Chapter 2

Discovering Novel Antigens

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Summary

This chapter aims to highlight the importance of autoantigen discovery for the development of immunological interventions following diagnosis and/or progression of type 1 diabetes, an autoimmune disease that specifically targets and destroys pancreatic β cells within the Islets of Langerhans. Both humoral (B-cell) and cell-mediated (T-cell) autoimmunity will be discussed, specifically focusing on the experimental models through which novel autoantigens have been discovered, characterized, and exploited for diagnostic purposes.

Key Words: ZnT8 zinc transporter 8, GAD65 glutamic acid decarboxylase, Insulin autoantibody, Proteomics, Expression library

INTRODUCTION

Type 1 autoimmune diabetes (T1D) is a progressive disease characterized by the targeted destruction of the insulin-secreting β-cells within the islets of Langerhans of the pancreas (*1*). It is generally accepted that T1D is a T-cell mediated disease and results from immune dysregulation with consequential loss of tolerance to β-cell antigens and ultimately insulitis, the infiltration of CD4+ and CD8+ lymphocytes into the islets. The major antigenic targets of autoimmunity have been defined via analysis of circulating autoantibodies, although they are not believed to be intrinsically diabetogenic. However, there is increasing evidence that autoantigen-specific B-cells play a significant role in the pathogenesis of T1D, as they are critical mediators of antigen presentation and comprise a large proportion of the mononuclear cells in the insulitic lesions of the pancreas (*2*). As the disease progresses and β-cell deterioration ensues, the disease manifests as impaired glucose tolerance and ultimately proceeds to symptomatic hyperglycemia (*2*).

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Despite the prevalence of sporadic disease (greater than 80%), there is a clear genetic predisposition associated with the development of β-cell autoimmunity. The majority of genetic determinants reside in the major histocompatibility loci, particularly HLA-DR and DQ, which account for greater than 40% of inheritability (*3, 4*). Furthermore, first-degree relatives of T1D individuals carry a significantly greater risk of developing T1D (6%) than individuals in the general population (0.4%) (*5, 6*). Thus, the disease presumably arises from failure to tolerate β-cell antigens in individuals with intrinsic (genetic) defects in critical immunomodulatory mechanisms (*7*). This underlying genetic predisposition combined with potential environmental triggers such as viral infections or dietary customs may initiate the process of autoimmune β-cell destruction, which can proceed for years without symptoms prior to overt clinical disease.

The prediction of T1D can be based on genetic susceptibility (*8, 9*), metabolic tests (*10–12*), or the detection of autoantibodies. A risk score based on a metabolic model including BMI, age, fasting C-peptide measurement, and post-challenge glucose and C-peptide levels from 2-hr oral glucose tolerance tests was indeed predictive of T1D; however, the subjects in the study were preselected as ICA positive (*11*). Currently, the measure of autoantibodies in circulation provides the most reliable preclinical indicator for diabetic autoimmunity, as their appearance can precede disease onset for years. Furthermore, the presence of multiple autoantibodies rather than their individual titer has the highest predictive value for progression to disease. The molecules recognized by autoantibodies generated in B-cells should likewise be targets for autoreactive diabetogenic T-cells as has been demonstrated for the established T1D antigens insulin, phogrin, and GAD65 (*13, 14*). Since the underlying mechanism required to generate antibodies requires the participation of T-cells, it is logical that B-and T-cells recognize the same antigens.

Identification of the autoantigens in T1D provides potential development of antigen-specific B-cell and T-cell therapies. Individual autoantibody profiling is vital to evaluate antigen-specific therapeutic interventions to prevent or delay clinical onset. Autoantibodies typically appear sequentially rather than concurrently during the course of the disease, and the prevalence and titer of autoantibodies varies with age (*15–17*). For instance, the titer and prevalence of insulin autoantibodies (IAA) display dramatic age dependency, as they are present in 90% of children who progress to disease onset prior to age 5, and from then on display an inverse correlation with age as disease develops (*18*). In adults, insulinoma autoantigen-2/protein tyrosine phosphatase autoantibodies (IA2A) and the 65-kD form of glutamate decarboxylase GAD65 autoantibodies (GADA) are most prevalent (*19, 20*). Prevalence of ZnT8A is low in young individuals, increases significantly after the age of 3,

and peaks in late adolescence (*21*). It is crucial to evaluate and discriminate potential antigen-based administration therapies. Given the long phase of asymptomatic autoimmunity characteristic of T1D, comprehensive autoantibody detection would allow identification of susceptible individuals at an earlier stage of disease when β-cell mass is still relatively high and when immunological-based therapies are most effective (as has been documented in animal models and clinical trials (*22, 23*)). This time-sensitive opportunity for treatment may be tailored to the autoantibody repertoire of the patient to prevent or delay T1D onset.

Given the ability to profile circulating autoantibodies in subjects, immunosuppression and immunomodulation therapy for T1D has potential. The development of the B cell depletion drug Rituximab as a lymphoma therapy has been useful for evaluating its success for other immune disorders. Rituximab is a humanized anti-CD20 monoclonal antibody that can induce B cell depletion for more than a year in peripheral blood lymphocytes and is generally well tolerated (*24*). In new onset diabetic non-obese (NOD) mice, anti-CD20 therapy reversed and prevented T1D diabetes, confirming the rationale for clinical trials (*25*). TrialNet, conducting the study "The effects of Rituximab on the progression of type 1 diabetes in new onset subjects," administers Rituximab within 3 months of diagnosis to individuals displaying residual β-cell function as measured by C-peptide levels.

Preliminary results imply that Rituximab may preserve C-peptide and thus β-cell mass (*26*). Similarly, a T-cell depletion immunosuppressive drug, Teplizumab, comprised of anti-CD3 monoclonal antibodies (mAbs), was initially shown to inhibit β-cell destruction in T1D subjects in the first year post onset and for another year thereafter when administered at diagnosis (*27*). Subsequent trials suggested β cells may be conserved for up to 5 years in drug-treated patients (*28*). These initial studies indicate that both B and T cells are appropriate targets for altering the immunologic course of T1D, and advances in immunomodulatory drugs will likely include targeting of B and T cell specific populations.

To date, the detection of circulating autoantibodies is the most reliable preclinical indicator and diagnostic test for diabetic autoimmunity. Currently, three established autoantibody assays based on fluid phase (radioimmunoassay, RIAs) are employed for IAA, GADA, and IA2A. Immune complexes are formed with ^{125}I-labeled insulin and ^{35}S-labeled GAD and IA-2, the latter generated by recombinant DNA production of antigen and subsequent incubation with antibodies (*29*). The combined biochemical detection of these autoantibodies reveals 80% of at-risk individuals or patients at disease onset. Measurement of these autoantibodies has been critical for identifying genetically at-risk individuals who have progressed to autoimmunity and their recruitment to therapeutic trials aimed at preventing a slow progression to T1D.

However, the assays fail to achieve the high sensitivity and specificity essential for detection of prediabetes in the general population, where only one in 300 individuals are identified even with additional consideration of genetic predisposition (HLA).

Combining the relatively new ZnT8 RIA with the established autoantibody assays for IAA, GADA, and IA2A significantly enhances prediction of T1D development (90% of newly diagnosed individuals) (*21*), but may not be adequate to determine risk in the general population, where specificity and sensitivity ideally demand 99 and 99%, respectively. The validity of a measure for prediction or diagnosis of disease is determined by its sensitivity (the number of individuals who are diabetic, possessing autoantibodies) divided by the number of individuals who develop the disease, specificity (the number of individuals without autoantibodies who do not develop T1D) divided by the number of subjects who do not progress to disease and finally is positive predictive value. Autoantibody detection has proven to be a valuable biomarker for T1D owing to the long prodromal phase between initial autoantibody detection and clinical onset and their high predictive indication. Comprehensive population screening also requires reliable, efficient, cost-effective, high-throughput protocols for corroboration among labs. Such improvements in disease prediction will hopefully be accompanied by advances in disease prevention. Antigen-specific immune therapy offers potential evasion or delay of disease onset, and may be further strengthened by the identification of novel autoantigens.

Islet Cell Autoantibody

Despite earlier speculation, it was not until the 1970s that T1D was generally accepted as an autoimmune disease (*30*). This realization stemmed largely from two sets of observations. First, insulin-dependent diabetes (IDD) was more frequently associated with other autoimmune conditions, such as Hashimoto's thyroiditis, pernicious anemia, and Addison's disease, than would be expected by chance (*31*). Second, lymphocytic infiltration of the islets of Langerhans was evident in some juveniles who had died shortly after onset of clinical disease (*32–34*). The presence of insulitis in subjects with functional islet abnormalities suggested that cell-mediated immunity might contribute to disease. Consistent with this hypothesis, two independent reports subsequently showed that some patients with IDD showed evidence of cellular hypersensitivity to islet extracts (*35, 36*).

Although it was evident that the serum of some IDD patients contained autoantibodies to the cytoplasm of thyroid gastric parietal and adrenal cortical cells, these individuals being clinically asymptomatic for the associated autoimmune diseases (*37–39*), initial attempts to demonstrate the presence of antibodies that recognized islet cells in the serum of IDD patients by indirect immunofluorescence were unsuccessful (*40*). However, a combination of technical improvements in microscopy and serendipity finally enabled

Bottazzo (*41*) and subsequently MacCuish (*42*) to confirm the predicted existence of disease-associated islet cell antibodies (ICAs). The breakthrough advanced from the use of frozen sections of human pancreas from blood group O donors, which provided a lower background, and sera from subjects with autoimmune polyendocrinopathies, which in retrospect were shown typically to have relatively high titers of ICAs. Both of these initial reports concluded that ICAs were rare and confined to only a small subset of patients with multiple autoimmune diseases (*36, 41*). However, it later became evident that this conclusion was erroneous, stemming mainly from the use of a relatively insensitive assay, and a failure to appreciate the transient nature of ICAs post-onset (*30*). It is now accepted that at least 70–80% of all T1D patients have ICAs at onset (*42*). Moreover, elevated ICAs are predictive of progression to T1D in at least a subset of asymptomatic genetically at risk individuals.

Although the identification of ICAs represented a major advance in understanding T1D, the assay has proved difficult to standardize and requires specifically trained personnel for accurate interpretation. Consequently, considerable effort has been devoted towards identifying the molecular targets of the autoantibodies and designing robust high-throughput biochemical assays for their measurement. Initial studies suggested that sialic acid-containing glycolipids were a major target of ICAs (*43*), although doubt was later cast on this conclusion by the observation that recombinant GAD65 could at least partially quench ICAs in many individuals (*44*). Indeed, it is now appreciated that ICAs are highly heterogeneous and react both with antigens common to all islet endocrine cell types, as well as to multiple cytoplasmic and membrane-associated epitopes restricted to particular cell types (*45*). Moreover, it is highly likely that a number of the targets of ICAs have yet to be characterized, evidenced by the presence of individuals with ICAs that do not react with any of currently defined T1D autoantigens. Consequently, the search for novel targets of ICAs is an ongoing area of significant research interest, especially since in addition to their value as diagnostic and predictive markers, autoantibodies can in most cases also identify the targets of disease causing autoreactive T-cells.

In the following sections, we survey both B cell and T-cell autoantigen discovery. The methods employed typically adhere to a "candidate gene" approach where knowledge of the molecular and cellular biology of the disease is exploited. These include: biochemical analyses of the constituents of T1D sera or β cells, RNA expression profiling of β cells, and microarray analysis of tissue-specific gene expression and splicing. However, the results from recent genome-wide association studies (GWAS) (*46*) and advances in epigenetics have highlighted the degree of interindividual variation, suggesting the need to develop novel strategies for biomarker discovery. To illustrate the utility of such approaches, we discuss the technologies that permitted the demonstration that ZnT8, a zinc transporter

localized to islet cell secretory granules, is a major target of humoral autoimmunity in T1D.

B CELL AUTOANTIGEN DISCOVERY

Many T1D autoantigens have been identified by screening genes via various expression scenarios with sera derived from T1D subjects (*47–53*). An assortment of successful expression systems have included: λ gt-11 expression libraries, recombinant fusion protein libraries, and Western blotting of islet lysates with T1D sera. We will consider each system separately below.

Screening λ gt-11 Expression Libraries

Protein expression libraries constructed from RNA specifically derived from human islets and screened with T1D sera have served as a rich resource for antigen discovery. Selection of clone ICA512 from a λ gt-11 human islet expression library using sera from T1D subjects revealed the ICA512 antigen (*47*). It encodes a neuroendocrine-specific transmembrane protein that contains a cryptic protein tyrosine phosphatase (PTP) catalytic domain and displays near identical amino acid sequence to the insulinoma antigen (IA-2), which was independently cloned from a human insulinoma library (*48*). IA-2/ICA512 was the second component of ICA to be identified, and was found to bind 70% of T1D sera in Western blots. Ongoing characterization of the GAD molecule revealed that tryptic peptides of the 64 kD molecule yielded the 50 kD GAD product as well as 37 and 40 kD peptides, the latter of which was identified as IA-2. Like preabsorption with recombinant GAD65, prebinding of recombinant IA-2 diminished ICA autoreactivity. However, as stated above, since not all of the ICA reaction can be accounted for with preabsorption to both molecules, other unidentified autoantigens contribute to the ICA reaction.

Like IA-2, ICA69 was identified as an autoantigen by screening a human islet λ gt-11 expression library with ICA-positive sera (*49*). Interestingly, ICA69 is the human counterpart to the cow's milk-related protein p69, and data exists to demonstrate the presence of circulating antibodies in a subset of T1D subjects to bovine serum albumin (BSA) (*50*). However, it should be noted that ICA69 and BSA share modest regions (five amino acids) of homology one of which is the "ABBOS" albumin peptide T-cell epitope. The relationship of ICA69 with T1D is apparent, however not exclusive, as such antibodies are also present in the sera of rheumatoid arthritis patients (*55*). The association of albumin (as an immunogen) and T1D is controversial.

Phogrin (phosphatase of granules of rat insulinoma), also referred to as IA-2β, was likewise cloned from an expression library screened with sera raised against a secretory-granule

membrane fraction from insulinoma cells (*51*). Subsequently, mouse IA-2β was cloned using degenerate PCR primers to amplify PTP family members (Lu 1998). Phogrin shares 80% amino acid identity with IA-2 in the PTP domain and was named the target for autoantibodies directed to the 37 kD tryptic peptide described above. Nearly all autoantibodies reacting with phogrin likewise react with IA-2 (typically in the PTP domain), although ~10% of T1D patients show autoreactivity only to IA-2 and not phogrin. In light of this, assays designed to detect IA-2 autoantibodies are sufficient to capture autoantibodies directed to the phogrin antigen. However, Achenbach (*56*) has shown that in ICA+IA2A+ individuals, autoantibodies to phogrin is associated with a very high risk for T1D.

The primary screen of the human islet λ gt-11 expression library that disclosed ICA512 as a T1D-associated antigen also revealed molecule ICA12, now identified as SOX13, a transcription factor. In enzyme linked immuno-absorption assays (ELISA) assays to SOX13, 18% T1D subjects display autoantibodies to ICA12, although it is uncertain whether a relationship with T1D exists, as these antibodies are also present (6.9%) in patients with other autoimmune disorders (lupus and rheumatoid arthritis). It is possible that these autoantibodies are the result of a common mechanism for development of autoimmunity to endocrine tissues. SOX13 autoantibodies tend to be more prevalent among older subjects (>20 years) and seem to persist after diagnosis. Its utility as a T1D marker would be specialized for detection of ICA+, GAD–, and IA2- adults. The low frequency of SOX13 autoantibodies in healthy control sera (2.5%) and T2D sera (3.4%) may be misleading, as it is not clear if these are age and HLA-matched controls. However, sera from 7.6% newly diagnosed T1D subjects were exclusively positive for SOX13 autoantibodies, but not IA-2, GAD65, or ICA, thus possibly representing a distinct autoantibody positive population (*57*).

Some sera from T1D individuals that contains autoantibodies to GAD65 was found to immunoprecipitate a 38 kD protein from pancreatic islets (*58*). Screening a cDNA expression library with high titer sera for the 38 kD protein and GAD65 identified clones encoding JunB, a nuclear transcription factor. T cells exhibit a pronounced response in proliferation assays challenged with JunB recombinant protein containing amino acids 1–180 in 71% of new-onset diabetic subjects and in 50% ICA+ first-degree relatives. However, 25% of sera derived from individuals with other autoimmune diseases also contain autoantibodies to JunB implying their presence may be related to a more general defect in the immune response rather than specifically to T1D (*58*).

Screening of islet λ gt-11 expression libraries with prediabetic sera disclosed carboxypeptidase H(E) as an autoantigen in T1D (*59*). Carboxypeptidase E is not islet specific, as it has been localized to both neuroendocrine cells and islet secretory granules.

Abundant in islet granules, it functions in processing proinsulin to insulin. Detection among newly diagnosed T1D individuals ranges from 5 to 10% and 25% in ICA+ sera. Assays for high-throughput screening for carboxypeptidase E have not been pursued yet.

Screening Fusion-Peptide Libraries

A random peptide library screened with sera from T1D subjects identified autoantigens osteopontin, expressed in the somatostatin producing δ islet cells, and importin β, in neuroendocrine cells. RIAs did not detect binding of antibodies to either of the recombinant protein probes, whereas ELISA assays revealed 60% T1D sera and 30% of sera derived from subjects with other autoimmune diseases was positive for the importin β antigen probe (*53*). Similarly, the osteopontin recombinant protein could detect autoantibodies in T1D sera only by ELISA. Often ELISA assays are deemed less sensitive than RIAs, albeit antigen dependent (*60*).

Western Blotting of Human Islet Extracts with T1D Sera

CD38 (ADP-ribosyl cyclase/cADPR hydrolase) autoantibodies have been reported at modest prevalence in T1D patients via Western blot with T1D sera against islet extracts (*61*). A 52 kD β cell-specific granule antigen sharing an epitope with the PC2 protein of the rubella virus has likewise been identified by the same strategy. It is notable that congenital rubella virus infection has been correlated to the development of T1D, and may suggest that the rubella virus capsid protein mimics a β-cell antigen that may sensitize at-risk individuals for an autoimmune response (*62*).

Immunoprecipitation of Islet Antigens with T1D Patient Sera

Immunoprecipitation of IgG autoantibodies from the sera of T1D subjects has identified a heavily glycosylated islet cell membrane-associated (GLIMA) protein of apparent molecular weight 38 kD, which is present in 38% of T1D patients and 35% of prediabetics. The discovery of GLIMA-38 illustrates the contribution of posttranslational modification as a criterion for some cases of immunoreactivity. GLIMA-38 displays the neuroendocrine tissue-specific expression pattern in islets and neuronal cell lines and is strongly associated (and possibly redundant) with the presence of IA-2 autoantibodies (*63, 64*).

Glycolipid Preabsorption of the ICA Reaction

Although peptide antigens are more abundant, lipid antigens are also apparent epitopes in autoimmune diabetes. Substantial effort to purify potential lipid antigens to test for autoantigenicity has been achieved using biochemical fractionation (*65*). Glycolipids extracted from pancreas tissue and separated from protein and lipid fractions have been shown to competitively inhibit the ICA reaction. Chromatographic separation of gangliosides indicated monosialoganglioside, GM-2, the major ganglioside from pancreatic islets, could effectively inhibit the ICA reaction (*65*). Circulating autoantibodies specifically to GM-2, in lieu of the other abundant pancreatic gangliosides, GD-3 and GM-3, have

been demonstrated in new onset and prediabetic subject sera (*66*) through an indirect immunoperoxidase assay on thin layer chromatography plates.

Screening Ganglioside ELISAs with T1D Sera

Binding of sera from new-onset T1D subjects to pancreatic gangliosides immobilized for ELISA analysis indicated that ganglioside GT3 is a target antigen for autoantibodies (~35%) (*67*). Human ganglioside GT3 also readily binds mouse mAbs reactive to polysialgangliosides and an anti-β cell-specific mouse monoclonal antibody (*67*). Although ganglioside autoantibodies are apparent, their mode of detection has not yet been standardized. More recently, the relationship between T1D, neuropathy, and ganglioside autoantibodies has been explored, as gangliosides are expressed in both peripheral nerves and the pancreas (*68*). Twelve of 16 diabetic individuals with neuropathy were found to have autoantibodies to ganglioside. Sera from subjects having other autoimmune diseases served as controls and was 10% positive for ganglioside antibodies. The prevalence of ganglioside autoantibodies among T1D patients versus controls might reflect similar criteria for antigens in peripheral nerve and pancreatic islet cells (*68*). However, nondisease, age-, and genotype-matched control sera was not monitored.

Once a contribution of antigenic lipids to the ICA was established (*65, 69*), a family of four lysophospholipids was identified as a component of the ICA (*70*). Using purified lipids in a chemiluminescent dot blot immunoassay, autoantibodies were identified in 61% of new-onset T1D sera. Interestingly, 65% of ICA new-onset sera was positive for lysophospholipid antigen. One member of the family of four lysophospholipids has been identified as lysophosphatidylmyoinositol (*70*).

Generation of Hybridomas of Islet Infiltrating B Lymphocytes and Identification of Their Cognate Antigen

To determine the antigenic array of islet-infiltrating B cells, hybridoma cell lines were generated from NOD mice developing insulitis. The majority of such hybridomas generated mAbs to the peripheral nervous system (PNS) components. The autoantigens recognized by the mAbs was determined to reside in a cytoskeletal fraction by biochemical fractionation of neuroblastoma cell extracts. Western blot analysis of neuroblastoma, insulinoma, and pancreatic islet extracts with the mAbs detected a 58 kD protein. Further characterization by two-dimensional gel electrophoresis and ultimately mass spectroscopy identified the antigen as peripherin. Most mAbs were found to recognize a major eptiope in the C-terminal domain of two peripherin isoforms (*71*).

Screening Nucleic Acid Programmable Protein Arrays

Nucleic Acid Programmable Protein Arrays (NAAPAs) have been employed to identify novel candidate autoantigens related to T1D. The arrays are constructed by depositing candidate recombinant DNAs encoding an affinity tag onto glass slides and subjecting the slides to an *in vitro* translation cocktail to generate

affinity-tagged and immobilized protein probes *in situ*. An array of 6,000 human proteins has been screened in a "proof of principle" study using three independent sera from T1D individuals. The initial "training" screen reduced the candidate antigens to 750, while a third screen narrowed the putative antigens to a set of 19, which could discriminate between T1D sera and healthy control sera. Among the unidentified antigens were the characterized autoantigens GAD65, IA-2, and insulin (*72*).

Candidate B Cell Gene Approach

Candidate genes for autoantigens have often been deduced from their association with T1D itself or other autoimmune disorders and their various afflictions. Insulin was the first T1D autoantigen characterized at the molecular level (*73*) and is predominantly a β cell-specific autoantigen, although modestly present in parts of the brain and thymus. Although an obvious autoantigen candidate, the potential development of antibodies to insulin within 2 weeks of treatment (a consequence of exogenous insulin therapy) deemed autoantibody measurement difficult. However, as sensitive assays developed, serum from T1D individuals was found to contain antibodies that bound insulin prior to the initiation of an insulin therapy regime. Currently, the autoantibody epitopes developed as a result of insulin treatment differ from those present prior to treatment and can be distinguished by their lower affinity to antigen (*74*). Methodologies have been explored to discriminate the epitope populations derived from these two antigen sources (*75*).

T1D is often associated with other endocrinopathies or autoimmune disorders. DNA topoisomerase I (TOPI) and II (TOPII) autoantibodies have been found to be associated with systemic sclerosis (TOPI) (*76*) and lupus juvenile rheumatoid arthritis, cryogenic fibrosing alveolitis, and arcoidosis (TOPII) (*77–79*). To determine whether TOPII is an autoantigen in T1D, overlapping DNA fragments spanning the gene were expressed as recombinant antigens to screen sera from T1D subjects. The redundant TOPII peptides were capable of detecting antibodies in 49.2% of T1D individuals. Interestingly, the reactive TOPII epitopes mimicked those of previously identified autoantigens (insulin, HSP65, and GAD) (*80*).

Expression of nephrin (*81*), densin, and filtrin have been localized to the kidney and pancreatic islets, thus elevating their candidacy as autoantigens. Recombinant autoantigen probes of densin and filtrin were employed in RIAs to evaluate their reactivity to sera from T1D patients. Indeed, autoantibodies were detected in 33 and 11% of sera screened with the densin and filtrin probes, respectively (*82*). Similarly, a recombinant nephrin antigen probe was found to be immunoprecipitated by 24% of sera from newly diagnosed T1D individuals (*83*).

Patients afflicted with autoimmune disease often develop an autoantibody response against molecular mediators of these diseases

(*84*). Recently, the chemokines have been shown to be inducers of the inflammatory process in various inflammatory autoimmune diseases. Specifically, CCL3 was recently reported to be a major antigen target for T1D. Ninety-five percent of first-degree relatives positive for at least one of the gold standard autoantibodies (insulin, ICA, GAD) were also positive for CCL3 autoantibodies (*85*).

Some of T1D autoantigens are not expressed solely in β cells, but are also prevalent in neuroendocrine tissues (GAD65, IA-2). Islet β cells and neurons share several phenotypic similarities, and there appears to be some link between islet and nervous system autoimmunity. Immunohistochemistry of pancreatic islets has shown that the peri-islet Schwann cells (pSCs), which form a mantle that envelops the endocrine islet tissue, are an autoimmune target among individuals at risk for T1D as well as NOD mice (*86*). The predominant proteins expressed in pSCs are glial fibrillary acidic protein (GFAP), a cytoskeletal element, and S100β, both of which are antigenic targets for CD8+ T cells and B cells in NOD mice. In humans, a component of the ICA reaction is assigned to autoantibodies that localize to pSCs. This autoimmune targeting of the pancreatic nervous system could conceivably precede β-cell destruction (*86*).

Stress proteins are often associated with autoimmune diseases (*87*); thus, HSP60 and HSP70, identified as autoantigens in lupus and multiple sclerosis, were similarly named as antigens in the sera of NOD mice via solid-phase RIA prior to the onset of diabetes (*88*). In NOD mice, anti-HSP60 T cells mediate the manifestations of T1D, and inhibition of the anti-HSP60 T-cell response can reduce the rate of β-cell destruction. The major epitope target, peptide 277 (p277), has been shown to elicit human T-cell proliferative responses (*89*), and T-cell proliferative responses to human HSP70 have likewise been confirmed (*90*). Furthermore, autoantibodies specific for human HSP60 (45%) and HSP70 (30%) have been described in the sera of T1D individuals by ELISAs.

Glutamic acid decarboxylase autoantibodies (GADA) were discovered by their clinical association with disease and were originally defined as a 65 kD protein in patients with a rare neurological disorder involving GABAergic neurons resulting in muscle spasms or rigidity (stiff-man syndrome, exhibits a high coincidence with T1D). GAD catalyzes gamma-aminobutyric acid synthesis in the nervous system and the islets. Sera from patients with stiff-man syndrome contain antibodies that cross-react with islet cells, and the same autoantibodies were subsequently identified in T1D patients. Anti-GAD autoantibodies were shown to account for a portion of the ICA reaction via preabsorption of sera with recombinant GAD65. Two highly homologous GAD isoforms are expressed in β cells and brain (GAD65 and GAD67), although autoantibodies against GAD65 are significantly more prevalent (80 vs. 16%, respectively) in T1D patient sera (*81*).

The association of T1D with stiffman syndrome and possibly Guillain–Barré syndrome (*91*) imply common antigens between endocrine pancreas and nervous tissue. Sulfatide, a glycosphingolipid, has been localized to the secretory granules of α and β cells of rat pancreatic islets as well as the neural system, specifically in association with myelin (*92*), but not exocrine tissue (*69*). Antisulfatide antibodies have been identified in both prediabetic and new-onset T1D individuals (88%) using ELISAs. Of those T1D patients positive for sulfatide autoantibodies, the majority (68%) were also ICA+ at diagnosis; however, sulfatide autoantibodies appear to be independent of ICA. Although none of the control group measured positive for sulfatide autoantibodies, the subjects were not age or HLA-matched. Bouchard (*69*) did not indicate if a subset of the individuals in the study also suffered from Guillain–Barré syndrome, where 65% are positive for the same sulfatide antigen. When mouse spleen cells are challenged with sulfatide *in vitro*, they exhibit a robust proliferative response (*93*). The appearance of sulfatide autoantibodies further illustrates the importance of modification of molecules as antigenic targets, as sulfatide displays both carbohydrate and sulfate moieties (*94*).

Recently, an extensive gene candidate screening of mostly secretory vesicle-associated proteins (37 of 56) was conducted to determine their capacity for autoreactivity in T1D using RIAs. For initial assays, a panel of 50 T1D sera that were previously determined to be positive for IA-2 or GAD65 was employed. A panel of 200 sera that were triple positive (IA2A, GADA, IAA) was used for validation assays with 200 age-matched controls. Two new minor autoantigens were identified through this study, vesicle-associated membrane protein 2 (VAMP2) and neuropeptide Y (NPY), detecting 23 and 25%, of sera respectively. Importantly, the previously identified antigens Sox13, JunB, Imogen38, IGRP, and S100β were shown to have autoantibody counterparts in sera derived from triple-positive individuals. However, several putative minor autoantigens reported to have autoantibody counterparts were not confirmed in this study (CPE, GFAP, HSP70, ICA69, or TOPII) (*95*). Since the sera employed in this study was preselected to be autoantibody positive, it is impossible to determine if these new autoantigens contribute to the power of detection. Screening a panel of sera negative for the current four major autoantibodies, IAA, IA-2, GADA, and ZnT8A, is necessary to evaluate the utility of new autoantigens to enhance detection. This study corroborates the hypothesis that secretory vesicle-associated proteins are vital, yet not exclusive antigenic targets in T1D.

Displaced Binding of mAbs by T1D Sera

A panel of mouse mAbs reacting with a rat insulinoma cell line was assembled to define novel autoantigens by their displacement of monoclonal antibody binding to insulinoma cells. T1D sera

specifically competed for binding of one monoclonal antibody (IA-2) in membrane extracts from rat insulinoma cells and the islets of various mammals including humans. The antibody was found to detect the 150 kD diabetes-associated protein 1 (DAP1), which is heavily glycosylated as determined by Western analysis. The autoantibodies are directed against the protein versus the glycosylation as measured with immunoprecipitation of *in vitro* translated probes. Similarly, sera from T1D subjects detects a 150 kD protein in Western analysis of rat brain lysates (*96*). Although autoantibodies to the human protein (138 kD) are present in a large proportion of T1D sera (87 vs. 4% control sera), they are likewise present in sera from first-degree relatives of T1D individuals (38%), and thus are either not specific or represent an early marker for T1D. Although there appeared to be no correlation for positive immunoreactivity to the138 kD antigen and HLA genotype, the control subjects were not HLA-matched, albeit age-matched. Autoantibodies to DAP1 appeared prior to clinical onset in 17/20 subjects. Although the competitive displacement assay for this antigen appears promising, with 86.9% sensitivity and 95.6% specificity, the characterization has not been reproduced.

Proteomics

An attribute of proteomics is its capacity to survey large numbers of proteins for quantitative differences in concentration in an unbiased fashion to ultimately identify candidate biomarkers. A pilot proteomic study of human plasma and sera from control and T1D individuals was conducted to evaluate the use of capillary liquid chromatography (LC) coupled with mass spectrometry (MS) to identify novel biomarkers in T1D (*97*). Initially, MC–MS/MS was performed on control plasma to establish a comprehensive peptide database. Subsequently, high-throughput and quantitative LC–MS analysis identified significant differences in the levels of five proteins in the sera of T1D individuals: α-2-glycoprotein 1 (zinc), corticosteroid-binding globulin, and lumican were twofold upregulated in T1D sera relative to control sera, while clusterin and serotransferrin were twofold upregulated in control samples relative to T1D sera (*97*). Further confirmation of these candidate biomarkers for T1D is required to determine their predictive and/or diagnostic value.

T CELL AUTOANTIGEN DISCOVERY

The targets for autoreactive diabetogenic T cells typically parallel the same antigens recognized by B cells. This hypothesis has been demonstrated in humans and animal models for insulin (*98*), GAD65 (*13*), phogrin (*14*), and IA-2 (*99*). However, not all T cell antigens have demonstrated B cell autoantibody-producing

counterparts. T cell "orphan" clones isolated from T1D patients that do not respond to the current collection of antigens do exist. These orphan clones were generated from lymphocytes isolated from pancreatic lymph nodes or islet infiltrates of spontaneous diabetes and then restimulated with whole islets or insulin granule membrane preparations. Presumably, their target antigens have yet to be defined.

T Cell Responses to Epitope Libraries

After sequential stimulation of T cells isolated from a T1D patient with a membrane fraction derived from extracts of rat insulinoma cells, a cytotoxic CD4+ mouse T-cell clone was isolated and determined to react to a 38 kD protein. The epitope of the 38 kD antigen was mapped by screening a subtracted cDNA expression library. Ultimately, the full-length human cDNA clone was isolated and determined to encode a mitochondrial protein, imogen 38; however, the human protein did not stimulate the mouse T-cell clone. Imogen 38 is highly, but not specifically, expressed in β cells. Its immunoreactivity may represent a bystander effect (*100*). Clones generated in the same study identified several ICA components and indicated that the PBMCs of T1D do indeed recognize β-cell proteins.

IGRP

The ligand for the NOD mouse T-cell clone, NY8.3 CD8+, was identified through a proteomic approach as islet-specific glucose-6-phosphatase-related protein (IGRP) (*101*). Specifically, H-2K^{d} class I MHC molecules from the NOD-derived pancreatic β-cell line were purified by immunoaffinity chromatography, fractionated by reverse-phase HPLC multiple times, and coupled to tandem mass spectrometry to define eluted peptides. Candidate peptides were tested in epitope reconstitution assays, and IGRP, identified in the NCBI database, was found to cause a response to clone 8.3.

IGRP was originally cloned by screening a cDNA library created by subtractive hybridization of mouse insulinoma β-cell cDNAs from mouse glucagonoma (α) cDNAs with a probe homologous to the mouse liver glucose-6-phosphatase (G-6-P). Two alternatively spliced isoforms distinguished by the presence of exon IV (118 bp) were characterized and found to be specifically expressed in pancreatic β cells, but not in cells or cell lines of nonislet neuroendocrine origin. In addition to being a major β cell-specific antigen targeted by an abundant, pathological population of CD8+ T cells of NOD mice, data has recently emerged identifying IGRP is also a CD8+ T-cell antigen in humans. Using interferon-gamma (IFN-γ) ELISPOT assays with four peptides to IGRP, 65% of T1D patients were found to be positive to at least one peptide. However, no IGRP autoantibodies have been detected either in NOD mice or in humans (*101, 102*).

Dystrophia Myotonica Kinase

Another orphan CD8+ T cell clone, AI4, was isolated from the earliest detectable infiltrates of the islets. By screening a recombinant peptide library in positional scanning format with subsequent pattern searches of the mouse protein database, the target antigen was determined to be a widely expressed ligand from dystrophia myotonica kinase (*103*).

ZnT8 AS A TARGET OF T1D AUTOIMMUNITY

The advent of technologies to compare differences in expression patterns of genes across multiple tissues, species, and conditions has advanced disease biomarker discovery. In particular, utilizing microarray expression analysis has allowed the survey and comparison of vast amounts of gene expression data, permitting the identification of novel disease-specific autoantigens in T1D. Discovery of such molecular targets is essential for the induction of antigen-specific immune tolerance therapies that may modulate or eliminate T and B cell populations of the immune system, and may eventually delay onset or prevent T1D altogether.

ZnT8 was originally identified as a candidate autoantigen using a combination of multidimensional analysis of microarray expression profiling data coupled with the premise that established β-cell autoantigens display a series of common features. T1D autoantigens typically display β cell-specific gene expression at moderate to high levels and are often localized to the insulin secretory granule itself, or play a role in the regulated secretory pathway. With the exception of insulin, most autoantigens are transmembrane proteins and contain at least one major cytoplasmic domain. Moreover, they often display tissue-specific alternative splicing.

Originally, our screen for novel candidate antigens interrogated the public domain multitissue custom array (*104*, *105*) using Shannon entropy plots that impart an index of tissue specificity based on tissue distribution and relative abundance. Once pancreas and islet specificity was established, our criteria focused on β cell-specific expression by comparison of microarray expression profiles among β TC3 insulinoma, α TC1–6 glucagonoma, and mPAC ductal cell lines as well as embryonic neurogenin 3 knockout mouse pancreas (lacking islets) (*106*), which permitted refinement of our candidate list from 300 to 68 antigens. Affirming the predictive value of our ranking algorithm was the appearance of all the major known T1D autoantigens: insulin (*106*), GAD65 (GAD2) (*81*), IA-2 (*107*), and phogrin (PTPRN2) (*108*), as well as the more peripheral autoantigens such as heat shock protein 90B (*109*), carboxypeptidase C (*59*), islet glucose-6-phosphatase catalytic subunit-related protein (IGRP) (*100*), pancreas associated protein

(Reg3a) (*110*), islet amyloid polypeptide (IAPP) (*111*), Imogen 38 (MRPS31) (*99*), ICA69 (*49*), peripherin (*112*), GAD67 (GAD1) (*113*), and SOX13 (*47*). Among the many genes that had not yet been proposed as autoantigens, the highest ranking was ZnT8 (*21*), a member of a family of genes that function in cation diffusion efflux from the secretory granule (Fig. 2.1).

ZnT8 Autoantibody Detection

RIAs for detection of ZnT8 autoantibodies were developed using ^{35}S-labeled protein derivatives of the ZnT8 gene generated in a coupled *in vitro* transcription/translation system followed by fluid-phase assays. Immune complexes were captured by protein A Sepharose beads followed by a series of washes in a 96-well filter plate format. Radioactive antigen probe trapped by sera bound to beads was then assessed by scintillation counting.

Nearly half of the ZnT8 molecule is confined to six putative hydrophobic transmembrane regions, which are inaccessible to antibodies and may interfere with proper protein folding in an *in vitro* translation system. The N-terminal and C-terminal domains, however, are believed to reside on the cytosolic face of the membrane, predicted to be hydrophilic, and they do indeed contain autoantibody epitopes. The C-terminal domain, when employed as an antigen probe, displays a striking 70% sensitivity at 99.5% specificity with new-onset T1D sera. Further optimization

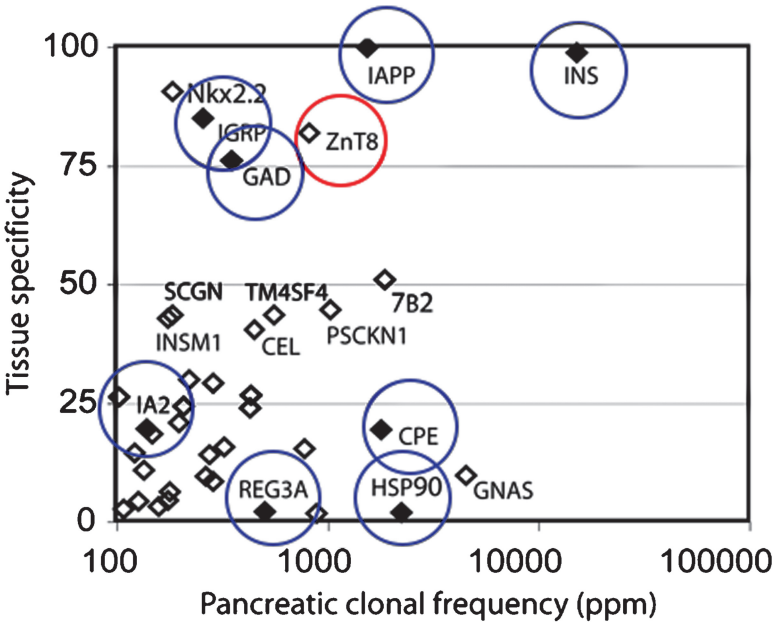


Fig. 2.1 Identification of candidate autoantigens in mouse. Pancreas specificity versus pancreatic abundance (number of pancreatic EST clones per all tissues). Approximate positions are shown for known autoantigens (*blue circles*) and ZnT8 (*red circle*).

of the probe design has extended the sensitivity of the assay to nearly 80% without conceding specificity. The sensitivity realized with the ZnT8 C-terminal probe is comparable to that achieved with the gold standard autoantibody of IA2A and exceeds that of IAA. ZnT8A measurement provides an independent measure of autoreactivity, as 25–30% of individuals classified as autoantibody negative possess ZnT8 autoantibodies. The ZnT8 measurement, when used in combination with classic assays, advances autoreactivity detection to greater than 90% (*21*). Consequently, individuals classified as single antibody positive can now be identified as double antibody positive, and are thus at increased risk for developing the disease.

Immunodominant Epitopes

Characterization of the epitopes recognized by autoantibodies is essential for the design of antigen-specific therapeutic agents as well as probe optimization. Alignment of the mouse and human ZnT8 C-terminal domains indicate that they are highly conserved (80% identical), differing only at 21 out of 102 amino acids. Although the spontaneous model of T1D, the NOD mouse, mimics many aspects of the human disease, they do not appear to harbor ZnT8 autoantibodies (Wenzlau and Hutton, unpublished data). The mouse C-terminal domain used as an antigenic probe in RIAs with sera that is highly reactive to the human C-terminal domain fails to immunoprecipitate despite its conservation with the human protein (Wenzlau and Hutton, unpublished data).

The crystal structure of the bacterial iron transporter Yiip has been solved and has been employed to model, via molecular threading of the human ZnT8 C-terminal domain (*114*). Noteworthy is that the amino acid differences between human and mouse lie at the predicted surface of the molecule. In fact, the nonsynonymous SNP that has been associated with susceptibility to T2D in GWAS (*46*) encodes residue 325, which is conspicuously positioned in a readily accessible amino-acid cluster. The single nucleotide polymorphism encodes either Arg (R_{325}) or Trp (W_{325}). The former, which confers risk of T2D, is present in 75% of European Caucasians, 98% African Americans, and 50% Asians.

A second nonsynonymous SNP exists in the second nucleotide of the same codon and encodes a Gln (Q_{325}), the residue existing in mZnT8, and occurs in less than 1% of Europeans, 9% of African Americans, and 1–2% of Asian populations (*115*). Substitution of the mZnT8 Q_{325} by the human R_{325} residue restored 12.5% autoreactivity (Wenzlau and Hutton, unpublished data).

Evaluation of autoreactivity of sera derived from newly diagnosed T1D to the three allelic variant forms of the ZnT8 antigen (R_{325}, W_{325}, and Q_{325}) indicated three epitopes residing in the C-terminal domain: one defined by R_{325} (30%), one for which

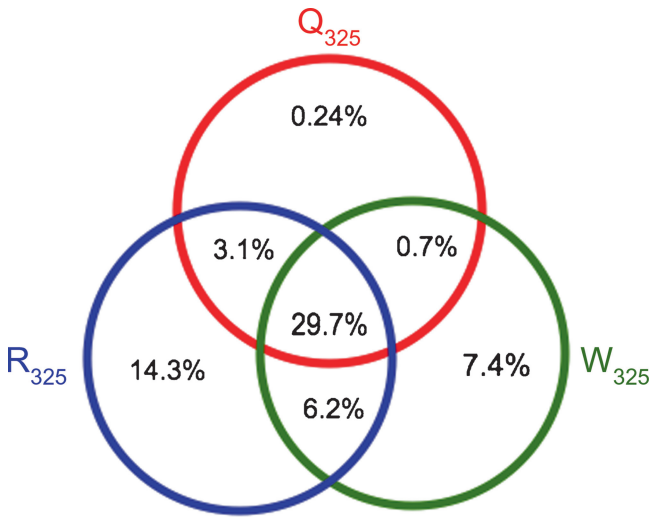


Fig. 2.2 Relationship between autoreactivity to R_{325}, W_{325}, and Q_{325} ZnT8 variants. Venn diagram illustrating the intersection of autoantibody reactivity with three polymorphic variant probes in a population of new-onset T1D individuals ($n = 420$). The percent of the total population reacting with each antigenic probe is indicated in the sectors.

W_{325} is essential (15%), and a third epitope at a topologically distinct location independent of the residue at position 325 (30%) (Fig. 2.2). Generally, 70% of new-onset T1D subjects react to one of the ZnT8 variant forms. The epitopes are conformational as recombinant epitope-specific protein, but not peptides, competitively inhibit binding to antigen probes. There is a strict correlation between genotype frequencies and autoreactivity to a given ZnT8 variant form (*116*) (Table 2.1). However, the functional relevance of the polymorphism to the β cell remains elusive.

Humoral responses to an antigen expressing a particular amino acid implies an oligoclonal response to self, and thus a restricted B-cell repertoire in T1D individuals. Within an oligoclonal response, it is probable that the surviving immunodominant clones will have arisen from a limited number of parental cells. This feature may enhance its suitability for therapeutic targeting, since a limited set of peptides or ligands may be administered to tolerize individuals to ZnT8 antigen. Furthermore, since ZnT8 expression is highly β cell specific, protocols to induce tolerance by effector or regulatory T cells will suffer fewer off-target effects than those directed to GAD65 or IA-2, which are more widely expressed in neuroendocrine cells. The presence of high-affinity, class-switched B cells specific for ZnT8 implies the likely presence of ZnT8-reactive T cells. Preliminary results from an ongoing study suggest that this is indeed the case (*117*).

Table 2.1
Levels of Autoreactivity Associated with rs13266634 SNP Genotype

		rs13266634			
	XX (351)	CC (194)	CT (127)	TT (30)	
Response	Mean ± SEM	Mean ± SEM	Mean ± SEM	Mean ± SEM	*P*
Q probe	0.108 ± 0.011	0.111 ± 0.016	0.100 ± 0.019	0.047 ± 0.018	ns
R probe	0.214 ± 0.018	0.269 ± 0.027	0.170 ± 0.026	0.050 ± 0.022	**[*]**
W probe	0.145 ± 0.014	0.100 ± 0.015	0.187 ± 0.028	0.253 ± 0.063	*[ns]**
Insulin	0.058 ± 0.010	0.057 ± 0.013	0.047 ± 0.011	0.110 ± 0.054	ns
GAD65	0.210 ± 0.018	0.202 ± 0.024	0.224 ± 0.033	0.205 ± 0.059	ns
IA2	0.585 ± 0.024	0.608 ± 0.031	0.539 ± 0.041	0.628 ± 0.088	ns

Sera from T1D individuals was measured by RIA with ZnT8 C-terminal polymorphic variant probes containing R_{325}, W_{325}, or Q_{325} or insulin, GAD65, or IA2 probes
P values between CC and CT/CT and TT/CC and TT genotypes were calculated by a Mann–Whitney nonparametric test
**P* = 0.01–0.05; ***P* = 0.01–0.001; ****P* < 0.001

CONCLUSIONS

The ability to predict ZnT8 as a major autoantigen in T1D can be attributed to advances in molecular engineering. Its discovery has enhanced the predictive value of autoantibodies for individuals' progression to disease and is an independent biomarker for β-cell mass and/or function in its own right. The uniqueness of a single amino acid determinant for specificity of antibodies is remarkable and speaks to the oligoclonality of the B-cell response. It is probable that some polymorphic variants may not be antigenic, further confirming reactivity to self. The circumstance of a nonsynonymous SNP conferring risk for T2D at the precise residue defining autoantibody specificity for T1D might appear to connect the diseases at the molecular level. However, T2D at least in part, involves β-cell dysfunction, whereas T1D arises following autoimmune attack on the β cell. Thus, while GWAS and autoantibody studies have linked the two distinct diseases via ZnT8 (*21, 46*), their association likely merely reflects the common target of the diseases, the pancreatic β cell.

The currently identified T1D autoantigens discussed above appear to sort into distinct associations, those that are specific to T1D and thus provide predictive value for disease, and those that are more broadly polyendocrine-related. Antigens with T1D disease association (or complications thereof) may be further distinguished by their tissue-specific, β-cell expression pattern (IAA,

ZnT8A) contrasted with wide tissue distribution (GADA, IA2A). As not all autoantigens define unique or significant groups of individuals at risk for T1D, their utility for detection and prediction of T1D is negligible, and they are thus classified as minor. Antigens reacting with less than 20% of new-onset T1D sera or whose specificity in a given assay is less than 95% are relatively insignificant. Such assays require optimization or alternate modes of autoantibody measurement. Yet, the existing set of antigens does not completely account for ICA, indicating that other autoantigens have yet to be defined.

The application of proteomics and molecular modification is in its infancy as applied to identification of novel antigens. The citrunillated autoantibodies associated with rheumatoid arthritis sets the precedent for the role of posttranslational modifications in autoimmune disease (*118*). Furthermore, the identity of the antigenic ligand for the BDC 2.5 T-cell clone is believed to be a modified peptide (*119*). It is possible that alternatively spliced variants of common genes or specific modifications of characterized proteins will contribute to the expanding list of autoantigens in T1D. As the pursuit of autoantigens proceeds, the "candidate" approach will excel as the technology for proteomics and genomics, epigenetics and posttranslational modification have yet to be rigorously applied. We will continue to identify novel autoantigens possibly among obvious, previously characterized, but slightly altered proteins.

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