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Autoimmunity against INS-IGF2 Protein Expressed in Human Pancreatic Islets*◆

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Background: Islet INS-IGF2 was examined as a possible autoantigen in type 1 diabetes.
Results: *INS-IGF2* expression was inversely related to donor HbA1c and glucose-stimulated insulin release. Autoantibodies doubly reactive with INS-IGF2 and insulin were more common in patients with type 1 diabetes than controls.
Conclusion: INS-IGF2 is recognized by autoantibodies in type 1 diabetes.
Significance: Autoantibodies doubly reactive with both INS-IGF2 and insulin may contribute to type 1 diabetes.

Insulin is a major autoantigen in islet autoimmunity and progression to type 1 diabetes. It has been suggested that the insulin B-chain may be critical to insulin autoimmunity in type 1 diabetes. INS-IGF2 consists of the preproinsulin signal peptide, the insulin B-chain, and eight amino acids of the C-peptide in addition to 138 amino acids from the *IGF2* gene. We aimed to determine the expression of *INS-IGF2* in human pancreatic islets and autoantibodies in newly diagnosed children with type 1 diabetes and controls. INS-IGF2, expressed primarily in beta cells, showed higher levels of expression in islets from normal compared with donors with either type 2 diabetes ($p = 0.006$) or high HbA1c levels ($p < 0.001$). INS-IGF2 autoantibody levels were increased in newly diagnosed patients with type 1 diabetes ($n = 304$) compared with healthy controls ($n = 355$; $p < 0.001$). Displacement with cold insulin and INS-IGF2 revealed that more patients than controls had doubly reactive insulin-INS-IGF2 autoantibodies. These data suggest that INS-IGF2, which contains the preproinsulin signal peptide, the B-chain, and eight amino acids of the C-peptide may be an autoantigen in type 1 diabetes. INS-IGF2 and insulin may share autoantibody-binding sites, thus complicating the notion that insulin is the primary autoantigen in type 1 diabetes.

Type 1 diabetes (T1D)[2] is strongly associated with a selective autoimmune destruction of the pancreatic beta cells (1, 2). The progressive loss of beta cells is often triggered at an early age (1, 3–5), and autoantibodies directed against islet autoantigens such as insulin, glutamic acid decarboxylase 65 (GAD65), islet antigen-2 (IA-2), and zinc transporter 8 (ZnT8) are currently the best markers of the islet autoimmunity that precede the clinical onset of T1D (6, 7). Insulin autoantibodies (IAA) may be the first islet autoantibodies to appear (8, 9). However, it is the least frequent (10) as IAA are particularly common in patients at a younger age of diagnosis (11). Persistent IAA in combination with any other islet autoantibody predicts T1D (12). Insulin iodinated with ^{125}I is primarily used to detect IAA (13, 14); however, IAA assays with iodinated insulin have proven difficult to standardize (15, 16). It has been speculated that the primary autoantigen is not matured insulin but rather proinsulin (17, 18) or perhaps preproinsulin (19, 20). It was hypothesized that beta cell endoplasmic reticulum fragments may become available to antigen-presenting cells following, for example, viral lysis of beta cells (21).

The immune recognition of (pro)insulin is not fully clarified as human studies are complicated by the fact that circulating T and B cells as well as antigen-presenting cells may not reflect an ongoing insulitis. T cells reactive with insulin have been detected in pancreatic lymph nodes from T1D patients. Proinsulin epitopes recognized by the T cell receptor (TCR) on CD4$^+$ T cells have been reported in children at HLA-risk for T1D prior to the development of IAA (22). CD4$^+$T cells seem preferentially to recognize (pro)insulin peptides from the B-chain when presented on HLA DR4 or DQ8 molecules (21). IAA have been associated with HLA-DQ8 that is in strong linkage disequilibrium with DR4 (23). The B cell receptor recognition may be broader as the binding site for IAA includes several B-chain amino acid residues (21). CD8$^+$ T cells were reported to express TCR recognizing B-chain residues 10–18 (20) and 9–23 (24) or signal peptide residues 15–24 (25, 26). These observations

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[2] The abbreviations used are: T1D, type 1 diabetes; T2D, type 2 diabetes; GAD65, glutamic acid decarboxylase 65; IA-2, islet antigen-2; ZnT8, zinc transporter 8; IAA, insulin autoantibody; TCR, T cell receptor; RBA, radiobinding assay; IGF2, insulin-like growth factor 2; GSIR, glucose-stimulated insulin release; DASP, Diabetes Antibody Standardization Program; GSIR, glucose-stimulated insulin release; CV, coefficient of variation; RBA, radiobinding assay.

suggest that the B-chain of insulin and perhaps also signal or B-C junction peptides may contribute to a preproinsulin autoimmune response. It is expected that this response would be reflected by the appearance of preproinsulin autoantibodies. The well established radiobinding assay (RBA) using coupled *in vitro* transcription-translation of autoantigen cDNA effectively detects a number of conformation-dependent autoantibodies (27–32). The aim of this study was to use coupled *in vitro* transcription-translation to test whether T1D patients may have conformation-dependent autoantibodies against the signal peptide-B-chain-B-C junction of preproinsulin.

The insulin gene (*INS*) and insulin-like growth factor 2 (*IGF2*) genes are located sequentially on chromosome 11 (33). The recently described 200-amino acid-long INS-IGF2 protein consists of the preproinsulin signal peptide (24 amino acids), the insulin B-chain (30 amino acids), and the C-peptide (8 amino acids) in addition to 138 amino acids coded for in the *IGF2* gene (34). However, expression of short *INS-IGF2* was restricted to human fetal pancreas and eye (34). The fact that the INS-IGF2 protein has the signal peptide-B-chain-B-C junction of preproinsulin makes INS-IGF2 a candidate autoantigen in T1D. Therefore, we first determined whether INS-IGF2 was expressed in adult human islets, and we then tested the hypothesis that INS-IGF2 autoantibodies measured by RBA were detected in newly diagnosed T1D patients and controls.

EXPERIMENTAL PROCEDURES

Human Pancreatic Islets—Islets from cadaver donors were provided by the Nordic Islet Transplantation Programme, Uppsala University, Uppsala, Sweden. Islets were obtained from 66 nondiabetic donors (30 females: age 59 ± 10; body mass index 25.9 ± 3.5; HbA1c 5.5 ± 1.1, and days of culture 3.5 ± 1.9 (mean values ± S.D.)) and 10 donors with type 2 diabetes (T2D) (four females and six males: age 60.7 ± 12; body mass index 28.1 ± 4.5; HbA1c 7.1 ± 1.2, and days of culture 2 ± 0.9). Purity of the islet preparations was assessed by dithizone staining and amounted to 60.1 ± 20% in the T2D and 70 ± 17% in the nondiabetic islets ($p = 0.10$). In addition, the contribution of exocrine and endocrine tissues was assessed by measuring expression of pancreatic lipase, α_2-amylase, and chymotrypsin 2 as markers of exocrine tissue and somatostatin and glucagon as markers of endocrine tissue (probes for insulin were unfortunately not included on the chip). Using this approach, the estimated contribution of islet endocrine tissue did not differ between nondiabetic (72%) and T2D (68%) donors ($p = 0.29$). We also measured insulin content as a surrogate marker for pancreatic islets beta cell mass in hyperglycemic (HbA1c >6.0%; 4.8 ± 3.2 ng/ml) and normoglycemic (HbA1c <6.0%; 5.6 ± 3.2 ng/ml; $p = 0.4$) donors, respectively. Prior to RNA isolation, the islets were cultured at 37 °C (5% CO_2) for 1–9 days in CMRL 1066 (ICN Biomedicals, Costa Mesa, CA) supplemented with 10 mmol/liter HEPES, 2 mmol/liter L-glutamine, 50 μg/ml gentamicin, 0.25 μg/ml Fungizone (Invitrogen), 20 μg/ml ciprofloxacin (Bayer Healthcare, Leverkusen, Germany), and 10 mmol/liter nicotinamide. All procedures were approved by Regional Ethics Boards at Uppsala and Lund Universities, respectively.

RNA Isolation—Total RNA was isolated with the AllPrep DNA/RNA mini kit (Qiagen, Hilden, Germany). RNA quality and concentration were measured using an Agilent 2100 Bio-Analyzer (Bio-Rad) and Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE).

Microarray Gene Expression in Human Pancreatic Islets—The microarrays were performed using the Affymetrix standard protocol. Briefly, 100–200 ng of total RNA was processed according to the instructions of the GeneChip® expression 3′-amplification reagents one-cycle cDNA synthesis kit (Affymetrix Inc., Santa Clara, CA) to produce double-stranded cDNA. This was used as a template to generate biotin-targeted cRNA following the manufacturer's specifications. A total of 15 μg of the biotin-labeled cRNA was fragmented to strands between 35 and 200 bases in length, 10 μg of which were hybridized onto the GeneChip® human gene 1.0 ST whole transcript-based assay overnight in the GeneChip® hybridization oven 6400 using standard procedures. The arrays were washed and stained in a GeneChip® Fluidics Station 450. Scanning was carried out with the GeneChip® Scanner 3000, and image analysis was performed using GeneChip® operating software. The array data were summarized and normalized with the Robust Multiarray Analysis method using the affy R package. All data are MIAME-compliant, and the raw data have been deposited in a MIAME database (GEO, accession numbers GSE 38642 and GSE 44035).

Glucose-stimulated Insulin Release (GSIR)—Islets were handpicked under a stereomicroscope at room temperature and first incubated for 30 min at 37 °C in Krebs-Ringer bicarbonate (KRB) buffer, pH 7.4, containing (in mmol/liter) 120 NaCl, 25 $NaHCO_3$, 4.7 KCl, 1.2 $MgSO_4$, 2.5 $CaCl_2$, 1.2 KH_2PO, 10 HEPES supplemented with 0.1% bovine serum albumin, HEPES (10 mmol/liter), and 1 mmol/liter glucose. Each incubation vial contained 12 islets in 1.0 ml of KRB buffer solution and was treated with 95% O_2, 5% CO_2 to obtain a constant pH 7.4 and oxygenation. After the first incubation, the buffer was changed to KRB buffer containing either 1 mM (basal secretion) or 16.7 mM glucose (stimulated secretion). The islets were then incubated for 1 h at 37 °C in a metabolic shaker (30 cycles/min). Immediately after incubation, an aliquot of the medium was removed for analysis of insulin using a radioimmunoassay kit (Euro-Diagnostica, Malmö, Sweden). Insulin content in homogenized human islets was assessed by ELISA (Mercodia, Uppsala, Sweden), and values were normalized to the total DNA in each sample as determined by a fluorometric assay (Quant-iT PicoGreen, Invitrogen).

Immunocytochemistry of Human Pancreas—Human pancreatic specimens taken during pancreatic surgery were used. Briefly, primary antibodies were diluted in PBS containing 0.25% bovine serum albumin and 0.25% Triton X-100 and applied overnight at 4 °C. The primary antibodies were mouse polyclonal anti-human INS-IGF2 (dilution 1:100, code BO1P, Abnova Corp., Taipei, Taiwan), guinea pig polyclonal anti-human proinsulin (dilution 1:5000, code 9003, Euro-Diagnostica, Malmö, Sweden), guinea pig polyclonal anti-human glucagon (dilution 1:5000, code 8708, Euro-Diagnostica), goat polyclonal anti-human somatostatin (dilution 1:800, code SC7819, Santa

TABLE 1
Characteristics of patient serum samples, frequencies of islet autoantibodies and HLA

	T1D (1–5 years)	T1D (15–18 years)	Controls
n	152	152	355
Median age (years) (Range)	4.4 (1.0–5.9)	16.1 (15.0–17.9)	12.6 (11.5–25.0)
Gender (% male)	49.3	66.4	48.5
HLA-DQ			
2/8	65 (43%)	34 (22%)	ND[a]
8/8, 8/*x*	54 (36%)	66 (43%)	ND
2/2, 2/*x*	17 (11%)	25 (16%)	ND
x/*x*	16 (11%)	27 (18%)	ND
Islet autoantibodies			
GAD65A	71 (47%)	101 (66%)	3/343 (0.9%)
IAA	87 (57%)	27 (18%)	2/180 (1.1%)
IA2A	113 (74%)	127 (84%)	0/343 (0%)
ZnT8WA	63 (41%)	78 (51%)	3/343 (0.9%)
ZnT8RA	72 (47%)	80 (53%)	3/343 (0.9%)
ZnT8QA	36 (24%)	46 (30%)	3/343 (0.9%)

[a] ND means not determined.

Cruz Biotechnology, Inc., Santa Cruz, CA), sheep polyclonal anti-human pancreatic polypeptide (dilution 1:640, code AHP 515, Serotec, Oxford, UK), or goat polyclonal anti-human ghrelin (dilution 1:1000, code SC10368, Santa Cruz Biotechnology). Secondary antibodies specific for mouse, guinea pig, or goat IgG and coupled to either Cy2, Texas Red, or 7-amino-4-methylcoumarin-3-acetic acid (Jackson ImmunoResearch) were applied for 1 h at room temperature. Immunofluorescence was examined in an epifluorescence microscope (BX60; Olympus, Lund, Sweden). Images were taken with a digital camera (DS-2Mv; Nikon, Lund, Sweden).

Patients—Serum samples were from 304 (54% male) patients with newly diagnosed T1D in the Better Diabetes Diagnosis study (10, 35). Patient sera were selected at random to represent one group of 152 patients with clinical onset at 1–5 years of age and another group of 152 patients at 15–18 years of age (Table 1). The classification of diabetes was clinically confirmed 6 months after onset with T1D (10, 35). HLA-DQ typing and analyses of islet autoantibodies against GAD65, insulin, IA-2, and ZnT8 (Table 1) were described in detail elsewhere (28, 35).

Controls—Serum samples were obtained from a total of 355 healthy controls (Table 1). Among these were 300 school children (47.3% males), 11.5–13.7 (range, median age 12.5) years old, as described previously (36). These children were previously analyzed for multiple autoantibodies (37, 38). In addition, 55 healthy adult blood donors (54.5% males, $n = 30$) aged 19.0–25.0 years (range, median age 23.0) were also investigated (39).

Preparation of INS-IGF2—*INS-IGF2* cDNA, based on the sequence in the National Center for Biotechnology Information (NCBI), was purchased in the pJ201 vector from DNA 2.0 Inc. (DNA 2.0 Inc. Menlo Park, CA).

Preparation of the pThINS-IGF2 Vector—The cDNA of *INS-IGF2* was cut from the pJ201 vector (DNA2.0 Inc.) with EcoRI and NotI (FastDigest™, Fermentas Sweden AB, Helsingborg, Sweden) using the FastDigest buffer 10× (Fermentas). Following 30 min of digestion at 37 °C, the pTNT™ vector was dephosphorylated with calf intestinal alkaline phosphatase (Fermentas) at a final concentration of 0.04 units/μl of DNA termini according to instructions from the supplier.

The linearized pTNT™ vector and cDNA of *INS-IGF2* were analyzed by gel electrophoresis in 1% agarose, and the two bands were extracted using a gel purification kit (QIAquick gel extraction kit, Qiagen AB, Solna, Sweden) according to the manufacturer's instructions. The ligation reaction with T4 ligase (New England Biolabs, Inc.), optimized to 1:3 (*INS-IGF2*) vector/insert molar ratio, was carried out at room temperature for 1 h. Subcloning efficiency DH5α-competent cells were used for transformation according to the manufacturer's instructions (Invitrogen). The ligation reaction (10 μl) was added to the cells (50 μl) and mixed gently, and after 30 min incubation on ice, the cells were heat-shocked for 90 s in a 42 °C incubator. The cells were immediately put on ice for 2 min and then incubated for 1 h at 37 °C in 500 μl of LB/ampicillin medium with shaking (300 rpm).

A total of 200 μl of cell suspension were plated onto LB-ampicillin plates and incubated overnight at 37 °C. White colonies were selected and transferred separately to a new set of LB-ampicillin plates for overnight culture before the cells were incubated for 16 h at 37 °C in 3 ml of LB-ampicillin medium. The bacterial cells were harvested by centrifugation for 10 min at 4 °C at 4000 rpm.

The pTh*INS-IGF2* plasmid DNA was extracted using the QIAprep spin miniprep kit (Qiagen AB). The insert was sequenced by GATC Biotech AG (Konstanz, Germany) using the 5′-TTA CGC CAG CCC GGA TCC-3′and 5′-AAG GCT AGA GTA CTT AAT ACG A-3′ as the reverse and forward primers (DNA Technology A/S, Risskov, Denmark), respectively.

Coupled in Vitro Transcription-Translation of pThINS-IGF2—The pTNT™ vector (Promega, Madison, WI) was used for *in vitro* coupled transcription-translation system in the presence of [^{35}S]methionine and cysteine (28). Briefly, the reaction mixture at a final volume of 100 μl contained 3 μg of pTh*INS-IGF2* vector, 50 μl of TNT® rabbit reticulocyte lysate, 4 μl of TNT® reaction buffer, 2 μl of amino acid mixture minus methionine and cysteine, 2 μl of RNasin® ribonuclease inhibitor, 2 μl of SP6 RNA polymerase (all from Promega), 4 μl of [^{35}S]methionine and cysteine (EasyTag™ EXPRESS^{35}S protein labeling mix, ^{35}S (NEG772, L-[^{35}S]methionine, 1175.0 Ci/mmol, L-[^{35}S]cysteine, 1075.0 Ci/mmol) from PerkinElmer Life Sciences), and nuclease-free water. The reaction mixture was incubated for 90 min at 30 °C with shaking at 300 rpm (Eppendorf Thermomixercomfort, Eppendorf AG, Hamburg, Germany).

The translation product was immediately subjected to gel filtration on Illustra™ NAP-5 columns (GE Healthcare). All fractions prior to the hemoglobin band were discarded. Subsequent fractions were one 300-μl fraction followed by several 100-μl fractions. Radioactivity incorporated into protein was determined (1450MicroBeta TriLuxMicroplate Scintillation-Luminescence Counter, PerkinElmer Life Sciences), and peak fractions of radioactivity were pooled.

Preparation of nonradioactive INS-IGF2 was carried out essentially as described above but without radioactive amino acids as 2 μl of Complete Amino Acid Mixture (Promega) was added to the TNT® rabbit reticulocyte lysate. The translation product was filtered on the NAP-5 columns, and fractions corresponding to the typical peak fractions of radioactivity labeling above were collected.

Electrophoresis of pThINS-IGF2 Translation Products—The labeled antigen produced in the *in vitro* transcription-translation kit was analyzed by SDS-PAGE (ClearPage™ precast 16%, VWR, West Chester, PA, according to the manufacturer's recommendations). The samples were incubated in sample buffer with reducing agent (ClearPage™ accessories, VWR) for 10 min at 70 °C and were loaded on the gel with a ^{14}C-labeled molecular weight markers (Protein Molecular Weight Markers, methyl-^{14}C-methylated (NEC811, 26.4 Ci/mg), PerkinElmer Life Sciences) and with unlabeled protein ladders (PageRuler™ Plus prestained protein ladder (SM1811) Fermentas). The gel was dried for 72 h at room temperature using a gel drying kit (Promega) according to the manufacturers' instructions.

The gel was placed in contact with x-ray-sensitive film (Hyperfilm™ ECL, GE Healthcare) for 72 h at room temperature. The film was treated with developing and fixation solutions following the manufacturer's instructions (DAB Dental, Malmö, Sweden).

RBA for INS-IGF2 Autoantibodies—The RBA for INS-IGF2 autoantibodies was carried out overnight at 4 °C in duplicate samples of 5 μl of serum incubated with 60 μl of labeled antigen at around 425 cpm/μl diluted in antigen buffer (150 mM NaCl, 20 mM Tris, 0.15% (v/v) Tween 20 (MP Biomedicals, LLC, Santa Ana, CA), 1% (v/v), Triton X-100 (Sigma), 0.1% (w/v) BSA (MP Biomedicals, LLC), pH 7.4). (All serum samples were diluted by adding 3 parts of antigen buffer to 1 part of serum before incubating with labeled antigen.) V-formed 96-well plates (Nunc V96 MicroWell™ plates, Nunc A/S, Roskilde, Denmark) were used. A total of 50 μl of reaction mixture was then incubated for 1 h at 4 °C with 50 μl of protein A-Sepharose 4B conjugated (20%, washed four times at 4 °C by sedimentation in antigen buffer) (Invitrogen) in a 96-well filtration plate (MultiScreen$_{HTS}$-DV Plate, Millipore AB, Solna, Sweden). The plate was then washed eight times with wash buffer (150 mM NaCl, 20 mM Tris, 0.15% Tween 20 (MP Biomedicals, LLC), pH 7.4) using a microplate washer (ELx50™ Microplate Strip Washer, Biotek Instruments, Inc., Winooski, VT). Antibody-bound radioactivity was counted in a β-counter (1450 MicroBeta® TriLux, PerkinElmer Life Sciences). Sepharose-bound radioactivity was converted into units/ml using individual standard curves generated by six-step doubling dilutions (1:128 to 1:4 dilutions) of high titer T1D patient's sera with high reactivity for the INS-IGF2 antigen. Three internal quality control samples, two from healthy subjects and one from a long term diabetes subject, were used in every assay. Intra-assay coefficient of variation (CV) and inter-assay CV for duplicate determinations were 4.1 and 5.1%, respectively.

RBA for GAD65A and IA-2A—These autoantibodies were analyzed as described in detail elsewhere (35). GAD65A and IA-2A levels were expressed as units/ml derived from the World Health Organization standard 97/550 (40). Samples were considered positive if GAD65A levels were >50 units/ml and IA-2A levels >10 units/ml. The intra-assay CV for duplicates in the GAD65A assay was 7% and in the IA-2A was 11%. In the Diabetes Autoantibody Standardization Program (DASP) 2010 workshop, the workshop sensitivity was 80% and specificity 99% for GAD65A and 60% sensitivity and 99% specificity for IA-2A.

RBA for ZnT8 Arg-, Trp-, and Gln-variant Autoantibodies—These autoantibodies were analyzed as described previously (28). The 2010 Diabetes Autoantibody Standardization Program workshop (41) showed that our laboratory had a workshop sensitivity of 52% for ZnT8RA, 50% for ZnT8WA, and 38% for ZnT8QA. The workshop specificity was 100% for all three variant autoantibodies.

RBA for Insulin Autoantibodies—Insulin autoantibodies were determined essentially as described (42). All samples were analyzed without and with cold insulin (2 IU/ml; Actrapid®, Novo Nordisk A/S, Bagsvaerd, Denmark) to control for nonspecific binding. Briefly, the RBA for IAA was carried out 48 h at 4 °C in duplicate samples of 7 μl of diluted serum samples incubated with 36 μl of human recombinant ^{125}I-insulin (2200 Ci/mmol from PerkinElmer Life Sciences) at around 40,000 cpm/36 μl diluted in antigen buffer (50 mM Tris, 1% (v/v) Tween 20 (Sigma), 1% (w/v) BSA (Sigma), pH 8.0). V-formed 96-well plates (Nunc V96 MicroWell™ plates, Nunc A/S, Roskilde, Denmark) were used. A total of 25 μl of reaction mixture was then incubated for 1.5 h at 4 °C with 50 μl of protein A-Sepharose 4B conjugated (40%, washed four times at 4 °C by sedimentation in antigen buffer) (Invitrogen) in a 96-well filtration plate (MultiScreen$_{HTS}$-DV Plate, Millipore AB). The plate was then washed 10 times with wash buffer (50 mM Tris, 1% Tween 20 (Sigma), pH 8.0) using a microplate washer (ELx50™ Microplate Strip Washer, Biotek Instruments, Inc.). Supermix scintillation solution (50 μl) was added to the wells after the plate had dried for 20 min. The radioactivity was measured in a β-counter (1450 MicroBeta® TriLux, PerkinElmer Life Sciences). Sepharose-bound radioactivity was converted into units/ml using individual standard curves generated by six-step doubling dilutions (1:512 to 1:8 dilutions) of high titer T1D patient's sera with high reactivity for IAA. Three internal quality control samples, two from healthy subjects and one from a long term diabetes subject, were used in every assay. Sepharose-bound radioactivity was converted into units/ml using individual standard curves generated by six-step doubling dilutions of high titer T1D patient's sera with high reactivity for each individual IAA.

Our assays showed comparable precision (intra-assay CV was 6.0% for IAA), and reproducibility (inter-assay CV) was 13.2%. Our laboratory participated in the DASP (14) to show 26% sensitivity and 100% specificity on the DASP samples.

Statistical Analysis—The IBM® SPSS® Statistics version 20 (IBM Corp., Armonk, NY) and GraphPad Prism version 5.03 for Windows (GraphPad Software, Inc., La Jolla, CA) were used for statistical analysis. Differences in titers of INS-IGF2 antibody between controls and patients were assessed by Mann-Whitney U test (two-tailed). Correlations were assessed by Spearman's ρ test and Fisher's exact test to compare frequencies.

RESULTS

Microarray Gene Expression in Human Pancreatic Islets—The *INS-IGF2* transcript (Fig. 1) was detected in human islets (Fig. 2). Levels of expression in islets from 66 normal donors were 2.5-fold higher than that of *IGF2* ($p < 0.001$; Fig. 2). The *IA-2* autoantigen gene, used for comparison, showed 3.2-fold higher levels than *INS-IGF2* ($p < 0.001$; Fig. 2*A*). In addition, in

pancreatic islets from 10 organ donors with T2D, the expression level of *INS-IGF2* was lower compared with the controls ($p = 0.006$). Islets from T2D organ donors also showed lower expression levels compared with controls of both *IGF2* ($p = 0.017$) and *IA-2* ($p = 0.009$). Among the 66 control donors there

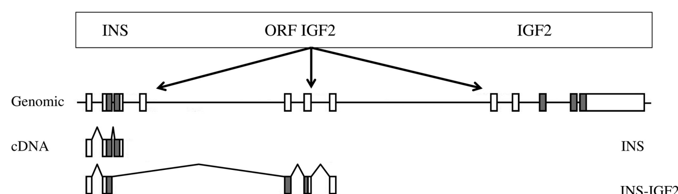

FIGURE 1. **Genomic structure of the preproinsulin (*INS*) and *IGF2* genes on chromosome 11.** The cDNAs for the *INS* and *INS-IGF2* genes are depicted essentially as described (34). The *INS-IGF2* cDNA is a splice variant of *INS* (signal peptide, B-chain and eight amino acids of the C-peptide) and the coding sequences of the two proximal ORFs of the *IGF2* gene. It should be noted that the *IGF2* gene ORFs are normally noncoding exons and thus insert a novel 138-amino acid C-terminal region unrelated to prepro-IGF2 (34). *Shaded boxes* represent ORFs.

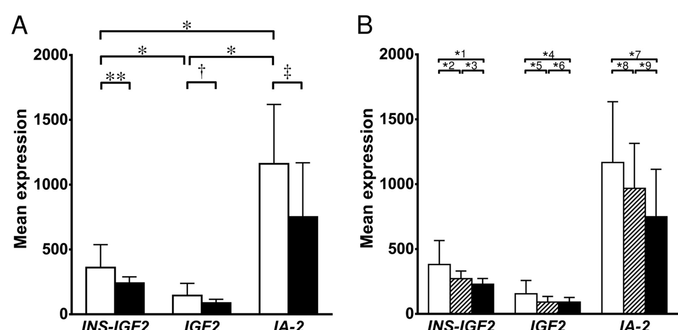

FIGURE 2. **Gene expression of *INS-IGF2, IGF2,* and *IA-2* in human pancreatic islets.** *A,* mean expressions (mean ± S.D.) of *INS-IGF2, IGF2,* and *IA-2* are shown in isolated human pancreatic islets obtained from nondiabetic controls ($n = 66$; *open columns*) and T2D patients ($n = 10$; *closed columns*). Gene expression, compared with controls, was lower in the T2D islet for *INS-IGF2* (**, $p = 0.006$), *IGF2* (†, $p = 0.017$), and *IA-2* (‡, $p = 0.009$). Differences in gene expression between the three genes are also indicated (*, $p < 0.001$). *B,* mean expressions (mean ± S.D.) of *INS-IGF2, IGF2,* and *IA-2* are shown for isolated human pancreatic islets obtained from donors with HbA1c being <6.0% ($n = 41$, *open bars*), 6.0–6.4% ($n = 12$, *hatched bars*), or ≥6.5% (T2D; $n = 10$; *closed bars*). Differences in gene expression between the three groups are also indicated as follows: **1*, $p < 0.001$; **2*, $p = 0.027$; **3*, $p = 0.059$; **4*, $p = 0.010$; **5*, $p = 0.015$; **6*, $p = 0.976$; **7*, $p = 0.006$; **8*, $p = 0.154$; and **9*, $p = 0.104$.

were 23 with HbA1c above 6.0% on the day of islet isolation (referred to as donors with hyperglycemia). The data in Fig. 2*B* demonstrate that *INS-IGF2* gene expression was reduced in islets from donors with 6.0–6.4% HbA1c ($n = 12$) and from donors with T2D who all had HbA1c ≥6.5% ($n = 10$) compared with donors with HbA1c <6% ($n = 43$).

Data on GSIR was available in human islets from 64 donors as determined in batch-type incubations (Fig. 3). As *INS-IGF2* gene expression was affected by donor HbA1c levels, it could not be excluded that a possible correlation between GSIR and *INS-IGF2* was similarly affected. The possible interrelation was therefore depicted in three-dimensional displays (Fig. 3). It can be seen from the data in Fig. 3*A* that the higher the *INS-IGF2* expression, the lower the HbA1c, and the higher the GSIR. This conclusion was supported by the observation (not illustrated) that GSIR was positively correlated with increased expression of *INS-IGF2* ($r = 0.284$; $p = 0.023$) primarily in donors with low HbA1c ($r = -0.446$; $p < 0.001$). Expression of *IGF2* in relation to GSIR and HbA1c showed a comparable pattern (Fig. 3*B*). However, *IGF2* expression was not correlated to GSIR ($r = 0.167$; $p = 0.186$) despite that the expression of *IGF2* was lower in hyperglycemic donors ($r = -0.394$; $p = 0.001$). *IA-2* expression in relation to both GSIR and HbA1c showed a pattern of higher GSIR in islets from donors with low HbA1c (Fig. 3*C*). Indeed, *IA-2* expression correlated positively with GSIR ($r = 0.501$; $p < 0.001$) especially in islets from normoglycemic donors ($r = -0.333$; $p = 0.008$).

Immunocytochemistry of Human Pancreas—The expression of immunoreactive INS-IGF2 in human islets was examined with a mouse polyclonal antiserum against full-length human INS-IGF2 (Fig. 4). Using triple-immunofluorescence and overlay, INS-IGF2 was detected in beta cells (Fig. 4, compare *A, B,* and *D*) and alpha cells (Fig. 4, compare *A, C,* and *D*), in a subpopulation of delta cells, but not in pancreatic polypeptide cells or ghrelin cells (data not shown).

In Vitro Transcription-Translation of INS-IGF2 cDNA—*INS-IGF2* cDNA, subjected to *in vitro* transcription-translation in the presence of both [^{35}S]methionine and [^{35}S]cysteine, showed an incorporation rate of 8–17% dependent on a batch of the rabbit reticulocyte lysate system compared with 2% when the

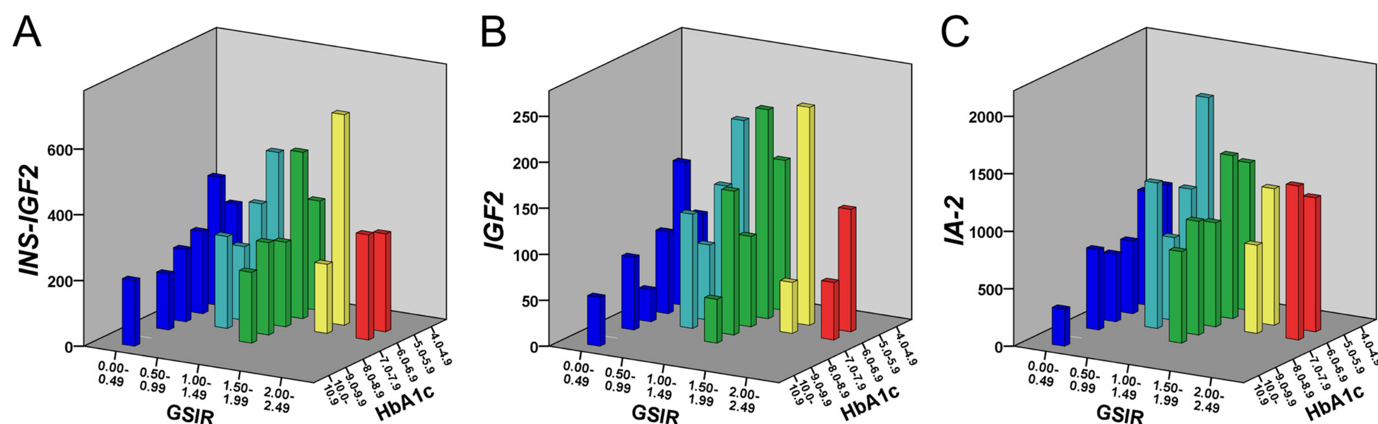

FIGURE 3. **Mean gene expression of *INS-IGF2, IGF2,* and *IA-2* in human pancreatic islets in relation to GSIR and HbA1c levels (%) of the islet donors.** Mean gene expression is shown for *INS-IGF2* (*A*), *IGF2* (*B*), and *IA-2* (*C*). The expression of *INS-IGF2* was correlated to GSIR ($r = 0.284$; $p = 0.023$) primarily in donors with low HbA1c ($r = 0.446$; $p < 0.001$). *IGF2* expression was not related to GSIR ($p = 0.186$) despite the lower expression in islets from donors with high HbA1c ($r = 0.394$; $p = 0.001$). Islets with increased *IA-2* expression showed higher GSIR ($r = 0.501$; $p < 0.001$) especially in islets from donors with low HbA1c ($r = 0.333$; $p = 0.008$).

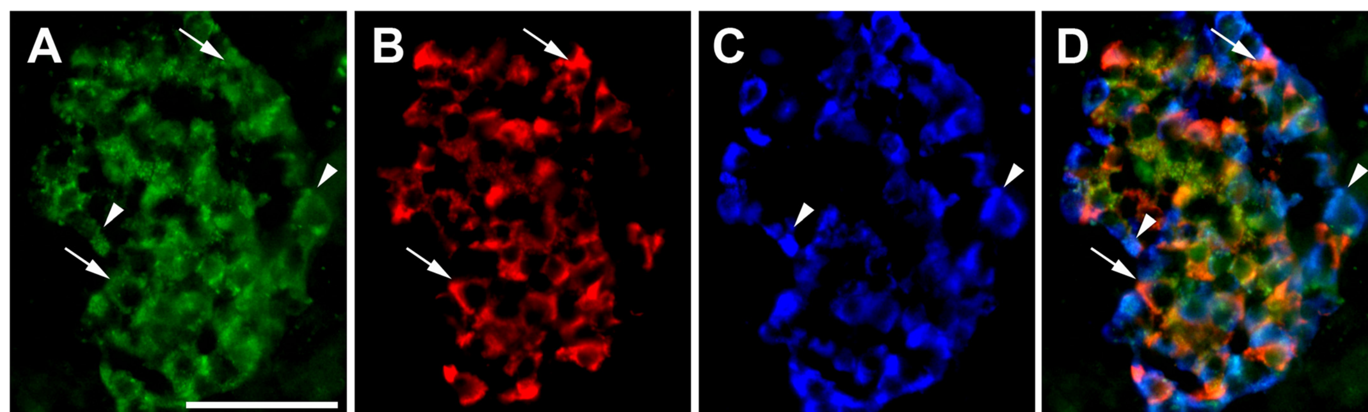

FIGURE 4. **Triple immunofluorescence photomicrographs of sections of human pancreas.** Immunostaining for INS-IGF2 (*A*), insulin (*B*), and glucagon (*C*) and the overlay show co-localization with both glucagon and insulin (*D*). Co-localization with insulin is indicated with *arrows*. Co-localization with glucagon is indicated with *arrowheads*. The *scale bar* indicates 50 μm.

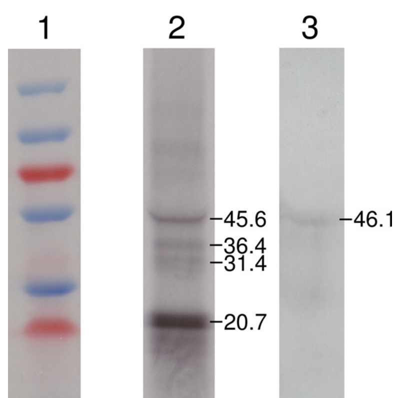

FIGURE 5. **SDS-gel electrophoresis and autoradiography of *in vitro* transcribed and translated INS-IGF2.** *Lane 1,* nonisotope protein ladder representing proteins of 250, 130, 100, 70, 55, 35, and 25 kDa. *Lane 2,* ^{35}S-INS-IGF2 generated by *in vitro* transcription-translation. The *major band* of 20.7 kDa represents INS-IGF2. The *minor bands* indicated may represent nonspecific components or an aggregate product. *Lane 3,* residual radioactivity after *in vitro* transcription-translation without added cDNA.

labeling reaction was run without added cDNA. SDS-gel electrophoresis and autoradiography revealed a major translation product at the expected M_r 21,500 for INS-IGF2 (Fig. 5, *lane 2*). Densitometric scanning showed that 27.1% of the total radioactivity was found in the major 21,500 band. The minor bands at higher molecular mass may represent multimers of INS-IGF2. There was a shadow of radioactivity when the labeling reaction was carried out without added cDNA (Fig. 5, *lane 3*).

RBA for INS-IGF2 Autoantibodies—Serum from patient MD, used as standard in the IAA assay showed the highest binding of ^{125}I-insulin at 1:2 dilution (Fig. 6*A*). Recombinant human insulin (2 IU/ml) fully displaced the binding of ^{125}I-insulin at all dilutions (Fig. 6*A*). Patient MD also showed binding of ^{35}S-INS-IGF2 in a dilution-dependent manner with maximal binding at a dilution of 1:4 (Fig. 6*B*). There was no further increase at a dilution of 1:2 (data not shown). The binding was displaced by about 50% at all serum dilutions by cold INS-IGF2 produced by *in vitro* transcription-translation (Fig. 6*B*). The data in Fig. 6*B* also demonstrate that cold insulin was less efficient than cold INS-IGF2 to displace the binding of INS-IGF2 to the serum of patient MD. The competition between ^{35}S-INS-IGF2 binding to the serum of patient MD (tested at a dilution of 1:4) and various concentrations of cold antigens showed that only cold INS-IGF2 was effective to displace the binding of ^{35}S-INS-IGF2 (Fig. 6*C*).

RBA for INS-IGF2 and Insulin Doubly Reactive Autoantibodies—Autoantibodies against INS-IGF2 (INS-IGF2A) were next examined in serum from healthy controls ($n = 355$) and newly diagnosed T1D patients ($n = 304$). To correct for nonspecific binding, all serum samples were displaced with either cold INS-IGF2 (specific INS-IGF2A) or cold insulin (insulin-specific INS-IGF2A) (Fig. 7). The median binding for specific INS-IGF2A showed that both 1–5-year-old ($p < 0.001$) and 15–18-year-old ($p < 0.001$) T1D patients differed from the controls. The specific INS-IGF2A did not differ between the two patient groups ($p = 0.204$). The displacement with cold insulin to reveal insulin-specific INS-IGF2A showed that both 1–5-year-old ($p < 0.001$) and 15–18-year-old ($p < 0.001$) T1D patients also differed from the controls (Fig. 7). However, the insulin-specific INS-IGF2A showed higher median levels in younger compared with older patients ($p < 0.001$).

Finally, specific INS-IGF2A (displaced with cold INS-IGF2) and insulin-specific INS-IGF2A (displaced with cold insulin) correlated in many but not all serum samples from controls ($r^2 = 0.195$, $p < 0.001$; Fig. 8*A*), T1D 1–5 year olds ($r^2 = 0.380$, $p < 0.001$; Fig. 8*B*), and T1D 15–18 year olds ($r^2 = 0.426$, $p < 0.001$; Fig. 8*C*). Samples displaced by both cold INS-IGF2 and by cold insulin are referred to as doubly reactive INS-IGF2A. The data in Fig. 8 suggest that doubly reactive INS-IGF2A was found in 85% (129/152) T1D 1–5 years old ($p < 0.001$) and in 74% (112/152) T1D 15–18 year olds ($p < 0.001$) compared with 48% (169/355) among the controls.

DISCUSSION

The major finding in this study is the possibility that INS-IGF2 is a novel autoantigen in T1D. This conclusion is based on several observations. First, INS-IGF2 could be specifically demonstrated in both insulin and glucagon cells by immunocytochemistry as well as by gene expression analyses of human islets from several organ donors. Second, the binding of ^{35}S-INS-IGF2 was higher in serum from T1D patients than in controls,

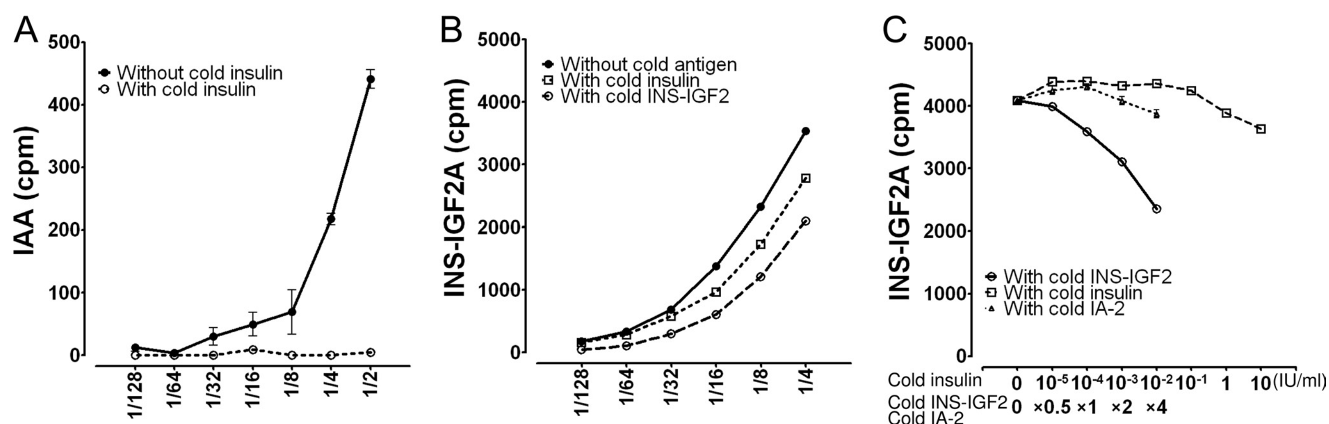

FIGURE 6. **Radiobinding assays for autoantibodies against insulin (IAA) and INS-IGF2 (INS-IGF2A) in type 1 diabetes serum (patient MD) used as in-house standard.** *A,* IAA assay with ^{125}I-insulin at various dilutions of the MD standard without (*closed circles*) and with an excess of cold insulin (2 IU/ml; *open circles*). Mean values ± S.E. ($n = 4$). *B,* INS-IGF2A assay with ^{35}S-INS-IGF2 at various dilutions of the MD standard without (*closed circles*) and with an excess of cold insulin (11 IU/ml; *open squares*) and cold INS-IGF2 (2× of the labeled INS-IGF2). Mean values ± S.E. ($n = 4$). *Error bars* are within the symbols. *C,* ^{35}S-INS-IGF2A in the MD standard serum diluted 1:4 in the presence of different concentrations of either cold INS-IGF2 (*open circles*), cold insulin (*open squares*), or cold IA-2 (*closed triangles*). *Error bars* are mostly within the symbols. Mean ± S.E. ($n = 6$).

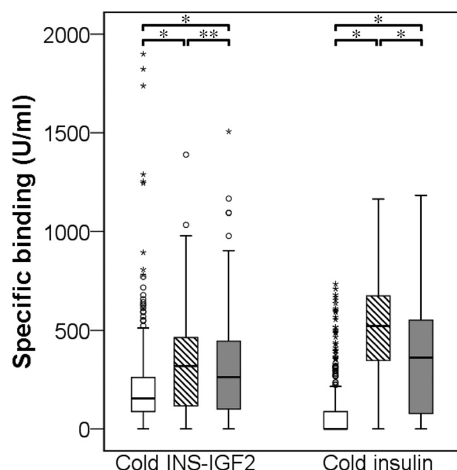

FIGURE 7. **Specific INS-IGF2 autoantibody levels (units/ml) after displacement with either cold INS-IGF2, generated by *in vitro* transcription-translation and used at a concentration representing twice the labeled INS-IGF2, or with 11 IU/ml cold insulin.** The *box plots* with whiskers and outliers show the median levels of specific binding for 355 controls (*open box*), 152 type 1 diabetes patients 1–5 years of age (*hatched box*), and 152 type 1 diabetes patients 15–18 years of age (*gray box*). Differences between the groups are indicated by *, $p < 0.001$, and **, $p = 0.204$.

and this binding could be displaced both by cold INS-IGF2 and cold insulin. Furthermore, our data suggest that not only patients but also some control subjects may have autoantibodies that were able to recognize both ^{125}I-insulin and ^{35}S-INS-IGF2. Indeed, the displacement analyses (Fig. 8) suggest that as many as 47% of controls compared with 85% among the 1–5 year olds and 74% among the 15–18 year old patients may have doubly reactive autoantibodies. The definition of a doubly reactive autoantibody sample would be that the sample is binding both ^{125}I-insulin and ^{35}S-INS-IGF2 and displaced by both cold insulin and cold INS-IGF2. The doubly reactive INS-IGF2A may be explained in part by the fact that insulin and INS-IGF2 share the complete B-chain sequence. The partial displacement of INS-IGF2 binding by cold insulin may be explained by such autoantibodies.

The fact that cold insulin in many samples did not fully displace binding is likely to be explained by autoantibody binding to non-B-chain epitopes of the INS-IGF2 molecule. As 47% (controls) and 85% (patients) had insulin-INS-IGF2 doubly reactive autoantibodies, it cannot be excluded that INS-IGF2 is a more immunogenic entity than insulin. This would be consistent with the report that T1D patients may have specific T cell reactivity against the signal peptide (19, 43) as well as C-peptide sequences (44, 45) that are part of both preproinsulin and INS-IGF2 but not insulin (Fig. 1). The insulin-INS-IGF2 doubly reactive sera complicate the understanding of insulin and INS-IGF2 as autoantigens. It is undisputable that they have sequence homologies. Although the autoimmunogenicity of the C-terminal end of INS-IGF2 remains to be determined, it cannot be excluded that also this part of the molecule contributes to the autoantibody binding.

Insulin autoantibodies were first described in 1983 using ^{125}I-insulin and acid charcoal to separate free from antibody-bund ^{125}I-insulin (46). In early serum exchange exercise workshops, only radiobinding assays but not ELISA tests were shown to detect disease-associated IAA (47–49). Despite efforts in assay improvements (42), the IAA as opposed to GADA, IA-2A, and also ZnT8A remained the least successful islet autoantibody assay to standardize (14, 39, 41, 50). We speculate that the inherent difficulties to standardize the IAA assay may be due to the presence of autoantibodies reactive with INS-IGF2.

It has not yet been possible to identify the mechanisms that trigger the formation of IAA. Current data suggest that IAA are more common in children developing T1D below the age of 5 compared with children older than 15 years of age (51). Indeed, in children born to parents with T1D who were followed from birth, IAA tends to be the first islet autoantibody. This early insulin autoantigenicity is still not understood, but it cannot be excluded that the combination of HLA-DQ (IAA are associated with HLA-DQ8) and insulin gene expression (reduced expression of insulin in the thymus is associated with risk for T1D through a genetic polymorphism in the *INS* gene) promote insulin autoreactivity particularly in young children. Our finding that INS-IGF2 was expressed in human islets would support

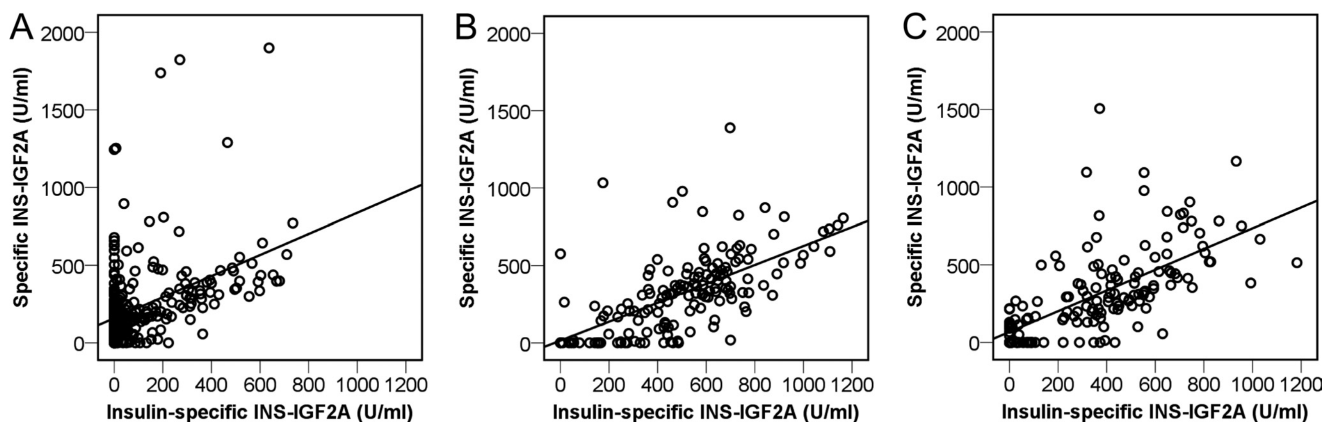

FIGURE 8. **Correlation between specific INS-IGF2A (units/ml) in sera from controls and T1D patients displaced with either cold INS-IGF2 or cold insulin (insulin-specific INS-IGF2) for specific binding.** *A*, correlation between specific INS-IGF2A and insulin-specific INS-IGF2A in control subjects (n = 355) ($r^2 = 0.195$, $p < 0.001$). *B*, correlation between specific INS-IGF2A and insulin-specific INS-IGF2A in 1–5-year-old T1D patients (n = 152) ($r^2 = 0.380$, $p < 0.001$). *C*, correlation between specific INS-IGF2A and insulin-specific INS-IGF2A in 15–18-year-old T1D patients (n = 152) ($r^2 = 0.426$, $p < 0.001$).

the notion that INS-IGF2 may also become an autoantigen based on similar mechanisms.

In a first report, *INS-IGF2* gene expression in human pancreas and eye was demonstrated (34). In this study, we therefore extend this finding to demonstrate *INS-IGF2* expression in human islets. Our observation that the splice variant expression was reduced in islets from T2D patients or in islets from organ donors with increased hemoglobin A1c suggests that the *INS-IGF2* gene regulation may be controlled by mechanisms comparable with that of *INS* itself. This is not surprising as the *INS* and *INS-IGF2* genes share the same promoter. This promoter also regulates *INS* gene expression in the thymus (52), and we expect similar control of the *INS-IGF2* expression. We noted that *INS-IGF2* expression was twice the expression of *IGF2* but comparable with *IA-2* (Fig. 1). The observation that the IA-2 autoantigen expression was similarly reduced in islets from T2D patients suggested that T2D-associated islet cell dysfunction may affect the expression of both autoantigens. It was noted that the expression of *INS-IGF2* was correlated to that of *IA-2*, both in healthy ($r^2 = 0.369$, $p < 0.001$) and in T2D ($r^2 = 0.888$, $p = 0.001$). Taken together, the gene expression of *INS-IGF2* in human islets and its regulation by mechanisms similar to that of the *INS* gene would be consistent with the possibility that INS-IGF2 is an autoantigen that may be important to T1D pathogenesis similar to that of insulin.

The analysis of IAA requires the use of ^{125}I-insulin (13, 42, 53) or possibly the use of electrochemiluminescence technology (54, 55). Strength to assays using *in vitro* transcription-translation of cDNA to label the autoantigen with radioactive amino acids has been the relative ease of these radiobinding assays to be standardized (15, 56, 57). Although we have not been able to transcribe *in vitro* and translate native as well as mutated variants of full-length human preproinsulin cDNA (data not shown), the present truncated *INS-IGF2* cDNA construct allowed us to obtain consistently labeled autoantigen.

The specificity of the binding was tested with increasing concentrations of insulin as well as nonradioactive INS-IGF2 generated by *in vitro* transcription-translation. In the absence of native or recombinant INS-IGF2 and a suitable antiserum, it has not been possible to establish a radioimmunoassay that could be used to determine the amount of cold INS-IGF2 generated. Although cold insulin could be used to fully block ^{125}I-insulin binding, this was not possible with INS-IGF2. This is a possible weakness to our study, and it cannot be excluded that we have underestimated the proportion of sera with doubly reactive autoantibodies. Another potential weakness is that we have not compared ^{125}I-labeled insulin with ^{125}I-labeled INS-IGF2. However, because ^{125}I-insulin for the detection of IAA requires insulin labeled at the A14 tyrosine, such comparison would indeed not be possible.

In future investigations, it will be important to synthesize or produce recombinant INS-IGF2 to determine maximal displacement of cold INS-IGF2 and insulin alone as well as together. It should then be possible to discern the contribution of IAA alone and INS-IGF2A alone along with doubly reactive sera. Examination of whether the autoreactivity to these two autoantigens with shared amino acid sequences occurs simultaneously or separately should be possible using sera from longitudinal studies of at-risk children followed from birth (The Environmental Determinants of Diabetes in the Young; Diabetes Prediction and Prevention; Diabetes Autoimmunity Study in the Young, and BABY DIAB). Similarly, if T and B cells have been collected in such studies, it will be of interest to determine whether the TCR of CD8$^+$ T cells express TCR recognizing B-chain residues 10–18 (20) or 9–23 (24) or signal peptide residues 15–24 (25, 26) from preproinsulin, INS-IGF2, or both.

This study demonstrates that INS-IGF2 may be a novel autoantigen in autoimmune T1D. Our displacement experiments suggest that autoantibodies against insulin and INS-IGF2 may share autoantibody-binding sites. Our findings may shed light on several recent reports that CD8$^+$T cells recognize signal peptide and C-peptide residues present in INS-IGF2 but not in mature insulin. In addition, any epitope recognized by T cells in the B-chain is likely to be shared between mature insulin and INS-IGF2. It cannot be excluded

therefore that INS-IGF2 may augment the ability of insulin to trigger the pathogenesis of T1D.

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