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An insulin-IAPP hybrid peptide is an endogenous antigen for CD4 T cells in the non-obese diabetic mouse

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Abstract

BDC-6.9, a diabetogenic CD4 T cell clone isolated from a non-obese diabetic (NOD) mouse, responds to pancreatic islet cells from NOD but not BALB/c mice. We recently reported that a hybrid insulin peptide (HIP), 6.9HIP, formed by linkage of an insulin C-peptide fragment and a fragment of islet amyloid polypeptide (IAPP), is the antigen for BDC-6.9. We report here that the core 12-mer peptide from 6.9HIP, centered on the hybrid peptide junction, is also highly antigenic for BDC-6.9. In agreement with the observation that BALB/c islet cells fail to stimulate the T cell clone, a single amino acid difference in the BALB/c IAPP sequence renders the BALB/c version of the HIP only weakly antigenic. Mutant peptide analysis indicates that each parent molecule—insulin C-peptide and IAPP—donates residues critical for antigenicity. Through mass spectrometric analysis, we determine the distribution of naturally occurring 6.9HIP across chromatographic fractions of proteins from pancreatic beta cells. This distribution closely matches the profile of the T cell response to the fractions, confirming that 6.9HIP is the endogenous islet antigen for the clone. Using a new MHC II tetramer reagent, 6.9HIP-tet, we show that T cells specific for the 6.9HIP peptide are prevalent in the pancreas of diabetic NOD mice. Further study of HIPs and HIP-reactive T cells could yield valuable insight into key factors driving progression to diabetes and thereby inform efforts to prevent or reverse this disease.

Keywords

non-obese diabetic (NOD) mouse; autoimmune type 1 diabetes; hybrid insulin peptides (HIPs); peptide fusion; tetramer

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1. Introduction

Autoimmunity arises from a failure of the immune system to establish or maintain self-tolerance. In the thymus, developing T cells are exposed to self-peptides derived from genetically encoded proteins, and self-reactive T cells are either deleted or develop a regulatory phenotype before venturing into the periphery [1–4]. The integrity of this checkpoint relies on the encounter between autoreactive T cells and their cognate self-antigen in the thymus. Post-translational modification (PTM) of self-peptides in the periphery may lead to the generation of neo-epitopes that are not displayed in the thymus. T cells specific for these modified peptides can bypass central tolerance mechanisms and escape into the periphery where they may potentially contribute to an autoimmune response [5–7]. PTM of proteins is well documented in several autoimmune diseases (e.g., rheumatoid arthritis, multiple sclerosis, celiac disease), but has been little characterized in autoimmune diabetes, with a few exceptions [8–13]. We have been investigating newly identified post-translationally modified peptide antigens with the view to better understanding the role of PTM in the disease, developing disease biomarkers, and devising strategies for intervention.

To facilitate the identification of disease-relevant autoantigens, we have used the BDC panel of islet-reactive, diabetogenic CD4 T cell clones that were isolated from spontaneously diabetic NOD mice [14]. These T cell clones produce IFN- γ in response to mouse islet cells or membrane preparations from beta cell tumors and rapidly induce disease by adoptive transfer into very young (<2 weeks of age) NOD recipients [14]. Unlike other T cell clones from the panel (such as BDC-2.5), which respond to islets from both NOD and BALB/c mice, the clone BDC-6.9 responds only to NOD islets. In a previous study to identify the genomic region harboring the gene encoding the BDC-6.9 antigen, NOD and BALB/c mice were crossed, and F₁ progeny were backcrossed with BALB/c mice. Islets of the offspring were tested with BDC-6.9 for antigenicity, and genetic linkage analysis revealed that the BDC-6.9 antigen was linked to two microsatellite regions on chromosome 6 [15]. Because the *Iapp* gene was identified within this locus, islet amyloid polypeptide (IAPP), a hormone produced by pancreatic beta cells, became the lead candidate for the BDC-6.9 antigen.

Two non-synonymous single-nucleotide differences exist between the coding sequences of *Iapp* in NOD and BALB/c mice [16,17]; each of these substitutions results in a single amino acid difference between the NOD and BALB/c IAPP molecules. Pro-IAPP is naturally processed in the secretory granules of beta cells to yield the peptides IAPP1, amylin, and IAPP2 [18]. One amino acid substitution is in the IAPP1 region, while the other is in the IAPP2 region. We postulated that the BDC-6.9 epitope was a peptide from one of these two regions of IAPP and that the NOD, but not the BALB/c, variant of the peptide would be antigenic. As screening of panels of overlapping peptides spanning the entirety of the NOD proIAPP sequence failed to reveal a peptide antigenic for BDC-6.9, we hypothesized that the natural epitope is derived from a post-translationally modified form of IAPP1 or IAPP2.

We recently identified hybrid insulin peptide (HIP) formation as a novel post-translational modification in the context of autoimmune diabetes [19]. HIPs are generated in the beta cell by fusion of the N-terminus of a peptide to the C-terminus of an insulin fragment via a

peptide bond. We reported that 6.9HIP, a hybrid between the first 26 residues of insulin C-peptide (C:1-26) [20–22] and the NOD IAPP2 peptide, was highly antigenic for BDC-6.9 and a second T cell clone, BDC-9.3, having the same T cell receptor (TCR) as BDC-6.9. In the present study, we further define and characterize the 6.9HIP as the antigenic ligand for BDC-6.9 and show that substituting the BALB/c sequence in the IAPP portion of the peptide markedly reduces antigenicity. We also demonstrate through the use of an MHC class II tetramer reagent that CD4 T cells specific for 6.9HIP are prevalent in the pancreas of diabetic NOD mice.

2. Materials and methods

2.1. Mice

BALB/c, NOD, NOD.RIP-TAg, NOD.IAPP^{−/−}, and NOD BDC-6.9 T cell receptor (TCR)-transgenic (NOD BDC-6.9 TCR-Tg) mice were bred and housed at National Jewish Health (Denver, CO) and University of Colorado Denver in specific pathogen-free conditions. Generation of BDC-6.9 TCR-Tg [23], NOD.RIP-TAg [24], and NOD.IAPP^{−/−} (IAPP^{−/−}) mice [25] was described previously. Mice were monitored for diabetes onset by urine glucose testing, and hyperglycemia was confirmed by blood glucose testing. Mice were considered diabetic when blood glucose levels were >15 mM (270 mg/dL) for at least two consecutive days. All experiments were conducted under protocols approved by the Institutional Animal Care and Use Committee.

2.2. Culture of T cell clones

Culture of T cell clones was described previously [26]. T cell clones used for these studies were the 6.9HIP-reactive T cell clones BDC-6.9 and BDC-9.3, the insulin B:9-23-reactive T cell clone BDC-4.38, and the insulin B:9-23-reactive T cell clone PD12-4.4, described by Daniel *et al* [27]. Prior to flow cytometry analysis, T cell clones were expanded in subculture for several days with additional IL-2.

2.3. Isolation of islets for antigen assay with T cell clones

To isolate pancreatic islet cells for assay with T cell clones, mice were euthanized and the pancreas was inflated with collagenase solution via the common bile duct. Following inflation, the pancreas was removed and incubated at 37°C to allow for digestion. Islets were then isolated by density centrifugation and were subsequently handpicked under a microscope. Islets were dissociated with trypsin to generate a single cell suspension and then islet cells were counted.

2.4. Peptides

The following peptides were obtained commercially at >95% purity from CHI scientific: SHLVEALYLVCGERG (B:9-23), EVEDPQVAQLELGGGPGAGDLQTLAL (insulin 2 C:1-26), NAARDPNRESLDFLLV (IAPP 2), EVEDPQVAQLELGGGPGAGDLQTLAL-NAARDPNRESLDFLLV (6.9HIP), LQTLALNAARDP (6.9HIP:core), LQTLALNAAGDP (6.9HIP:R→G), QTLALNAARDP, TLALNAARDP, LQTLALNAARD, LQTLALNAAR, LATLALNAARDP, LQTLALNAARAP, and LQTLALNAARRP. Hyphenation is used for

clarity when describing HIP sequences to denote the transition from insulin sequence to IAPP sequence.

2.5. T cell antigen assays

T cells (2×10^4 /well) were incubated with antigen and NOD thioglycollate-elicited peritoneal macrophages (2.5×10^4 /well) as antigen presenting cells (APCs) in culture medium in a 96-well plate overnight at 37°C. Following incubation, supernatant was collected and IFN- γ concentration was measured by sandwich ELISA.

2.6. T cell proliferation assays

Splenocytes from NOD BDC-6.9 TCR-Tg mice were stained with carboxyfluorescein succinimidyl ester (CFSE). Cells (1×10^6 /well) were transferred to a 96-well plate and α -CD28 and recombinant IL-2 were added to final concentrations of 100 ng/ml and 10 U/ml, respectively. Cells were tested with the indicated peptides or with α -CD3 (200 ng/ml) as a positive control. Cells were cultured 4 days at 37°C, harvested, counterstained with antibodies, and analyzed for CFSE dilution by flow cytometry.

2.7. Islet antigen purification

Beta cell tumors were harvested from NOD.RIP-TAg mice and homogenized through 40 μ m strainers. Cells were lysed by passing successively through 22, 27, and 30 gauge needles. Following removal of large cellular debris by low-speed centrifugation, secretory granules were pelleted by centrifugation at $18,400 \times g$ and then solubilized in 2% octyl- β -glucoside. Insoluble debris was removed by centrifugation. The soluble fraction was then resolved by size exclusion chromatography on a Superdex 200 16/60 column (Amersham Biosciences) at room temperature with PBS as a running buffer. Peak antigenic fractions were pooled and reduced with dithiothreitol (DTT; final concentration of 8 mM) for 1 hr. at 65°C. Reduced samples were fractionated on a reverse-phase high-performance liquid chromatography (RP-HPLC) Extend C18 column (Agilent) using a water/acetonitrile gradient (0.1% TFA). Solvents were removed by vacuum evaporation prior to analysis of fractions by T cell antigen assay or mass spectrometry.

2.8. Mass spectrometry

Proteins in chromatographic fractions were digested with AspN prior to analysis by mass spectrometry. Resulting peptides were resolved by online chromatography (C18 column, 1200 Series HPLC system) and then analyzed with a 6550 iFunnel Q-TOF LC/MS mass spectrometer (Agilent). MS/MS spectra were analyzed both manually and using Spectrum Mill software (Agilent). Spectral intensity was calculated from MS data using Mass Profiler Professional software (Agilent).

2.9. Ex vivo flow cytometry analysis

APC-conjugated I-Ag7 tetramers were obtained from the NIH tetramer core (HEL: AMKRHGLDNRYRGYSL; insp8G: HLYVERLYLVCGGEG; 6.9HIP: LQTLALNAARDP). Pancreas and spleen were harvested from NOD or NOD.IAPP $^{-/-}$ mice. Spleen samples were homogenized to yield single cell suspensions and pancreata were digested in 5 mg/ml

collagenase. Cells were stained with tetramer for 1 hr. at 37°C and then counterstained with antibodies. Samples were run on a FACSCalibur (BD) or CyAn (Beckman Coulter) flow cytometer. Data analysis was performed using FlowJo software (Tree Star).

2.10. Statistics

Data was analyzed using GraphPad Prism Version 7.0a software. Statistical significance was determined using the Friedman test and Dunn's multiple comparisons test (Fig. 4B,C) or the Mann-Whitney test (Fig. 4D,E). Statistical significance was defined as $p < 0.05$.

3. Results

3.1. The junction region of the insulin-IAPP hybrid 6.9HIP defines the epitope for BDC-6.9

Previous genetic linkage analysis implicated IAPP as the antigen for BDC-6.9. To confirm this, islets from NOD.IAPP^{-/-} mice were tested for antigenicity with BDC-6.9 and BDC-9.3, an additional T cell clone expressing the same TCR as BDC-6.9. The T cell clone BDC-4.38 [29], reactive to the insulin peptide B:9-23, was used as a control responder. All these clones responded to NOD islets, but only BDC-4.38 responded to islets from NOD.IAPP^{-/-} mice, indicating that expression of IAPP in the islets is required for generation of the BDC-6.9 antigen (Fig. 1A). As previously reported [15], BDC-6.9 did not respond to islets from BALB/c mice.

We demonstrated that the hybrid peptide 6.9HIP is highly antigenic for BDC-6.9 [19]. To better define the precise T cell epitope, we tested the core version of the hybrid peptide, LQTLAL-NAARDP (6.9HIP:core), which includes the insulin-IAPP junction at the center. This core peptide was still highly antigenic for both BDC-6.9 and BDC-9.3 (Fig. 1C,D), whereas the insulin-reactive clone BDC-4.38 did not respond to either full-length 6.9HIP or 6.9HIP:core (Fig. 1B). BDC-6.9 and BDC-9.3 were also tested with an unlinked combination of the C:1-26 and IAPP2 peptides, the two components of 6.9HIP. Failure of the clones to respond to this combination confirmed that the insulin C-peptide and IAPP2 sequences must be covalently linked in order to be recognized by the T cell clones. The sequences of the peptides tested are shown in Table 1. BDC-6.9 and BDC-9.3 did not respond to insulin 1 C:1-26, insulin 1 C-peptide, insulin 2 C-peptide, or the full NOD proIAPP molecule (data not shown).

3.2. A single amino acid difference in the sequence of BALB/c IAPP2 can account for the lack of antigenicity of BALB/c islets

BDC-6.9 and BDC-9.3 do not respond to islets from BALB/c mice (Fig. 1A), indicating that the antigen is either absent or present in very low quantities. We hypothesized that the BALB/c counterpart of 6.9HIP, in which glycine (G) would be substituted for an arginine (R), would not be antigenic for these clones. We tested BDC-6.9 and BDC-9.3 with the 6.9HIP:core sequence (LQTLAL-NAARDP) and the corresponding BALB/c core sequence 6.9HIP:R→G (LQTLAL-NAAGDP). 6.9HIP:R→G was only weakly antigenic for BDC-6.9 and BDC-9.3 (Fig. 1C,D). These results were confirmed using as responder T cells splenocytes from the NOD BDC-6.9 TCR-Tg mouse (Fig. 1E). Failure of 6.9HIP:R→G to

stimulate BDC-6.9 and BDC-9.3 at low concentrations provides a plausible explanation as to why these clones do not respond to BALB/c islets.

3.3. Residues from both insulin and IAPP are critical for antigenicity

To determine a minimal epitope for 6.9HIP-reactive T cell clones, the BDC-6.9 clone was tested with N- and C-terminally truncated versions of 6.9HIP:core. Removal of the N-terminal leucine (L) from 6.9HIP notably reduced antigenicity, whereas removal of the N-terminal leucine and glutamine (Q) entirely abolished antigenicity (Fig. 2A). Removal of the C-terminal proline (P) had little effect, but removal of both the proline and aspartic acid (D) eliminated activity (Fig. 2B). Therefore, the 11-mer peptide LQTLAL-NAARD appeared to be the minimal epitope for BDC-6.9.

To confirm the importance of the glutamine near the N-terminus of the peptide, the mutant peptide L~~A~~TLAL-NAARDP, in which the glutamine had been replaced with an alanine (A), was also tested. This substitution led to a complete loss of peptide antigenicity (Fig. 2C). The peptide LQTLAL-NAAR~~A~~P, in which aspartic acid (negatively charged side chain) had been replaced with an alanine (uncharged side chain), was less antigenic than the natural sequence (Fig. 2C). The peptide LQTLAL-NAARR~~P~~, in which aspartic acid had been replaced with arginine (R; positively charged side chain), was only weakly antigenic at very high concentrations (Fig. 2C), confirming that this position in the peptide is critical for antigenicity, and indicating that an amino acid with a negatively charged side chain may be favorable for activity.

3.4. Abundance of the insulin-IAPP hybrid in chromatographic fractions of pancreatic beta cell extract correlates with antigenicity for BDC-6.9/BDC-9.3

To provide further confirmation that the hybrid is the natural antigen for BDC-6.9 and BDC-9.3, we investigated whether the distribution of antigen across chromatographic fractions matched the distribution of the insulin-IAPP hybrid as determined by mass spectrometric analysis. As BDC-9.3, expressing the same TCR as BDC-6.9, provides a more robust response to antigen present in chromatographic fractions, BDC-9.3 was used for the following experiments. Beta cell tumors from NOD.RIP-Tag mice [24] were used as an abundant and readily attainable antigen source [30,31]. In these mice, beta cell-specific expression of the SV40 large T antigen, driven by the rat insulin promoter, results in the spontaneous formation of beta cell tumors. Tumors were homogenized to form a single cell suspension and were then mechanically disrupted by repeated passaging through needles. Secretory granules were enriched by differential centrifugation and solubilized in detergent to liberate secretory granule proteins. The sample was then fractionated by size-exclusion chromatography (SEC) and fractions were tested with BDC-9.3 for antigenicity (Fig. 3A). Peak antigenic fractions were pooled and further fractionated by reverse-phase high performance liquid chromatography (HPLC). HPLC fractions were then tested with the T cell clone for antigenicity (Fig. 3B). Antigenic fractions and flanking non-antigenic fractions were prepared for mass spectrometry analysis by digesting with the protease AspN, which cleaves proteins at the N-terminal side of aspartic acid residues. Cleavage by AspN of the full-length insulin-IAPP hybrid would generate the fragment DLQTLAL-NAAR. Peak antigenic samples were first analyzed on a Q-TOF mass spectrometer in MS/MS mode, and

data was searched against a custom database containing the hybrid peptide sequence using SpectrumMill software (Agilent). Using this approach, we confirmed that the hybrid peptide fragment DLQTLAL-NAAR was present in antigenic fractions (data not shown), as previously published [19]. Searching the data against the Swiss-Prot mouse protein database failed to identify a protein match for the spectrum of this peptide, indicating that the spectrum could only be described by the hybrid peptide sequence.

To determine the distribution of the insulin-IAPP hybrid across HPLC fractions, each AspN-digested sample was run twice in MS only mode and the average abundance of the DLQTLAL-NAAR parent ion from the two runs of each sample was determined. The distribution of the hybrid fragment DLQTLAL-NAAR closely matched the profile of the BDC-9.3 response (Fig. 3C).

3.5. CD4 T cells specific for 6.9HIP are prevalent in the pancreas of diabetic NOD mice

To examine the role of 6.9HIP-reactive CD4 T cells in the NOD mouse, we obtained a custom designed I-Ag7 tetramer loaded with 6.9HIP:core (6.9HIP-tet) from the NIH tetramer core. The reagent was first used to stain individual T cell clones. 6.9HIP-tet specifically stained BDC-6.9 and BDC-9.3 but not an insulin B:9-23-reactive clone, PD12-4.4 [27] (Fig. 4A). The insulin B:9-23 tetramer insp8G-tet [32] stained PD12-4.4 but not BDC-6.9 or BDC-9.3.

The tetramers were next used to interrogate the T cell repertoire in NOD mice. HEL-tet, a tetramer loaded with a hen egg lysozyme (HEL) peptide, was used as a negative control. As HEL is a foreign antigen, few, if any, T cells from the NOD mouse should stain with this tetramer. We began by examining diabetic mice. In the pancreas, which is the inflammatory site in autoimmune diabetes, cells staining with 6.9HIP-tet were far more abundant than insp8G-tet⁺ cells (Fig. 4B). On the other hand, in the spleen 6.9HIP-tet⁺ and insp8G-tet⁺ cells were present at comparable frequencies (Fig. 4C). The percentage of CD4 T cells that were 6.9HIP-tet⁺ was greatly reduced in the pancreas and spleen of diabetic NOD.IAPP^{-/-} mice (Fig. 4D,E), consistent with the observation that the BDC-6.9/BDC-9.3 antigen is absent from the islets of IAPP-deficient mice. The frequency of insulin-reactive CD4 T cells was the same in both NOD and NOD.IAPP^{-/-} mice. We then analyzed 12-13-week-old non-diabetic NOD mice. Cells staining with 6.9HIP-tet were present in both the pancreas (Fig. 4F) and spleen (Fig. 4G), indicating that CD4 T cells specific for 6.9HIP are present prior to disease onset.

4. Discussion

Hybrid insulin peptides, as a novel class of post-translationally modified autoantigens, hold exciting potential as a key to understanding how tolerance is breached during progression to disease. It is logical to assume that HIP-reactive cells are present in the periphery since HIPs, which are not genetically encoded, are not likely to be in the thymus. The observation that BDC-6.9 does not respond to insulin C-peptide or IAPP2 suggests that HIP-reactive cells do not cross-react with the unmodified fusion partners and are therefore unchecked by central tolerance mechanisms. We have discovered multiple HIP autoantigens in both

humans and mice, and we have identified multiple CD4 T cell clones responding to single HIPs, suggesting that these unique autoantigens may be dominant disease epitopes.

In this study, we sought to precisely define the epitope for the HIP-reactive clone BDC-6.9 and confirm the role of 6.9HIP as an endogenous antigen in the NOD mouse. Early work from our lab determined that the failure of BALB/c islets to stimulate BDC-6.9 was likely the result of one of two single amino acid differences in the pro-IAPP molecule expressed in these mice. We have now confirmed by testing NOD.IAPP^{-/-} islet cells that expression of IAPP is critical for generation of the BDC-6.9 antigen. We have demonstrated that the precise epitope for the HIP-reactive BDC-6.9 clone is a region centered on the hybrid peptide junction between an insulin C-peptide fragment and IAPP2. This epitope includes the amino acid that differs between the IAPP molecule in NOD and BALB/c mice, and introducing the BALB/c mutation into the hybrid (6.9HIP:R→G) greatly diminishes antigenicity. In our recent publication, we identified 6.9HIP in an antigenic chromatographic fraction purified from beta cells [19]. However, the same fraction contained numerous other peptides with potential to be the antigen. We have shown here by mass spectrometry that the distribution of 6.9HIP across chromatographic fractions of beta cell proteins matches the profile of the BDC-9.3 response to these fractions. This provides even stronger evidence that 6.9HIP is indeed the antigenic component of beta cells for BDC-6.9 and BDC-9.3.

CD4 T cells specific for an insulin-chromogranin A hybrid peptide, 2.5HIP, can be detected in the pancreas of diabetic NOD mice by staining with an MHC class II tetramer reagent [19]. Using the new 6.9HIP-tet reagent, we have shown that CD4 T cells specific for 6.9HIP are also present in the pancreas of diabetic NOD mice, pointing to an important role for HIP-reactive cells in pathogenesis. Furthermore, we have now demonstrated that 6.9HIP-reactive cells appear in the pancreas prior to the onset of diabetes, dismissing the possibility that these cells only appear after or shortly before disease onset.

Despite the important role of the insulin peptide B:9-23 as an epitope in the NOD mouse [33], little work has been done previously to characterize the CD4 T cell response to this antigen using MHC class II tetramers. We observed that a surprisingly low frequency of cells were detected in the pancreas of NOD mice using the B:9-23 tetramer insp8G-tet. One intriguing possibility is that many B:9-23-reactive T cells are specific for a modified form of the peptide and are therefore not detected by insp8G-tet. Thus, more than one tetramer may be needed to detect all B:9-23-reactive cells. Performing *ex vivo* analysis of T cells in isolated islets, rather than using the whole pancreas as was done in this study, might also yield higher frequencies of tetramer⁺ cells by excluding cells infiltrating the exocrine pancreas.

Although we do not yet know the mechanism of HIP formation, work by other groups has shown that the proteasome can generate neo-antigens for CD8 T cells by splicing non-contiguous peptide fragments from the same parent protein [34–38]. A similar mechanism, involving proteases present in beta cell secretory granules, may be responsible for HIP formation. Based on our previous data that other HIP-reactive T cell clones respond to islet antigen from multiple mouse strains, we hypothesize that HIPs form constitutively in all strains. Specifically, the CD4 T cell clones BDC-2.5 and BDC-10.1, which are both specific

for 2.5HIP [19], respond comparably to islet cells from either NOD or BALB/c mice [29]. If our hypothesis is correct, then differences in the nature of the immune response to HIPs, rather than HIP formation itself, could be a determining factor in the susceptibility of NOD mice to disease.

The work presented here confirms that 6.9HIP is the endogenous antigen for the diabetogenic CD4 T cell clones BDC-6.9 and BDC-9.3 and demonstrates that both peptide fusion partners donate essential amino acid residues. Our findings with 6.9HIP-tet indicate a robust response to 6.9HIP in the NOD mouse, warranting further investigation into the contribution of HIP-reactive cells to disease. Learning more about the role of hybrid insulin peptides and T cells responsive to these peptides could expand our understanding of the etiology and pathogenesis of type 1 diabetes, lead to the development of disease biomarkers, and inform the design of preventative or therapeutic strategies.

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References

1. Kyewski B, Derbinski J. Self-representation in the thymus: an extended view. *Nat. Rev. Immunol.* 2004; 4:688–698. doi:10.1038/nri1436. [PubMed: 15343368]
2. Mathis D, Benoist C. Back to central tolerance. *Immunity.* 2004; 20:509–516. doi:10.1016/S1074-7613(04)00111-6. [PubMed: 15142520]
3. Hogquist KA, Baldwin TA, Jameson SC. Central tolerance: learning self-control in the thymus. *Nat. Rev. Immunol.* 2005; 5:772–782. doi:10.1038/nri1707. [PubMed: 16200080]
4. Kyewski B, Klein L. A Central Role for Central Tolerance. *Annu. Rev. Immunol.* 2006; 24:571–606. doi:10.1146/annurev.immunol.23.021704.115601. [PubMed: 16551260]
5. Doyle HA, Mamula MJ. Posttranslational modifications of self-antigens. *Ann. N. Y. Acad. Sci.* 2005; 1050:1–9. doi:10.1196/annals.1313.001.
6. Dunne JL, Overbergh L, Purcell AW, Mathieu C. Posttranslational modifications of proteins in type 1, diabetes: The next step in finding the cure? *Diabetes.* 2012; 61:1907–1914. doi:10.2337/db11-1675. [PubMed: 22826307]
7. Klein L, Kyewski B, Allen PM, Hogquist KA. Positive and negative selection of the T cell repertoire: what thymocytes see (and don't see). *Nat. Rev. Immunol.* 2014; 14:377–91. doi:10.1038/nri3667. [PubMed: 24830344]
8. Mannering SI, Harrison LC, Williamson NA, Morris JS, Thearle DJ, Jensen KP, Kay TWH, Rossjohn J, Falk BA, Nepom GT, Purcell AW. The insulin A-chain epitope recognized by human T cells is posttranslationally modified. *J. Exp. Med.* 2005; 202:1191–7. doi:10.1084/jem.20051251. [PubMed: 16260488]
9. Delong T, Baker RL, He J, Barbour G, Bradley B, Haskins K. Diabetogenic T-cell clones recognize an altered peptide of chromogranin A. *Diabetes.* 2012; 61:3239–3246. doi:10.2337/db12-0112. [PubMed: 22912420]
10. Van Lummel M, Duinkerken G, Van Veelen PA, De Ru A, Cordfunke R, Zaldumbide A, Gomez-Tourino I, Arif S, Peakman M, Drijfhout JW, Roep BO. Posttranslational modification of HLA-DQ binding islet autoantigens in type 1 diabetes. *Diabetes.* 2014; 63:237–247. doi:10.2337/db12-1214. [PubMed: 24089515]

11. McGinty JW, Chow IT, Greenbaum C, Odegard J, Kwok WW, James EA. Recognition of posttranslationally modified GAD65 epitopes in subjects with type 1 diabetes. *Diabetes*. 2014; 63:3033–3040. doi:10.2337/db13-1952. [PubMed: 24705406]
12. Rondas D, Crèvecoeur I, D'Hertog W, Ferreira GB, Staes A, Garg AD, Eizirik DL, Agostinis P, Gevaert K, Overbergh L, Mathieu C. Citrullinated glucose-regulated protein 78 is an autoantigen in type 1 diabetes. *Diabetes*. 2015; 64:573–586. doi:10.2337/db14-0621. [PubMed: 25204978]
13. McLaughlin RJ, de Haan A, Zaldumbide A, de Koning EJ, de Ru AH, van Veelen PA, van Lummel M, Roep BO. Human islets and dendritic cells generate posttranslationally modified islet autoantigens. *Clin. Exp. Immunol.* 2016:133–140. doi:10.1111/CEI.12775. [PubMed: 26861694]
14. Haskins K. Pathogenic T-cell clones in autoimmune diabetes: more lessons from the NOD mouse. *Adv. Immunol.* 2005; 87:123–62. doi:10.1016/S0065-2776(05)87004-X. [PubMed: 16102573]
15. Dallas-Pedretti A, McDuffie M, Haskins K. A diabetes-associated T-cell autoantigen maps to a telomeric locus on mouse chromosome 6. *Proc. Natl. Acad. Sci. U. S. A.* 1995; 92:1386–1390. [PubMed: 7877988]
16. Keane TM, Goodstadt L, Danecek P, White MA, Wong K, Yalcin B, Heger A, Agam A, Slater G, Goodson M, Furlotte NA, Eskin E, Nellåker C, Whitley H, Cleak J, Janowitz D, Hernandez-Pliego P, Edwards A, Belgard TG, Oliver PL, McIntyre RE, Bhomra A, Nicod J, Gan X, Yuan W, van der Weyden L, Steward CA, Bala S, Stalker J, Mott R, Durbin R, Jackson IJ, Czechanski A, Guerra-Assunção JA, Donahue LR, Reinholdt LG, Payseur BA, Ponting CP, Birney E, Flint J, Adams DJ. Mouse genomic variation and its effect on phenotypes and gene regulation. *Nature*. 2011; 477:289–94. doi:10.1038/nature10413. [PubMed: 21921910]
17. Mouse Genomes Project. Sanger Institute; <http://www.sanger.ac.uk/science/data/mouse-genomes-project> [May 9, 2016]
18. Westermark P, Andersson A, Westermark GT. Islet amyloid polypeptide, islet amyloid, and diabetes mellitus. *Physiol Rev.* 2011; 91:795–826. doi:10.1152/physrev.00042.2009. [PubMed: 21742788]
19. Delong T, Wiles TA, Baker RL, Bradley B, Barbour G, Reisdorph R, Armstrong M, Powell RL, Reisdorph N, Kumar N, Elso CM, Denicola M, Bottino R, Powers AC, Harlan DM, Kent SC, Mannering SI, Haskins K. Pathogenic CD4 T cells in type 1 diabetes recognize epitopes formed by peptide fusion. *Science* (80). 2016; 351:711–714. doi:10.1126/science.aad2791.
20. Verchere CB, Paoletta M, Neerman-Arbez M, Rose K, Irminger JC, Gingerich RL, Kahn SE, Halban PA. Des-(27-31)C-peptide: A novel secretory product of the rat pancreatic beta cell produced by truncation of proinsulin connecting peptide in secretory granules. *J. Biol. Chem.* 1996; 271:27475–27481. doi:10.1074/jbc.271.44.27475. [PubMed: 8910330]
21. Paoletta M, Kahn S, Halban P. Truncated (des-[27-31]) C-peptide is not a major secretory product of human islets. *Diabetologia*. 2002; 45:1523–1527. doi:10.1007/s00125-002-0938-y. [PubMed: 12436335]
22. Ramström M, Hagman C, Tsybin YO, Markides KE, Håkansson P, Salehi A, Lundquist I, Håkanson R, Bergquist J. A novel mass spectrometric approach to the analysis of hormonal peptides in extracts of mouse pancreatic islets. *Eur. J. Biochem.* 2003; 270:3146–3152. doi: 10.1046/j.1432-1033.2003.03690.x. [PubMed: 12869189]
23. Pauza ME, Dobbs CM, He J, Patterson T, Wagner S, Anobile BS, Bradley BJ, Lo D, Haskins K. T-cell receptor transgenic response to an endogenous polymorphic autoantigen determines susceptibility to diabetes. *Diabetes*. 2004; 53:978–88. [PubMed: 15047613]
24. Hamaguchi K, Gaskins HR, Leiter EH. NIT-1, a pancreatic beta-cell line established from a transgenic NOD/Lt mouse. *Diabetes*. 1991; 40:842–9. [PubMed: 1647994]
25. Delong T, Baker RL, Reisdorph N, Reisdorph R, Powell RL, Armstrong M, Barbour G, Bradley B, Haskins K. Islet amyloid polypeptide is a target antigen for diabetogenic CD4 + T cells. *Diabetes*. 2011; 60:2325–2330. doi:10.2337/db11-0288. [PubMed: 21734016]
26. Haskins K, Portas M, Bergman B, Lafferty K, Bradley B. Pancreatic islet-specific T- cell clones from nonobese diabetic mice. *Proc. Natl. Acad. Sci.* 1989; 86:8000–8004. [PubMed: 2510155]
27. Daniel D, Gill RG, Schloot N, Wegmann D. Epitope specificity, cytokine production profile and diabetogenic activity of insulin-specific T cell clones isolated from NOD mice. *Eur. J. Immunol.* 1995; 25:1056–62. doi:10.1002/eji.1830250430. [PubMed: 7537670]

28. Haskins K, Portas M, Bradley B, Wegmann D, Lafferty K. Rapid Publication: T Lymphocyte Clone Specific for Pancreatic Islet Antigen. *Diabetes*. 1988; 37:1444–1448. [PubMed: 2458291]
29. Baker RL, Bradley B, Wiles TA, Lindsay RS, Barbour G, Delong T, Friedman RS, Haskins K. Cutting Edge: Nonobese Diabetic Mice Deficient in Chromogranin A Are Protected from Autoimmune Diabetes. *J. Immunol*. 2015; 196:39–43. doi:10.4049/jimmunol.1501190. [PubMed: 26608914]
30. Bergman B, McManaman JL, Haskins K. Biochemical Characterization of a Beta Cell Membrane Fraction Antigenic for Autoreactive T Cell Clones. *J. Autoimmun*. 2000; 14:343–351. doi: 10.1006/jaut.2000.0377. [PubMed: 10882061]
31. Bergman B, Haskins K. Islet-specific T-cell clones from the NOD mouse respond to beta-granule antigen. *Diabetes*. 1994; 43:197–202. [PubMed: 8288043]
32. Crawford F, Stadinski B, Jin N, Michels A, Nakayama M, Pratt P, Marrack P, Eisenbarth G, Kappler JW. Specificity and detection of insulin-reactive CD4+ T cells in type 1 diabetes in the nonobese diabetic (NOD) mouse. *Proc. Natl. Acad. Sci. U. S. A.* 2011; 108:16729–34. doi: 10.1073/pnas.1113954108. [PubMed: 21949373]
33. Nakayama M, Abiru N, Moriyama H, Babaya N, Liu E, Miao D, Yu L, Wegmann DR, Hutton JC, Elliott JF, Eisenbarth GS. Prime role for an insulin epitope in the development of type 1 diabetes in NOD mice. *Nature*. 2005; 435:220–3. doi:10.1038/nature03523. [PubMed: 15889095]
34. Hanada K-I, Yewdell JW, Yang JC. Immune recognition of a human renal cancer antigen through post-translational protein splicing. *Nature*. 2004; 427:252–256. doi:10.1038/nature02240. [PubMed: 14724640]
35. Vigneron N, Stroobant V, Chapiro J, Ooms A, Degiovanni G, Morel S, van der Bruggen P, Boon T, Van den Eynde BJ. An antigenic peptide produced by peptide splicing in the proteasome. *Science* (80). 2004; 304:587–590. doi:10.1126/science.1095522.
36. Warren EH, Vigneron NJ, Gavin MA, Coulie PG, Stroobant V, Dalet A, Tykodi SS, Xuereb SM, Mito JK, Riddell SR, Van den Eynde BJ. An antigen produced by splicing of noncontiguous peptides in the reverse order. *Science*. 2006; 313:1444–1447. doi:10.1126/science.1130660. [PubMed: 16960008]
37. Dalet A, Vigneron N, Stroobant V, Hanada K-I, Van den Eynde BJ. Splicing of distant peptide fragments occurs in the proteasome by transpeptidation and produces the spliced antigenic peptide derived from fibroblast growth factor-5. *J. Immunol*. 2010; 184:3016–24. doi:10.4049/jimmunol.0901277. [PubMed: 20154207]
38. Vigneron N, Van den Eynde BJ. Proteasome subtypes and the processing of tumor antigens: Increasing antigenic diversity. *Curr. Opin. Immunol*. 2012; 24:84–91. doi:10.1016/j.coi.2011.12.002. [PubMed: 22206698]

Highlights

- A hybrid insulin peptide (HIP) is highly antigenic for diabetogenic CD4 T cell clones.
- The T cell epitope is centered on the hybrid peptide junction.
- Each parent molecule of the HIP donates residues critical for antigenicity.
- Mass spectrometry confirms that the HIP is an endogenous beta cell antigen.
- HIP-reactive CD4 T cells are prevalent in the pancreas of diabetic NOD mice.

Figure 1. A core 12-mer peptide of 6.9HIP containing the NOD (but not BALB/c) IAPP sequence is highly antigenic for BDC-6.9

(A) T cell clones were cultured with APCs and 1×10^4 dissociated islet cells from NOD, BALB/c, or NOD.IAPP^{-/-} (IAPP^{-/-}) mice; IFN- γ production was measured by ELISA as a readout of T cell activation. The average $\pm$ S.D. of triplicate wells is reported. Results are representative of two independent experiments. (B-D) T cell clones were cultured with APCs and varying concentrations of the insulin 2 B chain peptide B:9-23, an unlinked combination of the insulin 2 C-peptide fragment C:1-26 and the NOD variant of IAPP2,

6.9HIP (EVEDPQVAQLELGGGPGAGDLQTLAL-NAARDPNRESLD~~F~~LLV), 6.9HIP:core (LQTLAL-NAARDP), or 6.9HIP:R→G (LQTLAL-NAAG~~D~~P). IFN- γ production was measured by ELISA. Results are representative of three independent experiments. **(E)** Splenocytes from BDC-6.9 TCR-Tg mice were labeled with CFSE and then cultured with varying concentrations of 6.9HIP:core or 6.9HIP:R→G. After four days, cells were analyzed by flow cytometry for CFSE dilution as an indicator of proliferation. The percentage of the original population that was induced to proliferate was calculated using the FlowJo (Tree Star) proliferation platform and is indicated in the figure. Results are representative of two independent experiments.

Figure 2. Amino acid residues in both the insulin and IAPP regions of 6.9HIP are critical for antigenicity

BDC-6.9 was cultured with APCs and varying concentrations of truncated or mutated 6.9HIP peptides to define a minimal epitope and residues critical for antigenicity. IFN- γ production was measured by ELISA. **(A)** Peptides truncated at the N-terminus. **(B)** Peptides truncated at the C-terminus. **(C)** Peptides with substitutions at critical residues. Results in (A-C) are from the same assay plate; accordingly, values for the LQTLAL-NAARDP peptide are identical in all three figures. Results are representative of three independent experiments. The peptide LQTLAL-NAARAP was only tested in two experiments.

Figure 3. The abundance of the insulin-IAPP hybrid in chromatographic fractions of beta cell proteins correlates with the magnitude of the BDC-9.3 response to the fractions
(A) Secretory granule proteins, isolated from pancreatic beta cell tumors of NOD.RIP-TAg mice, were fractionated by SEC. Fractions were then tested for antigenicity with BDC-9.3. In addition to measuring IFN- γ production, the absorbance at 280 nm was measured for each fraction as an indicator of total protein content. **(B)** Antigenic SEC fractions were pooled and further fractionated by reverse-phase HPLC; HPLC fractions were then tested for antigenicity with BDC-9.3. **(C)** Following digestion with the protease AspN, HPLC fractions were analyzed by mass spectrometry. Presence of the peptide DLQTLAL-NAAR

in antigenic fractions was confirmed by MS/MS analysis. Samples were then run in MS mode, and the spectral intensity of the parent ion corresponding to the peptide DLQTLAL-NAAR was analyzed using MassProfiler Professional software (Agilent). Reported spectral intensities represent the mean of two duplicate MS runs of the same biological sample. The IFN- γ response to each fraction, as shown in (B), is also displayed here for ease of comparison. Results for (A-C) are representative of two independent experiments.

Figure 4. CD4 T cells specific for 6.9HIP are prevalent in the pancreas of diabetic NOD mice
(A) The control clones PD12-4.4 (insulin B:9-23-reactive), BDC-6.9, and BDC-9.3 were stained with either 6.9HIP-tet or the control insulin tetramer insp8G-tet. Gated on CD4⁺ cells. (B,C) Cells from the (B) pancreas (n=15) and (C) spleen (n=15) of diabetic NOD mice were stained with antibodies and tetramer (HEL-tet, insp8G-tet, or 6.9HIP-tet) and analyzed by flow cytometry. Representative flow plots and summary statistics are shown for each tissue. Data is pooled from eight independent experiments. (D,E) The (D) pancreas (n = 5) and (E) spleen (n = 5) of diabetic NOD.IAPP^{-/-} mice were also analyzed. NOD.IAPP^{-/-}

data is pooled from three independent experiments. NOD data in (D) and (E) is the same as from (B) and (C), respectively. (F,G) Cells from the (F) pancreas (n = 7) and (G) spleen (n = 7) of non-diabetic 12-13-week old NOD mice were stained with antibodies and tetramer and analyzed by flow cytometry. Data is pooled from two independent experiments. (B-G) are gated on live CD4⁺CD45⁺CD8/CD11b/CD11c/CD19⁻ cells.

TABLE 1

PEPTIDES TESTED WITH T CELL CLONES.

<i>Peptide</i>	<i>Sequence</i>	<i>Reactive Clones</i>
insulin 2 B:9-23	SHLVEALYLVCGERG	BDC-4.38
insulin 2 C:1-26	EVEDPQVAQLELGGGPGAGDLQTLAL	
IAPP2	NAARDPNRESLDFLV	
6.9HIP	EVEDPQVAQLELGGGPGAGDLQTLAL-NAARDPNRESLDFLV	BDC-6.9/9.3
6.9HIP:core	LQTLAL-NAARDP	BDC-6.9/9.3
6.9HIP:R→G	LQTLAL-NAAGDP	(only weakly) BDC-6.9/9.3