Preptin derived from proinsulin-like growth factor II (proIGF-II) is secreted from pancreatic islet β-cells and enhances insulin secretion

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Pancreatic islet β-cells secrete the hormones insulin, amylin and pancreastatin. To search for further β-cell hormones, we purified peptides from secretory granules isolated from cultured murine βTC6-F7 β-cells. We identified a 34-amino-acid peptide (3948 Da), corresponding to Asp^{69}–Leu^{102} of the proinsulin-like growth factor II E-peptide, which we have termed 'preptin'. Preptin, is present in islet β-cells and undergoes glucose-mediated co-secretion with insulin. Synthetic preptin increases insulin secretion from glucose-stimulated βTC6-F7 cells in a concentration-dependent and saturable manner. Preptin infusion into the isolated, perfused rat pancreas increases the second phase of glucose-mediated insulin secretion by 30%, while anti-preptin immunoglobulin infusion decreases the first and second phases of insulin secretion by 29 and 26% respectively. These findings suggest that preptin is a physiological amplifier of glucose-mediated insulin secretion.

Key words: β-cell granules, perfused pancreas, proIGF-II E-peptide, protein mass spectrometry, secretory granules.

INTRODUCTION

Pancreatic islet β-cells play a major regulatory role in physiology, mainly through their secretion of insulin, a peptide hormone that exerts profound effects on intermediary metabolism [1]. In islet β-cells, pro-insulin is packaged in immature secretory granules, which undergo a maturation process involving proteolytic processing of pro-insulin to yield insulin. Mature granules contain electron-dense cores that are rich in insulin and zinc, while smaller amounts of insulin C-peptide, pro-insulin, proteases and other proteins are found in the surrounding granule matrix [2]. These granules are exocytosed in a regulated manner in response to fuels (e.g. glucose and amino acids) or neurohormonal stimuli.

Recent studies [3–5] have revealed that insulin is not the only metabolically important hormone stored in and secreted from the β-cell granule. A second β-cell hormone, amylin, may also contribute to β-cell regulatory function through its actions on insulin secretion and tissue insulin sensitivity [6,7], while pancreastatin, a β-cell specific proteolytic cleavage product of chromogranin-A, has also been found to modulate insulin secretion [8]. The presence of more than one hormone in the β-cell suggests that islet β-cell granules may contain further unknown regulatory peptides, which could contribute to the role of the β-cell in the regulation of metabolism.

Several studies have previously been undertaken to isolate and purify β-cell secretory granules in order to investigate the contents and machinery of this organelle [9–11]. To date, most studies have employed two-dimensional electrophoresis to separate granule proteins [9,12]. The study of Guest et al. [12] indicated that there are more than 100 individual granule-associated proteins detectable by two-dimensional electrophoresis. Furthermore, a high percentage of these proteins migrated with an apparent molecular mass of less than 14 kDa.

Due to the limited resolving power of standard two-dimensional electrophoresis in the lower molecular mass range, it is not surprising that only approx. 20 granule-associated proteins and peptides have been identified [2]. Consequently we developed a single-step method of purifying granules for subsequent analysis by reversed-phase HPLC (RP-HPLC), an alternative separation technique which is better able to separate smaller proteins and peptides.

In the present study we report the isolation of a novel peptide purified from βTC6-F7 islet β-cell granules that undergoes regulated co-secretion with insulin. We further demonstrate the presence of this peptide in physiological islet tissue, and its stimulatory effect on glucose-mediated insulin secretion.

MATERIALS AND METHODS

Transmission electron microscopy

βTC6-F7 cells were grown to 70% confluence in 24-well plates, washed with PBS and fixed with 3% (v/v) glutaraldehyde in Sörensen's phosphate buffer (pH 7.2) [12a]. Following fixation, cells were washed with 0.1 M Sörensen's buffer, post-fixed with 0.1% osmium tetroxide/0.1 M Sörensen's buffer, and serially dehydrated with increasing concentrations of ethanol. Cells were infiltrated for 1 h at room temperature (22±2 °C) with a 1:1 mixture of 812 epoxy resin/100% ethanol, followed by 100% epoxy resin overnight. The cells were then embedded with fresh 100% epoxy resin and cured for 48 h at 60 °C. Ultra-thin sections (70 nm) were cut using a diamond knife. Sections were collected on 200-mesh copper grids, before being stained with 2% (w/v) aqueous uranyl acetate and Reynolds' lead citrate. Sections were visualized using a Philips CM-12 model transmission electron microscope.

Isolation of β-cell secretory granules

βTC6-F7 cells [passages 55–60 cultured in Dulbecco's modified Eagle's medium (containing 5.5 mM glucose) with 15% (v/v)

Abbreviations used: FBS, fetal bovine serum; HBSS, Hanks balanced salt solution; KRB, Krebs–Ringer buffer; MALDI–TOF-MS, matrix-assisted laser-desorption ionization–time-of-flight MS; PLIM, preptin-like immunoreactive material; proIGF-II, proinsulin-like growth factor II; RP-HPLC, reversed-phase HPLC; TFA, trifluoroacetic acid.

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heat-inactivated horse serum and 2.5 % (v/v) fetal bovine serum (FBS)] were harvested by trypsinization (2.5–4.0 ml of cells), washed three times and suspended 1:5 in homogenization medium [0.3 M sucrose, 10 mM Mes K (Sigma), 1 mM potassium EGTA (Serva, Heidelberg, Germany) and 1 mM $MgSO_4$ (pH 6.5)] [9]. Cells were then homogenized on ice (24 strokes; ball bearing homogenizer [13]) before centrifugation (400 ***g*** for 10 min at 4 °C). Supernatants were overlaid on pre-formed continuous gradients [13–31 % (v/v) OptiPrep™ (Nycomed, Roskilde, Denmark) in homogenization medium] and ultracentrifuged (SW40Ti rotor; 160000 ***g*** for 16 h at 4 °C). Fractions were removed sequetially from the top to the bottom of the tubes (HaakeBuchler Auto Densi-Flow II), and fractions containing granules were combined (buoyant density of 1.10–1.11) and stored at −80 °C. Purity was confirmed by protein/enzyme-marker assays: aryl sulphatase (lysosomes) [14]; citrate synthase (mitochondria) [15]; insulin (granules). Granule integrity was confirmed using RIAs for insulin (granule core) and amylin (granule matrix).

Purification and identification of granule proteins

Granule fractions were lysed after a freeze–thaw cycle, and centrifuged (16000 ***g*** for 5 min at room temperature) to remove insoluble material. Soluble granule contents were then semi-purified by RP-HPLC [buffer A, 0.08 % (v/v) trifluoroacetic acid (TFA) in H_2O; buffer B, 80 % acetonitrile/20 % buffer A; 250 mm × 2 mm Jupiter C_{18} column (Phenomenex, Torrance, CA, U.S.A.); 250 μl/min; A_{214}]. Peptides were identified by N-terminal protein sequencing (ABI Procise™) and matrix-assisted laser-desorption ionization–time-of-flight MS (MALDI–TOF-MS; Hewlett–Packard G2025A).

Lys-C proteolysis

Purified granule-derived murine preptin was cleaved with 0.25 μg of endoprotease Lys-C (Boehringer Mannheim) in 50 mM Tris/2 mM EDTA buffer (pH 8.5) for 4.5 h at 37 °C, and peptides were separated by RP-HPLC.

Preptin RIA

Rat preptin was synthesized (Auspep Pty Ltd, Parkville, Victoria, Australia) using standard chemical methods. Ovalbumin-conjugated rat preptin (single-step glutaraldehyde coupling method, pH 7.0) was used to raise antisera in New Zealand White rabbits [16]. ^{125}I-labelled rat preptin (chloramine-T; 362 μCi/μg) [17] was purified [using Sephadex G-10 and 50 mM phosphate/1 % (w/v) BSA (pH 7.5)] and an RIA developed, with bound/free separation by polyethylene glycol-assisted secondary antibody (goat anti-rabbit) precipitation (final antiserum dilution = 1:30000; reference/total = 0.30; EC_{20} = 344 pM preptin; EC_{80} = 39 pM; zero cross-reactivity with insulin, amylin and IGF-II).

RP-HPLC/RIA

Conditioned medium

A 2 ml aliquot of βTC6-F7 24 h conditioned medium was acidified (with 0.1 ml of 4 M acetic acid) and applied to 1 ml C_{18} cartridges pre-equilibrated with 10 ml of 100 % methanol and 20 ml of 4 % (v/v) acetic acid. The cartridges were then washed [20 ml of 4 % (v/v) acetic acid] before elution (2 ml of 0.1 M acetic acid in 70 % methanol) and volume reduction (rotary evaporation; final volume of 150 μl).

Isolated rat islets

Pancreatic islets were isolated from normal adult male Wistar rats [18] (see below), and the contents were extracted with acid/ethanol [19]. The freeze-dried extract was reconstituted in 1.3 % (v/v) TFA in Milli Q-grade H_2O (resistivity = 18.2 MΩ). Extracts from both βTC6-F7 conditioned medium and rat islets were separated by RP-HPLC, and appropriate fractions were mass-analysed. All fractions were freeze-dried and reconstituted in RIA buffer [50 mM phosphate/0.3 % BSA (pH 7.4)] and then analysed (RIA). Samples from initial RP-HPLC granule fractions (see Figure 2B) were also analysed by RIA after 60-fold dilution in buffer [standard intragranular preptin-like immunoreactive material (PLIM)].

Competitive immunohistochemistry

Serial sections from normal adult mouse (FVB/N) pancreas were stained with haematoxylin and different specific antisera [diluted 1:40 in 1 % (w/v) BSA/PBS, for 1 h], then with goat anti-rabbit immunoperoxidase-labelled secondary antibodies [diluted 1:20 in 1 % (w/v) BSA/PBS, for 30 min]. Preptin (1 or 5 mg/ml) was pre-incubated with anti-preptin serum for 30 min before addition to sections.

Isolation of rat islets

Methods for pancreas digestion were adapted from published methods [18]. The common bile duct of male Wistar rats (220 ± 20 g) was cannulated and the pancreas removed en bloc. The duct was infused [10 ml of collagenase type V, Sigma; 1 mg/ml in Hepes/Hanks balanced salt solution (HBSS), pH 7.2], and the pancreas was transferred to a 50 ml Falcon tube containing 10 ml of the same solution and incubated in a shaking water bath (100 rev./min) at 37 °C for 35 min. Digestion was stopped by the addition of 30 ml of ice-cold Hepes/HBSS containing 0.35 % (w/v) BSA. Pancreatic material was then centrifuged (200 ***g*** for 1 min at 4 °C) and the supernatant was discarded. Disrupted pancreatic tissue was washed twice in the same buffer with repeated centrifugation and then sieved (500 μm mesh). The filtrate was centrifuged (200 ***g*** for 1 min at 4 °C) and the supernatant was discarded before the pellet was resuspended (final volume of pellet and Hepes/HBSS containing 0.35 % BSA was 10 ml). This mixture was underlaid with 1.25 ml of OptiPrep and centrifuged (200 ***g*** for 15 min at 4 °C), before 7.5 ml of supernatant was removed (being careful not to aspirate the band of disrupted pancreatic tissue). Tissue, OptiPrep and the supernatant were mixed before being overlaid with a continuous gradient [10–25 % (v/v) OptiPrep in Hepes/HBSS containing 0.35 % BSA], and then centrifuged for 25 min at 800 ***g*** (4 °C). Islets free of exocrine tissue float up into the OptiPrep gradient and are aspirated. Prior to use, purified islets were cultured for 48 h in RPMI 1640 containing 10 % (v/v) FBS and 8.3 mM glucose.

Preptin-stimulated insulin secretion from βTC6-F7 cells

Cells were subcultured (passage 52; 24-well plates; 4×10^5 cells/well), grown to 80 % confluence and washed twice in Krebs–Ringer buffer (KRB)/Hepes, pH 7.4. Preptin was serially diluted in incubation medium (KRB/Hepes containing 0.1 % BSA) containing 10 mM glucose to final concentrations of 0.1, 1, 5, 25, 75 and 150 nM, before 1 ml volumes were added to quadruplicate wells. Cells were incubated (at 37 °C for 2 h), medium aspirated and centrifuged (16000 ***g*** for 3 min) and the supernatant was analysed for insulin (RIA).

Insulin secretion from isolated, perfused rat pancreas: effects of preptin and anti-preptin immunoglobulins

Pancreases from fasted adult male Wistar rats (300 ± 25 g) were isolated and prepared [20]. Pancreases were perfused with Krebs–Henseleit buffer (112.8 mM NaCl, 4.4 mM KCl, 1.2 mM $MgSO_4$, 29.3 mM $NaHCO_3$, 1.5 mM KH_2PO_4 and 2.3 mM $CaCl_2$, pH 7.4) supplemented with 4 % (w/v) dextran, 0.5 % BSA, 3 mM arginine and 5.5 mM glucose. Perfusate was gassed with O_2/CO_2 (19:1) and infused using a peristaltic pump (2.7 ml/min without re-circulation). Pancreases were perfused and equilibrated for 20 min prior to each 70 min perfusion experiment; 10 min into the experiment either carrier buffer (0.1 % BSA in 0.9 % NaCl), preptin dissolved in carrier buffer, anti-preptin immunoglobulins or non-immunized rabbit immunoglobulins (both diluted in carrier buffer) were introduced via a side-arm infusion (final concentrations in perfusate: 75 nM preptin and 35 μg/ml immunoglobulins). In addition, at 25 min, glucose was infused for 20 min (final concentration in perfusate: 20 mM). Fractions were collected every minute, put on ice and assayed for insulin (RIA).

RESULTS

Purification and identification of β-cell secretory granule peptides

βTC6-F7 cells are a cultured murine β-cell line, subcloned from βTC6 cells [21], which contain large numbers of secretory granules and provide a ready source of granules (Figures 1A and 1B). Although cultured βTC6-F7 cells contain less insulin (0.2 units/mg of protein) than insulinoma tissue (0.7 units/mg of protein [9]) and normal islet β-cells (2 units/mg of protein; estimate based on yield from bovine pancreas [22]), cultured β-cells have proved useful for subcellular fractionation, since they provide a source of β-cells uncontaminated by fibroblasts, and glucagon and somatostain secreting cells [23].

We initially investigated published methods of granule purification [9,11,24], but found that these methods were not suitable for the purification of βTC6-F7 secretory granules. We also carried out some early granule preparations which included an additional step, washing the granules in Mes K-buffered sucrose and PMSF prior to storage (according to [25]); however, the introduction of this extra washing step greatly decreased total protein yield while chromatographic analysis of this granule material indicated no difference in A_{214} peak ratios (results not shown). As a result, this step was excluded from all subsequent preparations and we developed a single-step centrifugal purification protocol using OptiPrep, a 60 % (w/v) solution of iodixanol in water.

We confirmed separation of secretory granules from mitochondria and lysosomes by marker-protein analysis (Figure 2A and Table 1). Insulin RIAs were used to track purification of the granule cores, whereas amylin, which is present in the granule matrix [26], was measured to verify granule integrity (Figure 2A). Soluble granule components were then separated by RP-HPLC, using a gradient from 10–60 % buffer B (80 % acetonitrile in 0.08 % TFA/H_2O) over 25 min (Figure 2B). Purified peptides were identified by N-terminal sequencing and molecular masses were confirmed by MALDI–TOF-MS. In this manner we confirmed the presence of murine insulins I and II, C-peptides I and II, amylin and chromogranin fragments (Figure 2B).

No non-β-cell peptides were detected, and the molar ratios of amylin to insulin (1:23) and insulin-I to insulin-II (1:3) were equivalent to those of the murine pancreas [6,27]. Furthermore, no prohormones (i.e. pro-insulin or pro-amylin) were isolated, indicating that our granule purification protocol may have specifically isolated mature secretory granules. This is conceivable since insulin RIA was initially used to select granule-rich fractions, and pro-insulin has reduced cross-reactivity in this assay system [28].

A novel proinsulin-like growth factor II (proIGF-II)-derived peptide is isolated from β-cell secretory granules

A major peak that eluted immediately before insulin-I (Figure 2B) was purified to homogeneity, and the component peptide was shown by MS to have a molecular mass ($M + H^+$) of 3950 Da (Figure 2C). This molecule was digested with a lysine-specific protease, and the resulting peptides were separated by RP-HPLC (Figure 2D), prior to complete N-terminal protein sequencing. The complete sequence confirmed the identity of a previously unknown peptide, 34 amino acids in length, which corresponds to Asp^{69}–Leu^{102} of murine proIGF-II E-peptide (Figure 2E). We

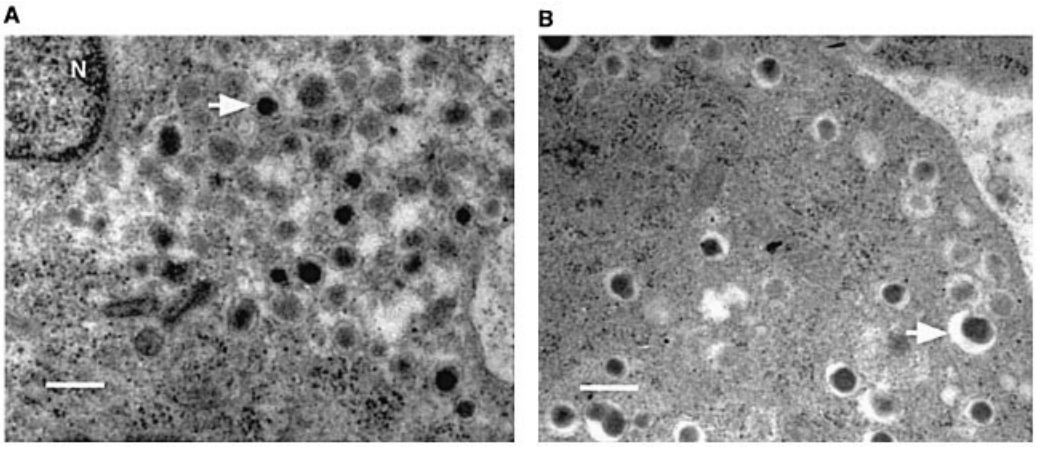

Figure 1 Electron micrographs of βTC6-F7 secretory granules

(**A**) Secretory granules in the perinuclear region showing characteristic membrane-limited granules in different stages of maturity. N = nucleus; bar = 300 nm. (**B**) Mature secretory granules near periphery of the cell showing characteristic electron-dense cores and electron-lucent haloes. Bar = 300 nm.

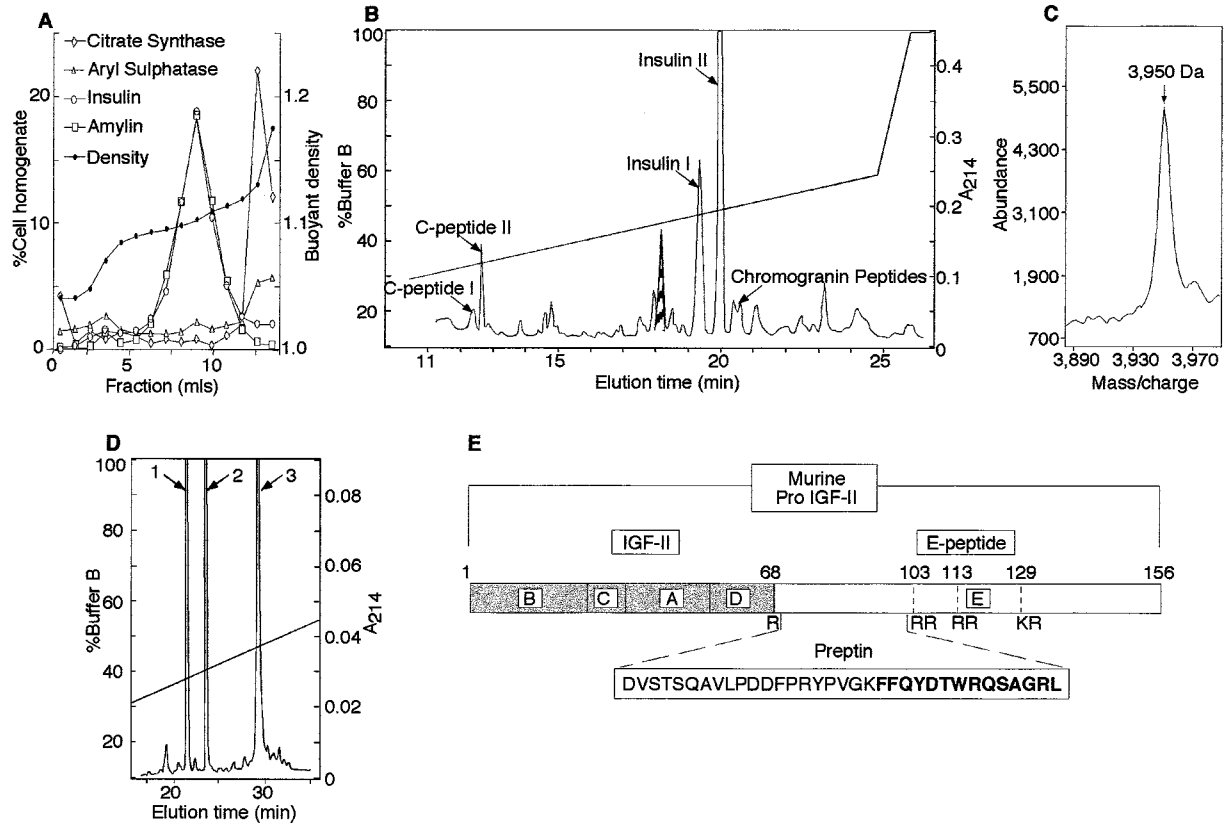

Figure 2 Purification and identification of preptin

(**A**) Assays for marker proteins indicating the localization of organelles from βTC6-F7 cells within a continuous OptiPrep gradient: granule-core (insulin); granule-matrix (amylin); lysosomes (aryl sulphatase); and mitochondria (citrate synthase). (**B**) Granule proteins purified by RP-HPLC. The indicated peak (hatched) was collected and further purified. (**C**) Purity and mass ($M+H^+$) of the major peptide from the hatched peak confirmed by MALDI–TOF-MS. (**D**) RP-HPLC profile of Lys-C digest of peptide purified from hatched peak: 1, N-terminal Lys-C fragment; 2, C-terminal Lys-C fragment; and 3, undigested peptide. (**E**) Structure of murine preptin as determined by sequencing of Lys-C-derived peptides from (**D**). The N-terminal Lys-C fragment is shown in normal font and the C-terminal Lys-C fragment is shown in bold font. Domains of proIGF-II (B, C, A, D and E) are indicated. The localization of murine preptin is also shown within the murine proIGF-II E-peptide. The recognized cleavage site at Arg^{68} is indicated and putative dibasic cleavage motifs are shown as broken lines.

Table 1 Percentage yield and relative specific activities of organelle marker proteins

Marker (compartment)	Initial cell supernatant	Isolated granules	Yield (%)	Relative specific activity
Insulin (granule core)	0.2 units/mg of protein	2.3 units/mg of protein	18.2	11.7
Amylin (granule matrix)	59.7 pmol/mg of protein	699.7 pmol/mg of protein	18.3	11.7
Aryl sulphatase (lysosome)	28.4 nmol/min per mg of protein	41.6 nmol/min per mg of protein	2.3	1.5
Citrate synthase (mitochondria)	14.2 pmol/min per mg of protein	8.6 pmol/min per mg of protein	0.9	0.6

Table 2 Comparison of amino acid sequences for murine, rat and human preptin

Residues in bold indicate differences from murine preptin. Flanking basic amino acids are shown in italics.

Species	Comparative preptin sequences	Identity (%)	Molecular mass (Da)
Mouse	R^{68}-DVSTSQAVLPDDFPRYPVGKFFQYDTWRQSAGRL-RR^{104}		3948.4
Rat	R^{68}-DVSTSQAVLPDDFPRYPVGKFF**KF**DTWRQSAGRL-RR^{104}	94	3932.4
Human	R^{68}-DVST**PPT**VLPD**N**FPRYPVGKFFQYDTW**K**QS**TQ**RL-RR^{104}	79	4030.5

have termed this peptide 'preptin'. Preptin is flanked N-terminally by a recognized arginine cleavage site, and C-terminally by a putative dibasic (Arg-Arg) cleavage motif [29] (Figure 2E). These basic residues are highly conserved between species ([30,31]; Table 2), and are likely to serve as post-translational processing signals.

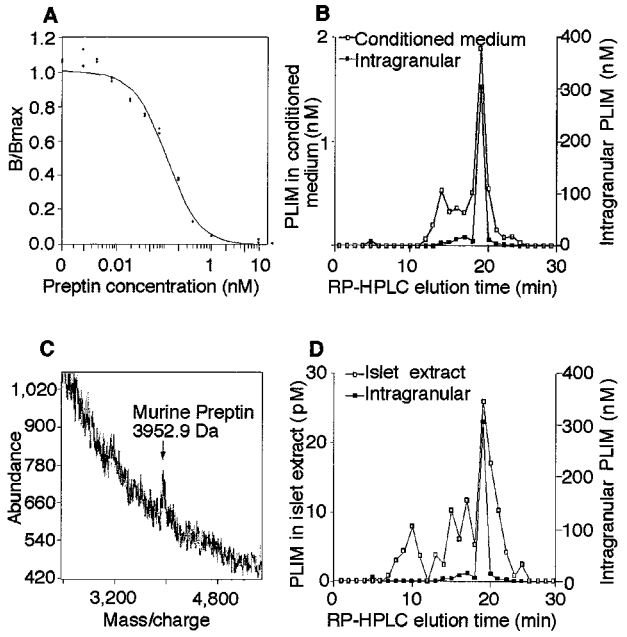

Figure 3 Characterization of preptin in βTC6-F7 cells and isolated rat islets

(**A**) Preptin RIA standard curve. B = bound (average c.p.m. minus blank c.p.m.); and Bmax = maximum bound (reference c.p.m. minus blank c.p.m.). (**B**) RIA characterization of PLIM in RP-HPLC fractions of βTC6-F7 24 h conditioned medium compared with intragranular fractions (1 min fractions from Figure 2B). (**C**) MALDI–TOF-MS of the major PLIM-containing HPLC fraction secreted from βTC6-F7 cells. The peak corresponds to the mass of murine preptin ($M+H^+$) $\pm$ 0.09%. (**D**) RIA characterization of PLIM in RP-HPLC fractions from rat islets compared with intragranular fractions (1 min fractions from Figure 2B).

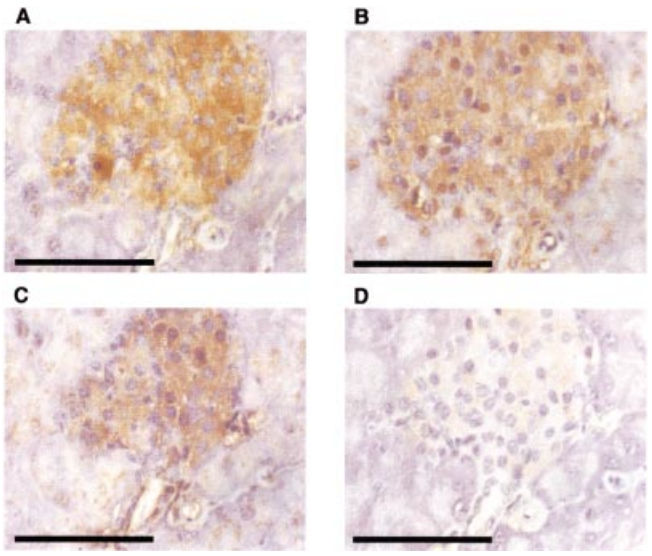

Figure 4 Immunohistochemistry of murine pancreas

Pancreases harvested from adult FVB/N mice were sectioned and stained with haematoxylin and polyclonal rabbit antisera using immunoperoxidase-conjugated goat anti-rabbit secondary antibodies. (**A**) Anti-insulin serum (diluted 1:40). (**B**) Anti-preptin serum (diluted 1:40). (**C** and **D**) Anti-preptin serum (diluted 1:40) pre-incubated with synthetic rat preptin at 1 mg/ml (**C**) or 5 mg/ml (**D**). Bars = 100 μm.

Although we did not isolate any proIGF-II from our granule preparations, proIGF-II (the precursor of preptin) has been detected in the endocrine β-cells of humans and rats by immunohistochemical techniques [32], and further immunohistochemical studies showed that proIGF-II was co-localized with insulin in the secretory granules of normal and diabetic rats [33].

Since preptin is a fragment of a larger precursor (proIGF-II), it was necessary to both qualitatively and quantitatively characterize any detectable PLIM. We generated preptin-specific antiserum, synthesized ^{125}I-labelled preptin using chloramine-T [17] and developed a sensitive preptin RIA in order to measure preptin in different biological samples. A typical standard curve is shown in Figure 3(A).

We then compared the PLIM in βTC6-F7 conditioned medium with the PLIM in intragranular fractions (from Figure 2B) by RP-HPLC/RIA. The major form of both intragranular and secreted PLIM co-eluted on RP-HPLC (Figure 3B). This confirmed that the hatched peak in Figure 2(B) is the only one containing substantial quantities of proIGFII-derived material. Furthermore, MALDI–TOF-MS of HPLC-purified material corresponding to the PLIM peak from βTC6-F7 conditioned medium showed the presence of a single species, with a molecular mass equivalent ($\pm$0.09%) to that of murine preptin (Figure 3C). This result confirms that the preptin purified from the βTC6-F7 cells did not occur simply as an artefact of proteolysis during purification, but exists and is secreted in this form from βTC6-F7 cells *in vivo*.

To qualitatively establish the identity of PLIM in normal islet tissue, we performed RP-HPLC/RIA of acid/ethanol extracts from islets isolated from adult rats. Relative to insulin, preptin levels were lower in islet tissue than in βTC6-F7 cells. However, the major peak of PLIM did co-elute with the standard intragranular preptin, indicating that preptin is the dominant component of PLIM in normal islets (Figure 3D). The low level of preptin in rat islet tissue relative to βTC6-F7 cells was not unexpected, since tumour-derived cells often contain higher amounts of growth factors, including IGF-II [34–36]. Furthermore, it should be noted that adult rats (the source of the pancreatic islets) show reduced IGF-II expression and low levels of IGF-II in the circulation [32]. This is in contrast with humans, who continue to produce high levels of IGF-II throughout their lifetime [37], from a number of tissues, including the pancreas [32,38].

To verify that preptin was present *in situ* we performed competitive immunohistochemical studies in normal murine pancreas. Insulin-like immunoreactive material and PLIM were co-localized in islet β-cells (Figures 4A and 4B). Competition studies showed that PLIM staining was suppressed in a concentration-dependent manner by preincubating preptin antiserum with synthetic preptin (Figures 4B–4D). It should be noted that while the anti-preptin antibodies are likely to cross-react with proIGF-II in this experimental system, the RP-HPLC/RIA results (Figure 3D) indicate that preptin is the dominant antigen present in islets. Taken together these studies suggest that preptin is present in pancreatic islet β-cells.

Preptin is co-secreted with insulin from βTC6-F7 cells and isolated rat islets in response to glucose stimulation

Given the co-localization of preptin and insulin within the β-cell secretory granule, experiments were undertaken to determine whether preptin and insulin are co-secreted in a regulated manner. Glucose-stimulated peptide secretion was studied according to published methods using both βTC6-F7 cells [21] and isolated rat islets [39]. The concentrations of both preptin and insulin were measured using specific RIAs.

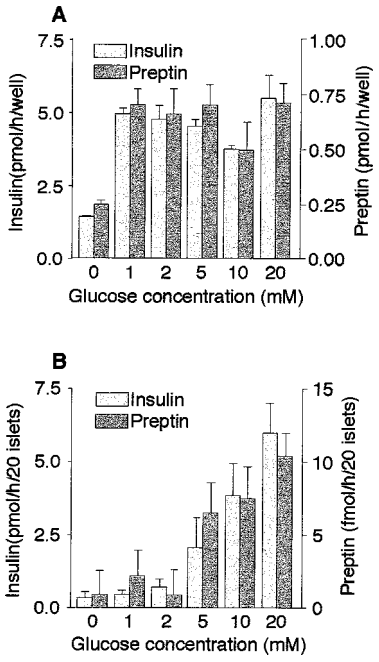

Figure 5 Preptin and insulin co-secretion from βTC6-F7 cells and isolated rat islets

(**A** and **B**) Glucose-mediated co-secretion of preptin with insulin measured following incubation for 2 h with various concentrations of glucose. (**A**) βTC6-F7 cells ($n = 8$) and (**B**) isolated rat islets ($n = 12$).

Although the βTC6-F7 cells were maximally stimulated by sub-physiological concentrations of glucose (< 5 mM), a clear pattern of insulin/preptin co-secretion was observed from both βTC6-F7 cells (Figure 5A) and normal rat islets (Figure 5B). The amount of preptin secreted from the isolated islets (preptin/insulin, 1:400) was lower than the level secreted from the βTC6-F7 cells (preptin/insulin, 1:8). This observation supported the HPLC/RIA results, which indicated lower levels of preptin in physiological rat tissue than in the cultured murine β-cells. Regardless of the different preptin/insulin ratios these results clearly indicated that preptin is co-secreted with insulin from both cultured and physiological β-cells in response to glucose stimulation.

Preptin significantly increases glucose-mediated insulin secretion from βTC6-F7 cells and the isolated, perfused rat pancreas

Since preptin is derived from proIGF-II, which also generates IGF-II, a member of the insulin family that regulates cell growth, differentiation and metabolism, we undertook studies to determine the biological activity of preptin. There is evidence that β-cell hormones, including insulin itself [40], IGF-II [41], amylin [42] and pancreastatin [4], modulate insulin secretion. We therefore first investigated the effects of preptin on insulin secretion from βTC6-F7 cells.

Cells were incubated in the presence of a maximally stimulating concentration of glucose (10 mM) and increasing concentrations of preptin. Medium was aspirated and centrifuged, and the supernatant was analysed for insulin (RIA).

Synthetic rat preptin had a direct effect on the β-cell, enhancing the maximal glucose-stimulated secretion of insulin from cultured βTC6-F7 cells, in a manner that was both concentration-dependent and saturable (Figure 6A). The calculated EC_{50} was 14 nM, and the maximally effective concentration approached 75 nM. This value of 75 nM is equivalent to that at which amylin elicits inhibition of insulin secretion [42]. The concentration-dependent and saturable stimulation of insulin secretion by preptin suggests that it elicits these effects by binding to a cell surface receptor.

To further probe the physiological relevance of preptin-mediated stimulation of insulin secretion, we measured the effect of infused synthetic rat preptin on maximal glucose (20 mM)-stimulated insulin secretion in the isolated, perfused rat pancreas. This model enables the study of islet β-cell function in the context of the whole organ.

Pancreases from male Wistar rats were isolated and subjected to a square-wave pulse of 20 mM glucose in a non-recirculating system, with a background infusion of preptin (using the maximally effective preptin concentration derived from the βTC6-F7 study, 75 nM) or carrier buffer (Figure 6B). In a separate set of experiments, a control peptide [(69–84)proIGF-II, equivalent to the first 16 N-terminal amino acids of preptin] was infused at the same concentration, and compared relative to the carrier buffer (Figure 6C).

All pancreases exhibited normal biphasic insulin secretion in response to 20 mM glucose infusion. Preptin exerted a small but insignificant increase in insulin secretion at basal glucose concentrations (5.5 mM), and significantly enhanced the second phase of insulin secretion, (average of 30% increase in secretion over controls; $P = 0.03$, two-tailed t-test of area under the curve; Figure 6B). However, the control peptide (69–84)proIGF-II, had no significant effect on insulin secretion, either at basal or stimulatory glucose concentrations (Figure 6C).

Investigation in βTC6-F7 cells showed that the level of insulin secretion was dependent on preptin concentration, while investigation in the whole organ indicated that preptin exerted a specific stimulatory effect on the second phase of insulin secretion. Therefore the results from both the *ex vivo* pancreas (Figure 6B) and the *in vitro* βTC6-F7 cell assay (Figure 6A) are consistent with the conclusion that preptin significantly increases insulin secretion in the presence of maximally stimulating concentrations of glucose. Furthermore, we can conclude that this activity is associated with the full-length preptin molecule and not the N-terminal fragment, (69–84)proIGF-II.

Removal of endogenous preptin significantly decreases insulin secretion from the isolated, perfused rat pancreas

To investigate the contribution of endogenous preptin to *in vivo* insulin secretion we needed to effectively remove endogenous preptin from the physiological intact organ. We achieved this by infusing the isolated pancreas preparation with anti-preptin antibodies. To eliminate the potential for interference from unspecified serum components, anti-rat preptin or control (non-immunized) rabbit γ-globulin fractions were first purified from whole serum by Protein A affinity chromatography [43]. The compositions of the two purified immunoglobulin fractions were confirmed by MALDI–TOF-MS, and their preptin-binding capacities determined under conditions simulating those of the antibody perfusion experiments [44]. The maximal amount of preptin completely bound by anti-preptin immunoglobulins under the perfused pancreas experimental conditions was 20 ng/min. Isolated, perfused pancreases were then infused with either anti-preptin or control immunoglobulins and subjected to a square-wave stimulation by 20 mM glucose (Figure 6D).

Secretion of insulin in both the first and second phases was significantly decreased by anti-preptin immunoglobulins (first phase: average of 29% inhibition compared with controls,

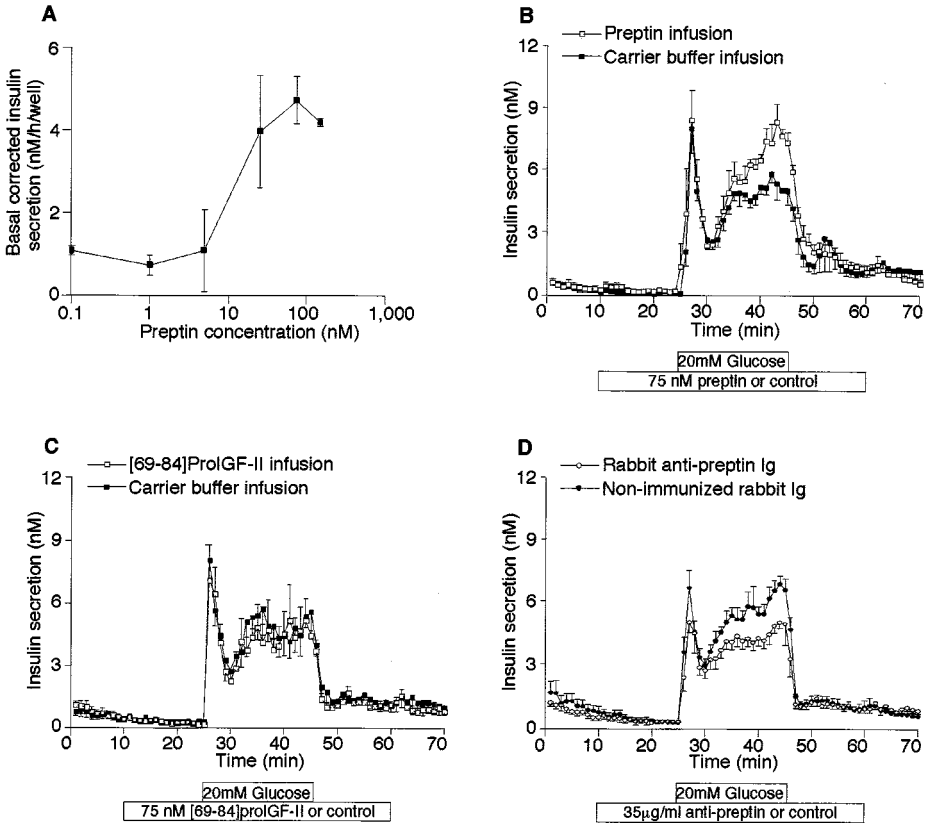

Figure 6 Effects of preptin on insulin secretion

(**A**) Effect of preptin on glucose (10 mM)-stimulated insulin secretion from βTC6-F7 cells. The graph illustrates increments in insulin concentration above basal (i.e. no added preptin and maximal glucose stimulation). Each point is the mean $\pm$ S.E.M. (duplicate analyses; $n = 4$). (**B**) Preptin-mediated insulin secretion from isolated, perfused rat pancreases stimulated with 20 mM glucose as indicated. Each point is the mean $\pm$ S.E.M. (duplicate analyses; four pancreases per curve; second phase of insulin secretion, $P = 0.03$, unpaired two-tailed *t*-test of area under the curve). (**C**) Effect of infusion of (69–84)proIGF-II or carrier buffer on insulin secretion from glucose-stimulated isolated, perfused rat pancreases. Each point is the mean $\pm$ S.E.M. (duplicate analyses; four pancreases per curve). (**D**) Effect of infusion of anti-preptin immunoglobulins or control (non-immunized rabbit immunoglobulins) on insulin secretion from glucose-stimulated isolated, perfused rat pancreases. Each point is the mean $\pm$ S.E.M. (duplicate analyses; five pancreases per curve; first phase, $P = 0.02$, one-tailed *t*-test of area under the curve; second phase, $P = 0.03$, one-tailed *t*-test of area under the curve).

$P = 0.02$, one-tailed *t*-test of area under the curve; second phase: average of 26% inhibition compared with controls, $P = 0.03$, one-tailed *t*-test of area under the curve). This experiment showed that infusion of anti-preptin antibodies was associated with a significant decrease in glucose-mediated insulin secretion.

These results gain further significance, since it could be argued that, in the previous preptin infusion experiment (Figure 6B), what are probably pharmacological concentrations of peptide were used. However, by removing endogenously produced preptin with specific antibodies and observing a reduction in insulin secretion, we have obtained clear evidence that preptin exerts an effect even at the physiological concentration secreted within the pancreas.

DISCUSSION

Preptin is a novel peptide derived from proIGF-II

Our work has identified a novel peptide, preptin, that is co-secreted with insulin and in turn increases the amount of insulin released from pancreatic islet β-cells.

It is well documented that multiple gene products can be derived from a single gene, either by selective processing at the level of transcription/translation (e.g. the calcitonin/calcitonin gene-related peptide [45]), or by post-translation modification (e.g. proteolytic processing of pro-opiomelanocortin [46]). Preptin is derived from proIGF-II and while it is possible that it is the product of an uncharacterized splice variant of the *IGF2* gene, it is more likely that it is generated by post-translational proteolytic processing at the conserved basic residues present in the E-domain of proIGF-II. The findings reported in the present study thus indicate that proIGF-II is the precursor not only for the recognized product IGF-II, but also for an additional peptide hormone, preptin.

Others have shown the existence of different proIGF-II E-peptide-derived peptides in cell culture medium and various mammalian biological fluids [47–49], but none have identified one equivalent to preptin. Previous studies nominate the conserved lysine residue present at position 88 as a cleavage site yielding both 'big IGF-II' (consisting of IGF-II with a 21-amino-acid C-terminal extension) and free E21-peptide [49–51]. While many of these studies relied on inexact size-exclusion liquid chromatography to determine molecular mass, we have characterized preptin by both N-terminal protein sequencing and MALDI–TOF-MS confirming that the E-domain-derived peptide isolated from β-cell granules is 34 amino acids in length, and terminates not at the commonly nominated cleavage point Lys^{88}, but at Leu^{102}.

Although preptin has never previously been described, the proIGF- II maturation model of Gammeltoft [52] predicted the formation of this fragment, and some further supporting evidence was derived from a study by Duguay et al. [52a]. The latter researchers characterized the post-translational processing of proIGF-II in human embryonic kidney cells, and observed that cleavage only occurred at Arg^{104}, which closely flanks the C-terminal Leu^{102} of preptin.

Furthermore, models of IGF-II maturation [51,52] predict that final processing of proIGF-II involves removal of any residual E-domain peptide by cleavage before Arg^{68}; however, N-terminal sequencing confirmed that preptin commenced at Asp^{69} of the E-domain. This phenomenon could result from two possible mechanisms, either cleavage at the C-terminus of Arg^{68} with subsequent carboxypeptidase activity on IGF-II, or cleavage at the N-terminus of Arg^{68} with subsequent aminopeptidase activity on preptin. Given the trypsin-like nature of proteolytic enzymes involved in prohormone production [53], and the presence of carboxypeptidase H/E in the β-cell secretory granule [54], it is most likely that the final step in IGF-II and preptin maturation involves the former cleavage scenario.

Preptin enhances glucose-mediated insulin secretion

The multiphasic secretion of insulin in response to a square-wave pulse of glucose is a well characterized phenomenon [55]. The first or 'rapid release' phase results in a transient spike in insulin secretion, while the second or 'priming' phase results from a progressive increase in insulin release. The most commonly held theory is that the first phase results from exocytosis of mature secretory granules near the periphery of the β-cell, while the second phase requires the transport and subsequent release of granules from deeper within the cell [56]. This hypothesis is supported by the experimental observation that colchicine, a chemical known to disrupt the microtubule transport system necessary for granule transport and margination [56,57], specifically inhibits the second phase of insulin secretion [58].

Preptin was found to enhance, but not initiate, insulin secretion. Furthermore, infusion of preptin into the isolated, perfused rat pancreas exerted a significant increase in the second phase of secretion, while removal of preptin from the experimental system by the addition of anti-preptin antibodies resulted in a decrease in both the first and second phases of insulin secretion. The mechanisms through which preptin could be seen to exert such a combination of effects are complex, and rely heavily upon theoretical explanations of secretory kinetics. It is known, however, that most non-nutrient modulators of insulin secretion effect changes in cAMP or inositol triphosphate levels [59]. Further, it is thought that factors which cause a targeted rise in the second phase of insulin secretion are involved in the priming of β-cells, and probably do not involve cAMP [60].

Other β-cell peptide hormones (insulin, amylin and pancreastatin) are thought to act through autocrine negative-feedback loops, mediated via binding to specific cell surface receptors. The concentration-dependent and saturable stimulation of insulin secretion by preptin suggests that it elicits these effects by binding to a cell surface receptor. The identity of this receptor is currently unknown, and we have initiated further studies to ascertain its identity and to elucidate the second messenger system through which the receptor is exerting its effects.

Both the *in vitro* cell secretion experiments and the *ex vivo* perfused pancreas experiments suggest that preptin might be a physiological regulator of glucose-stimulated insulin secretion, acting on β-cells via a feed-forward autocrine loop. This action may be similar to the feed-forward mechanism evoked in platelets by the thrombin-elicited release of thromboxane A_2 [61]. We therefore propose that preptin may function to counterbalance the inhibitory effects of other β-cell hormones on insulin secretion.

In summary, we report in the present study the discovery of preptin, a pancreatic islet β-cell hormone. Preptin is derived from the E-peptide of proIGF-II, is present in islet β-cell granules and is co-secreted with insulin in response to glucose. Preptin increases glucose-mediated insulin secretion, while the binding of endogenous preptin by anti-preptin antibodies decreases glucose-mediated insulin secretion. Our studies support the view that preptin is a proIGF-II derived peptide secreted from pancreatic islet β-cells that enhances insulin secretion.

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