Islet Autoantigens: Structure, Function, Localization, and Regulation

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Islet autoantigens associated with autoimmune type 1 diabetes (T1D) are expressed in pancreatic β cells, although many show wider patterns of expression in the neuroendocrine system. Within pancreatic β cells, every T1D autoantigen is in one way or another linked to the secretory pathway. Together, these autoantigens play diverse roles in glucose regulation, metabolism of biogenic amines, as well as the regulation, formation, and packaging of secretory granules. The mechanism(s) by which immune tolerance to islet-cell antigens is lost during the development of T1D, remains unclear. Antigenic peptide creation for immune presentation may potentially link to the secretory biology of β cells in a number of ways, including proteasomal digestion of misfolded products, exocytosis and endocytosis of cell-surface products, or antigen release from dying β cells during normal or pathological turnover. In this context, we evaluate the biochemical nature and immunogenicity of the major autoantigens in T1D including (pro)insulin, GAD65, ZnT8, IA2, and ICA69.

Pancreatic β cells are built for efficient regulated insulin secretion in response to acute changes in metabolic demand, which can exceed the rate at which new insulin can be synthesized. To accommodate this demand, β cells presynthesize insulin secretory granules that are accumulated in the cytoplasm, commonly referred to as the insulin storage pool. Although overall pancreatic insulin content changes relatively little during acute secretory stimulation (Poitout et al. 2004), insulin content in β cells responding to the secretory challenge acutely decreases, as detected by a decreased volume

density of secretory granules (Stefan et al. 1987). Thereafter, up-regulated biosynthetic activity in the endoplasmic reticulum (ER) and Golgi complex of those cells (Stefan et al. 1987), orchestrated in conjunction with granule membrane protein recycling (Vo et al. 2004; Torii et al. 2005; Wasmeier et al. 2005) to form new granules (Orci et al. 1985) restores secretory granule abundance to its homeostatic set point (Trajkovski et al. 2008). The net result of this “insulin factory” (Orci 1985) is that the entire β -cell secretory pathway is tuned to be iteratively responsive to meals and other stimuli. This

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paradigm constitutes the basic secretory cell biology of the pancreatic β cell, creating multiple opportunities for cell-surface exposure of many potential islet-cell autoantigens.

Type 1A diabetes (autoimmune T1D) results when autoreactive T cells become activated, resulting in destruction of insulin-secreting pancreatic β cells. Even before the process of T1D is first recognized clinically, islet-cell autoantibody responses also become detectable (Atkinson and Maclaren 1993)—indeed, onset of detectable islet autoantibodies can be used to predict the appearance of clinical T1D in otherwise healthy individuals (Orban et al. 2009). As the name indicates, immune autoreactivity is directed against self-antigens. Although we do not yet know if autoreactivity against endogenous β -cell protein antigens actually triggers onset of disease—this remains a leading hypothesis. The acknowledgment of autoantigens in pancreatic islet cells in patients with T1D (who may also have additional endocrinopathies) has been recognized for nearly 40 years—initially by immunofluorescence of human pancreas (Bottazzo et al. 1974)—and occasionally by cross-reaction in pancreatic β -cell lines in culture (in some cases even including β cells of other species [Dotta and Eisenbarth 1989; Karounos and Thomas 1990]). The islet autoantigens identified to date tend to be largely (but not exclusively) proteinaceous.

Curiously, the majority of identified islet autoantigens are located within the secretory pathway of pancreatic β cells. Indeed, most are located directly within the insulin secretory granule itself (the most abundant of these being insulin). It is not known why in T1D, secretory pathway proteins should be selected as antigens over proteins in other cellular compartments, or over other macromolecules such as RNAs or carbohydrates. However, the iterative surface exposure of T1D autoantigenic proteins, in conjunction with a susceptibility to autoimmunity in certain individuals, is a useful working hypothesis to explain these observations. One leading hypothesis for autoimmune susceptibility is a genetic predisposition to diminished thymic expression of islet-cell antigens, contributing to decreased self-tolerance. This could result in

one or another secretory pathway protein serving as a “primary antigen” to which T-cell (as well as autoantibody) reactivity is directed at the earliest stage during a sequential progression of islet autoimmunity (Krishnamurthy et al. 2006). Alternatively, once autoimmunity in T1D is initiated, β -cell injury or activation may expose further antigens, increasing the number of targeted islet autoantigens—so-called epitope spreading (Pietropaolo et al. 2008). The sheer abundance of the major secretory pathway proteins of pancreatic β cells makes them good candidates either as primary antigens or as secondary antigens involved in epitope spreading.

Using autoantibodies for immunoprecipitation, it has been possible to pursue the molecular identity of a number of major islet autoantigens—such an approach originally led to the identification of GAD65 (islet-cell antibodies to this antigen is now a standard clinical assay for the diagnosis of T1D), which encodes glutamic acid decarboxylase (Baekkeskov et al. 1990; Kaufman et al. 1992), an enzyme engaged in the synthesis of the neurotransmitter γ -aminobutyric acid (GABA, see below). In addition to GAD65, other major islet-cell autoantigens are now recognized, including proinsulin/insulin, ZnT8, IA2, and ICA69—in this work, we offer a brief perspective of each of these major β -cell autoantigens, their functions, and their exposure to the immune system at the time of initiation of T1D.

Because recent studies suggest that age at first presentation of clinical T1D is correlated with the mean levels of autoantibodies against insulin selectively (Steck et al. 2011), we consider this antigen first, and then proceed to review additional β -cell autoantigens.

INSULIN

Insulin is the central anabolic regulator of metabolic homeostasis, but for this discussion it is considered in its pathological role as a critical T1D autoantigen. Functional variation of the *INS* gene promoter conferred by the variable number of tandem repeats (VNTR) polymorphism, or mutations at other genetic loci, can lead to reduced insulin expression in the

thymus and lymphoid organs that may lead to loss of tolerance to insulin (Vafiadis et al. 2001; Durinovic-Bello et al. 2005) (and other self-antigens [Nagamine et al. 1997; Anderson et al. 2002; Ramsey et al. 2002]). Immune responses to antigenic peptides within insulin and its precursor proteins are likely to drive the pathogenesis of T1D in at least some patients and animal models—and as noted above, insulin autoantibodies usually precede T1D onset and can (along with other autoantibodies) predict development of the disease (Gottlieb and Eisenbarth 2002). Further, in susceptible mice, T-cell clones (both CD4 and CD8) recognizing insulin, can transfer the disease (Daniel et al. 1995; Wong et al. 2009).

Insulin biosynthesis (see below) is estimated at 0.4% of total pancreatic protein synthesis (Permutt et al. 1984) despite that islets make up only 1%–2% of total pancreatic mass (Jansson 1994). The net result is that the *INS* gene product can represent up to 50% of the total production of β -cell protein under stimulated conditions (Eizirik et al. 2009). In β cells, the initial site of insulin biosynthesis (and that of other insulin secretory granule proteins) is within the ER (Eskridge and Shields 1983) as preproinsulin, which includes the following contiguous peptides: a 24-residue signal peptide, the 30-residue B chain, the 31-residue C-peptide plus two sets of two basic flanking amino acids, and the 21-residue A chain. Delivery of preproinsulin into the lumen of the ER is initiated by signal peptide binding to the signal recognition particle (Okun et al. 1990), followed by docking and translocation at the ER membrane (Eskridge and Shields 1983), and cleavage of the signal peptide (Dodson and Steiner 1998) associated with completion of proinsulin biosynthesis (Sando et al. 1972). Interestingly, the preproinsulin signal peptide is one of the sites containing epitopes to which “insulin autoantibodies” (Berg et al. 1993) and cytotoxic T lymphocytes (Skowera et al. 2008; Toma et al. 2009) can be directed.

Subsequently, in the secretory pathway, coordinated proteolytic cleavages excise the C peptide of proinsulin, dependent on the subtilisin-like convertases PC1 and PC2 (Smeekens et al. 1992; Furuta et al. 1997; Zhu et al. 2002) in con-

junction with carboxypeptidase E (Naggert et al. 1995) to produce the mature two-chain hormone linked by two interchain disulfide bonds. The enzymic reactions converting proinsulin to insulin occur primarily within immature secretory granules beginning at the time of their emergence from the *trans*-Golgi network (Kuliawat and Arvan 1992; Huang and Arvan 1994).

It is back in the ER lumen where proinsulin folds into a globular protein that includes a natively like insulin moiety and C-peptide, which lacks an ordered structure (Yang et al. 2010). Although the amino-terminal and carboxy-terminal parts of the B chain are flexible (Zoete et al. 2004), the central B-chain structure includes three notable features: (1) the interchain disulfide bonds that link C(B7)–C(A7) and C(B19)–C(A20) as is conserved in multiple members of the insulin/IGF superfamily (Blundell et al. 1983); (2) a 10-residue α helix running from S(B9) to C(B19) (Kristensen et al. 1997); and (3) a β turn between residues G(B20) and G(B23) (Nakagawa et al. 2006). These features are especially interesting because proinsulin dimerizes (Derewenda et al. 1989)—possibly within the ER (Huang and Arvan 1995)—and the proinsulin dimerization interface precisely matches that for insulin dimers (Blundell et al. 1972). In these dimers, the side chains of residues S(B9)–G(B23) from each monomer contribute more than half of the surface area at the interface between the monomers (Fig. 1), and thus they are ordinarily buried when the properly folded protein resides within secretory compartments of the β cell. In contrast, on exocytosis (Gold and Grodsky 1984), insulin very rapidly dissociates to its component monomers (B chain disulfide linked to A chain is still considered a monomer) at which time S(B9)–G(B23) side chains become exposed. Indeed, the ability to form dimers is well correlated with the ability to activate insulin receptors (Nakagawa et al. 2000), in which the side chains of residues V(B12) and Y(B16) (Huang et al. 2004) as well as G(B23) (Baker et al. 1988) play significant roles.

T1D in nonobese diabetic (NOD) mice and in humans may have important differences, but NOD mice serve as one of the best animal models available for studies of autoimmune

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Figure 1. Insulin residues B9–B23 contribute to the dimerization interface between insulin monomers. (A) The crystal structure (Protein Data Bank code 2R34) of two insulin monomers is displayed. Atoms underlying the molecular surface are colored blue for nitrogen, red for oxygen, and green for carbon for monomer 1 A chain, cyan for carbon for monomer 1 B chain, yellow for carbon for monomer 2 B chain, and magenta for carbon for monomer 2 A chain. A chloride ion is depicted as a green sphere. A manganese (II) ion is depicted as a purple sphere. (B) The relative contribution of insulin B-chain residues from each monomer that contributes to the dimerization interface is shown. PROTORG was used to analyze the interfaces between insulin chains, which shows that B9–B23 residues participate in the dimerization interface. B16 tyrosine is buried at the interface contributing more (% interface surface accessible area) to the dimerization interface (21%) than other residues.

diabetes. Indeed, pathogenic T lymphocytes in NOD mice developing autoimmune diabetes recognize an immunodominant epitope contained within residues S(B9)–G(B23) of pro-insulin, and this is also true in patients with recent-onset T1D (Alleva et al. 2001). In T1D patients with the HLA-DRB1*0401 (DR4) DQ8 haplotype (conferring susceptibility to T1D), another immunodominant epitope has been reported within proinsulin, which includes the carboxy-terminal portion of the C-peptide running through the endoproteolytic cleavage site contiguous with the A chain (Congia et al. 1998). Characterization of human T cells reacting to this cleavage-site autoantigen were found to be CD4⁺ FoxP3-positive (regulatory) T cells (Durinovic-Bello et al. 2006) rather than pathogenic T cells. Still other studies have found that in T1D patients with HLA DR4, either clonally expanded T cells from draining pancreatic lymph nodes, or insulin autoantibodies, recognize one or more epitopes within the insulin

A1–A15 sequence (Achenbach et al. 2004). However, in many recent-onset and long-standing T1D patients, peripheral blood monocytes produce IFN- γ primarily in response to B-chain (rather than A-chain) peptides (Toma et al. 2005). Indeed, in NOD mice, injection/immunization with recombinant *Ins2* A chain fails to protect from development of T1D, yet injection of the recombinant *Ins2* B chain and, more narrowly, the B9–B23 peptide, does protect (Daniel et al. 1995; Muir et al. 1995). Curiously, with respect to subcutaneous “vaccination,” injection of the B9–B23 from the *Ins2* gene sequence [that begins with S(B9)]—but not the *Ins1* B9–B23 peptide [that begins with P(B9)]—significantly protects NOD mice from diabetes (Devendra et al. 2004). Indeed the only difference between the B9–B23 peptide sequence from mouse *Ins1* and *Ins2* gene products is at the B9 position (Table 1) (moreover, the mouse *Ins2* B9-B23 sequence is identical to that occurring in human insulin).

Table 1. Peptide sequences of the B9–B23 peptide of human and mouse (pro)insulins

B chain	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
Mouse- <i>Ins1</i>	P	H	L	V	E	A	L	Y	L	V	C	G	E	R	G
Mouse- <i>Ins2</i>	S	H	L	V	E	A	L	Y	L	V	C	G	E	R	G
Human <i>INS</i>	S	H	L	V	E	A	L	Y	L	V	C	G	E	R	G

Curiously, a complete *Ins2* knockout in the NOD background accelerates T1D onset (Thebault-Baumont et al. 2003); conversely, complete *Ins1* knockout diminishes T1D onset (Moriyama et al. 2003). Complete knockout of both mouse *Ins1* and *Ins2* but carrying a human preproinsulin transgene bearing endogenous S(B9) and a Y(B16)A point mutation provides sufficient insulin activity to avoid diabetes; more importantly, the animals do not develop T1D nor do they develop insulin autoantibodies (Nakayama et al. 2005). Altogether, the data seem to suggest that in NOD mice, specific structural features of the B chain are detected by the immune system with (pro)insulin-I (bearing a proline residue at position B9) triggering pathogenic T-cell immune responses leading to diabetes. In contrast, in humans, if insulin B9–B23 peptide serves as a primary autoantigen for T1D, then it must come from their one and only insulin gene product, which contains exclusively Ser at the B9 position.

GAD65

Early identification of L-glutamic acid decarboxylase (GAD) as a T1D autoantigen arose from immunoprecipitation studies (Baekkeskov et al. 1982) involving incubation of solubilized rat islets with sera from newly diagnosed T1D patients or controls, with the diabetic sera precipitating a 64-kDa protein. Impressively, immunoreactivity to the 64-kDa antigen was observed both in about 80% of new-onset T1D patients, and also in pre-T1D subjects. Thereafter, autoantibodies against the 64-kDa antigen were found to react with pancreatic β cells as well as GABAergic neurons in a rare condition termed stiff man syndrome (Solimena et al. 1988), facilitating subsequent identification of the 64-kDa autoantigen in T1D as GAD (Baekkeskov et al. 1990). Subsequent research on

GAD65 humoral autoimmunity in diabetes has led to the development of new assays to detect autoantibodies against GAD65, which are now used throughout the world (Grubin et al. 1994).

As noted above, GAD is the major enzyme required for production of the neurotransmitter, γ -amino butyric acid (GABA). GAD requires a cofactor, pyridoxal 5'-phosphate (i.e., activated vitamin B6) to catalyze this reaction. GAD and GABA are principally found in “GABA neurons” but are also found in extraneural tissues such as ovary and testis, and especially in pancreatic islet β cells (Wang et al. 2007). Indeed in islets, GABA is stored in the synaptic-like vesicles (SNLVs) of pancreatic β cells (Sorenson et al. 1991), but the functional consequence of this is not yet entirely clear—although GABA has been proposed to have a local paracrine effect in islets in regulation of glucagon secretion (Wendt et al. 2004). However, the GAD65^{-/-} mouse does not show any particular islet dysfunction (Kash et al. 1999).

There are actually two isoforms of GAD which share 65% homology at the primary amino acid sequence level: GAD65 and GAD67 (Erlander et al. 1991). Intriguingly, GAD65 and GAD67 have identical enzymatic activities but have subtle differences in their structure, with GAD65 having more flexibility in the carboxy-terminal region that is correlated with its far greater antigenicity (Fenalti and Buckle 2010). There is a well-documented body of data showing that early humoral autoimmune response to GAD65 in T1D is directed against epitopes primarily in the middle region of the molecule, and also includes the carboxyl terminus (Ronkainen et al. 2004, 2006). Additionally, intriguing structural crystallography studies aided by monoclonal antibody testing of antigenic determinants (Fenalti et al. 2008) have indicated that the more flexible carboxy-terminal region of GAD65

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shows a close grouping of autoantibody and T-cell antigenic determinants, raising the possibility that antigen–antibody complexes could contribute to GAD65-induced T-cell reactivity. Although the importance of the middle region and the carboxyl terminus bearing major immunoreactive epitopes is recognized, epitope spreading to the amino-terminal region of GAD65 can occur later (Bonifacio et al. 2000; Schlosser et al. 2005) although not all studies have substantiated this (Novak et al. 2000; Hampe et al. 2002). The knowledge gained from our understanding of the development of immunoreactive autoantigenic epitopes has provided a useful tool to more accurately identify risk and prediction to insulin-requiring stages in susceptible populations—not only in “classic” T1D but also in more recently characterized forms of the disease such as latent autoimmune diabetes of adulthood (LADA) (Zimmet et al. 1994; Pietropaolo et al. 2007).

Unlike the case for deletion of the *Ins1* gene, the cumulative incidence of autoimmune diabetes in NOD mice was reported to not be inhibited by GAD65 gene knockout (Yamamoto et al. 2004). However, autoimmune diabetes was inhibited by suppression of GAD expression in antisense-GAD transgenic/NOD females (Jun et al. 2002). Moreover, when splenocytes from female NOD/GAD65^{-/-} animals were transferred into female NOD-scid recipients, the onset of diabetes in the recipients was significantly delayed compared to recipients receiving splenocytes from ordinary NOD/GAD65^{+/+} females (Kanazawa et al. 2009). Thus, although the data establish that GAD65 is not an obligate antigen for T1D in the NOD background, GAD65-reactive T cells do seem to contribute to T1D onset in this model.

Unlike the majority of islet-cell antigens, GAD65 (in SNLVs) and GAD67 (in cytosol) are localized away from the other islet-cell autoantigens in β cells (Sorenson et al. 1991), i.e., not associated with dense-core insulin secretory granules (Fig. 2). Analogous to neuronal cells, both GAD65 and GAD67 are initially synthesized in the β -cell cytosol rather than the ER, and are subject to different acute translational regulation than proinsulin or other insulin se-

cretory granule autoantigens (Uchizono et al. 2007; Wicksteed et al. 2007). However, like for proinsulin, it has been reported that longer-term exposure to glucose (6–96 h) in isolated islets can specifically increase the transcription of GAD65 above that of total protein synthesis (Björk et al. 1992; Hagopian et al. 1993).

It has been postulated that GAD65 undergoes a two-step posttranslational palmitoylation on two cysteine residues in the amino-terminal region required for SNLV targeting (Christgau et al. 1992), whereas GAD67 remains persistently cytosolic. The palmitoylated GAD65 is inserted into SNLV membranes, leaving the vast majority of SNLV-associated GAD65 outwardly facing the cytosol. At this location GAD65 associates with a small protein complex that contains the vesicular GABA transporter (VGAT). This allows local production of GABA by GAD65 and then GABA's immediate transport by VGAT into the SNLV storage compartment in the β cell (Buddhala et al. 2009). GAD65 can be released from the membrane by an apparent enzyme activity in islets, suggesting that its membrane anchoring is potentially regulated. Unlike GAD67, the hydrophobic modifications and consequent membrane anchoring of GAD65 to GABA-containing SNLVs might be significant for its role as an islet autoantigen.

It is worth mentioning that human vascular endothelial cells (ECs) are capable of processing and presenting GAD65 epitopes to autoreactive T cells (Greening et al. 2003). In particular, in vitro transmigration across an EC monolayer by autoreactive T cells is markedly promoted by presentation of cognate peptide/HLA complexes on the EC surface, and is LFA-1 (lymphocyte function-associated antigen-1) dependent. These results suggest that presentation of autoantigens such as GAD65 by islet endothelium in vivo could promote islet transmigration of circulating autoantigen-specific T cells primed in regional lymph nodes.

ZNT8

β cells maintain an unusually high level of cellular zinc (Wijesekara et al. 2009), and they express several zinc transporters including ZnT5

Figure 2. Predominant intracellular distribution of major T1D autoantigens in pancreatic β cells. Two organelles of the β -cell secretory pathway are shown bearing autoantigens (in blue-green). The secretory granule contains primarily insulin (shown in black, similar to the appearance of the insulin crystal by transmission electron microscopy). A small fraction of unconverted proinsulin is also contained within secretory granules. The “clear space” surrounding the insulin granule core is thought to be enriched in the soluble C-peptide, which is not specifically denoted in the figure. The secretory granule membrane is the primary site of distribution of three additional β -cell autoantigens: ZnT8 is a polytopic membrane protein, IA-2 is a single-spanning transmembrane protein with both extensive luminal and cytosolic domains, and ICA69 is a type 1 transmembrane protein predominantly residing on the luminal side of the membrane. As noted in the text, GAD65 localizes away from these other autoantigens, residing primarily on the cytosolic side of the membrane of secretory microvesicles, also known as “synaptic-like vesicles” (SNLVs).

(Kambe et al. 2002) and ZnT3 (Clifford and MacDonald 2000). However, the most consistently expressed β -cell zinc transporter is ZnT8 (encoded by *SLC30A8*), a 369 amino acid polytopic, dimeric membrane protein whose pancreatic expression is concentrated in the islets (Chimienti et al. 2004; Murgia et al. 2009). In β cells (Chimienti et al. 2005), ZnT8 resides primarily in insulin secretory granules (Fig. 2) (Chimienti et al. 2006). ZnT8 has been found as an autoantigen in a high fraction of new-onset T1D patients (Wenzlau et al. 2007). Analysis of single-nucleotide polymorphisms has suggested that *SLC30A8* polymorphisms are associated with type 2 diabetes in Scandinavians (Hertel et al. 2008), other Europeans, and East Asians (Cauchi et al. 2010)—but also associated with T1D (Wenzlau et al. 2008b). Autoantibodies against ZnT8 (Kawasaki et al. 2008, 2010; Achenbach et al. 2009; Lampasona et al. 2010; Nielsen

et al. 2011) as well as T-cell responses (such as production of IFN- γ) against ZnT8 (Dang et al. 2010) are produced in patients that develop autoimmune diabetes. In turn, ZnT8 expression in β cells is down-regulated by cytokines (Egefjord et al. 2009) even as this down-regulation could result in β -cell dysfunction (El Muayed et al. 2010) and apoptosis leading to further ZnT8 antigen exposure that could exacerbate autoimmunity (Wenzlau et al. 2008a).

Especially given the existence of several different zinc transporters in β cells (Smidt et al. 2009), our understanding of the physiological role of ZnT8 in normal β -cell function is incomplete. However, substantial indirect evidence suggests that full ZnT8 function is needed for optimum insulin storage and secretion. Evidence suggests that TCF7L2 and PDX-1 (themselves both linked to normal versus pathological β -cell function) each contribute to regulation of

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ZnT8 expression (da Silva Xavier et al. 2009; Pound et al. 2010) and *db/db* mice down-regulate β -cell ZnT8 protein (Tamaki et al. 2009). In the hopes of establishing a mechanistic link between ZnT8 expression and diabetes, multiple groups have examined ZnT8-deficient mice. Isolated islets of such mice have measurably decreased glucose-stimulated insulin secretion (Pound et al. 2009) and—with some variation between reports—a glucose intolerance phenotype that can be elicited on feeding the ZnT8-deficient animals a diabetogenic diet (Lemaire et al. 2009; Nicolson et al. 2009). The phenotype is thought to be more obvious in animals with β -cell-selective deletion of ZnT8 (Wijesekara et al. 2010). Pancreatic β -cell lines with ZnT8 knockdown also show decreased glucose-stimulated insulin secretion (Fu et al. 2009), and double knockdown of ZnT8 and ZnT3 reportedly triggers cell death in pancreatic β -cell lines (Petersen et al. 2011)—perhaps owing to cytosolic or nuclear zinc intoxication. Altogether, these studies indicate that down-regulation of ZnT8 expression can contribute to β -cell dysfunction and diminished survival, whereas its exposure as an autoantigen can trigger immune responses.

IA-2 PHOSPHOTYROSINE PHOSPHATASE-RELATED PROTEINS

Two other common autoantigens, IA2 (also known as ICA512) and IA2 β (also known as phogrin) are also located to the insulin secretory granule membrane in β cells (Solimena et al. 1996; Torii 2009). Autoantibodies against both proteins can be detected decades before overt diabetes in first-degree relatives of T1D probands, and their presence is used to identify subjects at risk of progressing to the clinical onset of the disease (Kawasaki et al. 1996; Morran et al. 2010). IA-2 consists of a signal peptide (a.a. 1–24), extracellular (a.a. 25–576), transmembrane (a.a. 577–600), and intracellular (a.a. 601–979) domains. Its proform is a glycoprotein of 110 kDa that is processed by furin-like convertases during granule maturation (Mziaut et al. 2006). IA-2 is a granule membrane protein, whose cytosolic domain binds

β 2-syntrophin, an F-actin-associated protein, and is cleaved on granule exocytosis. The resulting cleaved cytosolic fragment, ICA512-CCF, is thought to reach the nucleus and up-regulate the transcription of granule genes (including insulin and ICA512) and may also dimerize with intact ICA512 on granules to influence granule mobility in the cytoplasm (Trajkovski et al. 2008).

IA2 and IA2 β have domains with close homology to protein phosphotyrosine phosphatases (PTPs), yet surprisingly they do not have any such enzyme activity. IA2 and IA2 β are both initially synthesized as proprotein precursors that are proteolytically processed likely by PC1/3 and/or PC2 in coordination with proinsulin. The mature IA2 and IA2 β proteins are type 1 integral membrane proteins, with the PTP domain oriented on the cytosolic side of the granule membrane and a short amino-terminal domain located on the inside of the granule. The actual function of IA2 and IA2 β remains unknown, although in β cells it has been postulated that they may play a role in regulating insulin secretory granule content and possibly regulation of β -cell growth (Torii 2009). In this regard the IA2 and IA2 β knockout mice are glucose intolerant with reduced insulin secretion (Saeki et al. 2002; Kubosaki et al. 2004). The double-knockout mouse has no worsening of this phenotype, although the insulin content of the islets is reduced by ~50% (Kubosaki et al. 2005). A recent report showed that deletion of IA-2 and/or IA-2 β results in a marked decrease in the number of dense-core vesicles (DCVs) in β cells and a decrease in β -cell $[Ca^{2+}]_i$ handling (Cai et al. 2011). In the single IA-2 knockout (KO) mice, the decrease in the insulin content and secretion correlated with a decrease in β -cell DCV number. In the case of the double IA-2 plus IA-2 β KO mice, the decrease in β -cell DCV number was proportionally greater than the decrease in insulin content and secretion. Interestingly, electron microscopy of islets from KO mice revealed a marked increase in the number and size of lysosomes and an increase in LC3 protein, suggesting that autophagy might be involved in the consumption of DCVs.

One might expect that secretion of insulin by granule exocytosis could increase the presentation of HLA class II allele-specific IA2 and IA2 β native epitopes on the β -cell plasma membrane, where antigenicity could then be manifested. Naturally processed epitopes of islet-cell autoantigens represent the targets of effector and regulatory T cells in controlling β -cell-specific autoimmune responses (Di Lorenzo et al. 2007). In particular, naturally processed HLA class II allele-specific epitopes recognized by CD4⁺ T cells, corresponding to the intracellular domain of IA2, were identified after native IA2 antigen was delivered to Epstein-Barr virus (EBV)-transformed B cells and peptides eluted and analyzed by mass spectrometry (Peakman et al. 1999). Dendritic cell subsets can facilitate the processing and presentation of soluble IA-2 antigen to CD4⁺ T cells. Specifically, at times near the onset of T1D, the plasmacytoid subset of dendritic cells is overrepresented in the blood, and these cells show a distinctive ability to capture islet autoantigenic immune complexes and enhance autoantigen-driven CD4⁺ T-cell activation in the presence of IA-2 autoantibody-positive patient serum (Allen et al. 2009). This may suggest a synergistic proinflammatory role for plasmacytoid dendritic cells and IA-2 autoantibodies in T1D. The field is therefore heading toward the ultimate identification of novel naturally processed IA-2 epitopes recognized by CD4⁺ T cells that may represent potential therapeutic agents, either in native form or as antagonistic altered peptide ligands, for the treatment of T1D.

ICA69 AND OTHER T1D-RELATED AUTOANTIGENS

Seminal studies by Atkinson et al. identified a subset of islet-cell antibodies (ICA) associated with a more clinically significant pancreatic β -cell injury in a subgroup of first-degree relatives of T1D probands. This subset of ICA was termed “non-GAD reactive” because ICA reactivity could only be partially blocked by GAD65 (Atkinson et al. 1993), implying that multiple additional islet autoantigens are recognized by T1D-specific humoral responses. We too found

that a subset of cytoplasmic ICA is associated to a more rapid progression to insulin-requiring diabetes in GAD65 and IA2 antibody-positive relatives as compared to relatives with GAD65 and IA2 antibodies without ICA—again suggesting additional unidentified ICA-recognizing autoantigen(s) (Pietropaolo et al. 2005). This may reflect “epitope spreading,” as solid observations indicate that islet autoantibody responses against multiple islet autoantigens are associated with progression to overt disease (Verge et al. 1996). With this in mind, a number of additional T1D-related autoantigens have been identified, which include islet cell autoantigen 69 kDa (ICA69), the islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP), chromogranin A (ChgA) the insulin receptor, heat shock proteins, the antigens jun-B,16, CD38 (Pietropaolo and Eisenbarth 2001), peripherin, and glial fibrillary acidic protein (GFAP) (Winer et al. 2003; Haskins and Cooke 2011).

Islet-cell autoantigen 69 (ICA69), a protein product of human *ICA1* or mouse *Ica1*, is predominantly expressed in pancreatic islets and neuroendocrine organs (Pietropaolo et al. 1993; Karges et al. 1996). This protein and its *Caenorhabditis elegans* homolog ric-19 are conserved regulators of neuroendocrine secretion (Pilon et al. 2000; Sumakovic et al. 2009). ICA69 is involved in DCV signaling and maturation, and it is recruited to Golgi membranes by activated Rab2 (Buffa et al. 2008). ICA69 is thought to be a T1D autoantigen based on the following two observations. First, autoantibodies to ICA69 can be detected in both first-degree relatives of T1D patients who are followed to overt diabetes and in newly diagnosed diabetic patients (Pietropaolo et al. 1993; Martin et al. 1995; Roep et al. 1996; Dosch et al. 1999; Song et al. 2003). Second, T cells autoreactive to ICA69 can be detected in newly diagnosed diabetic children and in NOD mice (Winer et al. 2000; Chen et al. 2001). We have recently found that polymorphisms within the NOD *Ica1* core promoter determines AIRE-mediated down-regulation of ICA69 expression in medullary thymic epithelial cells, thus providing a novel mechanistic explanation for the loss of immunologic tolerance to this

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self-antigen in autoimmunity in the NOD mouse model (Bonner et al. 2012).

IGRP is a member of the G6Pase family that is specifically expressed in pancreatic β cells, as an ER resident protein (Arden et al. 1999). This molecule is a target of a significant subset of islet-associated CD8⁺ T cells in NOD mice (Santamaria et al. 1995). These CD8⁺ T cells express an invariant α chain characterized by V α 17–J α 42 recombination (DiLorenzo et al. 1998) and undergo a process of avidity maturation during the progression of islet inflammation to overt diabetes. IGRP has been shown to be a target of autoreactive CD8⁺ T cells in human autoimmune diabetes (Mallone et al. 2007). Extrathymic *Aire*/IGRP-expressing cells (eTACs) may help reinforce immune tolerance by preventing the maturation of autoreactive T cells that escape thymic negative selection (Gardner et al. 2008).

Another component of insulin secretory granules, chromogranin A (ChgA), has been recently proposed as an autoantigen of T1D (Stadinski et al. 2010). ChgA is a member of the granin protein family and is found throughout neuroendocrine tissues including the adrenal medulla, central nervous system, and pancreas (Taupenot et al. 2003). ChgA may also be needed during the development of the endocrine pancreas, as evidenced by the fact that ChgA knockout mice (*ChgA*^{−/−}) have fewer islets and insulin-producing β cells (Porte 1991). Of autoantigenic significance, ChgA aids in the formation of secretory granules (Kim et al. 2001) and is processed to form smaller peptides (Taupenot et al. 2003). In regards to T1D in NOD mice, an antigen from β -cell secretory granules that activates the diabetic T-cell clone BDC-2.5 and two other clones was identified as ChgA (Stadinski et al. 2010). Stadinski et al. went on to identify a sequence in the carboxy-terminal region of ChgA, encoding the peptide WE-14, which activated all three T-cell clones. The results indicated that ChgA can trigger a T-cell-mediated immune response in NOD mice and that its WE-14 peptide is the antigenic epitope. In contrast, the role of ChgA as an autoantigen in human autoimmune diabetes remains to be elucidated.

Thought-provoking findings led to the hypothesis that early autoimmunity in spontaneous T1D can also target nervous system tissue elements in the pancreas, raising the concept that pathogenetic immune responses in T1D may also be non- β -cell exclusive (Winer et al. 2003). One of these molecular targets appears to be peripherin (Boitard et al. 1992). Peripherin is expressed in multiple endocrine tissues, including nerve fibers surrounding islets of Langerhans in the pancreas, adrenal medulla, nerve fibers in interstitial tissue between thyroid follicles, and nerve fibers adjacent to ovarian follicles (Chamberlain et al. 2010). Serologic responses to peripherin have been found in autonomic fibers in the pancreas, thyroid, and ovary, supporting clinical observations suggesting that neuronal elements may be a molecular target for immune-mediated injury in multiple forms of endocrine autoimmunity, including T1D (Chamberlain et al. 2010). However, it remains to be established as to whether or not the presence of peripherin antibodies, along with serologic responses to other putative neuronal elements, are predictive for the development of small fiber neuropathy (autonomic and/or somatic) and for the progression to overt diabetes.

PROTEIN MISFOLDING AS A CONTRIBUTOR TO AUTOANTIGENICITY

In addition to the physiological events of exocytotic protein exposure at the cell surface, β cells have additional opportunities for generating antigens from major islet secretory pathway proteins. Conceivably, the misfolding of proinsulin and other secretory pathway proteins, when linked to pathways of endoplasmic reticulum-associated degradation (ERAD) (McCracken and Brodsky 2003), results in proteasomal processing of these polypeptides that could increase major histocompatibility complex (MHC) class I loading with availability of autoantigenic peptides to antigen-presenting cells (Eizirik et al. 2008). Overexpression of class I molecules (HLA-A, B, and C) in pancreatic islets at the time of onset of T1D has long been recognized, in association with pancreatic infiltration with cytotoxic

CD8⁺ T cells surrounding damaged islets (Bottazzo et al. 1985). Increased MHC class I-related gene expression may be stimulated by cytokines but also under states of protein misfolding and cell stress, because MICA and MICB (MHC class I-related) are both regulated by heat-shock promoter elements similar to those of HSP70 genes (Groh et al. 1996). Thus, β -cell stress can potentially contribute to increased proteasomally derived antigen presentation, both by increasing ERAD and also by increasing expression of MHC class I-related gene products (Fig. 3).

Proinsulin, like other members of the IGF superfamily (Hober et al. 1994, 1999; Guo et al. 2002), is a protein predisposed to mispair its

disulfide bonds during folding either in vivo (Liu et al. 2005) or in vitro (Hua et al. 1995, 2002; Qiao et al. 2003). Proinsulin misfolding becomes a virtual certainty in the syndrome of mutant *INS* gene-induced diabetes of youth (MIDY), in which heterozygotes bear one of 26 different mutations in the preproinsulin coding sequence linked to autosomal-dominant diabetes, that presents often (but not exclusively) in neonatal life (Liu et al. 2010b). These mutant proinsulins not only misfold within the ER, but they also block in *trans* the folding and maturation of proinsulin coexpressed from a wild-type allele (Hodish et al. 2010). Blockade of intracellular transport appears to be coupled—at least in part—to the proteasomal degradation of the misfolded mutant proinsulin as well as the coexpressed wild-type proinsulin (Liu et al. 2010a). Although patients with MIDY are not autoantibody positive, the idea that proinsulin misfolding leads to potential proteasomally generated proinsulin-derived peptides that could be antigens, is plausible. Interestingly, of the preproinsulin domains (signal peptide, B chain, C-peptide, and A chain) the majority of MIDY mutations fall within the B chain, including seven such mutations within the S(B9)–G(B23) sequence. These data underscore the importance of this structurally sensitive region during the folding of proinsulin. This region is likely to remain structurally sensitive in mature insulin, because by X-ray crystallography, mature insulin monomers are likely to exist in two distinct states: the T state, in which the amino-terminal residues F(B1)–G(B8) lead into a β turn [C(B7)–H(B10)] thereby “masking” the α helix beginning at residue S(B9); and the R state in which these same amino-terminal residues are in an extended conformation, thereby “exposing” the S(B9)–C(B19) α helix (Baker et al. 1988). Unanue and coworkers have suggested that in islet β cells of NOD mice, the insulin B9–B23 peptide might be produced directly from processing of insulin derived from secretory granules (Mohan et al. 2010). Thus, there are multiple distinct changes in the conformational state of proinsulin and insulin—merely as one example—that may initiate its antigenicity.

Figure 3. Hypothesis: ERAD of misfolded secretory pathway proteins triggers MHC class I loading and presentation of autoantigens. In the case of misfolded secretory pathway proteins, retrotranslocation from the ER to the cytosol triggers degradation via the ubiquitin-proteasome system. The generation of small cleavage fragments and their transport back into the ER lumen allows for peptide loading of MHC class I (via the TAP/tapasin complex). Cell stress can promote ER misfolding of secretory and membrane proteins (Kuznetsov and Nigam 1998) and also may promote expression of major histocompatibility complex class I-related genes (Groh et al. 1996). Thus, it is a plausible hypothesis that the net result of these two effects is enhanced β -cell autoantigen presentation.

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REGULATION OF SECRETORY GRANULE AUTOANTIGEN PRODUCTION IN β CELLS

The biosynthesis of major secretory granule proteins is regulated by multiple nutrients, hormones, and neurotransmitters, the most notable of which is glucose (Rhodes 2004). Specifically, glucose coordinately regulates a subset of ~ 50 proteins in the β cell—most destined for insulin secretory granules (Guest et al. 1989; Rhodes 2004)—such as proinsulin and its processing endopeptidases proPC1/3 and proPC2 (Alarcón et al. 1993; Martin et al. 1994). This increase in granule protein biosynthesis is initiated rapidly, i.e., up to 10-fold within 60 min (Alarcón et al. 1993; Rhodes 2004), and this early response is controlled primarily at the translational level (Itoh and Okamoto 1980; Wicksteed et al. 2003). For prolonged periods of elevated glucose, there is a further effect on preproinsulin messenger RNA (mRNA) stability (Welsh et al. 1985) and increasing insulin gene transcription (Ohneda et al. 2000), which adds additional preproinsulin mRNA template potentially available for translation (Wicksteed et al. 2003). The mechanism for the specific glucose-induced increase in proinsulin translation is only partly understood, but it requires a highly conserved element in a 5'-untranslated region *cis*-element of the preproinsulin mRNA, called the preproinsulin glucose element (ppIGE) (Wicksteed et al. 2007). Interestingly, this ppIGE is also highly conserved in the 5'-untranslated region of the mRNA of other insulin secretory granule proteins whose biosynthesis is under the same specific glucose-induced translational control (Uchizono et al. 2007). In summary, the biosynthesis of insulin and selective other proteins is strongly and selectively up-regulated by glucose (Guest et al. 1991) so that protein constituents are coordinately regulated during secretory granule biogenesis.

In states of increased biosynthetic activity, proinsulin (and other protein) content in the ER increases (Eizirik et al. 2009), and under such conditions, the amount of misfolded proinsulin also increases (Liu et al. 2005). The ER stress response (also known as UPR) can initially protect β cells by expanding ER capacity to meet

the challenge of processing a physiological increase in proinsulin protein folding (Credle et al. 2005; Merksamer et al. 2008). However, in the face of sustained, high-level ER stress, chronic UPR activation may become cytotoxic when protective mechanisms in β cells fail (Papa et al. 2003). Although pancreatic β -cell-reactive T cells may initiate β -cell destruction in T1D, stress response mechanisms (Akirav et al. 2008) in β cells may actually accelerate the extent of disease progression. Specifically, not only can ER stress stimulate proteasomal generation of ERAD peptides (Fig. 3) but it may also directly promote β -cell death (Fig. 4). Indeed pancreatic β cells are highly susceptible to ER stress-induced cytotoxicity (Ron and Walter 2007) with a potential direct contribution to several forms of human diabetes (Oslowski and Urano 2011). With respect to T1D, dendritic cell interaction with, and processing of, dead or damaged β cells could lead to presentation of β -cell antigens to pathogenic T cells (Fig. 4) (Fonseca et al. 2009). However, autoimmune attack, by diminishing the β -cell population, may drive excessive translational activity in remaining β cells as they are forced to synthesize larger and larger amounts of proinsulin/insulin [with increased proinsulin misfolding (Liu et al. 2005)], thereby elevating ER stress levels in the remaining β cells (Todd et al. 2008). This in turn may promote further rounds of β -cell cytotoxicity while making additional antigen available for presentation to the immune system. Thus, both cytokine and secretory pathway stress could contribute to a vicious cycle in which secretory pathway antigens are connected to autoimmunity. For this reason, it is reasonable to postulate that cell compensatory responses might be critical modifiers of disease onset and activity, and might also be used as drug targets in T1D.

CONCLUDING REMARKS

There is still little understood about islet β -cell autoantigenicity. Are autoantigens the trigger of autoimmune attack, or collateral damage as part of an ongoing and premeditated immune destruction of a β cell? The β -cell homicide versus suicide debate is still not resolved

Figure 4. Simplified model of β -cell damage leading to antigen presentation. T cells can directly kill β cells through a cytotoxic process, but they can also influence β -cell destruction via release of mediators such as cytokines, chemokines, or perforin. Cytokine activation of inducible nitric oxide synthase can activate ER stress response signaling (Oyadomari et al. 2001)—pathways collectively known as the unfolded protein response (UPR). It is therefore conceivable that cell stress including UPR may be a potential contributor to β -cell toxicity in T1D. Processing of autoantigens within β cells generates peptides that are then taken up by antigen-presenting cells (APCs), either as whole dead β cells or β -cell fragments, for eventual further processing/presentation of these islet peptides to self-reactive T cells.

(Atkinson et al. 2011). But, with the possible exception of GAD65, it should be considered that the other major T1D autoantigens (pro)-insulin, ZnT8, IA2, and IA2 β , are coordinately regulated at the biosynthetic level. When metabolic demand is increased there will be increased production of these antigenic proteins, which in turn increases the chances of their misfolding with production of antigenic peptides. Moreover, there will be increased insulin secretory granule exocytosis, which will result in a greater frequency of antigen presentation at the β -cell surface. So at least, during the pathogenesis of T1D, which includes increased excursions of hyperglycemia, there would be more active β cells trying to produce and secrete more insulin to meet the demand, but this could also trigger β -cell demise. We conclude that in this particular situation, the β cell, in conjunction with autoimmune attack, is undergoing an assisted suicide. However, this is mere conjecture, and what is really needed is cooper-

ative research between immunologists and β -cell biologists to get at the root cause of T1D pathogenesis, and consequently to design new therapies that can help to avert the disease.

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