

Identification of Novel HLA-A*0201-Restricted Epitopes in Recent-Onset Type 1 Diabetic Subjects and Antibody-Positive Relatives

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Cytotoxic T-lymphocytes (CTLs) are considered to be essential for β -cell destruction in type 1 diabetes. However, few islet-associated peptides have been demonstrated to activate autoreactive CTLs from type 1 diabetic subjects. In an effort to identify novel epitopes, we used matrix-assisted algorithms to predict peptides of glial fibrillary acidic protein (GFAP), prepro-islet amyloid polypeptide (ppIAPP), and islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP) that likely bind to HLA-A*0201 with a strong affinity and contain a COOH-terminal proteasomal cleavage site. Seven peptides stabilized HLA-A*0201 expression in binding assays and were used to stimulate peripheral blood mononuclear cells and were evaluated for granzyme B secretion. We found that 5 of 13 type 1 diabetic subjects and 4 of 6 antibody-positive relatives exhibited greater numbers of granzyme B-secreting cells in response to at least one putative epitope compared with healthy control subjects. The most prevalent responses in antibody-positive and type 1 diabetic subjects were to ppIAPP(9-17). Other peptides recognized by type 1 diabetic or antibody-positive subjects included GFAP(143-151), IGRP(152-160), and GFAP(214-222). These data implicate peptides of ppIAPP, GFAP, and IGRP as CTL epitopes for a heterogeneous CD8⁺ T-cell response in type 1 subjects and antibody-positive relatives. *Diabetes* 55:3061-3067, 2006

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Ab, antibody; BIMAS, Bioinformatics and Molecular Analysis Section; CTL, cytotoxic T-lymphocyte; ELISpot, enzyme-linked immunospot; GFAP, glial fibrillary acidic protein; IA-2, insulinoma-associated antigen 2; IGRP, islet-specific glucose-6-phosphatase catalytic subunit-related protein; ppIAPP, prepro-islet amyloid polypeptide; PBMC, peripheral blood mononuclear cell.

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Type 1 diabetes is an autoimmune disease characterized by insulin insufficiency as a result of the cell-mediated destruction of pancreatic β -cells. At the time of diagnosis, it is estimated that 80% of the β -cells have been destroyed (1). In the well-studied nonobese diabetic (NOD) mouse model, cytotoxic T-lymphocytes (CTLs) are essential in that administration of anti-CD8 antibodies abrogates disease (2), and transfer of disease to NOD/scid mice requires both CD4⁺ and CD8⁺ splenocytes (3). Moreover, the presence of CTLs in the periphery with specificity for a mimetic peptide of islet-specific glucose-6-phosphatase catalytic subunit-related protein 206-214 [IGRP(206-214)] was found to be a positive predictor of diabetes onset in NOD mice (4). Several H-2K^d-restricted peptides have been identified as diabetes-associated epitopes in NOD mice (5), and, more recently, epitopes of proinsulin have been identified using novel techniques for epitope discovery (6). However, a definitive role for CD8⁺ CTLs in β -cell destruction has not been characterized in humans.

The functional importance of CTLs in the destruction of human β -cells is inferred from several observations. Post-mortem pancreas specimens taken from recent-onset diabetic subjects demonstrated the presence of a large CD8 cellular infiltrate within the islets concomitant with increased expression levels of HLA class I molecules on β -cells (7,8). Several HLA class I specificities, including HLA-A*0201 (HLA-A2) and HLA-A*2402, have been implicated as contributing to genetic susceptibility in antibody-positive populations (9-11). However, only a few HLA class I-restricted epitopes have been identified in type 1 diabetes. The first were peptides of GAD65 and insulin that were selected for study based on homology to known NOD epitopes (12,13). More recently, the availability of matrix-assisted algorithms for the prediction of HLA binding and proteasomal cleavage has enabled the identification of putative epitopes within a protein. By using algorithms to predict probable HLA binding, Panagiotopoulos et al. (14) identified a peptide of prepro-islet amyloid polypeptide (ppIAPP) that induced γ -interferon secretion by peripheral blood mononuclear cells (PBMCs) of recent-onset diabetic subjects. Another group identified several proinsulin epitopes spanning amino acids 28-64 by screening PBMCs of type 1 diabetic subjects against peptides predicted by proteasomal cleavage databases or generated after in vitro digestion with purified proteasomes (15).

In the current study, we sought to identify novel HLA

TABLE 1
HLA-A*0201⁺ subjects recruited in the study

Subject	Group	Age (years)*	Autoantibody†	Disease duration (days)‡	HLA-DRB1 haplotype
T466	Type 1 diabetic	29	Negative	62	*0101, *1101
T686	Type 1 diabetic	20	IA-2	787	*0301, *0901
T537	Type 1 diabetic	14	Negative	23	*0701, *1301
T160	Type 1 diabetic	24	GAD, IA-2, ICA	279	*0404, *0404
T473	Type 1 diabetic	26	GAD, IA-2	638	*0404, *1101
T363	Type 1 diabetic	27	GAD, IA-2	26	*0401, *1001
T620	Type 1 diabetic	9	GAD	3	*0301, *1301
T985	Type 1 diabetic	35	GAD	367	*0701, *0801
T339	Type 1 diabetic	14	GAD, IA-2	Unknown	*0404, *0901
T822	Type 1 diabetic	26	GAD, IA-2	688	*0401, *1302
T657	Type 1 diabetic	14	IA-2	551	*0701, *1201
T301	Type 1 diabetic	23	GAD, IA-2	60	*0301, *0401
T413	Type 1 diabetic	13	IA-2	501	*0301, *0401
A200	Antibody positive	17	GAD, IA-2, ICA	N/A	*0401, *0401
A901	Antibody positive	12	GAD, IA-2	N/A	*0301, *0404
A309	Antibody positive	13	GAD, IA-2	N/A	*0301, *0401
A372	Antibody positive	17	GAD, IA-2	N/A	*0401, *1301
A284	Antibody positive	36	GAD, IA-2	N/A	*0401, *0404
A519	Antibody positive	22	GAD	N/A	*1501, *1501
C387	Control	43	Negative	N/A	*1301, *0404
C552	Control	29	Negative	N/A	*0301, *0404
C530	Control	51	Negative	N/A	*1301, *1501
C573	Control	23	Negative	N/A	*1102, *1101
C363	Control	50	Negative	N/A	ND
C151	Control	31	Negative	N/A	*0901, *1602
C946	Control	50	Negative	N/A	ND
C860	Control	19	Negative	N/A	*1301, *1501

*Age at time of blood collection; †presence of autoantibodies to GAD65, IA-2, or islet cell Ab; ‡duration of disease at time of blood collection. ICA, islet cell Ab; ND, not determined at time of submission.

class I-restricted epitopes derived from islet proteins by using matrix-assisted algorithms, and we screened them using a highly sensitive enzyme-linked immunospot (ELISPOT) assay for the detection of antigen-specific cells. We analyzed healthy subjects with no familial history of autoimmunity, a population of nondiabetic antibody-positive relatives, and type 1 diabetic patients diagnosed within the preceding 2.5 years. By analyzing recent-onset diabetic patients and subjects considered to be at risk for developing type 1 diabetes, we endeavored to increase the probability of detecting epitope-specific T-cells before the contraction of the autoreactive CTL population resulting from the loss of islets.

Three proteins associated with disease in humans or NOD mice were chosen for analysis: ppIAPP, IGRP, and glial fibrillary acidic protein (GFAP). A major histocompatibility complex class I-restricted epitope from ppIAPP was previously identified in humans, and IGRP is a known epitope in NOD mice (14,16). GFAP is a type III intermediate filament expressed by peri-islet Schwann cells of the pancreas, and GFAP-reactive T-cells have been observed in prediabetic NOD mice (17). By using a combination of in silico analyses to substantially narrow the pool of potential peptides from these proteins and a highly sensitive technique for detection of antigen-specific T-cells, we identified several novel epitopes that induce granzyme B secretion by PBMCs of HLA-A2⁺ type 1 diabetic patients and antibody-positive relatives.

RESEARCH DESIGN AND METHODS

All subjects consented for study participation according to institutional review board-approved protocols. PBMCs from type 1 diabetic subjects ($n =$

13, male-to-female ratio 1:1.2, age [means \pm SD] 21 ± 7.7 years) were analyzed within 2.5 years of diagnosis. Antibody-positive subjects ($n = 6$, 1:1, 19.5 ± 8.8 years) were defined as first-degree relatives, except for subject A901, who was a second-degree relative, with at least one of the three diabetes-associated autoantibodies: GAD65 antibody (Ab), insulinoma-associated antigen 2 (IA-2) Ab, or islet cell Ab. Healthy control subjects ($n = 8$, 1:1.7, 37 ± 13.0 years) had no personal history of type 1 diabetes or other autoimmune disease and had no first- or second-degree relatives with diabetes. In addition, these subjects were negative for GAD65 Ab and IA-2 Ab (Table 1). Peripheral blood was collected by venous puncture into sodium heparin-containing tubes and stored overnight. PBMCs were isolated by Ficoll-hypaque density gradient centrifugation. PBMCs were frozen in a solution of 90% bovine calf serum and 10% DMSO and kept in liquefied nitrogen until use. Patient samples were haplotyped at the HLA-A allele by PCR-sequence-specific primer, using commercially available primers (One Lambda, Canoga Park, CA), by the Puget Sound Blood Center Immunogenetics laboratory or the British Columbia Child and Family Research Institute. All samples were provided for study in a blinded fashion.

In silico analyses. Matrix-assisted algorithms from the National Institutes of Health, Center for Information Technology, Bioinformatics and Molecular Analysis Section (BIMAS; available at http://bimas.dcrf.nih.gov/molbio/hla_bind/) and SYFPEITHI (available at <http://www.syfpeithi.de/>) were used to scan the complete amino acid sequences of ppIAPP, IGRP, and GFAP for nonameric peptides predicted to bind to HLA-A2 with a high affinity (18,19). Peptides predicted by each algorithm to bind HLA-A2 with the highest avidity (ranked in the top 2% of putative binders) were then evaluated for the probability of bearing a COOH-terminal proteasomal cleavage site, using the NetChop 2.0 program (available at <http://www.cbs.dtu.dk/services/NetChop/>) (20). Those peptides with a strongly predicted COOH-terminus and one or fewer internal cleavage sites were synthesized by the Benaroya Research Institute Peptide Synthesis Core Laboratory, using standard fluorenylmethoxycarbonyl chemistry. Three GFAP peptides—GFAP(386-394), GFAP(57-65), and GFAP(147-155)—that were each predicted to contain a COOH-terminal cleavage site, but not bind to HLA-A2 with a strong avidity, by SYFPEITHI or BIMAS algorithms were also synthesized as negative controls. All peptides were tested for purity by electrospray ionization mass spectrometry before use.

TABLE 2
Predicted HLA binding and proteasomal cleavage scores of putative epitopes

	Peptide	Sequence	SYFPEITHI score*	BIMAS binding score†	NetChop 2.0 score‡
1	IGRP(152-160)	FLWSVFWLI	20	5,676	0.849
2	GFAP(143-151)	NLAQDLATV	30	160	0.994
3	IGRP(215-223)	FLFAVGFYL	22	11,598	0.957
4	GFAP(192-200)	SLEEEIRFL	27	14	0.769
5	GFAP(214-222)	QLARQQVHV	24	70	0.914
6	ppIAPP(9-17)	FLIVLSVAL	27	98	0.904
7	IGRP(293-301)	RLLCALTSL	28	182	0.977
8	ppIAPP(12-20)	VLSVALNHL	26	84	0.998
9	IGRP(211-219)	NLFLFLFAV	27	13,045	0.955
10	GFAP(386-394)§	NLQIRETSL	22	21	0.998
11	GFAP(57-65)§	ALNAGFKET	20	14	0.942
12	GFAP(147-155)§	DLATVRQKL	23	1	0.969

*SYFPEITHI score was calculated by assigning values to each amino acid within a peptide based on similarity to residues of epitopes known to bind a given major histocompatibility complex molecule with a high avidity; †BIMAS binding score is the predicted half time of disassociation (in minutes) of an HLA molecule containing the indicated peptide sequence; ‡probability of carboxyl terminal proteasomal cleavage as calculated by NetChop 2.0; §peptides predicted to contain carboxyl terminal cleavage site but not bind strongly to HLA-A2 by either SYFPEITHI or BIMAS.

HLA-A2 stabilization assays. We incubated $174 \times$ CEM.T2 cells (1×10^6) in a 100 μ M solution of each peptide in 10% pooled human serum for 4 h at 37°C. The cells were then washed with 1% BSA in PBS and stained with 1 μ g fluorescein isothiocyanate-conjugated anti-HLA-A2 (clone BB7.2; BD Biosciences Pharmingen, San Diego, CA) for 20 min on ice. After an additional wash, surface levels of HLA-A2 on cells were analyzed using a FACScalibur flow cytometer (BD Biosciences Pharmingen). As negative controls, cells were incubated with either medium alone or with an HLA-A*2401-restricted Epstein-Barr virus Rta peptide (DYCNVLNKEF), which served as the background. High-affinity HLA-A2-restricted influenza MP(58-66) peptide (GILG-FVFTL) was used as a positive control to induce maximum HLA-A2 expression levels. Stabilization values induced by the putative epitopes are reported as the percentage of maximum HLA-A2 expression with the background subtracted.

ELISpot assays. Antigen-reactive T-cells from frozen PBMC samples were quantified using a granzyme B ELISpot assay (BD Biosciences Pharmingen). Frozen PBMC samples were rapidly thawed in a 37°C water bath and pelleted in a solution of 60% FCS and 1 unit/ml DNase (Sigma, St. Louis, MO). Cells were washed twice in 5% FCS in PBS and passed through a cell strainer to remove aggregates (Becton Dickinson Falcon, Franklin Lakes, NJ). ELISpot plates were coated with anti-granzyme B capture antibody (5 μ g/ml) overnight at 4°C and then washed once and blocked with 10% pooled human serum in RPMI 1640 for 2 h. Peptides were diluted to a concentration of 5 μ g/ml in 10% pooled human serum and added to wells of coated ELISpot plates. Influenza MP(58-66) and human cytomegalovirus pp65 (NLVPMVATV) epitopes were used as positive control peptides (21), and phytohemagglutinin (2.5 μ g/ml) was used as an additional positive control treatment. We also included the ppIAPP(5-13) epitope (KLQVFLIVL) as a control because this peptide has been demonstrated to activate CTLs of recent-onset diabetic subjects (14). HLA-A2-restricted PD5 peptide (KLSEGDLA) derived from pyruvate dehydrogenase was used as a negative control (22). PBMCs were added to wells at 1.0×10^6 per ml, and plates were incubated for 24 h at 37°C in 5.5% CO₂. Plates were washed twice with deionized distilled water and three times with 0.05% Tween 20 in PBS. Wells were coated with biotinylated anti-granzyme B detection antibody (2.0 μ g/ml) for 2 h at 25°C. After three washes with 0.05% Tween 20, a 1:100 dilution of streptavidin-conjugated horseradish peroxidase was added to wells for 1 h at 25°C. Wells were then washed three times with 0.05% Tween 20 and then three times with PBS before developing with 3-amino-9-ethylcarbazole substrate (AEC; Vector Laboratories, Burlingame, CA) according to the manufacturer's protocols. Plates were allowed to dry overnight at room temperature in the dark before quantifying spots per well, using an ImmunoSpot analyzer (Cellular Technology Ltd., Cleveland, OH), and all spot counts per well were also verified manually.

The number of spot-forming cells in wells containing the negative control PD5 epitope was subtracted from the spot-forming cell values of all other wells. Only mean spot-forming cell values with a coefficient of variation <0.4 were reported in order to control for random variation among wells. The cutoff of positivity for each epitope was defined as the mean number of spot-forming cells elicited by incubation of control subject PBMCs with the epitope + 3 SD of the mean. Fisher's exact test was used to compare the frequencies of epitope responsive samples in each group.

RESULTS

The matrix-assisted algorithms BIMAS and SYFPEITHI were used to compile an initial list of peptides that ranked in the top 2% of the strongest predicted HLA-A2-binding epitopes for GFAP, ppIAPP, and IGRP. Because the COOH-terminus of most class I-restricted epitopes is generated by the proteasome (23), we also analyzed the sequences of each protein using NetChop 2.0, a proteasomal cleavage predictive database that was trained using 1,100 published class I-restricted peptides (20). Those peptides that were predicted to contain a COOH-terminal cleavage site and one or fewer internal cleavage sites were included in the final panel of putative epitopes (Table 2). To test the accuracy of the HLA binding algorithms and ensure that we were not biasing the putative epitope panel based solely on these predictions, we included three peptides in the panel that were not predicted to be strong HLA-A2 binders by either algorithm but were predicted to each contain a COOH-terminal cleavage site: GFAP(386-394), GFAP(57-65), and GFAP(147-155).

Peptides were synthesized, and the ability of each to stabilize HLA-A2 expression on the TAP (transporter associated with antigen processing)-1- and -2-deficient $174 \times$ CEM.T2 cell line was analyzed. These cells express HLA-A2 at appreciable levels only in the presence of exogenously added high-affinity peptide; thus, HLA stabilization is correlated with peptide affinity (24). We normalized the stabilization values in reference to the maximum HLA-A2 expression levels induced by incubation with a high-affinity peptide of influenza, MP(58-66). Figure 1 shows the peptide stabilization values in rank order. Of the nine peptides predicted to bind HLA-A2, seven were able to stabilize HLA-A2 expression at levels $\geq 50\%$ of that induced by MP(58-66). The three peptides that were predicted to be weak binders by both algorithms—GFAP(386-394), GFAP(57-65), and GFAP(147-155)—stabilized HLA-A2 expression at the lowest levels. IGRP(211-219) was predicted to be a very strong binder by both algorithms, yet it stabilized HLA expression at the lowest level of all predicted binders. Only those peptides that stabilized HLA expression at $>50\%$ of the maximal level were used in subsequent experiments.

Each putative epitope was tested for its ability to induce

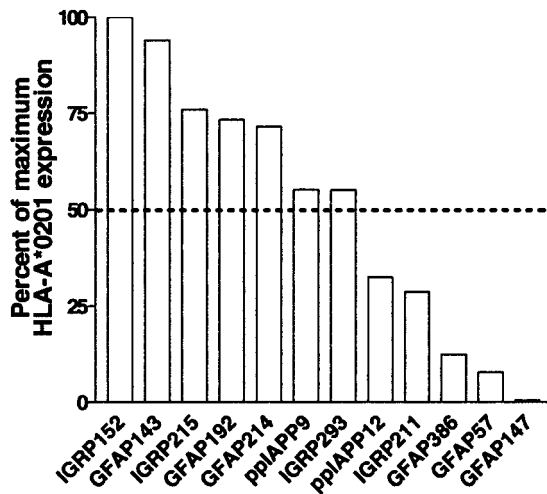


FIG. 1. Stabilization of surface HLA-A2 levels on $174 \times$ CEM.T2 cells induced by putative epitopes. Stabilization values are expressed as the percent of maximum HLA-A2 expression induced by incubation with the high-affinity HLA-A2-restricted influenza MP(58-66) peptide. The background HLA-A2 levels (induced by incubation with a non-HLA-A2-restricted Epstein Barr virus epitope) were subtracted from all values before calculation of percent maximum expression. The dashed line represents an arbitrary threshold for inclusion of peptides in subsequent experiments.

granzyme B secretion by PBMCs drawn from type 1 diabetic patients, antibody-positive relatives, or healthy subjects. Granzyme B is a direct mediator of cytotoxicity, and, unlike γ -interferon, it is more likely to be secreted by CTLs than bystander $CD4^+$ T-helper cells. Type 1 diabetic patients had been diagnosed within 2.5 years preceding the blood draw, and antibody-positive subjects were defined as first-degree relatives of type 1 diabetic patients who were positive for the presence of circulating autoantibodies with specificity for GAD65, IA-2, or islet cell Ab. An ELISpot assay was used to quantify granzyme B secretion by PBMCs in response to individual epitopes, and representative results from sample T466 are shown in Fig. 2. The threshold for determining a positive response was defined as 3 SDs above the control population's mean spot-forming cell value for each epitope. Of the 13 diabetic subjects, 5 recognized at least one novel epitope using this criterion. Three type 1 diabetic subjects (T466, T537, and T413) responded to pplAPP(9-17), which is an overlapping

peptide of the previously described pplAPP(5-13) epitope (14) (Fig. 3C). However, pplAPP(5-13) failed to elicit significant numbers of granzyme B-secreting cells from diabetic patients. Other epitopes recognized by multiple diabetic patient samples included GFAP(143-151) and GFAP(214-222). Two type 1 diabetic subjects (T466 and T985) responded to multiple putative epitopes (Table 3). We observed no correlation between the magnitude of response to the positive control influenza and cytomegalovirus epitopes and responses to putative epitopes (data not shown). We also tested pplAPP(12-20) and IGRP(211-219), peptides that failed to stabilize HLA expression at sufficient levels, for the ability to induce granzyme B secretion by a subset of type 1 diabetic and control subjects. No responses to these peptides were observed by either type 1 diabetic or control subjects (data not shown).

Analyses of PBMCs from antibody-positive relatives demonstrated that four of the six samples responded to pplAPP(9-17) (Fig. 3C). The frequency of pplAPP(9-17)-responsive antibody-positive subjects was significantly higher than in the control group ($P < 0.05$ by Fisher's exact test). Interestingly, pplAPP(9-17)-responsive diabetic patients and antibody-positive subjects demonstrated comparable numbers of granzyme B-secreting cells. Other epitopes that were recognized by both subject groups include GFAP(143-151), GFAP(214-222), and IGRP(152-160). Antibody-positive subject A901 responded to multiple epitopes, three of which were not recognized by diabetic patients (Table 3). Moreover, this subject also demonstrated substantially higher numbers of granzyme B-secreting cells to nearly all epitopes and developed diabetes 3 years subsequent to blood collection. There was no correlation observed between antibody status and epitope recognition by either diabetic or antibody-positive subjects. In addition, no relationship between age and epitope responsiveness was observed.

DISCUSSION

The identification of CTL epitopes in type 1 diabetes is essential for an understanding of disease pathogenesis and could provide additional markers for early detection and therapeutic intervention. The integration of epitope-predictive matrix-assisted algorithms with highly sensitive screening assays has been reported to be an accurate technique for the identification of novel CTL epitopes

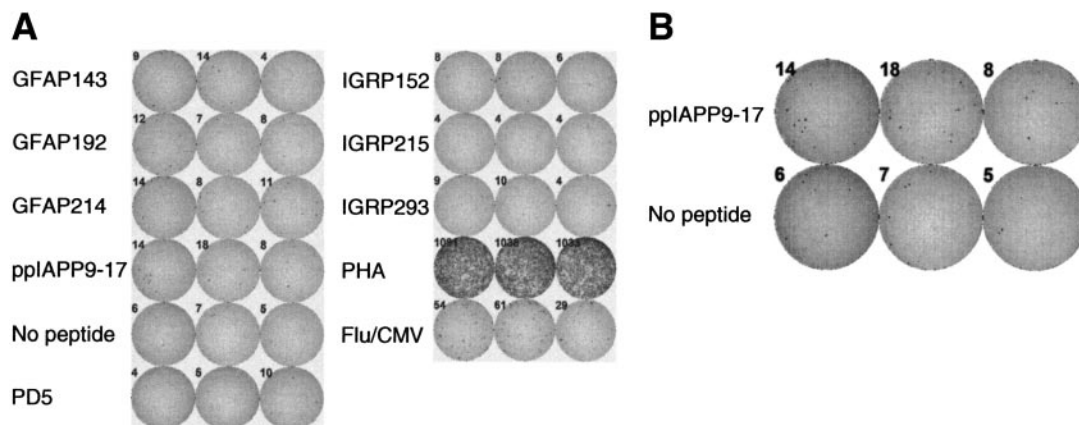


FIG. 2. Representative granzyme B ELISpot results derived from a diabetic patient (T466). A: The numbers of granzyme B-secreting cells per 6×10^5 PBMCs were calculated after a 24-h culture with indicated peptides in ELISpot plates. The number of spots per well were quantified using a CTL Immunospot Analyzer and indicated at the top left of each well. B: Magnification of wells (from A) containing PBMCs incubated with pplAPP(9-17) or diluent only to demonstrate spot morphology. CMV, cytomegalovirus; Flu, influenza; PHA, phytohemagglutinin.

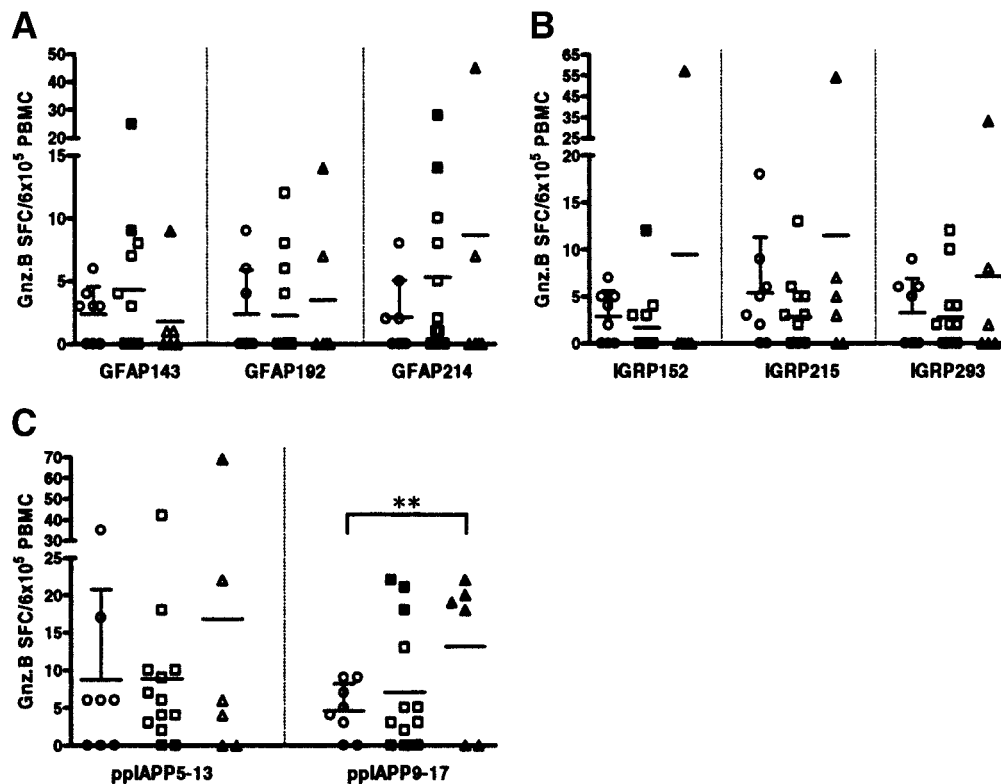


FIG. 3. Granzyme B secretion by PBMCs of healthy, diabetic, or Ab-positive subjects in response to putative epitopes. The number of granzyme (Gnz) B spot-forming cells (SFC) per 6×10^5 PBMCs elicited by incubation with putative epitopes from GFAP (A), IGRP (B), or ppIAPP (C) are shown. Horizontal bars represent the mean number of spot-forming cells, and error bars in the control group represent 1 SD of the mean. Filled symbols correspond to spot-forming cell values that are greater than the mean number of spot-forming cells of the control group + 3 SD of the mean. **Significant difference between the frequencies of responsive subjects to a given epitope in indicated groups ($P < 0.05$ by Fisher's exact test). ○, healthy; □, diabetic; △, Ab positive.

(25,26). In this study, we used SYFPEITHI and BIMAS algorithms to predict HLA-A2-binding sequences within GFAP, ppIAPP, and IGRP. Strongly predicted binders were analyzed for the presence of COOH-terminal proteasomal cleavage sites, using NetChop 2.0. Because the use of predictive algorithms to screen proteins is not sufficient, by itself, to identify epitopes (27), we tested predicted epitopes for the ability to stabilize HLA-A2 expression on the surface of $174 \times$ CEM.T2 cells. We failed to observe a direct correlation between the predicted binding scores generated by SYFPEITHI or BIMAS algorithms and the magnitude of HLA-A2 stabilization. This is not surprising because predicted HLA binding scores are calculated from

matrixes generated with peptide affinity data using cell-free systems rather than stabilization data, which represent a relative measure of peptide affinity. However, stabilization assays are the standard technique for assessing peptide binding strength to a given HLA molecule, and they are rapid and reproducible. Thus, the integration of in silico predictive data with an in vitro assay of peptide binding narrowed the pool of peptides for study and streamlined the protocol for selecting the most likely candidate epitopes.

The association between the stabilization value of a peptide and its ability to induce granzyme B secretion by CTLs was more apparent. Of the seven peptides that stabilized HLA-A2 expression at $\geq 50\%$ of maximum levels, four were recognized by PBMCs of at least one antibody-positive and type 1 diabetic subject. Interestingly, the peptide that was recognized by the highest number of type 1 diabetic and antibody-positive subjects, ppIAPP(9-17), stabilized HLA-A2 expression at modest levels, indicating a moderate to low affinity for this molecule. Self-peptides with a low affinity for HLA molecules have been reported as immunodominant epitopes in multiple sclerosis (28) and Goodpasture's disease (29). It has been hypothesized that the fast off-rate of these peptides impedes negative selection, thereby enabling the survival of autoreactive T-cells (30). It is plausible that the lower affinity of ppIAPP(9-17) for HLA-A2 might allow for the escape of antigen-specific CTLs from the thymus, resulting in later activation and expansion in the periphery. We also analyzed a subset of diabetic and control subjects for responsiveness to the overlapping ppIAPP(12-20) epitope and

TABLE 3
Summary of subjects responding to novel epitopes

Subject	Epitopes to which subject responded*
T466	GFAP214, ppIAPP9
T537	ppIAPP9
T620	GFAP143
T413	ppIAPP9
T985	GFAP143, GFAP214, IGRP152
A200	ppIAPP5-13, ppIAPP9
A901	GFAP192, GFAP214, ppIAPP9, IGRP152, IGRP215, IGRP293
A372	GFAP143, ppIAPP9
A284	ppIAPP9

*Novel epitopes that induced significantly increased granzyme B spot-forming cells per 6×10^5 PBMCs compared with responses by healthy control subjects.

found that it failed to induce granzyme B secretion (data not shown). It is likely that the affinity of this epitope for HLA-A2 is so low as to preclude presentation in the periphery.

Among the type 1 diabetic patients studied, disease duration was a relative predictor of epitope recognition in that four of the five subjects responding to novel epitopes had been diagnosed within ~1 year preceding blood collection. It is probable that the preferential responsiveness of these patients is the result of antigen presentation in the early stages of disease, before the complete loss of β -cells. Subjects with diabetes of longer duration likely present lower levels of islet antigens, resulting in the contraction of responsive CTLs. This phenomenon was observed in a report of insulin B peptide-specific T-cells that were detectable in patients experiencing recurrent autoimmunity secondary to islet allografts but that were not detectable in long-standing diabetic patients (31). However, in a report by Toma et al. (15), PBMCs from both short- and long-term type 1 diabetic subjects were activated by several proinsulin epitopes. It is plausible that CTLs with specificity for proinsulin peptides represent a memory population, and this accounts for their continued presence in long-term diabetic subjects. The authors used γ -interferon secretion as an indicator of CTL activation and did not report whether these peptides elicit a cytotoxic response to the epitopes. In our study the use of granzyme B ELISpot assays supports the conclusion that CTLs with specificity for multiple islet peptides may play a role in β -cell destruction in individuals with type 1 diabetes, although different subjects show considerable variation in this specificity. Within the samples evaluated in this study, this variation did not appear to correlate with other HLA genotypes present in the individual subjects, but larger studies are needed to fully assess this possibility.

Previously, ppIAPP(5-13) has been shown to elicit γ -interferon secretion by PBMCs of recently diagnosed type 1 diabetic patients but not patients with disease of a longer duration (14). We failed to observe significant numbers of granzyme B-secreting cells arising from recent-onset diabetic patient cultures with this epitope, and we did not assess the ability of novel epitopes to induce cytokine secretion. We are uncertain whether the lack of significant granzyme B response seen in cultures containing ppIAPP(5-13) was attributable to the fact that CTLs specific for this peptide represent a distinct phenotypic subset that differs from ppIAPP(9-17)-responsive cells (central versus effector memory), or whether responsive CTLs from diabetic patients were undetectable because of the high number of spot-forming cells seen in the control subject cultures (Fig. 3C). Further studies of larger populations will need to be performed to assess the differences in these two responsive populations. It is interesting to note that only one of seven subjects responding to ppIAPP(9-17) also recognized ppIAPP(5-13), demonstrating that these epitopes are not cross-reactive.

Two GFAP and one IGRP epitopes were recognized by at least two subjects. This is the first report demonstrating CTL responses in human diabetic or antibody-positive subjects to peptides of these proteins. In a recent report, NOD mice bearing a transgene encoding a chimeric HLA-A2 molecule were shown to generate T-cells with specificity for murine IGRP peptides (32). The predominant CTL response in these animals was directed to IGRP(228-236), and these CTLs were cross-reactive with the homologous human peptide. It is possible that

IGRP(228-236) serves as an additional epitope in type 1 diabetes; however, even in NOD mice, a considerable degree of heterogeneity in responses to this epitope was noted (32). We did not identify IGRP(228-236) as a putative epitope because it was predicted to be a low-affinity binder by both predictive algorithms (SYFPEITHI score: 22; BIMAS binding score: 54.6). Our aim in the current study was to rapidly and efficiently identify epitopes by integrating in silico and in vitro techniques. A more exhaustive analysis of peptides derived from ppIAPP, IGRP, and GFAP will likely elucidate additional epitopes that elicit CTL responses in at-risk and diabetic subjects.

Recently, Ouyang et al. (33) used similar techniques to identify epitopes in type 1 diabetes and demonstrated that ppIAPP(9-17), IGRP(152-160), and IGRP(215-223) induced γ -interferon secretion by PBMCs of recent-onset diabetic patients. The observation of CTL responses to identical ppIAPP and IGRP epitopes in separate patient populations, using two different assays of T-cell activation, likely substantiates the hypothesis that these epitopes play an important role in the development of type 1 diabetes. Future studies will be needed to more fully characterize the CTL responses to these epitopes in terms of disease pathogenesis.

Our data demonstrate that ppIAPP(9-17), GFAP(214-222), GFAP(143-151), and, to a lesser degree, IGRP(152-160) represent novel epitopes that induce granzyme B secretion by CTLs of antibody-positive relatives and recent-onset type 1 diabetic subjects. Based on this, we posit that these epitopes play a role in β -cell destruction. The potential use of T-cell responsiveness to these epitopes as an indicator of disease onset or progression will need to be delineated.

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REFERENCES

- Notkins AL, Lernmark A: Autoimmune type 1 diabetes: resolved and unresolved issues. *J Clin Invest* 108:1247-1252, 2001
- Wang B, Gonzalez A, Benoist C, Mathis D: The role of CD8+ T cells in the initiation of insulin-dependent diabetes mellitus. *Eur J Immunol* 26:1762-1769, 1996
- Bendelac A, Carnaud C, Boitard C, Bach JF: Syngeneic transfer of autoimmune diabetes from diabetic NOD mice to healthy neonates: requirement for both L3T4+ and Lyt-2+ T cells. *J Exp Med* 166:823-832, 1987
- Trudeau JD, Kelly-Smith C, Verchere CB, Elliott JF, Dutz JP, Finegood DT, Santamaria P, Tan R: Prediction of spontaneous autoimmune diabetes in NOD mice by quantification of autoreactive T cells in peripheral blood. *J Clin Invest* 111:217-223, 2003
- Panagiotopoulos C, Trudeau JD, Tan R: T-cell epitopes in type 1 diabetes. *Curr Diab Rep* 4:87-94, 2004
- Hassainya Y, Garcia-Pons F, Kratzer R, Lindo V, Greer F, Lemonnier FA, Niedermann G, van Endert PM: Identification of naturally processed HLA-A2-restricted proinsulin epitopes by reverse immunology. *Diabetes* 54:2053-2059, 2005
- Imagawa A, Hanafusa T, Tamura S, Moriwaki M, Itoh N, Yamamoto K, Iwahashi H, Yamagata K, Waguri M, Nanmo T, Uno S, Nakajima H, Namba M, Kawata S, Miyagawa JI, Matsuzawa Y: Pancreatic biopsy as a procedure for detecting in situ autoimmune phenomena in type 1 diabetes: close

- correlation between serological markers and histological evidence of cellular autoimmunity. *Diabetes* 50:1269–1273, 2001
8. Itoh N, Hanafusa T, Miyazaki A, Miyagawa J, Yamagata K, Yamamoto K, Waguri M, Imagawa A, Tamura S, Inada M, et al.: Mononuclear cell infiltration and its relation to the expression of major histocompatibility complex antigens and adhesion molecules in pancreas biopsy specimens from newly diagnosed insulin-dependent diabetes mellitus patients. *J Clin Invest* 92:2313–2322, 1993
 9. Fennessy M, Metcalfe K, Hitman GA, Niven M, Biro PA, Tuomilehto J, Tuomilehto-Wolf E: A gene in the HLA class I region contributes to susceptibility to IDDM in the Finnish population: Childhood Diabetes in Finland (DiMe) Study Group. *Diabetologia* 37:937–944, 1994
 10. Noble JA, Valdes AM, Bugawan TL, Apple RJ, Thomson G, Erlich HA: The HLA class I A locus affects susceptibility to type 1 diabetes. *Hum Immunol* 63:657–664, 2002
 11. Robles DT, Eisenbarth GS, Wang T, Erlich HA, Bugawan TL, Babu SR, Barriga K, Norris JM, Hoffman M, Klingensmith G, Yu L, Rewers M: Millennium award recipient contribution: identification of children with early onset and high incidence of anti-islet autoantibodies. *Clin Immunol* 102:217–224, 2002
 12. Kimura K, Kawamura T, Kadotani S, Inada H, Niihira S, Yamano T: Peptide-specific cytotoxicity of T lymphocytes against glutamic acid decarboxylase and insulin in type 1 diabetes mellitus. *Diabetes Res Clin Pract* 51:173–179, 2001
 13. Panina-Bordignon P, Lang R, van Endert PM, Benazzi E, Felix AM, Pastore RM, Spinosa GA, Sinigaglia F: Cytotoxic T cells specific for glutamic acid decarboxylase in autoimmune diabetes. *J Exp Med* 181:1923–1927, 1995
 14. Panagiotopoulos C, Qin H, Tan R, Verchere CB: Identification of a beta-cell-specific HLA class I restricted epitope in type 1 diabetes. *Diabetes* 52:2647–2651, 2003
 15. Toma A, Haddouk S, Briand JP, Camoin L, Gahery H, Connan F, Dubois-Laforgue D, Caillat-Zucman S, Guillet JG, Carel JC, Muller S, Choppin J, Boitard C: Recognition of a subregion of human proinsulin by class I-restricted T cells in type 1 diabetic patients. *Proc Natl Acad Sci U S A* 102:10581–10586, 2005
 16. Lieberman SM, Evans AM, Han B, Takaki T, Vinnitskaya Y, Caldwell JA, Serreze DV, Shabanowitz J, Hunt DF, Nathenson SG, Santamaria P, DiLorenzo TP: Identification of the beta cell antigen targeted by a prevalent population of pathogenic CD8⁺ T cells in autoimmune diabetes. *Proc Natl Acad Sci U S A* 100:8384–8388, 2003
 17. Winer S, Tsui H, Lau A, Song A, Li X, Cheung RK, Sampson A, Affifyan F, Elford A, Jackowski G, Becker DJ, Santamaria P, Ohashi P, Dosch HM: Autoimmune islet destruction in spontaneous type 1 diabetes is not beta-cell exclusive. *Nat Med* 9:198–205, 2003
 18. Parker KC, Bednarek MA, Coligan JE: Scheme for ranking potential HLA-A2 binding peptides based on independent binding of individual peptide side-chains. *J Immunol* 152:163–175, 1994
 19. Rammensee H, Bachmann J, Emmerich NP, Bachor OA, Stevanovic S: SYFPEITHI: database for MHC ligands and peptide motifs. *Immunogenetics* 50:213–219, 1999
 20. Kesmir C, Nussbaum AK, Schild H, Detours V, Brunak S: Prediction of proteasome cleavage motifs by neural networks. *Protein Eng* 15:287–296, 2002
 21. Currier JR, Kuta EG, Turk E, Earhart LB, Loomis-Price L, Janetzki S, Ferrari G, Birx DL, Cox JH: A panel of MHC class I restricted viral peptides for use as a quality control for vaccine trial ELISPOT assays. *J Immunol Methods* 260:157–172, 2002
 22. Kita H, Lian ZX, Van de WJ, He XS, Matsumura S, Kaplan M, Luketic V, Coppel RL, Ansari AA, Gershwin ME: Identification of HLA-A2-restricted CD8(+) cytotoxic T cell responses in primary biliary cirrhosis: T cell activation is augmented by immune complexes cross-presented by dendritic cells. *J Exp Med* 195:113–123, 2002
 23. Rock KL, Gramm C, Rothstein L, Clark K, Stein R, Dick L, Hwang D, Goldberg AL: Inhibitors of the proteasome block the degradation of most cell proteins and the generation of peptides presented on MHC class I molecules. *Cell* 78:761–771, 1994
 24. Nijman HW, Houbiers JG, Vierboom MP, van der Burg SH, Drijfhout JW, D'Amato J, Kenemans P, Melief CJ, Kast WM: Identification of peptide sequences that potentially trigger HLA-A2.1-restricted cytotoxic T lymphocytes. *Eur J Immunol* 23:1215–1219, 1993
 25. Standifer NE, Panagiotopoulos C, Tan R: Current approaches for the prediction and identification of CD8 T-cell epitopes in type 1 diabetes. *Curr Opin Endocrinol Diabetes* 12:298–302, 2005
 26. Larsen MV, Lundegaard C, Lambeth K, Buus S, Brunak S, Lund O, Nielsen M: An integrative approach to CTL epitope prediction: a combined algorithm integrating MHC class I binding, TAP transport efficiency, and proteasomal cleavage predictions. *Eur J Immunol* 35:2295–2303, 2005
 27. Pelte C, Cherepnev G, Wang Y, Schoenemann C, Volk HD, Kern F: Random screening of proteins for HLA-A*0201-binding nine-amino acid peptides is not sufficient for identifying CD8 T cell epitopes recognized in the context of HLA-A*0201. *J Immunol* 172:6783–6789, 2004
 28. Muraro PA, Vergelli M, Kalbus M, Banks DE, Nagle JW, Tranquill LR, Nepom GT, Biddison WE, McFarland HF, Martin R: Immunodominance of a low-affinity major histocompatibility complex-binding myelin basic protein epitope (residues 111–129) in HLA-DR4 (B1*0401) subjects is associated with a restricted T cell receptor repertoire. *J Clin Invest* 100:339–349, 1997
 29. Phelps RG, Turner AN, Rees AJ: Direct identification of naturally processed autoantigen-derived peptides bound to HLA-DR15. *J Biol Chem* 271:18549–18553, 1996
 30. Liu GY, Fairchild PJ, Smith RM, Prowle JR, Kioussis D, Wraith DC: Low avidity recognition of self-antigen by T cells permits escape from central tolerance. *Immunity* 3:407–415, 1995
 31. Pinkse GG, Tysma OH, Bergen CA, Kester MG, Ossendorp F, van Veelen PA, Keymeulen B, Pipeleers D, Drijfhout JW, Roep BO: Autoreactive CD8 T cells associated with beta cell destruction in type 1 diabetes. *Proc Natl Acad Sci U S A* 102:18425–18430, 2005
 32. Takaki T, Marron MP, Mathews CE, Guttman ST, Bottino R, Trucco M, DiLorenzo TP, Serreze DV: HLA-A*0201-restricted T cells from humanized NOD mice recognize autoantigens of potential clinical relevance to type 1 diabetes. *J Immunol* 176:3257–3265, 2006
 33. Ouyang Q, Standifer NE, Qin H, Gottlieb P, Verchere CB, Nepom GT, Tan R, Panagiotopoulos C: Recognition of HLA class I-restricted β -cell epitopes in type 1 diabetes. *Diabetes* 55:3068–3074, 2006