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Production and characterization of the recombinant Islet Neogenesis Associated Protein (rINGAP)

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ABSTRACT

Islet Neogenesis Associated Protein (INGAP) is implicated in pancreatic islet neogenesis. INGAP peptide, a pentadecapeptide comprising amino acids 104–118, reverses diabetes in rodents and improves glucose homeostasis in patients with diabetes. The mechanism of INGAP action is unknown, but such studies would benefit from the availability of the full-length recombinant protein (rINGAP).
Here we report the production of rINGAP from 293-SF cells following lentiviral transduction, and its characterization by MALDI-TOF and Q-TOF Mass Spectrometry, and HPLC.
Importantly, we show that rINGAP exhibits 100× the bioactivity of INGAP peptide on a molar basis in an *in vitro* assay of human islet regeneration.

Introduction

The World Health Organization estimates that 3.2 million deaths are attributable to diabetes every year. The number of people with diabetes mellitus, type 1 (T1DM) and type 2 (T2DM), will more than double over the next 25 years, to reach a total of 366 million people by 2030. In both types of diabetes, the functional beta-cell mass is reduced. In T1DM beta cells are destroyed by autoimmune reactivity, whereas in T2D apoptosis is suspected to be the cause of a 60% decrease in beta-cell volume [1,2]. The only therapies that restore the required mass of functional beta cells in diabetic patients are either pancreas or islet cell transplantation, both being limited by the shortage of compatible human donors and accompanied by a life-long immunosuppression. An alternative therapy to address all forms of diabetes would be to have a beta-cell source available that would meet the patient's needs either by engineering β-cell growth and differentiation *in vitro* prior to transplantation, or by inducing β-cell regeneration *in vivo*. INGAP[1] (Islet Neogenesis Associated Protein) has been shown to be such a neogenic agent.

INGAP is a 17 KDa member of the Reg3 family of proteins that was discovered in a model of partial pancreatic duct obstruction (PDO) in hamsters [3]. In this setting, animals developed an increased β-cell mass sufficient to reverse diabetes [4]. Initially, a crude pancreatic extract termed ilotropin, was prepared from the pancreata of PDO animals and this extract was shown to induce islet regeneration leading to reversal of chemically induced diabetes [5]. Subsequently, mRNA differential display comparison of PDO and control pancreata identified INGAP as being significantly upregulated in concert with the onset of islet neogenesis, hence its name [6]. Sequencing showed a region of the protein that was specific to INGAP compared to other known Reg proteins, comprising amino acids 104–118. This region was synthetically produced and termed INGAP peptide (or INGAP$^{104-118}$). Although hamster INGAP protein shares a high homology with Human Reg3α, there is no human counterpart of INGAP [7].

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[1] *Abbreviations used:* INGAP, Islet Neogenesis Associated Protein; T1DM, diabetes mellitus, type 1; T2DM, diabetes mellitus, type 2; PDO, pancreatic duct obstruction; DLS, duct-like structures; IMAC, immobilized-metal affinity chromatography; LV, lentivirus.

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The effect of INGAP peptide on islet beta-cell mass has been extensively studied. *In vivo* administration of INGAP peptide leads to the dose-dependent expansion of beta-cell mass in normoglycemic rodents and dogs [8], as well as a reversal of hyperglycemia in a mouse model of type 1 diabetes [9]. Studies of mice expressing INGAP peptide behind the elastase promoter reported increased β-cell mass, decreased cellular proliferation and resistance to chemically induced diabetes [10]. *In vitro* studies have shown that INGAP stimulates insulin secretion and upregulates a number of genes implicated in glucose stimulated insulin release in rat islets [11,12].

INGAP peptide has also been used to induce the *in vitro* differentiation of human islet-derived duct-like structures (DLS) back into glucose-responsive islets [13]. Unlike islets, DLS are highly proliferative, and therefore represent a population of expandable putative progenitors that could be used to increase beta-cell mass for the purposes of islet replacement by transplantation.

Most significantly, phase 2 clinical studies suggested that INGAP peptide administration increased endogenous insulin secretion and improved glycemic control [14–16].

To date, all INGAP studies have utilized the pentadecapeptide and little information about a putative receptor or signal transduction pathways has been obtained. Delineation of the mechanism(s) of INGAP action could be facilitated if the full-length protein were available.

We report here the cloning, expression, production and purification of recombinant full-length INGAP protein (rINGAP) using a method that allows rapid and efficient protein production in 293 cells [17].

Materials and methods

rINGAP protein plasmid construct (pcDNA3.1IngapHIS)

A full-length INGAP PCR product was generated from hamster (*Mesocricetus auratus*) pancreatic acinar-derived cysts [18] using the following primers: Forward: C ACC ATG ATG CTT CCC ATG AC and Reverse: TCA ATG ATG ATG ATG ATG ATG GAC CTT GAA TTT GCA GAT ATA GG and was cloned into pcDNA^{TM}3.1D/V5-His-TOPO® expression vector (Invitrogen). To exclude possible interference of a long V5-His Tag (47aa) with rINGAP function, a short construct containing only a 6-His C-terminal tag was generated by incorporating the 6-His coding sequence into the reverse primer (underlined) followed by a stop codon.

Construction of lentiviral plasmid

The plasmid LVR2CR5GFP was constructed by removing the CR5GFP fragment from JDCR5GFP by SphI/SalI digestion. The fragment was ligated into LVR2GFP [19] digested by the same enzymes. The plasmid LVR2CR5IngapHIS was constructed by removing the IngapHIS fragment from pcDNA3.1IngapHIS by BamHI/PmeI digestion. The fragment was ligated into pLVR2CR5GFP digested with SalI, blunted, and digested with BamHI.

Lentivirus production by transient transfection

293SFPacLV were transfected using PEI (PEI 25-kd linear, Polysciences, Warrington, PA) as described [20]. Four to six hours later, the medium was replaced with fresh medium supplemented with 1 μg/ml Dox and 10 μg/ml cumate. The supernatant containing the lentiviruses was collected at 48 and 72 h after transfection by low-speed centrifugation and filtered through 0.45-μm pore size–HT Tuffryn membrane (Pall Life Science, Ann Arbor, MI) The supernatant was used fresh, or frozen at −80 °C, and concentrated by centrifugation on a 20% sucrose cushion [21].

Lentiviral transduction of 293-SF cells

Polybrene (8 μg/ml) (Hexadimethrine Bromide, Sigma) was added to LV suspension and incubated for 30 min at 37 °C and then applied directly onto 293SF-CymR-rcTA [22,23] cells that had been plated for 24 h. The cells were then incubated overnight at 37 °C, after which fresh culture medium was added.

Cell lines

293SFPacLV [24], 293SF-CymR-rcTA [23] and 293SF-CymR-rcTA-Ingap were grown in Freestyle293 medium (Invitrogen). All the cell lines were maintained at 37°C in a 8% CO_2 humidified atmosphere.

Protein production

293SF-CymR-rcTA-Ingap cells were amplified in Freestyle 293 Medium in the presence of 1% Fetal Bovine Serum (Montreal Biotech). Cells were then switched to serum-free Freestyle 293 medium for 3 days prior to being treated with cumate to activate rINGAP transcription (50 μg/ml, Sigma, [23]). Treated cells were cultured on a culture shaker (120 rpm) at 37 °C and 8% CO2 for 7 days, after what media (cell supernatants) were collected and concentrated on Centricon column (Centricon Plus-70, NMWL-5K, Millipore), and then further processed for purification.

Purification of rINGAP

Protein purification by Immobilized-Metal Affinity Chromatography (IMAC) was carried out using a cobalt-loaded resin (Fractogel EMD Chelate (M), Merck) as described elsewhere [25]. The column (Qiagen-tip 500 Maxi kit, QIagen) was charged as follows; the column was packed with 4 ml of Fractogel. Five column-volumes of 0.5 M NaCl, four column-volumes of 200 mM CoCl2 and two column-volumes of 0.5 M NaCl (pH 5 ± 0.2) were successively added. Sample loading and recombinant protein elution were performed as follows; 10 column-volumes of PBS were added to equilibrate the column, then the concentrated medium was applied to the resin, and allowed to pass through the column by gravity, the resin was washed with 10 column-volumes of IMAC wash buffer 1 (300 mM NaCl, 50 mM sodium phosphate, pH 7.0) followed by 10 column-volumes of IMAC wash buffer 2 (8.3% (v/v) IMAC column elution buffer, 91.7% (v/v) IMAC wash buffer 1, final imidazole concentration: 25 mM). The purified protein was eluted by gravity flow in a four column-volumes of IMAC column elution buffer (300 mM imidazole, 300 mM NaCl, 50 mM sodium phosphate, pH 7.0) in 1-mL increments. The 1-mL fractions containing rINGAP were then pooled and concentrated on Amicon Ultra-4 columns (NMWL-3K, Millipore). The purification was completed by an exchange buffer extra step performed on Microcon columns (Microcon YM-3K Millipore) and the protein was frozen at −80 °C in 1× PBS.

SDS–PAGE and Western blot analysis of purified rINGAP

Five micrograms aliquots of purified rINGAP (Fig. 1B and C) or 1 μg of total proteins from cell supernatant after 1–9 days of culture (Fig. 3) were run on 12% (w/v) polyacrylamide gels and analyzed by staining with Coomassie Brilliant Blue G-250 stain (Sigma) (Fig. 1B) and by Western blot using mouse anti-Histidine antibody (Invitrogen) followed by horseradish-peroxidase–labeled goat anti-mouse IgG (Cell Signalling) and detection with an ECL reagent (Amersham Biosciences) (Figs. 1C and 3).

To assess the purification efficacy, 10 μl of cell supernatant and 10 μl of the purified protein were ran on a precasted Tris–glycine