVKKYLA AVL-NH ₂	183
PAC9:	HSDGIFTDKSYSRYRKQMAVKKYLA AVL-NH ₂	184
PAC10:	HSDGIFTDSKSYSRYRKQMAVKKYLA AVL-NH ₂	185
PAC11:	HSDGIFTDSYKRYRKQMAVKKYLA AVL-NH ₂	186
PAC12:	HSDGIFTDSYSEYRKQMAVKKYLA AVL-NH ₂	187
PAC13:	HSDGIFTDSYSRKQMAVKKYLA AVL-NH ₂	188
PAC14:	HSDGIFTDSYSRYEKQMAVKKYLA AVL-NH ₂	189
PAC15:	HSDGIFTDSYSRYREQMAVKKYLA AVL-NH ₂	190
PAC16:	HSDGIFTDSYSRYRKQMAVKKYLA AVL-NH ₂	191
PAC17:	HSDGIFTDSYSRYRKQKAVKKYLA AVL-NH ₂	192
PAC18:	HSDGIFTDSYSRYRKQMKVKKYLA AVL-NH ₂	193
PAC19:	HSDGIFTDSYSRYRKQMAKKYLA AVL-NH ₂	194
PAC20:	HSDGIFTDSYSRYRKQMAVEKYLA AVL-NH ₂	195
PAC21:	HSDGIFTDSYSRYRKQMAVKEYLA AVL-NH ₂	196
PAC22:	HSDGIFTDSYSRYRKQMAVKKKLA AVL-NH ₂	197
PAC23:	HSDGIFTDSYSRYRKQMAVKKYKA AVL-NH ₂	198
PAC24:	HSDGIFTDSYSRYRKQMAVKKYLA AVL-NH ₂	199
PAC25:	HSDGIFTDSYSRYRKQMAVKKYLA AVL-NH ₂	200

05555200-051500

270
216

PAC26:	HSDGIFTDSYSRYRKQMAVKYLAALK-NH ₂	201
PAC27:	HSDGIFTDSYSRYRKQMAVKYLAALK-NH ₂	202

Example 8. Insulin secretion by rat islets

Peptide R3P3 (0.1-100 nM) stimulates insulin secretion from isolated rat islets in a glucose-dependent fashion. These studies compare the effects on islet cells of R3P3 peptide and GLP-1.

Rat islets were isolated and treated with GLP-1 or R3P3 at either 3 or 8 mM glucose in the medium in accordance with the rat islet protocol described above in example 1. As shown by Figures 4A-4B, peptide R3P3 significantly enhances the release of insulin from islets in a concentration-dependent fashion and this effect is similar to that of GLP-1, a known insulin secretagogue.

Example 9. In vivo insulin and glucose response.

As shown in Tables 2 and 3 below, polypeptides that activate the R3 receptor also potentiate glucose-induced increase in plasma insulin levels when compared to glucose alone. This increase in insulin causes concomitant decrease in plasma glucose.

In accordance with the protocol of example 2 above, overnight fasted Wistar rats were anesthetized with pentobarbital; I.v. injected with glucose $\pm$ peptide and eye bled after 1 minute. N=12 rats/group. The graph in Figure 5 shows that the polypeptide R3P3 causes an increase in glucose disposal that accompanies the increase in insulin secretion. The peptide or vehicle was given I.v. followed by a glucose load given I.p. in accordance with example 3. The plasma glucose was followed over the time period indicated. As shown in the graph, R3P3 markedly accelerated the disposal of blood glucose.

Example 10. Diarrhea side effects.

As described in the protocol of example 4 above, fasted rats were injected s.c. with the indicated peptide (5 nmol/kg or 22-24 nmol/kg). Five minutes after the injection, 0.3 ml of water was given p.o. Five min after the water dose, the animals were euthanized and the water content in the small intestine determined. As shown in Figure 6, VIP injections at the two doses caused a marked increase in the water content of the small intestine lumen over the vehicle (saline) control. At the highest dose, the R3 peptides caused only a very small increase (i.e., roughly 10%) in comparison to VIP. At the 5 nmol/kg dose, the peptides did not produce any change in water content in the small gut. The degree of water retention was used as an index of R2 activation *in vivo*.

Example 11. Effect of Peptides on Intraperitoneal Glucose Tolerance in Rats

Wistar rats were fasted overnight and then anesthetized with Pentobarbital. The rats were eye-bled (zero time) and the peptide (in 1% human albumin) was injected subcutaneously. Five minutes later, 1 g/kg of glucose (in saline) was injected

221
217

Intraperitoneally, and the rats were eye-bled after 30 minutes. Plasma glucose levels were determined using the Axon autoanalyzer, and are shown in Figure 7.

Figure 7 shows that plasma glucose elevated to 160 mg/dl above basal (100mg/dl) in rats treated with vehicle 30 minutes after IPGTT (IP Glucose Tolerance Test). In rats injected with peptides R3P3, R3P12 and R3P13, this elevation of plasma glucose was significantly reduced, verifying the insulin-producing effect. At 1 nmole/kg s.c., the glucose-lowering effect of each peptide was similar to that observed with an equivalent dose of GLP-1.

Example 12. Glucose-dependent insulin secreting polypeptides.

Table 2 contains a list of the peptides that stimulated release of insulin in vivo in the IPGTT assay, or in vitro through the rat islet assay. As shown by the data, the peptides enhance glucose-mediated insulin release in vivo and in vitro.

Plasma insulin: Data are expressed as % of plasma insulin one minute after IVGTT (0.4g/kg glucose) with either saline, 0.1nmol/kg P51, P55, P60, P66 or 1 nmole/kg of the other peptides in Wistar rats. Blood was drawn from the eye and insulin was measured with the rat insulin RIA kit (Linco Research, Inc., St. Charles, MO).

Plasma glucose: Data are expressed as % of vehicle of plasma glucose area under the curve after IPGTT (1 g/kg glucose) after treatment with 1 nmol/kg dose of the peptide. PACAP27 has been reported to induce insulin secretion but does not affect plasma glucose levels (Filipsson, K. et al., J. Clin. Endocrin. & Metabolism 82: 3093-3098 (1997)). The present inventors have shown for the first time that this is because the effects of R2 and R3 on glucose tend to cancel each other out. The R2-selective agonist [K15, R16, L27]VIP(1-7)/GRF(8-27) (Gourlet, P. et al., Peptides 18:1539-45 (1997)), defined as R2P1, increases plasma glucose level by 14% while R3-selective agonists lower plasma glucose level by ~20%. Therefore, R3-selectivity appears to be a desirable attribute for medicaments which are to be employed in achieving blood glucose reduction, for treatment of type 2 diabetes.

Islet insulin release: Rat islets were isolated from Sprague-Dawley rats as described in example 1 and treated with either vehicle or specified peptide (10nM) for 2hrs. Insulin concentration in the medium was measured with the Linco rat insulin RIA kit. Glucose concentration in the medium was 8mM. The data are expressed as % of [insulin] in 8mM glucose alone. No polypeptide-induced increases in insulin concentration were observed at 3 mM glucose; thus, the insulin releasing activity of these polypeptides are glucose-dependent.

[illegible]

Example 13. Pharmaceutical composition – IV formulation

A sterile injectable formulation is made from 4 mg of a polypeptide of SEQ ID NO: 72 and 1 liter of sterile saline, using a manufacturing process well known in the art.

Example 14. Pharmaceutical composition – IV formulation:

A sterile injectable formulation is made from 400 mg of a polypeptide of SEQ ID NO 174 and 1 liter of sterile saline, using a manufacturing process well known in the art.

Example 15: Effect of PACAP27, VIP and PACAP receptor-selective peptide agonists on heart rate in conscious dogs

Protocol:

Beagle dogs were put into a sling where they have been trained to stand for up to 3 hours. The cuff of the heart rate monitor was placed around the tail of the dog.

Saline was injected into the cephalic vein and heart rate monitored every 2 minutes to establish a baseline. After 10 minutes, peptide was injected and heart rate monitored every 2 minutes for the next 20 minutes. If heart rate was normal at that time, a higher dose was given and heart rate monitored for 20 minutes. The area under the curve (AUC) for the first 10 minutes of heart rate change induced by the polypeptide was plotted as % over vehicle AUC against polypeptide concentration. PACAP27 and the R1-selective agonist maxadilan (Moro, O., J. Biol. Chem. 272:966-970 (1997)) possess similar potency in heart rate increase. VIP which activates both R2 and R3 but not R1, R2-selective agonist R2P1, and R3 selective agonists R3P0, R3P3, R3P19, R3P36, R3P51, and R3P53 are at least 10-fold less potent than PACAP27 or maxadilan. Thus, the cardiovascular effect of PACAP27 can be mostly attributed to PACAP-R1 activation. All peptides are full agonists at their

respective receptors with comparable affinities. R3P0 is the Roche analog RO 25-1553 that has been shown to display selectivity for PACAP-R3 of at least 100-fold over R1 and R2 (Gourlet et al, Peptides 18:4030408 (1997)).

5

Example 16. Cyclic AMP SPA

CHO cells expressing the PACAP R3 were plated in 96-well plates (Costar) at 8×10^4 cell/well and grown at 37°C for 24 hours in α MEM + nucleosides + glutamine (Gibco BRL), 10% FBS, 100 μ g/ml Pen/Strep, 0.3 mg/ml glutamine, 1 mM HEPES, 0.5 mg/ml Geneticin (Gibco BRL). The media was removed and the plates were washed with PBS. The cells were incubated with a peptide, in Hopes-PBS-BSA with 0.4mg/ml Soybean Trypsin Inhibitor, 0.5 mg/ml Bacitracin, 100uM IBMX, for 15 min at 37 C. Cyclic AMP in the cell extracts was quantitated using the cAMP SPA direct screening assay system (Amersham Pharmacia Biotech Inc, Piscataway, NJ,). The peptides shown in Table 3 were assayed for cAMP activity.

15

20

25

Table 3 reports *in vitro* cAMP SPA results on CHO cells transfected with PACAP-R2 or PACAP-R3. EC50 is defined as the concentration of the polypeptide at which 50% of maximum PACAP27 activity is achieved. "NA" denotes no detectable activities. "R3 Selectivity" is derived from the ratio of EC50 at R2 versus EC50 at R3. Most of the following polypeptides are designed based on VIP, which has been shown to lack activity at R1 (Vaudry D. et al., 2000, Pharmacological Reviews, 52: 289-324). Therefore, it is believed that these polypeptides do not possess appreciable activity at R1. Peptides designed based on VIP include SEQ ID NO: 6-53, 62-65, and 70-175.

Table 3

Peptide	Seq ID NO	R2 EC50, nM	R3 EC50, nM	R3 Selectivity
VIP	1	0.1	0.08	1.3
R3P0	5	>100	0.4	>250
R3P1	6	>100	2	>50
R3P2	7	>1000	3	>300
R3P3	8	100	0.75	130
R3P5	10	200	7	30
R3P6	11	2	1.4	1.5
R3P9	12	40	2	20
R3P10	13	3	11	0.3
R3P11	14	10	3	3
R3P12	15	>100	0.5	>200
R3P13	16	163	5	33
R3P14	17	50	17	3
R3P19	18	42	1.4	30
R3P20	19	330	1.4	230
R3P21	20	17	0.3	60
R3P22	21	38	1.6	24

5053P1

224
220

R3P24	22	45	1.3	34
R3P25	23	15	0.7	20
R3P26	24	>100	0.55	>180
R3P29	25	>100	0.5	>200
R3P30	26	20	0.4	50
R3P31	27	>100	0.3	>300
R3P32	28	84	0.4	200
R3P33	29	>100	0.5	>200
R3P34	30	>100	1.4	>70
R3P35	31	>100	2.8	>40
R3P36	32	>80	0.4	>200
R3P41	33	8	0.08	100
R3P42	34	0.3	0.03	10
R3P43	35	0.8	0.08	10
R3P44	36	8	1.3	6
R3P45	37	NA	NA	
R3P46	38	0.4	0.06	7
R3P47	39	0.7	0.12	8
R3P48	40	1	0.13	8
R3P49	41	8	0.27	30
R3P50	42	0.7	0.13	6
R3P51	43	23	0.26	90
R3P52	44	>100	0.4	>250
R3P53	45	500	0.2	2500
R3P54	46	>100	0.5	>200
R3P55	47	50	0.17	280
R3P56	48	40	0.4	100
R3P57	49	42	0.23	190
R3P58	50	55	0.32	170
R3P59	51	10	0.15	70
R3P60	52	120	1	120
R3P61	53	110	0.8	140
R3P6	57	220	80	3
R3P7	58	2	4	0.5
R3P15	59	12	5	2
R3P16	60	14	10	1.4
R3P17	61	9	4	2
R3P18	62	NA	NA	
R3P23	63	10	0.43	23
R3P27	64	6.4	0.8	8
R3P28	65	8	7	1
R3P37	66	71	4	18
R3P38	67	17	2.3	7
R3P39	68	1	0.5	2
R3P40	69	1.4	0.7	2
R3P62	70	180	1.8	100
R3P65	71	80	0.6	130
R3P66	72	100	0.4	250
R3P67	73	100	0.3	330
R3P68	74	130	0.5	260
R3P69	75	90	0.5	190
R3P70	76	>100	0.4	>250
R3P71	77	56	0.09	680

75
21

005430-0825656

R3P72	78	>100	0.18	>600
R3P73	79	50	0.3	170
R3P74	80	90	0.6	150
R3P75	81	>150	0.3	>500
R3P76	82	>150	0.1	>1500
R3P77	83	200	0.4	500
R3P78	84	250	1.1	230
R3P79	85	100	0.5	200
R3P80	86	88	0.44	200
R3P81	87	50	0.5	100
R3P82	88	10	0.4	23
R3P83	89	5	0.08	60
R3P84	90	2.5	0.06	40
R3P85	91	5	0.18	30
R3P86	92	90	0.8	110
R3P87	93	0.8	0.2	3
R3P88	94	0.9	0.08	12
R3P89	95	0.9	0.06	15
R3P92	96	2	0.02	100
R3P93	96	6	0.14	40
R3P94	98	9	0.09	100
R3P98	101	NA	NA	
R3P99	102	40	1.0	40
R3P100	103	10	0.3	30
R3P101	104	400	1.0	400
R3P102	105	300	60.0	5
R3P103	106	0.4	0.05	8
R3P104	107	100	0.2	500
R3P105	108	1	0.3	3
R3P106	109	2	0.1	20
R3P107	110	1000	200.0	5
R3P108	111	1000	300.0	3
R3P109	112	1000	10.0	100
R3P110	113	100	0.5	200
R3P111	114	1000	3.0	300
R3P112	115	4	0.1	40
R3P113	116	3	0.3	10
R3P114	117	20	2.0	10
R3P115	118	300	6.0	50
R3P116	119	20	1.0	20
R3P118	121	15	0.5	30
R3P119	122	15	0.5	30
R3P120	123	10	0.2	50
R3P121	124	50	1.0	50
R3P122	125	40	0.2	50
R3P123	126	5	0.1	50
R3P124	127	3	0.2	15
R3P125	128	60	2.0	30
R3P126	129	200	1.0	200
R3P127	130	300	0.5	600
R3P128	131	60	0.3	200
R3P129	132	50	1.0	50
R3P130	133	40	1.0	40

5053P1

276
222

R3P131	134	20	0.3	70
R3P132	135	10	0.2	50
R3P133	136	8	0.1	80
R3P134	137	40	0.4	100
R3P136	139	20	0.3	70
R3P137	140	30	0.3	100
R3P139	142	20	0.4	50
R3P140	143	15	0.3	50
R3P141	144	20	0.2	100
R3P142	145	6	0.2	30
R3P143	146	6	0.2	30
R3P144	147	300	3.0	100
R3P145	148	50	0.5	100
R3P146	149	30	0.3	100
R3P147	150	100	0.2	500
R3P148	151	30	0.3	100
R3P149	152	40	0.5	80
R3P150	153	40	0.8	50
R3P153	156	40	0.2	200
R3P155	157	40	0.4	100
R3P156	158	100	1.0	100
R3P157	159	50	0.3	170
R3P158	160	8	0.07	90
R3P159	161	200	0.5	400
R3P160	162	100	0.2	500
R3P161	163	80	0.2	300
R3P162	164	20	0.1	200
R3P163	165	40	0.2	200
R3P164	166	100	0.3	300
R3P165	167	150	0.5	300
R3P166	168	50	0.1	500
R3P167	169	300	1.0	300
R3P168	170	60	0.2	300
R3P169	171	60	0.2	300
R3P170	172	20	0.1	200
R3P171	173	80	0.4	200
R3P172	174	77	2.6	29
R3P173	175	NA	200	
PAC1	176	970	10	97
PAC2	177	NA	34	
PAC3	178	NA	NA	
PAC4	179	45	7	6
PAC5	180	1.8	0.7	2
PAC6	181	NA	NA	
PAC7	182	NA	NA	
PAC8	183	43	47	1
PAC9	184	0.9	0.7	1
PAC10	185	110	27	4
PAC11	186	10	140	0.1
PAC12	187	150	3	50
PAC13	188	3	0.5	6
PAC14	189	110	1.6	70
PAC15	190	2.4	0.2	12

227
223

PAC16	191	0.2	0.2	1
PAC17	192	0.25	0.15	1.8
PAC18	193	0.7	1.1	0.7
PAC19	194	8	0.4	20
PAC20	195	20	0.7	30
PAC21	196	2.5	0.24	10
PAC22	197	2	15	0.1
PAC23	198	170	13	13
PAC24	199	0.3	0.2	1.5
PAC25	200	0.13	0.04	3
PAC26	201	0.25	0.3	1
PAC27	202	1.5	1.1	1.4

Example 17. Polyclonal antibody production

5 Synthesis of the peptide Ac-CRKQVAANKYLQSIKNKRY COOH was performed on
an Applied Biosystems 430A peptide synthesizer using fmoc chemistry with HBTU activation
of amino acids. The peptide was cleaved using a 84.6% TFA, 4.4% phenol, 4.4% water,
4.4% thioanisole, and 2.2% ethanedithiol cocktail. The crude peptide was purified using a C18
reverse phase column with a 0.1%TFA/CH3CN gradient. Evaluation of purity was performed
on a PerSeptive V Biosystems Voyager DE Pro MALDI mass spectrometer. The cysteine
10 residue was coupled to KLH using the Pierce Inject Maleimide Activated mKLH kit and
protocol (Pierce , Rockford, IL). Rabbits were immunized using the following polyclonal
antiserum immunization schedule:

- Day 0 - 10 ml prebleed for baseline serum
250ug each peptide in 1 ml emulsion of Complete Freund's Adjuvant,
15 0.1 ml/site X 10 sites subcutaneously
Day 14 - Boost 250 µg each peptide in 1 ml emulsion of Incomplete Freund's Adjuvant
0.1 ml/site X 10 sites subcutaneously
Day 21 - 35 ml bleed
Day 35 - Boost 250 µg each peptide in 1 ml emulsion of Incomplete Freund's Adjuvant
20 0.1 ml/site X 10 sites subcutaneously
Day 42 - 35 ml bleed
Day 56 - Boost 250 µg each peptide in 1 ml emulsion of Incomplete Freund's Adjuvant
0.1 ml/site X 10 sites subcutaneously
Day 63 - 35 ml bleed
25 Day 77 - Boost 250 µg each peptide in 1 ml emulsion of Incomplete Freund's Adjuvant
0.1 ml/site X 10 sites subcutaneously
Day 84 - Terminal bleed

Antibodies were characterized using the following protocol:

- 30 Immulon III plate (DYNATECH LABORATORIES, INC, Chantilly, Virginia) was
coated with P66 peptide (0.3-100ng range) in 100ul of EIA coating buffer 1.6L of 0.1M

005053P1-00000000

NaHCO₃ + 0.4L of 0.1M Na₂CO₃ pH9.5) for 3 hours at room temperature (RT). The plate was blotted with 100ul of 5% milk TBS Tween 20 (10mM tris pH8.0, 150mM NaCl, 0.05% Tween-20 (SIGMA P-1379)) for 1 hour at RT and washed 3 times with TBS Tween. R3P66 antibody was added to the well in 100ul of 5% bloto (TBS- Tween 20 + 5% milk) for 2 hours
 5 RT followed by a wash with TBS Tween (repeat wash 5 times). Secondary antibody (BioRad goat anti rabbit Alkaline Phosphatase conjugate) was added to the well at 1:1000 dilution in 100ul of 5% bloto for 1 hour. The plate was washed 5 times with TBS Tween. p-Nitrophenyl Phosphate (SIGMA 104-105) 0.5mg/ml of substrate buffer (1M Diethanolamine, 0.5uM MgCl₂*6H₂O, pH 9.8 w/HCl) in 100ul and incubated at RT for 1 hour to O/N. The plate was
 10 read at OD 405 in SPECTRAMax 250 (Molecular Devices Corporation, Sunnyvale, CA).

To determine if the antibodies produced in rabbits to the peptide Ac-CRKKQVAAIKKYLQSIKIKRY-COOH in accordance with example 17 recognize the peptide R3P66 (SEQ ID NO 72), the enzyme-linked immunosorbent assay (ELISA) was
 15 performed. When antibodies recognize a peptide, a signal at OD405 is detected. Figure 10 shows that these antibodies recognize R3P66 but do not interact with homologous peptides PACAP-27 or VIP up to 30ug of peptide concentration.

20 All publications and patents mentioned in the above specification are incorporated herein by reference. Various modifications and variations of the described compositions and methods of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection
 25 with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the above-described modes for carrying out the invention which are obvious to those skilled in the field of molecular biology or related fields are intended to be within the scope of the following claims. Those skilled in the art will recognize, or be able to ascertain using no
 30 more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

929
225

We claim:

- 5 1. A polypeptide selected from the group consisting of SEQ ID NOs:11 through 14, SEQ ID NO:18, SEQ ID NOs: 21 through 28, SEQ ID NOs: 32 through 38, SEQ ID NOs: 40 through 53, SEQ ID NOs: 57 through 61, SEQ ID NOs: 63 through 99, SEQ ID NOs: 102 through 119, SEQ ID NOs: 121 through 137, SEQ ID NOs: 139 through 177, SEQ ID NOs: 179, 180, SEQ ID NOs: 183 through 202 and fragments, derivatives and variants thereof.

005130-3825050

230
226

Abstract

5 This invention provides novel peptides that function in vivo to stimulate insulin
release from pancreatic beta cells in a glucose-dependent fashion. These insulin
secretagogue peptides are shown to stimulate insulin release in rat islet cells in vitro, and in
10 vivo. The peptides of the present invention provide a new therapy for patients with
decreased endogenous insulin secretion, in particular type 2 diabetics. In particular, the
invention is a polypeptide selected from a specific group of VIP/PACAP-related polypeptides,
or functional equivalents thereof. The invention is also directed to a method of treating a
15 metabolic disease in a mammal comprising administering a therapeutically effective amount
of the insulin secretagogue peptides to said mammal. Also disclosed are methods of making
the peptides, both recombinant and synthetic.

00505280.034500

271
227

00505000-001500

					SEQ. ID NO.
	R3P8	1	HSDAVFTDNYTRLRKQMAVKKYLNLSIKK-NH ₂	28	11
5	R3P9*	1	HSDAVFTDNYTRLRKQMAVKKYLNLSIKKGGT	31	12
	R3P10	1	HSDAVFTENYTKLRKQLAAKKYLNLDLNGGT	31	13
	R3P11	1	HSDAVFTDNYTKLRKQLAAKKYLNLDLNGGT	31	14
	R3P19	1	HSDAVFTENYTKLRKQLAAKKYLNLDLKKGGTSWCEPGWCR	39	18
	R3P22	1	HSDAVFTDNYTRLRKQLAAKKYLNLSIKKGGT	31	21
10	R3P24	1	HSDAVFTDNYTRLRKQLAAKKYLNLDKNGGT	31	22
	R3P25	1	HSDAVFTDNYTRLRKQLAVKKYLNLSIKKGGT	31	23
	R3P28	1	HSDAVFTDNYTRLRKQMAAKKYLNLSIKKGGT	31	24
	R3P29	1	HSDAVFTDNYTRLRKQLAVKKYLNLDKNGGT	31	25
	R3P30	1	HSDAVFTDNYTRLRKQLAAKKYLNLSIKKGGT	31	26
15	R3P38	1	HSDAVFTDNYTRLRKQLAAKKYLNLDIKKKRY	31	32
	rR3P41	1	HSDAVFTDNYTRLRKQMAVKKYLNLSIKK	28	33
	rR3P42	1	HSDAVFTDNYTRLRKQMAVKKYLNLSIKN	28	34
	rR3P43	1	HSDAVFTDNYTRLRKQMAVKKYLNLSILK	28	35
	rR3P44	1	HSDAVFTDNYTELKQMAVKKYLNLSILN	28	36
20	rR3P48	1	HSDAVFTDNYTRLRKQMAVKKYLNLDILN	28	40
	rR3P49	1	HSDAVFTDNYTRLRKQMAAKKYLNLSIKN	28	41
	rR3P50	1	HSDAVFTDNYTRLRKQMAAKKYLNLSILK	28	42
	rR3P51*	1	HSDAVFTDNYTRLRKQMAAKKYLNLSIKK	28	43
	rR3P52	1	HSDAVFTDNYTRLRKQMAAKKYLNLSIKKKRY	31	44
25	rR3P53*	1	HSDAVFTDNYTRLRKQMAAKKYLNLSIKKKR	30	45
	rR3P54	1	HSDAVFTDNYTRLRKQMAAKKYLNLSIKK	29	46
	rR3P55*	1	HSDAVFTDNYTRLRKQMAAKKYLNLSIKNKRY	31	47
	rR3P58	1	HSDAVFTDNYTRLRKQMAVKKYLNLSIKKKRY	31	48
	rR3P57	1	HSDAVFTDNYTRLRKQMAVKKYLNLSIKKKR	30	49
30	rR3P58	1	HSDAVFTDNYTRLRKQMAVKKYLNLSIKK	29	50
	rR3P59	1	HSDAVFTDNYTRLRKQMAVKKYLNLSIKNKRY	31	51
	rR3P60	1	HSDAVFTDNYTRLRKQVAAKKYLSIKK	28	52
	rR3P61	1	HSDAVFTDNYTRLRKQIAAKKYLTIKK	28	53
35	R3P6	1	HSDGIFTESYSRYRKQMAVKKYLAALKKKRYKQRVKKNK	38	57
	R3P7	1	HSDAVFTENYTRLRKQMAVKKYLNLSLKK-NH ₂	28	58
	R3P15	1	HSDGIFTDSYSRYRKQMAVKKYLSAVRHGGT-NH ₂	31	59
	R3P16	1	HSDGIFTDSYSRYRKQMAVKKYLAAVKQGGT-NH ₂	31	60
	R3P17	1	HSDGIFTDSYSRYRKQMAVKKYLAAVKKYLAAVRHG-NH ₂	38	61
	R3P23	1	HSDAVFTDNYTRLRKQLAAKKYLNLDLKGGT	31	63
40	R3P27	1	HSDAVFTDNYTRLRKQLAAKKYLNLDLNGGT	31	64
	R3P28	1	HSDAVFTDNYTRLRKQLAVKKYLNLDLKGGT	31	65
	R3P37	1	HSDGIFTDSYSRYRKQLAAKKYLADVKKGGT	31	66
	R3P38	1	HSDGIFTDSYSRYRKQLAAKKYLADVKK	28	67
	R3P39	1	HSDGIFTDSYSRYRKQLAVKKYLAAVKK	28	68
45	R3P40	1	HSDGIFTDSYSRYRKQMAVKKYLAAVKK	28	69
	R3P62	1	HSDAVFTDNYTRLRKQVAAKKYLNLSIKK	28	70
	R3P65	1	HSDAVFTDNYTRLRKQMAAKKYLNLSIKNKR	30	71
	R3P66	1	HSDAVFTDNYTRLRKQVAAKKYLSIKNKRY	31	72
	R3P67	1	HSDAVFTDNYTRLRKQLAAKKYLNLTIKNKRY	31	73
50	R3P68	1	HSDAVFTDNYTRLRKQVAAKKYLNLSIKNKRY	31	74
	R3P69	1	HSDAVFTDNYTRLRKQMAAKKYLSIKNKRY	31	75
	R3P70	1	HSDAVFTDNYTRLRKQMAAKKYLNLTIKNKRY	31	76
	R3P71	1	HSDAVFTDQYTRLRKQMAAKKYLNLSIKNKRY	31	77
	R3P72	1	HSDAVFTDQYTRLRKQLAAKKYLNLTIKNKRY	31	78
55	R3P73	1	HSDAVFTDNYTRLRKQMAAHKYLNLSIKNKRY	31	79
	R3P74	1	HSDAVFTDNYTRLRKQMAAHKYLNLSIKNKRY	31	80
	R3P75	1	HSDAVFTDQYTRLRKQLAAHKYLNLTIKNKRY	31	81
	R3P76	1	HSDAVFTDQYTRLRKQLAAHKYLNLTIKNKRY	31	82
	R3P77	1	HSDAVFTDNYTRLRKQVAAKKYLSIKKKR	30	83

	R3P78	1	HSDAVFTDNYTRLRKQVAAKKYLSIKKKR	30	84
	R3P79	1	HSDAVFTDNYTRLRKQVAAKKYLSIKNKRY	31	85
	R3P80	1	HSDAVFTDNYTRLRKQVAVKKYLSIKKKR	30	86
	R3P81	1	HSDAVFTDNYTRLRKQVAVKKYLSIKKK	29	87
5	R3P82	1	HSDAVFTDNYTRLRKQVAVKKYLSIKNKRY	31	88
	R3P83	1	HSDAVFTDNYTRLRKQVAAKKYLSILKKRY	31	89
	R3P84	1	HSDAVFTDNYTRLRKQVAAKKYLSILKKR	30	90
	R3P85	1	HSDAVFTDNYTRLRKQVAAKKYLSILKK	29	91
	R3P86	1	HSDAVFTDNYTRLRKQVAAKKYLSIKNK	29	92
10	R3P87	1	HSDAVFTDNYTRLRKQVAVKKYLSILKKRY	31	93
	R3P88	1	HSDAVFTDNYTRLRKQVAVKKYLSILKKR	30	94
	R3P89	1	HSDAVFTDNYTRLRKQVAVKKYLSILKK	29	95
	R3P91	1	HSDAVFTDNYTRLRKQVAVKKYLSIKNK	29	96
	R3P92	1	HSDAVFTDNYTRLRKQVAAKKYLSILNKRY	31	97
15	R3P93	1	HSDAVFTDNYTRLRKQVAAKKYLSILNKR	30	98
	R3P94	1	HSDAVFTDNYTRLRKQVAAKKYLSILNK	29	99
	rR3P99	1	HSDAVFTDNYTRLRKQMAEKKYLSIKNKR	30	102
	rR3P100	1	HSDAVFTDNYTRLRKQMAFKKYLSIKNKR	30	103
	rR3P101	1	HSDAVFTDNYTRLRKQMAKKKYLSIKNKR	30	104
20	rR3P102	1	HSDAVFTDNYTRLRKQMAHKKYLSIKNKR	30	105
	rR3P103	1	HSDAVFTDNYTRLRKQMAIKKYLSIKNKR	30	106
	rR3P104	1	HSDAVFTDNYTRLRKQMAKKKYLSIKNKR	30	107
	rR3P105	1	HSDAVFTDNYTRLRKQMALKKYLSIKNKR	30	108
	rR3P106	1	HSDAVFTDNYTRLRKQMAKKKYLSIKNKR	30	109
25	rR3P107	1	HSDAVFTDNYTRLRKQMANKKYLSIKNKR	30	110
	rR3P108	1	HSDAVFTDNYTRLRKQMAPKKYLSIKNKR	30	111
	rR3P109	1	HSDAVFTDNYTRLRKQMAQKKYLSIKNKR	30	112
	rR3P110	1	HSDAVFTDNYTRLRKQMARKKYLSIKNKR	30	113
	rR3P111	1	HSDAVFTDNYTRLRKQMASKKYLSIKNKR	30	114
30	rR3P112	1	HSDAVFTDNYTRLRKQMATKKYLSIKNKR	30	115
	rR3P113	1	HSDAVFTDNYTRLRKQMAVKKYLSIKNKR	30	116
	rR3P114	1	HSDAVFTDNYTRLRKQMAWKKYLSIKNKR	30	117
	rR3P115	1	HSDAVFTDNYTRLRKQMAYKKYLSIKNKR	30	118
	rR3P116	1	HSDAVFTDNYTRLRKQMAAKKYLSIANKR	30	119
35	rR3P118	1	HSDAVFTDNYTRLRKQMAAKKYLSIDNKR	30	121
	rR3P119	1	HSDAVFTDNYTRLRKQMAAKKYLSIENKR	30	122
	rR3P120	1	HSDAVFTDNYTRLRKQMAAKKYLSIFNKR	30	123
	rR3P121	1	HSDAVFTDNYTRLRKQMAAKKYLSIGNKR	30	124
	rR3P122	1	HSDAVFTDNYTRLRKQMAAKKYLSIHNR	30	125
40	rR3P123	1	HSDAVFTDNYTRLRKQMAAKKYLSIINKR	30	126
	rR3P124	1	HSDAVFTDNYTRLRKQMAAKKYLSIMNKR	30	127
	rR3P125	1	HSDAVFTDNYTRLRKQMAAKKYLSINNKR	30	128
	rR3P126	1	HSDAVFTDNYTRLRKQMAAKKYLSIPNKR	30	129
	rR3P127	1	HSDAVFTDNYTRLRKQMAAKKYLSIQNKR	30	130
45	rR3P128	1	HSDAVFTDNYTRLRKQMAAKKYLSIRNKR	30	131
	rR3P129	1	HSDAVFTDNYTRLRKQMAAKKYLSISNKR	30	132
	rR3P130	1	HSDAVFTDNYTRLRKQMAAKKYLSITNKR	30	133
	rR3P131	1	HSDAVFTDNYTRLRKQMAAKKYLSIVNKR	30	134
	rR3P132	1	HSDAVFTDNYTRLRKQMAAKKYLSIWNKR	30	135
50	rR3P133	1	HSDAVFTDNYTRLRKQMAAKKYLSIYNKR	30	136
	rR3P134	1	HSDAVFTDNYTRLRKQMAAKKYLSIKNAR	30	137
	rR3P136	1	HSDAVFTDNYTRLRKQMAAKKYLSIKNDR	30	138
	rR3P137	1	HSDAVFTDNYTRLRKQMAAKKYLSIKNER	30	140
	rR3P138	1	HSDAVFTDNYTRLRKQMAAKKYLSIKNFR	30	141
55	rR3P139	1	HSDAVFTDNYTRLRKQMAAKKYLSIKNGR	30	142
	rR3P140	1	HSDAVFTDNYTRLRKQMAAKKYLSIKNHR	30	143
	rR3P141	1	HSDAVFTDNYTRLRKQMAAKKYLSIKNIR	30	144
	rR3P142	1	HSDAVFTDNYTRLRKQMAAKKYLSIKNLR	30	145
	rR3P143	1	HSDAVFTDNYTRLRKQMAAKKYLSIKNMR	30	146

233
229

0055200.051500

	rR3P144	1	HSDAVFTDNYTRLRKQMAAKKYLNSIKNNR	30	147
	rR3P145	1	HSDAVFTDNYTRLRKQMAAKKYLNSIKNPR	30	148
	rR3P148	1	HSDAVFTDNYTRLRKQMAAKKYLNSIKNQR	30	149
	rR3P147	1	HSDAVFTDNYTRLRKQMAAKKYLNSIKNRR	30	150
5	rR3P148	1	HSDAVFTDNYTRLRKQMAAKKYLNSIKNSR	30	151
	rR3P149	1	HSDAVFTDNYTRLRKQMAAKKYLNSIKNTR	30	152
	rR3P150	1	HSDAVFTDNYTRLRKQMAAKKYLNSIKNVR	30	153
	rR3P151	1	HSDAVFTDNYTRLRKQMAAKKYLNSIKNWR	30	154
	rR3P152	1	HSDAVFTDNYTRLRKQMAAKKYLNSIKNYR	30	155
10	rR3P153	1	HSDAVFTDNYTRLRKQMAAKKYLNSIKNKA	30	156
	rR3P155	1	HSDAVFTDNYTRLRKQMAAKKYLNSIKNKD	30	157
	rR3P156	1	HSDAVFTDNYTRLRKQMAAKKYLNSIKNKE	30	158
	rR3P157	1	HSDAVFTDNYTRLRKQMAAKKYLNSIKNKF	30	159
	rR3P158	1	HSDAVFTDNYTRLRKQMAAKKYLNSIKNKG	30	160
15	rR3P159	1	HSDAVFTDNYTRLRKQMAAKKYLNSIKNKH	30	161
	rR3P160	1	HSDAVFTDNYTRLRKQMAAKKYLNSIKNKI	30	162
	rR3P161	1	HSDAVFTDNYTRLRKQMAAKKYLNSIKNKK	30	163
	rR3P162	1	HSDAVFTDNYTRLRKQMAAKKYLNSIKNKL	30	164
	rR3P163	1	HSDAVFTDNYTRLRKQMAAKKYLNSIKNKM	30	165
20	rR3P164	1	HSDAVFTDNYTRLRKQMAAKKYLNSIKNKN	30	166
	rR3P165	1	HSDAVFTDNYTRLRKQMAAKKYLNSIKNKP	30	167
	rR3P166	1	HSDAVFTDNYTRLRKQMAAKKYLNSIKNKQ	30	168
	rR3P167	1	HSDAVFTDNYTRLRKQMAAKKYLNSIKNKS	30	169
	rR3P168	1	HSDAVFTDNYTRLRKQMAAKKYLNSIKNKT	30	170
25	rR3P169	1	HSDAVFTDNYTRLRKQMAAKKYLNSIKNKV	30	171
	rR3P170	1	HSDAVFTDNYTRLRKQMAAKKYLNSIKNKW	30	172
	rR3P171	1	HSDAVFTDNYTRLRKQMAAKKYLNSIKNKY	30	173
	R3P172	1	HSDAVFTDNYTRLRKQVAACKYLSIKNKRYSWCEPGWCR	39	174
	R3P173	1	HSDAVFTDNYTRLRKEVAACKYLESIKDKRY	31	175
30	PAC1:	1	ESDGIFTDSYSRYRKQMAVKKYLA AVL-NH ₂	27	176
	PAC2:	1	HKDGIFTDSYSRYRKQMAVKKYLA AVL-NH ₂	27	177
	PAC4:	1	HSDGIFTDSYSRYRKQMAVKKYLA AVL-NH ₂	27	179
	PAC5:	1	HSDGIFTDSYSRYRKQMAVKKYLA AVL-NH ₂	27	180
	PAC8:	1	HSDGIFTDSYSRYRKQMAVKKYLA AVL-NH ₂	27	183
35	PAC9:	1	HSDGIFTDSYSRYRKQMAVKKYLA AVL-NH ₂	27	184
	PAC10:	1	HSDGIFTDSYSRYRKQMAVKKYLA AVL-NH ₂	27	185
	PAC11:	1	HSDGIFTDSYSRYRKQMAVKKYLA AVL-NH ₂	27	186
	PAC12:	1	HSDGIFTDSYSRYRKQMAVKKYLA AVL-NH ₂	27	187
	PAC13:	1	HSDGIFTDSYSRYRKQMAVKKYLA AVL-NH ₂	27	188
40	PAC14:	1	HSDGIFTDSYSRYRKQMAVKKYLA AVL-NH ₂	27	189
	PAC15:	1	HSDGIFTDSYSRYRKQMAVKKYLA AVL-NH ₂	27	190
	PAC16:	1	HSDGIFTDSYSRYRKQMAVKKYLA AVL-NH ₂	27	191
	PAC17:	1	HSDGIFTDSYSRYRKQMAVKKYLA AVL-NH ₂	27	192
	PAC18:	1	HSDGIFTDSYSRYRKQMAVKKYLA AVL-NH ₂	27	193
45	PAC19:	1	HSDGIFTDSYSRYRKQMAVKKYLA AVL-NH ₂	27	194
	PAC20:	1	HSDGIFTDSYSRYRKQMAVKKYLA AVL-NH ₂	27	195
	PAC21:	1	HSDGIFTDSYSRYRKQMAVKKYLA AVL-NH ₂	27	196
	PAC22:	1	HSDGIFTDSYSRYRKQMAVKKYLA AVL-NH ₂	27	197
	PAC23:	1	HSDGIFTDSYSRYRKQMAVKKYLA AVL-NH ₂	27	198
50	PAC24:	1	HSDGIFTDSYSRYRKQMAVKKYLA AVL-NH ₂	27	199
	PAC25:	1	HSDGIFTDSYSRYRKQMAVKKYLA AVL-NH ₂	27	200
	PAC26:	1	HSDGIFTDSYSRYRKQMAVKKYLA AVL-NH ₂	27	201
	PAC27:	1	HSDGIFTDSYSRYRKQMAVKKYLA AVL-NH ₂	27	202

Figure 1

234
230

RSP3
 RSP12
 RSP13
 HP
 ACP38
 GLP-1
 Cauda-4

Fig. 2

5

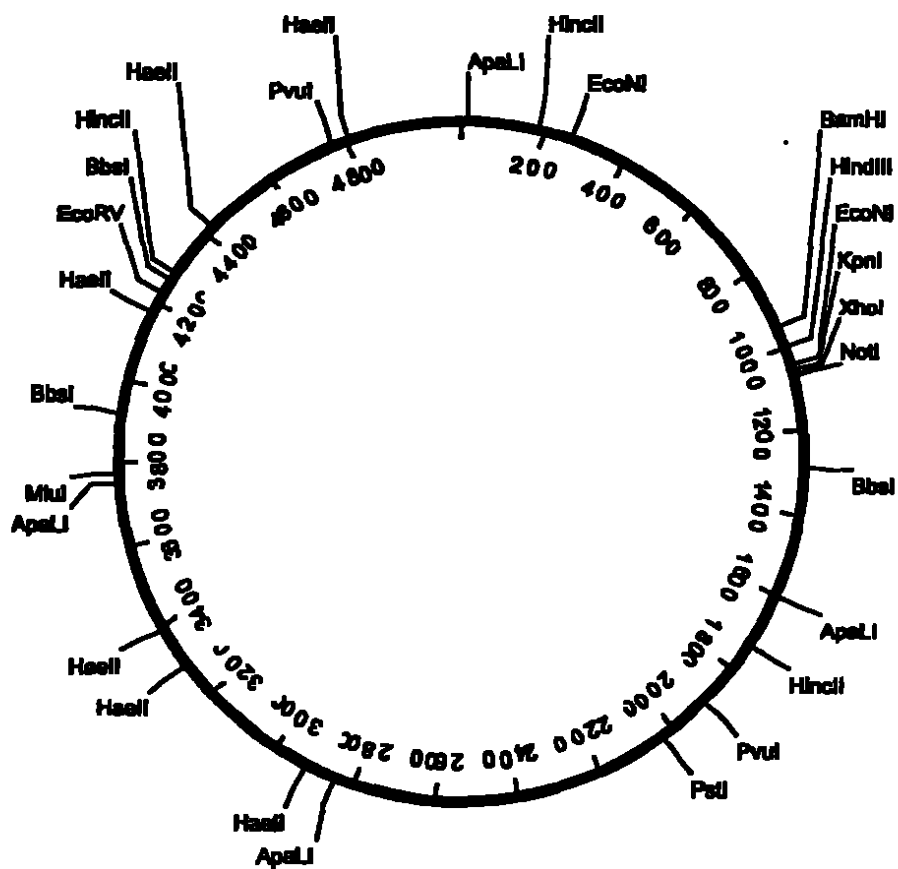


Figure 3

[illegible]

275
231

005190-00250500

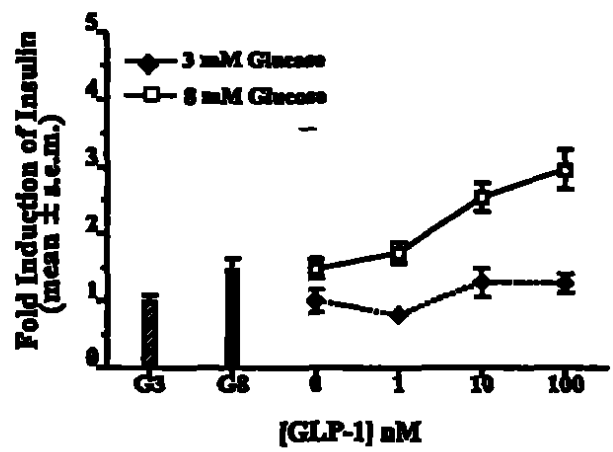


Figure 4A

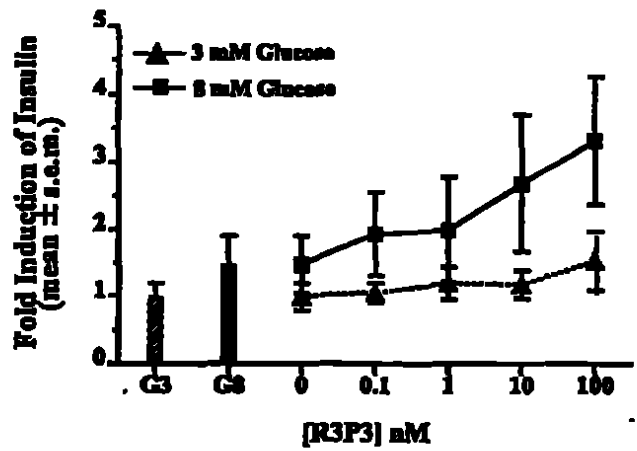


Figure 4B

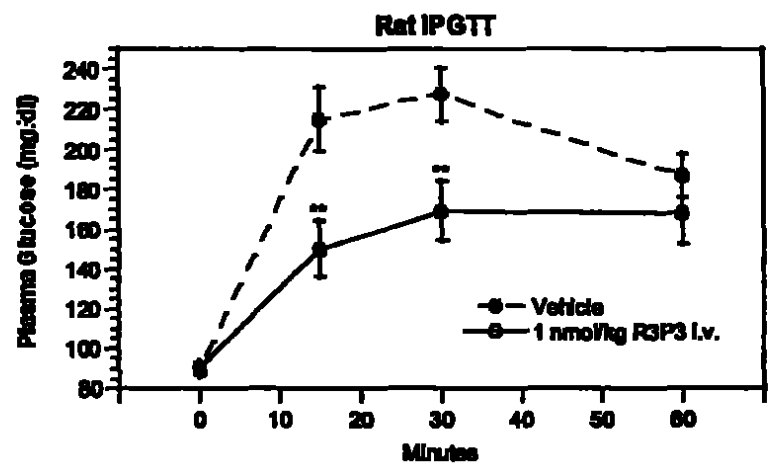


Fig. 5

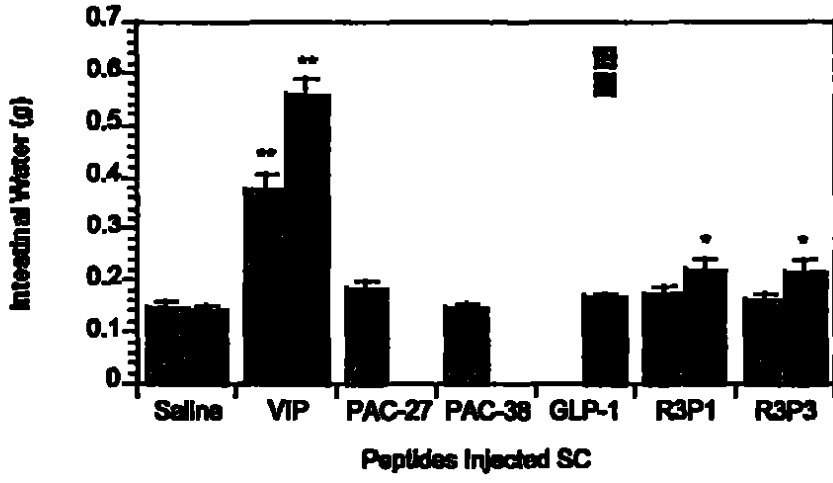


Fig. 6

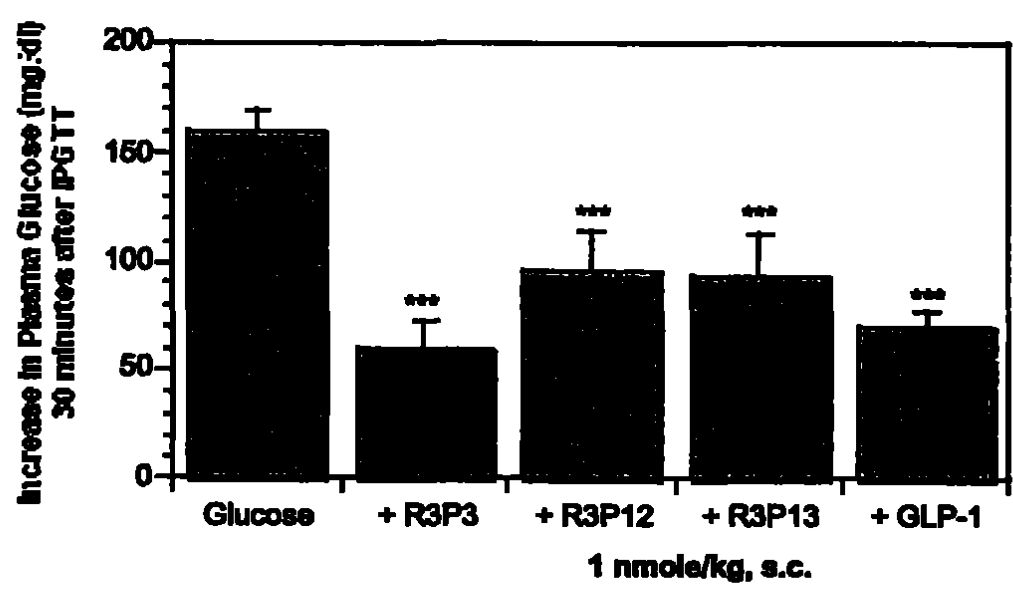


Fig. 7

5 rPACAP 27 (SEQ ID NO: 203)
GGATCCATCGAAGGTCGTCACTCCGATGGTATCTTCACCGACTCCTACTCGAGGTACC
GCAAGCAGATGGCTGTAAAGAAATATCTGGCTGCAGTTCTGTAATGACTCGAG

10 rPACAP 38 (SEQ ID NO: 54)
GGATCCATCGAAGGTCGTCACTCCGATGGTATCTTCACCGACTCCTACTCTCGGTACCG
CAAGCAGATGGCTGTAAAGAAATATCTGGCTGCAGTCCTAGGCAAACGTTACAAGCAAC
GCGTTAAAAACAAGTAATGACTCGAG

15 rVIP (SEQ ID NO: 55)
GGATCCATCGAAGGTCGTCACTCCGACGCTGTTTTACCGACAACCTACACGCGTCTGC
GTAAACAGATGGCTGTAAAGAAATACCTGAATTCCATCCTGAACTAATGACTCGAG

20 rR3P3 (SEQ ID NO: 58)
GGATCCATCGAAGGTCGTCACTCCGATGCTGTTTTACCGAAAACCTACACCAAGCTTCG
TAAACAGCTGGCAGCTAAGAAATACCTCAACGACCTGAAAAAGGGCGGTACCTAATGAC
TCGAG

25 rR3P9 (SEQ ID NO: 204)
GGATCCATCGAAGGTCGTCACTCCGACGCTGTTTTACCGACAACCTACACGCGTCTGC
GTAAACAGATGGCTGTAAAGAAATACCTGAATTCCATCAAGAAAGGGCGGTACCTAATGA
CTCGAG

30 rR3P12 (SEQ ID NO: 205)
GGATCCATCGAAGGTCGTCACTCCGACGCTGTTTTACCGACAACCTACACGCGTCTGC
GTAAACAGCTGGCTGCTAAGAAATACCTGAACGACATCAAGAAAGGTGGCACCTAATGA
CTCGAG

35 rR3P33 (SEQ ID NO: 206)
GGATCCATCGAAGGTCGTCACTCCGACGCTGTTTTACCGACAACCTACACGCGTCTGC
GTAAACAGCTGGCTGCTAAGAAATACCTGAACGACATCAAGAAATAATGAC

40 rR3P41 (SEQ ID NO: 207)
GGATCCATCGAAGGTCGTCACTCCGACGCTGTTTTACCGACAACCTACACGCGTCTGC
GTAAACAGATGGCTGTAAAGAAATACCTGAATTCCATCAAGAAATAATGACTCGAG

45 rR3P42 (SEQ ID NO: 208)
GGATCCATCGAAGGTCGTCACTCCGACGCTGTTTTACCGACAACCTACACGCGTCTGC
GTAAACAGATGGCTGTAAAGAAATACCTGAATTCCATCAAGAACTAATGACTCGAG

50 rR3P43 (SEQ ID NO: 209)
GGATCCATCGAAGGTCGTCACTCCGACGCTGTTTTACCGACAACCTACACGCGTCTGC
GTAAACAGATGGCTGTAAAGAAATACCTGAATTCCATCCTGAAATAATGACTCGAG

55 rR3P44 (SEQ ID NO: 210)
GGATCCATCGAAGGTCGTCACTCCGACGCTGTTTTACCGACAACCTACACGGAACCTGC
GTAAACAGATGGCTGTAAAGAAATACCTGAATTCCATCCTGAACTAATGACTCGAG

rR3P45 (SEQ ID NO: 211)
GGATCCATCGAAGGTCGTCACTCCGACGCTGTTTTACCGACAACCTACACGCGTCTGC
GTGAACAGATGGCTGTAAAGAAATACCTGAATTCCATCCTGAACTAATGACTCGAG

rR3P46 (SEQ ID NO: 212)
GGATCCATCGAAGGTCGTCACTCCGACGCTGTTTTACCGACAACCTACACGCGTCTGC
GTAAACAGCTGGCTGTAAAGAAATACCTGAATTCCATCCTGAACTAATGACTCGAG

5 rR3P47 (SEQ ID NO: 213)
GGATCCATCGAAGGTCGTCACTCCGACGCTGTTTTACCGACAACCTACACGCGTCTGC
GTAAACAGATGGCTGCAAAGAAATACCTGAATTCCATCCTGAACTAATGACTCGAG

10 rR3P48 (SEQ ID NO: 214)
GGATCCATCGAAGGTCGTCACTCCGACGCTGTTTTACCGACAACCTACACGCGTCTGC
GTAAACAGATGGCTGTTAAGAAATACCTGAATGACATCCTGAACTAATGACTCGAG

15 rR3P49 (SEQ ID NO: 215)
GGATCCATCGAAGGTCGTCACTCCGACGCTGTTTTACCGACAACCTACACGCGTCTGC
GTAAACAGATGGCTGCAAAGAAATACCTGAATTCCATCAAGAACTAATGACTCGAG

20 rR3P50 (SEQ ID NO: 216)
GGATCCATCGAAGGTCGTCACTCCGACGCTGTTTTACCGACAACCTACACGCGTCTGC
GTAAACAGATGGCTGCAAAGAAATACCTGAATTCCATCCTGAAATAATGACTCGAG

25 rR3P51 (SEQ ID NO: 217)
GGATCCATCGAAGGTCGTCACTCCGACGCTGTTTTACCGACAACCTACACGCGTCTGC
GTAAACAGATGGCTGCAAAGAAATACCTGAATTCCATCAAGAAATAATGACTCGAG

30 rR3P52 (SEQ ID NO: 218)
GGATCCATCGAAGGTCGTCACTCCGACGCTGTTTTACCGACAACCTACACGCGTCTGC
GTAAACAGATGGCTGCAAAGAAATACCTGAATTCCATCAAGAAAAAGCGTTACTAATGA
CTCGAG

35 rR3P53 (SEQ ID NO: 219)
GGATCCATCGAAGGTCGTCACTCCGACGCTGTTTTACCGACAACCTACACGCGTCTGC
GTAAACAGATGGCTGCAAAGAAATACCTGAATTCCATCAAGAAAAAGCGTTAATGACTC
GAG

40 rR3P54 (SEQ ID NO: 220)
GGATCCATCGAAGGTCGTCACTCCGACGCTGTTTTACCGACAACCTACACGCGTCTGC
GTAAACAGATGGCTGCAAAGAAATACCTGAATTCCATCAAGAAAAAGTAATGACTCGAG

45 rR3P55 (SEQ ID NO: 221)
GGATCCATCGAAGGTCGTCACTCCGACGCTGTTTTACCGACAACCTACACGCGTCTGC
GTAAACAGATGGCTGCAAAGAAATACCTGAATTCCATCAAGAACAAAGCGTTACTAATGA
CTCGAG

50 rR3P56 (SEQ ID NO: 222)
GGATCCATCGAAGGTCGTCACTCCGACGCTGTTTTACCGACAACCTACACGCGTCTGC
GTAAACAGATGGCTGTTAAGAAATACCTGAATTCCATCAAGAAAAAGCGTTACTAATGAC
TCGAG

55 rR3P57 (SEQ ID NO: 223)
GGATCCATCGAAGGTCGTCACTCCGACGCTGTTTTACCGACAACCTACACGCGTCTGC
GTAAACAGATGGCTGTTAAGAAATACCTGAATTCCATCAAGAAAAAGCGTTAATGACTC
GAG

60 rR3P58 (SEQ ID NO: 224)
GGATCCATCGAAGGTCGTCACTCCGACGCTGTTTTACCGACAACCTACACGCGTCTGC
GTAAACAGATGGCTGTTAAGAAATACCTGAATTCCATCAAGAAAAAGTAATGACTCGAG

65 rR3P59 (SEQ ID NO: 225)
GGATCCATCGAAGGTCGTCACTCCGACGCTGTTTTACCGACAACCTACACGCGTCTGC
GTAAACAGATGGCTGTTAAGAAATACCTGAATTCCATCAAGAACAAAGCGTTACTAATGAC
TCGAG

rR3P60 (SEQ ID NO: 226)
GGATCCATCGAAGGTCGTCACTCCGACGCTGTTTTACCGACAACCTACACGCGCTTAAG
AAAACAGGTTGCTGCAAAGAAATACCTGCAGTCCATCAAGAAATAATGACTCGAG

5 rR3P61 (SEQ ID NO: 227)
GGATCCATCGAAGGTCGTCACTCCGACGCTGTTTTACCGACAACCTACACGCGCTTAAG
AAAACAGATCGCTGCAAAGAAATACCTGCAGACTATCAAGAAATAATGACTCGAG

10 rR3P62 (SEQ ID NO: 228)
GGATCCATCGAAGGTCGTCACTCCGACGCTGTTTTACCGACAACCTACACGCGCTTAAG
AAAACAGGTTGCTGCAAAGAAATACCTGAATTCATCAAGAAATAATGACTCGAG

15 rR3P65 (SEQ ID NO: 228)
GGATCCATCGAAGGTCGTCACTCCGACGCTGTTTTACCGACAACCTACACGCGTCTGC
GTAAACAGATGGCTGCAAAGAAATACCTGAACTCCATCAAGAACAAGCGTTAATGAGAA
TTC

20 rR3P71 (SEQ ID NO: 230)
GGATCCATCGAAGGTCGTCACTCCGACGCTGTTTTACCGACAACCTACACGCGTCTGC
GTAAACAGATGGCTGCAAAGAAATACCTGAACTCCATCCTGAACAAGCGTTAATGAGAA
TTC

25 rR3P88 (SEQ ID NO: 231)
GGATCCATCGAAGGTCGTCACTCCGACGCTGTTTTACCGACAACCTACACGCGCTTAAG
AAAACAGATGGCTGACAAGAAATACCTGAACTCCATCAAGAACAAGCGTTAATGAGAAT
TC

30 rR3P89 (SEQ ID NO: 232)
GGATCCATCGAAGGTCGTCACTCCGACGCTGTTTTACCGACAACCTACACGCGCTTAAG
AAAACAGATGGCTGAGAAGAAATACCTGAACTCCATCAAGAACAAGCGTTAATGAGAAT
TC

35 rR3P100 (SEQ ID NO: 233)
GGATCCATCGAAGGTCGTCACTCCGACGCTGTTTTACCGACAACCTACACGCGCTTAAG
AAAACAGATGGCTTTCAAGAAATACCTGAACTCCATCAAGAACAAGCGTTAATGAGAATT
C

40 rR3P101 (SEQ ID NO: 234)
GGATCCATCGAAGGTCGTCACTCCGACGCTGTTTTACCGACAACCTACACGCGCTTAAG
AAAACAGATGGCTGGCAAGAAATACCTGAACTCCATCAAGAACAAGCGTTAATGAGAAT
TC

45 rR3P102 (SEQ ID NO: 235)
GGATCCATCGAAGGTCGTCACTCCGACGCTGTTTTACCGACAACCTACACGCGCTTAAG
AAAACAGATGGCTCACAAGAAATACCTGAACTCCATCAAGAACAAGCGTTAATGAGAAT
TC

50 rR3P103 (SEQ ID NO: 236)
GGATCCATCGAAGGTCGTCACTCCGACGCTGTTTTACCGACAACCTACACGCGCTTAAG
AAAACAGATGGCTATCAAGAAATACCTGAACTCCATCAAGAACAAGCGTTAATGAGAATT
C

55 rR3P104 (SEQ ID NO: 237)
GGATCCATCGAAGGTCGTCACTCCGACGCTGTTTTACCGACAACCTACACGCGCTTAAG
AAAACAGATGGCTAAAAGAAATACCTGAACTCCATCAAGAACAAGCGTTAATGAGAATT
C

5 rR3P105 (SEQ ID NO: 238)
GGATCCATCGAAGGTCGTCACTCCGACGCTGTTTTACCGACAACCTACACGCGCTTAAG
AAAACAGATGGCTCTGAAGAAATACCTGAACTCCATCAAGAACAAGCGTTAATGAGAAT
TC

10 rR3P106 (SEQ ID NO: 239)
GGATCCATCGAAGGTCGTCACTCCGACGCTGTTTTACCGACAACCTACACGCGCTTAAG
AAAACAGATGGCTATGAAGAAATACCTGAACTCCATCAAGAACAAGCGTTAATGAGAAT
TC

15 rR3P107 (SEQ ID NO: 240)
GGATCCATCGAAGGTCGTCACTCCGACGCTGTTTTACCGACAACCTACACGCGCTTAAG
AAAACAGATGGCTAACAAGAAATACCTGAACTCCATCAAGAACAAGCGTTAATGAGAAT
TC

20 rR3P108 (SEQ ID NO: 241)
GGATCCATCGAAGGTCGTCACTCCGACGCTGTTTTACCGACAACCTACACGCGCTTAAG
AAAACAGATGGCTCCGAAGAAATACCTGAACTCCATCAAGAACAAGCGTTAATGAGAAT
TC

25 rR3P109 (SEQ ID NO: 242)
GGATCCATCGAAGGTCGTCACTCCGACGCTGTTTTACCGACAACCTACACGCGCTTAAG
AAAACAGATGGCTCAGAAGAAATACCTGAACTCCATCAAGAACAAGCGTTAATGAGAAT
TC

30 rR3P110 (SEQ ID NO: 243)
GGATCCATCGAAGGTCGTCACTCCGACGCTGTTTTACCGACAACCTACACGCGCTTAAG
AAAACAGATGGCTCGCAAGAAATACCTGAACTCCATCAAGAACAAGCGTTAATGAGAAT
TC

35 rR3P111 (SEQ ID NO: 244)
GGATCCATCGAAGGTCGTCACTCCGACGCTGTTTTACCGACAACCTACACGCGCTTAAG
AAAACAGATGGCTTCCAAGAAATACCTGAACTCCATCAAGAACAAGCGTTAATGAGAAT
TC

40 rR3P112 (SEQ ID NO: 245)
GGATCCATCGAAGGTCGTCACTCCGACGCTGTTTTACCGACAACCTACACGCGCTTAAG
AAAACAGATGGCTACCAAGAAATACCTGAACTCCATCAAGAACAAGCGTTAATGAGAAT
TC

45 rR3P113 (SEQ ID NO: 246)
GGATCCATCGAAGGTCGTCACTCCGACGCTGTTTTACCGACAACCTACACGCGCTTAAG
AAAACAGATGGCTGTGAAGAAATACCTGAACTCCATCAAGAACAAGCGTTAATGAGAAT
TC

50 rR3P114 (SEQ ID NO: 247)
GGATCCATCGAAGGTCGTCACTCCGACGCTGTTTTACCGACAACCTACACGCGCTTAAG
AAAACAGATGGCTTGAAGAAATACCTGAACTCCATCAAGAACAAGCGTTAATGAGAAT
TC

55 rR3P115 (SEQ ID NO: 248)
GGATCCATCGAAGGTCGTCACTCCGACGCTGTTTTACCGACAACCTACACGCGCTTAAG
AAAACAGATGGCTTACAAGAAATACCTGAACTCCATCAAGAACAAGCGTTAATGAGAAT
C

5 rR3P118 (SEQ ID NO: 249)
GGATCCATCGAAGGTCGTCACTCCGACGCTGTTTTACCGACAACCTACACGCGTCTGC
GTAAACAGATGGCTGCAAAGAAATACCTGAACTCCATCGCGAACAAGCGTTAATGAGAA
TTC

10 rR3P118 (SEQ ID NO: 250)
GGATCCATCGAAGGTCGTCACTCCGACGCTGTTTTACCGACAACCTACACGCGTCTGC
GTAAACAGATGGCTGCAAAGAAATACCTGAACTCCATCGACAACAAGCGTTAATGAGAA
TTC

15 rR3P119 (SEQ ID NO: 251)
GGATCCATCGAAGGTCGTCACTCCGACGCTGTTTTACCGACAACCTACACGCGTCTGC
GTAAACAGATGGCTGCAAAGAAATACCTGAACTCCATCGAGAACAAGCGTTAATGAGAA
TTC

20 rR3P120 (SEQ ID NO: 252)
GGATCCATCGAAGGTCGTCACTCCGACGCTGTTTTACCGACAACCTACACGCGTCTGC
GTAAACAGATGGCTGCAAAGAAATACCTGAACTCCATCTTCAACAAGCGTTAATGAGAA
TTC

25 rR3P121 (SEQ ID NO: 253)
GGATCCATCGAAGGTCGTCACTCCGACGCTGTTTTACCGACAACCTACACGCGTCTGC
GTAAACAGATGGCTGCAAAGAAATACCTGAACTCCATCGGCAACAAGCGTTAAAGAGAA
TTC

30 rR3P122 (SEQ ID NO: 254)
GGATCCATCGAAGGTCGTCACTCCGACGCTGTTTTACCGACAACCTACACGCGTCTGC
GTAAACAGATGGCTGCAAAGAAATACCTGAACTCCATCCACAACAAGCGTTAATGAGAA
TTC

35 rR3P123 (SEQ ID NO: 255)
GGATCCATCGAAGGTCGTCACTCCGACGCTGTTTTACCGACAACCTACACGCGTCTGC
GTAAACAGATGGCTGCAAAGAAATACCTGAACTCCATCATGAACAAGCGTTAATGAGAA
TTC

40 rR3P124 (SEQ ID NO: 256)
GGATCCATCGAAGGTCGTCACTCCGACGCTGTTTTACCGACAACCTACACGCGTCTGC
GTAAACAGATGGCTGCAAAGAAATACCTGAACTCCATCATGAACAAGCGTTAATGAGAA
TTC

45 rR3P125 (SEQ ID NO: 257)
GGATCCATCGAAGGTCGTCACTCCGACGCTGTTTTACCGACAACCTACACGCGTCTGC
GTAAACAGATGGCTGCAAAGAAATACCTGAACTCCATCAACAACAAGCGTTAATGAGAA
TTC

50 rR3P126 (SEQ ID NO: 258)
GGATCCATCGAAGGTCGTCACTCCGACGCTGTTTTACCGACAACCTACACGCGTCTGC
GTAAACAGATGGCTGCAAAGAAATACCTGAACTCCATCCGAACAAGCGTTAATGAGAA
TTC

55 rR3P127 (SEQ ID NO: 259)
GGATCCATCGAAGGTCGTCACTCCGACGCTGTTTTACCGACAACCTACACGCGTCTGC
GTAAACAGATGGCTGCAAAGAAATACCTGAACTCCATCCAGAACAAGCGTTAATGAGAA
TTC

rR3P128 (SEQ ID NO: 260)

GGATCCATCGAAGGTCGTCACCTCCGACGCTGTTTTACCGACAACCTACACGCGTCTGC
GTAAACAGATGGCTGCAAAGAAATACCTGAACTCCATCAGGAACAAGCGTTAATGAGAA
TTC

5

rR3P129 (SEQ ID NO: 261)

GGATCCATCGAAGGTCGTCACCTCCGACGCTGTTTTACCGACAACCTACACGCGTCTGC
GTAAACAGATGGCTGCAAAGAAATACCTGAACTCCATCAGGAACAAGCGTTAATGAGAA
TTC

10

rR3P130 (SEQ ID NO: 262)

GGATCCATCGAAGGTCGTCACCTCCGACGCTGTTTTACCGACAACCTACACGCGTCTGC
GTAAACAGATGGCTGCAAAGAAATACCTGAACTCCATCAGGAACAAGCGTTAATGAGAA
TTC

15

rR3P131 (SEQ ID NO: 263)

GGATCCATCGAAGGTCGTCACCTCCGACGCTGTTTTACCGACAACCTACACGCGTCTGC
GTAAACAGATGGCTGCAAAGAAATACCTGAACTCCATCAGGAACAAGCGTTAATGAGAA
TTC

20

rR3P132 (SEQ ID NO: 264)

GGATCCATCGAAGGTCGTCACCTCCGACGCTGTTTTACCGACAACCTACACGCGTCTGC
GTAAACAGATGGCTGCAAAGAAATACCTGAACTCCATCAGGAACAAGCGTTAATGAGAA
TTC

25

rR3P133 (SEQ ID NO: 265)

GGATCCATCGAAGGTCGTCACCTCCGACGCTGTTTTACCGACAACCTACACGCGTCTGC
GTAAACAGATGGCTGCAAAGAAATACCTGAACTCCATCAGGAACAAGCGTTAATGAGAA
TTC

30

rR3P134 (SEQ ID NO: 266)

GGATCCATCGAAGGTCGTCACCTCCGACGCTGTTTTACCGACAACCTACACGCGTCTGC
GTAAACAGATGGCTGCAAAGAAATACCTGAACTCCATCAGGAACAAGCGTTAATGAGAA
TTC

35

rR3P136 (SEQ ID NO: 267)

GGATCCATCGAAGGTCGTCACCTCCGACGCTGTTTTACCGACAACCTACACGCGTCTGC
GTAAACAGATGGCTGCAAAGAAATACCTGAACTCCATCAGGAACAAGCGTTAATGAGAA
TTC

40

rR3P137 (SEQ ID NO: 268)

GGATCCATCGAAGGTCGTCACCTCCGACGCTGTTTTACCGACAACCTACACGCGTCTGC
GTAAACAGATGGCTGCAAAGAAATACCTGAACTCCATCAGGAACAAGCGTTAATGAGAA
TTC

45

rR3P138 (SEQ ID NO: 269)

GGATCCATCGAAGGTCGTCACCTCCGACGCTGTTTTACCGACAACCTACACGCGTCTGC
GTAAACAGATGGCTGCAAAGAAATACCTGAACTCCATCAGGAACAAGCGTTAATGAGAA
TTC

50

rR3P139 (SEQ ID NO: 270)

GGATCCATCGAAGGTCGTCACCTCCGACGCTGTTTTACCGACAACCTACACGCGTCTGC
GTAAACAGATGGCTGCAAAGAAATACCTGAACTCCATCAGGAACAAGCGTTAATGAGAA
TTC

55

CG505280 - 01-1500

5 rR3P140 (SEQ ID NO: 271)
GGATCCATCGAAGGTCGTCACTCCGACGCTGTTTTACCGACAACCTACACGCGTCTGC
GTAAACAGATGGCTGCAAAGAAATACCTGAACTCCATCAAGAACCACCGTTAATGAGAA
TTC

10 rR3P141 (SEQ ID NO: 272)
GGATCCATCGAAGGTCGTCACTCCGACGCTGTTTTACCGACAACCTACACGCGTCTGC
GTAAACAGATGGCTGCAAAGAAATACCTGAACTCCATCAAGAACATCCGTTAATGAGAA
TTC

15 rR3P142 (SEQ ID NO: 273)
GGATCCATCGAAGGTCGTCACTCCGACGCTGTTTTACCGACAACCTACACGCGTCTGC
GTAAACAGATGGCTGCAAAGAAATACCTGAACTCCATCAAGAACGTCGTTAATGAGAA
TTC

20 rR3P143 (SEQ ID NO: 274)
GGATCCATCGAAGGTCGTCACTCCGACGCTGTTTTACCGACAACCTACACGCGTCTGC
GTAAACAGATGGCTGCAAAGAAATACCTGAACTCCATCAAGAACATGCGTTAATGAGAA
TTC

25 rR3P144 (SEQ ID NO: 275)
GGATCCATCGAAGGTCGTCACTCCGACGCTGTTTTACCGACAACCTACACGCGTCTGC
GTAAACAGATGGCTGCAAAGAAATACCTGAACTCCATCAAGAACAACCGTTAATGAGAA
TTC

30 rR3P145 (SEQ ID NO: 276)
GGATCCATCGAAGGTCGTCACTCCGACGCTGTTTTACCGACAACCTACACGCGTCTGC
GTAAACAGATGGCTGCAAAGAAATACCTGAACTCCATCAAGAACCCGCGTTAATGAGAA
TTC

35 rR3P146 (SEQ ID NO: 277)
GGATCCATCGAAGGTCGTCACTCCGACGCTGTTTTACCGACAACCTACACGCGTCTGC
GTAAACAGATGGCTGCAAAGAAATACCTGAACTCCATCAAGAACCAGCGTTAATGAGAA
TTC

40 rR3P147 (SEQ ID NO: 278)
GGATCCATCGAAGGTCGTCACTCCGACGCTGTTTTACCGACAACCTACACGCGTCTGC
GTAAACAGATGGCTGCAAAGAAATACCTGAACTCCATCAAGAACC GCCGTTAATGAGAA
TTC

45 rR3P148 (SEQ ID NO: 279)
GGATCCATCGAAGGTCGTCACTCCGACGCTGTTTTACCGACAACCTACACGCGTCTGC
GTAAACAGATGGCTGCAAAGAAATACCTGAACTCCATCAAGAACAGCCGTTAATGAGAA
TTC

50 rR3P149 (SEQ ID NO: 280)
GGATCCATCGAAGGTCGTCACTCCGACGCTGTTTTACCGACAACCTACACGCGTCTGC
GTAAACAGATGGCTGCAAAGAAATACCTGAACTCCATCAAGAACACCCGTTAATGAGAA
TTC

55 rR3P150 (SEQ ID NO: 281)
GATCCATCGAAGGTCGTCACTCCGACGCTGTTTTACCGACAACCTACACGCGTCTGCGT
AAACAGATGGCTGCAAAGAAATACCTGAACTCCATCAAGAACGTCGTTAATGAGAA
TTC

245
241

rR3P151 (SEQ ID NO: 282)
GGATCCATCGAAGGTCGTCACTCCGACGCTGTTTTACCGACAACCTACACGCGTCTGC
GTAAACAGATGGCTGCAAAGAAATACCTGAACTCCATCAAGAAGTGGCGTTAATGAGAA
TTC

5

rR3P152 (SEQ ID NO: 283)
GGATCCATCGAAGGTCGTCACTCCGACGCTGTTTTACCGACAACCTACACGCGTCTGC
GTAAACAGATGGCTGCAAAGAAATACCTGAACTCCATCAAGAAGTACCGTTAATGAGAA
TTC

10

rR3P153 (SEQ ID NO: 284)
GGATCCATCGAAGGTCGTCACTCCGACGCTGTTTTACCGACAACCTACACGCGTCTGC
GTAAACAGATGGCTGCAAAGAAATACCTGAACTCCATCAAGAACAAGGCGTAATGAGAA
TTC

15

rR3P155 (SEQ ID NO: 285)
GGATCCATCGAAGGTCGTCACTCCGACGCTGTTTTACCGACAACCTACACGCGTCTGC
GTAAACAGATGGCTGCAAAGAAATACCTGAACTCCATCAAGAACAAGGACTAATGAGAA
TTC

20

rR3P156 (SEQ ID NO: 286)
GGATCCATCGAAGGTCGTCACTCCGACGCTGTTTTACCGACAACCTACACGCGTCTGC
GTAAACAGATGGCTGCAAAGAAATACCTGAACTCCATCAAGAACAAGGAGTAAAGAGAA
TTC

25

rR3P157 (SEQ ID NO: 287)
GGATCCATCGAAGGTCGTCACTCCGACGCTGTTTTACCGACAACCTACACGCGTCTGC
GTAAACAGATGGCTGCAAAGAAATACCTGAACTCCATCAAGAACAAGTTCTAATGAGAA
TTC

30

rR3P158 (SEQ ID NO: 288)
GGATCCATCGAAGGTCGTCACTCCGACGCTGTTTTACCGACAACCTACACGCGTCTGC
GTAAACAGATGGCTGCAAAGAAATACCTGAACTCCATCAAGAACAAGGCGTAATGAGAA
TTC

35

rR3P159 (SEQ ID NO: 289)
GGATCCATCGAAGGTCGTCACTCCGACGCTGTTTTACCGACAACCTACACGCGTCTGC
GTAAACAGATGGCTGCAAAGAAATACCTGAACTCCATCAAGAACAAGCACTAATGAGAA
TTC

40

rR3P160 (SEQ ID NO: 290)
GGATCCATCGAAGGTCGTCACTCCGACGCTGTTTTACCGACAACCTACACGCGTCTGC
GTAAACAGATGGCTGCAAAGAAATACCTGAACTCCATCAAGAACAAGATCTAATGAGAA
TTC

45

rR3P161 (SEQ ID NO: 291)
GGATCCATCGAAGGTCGTCACTCCGACGCTGTTTTACCGACAACCTACACGCGTCTGC
GTAAACAGATGGCTGCAAAGAAATACCTGAACTCCATCAAGAACAAGTAATGAGAA
TTC

50

rR3P162 (SEQ ID NO: 292)
GGATCCATCGAAGGTCGTCACTCCGACGCTGTTTTACCGACAACCTACACGCGTCTGC
GTAAACAGATGGCTGCAAAGAAATACCTGAACTCCATCAAGAACAAGCTGTAATGAGAA
TTC

55

CGCGTCTGC

246
242

5
rR3P163 (SEQ ID NO: 293)
GGATCCATCGAAGGTCGTCACCTCCGACGCTGTTTTACCGACAACCTACACGCGTCTGC
GTAAACAGATGGCTGCAAAGAAATACCTGAACTCCATCAAGAACAAGATGTAATGAGAA
TTC

10
rR3P164 (SEQ ID NO: 294)
GGATCCATCGAAGGTCGTCACCTCCGACGCTGTTTTACCGACAACCTACACGCGTCTGC
GTAAACAGATGGCTGCAAAGAAATACCTGAACTCCATCAAGAACAAGAACTAATGAGAA
TTC

15
rR3P165 (SEQ ID NO: 295)
GGATCCATCGAAGGTCGTCACCTCCGACGCTGTTTTACCGACAACCTACACGCGTCTGC
GTAAACAGATGGCTGCAAAGAAATACCTGAACTCCATCAAGAACAAGCCGTAATGAGAA
TTC

20
rR3P166 (SEQ ID NO: 296)
GGATCCATCGAAGGTCGTCACCTCCGACGCTGTTTTACCGACAACCTACACGCGTCTGC
GTAAACAGATGGCTGCAAAGAAATACCTGAACTCCATCAAGAACAAGCAGTAATGAGAA
TTC

25
rR3P167 (SEQ ID NO: 297)
GGATCCATCGAAGGTCGTCACCTCCGACGCTGTTTTACCGACAACCTACACGCGTCTGC
GTAAACAGATGGCTGCAAAGAAATACCTGAACTCCATCAAGAACAAGAGCTAATGAGAA
TTC

30
rR3P168 (SEQ ID NO: 298)
GGATCCATCGAAGGTCGTCACCTCCGACGCTGTTTTACCGACAACCTACACGCGTCTGC
GTAAACAGATGGCTGCAAAGAAATACCTGAACTCCATCAAGAACAAGACCTAATGAGAA
TTC

35
rR3P169 (SEQ ID NO: 299)
GGATCCATCGAAGGTCGTCACCTCCGACGCTGTTTTACCGACAACCTACACGCGTCTGC
GTAAACAGATGGCTGCAAAGAAATACCTGAACTCCATCAAGAACAAGGTGTAATGAGAA
TTC

40
rR3P170 (SEQ ID NO: 300)
GGATCCATCGAAGGTCGTCACCTCCGACGCTGTTTTACCGACAACCTACACGCGTCTGC
GTAAACAGATGGCTGCAAAGAAATACCTGAACTCCATCAAGAACAAGTGGTAATGAGAA
TTC

45
rR3P171 (SEQ ID NO: 301)
GGATCCATCGAAGGTCGTCACCTCCGACGCTGTTTTACCGACAACCTACACGCGTCTGC
GTAAACAGATGGCTGCAAAGAAATACCTGAACTCCATCAAGAACAAGTACTAATGAGAA
TTC

Figure 8

005195-0826500

247
243

5

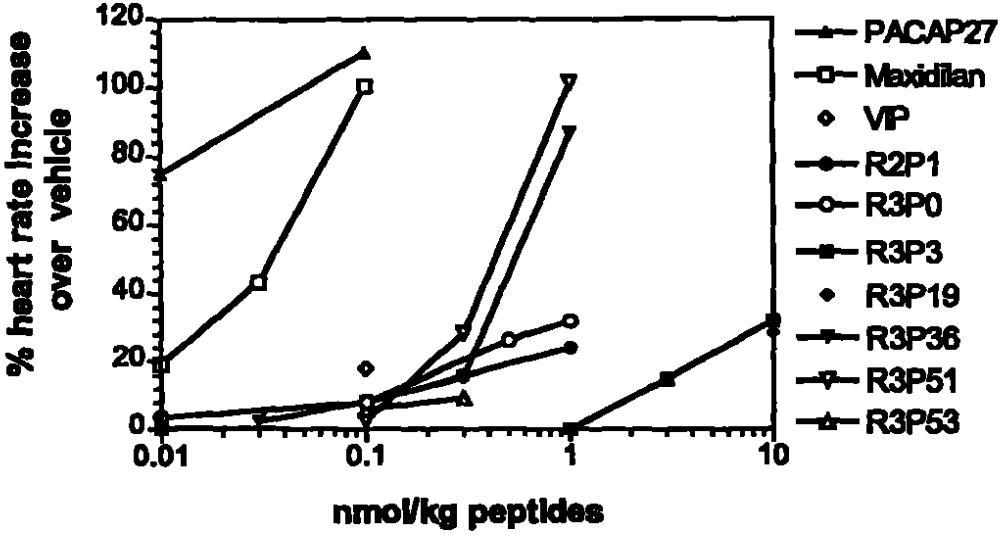


Figure 9

10

005730-08250500

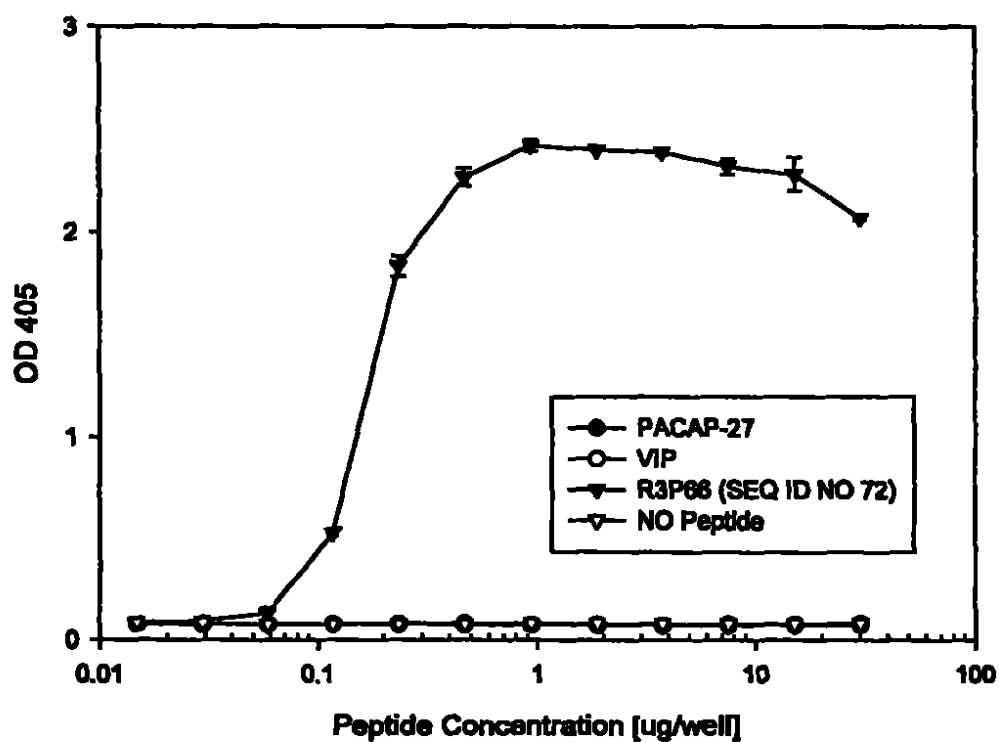


Fig. 10

249
245

DECLARATION FOR UTILITY OR DESIGN
PATENT APPLICATION

Attorney Docket Number: 5053P1
First Named Inventor: Clark Pan

☒ Declaration submitted with original filing

or

☐ Declaration submitted after initial filing

Complete if known:

Application Number:

Filing Date: 15 June 2000

Group Art Unit:

Examiner Name:

As an inventor named below, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or, an original, first and joint inventor (if more than one name is listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

PITUITARY ADENYLATE CYCLASE ACTIVATING PEPTIDE (PACAP) RECEPTOR 3 (R3) AGONISTS
AND THEIR PHARMACOLOGICAL METHODS OF USE

the specification of which:

- ☒ is attached hereto
☐ was Express Mailed on _____, Serial No. not yet known
☐ was filed on _____ as United States Application Serial No. _____
☐ was amended on _____
☐ was described and claimed in PCT International Application No. _____
filed on _____, and as amended under PCT Article 19 on _____

I hereby state that I have reviewed and understand the contents of the specification identified above, including the claims, as amended in any amendment referred to above.

I acknowledge my duty to disclose to the United States Patent Office all information which is known to me to be material to patentability as defined in 37 CFR §1.56.

Priority Claim under 35 USC §119(e):

I hereby claim the benefit under 35 USC §119(e) of any United States provisional application(s) listed below.

☒ None

Application Number

Day/Month/Year Filed

1. A 37 CFR §1.53(c)(2) petition to convert nonprovisional application No. _____ to the provisional application from which priority is claimed was filed on _____ as Express Mail Label No. _____.

2.

8/10/98

Docket No. 5053P1

c:\patents\forms\declaration.wpd

750
246

Priority claim under 35 USC §120 or §365(c):

I hereby claim the benefit under 35 USC §120 of any previously filed United States non-provisional application(s), including original, divisional, continuation and continuation-in-part application(s), or claim the benefit under 35 USC §365(c) of any PCT International application(s) designating the United States that are listed below.

☐ None

	<u>Parent Application Number</u>	<u>Parent Filing Date (Day/Month/Year)</u>	<u>Status</u>
1.	US 09/407,832	28 September 1999	Pending
2.			

Insofar as the subject matter of each of the claims of this application is not disclosed in the above prior United States or PCT International applications in the manner provided by the first paragraph of 35 USC §112, I acknowledge my duty to disclose to the United States Patent Office all information which is known to me to be material to patentability as defined in 37 CFR §1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application.

Priority claim under 35 USC §119(a)(b) and (d) or §365(a) and (b):

I hereby claim foreign priority benefits under 35 USC §119(a)(b) and (d) and §365(a) and (b) of any foreign application(s) for patent or inventor's certificate, or any PCT International application(s) designating at least one country other than the United States, listed below.

☒ None

	<u>Application Number</u>	<u>Country</u>	<u>Filing Date (Day/Month/Year)</u>
1.			
2.			

Foreign Application(s) filed before the priority document(s) under 35 USC §119(c) and 37 CFR §1.63(c):

I have also identified below any foreign application for patent or inventor's certificate, or PCT International application designating at least one country other than the United States filed by me on the same subject matter and having a filing date before that of the application(s) from which foreign priority is claimed, whether abandoned or not.

☒ None

	<u>Application Number</u>	<u>Country</u>	<u>Day/Month/Year Filed</u>
1.			

As a named inventor, I hereby appoint the following attorney(s) to prosecute this application and transact all business in the United States Patent and Trademark Office connected therewith.

Jeffrey M. Greenman
Barbara A. Shimei
William F. Gray
Alice A. Brewer
Jenife L. Chiu

Reg. No. 20552
Reg. No. 29082
Reg. No. 31018
Reg. No. 32888
Reg. No. 41670

Direct all telephone calls to Alice A. Brewer at (203) 812-2705.

Address all written correspondence to:

Mr. Jeffrey M. Greenman
Vice President, Patents & Licensing
Bayer Corporation
400 Morgan Lane
West Haven, Connecticut 06516

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 USC §1001, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Full name of sole or first inventor: Clark Pan
Given/first name Middle name/initial Family/last name
Inventor's signature: Clark Pan Date: 5/31/00
Residence: City Castro Valley State California Country U.S.A.
Post Office Address: 22362 Princeton Place, Castro Valley, California 94552
Country of Citizenship: United States of America

Full name of second joint inventor: Manami Teutsumi
Given/first name Middle name/initial Family/last name
Inventor's signature: Manami Teutsumi Date: 5/31/2000
Residence: City Stratford State Connecticut Country U.S.A.
Post Office Address: 70 Maureen Street, Stratford, Connecticut 06815
Country of Citizenship: Japan

Full name of third joint inventor: Arman B. Shanafelt
Given/first name Middle name/initial Family/last name
Inventor's signature: Arman B. Shanafelt Date: 31 MAY 00
Residence: City Moraga State California Country U.S.A.
Post Office Address: 1309 Rimer Drive, Moraga, California, 94556
Country of Citizenship: United States of America



LOS ESTADOS UNIDOS DE AMERICA

**A TODOS AQUELLOS A QUIENES LA PRESENTE CONCIERNE:
DEPARTAMENTO DE COMERCIO DE LOS ESTADOS UNIDOS**

Oficina de Patentes y Marcas de los Estados Unidos

1 de diciembre de 2000

**LA PRESENTE ES PARA CERTIFICAR QUE LA ANEXA A ESTA ES UNA COPIA FIEL
DE LOS REGISTROS DE LA OFICINA DE PATENTES Y MARCAS DE LOS ESTADOS
UNIDOS DE LOS DOCUMENTOS DE LA SOLICITUD DE PATENTE IDENTIFICADA MAS
ADELANTE QUE SATISFACEN LOS REQUISITOS PARA QUE SE OTORGUE UNA FECHA
DE PRIORIDAD BAJO 35 USC 111..**

SOLICITUD NUMERO: 09/595,280

FECHA DE PRESENTACION: 15 de junio de 2000



**Por la Autoridad del
COMISIONADO DE PATENTES Y MARCAS**

**Firmado por
T. WALLACE**

Funcionario Certificador

754
249

REIVINDICACION

1. Un polipéptido seleccionado del grupo que consiste de SEQ ID NOS: 11 a 14, SEQ ID NO:18, SEQ ID NOS: 21 a 26, SEQ ID NOS: 32 a 36, SEQ ID NOS: 40 a 53, SEQ ID NOS: 57 a 61, SEQ ID NOS:63 a 99, SEQ ID NOS:102 a 119, SEQ ID NOS: 121 a 137,SEQ ID NOS: 139 a 177, SEQ ID NOS:179,180, SEQ ID NOS: 183 a 202 y fragmentos, derivados y variantes de las mismas.

COLO 09597-801-017-1

JJC-ID-174