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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.

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,

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and F_1 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), EVEDPQVAQLELGGGPGAGDLQTLALNAARDPNRESLDFLLV (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 μm 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*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 dithiotreitol (DTT; final concentration of 8 mM) for 1 h 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.