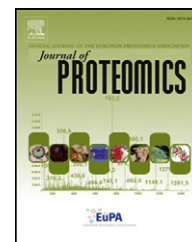


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Serological Proteome Analysis (SERPA) as a tool for the identification of new candidate autoantigens in type 1 diabetes

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ABSTRACT

Type 1 diabetes (T1D) is an autoimmune disease characterized by the presence of circulating autoantibodies directed against proteins of islet beta-cell. Autoantibody testing is used for diagnostic purposes; however, up to 2–5% of patients who are clinically diagnosed with T1D are found negative for known antibodies, suggesting that the T1D autoantigen panel is incomplete. With the aim of identifying new T1D autoantigen(s), we used sera from subjects clinically diagnosed with T1D, but who tested negative for the four T1D autoantibodies currently used in clinical practice and for genes responsible for sporadic cases of diabetes.

Sera from these patients were challenged by Western blot against the proteome from human pancreatic beta-cells resolved by 2DE. Eleven proteins were identified by MS. A radiobinding assay (RBA) was developed to test the reactivity to Rab GDP dissociation inhibitor beta (GDI β) of T1D sera using an independent method. Depending on the construct used (open reading frame or COOH-terminus) 22% to 32% of fifty T1D sera showed increased binding to GDI β by RBA. In addition, 15% of patients with celiac disease had raised binding to the COOH-terminus GDI β .

Abbreviations: T1D, Type 1 diabetes; PNDM/MDI, Permanent Neonatal Diabetes Mellitus/Monogenic Diabetes of Infancy; GAD65A, Glutamic acid decarboxylase 65 kDa auto antigen; IA-2A, Tyrosine phosphatase-like insulinoma Ag 2 auto antigen; IAA, Insulin auto antigen; IMP, Immunoproteomics; ZnT8A, Cation Efflux Zinc Transporter 8 auto antigen; RBA, Radioimmunobinding assay; CMEF, Cytoplasmic membrane enriched fraction; GDI β , Rab GDP dissociation inhibitor beta; PPIA, Peptidylprolyl cis-trans isomerase A; SERPA, Serological Proteome Analysis.

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These results indicate that immunoproteomics is a feasible strategy for the identification of candidate T1D autoantigens.

Biological significance

Several approaches have been previously used to look for new type 1 diabetes autoantigens. With the present work we show that carefully selected sera from rare patients with diabetes both negative for the 5 autoantibodies currently used in clinical practice and for genes responsible for sporadic cases of diabetes, may be exploited in experiments utilizing human pancreatic islets extracts as a target for SERPA to identify novel candidate T1D autoantigens.

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1. Introduction

Type I diabetes (T1D), an autoimmune, T cell-dependent disease due to a chronic, progressive destruction of insulin-producing islet beta-cells [1–3], is the main cause of diabetes in childhood [4]. For unknown reasons, median age at onset of T1D is constantly decreasing [4], and it is becoming common to diagnose T1D in children under two years of age. Serological markers of T1D, i.e. autoantibodies (autoAbs) to insulin (IAA), glutamic acid decarboxylase (GAD65A), islet autoantigen 2 (IA-2A) and islet beta-cell zinc cation efflux transporter (ZnT8A), are currently used to confirm the diagnosis. [5]. It is known however, that a small percentage (between 2 and 4%) of patients clinically classified as T1D sufferers are negative for these islet antibody markers. Recently, mutations in three genes (*KCNJ11*, *INS*, and *ABCC8*) have been described as the most frequent cause of autoAbs-negative, permanent diabetes presenting within 6 months of birth (Permanent Neonatal Diabetes Mellitus, PNDM/Monogenic Diabetes of Infancy, MDI) [6]. In addition, mutations in these genes are rarely found in patients with diabetes onset during childhood or adulthood [7–9]. Interestingly, most cases with PNDM/MDI are sporadic, because they originate from spontaneous mutations [6], though familial cases have also been reported [7]. Thus, from the clinical standpoint it is not easy to determine whether a patient with autoAbs-negative diabetes with onset in childhood has T1D or monogenic diabetes.

It is important to discern whether a patient's diabetes is autoimmune or genetic in origin for several reasons, the most obvious of which are the implications for genetic counselling, but also the fact that some genetic forms of diabetes (e.g. those associated with mutations of *KCNJ11*, *ABCC8*, *TCF1* and *HNF4A* genes) may respond to a treatment with sulfonylureas [9,10]. In a study aimed at identifying mutations of *INS* gene in patients clinically classified as T1D, but negative for IAA, GAD65A, IA-2A and ZnT8A, we found 2 *INS* mutations out of 7 patients screened [9]. The remaining 5 patients were also negative for the search for mutations in *KCNJ11* and *ABCC8* genes, and might either carry a mutation in a yet-to-be-discovered gene or rather be autoimmune and have developed autoantibody/ies different from known T1D serological markers. HLA testing of 4 of these patients showed T1D predisposing haplotypes, suggesting that the most plausible cause of diabetes in these patients may be organ-specific autoimmunity. We thus decided to exploit these very special patients for the – possible – identification of new autoantigens/autoantibodies. To this end, the sera of these patients (hereafter called Neg4) were used for immunoblotting

against human pancreatic islet and exocrine proteins resolved by 2DE, in an immunoproteomics approach (Serological Proteome Analysis; SERPA).

Here we show that several islet proteins reacted in SERPA experiments and were subsequently identified by mass spectrometry. We conclude that immunoproteomics is a robust tool for the identification of new candidate autoantigens/autoantibodies of type 1 diabetes.

2. Materials and methods

2.1. Reagents and media

All chemicals were from Sigma Aldrich, unless otherwise specified. All media and cell supplements were from Invitrogen.

2.2. Human islets and exocrine pancreas

Pancreata were obtained from heart-beating cadaveric multiorgan donors with the approval of the Ethical Committee of Ca' Granda Niguarda Hospital (Milan). Islets were isolated according to the automated method [11], purified by continuous gradient with refrigerated COBE processor as previously described [12] and maintained in M199 medium, supplemented with serum and antibiotics, at 37 °C, 5%CO₂ until use. Exocrine tissue was recovered from the bottom layer of gradient media after successful COBE purification. Beta cell contamination was assessed by dithizone staining and was <1%.

2.3. Sera for immunoproteomics

We collected sera from 25 Italian subjects, divided into groups as follows (Table 1): Neg4 (study group), composed of 5 subjects diagnosed with T1D from clinical symptoms, negative to all well characterized T1D autoantibodies and for common PNDM/MDI genes (*KCNJ11*, *INS*, and *ABCC8*); MUTins (negative control), 4 subjects with diabetes due to a mutation of *INS* gene [7,8]; and 16 T1D patients positive for a single autoantibody: ZnT8Apos (4 subjects); IAAPos, (4 subjects) and GAD65Apos (8 subjects). T1D sera from the latter three groups were considered as positive controls. Neg4 subjects belong to a group of patients followed by R.B. at H San Raffaele Hospital, a long-standing participant of the Diabetes Antibody Standardization Program (DASP); cut-offs utilized to establish negativity were previously published [8]. Neg4 patients were also negative to islet cell autoantibodies (ICA). The autoantibody status of all groups was

Table 1 – Clinical features of patients and reactivity of their sera to GD1 β and PPIA.

Pat. subgroup	Patient number	Sex	Diagnosis	Age at onset (years, months)	Diabetes duration at the time of sample collection	Mutation in KCNJ11, INS or ABCC8	IMP GDI β	RBA GDI β ORF	RBA COOH-GDI β	IMP PPIA
Neg 4	PZ1	M	T1D	9 year	3 year	None	NEG	NEG	NEG	NEG
	PZ2	F	T1D	4 year	3 year	None	POS	NEG	NEG	POS
	PZ3	M	T1D	10 years	3 years	None	POS	POS	NEG	POS
	PZ4	F	T1D	10 years	At onset	None	POS	NEG	NEG	POS
	PZ5	F	T1D	9 years	At onset	None	POS	NEG	NEG	NEG
MutINS	PZ6	M	PNDM/MDI	4 months	11 months	INS	NEG	NEG	NEG	NEG
	PZ7	M	PNDM/MDI	2 years, 7 months	3 year	INS	NEG	NEG	NEG	NEG
	PZ8	M	PNDM/MDI	6 years, 8 months	7 years, 7 months	INS	NEG	NEG	NEG	NEG
	PZ9	F	PNDM/MDI	6 months	1 year, 2 months	INS	NEG	NEG	NEG	NEG
ZnT8APOS	PZ10	M	T1D	4 years	3 years	Not done	NEG	NEG	NEG	NEG
	PZ11	M	T1D	4 years	1 years	Not done	NEG	NEG	NEG	POS
	PZ12	M	T1D	7 years	1 years	Not done	POS	NEG	NEG	NEG
	PZ13	F	T1D	7 years	2 years	Not done	POS	NEG	NEG	NEG
IAAPOS	PZ14	F	T1D	2 years, 2 months	At onset	Not done	NEG	NEG	NEG	NEG
	PZ15	M	T1D	1 years, 9 months	At onset	Not done	NEG	NEG	NEG	NEG
	PZ16	F	T1D	3 years, 5 months	At onset	Not done	NEG	NEG	NEG	NEG
	PZ17	M	T1D	7 years	At onset	Not done	POS	NEG	NEG	NEG
GAD65A	PZ18	F	T1D	6	At onset	Not done	NEG	Not done	Not done	NEG
	PZ19	F	T1D	15	At onset	Not done	POS	NEG	NEG	NEG
	PZ20	M	T1D	5	At onset	Not done	POS	POS	POS	NEG
	PZ21	F	T1D	9	At onset	Not done	NEG	Not done	Not done	NEG
	PZ22	M	T1D	13	At onset	Not done	NEG	NEG	NEG	NEG
	PZ23	M	T1D	2	At onset	Not done	POS	NEG	NEG	NEG
	PZ24	F	T1D	6	At onset	Not done	NEG	Not done	Not done	NEG
	PZ25	F	T1D	5	At onset	Not done	NEG	Not done	Not done	NEG

IMP = immunoproteomics.

RBA = radiobinding assay.

T1D = type 1 diabetes.

PNDM/MDI = Permanent Neonatal Diabetes Mellitus/Monogenic Diabetes of Infancy.

assessed at diagnosis. However, not all sera utilized in our study were an aliquot of the same sample obtained at diagnosis: in the case of MUTins, ZnT8apos and 3 patients of Neg4 groups sera were collected later, at variable times from diagnosis (from 7 months to 3 years), as indicated in Table 1.

Age and gender were comparable among groups, but MUTins, with two subjects with onset ≤ 6 months (Table 1) and Neg4 patients being older than IAA patients ($p = 0.0358$ by unpaired Mann Whitney test). All patients were negative for celiac disease and thyroiditis. Four subjects of Neg4 group showed HLA predisposing alleles to T1D (not shown). Signed written consent forms were obtained from all participants or their parents.

2.4. 2DE, Western blot and image analysis

Human islets and exocrine cells were maintained in M199 medium for 2 days, collected by centrifugation, homogenated by Dounce (60 strokes) on ice and passed through needles of different sizes (19G; 22G and 27G, 20 strokes/each on ice); nuclei and microsomal granular fractions were discarded by two successive centrifugations [13,14]. The protein fraction enriched in cytoplasm and membrane (CMEF) was the only one considered in this study, taking into consideration that three out of four of well characterized T1D autoantigens are cytoplasmic/membrane proteins. For 2DE analysis 200 μ g of acetone-precipitated proteins from CMEF were resuspended

in a RB-Thio buffer (6 mM Urea, 2 mM Thio-Urea, 4% w/v CHAPS, 65 mM DTT), 0.2% v/v IPG buffer 3–10 NL (GE Healthcare) plus 0.05% bromophenol blue and applied on 7 cm IPG strips 3–10 NL (GE Healthcare) by in-gel rehydration at 18 °C. Focusing and second dimension were performed as described [13]. Proteins were visualized by staining with Sypro Ruby (Bio Rad) or by Western blot. Human sera were diluted 1/1000 in 5% non fat milk in TTBS (100 mM Tris, 150 mM NaCl pH 7.5, 0.1% Tween 20). Reactivity was revealed by incubation with anti-human HPR Fc IgG (Sigma) diluted 1/1000 in 5% non fat milk in TTBS followed by ECL (GE Healthcare) reaction. Gel and blot images were acquired using a Personal SI Laser Densitometer (GE Healthcare) and 2DE protein patterns analyzed using Progenesis PG240 (Nonlinear Dynamics).

Western blot validations were performed with anti-GAD65, anti-GDI β (Santa Cruz) and anti-PPIA (Millipore) antibodies, revealed by anti-rabbit IG-HRP conjugated antibody (Dako).

2.5. Criteria for the selection of the spots

The spots that fulfilled one of the following criteria were chosen for mass spectrometry identification: 1) spots present only in Neg4 blots, or 2) spots shared by Neg4, ZnT8Apos and IAApos. We fixed the second criterion on the basis that almost all patients affected by T1D (>90%) show antibodies to multiple islet autoantigens [5]. The GAD65Apos group was excluded as a consequence of the very high reactivity that made a reliable

comparison difficult. This group of sera was used only for subsequent validation.

2.6. Protein identification

Protein spots were excised from 2DE gels with EXQuest™ spot cutter (Bio-Rad), processed and gel-digested as described [15]. Peptide mass fingerprinting and MS/MS analysis were done on a 4800 MALDI TOF/TOF™ mass spectrometer (Applied Biosystems). The mass spectra were internally calibrated with trypsin autolysis fragments. The five most abundant precursor ions, from the exclusion mass list (ions from human keratin and trypsin), were selected for MS/MS analysis. The combined MS and MS/MS data were submitted to the MASCOT database search engine (Version 2.1, Matrix Science) using GPS Explorer v.3.6 software (Applied Biosystems) for protein identification. A search was performed with the following parameters: Swissprot 55.2x database over all *Homo sapiens* protein sequences deposited with no fixed modifications, but allowing the following as possible modifications: a) carboamidomethylation of cysteine and oxidation of methionine, b) one missed trypsin cleavage, c) a mass tolerance of $\pm 0.1D$ for the peptide mass values and of $\pm 0.3D$ for the MS/MS fragment ion mass values. A protein was regarded as identified if the MASCOT score, based on combined MS and MS/MS data, was above the 5% significance threshold for the database (score >56) [16].

2.7. Radioimmunobinding assay (RBA)

Two different constructs for GDI β which correspond to the open reading frame (ORF; 1–445 aminoacids,) and to the COOH terminal domain (COOHterm, 338–445 aminoacids) were synthesized starting from human cDNAs cloned into pENTR₂₂₁ vector (Invitrogen) using primers that generate EcoRI and SalI insertion site (see Table S1 supplementary information). Constructs were sequenced to exclude the presence of mutations. An assay was developed and performed as described by others [17,18] using an *in vitro* coupled transcription/translation kit (TNTQuick SP6 promoter; Promega) that uses the pTNT™Vector. Translation was performed in the presence of 30 μ Ci of [³⁵S] methionine (Perkin Elmer) and 1.4 μ g pTNT-constructs according to the manufacturer's instructions. The [³⁵S] methionine not incorporated in the *in vitro* translated protein was removed by using NP5 column (GE Healthcare) equilibrated with 10 ml of Wash Buffer (20 mM Tris, 150 mM NaCl, Tween 0.15 %, 0.1% BSA pH 7.4), and translation efficiency was evaluated as a percentage of cpm incorporated into protein, considering 100% as the cpm used for the translation. The labelling efficiency was in the range of 25–30% in all the experiments. For each construct, 50,000 cpm resuspended in Wash Buffer were mixed with 5 μ l of patients or control sera in a total reaction volume of 50 μ l and incubated on a shaker 20 h at 4 °C. Then, 25 μ l of slurry Protein A Sepharose beads (GE-Healthcare) (vol/vol Wash Buffer) were added to the mix and incubated a further 2 h at 4 °C. After washing five time with Wash Buffer, beads were resuspended with 200 μ l of Wash Buffer transferred to vials containing 2.5 ml of scintillation liquid (Hewlett-Packard) and cpm recorded in beta-Counter (Top Count NXT, Hewlett-Packard). Nonspecific binding was evaluated as the cpm recorded in samples incubated in the absence of sera, and the

resulting value was subtracted from each sample. Sera were analyzed in duplicate in each experiment, and the results are the average of three independent experiments.

2.8. Statistical analysis

Statistical analysis of continuous data was evaluated by unpaired Mann Whitney test; two-tailed p value was used for the comparison of two means and their standard error. In all analyses, a $p < 0.05$ was considered statistically significant. Statistical analysis of categorical data was performed by 2×2 contingency table using Fisher's exact test and two-tailed p value. The statistical analysis was performed with Prism V4.03 software (GraphPad Inc., San Diego, CA). Sera were considered positive when they exceeded the average result for the negative control group (obese subjects) plus three standard deviations. Before any statistical calculation the cpm values obtained in the RBA without sera were subtracted from the cpm values of each sample. An intra assay coefficient of variation (CV) was also calculated from the duplicate measurements.

2.9. Sera for RBA

Sera from different groups collected for immunoproteomics were also used in RBA together with: a) sera from 50 patients with T1D with an average diabetes onset below 5 years of age, randomly chosen regardless their autoantibody characteristics; 10 of these sera were obtained within 3 years from diagnosis, the other 40 within 10 years, b) 50 sera from non-diabetic, and otherwise healthy obese subjects whose sera were collected at a median age of 11 years (range 5–19), c) 100 sera from non-diabetic, GADA negative celiac subjects who received diagnosis by the age of 6 years.

3. Results

3.1. Identification of new potential autoantigens

With the aim of creating “average blots” needed for proper comparison among different groups of sera (i.e. Neg4 vs IAApos, etc.) we performed spot detection and matching of 2DE-Western blots obtained with each serum of a given group (e.g. the average blot of IAApos group results from the matching of four 2DE-Western) using Progenesis PG240 software. The average blot from the four MUTins Western blots was considered “basal” reactivity, and subtracted from average blots obtained for each different group of sera. The same procedure/logic was followed to highlight auto-immunity specifically directed against pancreatic islets by “filtering” average blots of islet CMEF against that of exocrine pancreas.

Finally, a comparison among Neg4, ZnT8Apos and IAApos average blots was performed to look for spots fulfilling criteria as described in [Materials and methods](#). This comparison is summarized in [Fig. 1H](#). On average, 68 spots were unique to the Neg4 sera group, while ZnT8Apos and IAApos sera recognized respectively 19 and 8 spots of islet CMEF, which can be considered “specific” to these two groups. In addition to shared reactivity between groups when matched one to

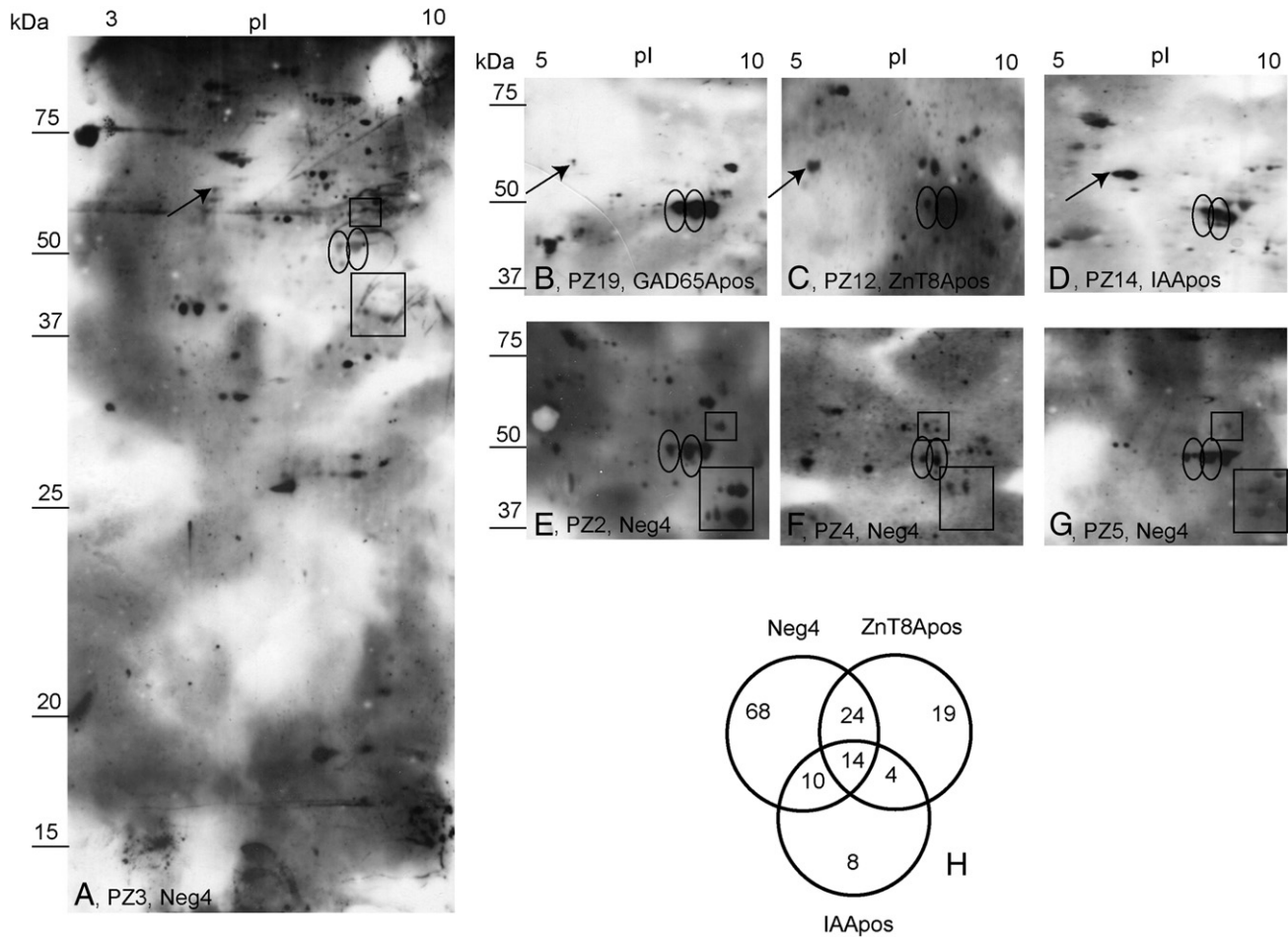


Fig. 1 – Western blot matching and Progenesis PG240 analysis: Western blot of 200 µg CMEF proteins from human pancreatic islets resolved by 2DE. 1st dimension on 7 cm strip pH 3–10 NL. 2nd dimension on 10% acrylamide 7 × 7 cm gel. Human sera diluted 1/1000. Blots exposed 5'. **A:** PZ3, Neg4 blot (“reference”). **B–D:** A representative blot from GAD65Apos group (**B**), ZnT8Apos group (**C**) and IA Apos group (**D**) are shown. **E–G:** Blots of PZ2, PZ4 and PZ5 of the Neg4 group. The ovals and arrows identify common spots to all sera. Spots unique to Neg4 group, are identified by squares. **H:** Summary of Progenesis PG240 analysis comparing Neg4, IA Apos and ZnT8Apos sera groups.

one, PG240 analysis showed that Neg4, ZnT8Apos and IA Apos average blots had 14 spots in common (Fig. 1H).

3.2. Protein identification

To pursue this goal, the average Western blots of Neg4, IA Apos and ZnT8Apos groups were compared to Sypro Ruby stained gels on which CMEF proteins from pancreatic islets were resolved by 2DE. This analysis allowed 55 of the 82 (68 + 14) spots previously selected to be positioned and picked. Among these 55, eleven proteins were identified by MALDI TOF/TOF with a score > 56 (Table 2 and Table S2 supplementary information; Fig. 2A), eight of these were common to Neg4, ZnT8Apos and IA Apos (Group 1), while three were unique to Neg4 group (Group 2). Among these eleven, two were chosen for further studies: Rab GDP dissociation inhibitor beta (GDIβ, Group 1), which is involved in the regulation of Rab proteins, which act in the final part of fusion to the plasma membrane of insulin granules [19], and Peptidylprolyl cis-trans isomerase A (PPIA, Group 2), which has been associated with IA-2A [20]. Other identified proteins

were carbonic anhydrase 2 and protein disulfide isomerase, already proposed as T1D autoantigens [21,22], and tubulins [23,24] (Table 2). However, none of the proteins we identified by MS had an expression pattern restricted to the pancreatic islet.

3.3. Validation of identified proteins with commercial antibodies

Identification of GDIβ and PPIA was confirmed using commercial antibodies on the same Western blots reacted with human sera. First we validated the procedure using a commercial antibody against GAD65. Fig. 2B shows that the commercial antibody reveals a couple of spots of 65–68 kDa and 6.6 pI (6.45 pI calculated) on the same blot of GAD65Apos sera (Fig. 2C), while the GAD65 spot is always absent in Neg4, ZnT8Apos and IA Apos (Fig. 2D) and in all exocrine blots, as expected.

Fig. 2E–G shows the validation for PPIA identification. The more basic of the two spots for PPIA revealed by the commercial antiserum (calculated pI 7.68, MW, 18 kDa; Fig. 2E) corresponds

Table 2 – Proteins of in human pancreatic islets identified by mass-spectrometry.

Protein name	AC ^a	Mr (pred/exp) ^b	pI (pred/exp)	Matched ^c	Coverage (%) ^d	Prot. Score ^e	Best ion score	Peptide sequence	Serum Positivity
Retinal dehydrogenase 1	P00352	54862/60000	6.30/6.0	15	28.5	168	54	R.IFVEESYDEFVR.R	Neg.4
Peptidylprolyl cis-trans isomerase A	P62937	18012/18000	7.68/7.50	12	63.6	126	47	M.VNPTVFFDIAVDGEPLGR.V	Neg.4
Rab GDP dissociation inhibitor beta	P50395	50663/50000	6.10/7.5	19	32.8	104	25	K.FLVVYVANFDEKDPR.T	ZnT8pos IAApos Neg.4
UDP-glucose 6-dehydrogenase	O60701	55024/60000	6.73/6.0	28	58	83	22	K.LAANAFLAQR.I	Neg.4
Tubulin alpha chain 1C	Q9BQE3	49895/55000	4.96/4.9	10	24.3	81	46	R.AVFVDLEPTVIDEVR.T	ZnT8pos IAApos Neg.4
Protein disulfide isomerase A3	P30101	56782/60000	5.98/5.6	23	44.3	71			Neg.4
Carbonic anhydrase 2	P00918	29246/37000	6.87/6.2	6	17.7	66	31	K.SADFTNFDPR.G	Neg.4
Glyceraldehyde-3-phosphate dehydrogenase	P04406	36053/37000	8.57/8.9	8	13.1	66	26	K.LVINGNPITIFQERDPSK.I	Neg.4
Peroxiredoxin-6	P30041	25035/37000	6.00/5.9	10	38.8	59	19	K.LPFPPIIDDR.N	Neg.4
Vimentin	P08670	53652/60000	5.05/4.9	17	39.6	59			Neg.4
Tubulin beta chain 2C	P07437	49671/53000	4.78/4.9	17	25.4	57	18	R.FPGQLNADLR.K	ZnT8pos IAApos Neg.4

The proteins that had a statistically significant ($p < 0.05$) protein score (>56), were considered successfully identified.

^a = AC, accession numbers from SwissProt database.

^b = predicted/experimental.

^c = matched, the number of peaks that match to the theoretical peptide mass fingerprinting.

^d = sequence coverage as the number of amino acids spanned by the assigned peptides divided by the sequence length.

^e = protein score based on combined MS and MS/MS spectra from MALDI-TOF/TOF analysis using MASCOT search engine (Matrix Science).

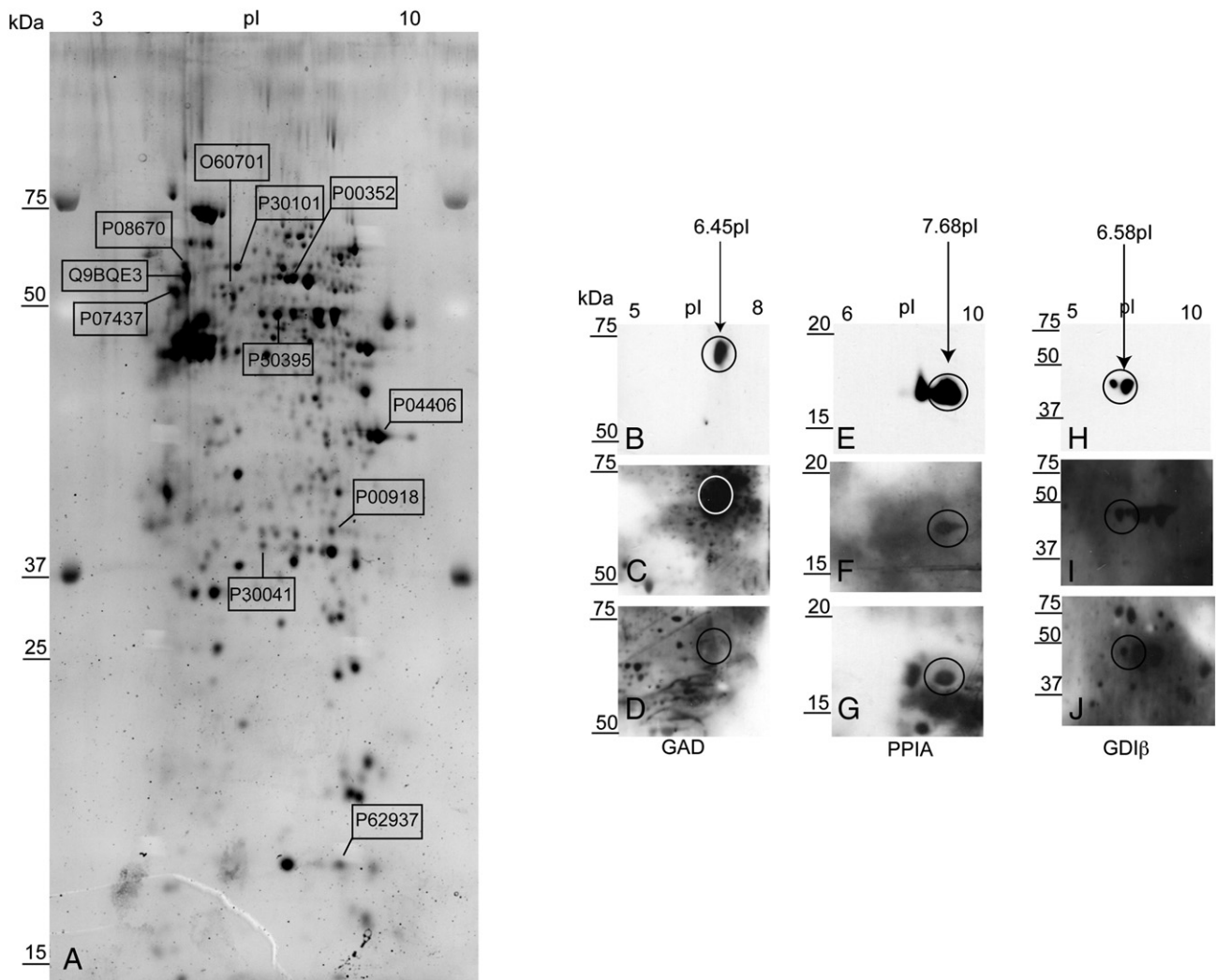


Fig. 2 – Spot identification and validation: A: 200 µg CMEF proteins from human pancreatic islets resolved by 2DE. Gel stained with Sypro Ruby. Identified spots indicated by SwissProt number. B–D: GAD65 validation. Comparison between the reaction of a commercial antibody (B) and GAD65Apos serum (C, one out of eight). Neg4 sera were always negative to GAD65. (D). E–G: PPIA validation. Comparison among the reaction of a commercial antibody (E), Neg4 serum (F, one out of three positive blots) and ZnT8Apos serum (G, one out of two positive blots). H–J: GDIβ validation. Comparison among the reaction of a commercial antibody (H), Neg4 serum (I, one out of two positive blots) and GAD65Apos serum (G, one out of three positive blots). All blots exposed 5 min.

to the spot revealed by one of the Neg4 sera (pI 7.50; Fig. 2F). Furthermore, we found that the reaction against PPIA was present in two other Neg4 blots, totalling three blots out of five; Fig. 2F shows the blot with the strongest reaction. A PPIA spot was also revealed by one ZnT8Apos serum (out of four) that was probably missed by initial analysis with PG240 (Fig. 2G). In contrast, the spot that corresponds to PPIA was absent in the GAD65Apos, IAApos and exocrine blots (Table 1).

The validation of the GDIβ (calculated pI 6.58, MW 50.6 kDa; Fig 2H–J) was performed in the same way. GDIβ was found positive in two Neg4 blots (out of five), two ZnT8Apos blots (out of four), three GAD65Apos blots (out of eight) and one IAApos blot (out of four), and was always absent on the exocrine blots (Table 1).

3.4. RBA as independent method to confirm candidate autoantigens

To test GDIβ antibodies by RBA we used sera from a group of 50 patients with T1D; sera from patients with simple obesity and from 2 age-matched patients with mutations in the insulin gene (MUTins) (Table 1) were utilized as negative controls. T1D sera had been collected at different clinical stages of the disease, i.e. close to the onset or later and were not selected for antibody type or titer.

Two different constructs of GDIβ were utilized in RBA. First, we tested full-length (ORF)-GDIβ in three different groups of samples: sera of patients with T1D, sera initially used for immunoproteomics, and sera of four subjects classified as

Neg4; samples that were 3 SD above the mean of counts of individuals with simple obesity were considered positive. We found that 11/50 (22%) of T1D group tested positive as compared to 0% (0 out of 50) of negative controls ($p = 0.0005$) (Fig. 3A). In addition, one serum of each Neg4 (1/5), and GAD65Apos (1/4) groups and not all the sera that reacted with GDI β in immunoproteomics experiment, were positive to (ORF)-GDI β . Complete statistics are reported in Table 3.

Results of RBA with COOHterm-GDI β showed a higher percentage of T1D patients testing positive (16/50 or 32%; $p = 0.0001$) (Table 3). However, four T1D samples that were positive to ORF-GDI β resulted negative to COOHterm-GDI β and vice-versa eleven T1D who were negative to ORF-GDI β scored positive to COOHterm-GDI β . Thus, the two RBA combined identified 20 (4 + 16, or 40%) T1D patients positive to GDI β . In addition a single GAD65Apos was positive to this test. None of the negative controls (obese subjects) tested positive.

Because T1D autoantibodies may appear in patients with celiac disease [25,26] and individuals with type 1 diabetes are often diagnosed with celiac disease [27–29], we tested sera of subjects with serologically and histologically diagnosed celiac disease, but with no clinical evidence of diabetes, with COOHterm-GDI β RBA. Sera from this group of patients were found positive in 15% of cases (15/100; $p = 0.0026$) (Fig. 3B; Table 3). The assay reproducibility was assessed by intra-assay coefficient of variation (CV) calculated from the duplicate measurements for each experiment and found to be <10 (CVexp1 = 9.68; CVexp2 = 8.18 and CVexp3 = 5.65).

To further confirm binding specificity to this protein, we immunodepleted cytoplasmic fraction of human pancreatic islets with two different T1D sera RBA-positive to GDI β (both ORF and COOH-term or two pools (five sera each) of GDI β -negative celiac patients or a commercial rabbit polyclonal anti-GDI β antibody and then challenged by Western blot the immunoprecipitated material with or the same commercial anti-GDI β antibody utilized for immunodepletion. This

experiment clearly shows that a single protein of 50 kDa immunoprecipitated by T1D sera RBA-positive to GDI β reacts with rabbit anti-GDI β antibody, and not with pools of sera from GDI β negative celiac patients (Fig. S1).

4. Discussion

The search for new type 1 diabetes autoantigen(s)/autoantibody(ies) is of interest for several reasons, among which are the following: 1) a new, well-validated autoantibody may help in predicting type 1 diabetes in relatives positive to standard autoantibodies [30], thus increasing the number of subjects who could potentially take part in primary prevention trials [31], 2) a new autoantigen may potentially be exploited for the induction of active or passive tolerance in people with recent onset T1D [31] and 3) a new autoantibody may be useful for diagnostic purposes in the 2–4% of patients clinically classified as affected by T1D, but negative to all currently available biochemical markers [5]. The aim of our project was to test the feasibility of an immunoproteomic approach in order to identify (novel) autoantigens in patients with clinical symptoms of T1D, and with HLA alleles predisposing to the disease, but negative for the four autoantibodies now in use to confirm the diagnosis (i.e. Neg4). In a previous study seven Neg4 patients were subjected to a molecular screening that identified two *INS* gene mutations [8], and we reasoned that sera from the remaining 5 (also negative to *KCNJ11* and *ABCC8* screening) might be a tool for the identification of new T1D autoantigen(s)/autoantibody(ies). Immunoproteomics has been employed to look for autoantibodies in many autoimmune diseases [32,33] but – even if advocated [34] – never applied in type 1 diabetes. In contrast, a number of methods have been employed in T1D to confirm the presence and evaluate the titers of autoantibodies in patients' sera [17,18,35] often in combination with RIA or RBA [18].

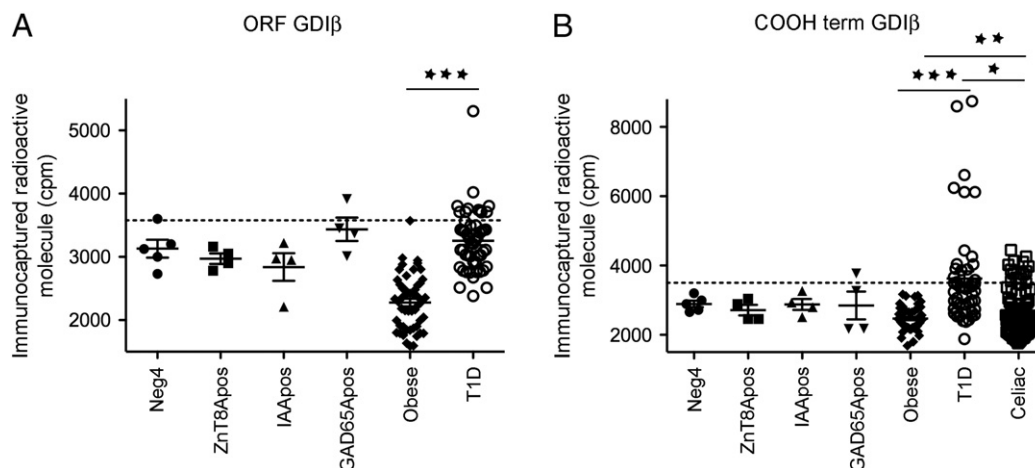


Fig. 3 – Radio binding assay. RBA performed with patients' and controls' sera against *in vitro* translated radiolabeled molecules (ORF GDI β and COOHterm GDI β); the values of immuno-captured molecules are reported as cpm. Dotted lines are referred to the arbitrary threshold fixed as the average of negative controls plus three standard deviations, defining subjects that were considered positive for the presence of specific autoantibodies (Table 3). Statistical analysis is referred to positive subjects compared to control obese group and were done by Fisher's test (* = $p < 0.05$; ** = $p < 0.001$; *** = $p < 0.0001$). A: RBA of ORF GDI β . B: RBA of COOHterm GDI β .

Table 3 – Radio binding assay (RBA) of GDI β occurrence of autoantibodies in Neg4, subgroups of patients with type 1 diabetes, and celiac and obese (negative control) subjects.

	Neg4	ZnT8Apos	IAApos	GAD65Apos	T1D	Obese	Celiac
RBA, cpm (Mean $\pm$ sd)							
GDI β ORF	3131 $\pm$ 317.	2973 $\pm$ 167	2839 $\pm$ 436	3436 $\pm$ 372	3256 $\pm$ 493	2278 $\pm$ 433	ND
GDI β COOHterm	2893 $\pm$ 217	2714 $\pm$ 300	2878 $\pm$ 315	2848 $\pm$ 803	3626 $\pm$ 1445	2470 $\pm$ 344	2634 $\pm$ 644
Positive sera ($> m \pm 3sd$ obese)							
GDI β ORF	1/5	0/4	0/4	1/4	11/50	0/50	NA
GDI β COOHterm	0/5	0/4	0/4	1/4	16/50	0/50	15/100
Fischer's test (p)							
	Neg4	ZnT8Apos	IAApos	GAD65Apos	T1D	T1D	Obese
	Vs	vs	vs	vs	vs	vs	vs
	Obese	Obese	Obese	Obese	Obese	Celiac	Celiac
GDI β ORF	0.0909	1	1	0.0741	0.0005	NA	NA
GDI β COOHterm	1	1	1	0.0741	0.0001	0.0193	0.0026
Frequency of auto-Ab occurrence							
GDI β ORF	20%	0%	0%	25%	22%	0%	NA
GDI β COOHterm	0%	0%	0%	20%	32%	0%	15%

Neg4 (n = 5); ZnT8Apos (n = 4); IAApos (n = 4); GAD65Apos (n = 4); T1D patients (n = 62); Celiac (N = 100); obese (n = 50).
 ND = not-done.
 NA = not-applicable.

Immunoproteomics combined with MS allowed the identification of eleven proteins of cytoplasmic and membrane enriched fractions of human islets that had reacted with the sera of patients used in SERPA. Interestingly some of these proteins, such as carbonic anhydrase, PDI and tubulins, have already been considered as potential auto-antigens [21–24], also in T1D [21,22]. Our results, by independently replicating findings obtained with different techniques [21,22], strongly indicates the feasibility of a proteomic approach in the search for novel T1D autoantigen(s). In the absence of a strong candidate, i.e. a protein with an expression pattern restricted to the pancreatic islet, the choice of PPIA and GDI β for further characterization was made because these two proteins are involved in insulin granule secretion [19,20]. In addition, though GDI β and PPIA are expressed both in pancreatic islets and in exocrine pancreas, our results showed that sera only reacted with CMEF proteins derived from the former tissue (Table 2). Our interpretation of this result is that GDI β and PPIA are likely to be subject to different posttranslational modifications in endocrine and exocrine pancreas. Whatever the case, commercial antibodies confirmed the oneness of these two proteins detected by sera in human pancreatic islet and this in turn suggested that both may be part of the T1D autoantigens repertoire. GDI β -RBA was developed to check by an independent method whether sera from randomly selected T1D patients may react with this protein. We found that ORF-GDI β identified a positive reactivity in 22/50 T1D sera; and that COOHterm-GDI β , developed in the attempt to roughly map GDI β epitopes, moderately improved the performance of the test to 32% positivity in T1D group (Table 1), with a positivity of 40% when the two tests combined. These results, though confirmative of SERPA (Table 2) and Western blot by commercial antibody (Fig. 2), are not readily comparable to those obtained with ZnT8, a beta cell-restricted

protein, which elicited a positive response in 26% of sera negative to IAA, GAD65A and IA2A and ICA, in 60–80% of new-onset T1D and less 2% of negative controls [17]. A possible explanation of this discrepancy, and we acknowledge that this is a limitation of our work, is that very few sera of our T1D group were collected at diagnosis; this may account for the relatively low percentage of positives to GDI β -RBA. Whether GDI β autoreactivity is frequently present in patients with newly diagnosed type 1 diabetes or can be identified in first-degree relatives of individuals with T1D will be the subject of further studies. Interestingly, the brain-specific sister protein GDI α has been identified with immunoproteomic experiments as an autoantigen in patients with neuropsychiatric systemic lupus erythematosus [36]. Moreover, fifteen percent of sera from celiac, non-diabetic subjects scored positive when tested with COOHterm-GDI β RBA (Table 3). This result is roughly comparable with that obtained in untreated, normoglycemic celiac patients whose sera tested positive to the T1D autoantigens (GAD65A, IA-2A, IAA, and ZnT8A) in more than 15% of cases [17,26]. The association between celiac disease and T1D could be explained by shared common genetic factors (e.g. HLA DQ2 alleles, DQA1*05/DQB1*02) or by one disease being the pathogenic consequence of the other. In this regard, some of these autoantibodies (GAD65A, IA-2A, and IAA), may be gluten dependent, because we found that they disappeared in patients following a gluten-free diet [26].

5. Conclusions

In conclusion, the data reported here support the notion that the immunoproteomic approach is feasible in the search for new T1D autoantigens/autoantibodies. However, further studies are needed to evaluate if any of the proteins identified may be included in the T1D autoantigens repertoire.

An additional conclusion that can be drawn from the present study is that those Neg4 patients, who share reactivity to (candidate) autoantigens with patients with T1D (i.e. ZnT8Apos, IAApos) and also carry HLA genotypes predisposing to T1D, are likely to have autoimmune diabetes.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.jprot.2013.02.030>.

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