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Unravelling beta cell destruction in type 1 diabetes

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CHAPTER 6

Expression of alternative insulin gene-derived proteins in human pancreatic islets

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ABSTRACT

Blood glucose homeostasis is tightly regulated by the endocrine cells within the islet of Langerhans. Protein Expression of insulin, glucagon, and somatostatin is restricted to beta, alpha, and delta cells, respectively. Yet, recent transcriptome analyses have revealed that insulin mRNA is also detected in non-beta endocrine cells. We studied alternative splicing of human insulin mRNA in pancreatic islets and identified the presence of an insulin mRNA isoform. This transcript, encoding the complete preproinsulin signal peptide and B-chain, yields an altered C-terminus due to the disruption of the original reading frame. The resulting polypeptide is largely identical with the previously identified insulin-derived defective ribosomal protein (INS-DRiP). Immunohistochemistry on pancreatic sections using antisera raised to detect these alternative insulin gene-derived products shows the presence of INS-DRiP in insulin-producing beta cells and the product of a splice variant primarily in somatostatin-producing delta cells. Although the role of this insulin splice variant has not yet been defined, its presence in secretory granules of somatostatin-positive cells, as revealed by electron microscopy, suggests an endocrine or paracrine function.

INTRODUCTION

While polyhormonal endocrine cells have been shown to reside in human foetal pancreatic islets and in individuals with chronic pancreatitis, fully differentiated endocrine cells are classically dedicated to producing a single hormone; i.e. glucagon, insulin, somatostatin production by alpha, beta, and delta cells, respectively[1-3]. Under this definition, insulin gene expression is restricted to the pancreatic beta cells. However, accumulating data indicate mature human beta cells are more plastic than previously assumed[4]. Whereas the differentiated state is maintained by reinforcement of beta cell-specific gene regulatory networks and repression of other transcriptional programs[5-8], metabolic and mechanical stress have been shown to cause spontaneous dedifferentiation and transdifferentiation of human beta cells. Conversion of beta cells to alpha and delta cell-like states observed in T2D patients has been proposed to cause beta cell failure[9]. In addition, *in vitro* reaggregation of human islet cells induced spontaneous beta cell conversion into glucagon-producing cells[10]. This endocrine plasticity has been proposed to allow dysfunctional beta cells to escape apoptosis due to environmental stress as well as replenish the beta cell population[11, 12]. In situ hybridisation and single-cell transcriptome analysis of human islet cells have confirmed the presence of insulin mRNA in alpha and delta cells[13, 14]. Approximately 46% of islets cells were found to express more than one hormonal transcript type per cell, with a considerable portion of endocrine cells containing both insulin and somatostatin transcripts[15].

Alternative splicing increases proteome diversity by generation of multiple mRNA transcripts from a single gene that differ in assembly of exons and introns. It is estimated that approximately 95% of the human transcriptome is alternatively spliced[16]. Tissue-specific splicing patterns allow expression of similar genes in different cell types to produce different proteins which differ in biologic composition and activity[17]. Alternative splicing networks are implicated in a broad variety of biological processes, including: maintenance of pluripotency, directing cell differentiation, and cell lineage commitment[18-21]. Furthermore, splicing patterns are highly dynamic and therefore provide a mechanism to swiftly adapt to changes in the local microenvironment[22, 23]. The beta cell transcriptome was shown to be highly impacted by inflammatory and metabolic insults[24, 25]. Experiments conducted in HEK293T cells overexpressing the human insulin gene demonstrated the presence of cryptic splice sites in insulin, as multiple insulin isoforms were detected[26, 27].

In this study, we investigated insulin gene splicing in human pancreatic islets. We generated antisera directed against these alternative insulin gene products and demonstrate that an insulin isoform protein can be detected in delta cells. More specifically, high resolution electron microscopy and immune-labelling revealed that this variant insulin gene product localized to the secretory granules.

RESULTS

Evidence of alternative insulin RNA splicing in human islets

Human insulin is encoded by a small gene with only three exons and two introns. Yet, PCR analyses performed on RNA isolated from human islets of three different donors show the presence of two insulin transcript isoforms (Fig. 1A). Nucleotide sequencing of the PCR fragments indicated that the larger, most abundant isoform represents wild-type insulin (INS-wt) in which intron 1 and 2 have been fully spliced out. We recently showed that this mRNA, known to encode preproinsulin (PPI), generates a defective ribosomal product (DRiP) that is target of islet autoimmunity and associated with type 1 diabetes (T1D) pathology[28]. The shorter isoform results from a cryptic splicing site within exon 3 at position 1338, predicted from *in silico* analysis (supplementary Fig. 1). The altered open reading frame that is formed by alternative splicing may lead to the generation of a polypeptide during translation in which the signal peptide and B-chain of the canonical PPI are intact but the C-terminal end of the molecule is different (referred to as INS-splice). Coincidently this C-terminal region would be identical to the INS-DRiP with except of the first 10 N-terminal amino acids that contains the diabetogenic T cell epitope (Fig. 1B)[28].

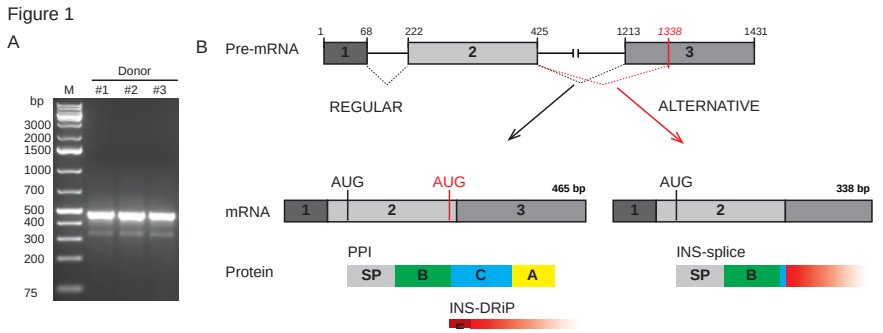

Figure 1: Insulin gene-derived products. A) Analysis of insulin gene derived transcript splicing by PCR on RNA derived from human pancreatic islets of 3 different donors (#1, 2 and 3) visualized on a DNA gel. The DNA marker is indicated by the M. B) Schematic overview of

the human insulin pre-mRNA with the exons annotated by numbers (1-3) and the intronic regions represented by a black solid line. Normal insulin splicing and alternative insulin splicing are indicated by black and red dashed lines, respectively. The resulting mRNA products with translation initiation sites are depicted underneath. For each mRNA molecule the potential protein products are displayed, including preproinsulin (PPI) with the signal peptide (SP, grey), B-chain (B, green), C-peptide (C, blue), and A-chain (A, yellow) specified. In red the INS-DRiP with the E, indicating the previously identified CD8 T-cell epitope and an insulin splice variant (also known as INS-splice). Corresponding amino acid sequences are indicated with corresponding colours, letters indicate the presence of the complete chain.

Alternatively spliced insulin is a template for translation in human islets

In order to investigate these alternative insulin gene-derived proteins, rabbits were immunized with a short synthetic polypeptide unique to INS-DRIP (DRiP1-13) and a short synthetic polypeptide of the C-terminus common to both predicted alternative insulin gene-derived products INS-DRiP and INS-splice (FS-c). The peptides were selected from analysis of the UniProt human protein knowledgebase using the basic local alignment search tool (BLAST) to avoid cross reactivity to other known proteins (data not shown). Serum specificity was confirmed by ELISA using recombinant PPI, INS-DRiP and INS-splice. As expected, neither sera cross reacted with PPI (Fig. 2) and while the anti-DRiP1-13 serum specifically detected the INS-DRiP polypeptide, the anti-FS-c antiserum recognized both alternative INS-derived recombinant proteins (Supplementary Fig. 2).

To investigate whether the insulin gene-derived polypeptides are generated by islets, human pancreatic sections were stained with either the pre-immunization or post-immunization antisera. The localisation of the N-terminal INS-DRiP polypeptide within beta cells is in line with our previous findings and supports beta cell destruction by CTL directed against the DRiP (Fig. 3A). Yet, the antiserum raised to the C-terminus common to INS-DRiP and INS-splice did not colocalize with insulin, indicating FS-c positive (FS-c^+) cells are not beta cells (Fig. 3B). To assess the identity of these FS-c^+ cells, human pancreatic sections were analysed for various endocrine cell markers (i.e. insulin, glucagon, and somatostatin). Our results show that the staining of the FS-c epitope was restricted to delta cells as indicated by its colocalization with somatostatin (Fig. 3C). Of note, staining of other endocrine tissues demonstrates that the expression of the INS-splice polypeptide was specific to pancreatic islets (Supplementary Fig. 3).

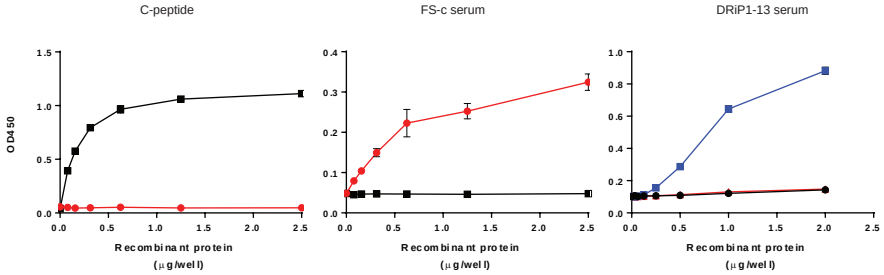

Figure 2: Validation of DRiP1-13 and FS-c antisera. Validation of serum responses in an indirect ELISA assay to serial dilutions of recombinant PPI (black curves) and recombinant INS-splice (red curve) and INS-DRiP (blue) using c-peptide antibody (left panel), FS-c antiserum (middle panel) and DRiP1-13 antiserum (right panel).

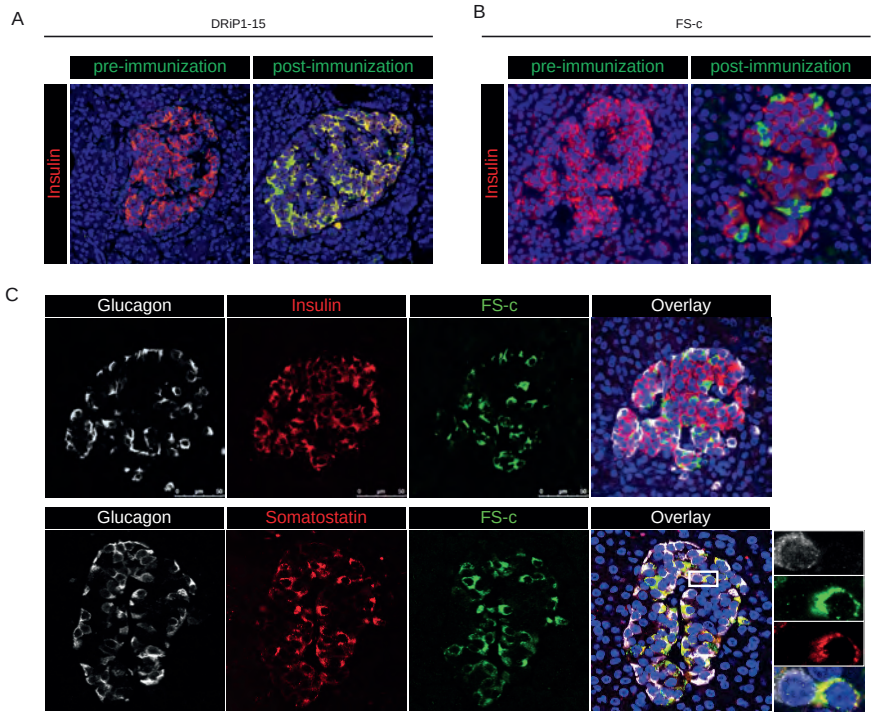

Figure 3: FS-c antiserum exclusively labelled somatostatin-producing delta cells. Immunostaining of human pancreatic sections with pre-immunization serum (left panel) and post-immunization serum (right panel) (green) in combination with insulin (red). A) Serum derived from DRiP1-13 immunized mice. B) Serum derived from FS-c immunized mice. C) Human pancreatic sections stained for glucagon (white) and insulin (red) (middle panels) and glucagon (white) and somatostatin (red) (lower panels) in combination with FS-c antiserum (green). At the lower right enlarged images of the white enclosure are depicted. Nuclei are visualized by DAPI staining (blue).

The FS-c antiserum does not cross react with somatostatin

To validate the presence of an insulin gene-derived product and to exclude cross reactivity with somatostatin, we generated 293T cells expressing the human insulin gene. In these cells, expression of the gene leads to expression of two insulin transcript variants, as observed in human islets (Supplementary Fig. 4A). Western blot analysis of the cell lysates of surrogate beta cells, indicated that PPI is expressed, as well as an alternative insulin-derived product (Fig. 4A). To confirm that the spliced isoform is detected with the antiserum, both transcript isoforms were isolated and cloned into different expression plasmids. Western blot analysis of lysates of 293T cells transfected with the WT insulin cDNA or the spliced cDNA demonstrates that the serum specifically detects the spliced isoform. The C-peptide is only detected in lysates of cells transfected with the INS-wt (Fig. 4B, supplementary Fig. 4B).

Cross reactivity of the serum to delta cell-specific protein somatostatin was excluded by FS-c specificity tested on recombinant somatostatin (Fig. 4C). Furthermore, antibody blocking assays using recombinant somatostatin did not alter detection of recombinant INS-splice, while antibody blocking with recombinant INS-splice markedly reduced INS-splice detection (Fig. 4D). Similarly, antibody blocking using immunization peptide reduced the mean fluorescence of the FS-c^+ islet cell population compared to irrelevant peptide (Fig. 5A). RNA isolation from the FS-c^+ population showed an enrichment of somatostatin and INS-splice RNA in these cells when compared to the FS-c^- cells, confirming our previous finding. (Fig. 5B, Supplementary Fig.4C).

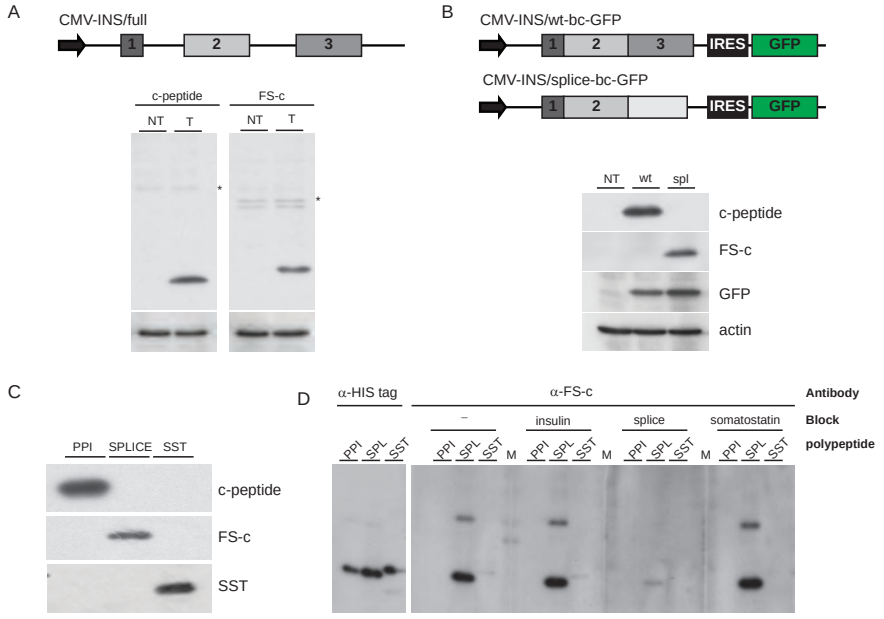

Figure 4: INS-splice is insulin gene-derived and serves as template for translation in 293T cells. A) Schematic representation of the plasmids CMV-INS/full, encoding all exons (annotated by numbers 1-3) and introns (represented by the solid black line) of insulin driven by a CMV promotor. Western blot analysis of 293T cell lysates transfected with CMV-insulin/full (T) and non-transfected cells (NT). Lysates were made 48 hours post transfection and analysed with anti-c-peptide (left panel), anti-FS-c (right panel) and anti-actin (bottom panels). Unspecific background bands (*). B) Schematic representation of CMV-INS/wt-bc-GFP, encoding the mRNA of normally spliced insulin, and CMV-INS/splice-bc-GFP, encoding the mRNA of alternatively splicing insulin in exon 3. With underneath the western blot analysis of 293T cell lysates transfected with CMV-INS/WT-bc-GFP (wt) and CMV-INS/SPLICE-bc-GFP (spl) or non-transfected (NT). Lysates were made 48 hours post transfection and analysed with anti-c peptide (first panel), anti-FS-c (second panel), anti-GFP (third panel) and anti-actin (fourth panel). C) Western blot analysis of recombinant PPI (PPI), recombinant INS-splice (SPLICE) and recombinant somatostatin (SST) with c-peptide antibody (upper panel), FS-c antiserum (middle panel) and somatostatin antibody (lower panel). D) Western blot analysis of recombinant polypeptides with FS-c antiserum that has previously been blocked overnight with recombinant PPI, INS-splice or somatostatin. 15% SDS-polyacrylamide gels were loaded with 50 ng recombinant polypeptide. Antibody blockings were performed with 5 µg recombinant polypeptide. All membranes were developed simultaneously on a single film to exclude differences in band intensity due to differences in exposure time. The M indicate the position of the protein marker were membranes were cut after blotting and put together prior development. Loading of recombinant polypeptides was demonstrated by the presence of the HIS-tag (right panel).

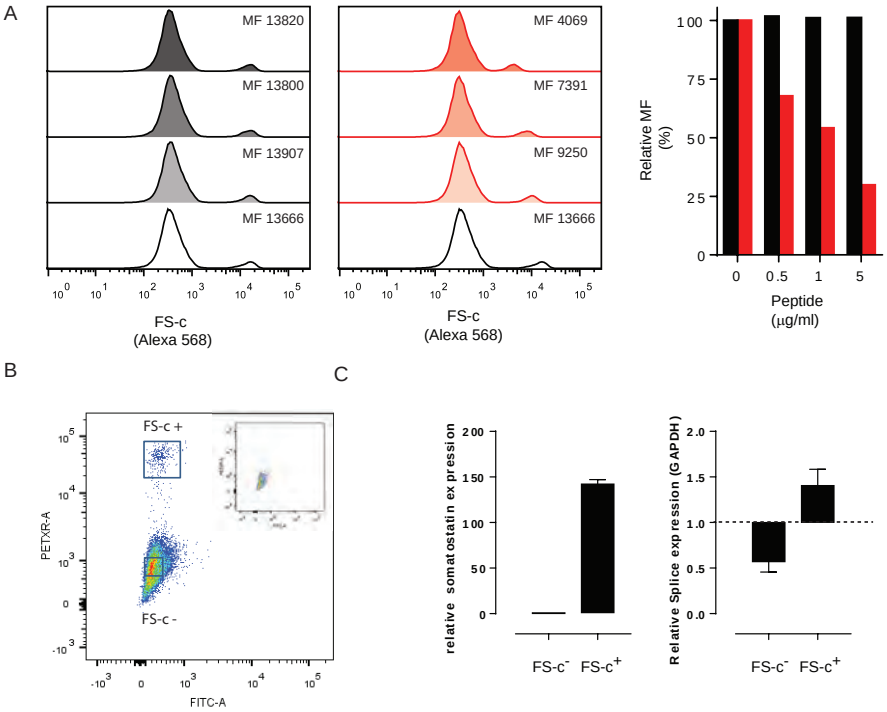

Figure 5: In depth analysis of human pancreatic FS-c$^+$ cells. A) Flow cytometric analysis of human pancreatic islets stained with FS-c serum after antibody blocking with an increasing amount of irrelevant peptide (PPI15-24) (black histograms) and the peptide used for immunization (red histograms). On the top right the mean fluorescence (MF) of the FS-c$^+$ cell population is depicted. The relative MF of the FS-c$^+$ cell population compared to unblocked antiserum (0 peptide) of antibody blocked with irrelevant peptide (black) and relevant peptide (red) (left graph). B) Sorting of FS-c$^+$ human pancreatic islet cells. Inset depicts unstained cells. C) Transcriptome analysis of FS-c$^-$ and FS-c$^+$ human islet cells for relative somatostatin and relative INS-splice expression.

Expression of INS-splice in delta cell granules

Since the alternatively spliced insulin transcript-encoded isoform has the same N-terminus as insulin, we wondered to what extent the presence of the signal peptide contributes to the posttranslational processing and localization of the isoform protein. Detailed examination of pancreatic coupes by high-resolution electron microscopy with immunogold labelled anti-serum demonstrates that the polypeptide localizes within secretory granules of delta cells (Fig. 6A,B)[29]. This confirms that INS-splice is transported to the delta cell granules and suggest it to be a secreted protein.

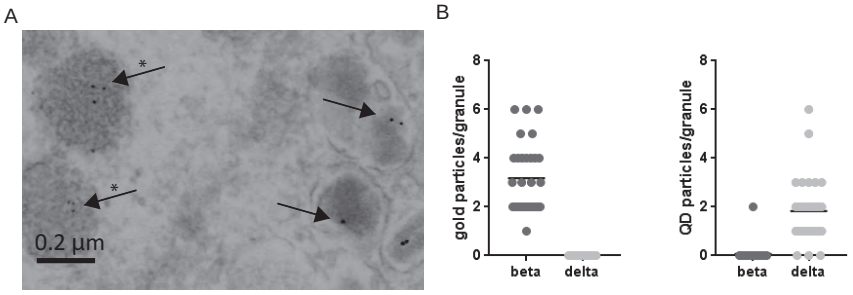

Figure 6: INS-splice is targeted to the delta cell granules. A) Electron microscopic images of human pancreatic sections labelling for INS-SPLICE (quantum dots, black * arrow) and insulin (immunogold, black arrow) visible as black dots. The granules were identified by the morphology of the secretory granules. B) Quantification of the gold$^+$ and QD$^+$ granules in beta and delta cells. Each granule is represented as a point. The graphs represent the mean of 30 beta and 30 delta cell granules.

DISCUSSION

Insulin production and secretion by beta cells is crucial for the maintenance of glucose homeostasis. Paradoxically, this hormone is also a major target in the CD4$^+$ and CD8$^+$ T cell-mediated destruction of beta cells in type 1 diabetic patients [30]. While insulin is widely studied for its role in glucose homeostasis and islet autoimmunity in T1D, little is known about other insulin gene-derived products or splicing isoforms. In this study, we investigate two insulin gene-derived polypeptides; the INS-DRiP, a defective ribosomal product, and INS-splice, a protein encoded by an alternatively spliced insulin gene-derived transcript that is partly identical to the PPI and INS-DRiP proteins.

We have previously shown that INS-DRiP may participate to T1D pathology as target of CD8$^+$ T cells[28]. Supporting our previous data, the N-terminus of DRiP, containing the CD8$^+$ T cell epitope that is lacking in INS-splice, can be detected in pancreatic beta cells by immunohistochemistry. The absence of the C-terminus may imply that this protein is rapidly targeted for degradation during translation, which is in line with the classic degradation process for proteins that lack an in frame stop codon[31].

We also report that alternative splicing of insulin pre-mRNA results in a translational product that has an N-terminal region identical to PPI and a C-terminal portion that is identical to that of the INS-DRiP protein. Similar to wild type insulin mRNA, the alternatively spliced mRNA can be found abundantly in both beta and delta cells by single-cell transcriptome analyses of human pancreatic islets (available online)[32] while hormone production

appears to be cell-type exclusive. This could point to the participation of a cell type-specific translation machinery in adult endocrine cells. Yet the relevance of the insulin gene-derived mRNA and possible translates thereof in endocrine cells other than beta cells remains unclear. Several studies suggest that delta cells have an important role in beta cell development during organogenesis[33, 34]. Also, human pancreatic beta cells have been shown to change cell identity via dedifferentiation and transdifferentiation, presumably as a manner to evade harsh environmental conditions that could result in their death[11]. Alternative splicing is involved in maintaining lineage differentiation as well as maintenance of cell pluripotency and is influenced by the microenvironment [20, 35]. Whether insulin promoter activity in endocrine cells other than beta cells is a phenotypic remnant of their common progenitor cell or contributes to maintaining their endocrine cell plasticity in adolescence remains to be elucidated.

Both the INS-DRiP and INS-splice open reading frames lacks a STOP codon. Nevertheless, the INS-splice product seems to be fully conventionally synthesized and processed as it was detectable in delta cell granules specifically. The presence of the signal peptide may target the co-translational translocation of the polypeptide to the ER lumen and subsequently secretory granules [36]. Nonstop proteins targeted to the endoplasmic reticulum and mitochondria can block translocon channels and should be rapidly cleared to allow continued cellular functions. This allows the release of nonstop proteins into the organelle and to escape proteasomal degradation[37, 38]. Although other quality control and clearance mechanisms within these organelles are likely, we propose that the cotranslational translocation allows the maturation of INS-splice, explaining the detection of the C-terminal sequence in delta cells.

The observation that this insulin isoform is exclusively detected in delta cell is intriguing. Furthermore, this suggests the presence of the preproinsulin signal peptide and B-chain in delta cells, which are major targets for islet autoimmunity in T1D[39-41]. The fate of delta cells in context of type 1 diabetes is poorly investigated. While, impaired delta cell function has been reported[42], we are not aware of any reports demonstrating evidence for delta cell destruction by autoreactive CTLs. While the function of the INS-splice polypeptide remains enigmatic, its presence in secretory granules implies that this protein is secreted and could have paracrine or endocrine functions in humans.

MATERIALS AND METHODS

Cell culture

HEK293T cells were maintained in high glucose Dulbecco's modified Eagle's medium (DMEM, Gibco-BRL, Breda, The Netherlands) supplemented with 8% fetal bovine serum (FBS) (Gibco-BRL), 100 units/ml Penicillin and 100 µg/ml Streptomycin (Gibco-BRL).

DNA constructs

To generate insulin expressing vectors, PCR on human genomic DNA was isolated from HEK293T cells. The human insulin gene was cloned by PCR using the following primers: INS Fw 5'-AGCCCTCCAGGACAGGC-3' and INS Rv 5'-TTTTGCTGGTTCAAGGGCTTTATT-3'. The PCR fragment was cloned into pJet2.1 vector and subcloned into pRRL-CMV vector to generate a pLV-CMV-INS/full expressing the human INS gene. The same PCR primers were used on RNA isolated from purified human islets. The two PCR fragments obtained were cloned into pJET2.1 and subsequently cloned into pRRL-CMV-IRES GFP to generate a pLV-CMV-INS/wt-bc-GFP and pLV-CMV-INS/splice-bc-GFP. The constructs were verified by Sanger sequencing using primer located into the CMV promoter.

Polyethlenimine transfection

HEK293T cells were transfected in suspension using polyethylenimine (PEI). A transfection mixture for a 6-well consisted of 2 µg plasmid DNA and 6 µg PEI (pH 7.4) supplemented with Opti-MEM I reduced serum medium (Gibco) to a final volume of 200 µl. Mixtures were thoroughly mixed and pre-incubated for 10 min at room temperature before adding to the cell suspension, 48 hours post transfection cells were harvested for further analysis.

Reverse transcription PCR and real time analysis

RNA was isolated with TRIzol reagent (15596018, Invitrogen). To ensure RNA quality all samples were DNase treated (Invitrogen). Reverse transcription was performed using 500 ng RNA using Superscript RT III kit (Invitrogen, Karlsruhe, Germany). Gene expression was determined using the following primers: Exon 2: Fw 5'-GGCTTCTTCTACACACCCAAG -3', Rv 5'-CGGGTCTTGGGTGTGTAGAAG-3';
Splice:

Fw 5′-GGCTTCTTCTACACACCCAAG-3′,
Rv 5′-GCAGTAGTTCTCCAGCCTGCAGGTCC-3′;
Somatostatin: Fw 5′-CGTCAGTTTCTGCAGAAGTCCCTGGCT-3′,
Rv 5′-CCATAGCCGGGTTTGAGTTAGCAGATC-3′ and GAPDH;
Fw 5′-ACAGTCAGCCGCATCTTCTT-3′, Rv 5′-AATGAAGGGGTCATTGATGG-3′.
Quantitative PCR analysis was performed using the SybrGreen master mix kit (Applied Biosystems, Foster City, CA) in triplicate on an Applied Biosystems Step One Plus machine.

Generation of custom polyclonal antisera

Custom polyclonal antisera (Eurogentec, secure.eurogentec.com/speedy, Belgium) were generated by immunization of rabbits using synthetic peptides, MLYQHLLPLPAGEC (DRiP1-13, cysteine serves as anchor residue for the carrier) and LLHRERWNKALEPAK (FS-c). During the speedy immunisation programme of 28 days the animals were repeatedly injected with synthetic peptide and bled prior immunization and post immunization. Immune responses of the bleeds were tested for antigen reactivity by ELISA performed by the manufacturer.

Recombinant polypeptides

Human recombinant polypeptides were synthesized as previously described[43]. Protein encoding cDNA was obtained from human pancreatic islets by PCR and cloned in pDest17 for protein production in *Escherichia Coli* using gateway cloning technology (Invitrogen, Carlsbad, CA, USA). Recombinant proteins were purified by the His6 affinity purification tag and freeze dried. Purified polypeptides were dissolved in 0.05% acetic acid in MQ/PBS to a stock concentration of 1 mg/ml.

Enzyme-linked immunosorbent assay

Nunc maxisorp 96 well plates (Sigma-Aldrich) were coated with 1 µg/well of the desired recombinant polypeptide diluted in PBS to reach a final volume of 100 µl/well and incubated overnight at 4C. After coating, the wells were blocked with 200 µl/well 2% BSA in PBS for 1 h at room temperature (RT), followed by 2 h incubation with the primary antibody (i.e. DRiP1-13 (1:1000), FS-c (1:1000) or ms-α-C-peptide (1:2500)) of interest and subsequently 2 h with HRP-conjugated secondary antibody (i.e. anti-rabbit and anti-mouse (Santa Cruz)) for visualization. Antibodies were diluted in 1% BSA in PBS to reach a final

volume of 100 µl/well. For detection, 100 µl/well of freshly prepared substrate, 0.4 mg/ml O-phenylenediamine (P9029, Sigma) in 0.05M phosphate-citrate buffer pH 5.0, was used. After 15 minutes incubation the absorbance at 450 nm was measured. After each step, wells were aspirated and extensive washed using 200 µl/well Wash buffer (0.05% Tween-20 in PBS). This was repeated for a total of 5 washes with 1 minute soaking to increase the effectiveness of the washes.

Human islet isolation

Pancreatic islets were obtained from non-diabetic human cadaveric donor pancreata, given the islets could not be used for transplantation and research consent was present. The isolations were performed in the GMP-facility of LUMC according to the previously described protocol[44]. For experimental use, human islets were maintained in ultra-low attachment plates (Corning, NY 14831) in low glucose DMEM supplemented with 10% FBS, 100 units/ml Penicillin and 100 µg/ml Streptomycin (Gibco-BRL).

Immunohistochemistry and confocal microscopy

Paraffin embedded tissues were cut into 4 µm sections. Tissues were deparaffinized in xylene and rehydrated in a series of ethanol decreasing in concentration. Prior antibody incubation antigen retrieval was performed by autoclavation in citric buffer (pH 6.0). Sections were blocked for 1 hour with 2% normal donkey serum in PBS. Primary and secondary antibodies diluted in PBS/1%BSA were incubated subsequently for 1 hour at room temperature with additional washing steps in between. Primary antibodies against insulin (1:100), C-peptide (1:500, CBL94 Millipore), glucagon (1:200 ab10988 Abcam), somatostatin (1:500, 13-2366 Emelca Bioscience), DRiP1-13 (1:500) and FS-c (1:500). The appropriate Alexa-conjugated antibodies were used (anti-mouse, anti-rabbit, and anti-sheep). Nuclei were stained with DAPI vectashield (Vector laboratories #H-1500). Immunofluorescence was detected with a Leica SP8 confocal microscope.

Flow cytometric analysis of human pancreatic islet cells

Prior flow cytometric analysis of human pancreatic islets, a single cell suspension was prepared by dispersion using trypsin and filtration. Thereafter, the islets cells were fixed and permeabilized with 4% PFA, 0.1% saponin in PBS for 30 min

at 4 degrees. Islet cells were subsequently stained with anti-FS-c as primary antibody (1:500) and Alexa-568 conjugated secondary antibodies against rabbit (Life technologies 1:500). Antibodies were diluted in 0.1% saponin, 1% BSA in PBS and incubation time of 1 hour cold. Islet cell populations were analysed and sorted using a FACS Aria II (BD biosciences).

To isolate RNA from sorted cells, fixation, permeabilization and staining were performed in presence of RNase inhibitor following the MARIS method described by Hrvatin et al[45].

Western blot Sample preparation and immunoblotting

Cell lysates were made using Tropix lysis mix (Applied Biosystems) and the protein content was determined by Bradford reagent assay (Biorad, Veenendaal, The Netherlands). For analysis 50 µg protein sample was used. Samples were boiled with sample buffer (10% glycerol, 2% SDS, 50 mM Tris-HCl pH 6.8, 0.1% Bromophenol blue and 1% β-mercaptoethanol) for 5 minutes before loading the samples onto a 15% SDS-polyacrylamide gel. The proteins were subsequently transferred to 0.2 µm pore size Immobilon-P (Merk Millipore) and visualized by standard protocols with anti-insulin (1:1000, H-86 sc-9168), anti-C-peptide (1:1000, CBL94 Millipore) ,anti-actin (1:5000, C4 MAB1501 Merck Millipore), anti-GFP (1:2000, A11122 Invitrogen), anti-FS-c (1:1000), anti-somatostatin (1:1000, 13-2366 Emelca Bioscience).

For antibody blocking assays, the primary antibody was blocked with 5 µg recombinant polypeptide overnight at 4°C prior addition to the membranes. After this standard western blot protocols were followed.

Donor samples for electron microscopy (EM)

Electron microscopy datasets were created from nPOD donors. Additional donor details can be obtained through the JDRF nPOD online pathology database. Tissues were recovered following informed research consent from next of kin in the United States and shipped to the nPOD program at the University of Florida for processing as previously described[46-48]. All experiments were conducted under the approval of the University of Florida Institutional Review Board and the current study fulfills all requirements for tests as approved by the medical ethical review board of the University Medical Center Groningen.

Pancreas sample electron microscopy processing

Pancreas samples were fixed in cold, freshly prepared 2% paraformaldehyde-1% glutaraldehyde for 48 hours followed by transfer to phosphate-buffered saline for storage at 4°C before shipment to the Netherlands[48]. Tissue vibratome sections (~50 µm; Microm HM 650V) were post-fixed in osmium tetroxide/ potassium ferrocyanide, followed by dehydration and flat-embedding as previously reported[49]. Next, regions with islets were selected from toluidine stained 1 µm sections using light microscopy. Subsequent ultrathin (80 nm) sections were cut (UC7 ultramicrotome, Leica Microsystems, Vienna, Austria) and placed on formvar coated copper grids (Electron Microscopy Sciences, Hatfield, Pennsylvania). Finally, sections were contrasted with uranyl acetate as previously described[49, 50].

Immuno-EM

Post-embedding immunolabeling on epon with gold or quantum dots was carried out as described before[51], using rabbit-α-FSc serum.

EM acquisition and image processing (nanotomy)

Data were acquired on a Supra 55 scanning EM (SEM; Zeiss, Oberkochen, Germany) using a scanning transmission EM (STEM) detector at 28kV with 2.5 nm pixel size with an external scan generator ATLAS 5 (Fibics, Ottawa, Canada) as previously described[50, 52]. After tile stitching, data were exported as an html file and uploaded to www.nanotomy.org.

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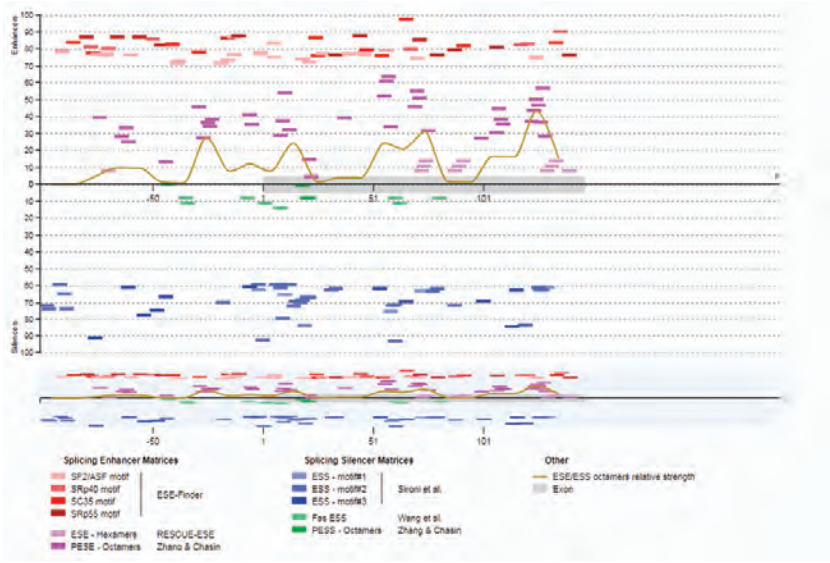
SUPPLEMENTARY INFORMATION

A



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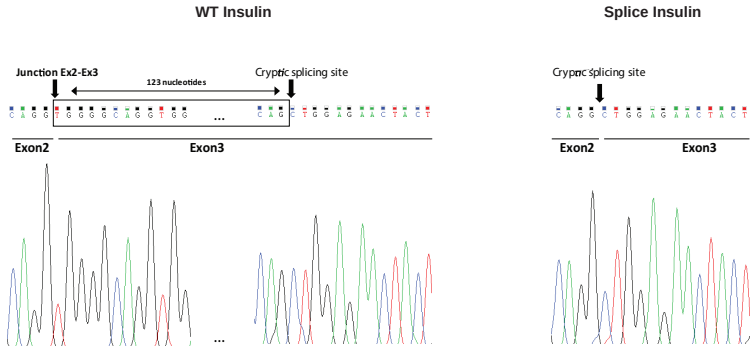


Supplementary figure 1: Alternative splice site analysis of the human insulin gene. A) Splicing analysis of the human insulin exon 3 with Human Splice Finder and ESE finder enhancers. The exon is schematically indicated by the grey bar, the intronic regions flanking the exon in a solid black line. Splicing enhancer and silence motifs are indicated by different colour bars along the sequence and further explained in the legend. The yellow line indicates the ESE/ESS relative strength. B) DNA sequence analysis of the retrieved PCR fragment spanning the splicing between 5′ donor site in exon 2 and the cryptic 3′ acceptor site in exon 3.

A

Recombinant PPI

```
  1 MSYYHHHHHH LESTSLYKKA GFTMALWMRL LPLLALLALW GPDPAAAFVN QHLCGSHLVE  ALYLVCGERG FFYTPKTRRE
 81 AEDLQVGQVE LGGGPGAGSL QPLALEGSLQ KRGIVEQCCT SICSLYQLEN YCN*
```

Recombinant INS-DRiP

```
  1 MSYYHHHHHH LESTSLYKKA GFTMLYQHLL PLPAGELLQL DAARRQPPTR RLLHRERWNK ALEPAK*
                                   DRiP1-13                 FS-c
```

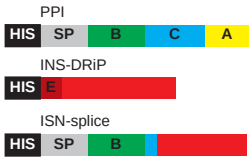
Recombinant INS-splice

```
  1 MSYYHHHHHH LESTSLYKKA GFTMALWMRL LPLLALLALW GPDPAAAFVN QHLCGSHLVE ALYLVCGERG FFYTPKTRRE
 81 AEDLQAGELL QLDAARRQPP TRRLLHRERW NKALEPAK*
                                   FS-c
```

Recombinant proSST

```
  1 MSYYHHHHHH LESTSLYKKA GFTMAPSDPR LRQFLQKSLA AAAGKQELAK YFLAELLSEP NQTENDALEP EDLSQAAEQD
 81 EMRLELQRSA NSNPAMAPRE RKAGCKNFFW KTFTSC*
```

B



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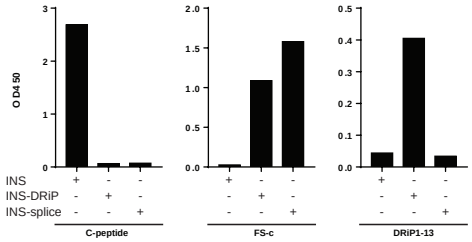
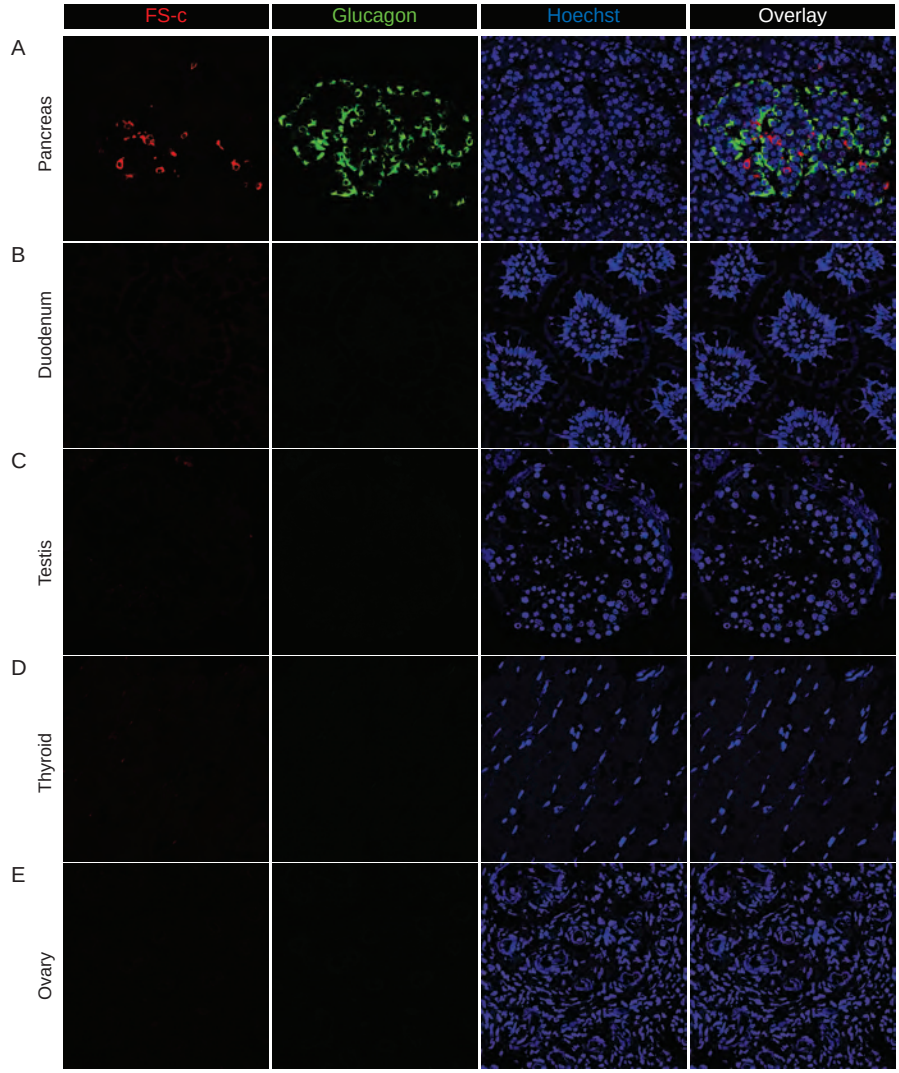
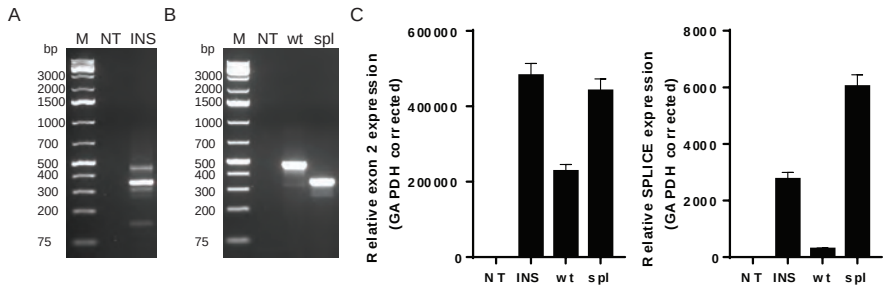


Supplementary figure 2: Recombinant polypeptides. A) Amino acid sequence of the recombinant polypeptides. Red underlined the location of the immunization peptides used for antiserum development are indicated. B) Schematic representation of the recombinant polypeptides. The His tag (black box), signal peptide (SP, grey), B-chain (B, green), C-peptide (C, blue), A-chain (A, Yellow) frame shift of INS-DRiP and INS-splice (red) are separately indicated. Corresponding amino acid sequences are indicated with corresponding colours, letters indicate the presence of the complete chain. The E indicates the INS-DRiP-specific CD8 T-cell epitope. C) Validation of serum specificity in an indirect ELISA assay to recombinant PPI, INS-DRiP and INS-splice using c-peptide antibody (left panel), FS-c antiserum (middle panel) and DRiP1-13 antiserum (right panel).



Supplementary figure 3: INS-splice is exclusively expressed in pancreatic tissue. Immunohistochemistry analysis of a variety of endocrine tissue for ins-splice (red) and glucagon (green). A) pancreas, B) Duodenum, C) Testis, D) Thyroid and E) Ovary. Nuclei are visualized by hoechst staining (blue).



Supplementary figure 4: Insulin splicing in transfected 293T cells. A) Analysis of insulin gene-derived transcript splicing in 293T cells after transfection with CMV-INS/full (INS) by PCR using the same primer set as described in Fig 1. B) Splicing analysis of transfected 293T cells with CMV-INS/wt-bc-GFP (wt) and CMV-INS/splice-bc-GFP (spl). Splicing patterns are compared to non-transfected 293T cells (NT). The DNA marker is indicated by the M. C) Primer specificity tested on RNA isolated from transfected 293T cells with CMV-INS/full (INS), CMV-INS/wt-bc-GFP (wt) and CMV-INS/splice-bc-GFP (spl) for exon 2 (right graph) and splice (left graph). All data are expressed as relative expression to non-transfected cells (NT) and GAPDH corrected.