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Novel Diabetes Autoantibodies and Prediction of Type 1 Diabetes

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Abstract

Autoantibodies are currently the most robust biomarkers of type 1 diabetes and are frequently used to establish entry criteria for the participation of genetically at-risk individuals in secondary prevention/intervention clinical trials. Since their original description almost 40 years ago, considerable efforts have been devoted toward identifying the precise molecular targets that are recognized. Such information can have significant benefit for developing improved metrics for identifying/stratifying of at-risk subjects, developing potential therapeutic targets, and advancing understanding of the pathophysiology of the disease. Currently, four major molecular targets ([pro]insulin, GAD65, IA-2, and ZnT8) have been confirmed, with approximately 94% of all subjects with a clinical diagnosis of type 1 diabetes expressing autoantibodies to at least one of these molecules at clinical onset. In this review, we summarize some of the salient properties of these targets that might contribute to their autoantigenicity and methods that have been used in attempts to identify new components of the humoral autoresponse.

Keywords

Autoantigen; Autoantibody; Zinc transporter 8; Type 1 diabetes

Introduction

Autoimmune diabetes (T1D) is a chronic progressive disease characterized by the targeted destruction of the insulinsecreting β cells within the pancreatic islets [1]. The precise etiology remains uncertain; however, there is a general consensus that T1D is a T-cell-mediated disease that results from immune dysfunction, with subsequent loss of tolerance to β cell antigens and destructive lymphocytic infiltration of the islets. This, in turn, leads to insulin deficiency, impaired glucose homeostasis, and symptomatic hyperglycemia [2]. Although unlikely to be intrinsically diabetogenic, circulating autoantibodies targeting pancreatic β cell proteins have been defined. Monitoring such systemic autoantibodies is currently the most reliable biomarker in the prodromal phase of T1D, since their appearance typically precedes overt T1D onset for years or even decades [3]. This provides a window

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for therapeutic intervention, and considerable effort has been devoted to identification of autoantigens as biomarkers, both as an avenue to antigen-specific B-cell and T-cell therapies (since they often share antigens) and as a measure of treatment efficacy [4–6]. Most important, the prevalence of multiple types of autoantibodies identifies individuals with the highest risk of progression to clinical disease.

Features of Autoantigens

The four *major* humoral autoantigens in T1D identified to date ([pro]insulin, the 65kDa form of glutamic acid decarboxylase [GAD65], insulinoma antigen 2 [IA-2], and zinc transporter 8 [ZnT8]) share a subset of common features.

1. They are components of the regulated secretory pathway. Most major T1D autoantigens in the pancreatic β cell are associated with the regulated secretory pathway, and some intimately with the secretory granule itself [7]. Insulin is the prototypical secretory polypeptide; after passage through the endoplasmic reticulum and Golgi apparatus, it is sorted into regulated secretory granules for proteolytic processing/maturation, storage, and subsequent release in response to appropriate stimuli. It is the chief secretory product of pancreatic beta cell making up 50% of the total cell protein [8, 9] within the $\sim 10,000$ secretory granules of the β cell. IA-2, and its close homolog phogrin (I-A2 β), with which it shares 80% amino acid identity, play pivotal roles in insulin secretion. They are both members of the conserved protein tyrosine phosphatase (PTP) family albeit catalytically inactive [9]. ZnT8 functions to channel zinc into the secretory granule itself, where it is instrumental in the formation of insulin hexamers for crystallization, the “dense core” of the vesicle [10, 11]. Zinc is released along with insulin upon fusion of the granule with the plasma membrane. In contrast to the other major T1D autoantigens that are closely associated with the granule, GAD65 is a peripheral membrane protein associated with synaptic-like microvesicles within the β cell and the enzyme responsible for gamma aminobutyric acid synthesis.
2. Most are membrane proteins. The autoimmune profile of T1D closely reflects that of β -cell membrane-bound proteins. Insulin, although not membrane-bound, is the primary cargo of the secretory granule, while IA-2, phogrin, and ZnT8 are integrated in the granule membrane itself [12]. The former PTPs contain single transmembrane regions with relatively large cytosolic tails (~ 378 aa), where the vast majority of autoantibody epitopes reside [12]. In contrast, ZnT8 is polytopic, spanning the granule membrane 6 times with the N- and C-terminal domains residing on the membrane's cytoplasmic face, while GAD65 is principally localized to the cytoplasmic face of synaptic-like microvesicles by virtue of palmitoylation [13].
3. Most show tissue-specific gene expression. T1D islet autoantigens typically display elevated levels of transcription specifically in the pancreatic β cell, although several are also expressed in a wider spectrum of neuronal and neuroendocrine tissues. Thus, insulin is predominantly expressed in pancreatic β cells but displays trace expression in various other tissues, such as the brain and thymus [14]. In contrast, whereas IA-2 and phogrin are enriched in β -cells within the pancreas [12, 15], they are also expressed at significant levels in a restricted population of neurons and other neuroendocrine tissues and at detectable levels in the thymus and spleen [16]. GAD65 shows broad expression within the nervous system, in all islet cells, including alpha, beta, and delta cells, and to a smaller degree, in the lung [17]. ZnT8 is the most highly expressed zinc transporter in the beta cell, while lower levels of expression have been reported in the glucagon (α) and somatostatin (δ)

producing cells of the islet [18], as well as a restricted population of extrapancreatic cells.

4. All show evidence of alternative splicing. At present, the mechanisms responsible for the breakdown in tolerance to diabetes autoantigens remains unclear, although one plausible theory is that differential expression in the thymus could allow potentially pathogenic T cells to escape negative selection. Consistent with this hypothesis splice variants for all four major autoantigens insulin;3, IA-2;3, GAD65;2, and ZnT8;5 have been reported [19–21], although not all encode distinct proteins. The biological relevance of variations in the ratios of thymic versus β cell expression of T1D autoantigen splice forms is an area of active investigation.

Methods for T1D Autoantigen Discovery

Immunofluorescence of pancreatic sections treated with sera from individuals with T1D provided the first evidence of islet autoimmunity and is the basis of the classic islet cell autoantibody (ICA) test [22]. Subsequently, a myriad of strategies designed to identify the components of the immunoreactivity displayed by the ICA test appeared in the T1D literature. GAD65 autoantibodies were identified by their clinical association with the rare neurological disorder stiff person syndrome, evidenced by the fact that sera from such patients contain antibodies that cross-react with islet cells; related autoantibodies were subsequently identified in the sera of T1D patients [23]. Biochemically, GAD65 was immunoprecipitable from isotopically tagged rat islets with sera from individuals with T1D [24]. Preabsorption of the sera with recombinant GAD65 demonstrated its contribution to the ICA test (81). In contrast, expression cloning of human islet and insulinoma cDNA libraries screened with T1D sera facilitated the discovery of IA-2 and phogrin, which represent another component of ICA [12, 25, 26].

Independently of the ICA test, autoantibodies to the prime candidate insulin were first demonstrated in sera that were obtained from patients with newly diagnosed T1D prior to the commencement of treatment with exogenous insulin, by immunoprecipitation of ^{125}I -labeled antigen [27]. Other methods used to examine autoreactivity to candidate gene products include immunoprecipitation (IPP) of islet extracts, western blotting using T1D sera for detection, ELISA, 2D gel electrophoresis coupled with mass spectrometry, high throughput IPPs, competitive binding of sera from diabetic subjects to monoclonal antibodies reactive to insulinoma cell lines, and proteomics. Autoantibodies to the targets thus identified have significantly varying prevalences (5%–88%) in recently diagnosed diabetic individuals (reviewed in [28]).

Novel Autoantigens in T1D

More recently, autoantibodies to Pdx1 and Reg1A have been demonstrated with reported prevalences of 24% and 47%, respectively [29, 30]. In both cases, the targets were identified by probing Western blots with sera from diabetic patients. It should be noted that Reg1A is classified as “undetermined” in our fluid phase RIA (Table 1), since the assay gave high background signals with control sera that were indistinguishable from the test samples. This may serve to illustrate the limitations of relying solely on the RIA technique. The cytokine CCL3 has also been reported to be a frequent target of autoantibodies in T1D [31], although this observation has not been widely replicated [32] (Table 1). At present, the reasons for the discrepancy are unclear but may reflect differences in the demographics of the populations tested.

Autoantibodies to a 9 aa epitope shared by three otherwise unrelated proteins, golgin-160, voltage-gated potassium channel, and disulfide isomerase, were detected following

screening of human islet expression libraries with sera from patients and controls [33], although the prevalence was not reported. Similarly, serological proteome analysis demonstrated that autoantibodies to Rab GDP dissociation inhibitor beta (GDIb) are present in 22%–32% of subjects with T1D, depending on the format of the antigen probe [34]. In this study, sera from patients previously demonstrated to be negative for the four established autoantigens were used to probe Western blots of an abbreviated human β cell proteome that had been resolved via 2D electrophoresis, and immuno-reactive proteins identified by mass spectrometry. Eleven proteins were identified and assayed with custom RIAs, with GDIb displaying the most significant immunoreactivity.

Identification of Zinc Transporter 8 as a Major Autoantigen in T1D

Discovery of the fourth major humoral autoantigen, ZnT8, the most recent identified to date, involved a novel bioinformatics strategy based on the aforementioned common features of the previously established major autoantigens: association with the secretory pathway, enhanced β -cell expression, and alternative splicing coupled with multidimensional analyses of microarray-based mRNA expression profiling [35]. An algorithm was developed that calculated an index of tissue specificity based on distribution and abundance of transcripts. The ratios of messages in the whole pancreas versus islets, insulinoma versus glucagonoma cell lines, and wild type versus Ngn3 knockout mice were distinguishing variables. The list of antigen candidate genes contained all the existing primary T1D autoantigens, in addition to many minor ones that had already been documented. Among the remaining potential autoantigens, the top candidate was SLC30A8 (ZnT8).

ZnT8 Radioimmunoassay Development

To examine potential autoreactivity, Wenzlau and colleagues [35] optimized radioimmunoassays (RIAs) for detection of ZnT8 autoantibodies by cloning human ZnT8 cDNA (or derivatives thereof) into a vector suitable for in vitro transcription and translation in the presence of ^{35}S -labeled methionine to generate antigen probes, which were subsequently incubated with human T1D sera. Initially, 60%–80% of sera from subjects with new onset T1D were found to contain ZnT8 antibodies, and most remarkable, 26% were sera classified as autoantibody negative, using the established assays. Thus, some individuals previously categorized as single antibody positive were double antibody positive, and likewise, double autoantibody positive individuals may be triply positive. Subsequent studies suggested that ZnT8 autoantibody positive “prediabetic” individuals who are also positive for any one other T1D autoantibody are at greater risk for developing disease than are those who have two autoantibodies that do not include ZnT8.

Analysis of the predicted structure of the ZnT8 C-terminal (cytosolic) domain, where the vast majority of immunoreactivity has been mapped, has permitted the design of epitopespecific ZnT8 probes to optimize autoantibody detection. Probes yielding the highest sensitivity and specificity omit the (6) hydrophobic transmembrane regions and extraneous amino acid stretches at the N- and C-termini of the cytoplasmic region, provided that they maintain proper protein folding and, thus, epitope conformation [36].

Dissection of the ZnT8 epitopes highlighted the influence of a common nonsynonymous SNP (rs13266634) encoding amino acid (aa) 325 (Arg or Trp), which have since been demonstrated as single determinants of immunoreactivity, yet not a risk for T1D. Yet another SNP encodes Gln at the same position but is relatively rare (1% Europeans, 1%–2% Asians, 9% Africans). The specificity of ZnT8 autoreactivity in some individuals is exclusively dictated by the 325 variant encoded by their genes, albeit in others autoreactivity reflects a more complex pattern, with sera precipitating multiple ZnT8 Arg, Trp, or Gln variants [37]. The SNP-encoded amino acid residue determines the binding specificity of a

subset of ZnT8A. Remarkably, genome-wide association studies have shown that the major allele encoding the Arg variant is associated with increased susceptibility to type 2 diabetes, but no direct association between the polymorphism and overall T1D risk has been shown. The major allele may confer an increased susceptibility for developing autoimmune diabetes at a younger age, and homozygosity at this locus (either Arg or Trp) can stratify risk in ZnT8A+, but not ZnT8A-, individuals.

Contribution of ZnT8 Autoantibody Measurement

Assessment of the prevalence of ZnT8 autoantibodies in recently diabetic subjects, in combination with monitoring of GAD65, IA-2, and insulin autoantibodies, allows autoimmunity to be confirmed in more than 96% of cases [6, 38, 39, 40••] (Fig. 1).

Probability of Additional T1D Autoantigens

There is a strong likelihood that additional targets of ICAs have yet to be characterized. This is evidenced by the presence of individuals with positive ICA tests that do not show immunoreactivity with any of the major currently defined T1D autoantigens [6], although it remains uncertain whether this represents reactivity to a plethora of minor targets or to one or more with highly prevalent specificity. Currently, reliable autoantibody assays are available for antibody detection to insulin, GAD65, IA-2/phogrin, and ZnT8, which have each been validated by the Diabetes Autoantibody Standardization Program [41•]. Assessment of these autoantibodies is crucial for identifying genetically at-risk individuals who display autoimmunity, thereby prompting their recruitment to therapeutic interventions to slow progression to T1D. Unfortunately, despite considerable efforts, the current protocols still fall short of the necessary degrees of sensitivity and specificity required for cost-effective screening of prediabetes in the general population. Consequently, the pursuit for new targets of ICAs persists, especially since, in addition to their value as diagnostic and predictive biomarkers, autoantibodies typically identify the targets of pathogenic autoreactive T cells.

Screening Antigen Candidates by RIA

To assess the immunoreactivity of new candidate antigens, it is best to screen with sera derived from newly diagnosed subjects (within 2 weeks of onset), when the autoantibody titers are likely to be highest, since titer and, hence, detectability typically tend to wane postonset, likely as a reflection of the continuing loss of residual β cell mass [42]. To date, we have investigated 36 additional candidates (Table 1) using the protocol described below. Full length cDNAs are generated via RT-PCR, with RNA isolated from human islets or existing cDNA clones (if available) as templates, and are introduced into suitable expression vectors that will enable radiolabeled antigen probes to be generated by coupled in vitro transcription/translation. Subsequently, a panel of sera (typically from ~100 recently diabetic subjects and 20 unaffected controls) are tested using the ^{35}S -methionine labeled antigenic probe.

In many instances, autoantibodies recognize conformational rather than linear epitopes. This may cause problems with those autoantigens that are membrane-bound, where use of the full-length protein may be inappropriate due to misfolding or insolubility. In such cases, it is possible to interrogate the primary structure with programs that predict probable molecular topology to identify extramembranous stretches of amino acids with the potential to form epitopes. These regions can then be modified to include artificial initiation codons in the context of a Shine-Delgano or Kozak consensus site (as appropriate for the expression system) and subcloned independently for further consideration [35]. Our experience with ZnT8 exemplifies this approach: The full-length protein (including six transmembrane

domains) was poorly soluble, giving a high background signal, and thus was able to detect positivity in only ~20% of sera from recently diabetic subjects, whereas the C-terminal 93 aa gave minimal background signals and could detect autoreactivity in ~70% of the same sera. A second illustration of the success of this strategy is provided by a sensitive and specific RIA for detection of autoantibodies indicative of autoimmune gastritis/pernicious anemia (PA) that we recently developed [43, 44]. The major antigen recognized by parietal-cell autoantibodies is gastric H^+/K^+ -ATPase, a heterodimer composed of α and β subunits (SU). The α SU is a multipass transmembrane protein with three major cytoplasmic loops [45]. These were monitored separately as antigen probes with sera from relevant individuals. One of the three domains displayed significant immunoreactivity that, after further optimization, provided a probe that achieved 95% sensitivity at 100% specificity, using a panel of sera from subjects whose diagnosis had been confirmed by biopsy [41•].

It should be noted however, that a potential caveat to the domain-specific approach is that recombinant proteins containing only intracellular and/or extracellular loops may fail to form conformational epitopes, because they either lack the structural constraints imposed by the membrane anchors or require contributions from multiple loops/domains. This problem can be addressed by incorporating the full-length protein into synthetic nano-discs containing phospholipid and apolipoproteins [46] that allow correct topology to be established while maintaining the protein in a soluble state that allows access of autoantibodies to both external and internal domains.

Another potential problem is that, in some instances, full-length candidate antigens or derivatives do not contain a sufficient number of methionines for high specific activity isotopic labeling. In this case, probes containing in-frame concatenated multimers and/or the addition of methionines to the N or C termini can be generated to enhance the specific activity of the resulting probes. Multimerization may also increase functional avidity of the probe and, hence, sensitivity. Again, as demonstrated with our optimization of the ZnT8 RIA, where dimeric probes containing the major epitopes in the C-terminus further enhanced the sensitivity and specificity by reducing the signal-to-noise ratio and/or, potentially, by allowing detection of epitopes dependent on dimerization, the endogenous conformation of ZnT8 [41•] (Fig. 2).

The results from screening the antigen candidates listed in Table 1 are segregated according to signature of the assay. For individual assays, 20,000 cpm of gel-filtered probe are incubated with each serum sample, and at least 100 T1D sera are monitored simultaneously. The cutoff for positivity is specified as 3 standard deviations above the mean of the internal controls for each assay. Where possible, a commercial positive control serum is also included. Our stratification criteria were that RIAs displaying higher than 10% prevalence were considered significant T1D antigens (ZnT8). Candidates with less than 10% positives in the test sera samples were classified as “low prevalence.” Finally, as was discussed above, not unexpectedly several candidates displayed high backgrounds precipitating >1,000 cpm with the control sera. In such cases, only high-affinity antibodies would be detected, since low signals would be masked by background binding. Consequently, this group is classified as “undetermined.” Despite the initial success of the bioinformatic algorithm in identifying ZnT8 as a major antigen in T1D, it is apparent from the results presented in Table 1 that not all potential candidates are actually targets of autoimmunity, although it should be noted that not all Tcell targets generate autoantibodies, as appears to be the case for IGRP, and those classified as “undetermined” could actually generate autoantibodies that we have so far failed to detect.

In general, we assume the high background signals among negative controls originate from improper protein folding and consequential formation of aggregates that precipitate during

the collection of immune complexes. This is most likely to occur with membrane proteins; however, it could also result from the absence of key post-translational modifications that are absent in probes generated by the in vitro system we generally employ. Moreover, it is becoming apparent that, in some instances, epitopes depend on the presence of post-translational modifications, be they native to the endogenous protein or a consequence of pathological modification. To address the former concern, antigen probes expressed as fusion proteins with derivatives of luciferase targeted for secretion from transfected mammalian cells can be generated and harvested from the media. An added attribute of this system is the avoidance of radioactivity, since the read-out is bioluminescent signal intensity (Hutton et al., unpublished data). Alternatively, probes can be treated with enzymes, such as peptidyl arginine deiminase or transglutaminase, or with oxidants to introduce potential pathogenic changes [47].

Conclusions

The bioinformatics algorithm that prompted both our initial studies with ZnT8 and subsequent consideration of many of the candidates listed in Table 1 defined specificity on the basis of the expression of the primary transcript in pancreatic β cells, an assumption with limitations. It did not consider the possibility of β cell enriched, alternatively spliced variants, since that data resource was not widespread at the time of the algorithm design. With the increasing application of exon arrays and RNAseq, it is now apparent that a high percentage of genes are alternatively spliced, although it remains uncertain how many such transcripts reflect stable proteins that could potentially be targeted by autoantibodies. Precise characterization of the β cell proteome, from which all primary targets of diabetic autoimmunity must by definition be drawn, is further complicated by the wide spectrum of posttranslational modifications that individual gene products can undergo, some of which may be tissue specific, and others the consequence of cellular responses to various stresses. Thus, it can be expected that the number of targets of diabetes-associated humoral autoimmunity will increase as our knowledge of the “normal” and “stressed” proteomes grows, which may permit major advances in future diagnostic and therapeutic approaches.

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References

Papers of particular interest, published recently, have been highlighted as:

- Of importance
 - Of major importance
1. Rosmalen JG, van Ewijk W, Leenen PJ. T-cell education in autoimmune diabetes: teachers and students. *Trends Immunol.* 2002; 23(1):40–6. [PubMed: 11801453]
 2. Pihoker C, Gilliam I, Hampe C, Lernmark A. Autoantibodies in diabetes. *Diabetes.* 2005; 54:S52–61. [PubMed: 16306341]
 3. Eisenbarth GS. Type I diabetes mellitus. A chronic autoimmune disease. *N Engl J Med.* 1986; 314(21):1360–8. [PubMed: 3517648]
 4. Oling V, Marttila J, Ilonen J, Kwok WW, Nepom G, Knip M, et al. GAD65- and proinsulin-specific CD4+ T-cells detected by MHC class II tetramers in peripheral blood of type 1 diabetes patients and at-risk subjects. *J Autoimmun.* 2005; 25(3):235–43. [PubMed: 16263242]

5. Kelemen K, Gottlieb PA, Putnam AL, Davidson HW, Wegmann DR, Hutton JC. HLA-DQ8-associated T cell responses to the diabetes autoantigen phogrin (IA-2 beta) in human prediabetes. *J Immunol.* 2004; 172(6):3955–62. [PubMed: 15004204]
6. Pietropaolo M, Towns R, Eisenbarth GS. Humoral autoimmunity in type 1 diabetes: prediction, significance, and detection of distinct disease subtypes. *Cold Spring Harb Perspect Med.* 2012 1101/cshperspect.a012831.
7. Arvan P, Pietropaolo M, Ostrov D, Rhodes CJ. Islet autoantigens: structure, function, localization, and regulation. *Cold Spring Harb Perspect Med.* 2012 10.1101/cshperspect.a007658
8. Eizirik DL, Colli ML, Ortis F. The role of inflammation in insulinitis and beta-cell loss in type 1 diabetes. *Nat Rev Endocrinol.* 2009; 5(4):219–26. [PubMed: 19352320]
9. Bonifacio E, Lampasona V, Genovese S, Ferrari M, Bosi E. Identification of protein tyrosine phosphatase-like IA2 (islet cell antigen 512) as the insulin-dependent diabetes-related 37/40K autoantigen and a target of islet-cell antibodies. *J Immunol.* 1995; 155(11):5419–26. [PubMed: 7594559]
10. Chimienti F, Favier A, Seve M. ZnT-8, a pancreatic beta-cell-specific zinc transporter. *Biomaterials.* 2005; 18(4):313–7. [PubMed: 16158222]
11. Chimienti F, Devergnas S, Pattou F, Schuit F, Garcia-Cuenca R, Vandewalle B, et al. In vivo expression and functional characterization of the zinc transporter ZnT8 in glucose-induced insulin secretion. *J Cell Sci.* 2006; 119:4199–206. [PubMed: 16984975]
12. Wasmeier C, Hutton JC. Molecular cloning of phogrin, a proteintyrosine phosphatase homologue localized to insulin secretory granule membranes. *J Biol Chem.* 1996; 271(30):18161–70. [PubMed: 8663434]
13. Christgau S, Aanstoot HJ, SchierbeckH, Begley K, Tullin S, Hejnaes K, et al. Membrane anchoring of the autoantigen GAD65 to microvesicles in pancreatic beta-cells by palmitoylation in the NH2-terminal domain. *J Cell Biol.* 1992; 118(2):309–20. [PubMed: 1321158]
14. Kutlu B, Burdick D, Baxter D, Rasschaert J, Flamez D, Eizirik DL, et al. Detailed transcriptome atlas of the pancreatic beta cell. *BMC Med Genomics.* 2009; 2:3. [PubMed: 19146692]
15. Mziaut H, Trajkovski M, Kersting S, Ehninger A, Altkrüger A, Lemaitre RP, et al. Synergy of glucose and growth hormone signalling in islet cells through ICA512 and STAT5. *Nat Cell Biol.* 2006; 8(5):435–45. [PubMed: 16622421]
16. Diez J, Park Y, Zeller M, Brown D, Garza D, Ricordi C, et al. Differential splicing of the IA-2 mRNA in pancreas and lymphoid organs as a permissive genetic mechanism for autoimmunity against the IA-2 type 1 diabetes autoantigen. *Diabetes.* 2001; 50(4):895–900. [PubMed: 11289059]
17. Wang C, Mao R, Van de Casteele M, Pipeleers D, Ling Z. Glucagon-like peptide-1 stimulates GABA formation by pancreatic beta-cells at the level of glutamate decarboxylase. *Am J Physiol Endocrinol Metab.* 2007; 292(4):E1201–6. [PubMed: 17190904]
18. Tamaki M, Fujitani Y, Uchida T, Hirose T, Kawamori R, Watada H. Downregulation of ZnT8 expression in pancreatic β -cells of diabetic mice. *Islets.* 2009; 1(2):124–8. [PubMed: 21099260]
19. Marchand L, Polychronakos C. Evaluation of polymorphic splicing in the mechanism of the association of the insulin gene with diabetes. *Diabetes.* 2007; 56(3):709–13. [PubMed: 17327440]
20. Diez J, Park Y, Zeller M, Brown D, Garza D, Ricordi C, et al. Differential splicing of the IA-2 mRNA in pancreas and lymphoid organs as a permissive genetic mechanism for autoimmunity against the IA-2 type 1 diabetes autoantigen. *Diabetes.* 2001; 50(4):895–900. [PubMed: 11289059]
21. Liu H, Wang Z, Li S, Zhang Y, Yan YC, Li YP. Utilization of an intron located polyadenylation site resulted in four novel glutamate decarboxylase transcripts. *Mol Biol Rep.* 2009; 36(6):1469–74. [PubMed: 18758993]
22. Bottazzo GF, Florin-Christensen A, Doniach D. Islet-cell antibodies in diabetes mellitus with autoimmune polyendocrine deficiencies. *Lancet.* 1974; 2(7892):1279–83. [PubMed: 4139522]
23. Baekkeskov S, Aanstoot HJ, Christgau S, Reetz A, Solimena M, Cascalho M, et al. Identification of the 64K autoantigen in insulin-dependent diabetes as the GABA-synthesizing enzyme glutamic acid decarboxylase. *Nature.* 1990; 347(6289):151–6. [PubMed: 1697648]
24. Hagopian WA, Michelsen B, Karlsen AE, Larsen F, Moody A, Grubin CE, et al. Autoantibodies in IDDM primarily recognize the 65,000-M(r) rather than the 67,000-M(r) isoform of glutamic acid decarboxylase. *Diabetes.* 1993; 42(4):631–6. [PubMed: 8454115]

25. Rabin DU, Pleasic SM, Palmer-Crocker R, Shapiro JA. Cloning and expression of IDDM-specific human autoantigens. *Diabetes*. 1992; 41(2):183–6. [PubMed: 1733807]
26. Lan MS, Lu J, Goto Y, Notkins AL. Molecular cloning and identification of a receptor-type protein tyrosine phosphatase, IA-2, from human insulinoma. *DNA Cell Biol*. 1994; 13(5):505–14. [PubMed: 8024693]
27. Palmer JP, Asplin CM, Clemons P, Lyen K, Tatpati O, Raghu PK, et al. Insulin antibodies in insulin-dependent diabetics before insulin treatment. *Science*. 1983; 222(4630):1337–9. [PubMed: 6362005]
28. Wenzlau, JM.; Sheridan, L.; Hutton, JC. *Immunoendocrinology: Scientific and Clinical Aspects 2010*, Book Chapter. Springer Publishing; *Discovering Novel Antigens in Type 1 Diabetes*.
29. Astorri E, Guglielmi C, Bombardieri M, Alessandri C, Buzzetti R, Maggi D, et al. Circulating Reg1 α proteins and autoantibodies to Reg1 α proteins as biomarkers of β -cell regeneration and damage in type 1 diabetes. *Horm Metab Res*. 2010; 42(13):955–60. [PubMed: 20972946]
30. Li SW, Koya V, Li Y, Donelan W, Lin P, Reeves WH, et al. Pancreatic duodenal homeobox 1 protein is a novel beta-cell-specific autoantigen for type I diabetes. *Lab Invest*. 2010; 90(1):31–9. [PubMed: 19901909]
31. Shehadeh N, Pollack S, Wildbaum G, Zohar Y, Shafat I, Makhoul R, et al. Selective autoantibody production against CCL3 is associated with human type 1 diabetes mellitus and serves as a novel biomarker for its diagnosis. *Immunol*. 2009; 182(12):8104–9.
32. Huang G, Mo X, Li M, Xiang Y, Li X, Luo S, et al. Autoantibodies to CCL3 are of low sensitivity and specificity for the diagnosis of type 1 diabetes. *Acta Diabetol*. 2012; 49(5):395–9. [PubMed: 22350136]
33. Fierabracci A, Saura F. Identification of a common autoantigenic epitope of protein disulfide isomerase, golgin-160 and voltage-gated potassium channel in type 1 diabetes. *Diabetes Res Clin Pract*. 2010; 88(2):e14–6. [PubMed: 20170975]
34. Massa O, Alessio M, Russo L, Nardo G, Bonetto V, Bertuzzi F, et al. Serological Proteome Analysis (SERPA) as a tool for the identification of new candidate autoantigens in type 1 diabetes. *J Proteomics*. 2013; 82C:263–73. [PubMed: 23500132]
35. Wenzlau JM, Juhl K, Yu L, Moua O, Sarkar S, Gottlieb P, et al. The cation efflux transporter ZnT8 (Slc30A8) is a major autoantigen in human type 1 diabetes. *Proc Natl Acad Sci U S A*. 2007; 104:17040–5. [PubMed: 17942684]
36. Wenzlau, JM.; Gardner, TJ.; Yu, L.; Eisenbarth, GS.; Davidson, HW.; Hutton, JC. Composite multi-antigen probes enhance detection of type 1 diabetes autoantibodies. Poster presentation at the 45th Annual Meeting of the European Association for the Study of Diabetes (EASD); Vienna, Austria. Sept. 8–11, 2009;
37. Wenzlau JM, Liu Y, Yu L, Moua O, Fowler KT, Rangasamy S, et al. A Common Non-synonymous Single Nucleotide Polymorphism in the Slc30A8 Gene Determines ZnT8 Autoantibody Specificity in Type 1 Diabetes. *Diabetes*. 2008; 57(10):2693–7. The study shows ZnT8 variant specific autoimmunity demonstrating a true immune response against self molecules. [PubMed: 18591387]
38. Kordonouri O, Hartmann R, Charpentier N, Knip M, Danne T, Ilonen J. Genetic risk markers related to diabetes-associated autoantibodies in young patients with type 1 diabetes in berlin, Germany. *Exp Clin Endocrinol Diabetes*. 2010; 118(4):245–9. [PubMed: 20140847]
39. Brorsson C, Vaziri-Sani F, Bergholdt R, Eising S, Nilsson A, Svensson J, Pociot F, et al. Danish Study Group of Childhood Diabetes. Correlations between islet autoantibody specificity and the SLC30A8 genotype with HLA-DQB1 and metabolic control in new onset type 1 diabetes. *Autoimmunity*. 2011; 44(2):107–14. [PubMed: 20836749]
- 40•. Yu L, Miao D, Scrimgeour L, Johnson K, Rewers M, Eisenbarth GS. Distinguishing persistent insulin autoantibodies with differential risk: nonradioactive bivalent proinsulin/insulin autoantibody assay. *Diabetes*. 2012; 61(1):179–86. The paper documents firm establishment of a consistent, reliable and sensitive insulin autoantibody assay. [PubMed: 22124462]
- 41•. Lampasona V, Schlosser M, Mueller PW, Williams AJ, Wenzlau JM, Hutton JC, et al. Diabetes antibody standardization program: first proficiency evaluation of assays for autoantibodies to

- zinc transporter 8. *ClinChem*. 2011; 57(12):1693–702. External validation of the ZnT8 RIA, leading to eventual implementation for clinical diagnosis of T1D.
42. Wenzlau JM, Walter M, Gardner TJ, Frisch LM, Yu L, Eisenbarth GS, et al. Kinetics of the post-onset decline in zinc transporter 8 autoantibodies in type 1 diabetic human subjects. *J Clin Endocrinol Metab*. 2010; 95(10):4712–9. [PubMed: 20610599]
 43. Ungar B, Stocks AE, Martin FI, Whittingham S, Mackay IR. Intrinsic-factor antibody, parietal-cell antibody, and latent pernicious anaemia in diabetes mellitus. *Lancet*. 1968; 2(7565):415–7. [PubMed: 4174152]
 44. Wenzlau JM, Gardner TJ, Frisch LM, Davidson HW, Hutton JC. Development of a novel autoantibody assay for autoimmune gastritis in type 1 diabetic individuals. *Diabetes Metab Res Rev*. 2010; 27(8):887–90. [PubMed: 22069279]
 45. Maeda M. Gastric proton pump (H⁺/K⁺)-ATPase): structure and gene regulation through GATA DNA-binding protein(s). *J Biochem*. 1994; 115(1):6–14. [PubMed: 8188638]
 46. Shenkarev ZO, Lyukmanova EN, Butenko IO, Petrovskaya LE, Paramonov AS, Shulepko MA, et al. Lipid-protein nanodiscs promote in vitro folding of transmembrane domains of multi-helical and multimeric membrane proteins. *Biochim Biophys Acta*. 2013; 1828(2):776–84. [PubMed: 23159810]
 47. Khan MW, Sherwani S, Khan WA, Ali R. Characterization of hydroxyl radical modified GAD65: a potential autoantigen in type 1 diabetes. *Autoimmunity*. 2009; 42(2):150–8. [PubMed: 19085183]

Fig. 1.

Combining ZnT8A with IAA, GADA, and IA2A assays increases the sensitivity of autoimmune detection. Sera obtained from recently diabetic subjects ranging in age from 2 to 40 years were assayed in parallel with RIAs to insulin (INS), GAD65 (GAD), IA-2, and ZnT8. Overall prevalence is shown for each antigen, although it should be noted that this can vary significantly with age of onset, especially for IAA. In this cohort, the combined measurement of GADA, IAA, and IA2A detected autoimmunity in ~86% of subjects. Inclusion of ZnT8A increased this to ~96%, and ~25% reduction in the number of autoantibody negative individuals

Fig. 2. Homo-dimeric ZnT8 antigen probes enhance sensitivity. Sera from newly diabetic subjects were assayed in parallel with monomeric and homo-dimeric ZnT8 variant probes. Detected prevalences with monomers versus dimers were $X \text{ v } Y$, $X1 \text{ v } Y1$, and $X2 \text{ v } Y2$ for the R, W, and Q probe pairs, respectively. Red lines indicate cutoff values

Table 1
Humoral autoimmunity to T1D candidate antigens identified using a bioinformatics approach

Gene Symbol	Common Name	Subdomain(s)	Prevalence (%)	Notes	Rank
<i>BETACAM</i>	hepaCAM		<1		
<i>CEL</i>	carboxy ester lipase		undetermined	high BG	11
<i>CCL3</i>	chemokine C-C motif 3		<1		
<i>CFC1</i>	cryptic family 1		<1		
<i>CHGA</i>	chromogranin A	3	<1		30
<i>CHGA</i>		3SD of SD1	<1		30
<i>CPE</i>	carboxypeptidase E		3		7
<i>FXYD2</i>	FXY domain ion transporter		undetermined	high BG	35
<i>GABA</i>	glyceraldehyde-3-phosphate dehydrogenase A		3	low prevalence	
<i>GCK</i>	glucokinase		<1		
<i>GPCT</i>	glutamyl peptide cyclotransferase	SV1	<1		
<i>GPCT</i>		SV2	<1		
<i>GNAS</i>	guanine nucleotide binding protein alpha stimulating	SV1	4	low prevalence	6
<i>GNAS</i>		SV2	2	low prevalence	6
<i>GNAS</i>		SV3	3	low prevalence	6
<i>G6PT</i>	glucose-6-phosphatase		5	low prevalence	
<i>HK</i>	hexokinase		<1		
<i>IGRP</i>	islet-specific glucose-6-phosphatase catalytic subunit-related protein	SV1	4	low prevalence	10
<i>IGRP</i>		SV2	<1		10
<i>IGRP</i>		SV3	<1		10
<i>IGRP</i>		SV4	<1		10
<i>IGRP</i>		SV5	<1		10
<i>IAPP</i>	islet amyloid polypeptide		<1		2
<i>IMOGEN38</i>	Islet mitochondrial antigen of 38KD		1	low prevalence	43
<i>KCNJ10</i>	potassium inwardly-rectifying channel		2	low prevalence	
<i>KCNMB2</i>	potassium inwardly-rectifying channel		5	low prevalence	31
<i>NKX2.2</i>	NK transcription factor		4	low prevalence	12
<i>NPY</i>	neuropeptide Y receptor		undetermined	high BG	46
<i>PAM</i>	peptidylglycine alpha-amidating monooxygenase		<1		

Gene Symbol	Common Name	Subdomain(s)	Prevalence (%)	Notes	Rank
<i>PA M</i>		C-term	<1		
<i>PC2</i>	protein convertase 2	<1		15	
<i>PSKN1</i>	protein convertase 1		<1		20
<i>PDX1</i>	pancreas and duodenal homeobox 1		1	low prevalence	
<i>PDX1</i>		SD1	<1		
<i>REG1A</i>	regenerating islet derived 1A		undetermined	high BG	
<i>REG3A</i>	regenerating islet derived 3A		9	low prevalence	35
<i>REG3G</i>	regenerating islet derived 3G		<1		32
<i>GRCH37</i>	serpin B13		<1		
<i>SEZ6L2</i>	seizure-related 6 homolog (mouse)-like 2		undetermined	high BG	33
<i>SGNE</i>	7B2		undetermined	high BG	3
<i>STYL4</i>	granuphilin		<1		66
<i>TMED6</i>	transmembrane emp24 protein transport domain containing 6		1	low prevalence	
<i>TMED6</i>		luminal domain	<1		
<i>TMED6</i>		SD1	1	low prevalence	
<i>TM4SF4</i>	transmembrane 4 L		<1	high BG	
<i>TM4SF4</i>		SD1	<1		
<i>VEGF</i>	nerve growth factor inducible	SV1	<1	high BG	
<i>VEGF</i>		SV2	<1		
<i>VMAT2</i>	vesicle monoamine transporter 2		undetermined	high BG	
<i>WFM</i>	wolfram	SD1	<1		
<i>WFM</i>	wolfram	SD2	undetermined	high BG	
<i>SLC30A8</i>	zinc transporter 8	C-terminal domain	60-80	high prevalence	4
<i>SLC30A8</i>	zinc transporter 8		20	high prevalence	4

Note. A list of candidate antigens was generated using a previously described bioinformatics-based algorithm (ref 35), and recognition of 36 of these potential targets by patient or control sera tested using custom RIAs. Only 1 target (ZnT8) showed evidence of autoimmunity at high prevalence and 15 at low prevalence, and the remainder were either negative or classified as undetermined due to high background signals (>5% input radiolabel). SD, subdomain; SV, splice variant; BG, background.