Rapid Publication

Identification of a β-Cell–Specific HLA Class I Restricted Epitope in Type 1 Diabetes

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Type 1 diabetes is an autoimmune disease in which pancreatic β-cells are destroyed by cytotoxic T-cells that recognize peptide epitopes presented by HLA class I molecules. The identification of human β-cell epitopes may significantly improve the prospects for immunodiagnosis and immunotherapy in type 1 diabetes. Using algorithms to predict nonameric β-cell peptides that would bind to the common HLA allele, HLA-A*0201, we identified a potential epitope from the leader sequence of islet amyloid polypeptide (human islet amyloid polypeptide [IAPP] precursor protein [preproIAPP] 5-13: KLQVFLIVL). Peripheral blood mononuclear cells (PBMCs) were isolated from 18 HLA-A*0201 patients with type 1 diabetes (9 with recent-onset [<180 days; range, 1–120 days] and 9 with long-standing diabetes [>180 days; range, 183–3,273 days]) and 9 healthy, nondiabetic control subjects. PBMCs were screened for peptide recognition using interferon-γ enzyme-linked immunospot (ELISpot) assays. Of the nine patients with recent-onset type 1 diabetes, six had ELISpot responses to preproIAPP 5-13 that were >3 SDs above the mean of the nondiabetic control subjects ($P = 0.002$). In contrast, no patients with type 1 diabetes for >180 days had a response above this threshold. In summary, preproIAPP 5-13 is a novel HLA class I epitope recognized by a significant proportion of cytotoxic T-cells from HLA-A*0201 patients with recent-onset type 1 diabetes and may prove to be a useful tool for the prediction and/or prevention of this disease. *Diabetes* 52: 2647–2651, 2003

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Received for publication 15 July 2003 and accepted in revised form 19 August 2003.

Posted on the World Wide Web at http://diabetes.diabetesjournals.org on 5 September 2003.

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ELISpot, enzyme-linked immunospot; HCV, hepatitis C virus; IAPP, islet amyloid polypeptide; MHC, major histocompatibility complex; PBMC, peripheral blood mononuclear cell; PHA, phytohemagglutinin; preproIAPP, IAPP precursor protein.

Type 1 diabetes is an autoimmune disease in which the death of islet β-cells is mediated by the cellular immune system, including the actions of β-cell–specific cytotoxic T-cells (1). Autoreactive cytotoxic T-cells recognize peptide epitopes displayed on the β-cell surface in the context of HLA class I molecules. These 8–10 amino acid epitopes are considered derived primarily from β-cell proteins, but their identity remains largely unknown in humans (2). Identification of the β-cell proteins and, in particular, the peptide epitopes that are targets of cytotoxic T-cell killing in human type 1 diabetes could have wide-ranging implications for prognostic, preventive, and therapeutic applications. For example, a peptide sequence important in cytotoxic T-cell–mediated β-cell killing in humans might be used to develop assays for predicting disease (3,4) or in the design of peptide-specific immunotherapies (5,6).

In the nonobese diabetic (NOD) mouse, a model of autoimmune diabetes, two peptide epitopes recognized by autoreactive cytotoxic T-cells have been described thus far. One is a nine–amino acid peptide mimotope known as NRP (KYNKANWFL) that was identified by screening combinatorial peptide libraries against a diabetogenic cytotoxic T-cell clone derived from NOD mice (7). The endogenous β-cell counterpart of NRP has been recently reported to be VYLKTNVFL, a peptide derived from the islet-specific glucose-6-phosphatase catalytic subunit–related protein (8,9). A second endogenous epitope recognized by a NOD-derived cytotoxic T-cell clone is a peptide derived from the B chain of insulin (insulin 15-23) (10).

The successful detection of these T-cell autoepitopes in NOD mice relied on access to islet cells before the onset of overt disease, allowing the generation of islet-derived T-cell lines or clones that were used to screen candidate peptide epitopes. This approach, however, is not feasible for the generation of β-cell–specific cytotoxic T-cell lines from humans because of the difficulties in acquiring pancreatic tissue from pre-diabetic and diabetic subjects. An alternate approach that is suitable for identifying epitopes in human autoimmune disease relies on predicting peptide epitopes based on the HLA type of individuals with the disease and screening the candidate peptides using mononuclear cells present in the peripheral blood of these subjects. This methodology has proven useful for predicting virus-derived HLA class I epitopes for potential use as vaccine components (11) and has also led to the identifi-

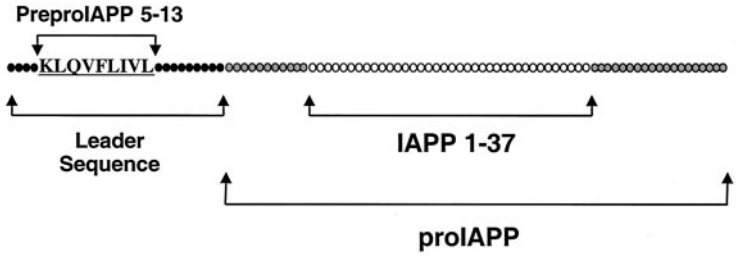

FIG. 1. Schematic of human preproIAPP molecule showing the location of the candidate epitope (preproIAPP 5-13) within the leader sequence.

cation of candidate HLA class I epitopes in other autoimmune diseases, including vitiligo (12), primary biliary cirrhosis (13), and most recently, celiac disease (14).

The present study utilized this approach to identify novel epitopes in type 1 diabetes for the class I allele, HLA-A*0201. This common allele has been shown to confer additional risk to the development of type 1 diabetes in patients who have the high-risk class II alleles DR3/4 (15,16). Also, in the NOD mouse, the transgenic expression of HLA-A2.1 major histocompatibility complex (MHC) class I molecules leads to accelerated onset of diabetes, with evidence of A2-restricted T-cell responses against pancreatic β-cells (17). Given the high frequency of this allele in our patient population of type 1 diabetes, we therefore limited our initial studies to patients carrying the HLA-A2 allele. Our findings indicate that a peptide derived from the leader sequence of the β-cell peptide, islet amyloid polypeptide (IAPP or amylin), is an HLA-A*0201 class I restricted epitope recognized by cytotoxic T-cells in individuals with recent-onset type 1 diabetes.

RESEARCH DESIGN AND METHODS

Peptide epitope prediction and synthesis. To predict nonameric β-cell peptides that would bind to the common MHC allele, HLA-A*0201, we used computer-based programs available at the websites for BioInformatics & Molecular Analysis Section (BIMAS) HLA Peptide Binding Predictions (http://www.bimas.dcrt.nih.gov) and SYFPEITHI, Institute of Cell Biology, University of Tuebingen (http://syfpeithi.bmi-heidelberg.com). All peptides were synthesized by the Nucleic Acid Peptide Synthesis (NAPS) Laboratory (University of British Columbia, Canada), and peptide purity was assessed by analytical high-performance liquid chromatography and mass spectrometry.

MHC stabilization assay. T2 cells (5×10^5 cells/well) lacking stable HLA-A*0201 surface expression (unless bound to peptides) were incubated for 16 h at 26°C with synthetic human IAPP precursor protein (preproIAPP) 5-13 (100 μg/ml) or equimolar amounts of a peptide known to bind to HLA-A*0201 (Epstein-Barr virus, EBV BMLF1 lytic cycle antigen, GLCTLVAML) or a control peptide that is known to bind to HLA-B*0801 but not HLA-A*0201 (EBV BZLF1 antigen, RAKFKQLL). The cells were then stained with fluorescein isothiocyanate–labeled anti–HLA-A*0201 (BD Pharmingen) and analyzed on a FACScalibur flow cytometer (BD Biosciences, San Jose, CA) to determine stabilization of HLA expression.

Patient recruitment. Peripheral blood was obtained from type 1 diabetic HLA-A*0201 patients and nondiabetic HLA-A*0201 healthy control subjects. Study participants were typed at the HLA-A locus by allele-specific DNA amplification (PEL-FREEZ Clinical Systems, Milwaukee, WI). Parents of all participants provided informed written consent, and patients provided written assent. The study protocol was approved by the Clinical Research Ethics Board of the University of British Columbia.

Enzyme-linked immunospot assays. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll density centrifugation and screened for peptide recognition using interferon-γ enzyme-linked immunospot (ELISpot) assays (Mabtech, Nacka, Sweden), as previously described (18). In brief, PBMCs (2×10^4 cells/well in duplicate) were incubated overnight with 5 μg/ml preproIAPP 5-13, media (negative control), phytohemagglutinin (PHA, positive control), or nucleocapsid epitope of hepatitis C virus (HCV, negative control, DLMGYIPLV) before transfer to 96-well polyvinylidene fluoride plates (MAIP N 45; Millipore, Bedford, MA). ELISpots were developed (18), scanned at high resolution, and magnified to allow unequivocal identification of spots. The frequency of preproIAPP 5-13 reactive cytotoxic T-cells was calculated by subtracting the number of spots present in wells containing the control HCV epitope from the number of spots in the preproIAPP 5-13 well. Statistical comparison between the three groups was performed using the Mann-Whitney test.

RESULTS

IAPP is a β-cell peptide that is cosecreted with insulin from β-cell secretory granules (19). In human type 1 diabetes, IAPP has attracted little attention as a possible autoantigen because studies have demonstrated no difference in the prevalence of autoantibodies against synthetic IAPP 1-37 in type 1 diabetic patients compared with type 2 diabetic control subjects (20). Interestingly, IAPP has been previously suggested as a candidate autoantigen in the NOD mouse because a CD4+ T-cell clone derived from these animals was shown to recognize a unique autoantigen that mapped to the telomeric region of mouse chromosome 6, where the IAPP gene resides (21).

We therefore revisited the possibility that IAPP, or its precursor molecule, preproIAPP, may be an autoantigen in type 1 diabetes. To this end, the amino acid sequence of human preproIAPP (Fig. 1) was analyzed using the SYFPEITHI and BIMAS algorithms to yield nonameric peptides that might bind to HLA-A*0201 (Table 1). In our experience and those of others (13,14), scores ≥60 (BIMAS) or ≥23 (SYFPEITHI) have the highest potential to bind to the class I heavy chain. To give consideration to the score from each ranking system with similar weighting, we used the product of these two scoring systems and then ranked the peptides according to their weighted score. By this analysis, a peptide within the leader sequence of IAPP (human preproIAPP 5-13: KLQVFLIVL) was found to receive a markedly higher score than any other peptide within preproIAPP (Table 1) and was chosen for further study.

To verify first that preproIAPP 5-13 would bind to HLA-A*0201 molecules, an MHC stabilization assay using T2 cells was performed. PreproIAPP 5-13, but not the negative control peptide, was able to stabilize HLA-A*0201 equally as well as the positive control peptide (Fig. 2),

TABLE 1
The protein sequence of preproIAPP was submitted to BIMAS and SYFPEITHI, and the scores that were returned were multiplied to obtain a weighted score. The peptides were ranked according to the weighted score.

Position	Sequence	SYFPEITHI	BIMAS	Score
5	KLQVFLIVL	26	268	6,966
9	FLIVLSVAL	27	98	2,653
12	VLSVALNHL	26	84	2,172
2	GILKLQVFL	24	60	1,435

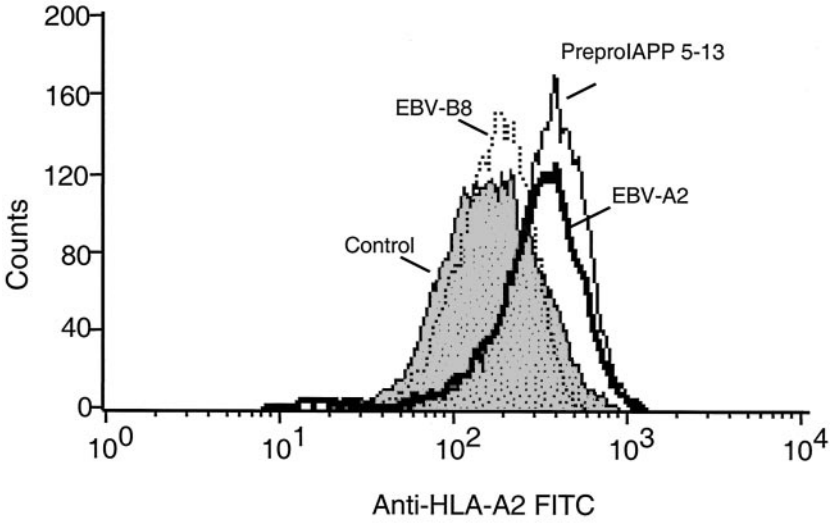

FIG. 2. HLA-A*0201 is stabilized on the cell surface by preproIAPP 5-13. PreproIAPP 5-13 stabilizes HLA molecules on the surface of T2 cells expressing HLA-A*0201. Fluorescence in the presence of preproIAPP 5-13 is similar to that observed in the presence of the known HLA-A2 epitope, EBV-A2, and significantly greater than that observed in the absence of peptide or in the presence of the HLA-B8 epitope, EBV-B8.

indicating that preproIAPP 5-13 binds to and stabilizes HLA-A*0201 on the cell surface.

After obtaining informed consent, we collected peripheral blood from 45 patients with type 1 diabetes, of whom 18 patients were shown to be HLA-A*0201 positive for at least one allele. Of the 18 HLA-A*0201 patients with type 1 diabetes forming the study population, 9 had recent-onset diabetes, which is defined as <180 days duration (duration of diabetes for each patient: 1, 1, 1, 2, 3, 26, 32, 45, and 120 days). The ages of these recent-onset patients were 4.8, 5.1, 6.9, 7.9, 8.1, 13.2, 13.9, 15.2, and 16.6 years. Nine patients had long-standing diabetes, which is defined as >180 days (duration of diabetes for each patient: 183, 185, 279, 354, 1,233, 1,586, 2,353, 1,650, and 3,273 days). The ages of these long-duration patients were 4.1, 6.5, 11.9, 12.2, 12.6, 13.0, 13.1, 13.4, and 17.3 years. Their insulin requirements ranged from 0.9 to 2.0 units $\cdot$ kg^{-1} $\cdot$ day^{-1}, and HbA_{1c} values ranged from 7.2 to 9.1%, suggesting that these long-duration patients had minimal endogenous β-cell function. Nine healthy, nondiabetic, non–age-matched HLA-A*0201 control subjects were also enrolled (ages: 18, 24, 25, 25, 26, 30, 38, 40, and 43 years).

Representative ELISpot assays performed on PBMCs from a patient with type 1 diabetes of recent onset, one of long duration, and one control subject without diabetes are presented in Fig. 3*A*. The recent-onset type 1 diabetic patient had a marked number of PBMCs responding to preproIAPP 5-13 in contrast to those from the long-standing diabetic patient and the nondiabetic control subject. Neither the diabetic patients nor the control subject had PBMCs that responded to either the negative control peptide (HCV epitope) or medium alone. A summary of all the ELISpot data are presented in Fig. 3*B*. Of the nine patients with recent-onset type 1 diabetes (<180 days), six had ELISpot responses to preproIAPP 5-13 that were at least 3 SDs above the mean of the nondiabetic

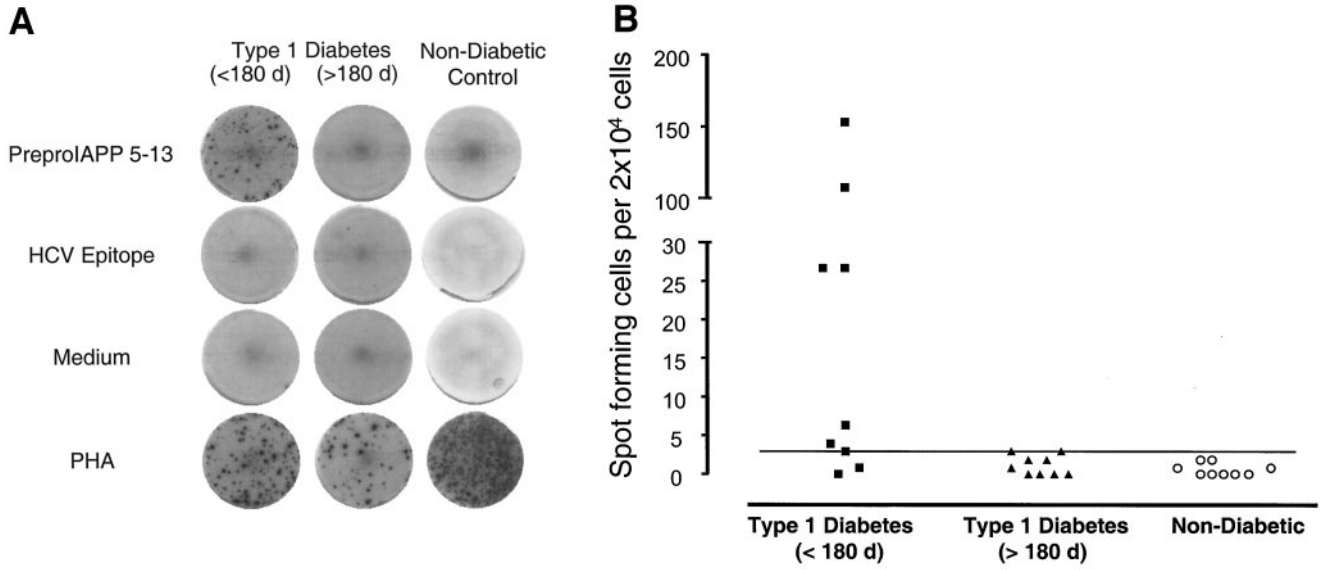

FIG. 3. Cytotoxic T-cells from HLA-A*0201 patients with type 1 diabetes recognize preproIAPP 5-13. *A*: Representative ELISpot assays showing the response of PBMCs from HLA-A*0201 diabetic patients of short (<180 days) or long (>180 days) duration and from nondiabetic HLA-A*0201 control subjects to preproIAPP 5-13, HCV epitope (negative control), media (negative control), and PHA (positive control). *B*: Summary of quantification of ELISpot assays performed on PBMCs from nine HLA-A*0201 diabetic patients of short (<180 days) duration, nine of long (>180 days) duration, and from nine nondiabetic HLA-A*0201 control subjects. The horizontal line represents 3 SDs above the mean for control subjects.

control subjects ($P = 0.002$), although two of these responders were only slightly above this threshold. In contrast, no patients with diabetes for >180 days had a response above this threshold.

DISCUSSION

In autoimmune diabetes affecting both humans and NOD mice, T-cells and, in particular, CD8+ cytotoxic T-cells are mediators of β-cell destruction (1). Although epitopes recognized by CD4+ T-cells and autoantibodies have been described in type 1 diabetes (22), the nature of the HLA class I epitopes remains largely unknown; one other HLA-A*0201–restricted epitope, derived from glutamic acid decarboxylase, has been previously described (2). In this study, we show that a significant proportion of cytotoxic T-cells from HLA-A*0201 patients with recent-onset type 1 diabetes recognize a peptide derived from the leader sequence of preproIAPP. These cells were not detected in patients who had a longer duration of diabetes, which is consistent with our finding in NOD mice that in the absence of continued antigenic stimulus due to loss of β-cells, the frequency of responding cytotoxic T-cells is low (3).

Like most secretory proteins, the IAPP precursor protein contains a hydrophobic leader sequence to direct its synthesis into the lumen of the endoplasmic reticulum. Once inside the endoplasmic reticulum, leader sequences are trimmed and normally degraded. However, because of their presence in the endoplasmic reticulum where HLA class I molecules are assembled, such sequences may make ideal self-peptides for binding to HLA heavy chains that prefer hydrophobic anchor residues. This mechanism of leader-derived peptide loading has been previously described for peptides binding to HLA-E and QA-1 in humans and mice, respectively (23,24).

In NOD mice, prediction of disease development by quantification of β-cell–specific cytotoxic T-cells (3) and prevention of clinical disease by peptide immunization (1) have been possible because of the identification of immunodominant MHC class I β-cell epitopes. Our identification of an HLA class I β-cell epitope in type 1 diabetes may now enable investigation of these diagnostic and therapeutic approaches in patients. Quantification of autoreactive cytotoxic T-cells may complement current approaches to predicting type 1 diabetes that include detection of both autoantibodies to β-cell proteins (22) and autoreactive CD4+ T-cells (25). In addition, altered or endogenous versions of preproIAPP 5-13 may have therapeutic value, as shown previously for an HLA class II epitope (6). Given the HLA class I heterogeneity of patients with type 1 diabetes, preproIAPP 5-13 is likely to be one of many epitopes recognized by autoreactive cytotoxic T-cells. The development of A*0201 tetramers to recognize preproIAPP 5-13–specific cytotoxic T-cells and the generation of peptide-specific cytotoxic T-cell lines will facilitate future studies aimed at delineating the importance of this epitope in type 1 diabetes pathogenesis. The success of this method suggests that further HLA class I β-cell epitopes may be identified using a similar approach.

ACKNOWLEDGMENTS

This study was funded by a Proof-of-Principle grant (to R.T. and C.B.V.) and a New Investigator Award (to C.B.V.) by the Canadian Institutes of Health Research. C.P. received salary support from the Canadian Pediatric Endocrine Group sponsored by Eli Lilly.

We are grateful to I-Fang Lee for assistance with HLA typing. We are indebted to Dr. Jan Dutz for his thoughtful review of this study and Jacqueline Trudeau for her input into study methodology. We thank Drs. Jean-Pierre Chanoine, Dan Metzger, and Laura Stewart for assistance with patient recruitment.

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