First Signature of Islet \( \beta \)-Cell-Derived Naturally Processed Peptides Selected by Diabetogenic Class II MHC Molecules

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First Signature of Islet β-Cell-Derived Naturally Processed Peptides Selected by Diabetogenic Class II MHC Molecules

Anish Suri,* James J. Walters,† Henry W. Rohrs,† Michael L. Gross,* † and Emil R. Unanue2*

The diversity of Ags targeted by T cells in autoimmune diabetes is unknown. In this study, we identify and characterize a limited number of naturally processed peptides from pancreatic islet β-cells selected by diabetogenic I-A\(^{\text{b}}\) molecules of NOD mice. We used insulinomas transfected with the CIITA transactivator, which resulted in their expression of class II histocompatibility molecules and activation of diabetogenic CD4 T cells. Peptides bound to I-A\(^{\text{b}}\) were isolated and examined by mass spectrometry: some peptides derived from proteins present in secretory granules of endocrine cells, and a number were shared with cells of neuronal lineage. All proteins to which peptides were identified were expressed in β cells from normal islets. Peptides bound to I-A\(^{\text{b}}\) molecules contained the favorable binding motif characterized by acidic amino acids at the P9 position. The draining pancreatic lymph nodes of prediabetic NOD mice contained CD4 T cells that recognized three different natural peptides. Furthermore, four different peptides elicited CD4 T cells, substantiating the presence of such self-reactive T cells. The overall strategy of identifying natural peptides from islet β-cells opens up new avenues to evaluate the repertoire of self-reactive T cells and its role in onset of diabetes. The Journal of Immunology, 2008, 180: 3849–3856.

A

untoimmune diseases such as type 1 diabetes mellitus (T1DM)\(^3\) exhibit strong linkage to genes encoding for MHC molecules. In both human and NOD mice, a widely used animal model for T1DM, the diabetogenic class II MHC molecules share the unique feature of presence of a non-Asp amino acid at position 57 of the β-chain (1, 2). Structural studies of the NOD class II MHC molecules, I-A\(^{\text{b}}\) (β57 Ser), and the human diabetogenic MHC molecule, HLA-DQ8 (β57 Ala), indicated similarities in the P9 pocket between the two (3–5). Analysis of naturally processed self-peptides from I-A\(^{\text{b}}\) and DQ8 demonstrated that: 1) both I-A\(^{\text{b}}\) and DQ8 selected for similar peptides that contained multiple acidic amino acids toward their C-termini (6–9); 2) the acidic amino acids interacted with the P9 binding pocket and influenced binding affinity to the MHC molecule (7); and 3) the specificity of the P9 pocket was most crucial in determining the final outcome of peptide selection by APCs (7, 9). Taken together, these results established the preferred peptide-binding motif for diabetogenic MHC molecules.

Although T1DM results from autoimmune destruction of insulin-producing β cells, the precise nature and identity of the target autoantigens still remains to be determined, although progress is now evident (10). The paucity of this information makes it difficult to address issues relating to the specificity and diversity of the autoreactive T cell repertoire, and the temporal features of autoimmune immunity against β-cell Ags during various stages of diabetogenesis. Furthermore, the identification of T cell epitopes derived from β cells has several limitations that make it difficult to identify natural peptides. Primary β cells do not express class II MHC molecules. The mode of presentation of β-cell Ags is primarily via Ag-transfer mechanisms wherein the β-cell Ags are taken up by APCs, which process and present them to T cells (11–13). Moreover, the numbers of β cells in pancreas is limited (~10\(^5\) β cells per mouse), so using them for Ag transfer studies is difficult.

To circumvent these issues, we engineered an insulinoma into a line that expressed β cells along with the diabetogenic I-A\(^{\text{b}}\) molecule. A number of peptides were isolated that stimulated spontaneous CD4 T cells found in the draining lymph nodes. These studies establish the feasibility of the approach and provide an initial description of naturally processed β cell epitopes.

Materials and Methods

Generation of NitCIITA APC line and FACS analysis

The murine CIITA gene was cloned from the C3.G7 B cell lymphoma line, used in previous biochemical analysis of naturally processed peptides selected by I-A\(^{\text{b}}\) (7, 9). The CIITA gene was cloned into a lentiviral expression construct (ViraPower Lentiviral Expression System Invitrogen), and cotransfected with the packaging mix (ViraPower Packaging mix; Invitrogen) into the 293FT cell line to produce a lentiviral stock. The lentiviral stock was used to transduce the Nit-1 cells, which were then screened for growth under Zocin antibiotic selection. After 2–3 days post transduction, the surviving Nit-1 cells, now termed NitCIITA, were analyzed by FACS analysis for expression of cell surface I-A\(^{\text{b}}\) molecules. Subsequently, the NitCIITA cells were FACS sorted successively for four to five rounds to obtain a stable cell line that expressed high levels of I-A\(^{\text{b}}\) molecules on the cell surface. Biotinylated Ags2.42.7 and SF1.1.1 mAbs were used to detect I-A\(^{\text{b}}\) and K\(^{\text{b}}\), respectively, followed by staining with secondary streptavidin PE (Fig. 1).

Isolation and identification of naturally processed peptides from NitCIITA

The NitCIITA cells were expanded in ~300 tissue culture flasks (162 cm\(^2\)), with each containing 100 ml of DMEM supplemented with 10% FCS (FCS). A total of ~5–10 \times 10\(^5\) cells were obtained, which were then lysed in detergent, as described previously (7–9). Following immunoprecipitation of I-A\(^{\text{b}}\)-peptide complexes using mAbs conjugated to Sepharose beads, the peptides were released from class II MHC molecules via treatment with 0.01% trifluoroacetic acid (7–9). The peptides were separated

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3 Abbreviations used in this paper: T1DM, type 1 diabetes mellitus; RP, reverse phase; FA, formic acid; MS, mass spectrometry; PPLN, peri-pancreatic lymph node.

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from the α- and β-chains of I-A\(^{\beta}\) using size-exclusion columns and then analyzed by mass spectrometry as described below.

**Liquid chromatography-mass spectrometry**

MHC peptide extracts from the cells were analyzed by on-line reverse phase (RP) LC mass spectrometry (MS)/MS or by off-line strong cation exchange LC, followed by on-line RP-LC MS/MS (2D-LC-MS). For off-line strong cation exchange analysis, samples were reconstituted in Solvent C (30% CH\(_3\)CN, 0.1% formic acid (FA)). Ten microliters were injected using an Ultra-Plus II LC with flow split to 5 \(\mu\)L/min and a PolyBioethyl (15 cm by 320 \(\mu\)m; Microtech Scientific). The gradient was from 0% D (30% CH\(_3\)CN, 0.1% FA, 1 M ammonium acetate) to 25% D in 35 min, to 50% D at 50 min, to 100% D at 65 min. Fifteen 5-min fractions were collected, dried down, and reconstituted in 10 \(\mu\)L solvent A (3% CH\(_3\)CN and 0.1% FA). A total of 5 \(\mu\)L was injected for on-line RP-LC ESI-MS as described below using data-dependant MS/MS scanning.

For the on-line reverse phase analyses, a nanoflow HPLC (Eksigent) was operated at 260 nL/min. Five microliters of sample was loaded onto a silica capillary column with a Picofrit tip (New Objective) packed in-house with C18 reverse-phase material (Delta-Pak, 0.075 \times 100 mm, 5-\(\mu\)m, 300-A, Waters Co.). The gradient was from 0% solvent B (97% CH\(_3\)CN, 3% H\(_2\)O, 0.1% FA) to 50% solvent B over 70 min. The source was a Picoview PV500 (New Objective) nanospray source. Data were acquired with a LCQ Deca ion trap mass spectrometer (Thermo Fisher) or an LTQ-FT mass spectrometer (Thermo Fisher). Details of the ion trap operation can be found in previous publications (8, 9). The LTQ-FT experiments used a Picoview PV500 (New Objective) nanospray source. The MS parameters were similar to those used with the LCQ-Deca system; however, the relative collision energy was 30%, and the eight most abundant ions were selected for MS/MS analysis.

Product-ion spectra were automatically compared with the theoretical spectra using Mascot (Matrix Science) to search against the NCBI non-redundant database (version 20060209). Only peptides meeting the homology or identity criteria in Mascot were accepted (14). Furthermore, the accurate masses of the precursor ions of all peptides identified herein were within ± 5 ppm, as obtained with the LTQ-FT mass spectrometer. All the sequences were also manually verified by studying the experimental product-ion spectra. Although adhering to criteria used in manual verification was difficult because product-ion spectra show considerable variability from one peptide to another, the following were applied for most identifications: 1) the signal-to-noise ratio in the product-ion spectrum was 15:1; 2) sequence stretches were comprised of three or more y or b ions; 3) at least three or four of the dominant ion peaks were attributable to sequence ions; 4) peptides containing P showed an N-terminal cleavage at P to give a dominant y ion; 5) peptides containing S and T fragmented by losses of water; and 6) peptides containing R, N, and Q fragmented by losses of ammonia.

**Binding reactions and analysis of messenger RNA**

Synthetic peptides having the same sequence as that of the natural peptides were tested for binding to soluble I-A\(^{\beta}\) molecules generated in insect cells as described before (4, 7–9). In brief, varying amounts of test peptides were incubated with I-A\(^{\beta}\) molecules in presence of \(^{125}\)I-labeled test peptide (GKKVATTVHAGYG). Binding reactions were incubated overnight at 25°C in 30- to 32-\(\mu\)L volumes. Complexes were purified from free peptide by gel filtration Bio-Spin columns (Bio-Rad). The percentage of bound peptide was evaluated by gamma counting. <0.5% of peptides nonspecifically passed through the Bio-spin columns. Individual binding results varied from the averaged value. All binding reactions were performed in triplicate. The final concentration of Nit-1 insulinoma cells was 1.5 \times 10^6/ml. Complexes were purified from free peptide by gel filtration Bio-Spin columns (Bio-Rad). The percentage of bound peptide was evaluated by gamma counting. <0.5% of peptides nonspecifically passed through the Bio-spin columns. Individual binding results varied from the averaged value. All binding reactions were performed in triplicate.

**Isolation of spontaneous T cells and T cell assays**

Naturally occurring T cell clones reactive with the selected peptides were isolated from peri-pancreatic lymph node (PPLN) cells of 10–14 wk NOD mice. All mouse studies were reviewed and approved by our institutional review committee. In brief, in a 96-well round-bottom plate, 1–4 \times 10^5 PPLN cells/well were stimulated with 2.5 \times 10^5 irradiated NOD spleen cells in the presence of 50U/ml IL-2 and 10 \(\mu\)M of peptide pool (each pool contained between four and nine individual peptides) in a final volume of 200 \(\mu\)L DMEM 10% FCS. After 7 days at 37°C, the growth positive wells were tested for their ability to respond to the stimulatory peptide pool. The Ag-specific T cells clones from this assay were further expanded into six wells using the same conditions as described above. After the last expansion, the T cells were tested in a proliferation assay with each of the peptides that constituted the original pool.

For primary T cell proliferation assays, 1–2 \times 10^5 T cell clones were stimulated with 2.5 \times 10^5 irradiated NOD spleen cells in the presence of titering amounts of peptides in 200 \(\mu\)L of DMEM 10% FCS in a 96-well round-bottom plate. After 72 h at 37°C, each well was pulsed with \[^{3}H\]thymidine for 12–18 h, following which the plates were harvested for thymidine incorporation. In other experiments, the T cell clones were fused to the BW5147 thymoma partner to generate T hybridomas, which were used in T cell assays. Briefly, 2.5 \times 10^5 T hybridomas per well were stimulated with APCs (either 5 \times 10^{-4} C3.G7 APCs/well or 2.5 \times 10^5 irradiated NOD spleen cells/well) in the presence of titering amounts of Ag. All assays were done in 96-well flat-bottom plates in a final volume of 200 \(\mu\)L DMEM 10% FCS. In other assays, T hybridomas were stimulated by titering numbers of NitCIITA insulinoma APCs. Following 24 h at 37°C, the supernatant from each well was harvested and tested for the presence of IL-2 using either ELISA (mouse IL-2 ELISA Set, BD OptEIA; BD Biosciences) or IL-2-dependent CTLL cell line. In one set of experiments described in Fig. 6, male NOD mice of 8–10 wk of age were immunized with 10 nmole of peptides in complete Freund’s adjuvant. Mice were bled seven days later and the draining lymph node were collected and T cells placed in culture with 5 \(\mu\)M peptide for seven days, after which aliquots were tested for proliferation as described above.

**Results**

**Generation and characterization of the insulinoma APC line**

Isolation and identification of MHC-bound naturally processed peptides requires large numbers of APCs (10^{7}-10^{10} cells) expressing cell surface MHC molecules. To this end, we made use of the Nit-1 insulinoma cell line, an SV40 transformed insulinoma generated by Leiter’s laboratory (15). Nit-1 cells, which are known not to express I-A\(^{\beta}\), were infected with a lentivirus encoding for the murine CIITA gene (referred to as ‘NitCIITA’) and then screened by FACS analyses for induction of I-A\(^{\beta}\) molecules on their cell surface. As shown in Fig. 1, introduction of murine CIITA resulted in expression of high levels of cell surface I-A\(^{\beta}\) molecules in NitCIITA cells. NitCIITA but not Nit expressed H2-DM and Invariant chain genes by RT-PCR (data not shown). The NitCIITA, along with the original Nit-1 insulinomas and a control I-A\(^{\beta}\) expressing B cell line, C3G7, were tested in functional assays for activation of a number of CD4 T cells, including the diabetogenic BDC2.5 CD4+ T cell (from here on referred to as ‘BDC T cell’) (12, 16). Our strategy consisted of isolating peptides bound to the
I-A\textsuperscript{g7} molecules, characterizing them insofar as binding parameters and then searching for spontaneous T cells to them in the PPLN.

The NitCIITA cells were tested functionally with the diabetogenic CD4 BDC T cell clone previously shown to respond to an unidentified Ag derived from \(/H9252\) cells (12, 16). As shown in Fig. 2, the NitCIITA cells activated the BDC T cell—between 1000 and 3000 elicited a detectable T cell response. The BDC T cell activation was entirely dependent upon recognition of an I-A\textsuperscript{g7}-peptide complex on the surface of NitCIITA APCs. T cell response was inhibited by a blocking Ab to I-A\textsuperscript{g7} but not to I-A\textsuperscript{d}. In this experiment, 10\textsuperscript{5} NitCIITA APCs were tested with 2.5 × 10\textsuperscript{4} BDC T cells in the presence of 1 μg of the indicated Abs. In B, “NitCIITA,” T cells were not added to the culture.

We also tested islet-infiltrating T cells whose Ag specificities were unknown (they did not respond to any peptides from known autoantigens, including insulin and glutamic acid decarboxylase). These T cells responded to freshly isolated \(/H9252\)-cell reactive T cells activated by the NitCIITA, but not by C3G7 or Nit-1-g7 cells.

Characterization of naturally processed I-A\textsuperscript{g7}-peptides from NitCIITA

The NitCIITA cells were expanded in tissue culture flasks to obtain 5–10 × 10\textsuperscript{9} cells, which were lysed in detergent—the peptides bound to I-A\textsuperscript{g7} were isolated and analyzed by MS/MS.
from NOD islet cells are denoted by the letter “b.” Eight specific genes were analyzed: 1) secretograninII; 3) ODZ; 4) secretograninIII; 5) synapse associated protein; 6) Gabarap; 7) synaptotagmin 11; 8) chromogranin A; and 9) HPRT control.

Table I. *Tissue-restricted naturally processed peptides selected by I-A<sup>α</sup> molecules from the NitCIITA insulinoma APC line*  

<table>
<thead>
<tr>
<th>Peptide</th>
<th>P1</th>
<th>P4</th>
<th>P6</th>
<th>P9</th>
<th>Binding IC&lt;sub&gt;50&lt;/sub&gt; (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Synaptogranin 11 177–189 (63)&lt;sup&gt;*&lt;/sup&gt;</td>
<td>I</td>
<td>Q</td>
<td>E</td>
<td>A</td>
<td>H</td>
</tr>
<tr>
<td>177–191 (70)</td>
<td>I</td>
<td>Q</td>
<td>E</td>
<td>A</td>
<td>H</td>
</tr>
<tr>
<td>174–191 (62)</td>
<td>V</td>
<td>V</td>
<td>T</td>
<td>I</td>
<td>Q</td>
</tr>
<tr>
<td>ODZ 837–849 (58)</td>
<td>T</td>
<td>P</td>
<td>S</td>
<td>Q</td>
<td>A</td>
</tr>
<tr>
<td>836–849 (56)</td>
<td>Q</td>
<td>T</td>
<td>P</td>
<td>S</td>
<td>Q</td>
</tr>
<tr>
<td>835–849 (55)</td>
<td>L</td>
<td>Q</td>
<td>T</td>
<td>P</td>
<td>S</td>
</tr>
<tr>
<td>Neuromodulin 184–201 (78)</td>
<td>Q</td>
<td>P</td>
<td>T</td>
<td>E</td>
<td>T</td>
</tr>
<tr>
<td>262–279&lt;sup&gt;++&lt;/sup&gt;</td>
<td>S</td>
<td>A</td>
<td>P</td>
<td>K</td>
<td>V</td>
</tr>
<tr>
<td>189–204 (51)</td>
<td>S</td>
<td>A</td>
<td>P</td>
<td>K</td>
<td>V</td>
</tr>
<tr>
<td>SecretograninII 229–244 (62)</td>
<td>I</td>
<td>P</td>
<td>E</td>
<td>K</td>
<td>V</td>
</tr>
<tr>
<td>Axonal Transporter of synaptic vesicles 885–898 (55)</td>
<td>V</td>
<td>A</td>
<td>V</td>
<td>Q</td>
<td>A</td>
</tr>
<tr>
<td>Beta-site APP-Cleaving Enzyme (BACE)-2 112–124 (53)</td>
<td>F</td>
<td>A</td>
<td>V</td>
<td>A</td>
<td>G</td>
</tr>
<tr>
<td>113–126 (31)</td>
<td>A</td>
<td>V</td>
<td>A</td>
<td>G</td>
<td>A</td>
</tr>
<tr>
<td>112–125 (49)</td>
<td>F</td>
<td>A</td>
<td>V</td>
<td>A</td>
<td>G</td>
</tr>
<tr>
<td>112–126 (47)</td>
<td>F</td>
<td>A</td>
<td>V</td>
<td>A</td>
<td>G</td>
</tr>
<tr>
<td>112–127 (35)</td>
<td>F</td>
<td>A</td>
<td>V</td>
<td>A</td>
<td>G</td>
</tr>
<tr>
<td>110–126 (52)</td>
<td>S</td>
<td>N</td>
<td>F</td>
<td>A</td>
<td>V</td>
</tr>
<tr>
<td>109–125 (33)</td>
<td>S</td>
<td>N</td>
<td>F</td>
<td>A</td>
<td>V</td>
</tr>
<tr>
<td>109–126 (61)</td>
<td>S</td>
<td>N</td>
<td>F</td>
<td>A</td>
<td>V</td>
</tr>
<tr>
<td>Synaptic cell adhesion molecule 204–218 (32)</td>
<td>V</td>
<td>T</td>
<td>S</td>
<td>Q</td>
<td>L</td>
</tr>
<tr>
<td>203–218 (30)</td>
<td>V</td>
<td>T</td>
<td>S</td>
<td>Q</td>
<td>L</td>
</tr>
<tr>
<td>SecretograninII 234–248 (34)</td>
<td>D</td>
<td>V</td>
<td>Y</td>
<td>K</td>
<td>T</td>
</tr>
<tr>
<td>SecretograninII 420–434 (38)</td>
<td>A</td>
<td>P</td>
<td>G</td>
<td>R</td>
<td>G</td>
</tr>
<tr>
<td>Chromogranin A 407–423 (31)</td>
<td>R</td>
<td>P</td>
<td>S</td>
<td>S</td>
<td>R</td>
</tr>
<tr>
<td>NMDA 2A 36–46 (40)</td>
<td>0</td>
<td>I</td>
<td>A</td>
<td>V</td>
<td>L</td>
</tr>
<tr>
<td>Gabarap 29–45&lt;sup&gt;++&lt;/sup&gt;</td>
<td>V</td>
<td>P</td>
<td>V</td>
<td>I</td>
<td>V</td>
</tr>
<tr>
<td>Carboxypeptidase H (CPH) 348–363 (41)</td>
<td>K</td>
<td>F</td>
<td>P</td>
<td>P</td>
<td>P</td>
</tr>
<tr>
<td>Lisch 7 491–509 (70)</td>
<td>S</td>
<td>G</td>
<td>R</td>
<td>P</td>
<td>R</td>
</tr>
<tr>
<td>Amyloid beta A4 237–249 (55)</td>
<td>K</td>
<td>S</td>
<td>E</td>
<td>F</td>
<td>P</td>
</tr>
<tr>
<td>Amyloid beta A4 479–489 (55)</td>
<td>V</td>
<td>A</td>
<td>E</td>
<td>E</td>
<td>I</td>
</tr>
<tr>
<td>476–489 (92)</td>
<td>V</td>
<td>P</td>
<td>A</td>
<td>V</td>
<td>A</td>
</tr>
<tr>
<td>475–489 (99)</td>
<td>N</td>
<td>V</td>
<td>P</td>
<td>A</td>
<td>V</td>
</tr>
<tr>
<td>Amyloid beta A4 524–539 (34)</td>
<td>E</td>
<td>T</td>
<td>K</td>
<td>T</td>
<td>T</td>
</tr>
<tr>
<td>Scl12a7 6–18 (44)</td>
<td>T</td>
<td>V</td>
<td>P</td>
<td>V</td>
<td>E</td>
</tr>
<tr>
<td>Reticulon 4 receptor-like 1 366–380 (50)</td>
<td>R</td>
<td>N</td>
<td>Q</td>
<td>I</td>
<td>S</td>
</tr>
</tbody>
</table>

<sup>*</sup>The table contains peptides isolated from NitCIITA. Peptides were aligned according to the I-A<sup>α</sup> binding motif: indicated are the P1, P4, P6, and P9 residues. In Table II, three of the peptides were tested for the role of the P9 residue in binding. Binding values represents 50% inhibitory dose.

<sup>++</sup>Parenthesis following the peptide indicates ion scores obtained by Mascot (14), see Materials and Methods section for more details. The higher the score, the greater is the probability of a correct match. The scores exceed in all cases the homology and, in many cases, the identity score (14).

A total of 320 peptides representing 120 distinct families were identified. Class II MHC-associated peptides are often selected in “families,” wherein all members share a common 9-mer core that spans residues P1-P9 along with varying lengths of flanking residues in the C- and N-termini (17). As expected, most of the peptide families were derived from ubiquitous self-proteins; and many had been found in our previous reports and are not shown here (7–9).

Table I lists a total of 36 peptides belonging to 21 distinct families derived from proteins that exhibited a tissue-restricted expression pattern. Some were derived from proteins associated with neuronal or neuro-endocrine cell types (e.g., synaptotagmin, neuromodulin, and amyloid β), whereas others were derived from proteins associated with secretory granules (e.g., secretogranin and chromogranin). Although the finding that many of these epitopes were shared with neuronal cells was surprising, recent studies have underscored the crosstalk between endocrine and neuronal cells in the pathophysiology of T1DM (18–22).

All the eight genes tested were positive in both NitCIITA cells and in NOD islets (Fig. 4). However, neither the amount of

FIGURE 4. Messenger RNA analysis from NOD islet β cells and NitCIITA APCs. RNA samples from NitCIITA are denoted by the letter “n,” whereas those from NOD islet cells are denoted by the letter “b.” Eight specific genes were analyzed: 1) β-site amyloid precursor protein-cleaving enzyme (BACE)-2; 2) secretograninIII; 3) ODZ; 4) secretograninIII; 5) synapse associated protein; 6) Gabarap; 7) synaptotagmin 11; 8) chromogranin A; and 9) HPRT control.
message RNA nor protein has been quantitated and whether there are differences was not determined.

**Binding analysis of naturally processed peptides**

Synthetic peptides derived from the natural peptide sequences were tested in binding assays to soluble I-A<sup>g7</sup> molecules. As shown in Table I, all peptides bound to I-A<sup>g7</sup>, albeit with varying affinities, confirming that these were indeed ‘true’ I-A<sup>g7</sup>-bound epitopes. Of the 21 peptides tested, 12 (or 57%) bound with relatively good affinity (IC<sub>50</sub> < 1.0 μM), whereas 8 (or 38%) bound moderately (IC<sub>50</sub> 1.0 – 10.0 μM). One peptide, an epitope from NMDA 2A 36 – 46, bound poorly with an IC<sub>50</sub> value of >30.0 μM. This peptide contains a histidine at the P6 position, which is an unfavorable anchor residue due to the small size of the P6 pocket of I-A<sup>g7</sup> (3, 4, 7, 9).

Most natural peptides contained acidic amino acids toward their C terminus, in agreement with our previous studies (7, 8). Alignment of the 21 families of natural peptides, as shown in Table I, revealed that 19 (90%) contained either an aspartic or glutamic acid at P9, whereas two contained either a glycine or an alanine. All these residues can be accommodated into the P9 pocket (Table I). To confirm that the acidic amino acids interacted at the P9 pocket, the binding of each of three peptides to I-A<sup>g7</sup> was compared with one in which only the corresponding P9 amino acid was changed to a lysine (Table II). The choice of a lysine substitution was based on our earlier structural and biochemical studies that demonstrated a loss of binding upon this mutation (4, 7, 8). As shown in Table II, all three peptides (carboxypeptidase H 348–362; Lisch 7 491–509; and amyloid β A4 237–249) contained either a glutamic acid or an aspartic acid at P9, which when changed to a lysine resulted in a notable loss of binding.

**Searching for T cells to the selected peptides**

To decipher whether any of the natural peptides were targets of CD4<sup>+</sup> T cells, we screened the PPLNs under clonal limiting dilution conditions. The PPLNs are the site for initial priming and expansion of diabetogenic T cells (23–25). Pools of natural peptides, each containing 4 –9 different peptides, were used to stimulate PPLN cells in the presence of NOD splenic APCs and IL-2. The growth positive wells were tested for reactivity against the peptide pool, and those that responded were expanded and then tested singly with each of the peptides that constituted the pool. An example

![Figure 5](http://www.jimmunol.org/)

**FIGURE 5.** A, Example of a growth-positive T cell clone from pancreatic lymph nodes that was first identified based upon reactivity with peptide pool B. Subsequently, this T cell clone was expanded and tested singly with each of the peptides that constituted pool B, which revealed that an epitope from synapse associated protein (SynAP) was the target of this particular T cell clone (B). C and D, Two other CD4<sup>+</sup> T cells isolated from PPLNs that recognized peptides from secretogranin III and Gaba-receptor associated protein (Gabarap). E, Dose-response curves to the peptides of the three CD4<sup>+</sup> T cells using bone marrow derived dendritic cells as APCs.

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**Table II. Binding and mutational analysis to identify the P9 anchor of naturally processed peptides**

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Amino Acid Sequence</th>
<th>Binding IC&lt;sub&gt;50&lt;/sub&gt; (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carboxypeptidase H 348–362</td>
<td>K F P P E T L K S Y W E D N K</td>
<td>0.6</td>
</tr>
<tr>
<td>Lisch 7 491–509</td>
<td>S G R P R A R S V D A L D I N R P G</td>
<td>13.8</td>
</tr>
<tr>
<td>Amyloid beta A4 237–249</td>
<td>K S E F P E T A D L E D F</td>
<td>&gt;30.0</td>
</tr>
</tbody>
</table>

*Binding analysis to I-A<sup>g7</sup> of the indicated peptides. The same peptides but having a lysine at the putative P9 anchor residues bound considerably less. In bold is indicated the nine amino acid core segment (17).
of one such T cell clone, 2522–113N, that recognizes an epitope from the synapse-associated protein (SynAP 262–279) (Table I) is shown in Fig. 5. Note here that in the initial screen, 2522–113N reacted with the peptide pool containing nine distinct peptides; however, when the peptide pool was deconvoluted, the same T cell clone only reacted to a single peptide from the pool (Fig. 5A).

Using this method, we identified three different T cells that reacted with peptides from NitiCIITA, indicated in bold in Table I. The other two T cells were reactive to peptides derived from secretogranin III, an islet granule protein, and from the GABA-receptor associated protein (Gabarap 29–45) (Fig. 5, C–E). The dose-response curve for three T cells is shown in Fig. 5E: 2522–113N and 2533–30 T cell clones, which were specific for SynAP 262–279 and secretogranin III 229–224, respectively, were ~4–6-fold more sensitive than the Gabarap 29–45-specific 2535–3 T cell.

Next, we conducted a detailed mapping of the TCR contacts for each peptide, i.e., P2, P5, and P8, were mutated to an alanine: for the 2522–113N T cell, changing the central P5 lysine abrogated T cell response while changing the P2 serine or P8 glutamic acid had no effect on T cell response. In fact, changing P2 or P8 to an alanine generated ligands that stimulated this T cell clone more vigorously than the wild-type peptide (Table III). However, for 2533–30 and 2535–3 T cell clones, all three TCR contacts were needed for activation – changing any of these to alanines abolished T cell recognition (Table III).

Although the function of these three autoreactive T cells is not known at this time, the fact that 3/21 peptide families (14%) were reactive with PPLN T cells confirms that the repertoire of naturally occurring T cells, as discussed in the previous section (Fig. 5). The other three epitopes were: secretogranin II 234–248, chromogranin A 407–423, and secretogranin II 420–434. The choice of using these particular peptides was largely based on the fact that these derived from proteins of secretory granules, and hence may be attractive targets for islet-reactive T cells. Except for secretogranin II 420–434, which gave a response of dubious significance, all other four peptides elicited T cells, albeit with varying degrees of reactivity. The two strongest responding T cells were against secretogranin III 229–244 and gabarap 29–45, the same two peptides recognized by the naturally occurring T cells in NOD mice (Fig. 6).

Discussion

Identifying the autologous peptides presented by class II MHC molecules is important for understanding autoimmune diseases: it can establish the biochemical base from which the autoreactive T cells are selected. The justification for examining the natural I-A<sup>+</sup> β cell peptidome are the findings that class II MHC-bound peptides are heterogeneous and vary regarding flanking residues and amounts displayed, all of which play a significant role in the selection of the T cell repertoire. We prove here that using an insulinoma expressing Ag presentation molecules is an effective way to identify natural peptides presented by class II MHC molecules and to search for the repertoire of autoreactive CD4<sup>+</sup> T cells. Because of the nature of β cell Ag presentation, which involves Ag transfer from a donor cell, the β cell, to an APC, the logistics for identifying natural peptides by using fresh β cells mixed with APC, or by testing directly pancreatic APC, is simply not feasible.

This initial study identified 21 peptide families (a total of 36 peptides) of restricted expression, and three spontaneous T cells.
As expected, the naturally processed peptides from β cells contained the previously described I-A^g7 binding motif. Although most peptides were derived from ubiquitous self-proteins, a significant fraction was from proteins with tissue-restricted expression, especially from cells of the neuro-endocrine lineage. Some of these proteins are present in granules; or involved in granule-exocytosis. An important lesson is that without H-2DM, some proteins were not presented (Figs. 2 and 3). In experiments to be reported we examined the I-A^7 peptide from APC bearing I-A^7 with or without H-2DM, and found a large differences in the peptides selected: without H-2DM the selection of peptides with preference for acidic C-terminal residues was lost.

The feasibility of this first analysis encourages us to a more extensive examination of the β cell-I-A^g7 peptide. Clearly we need to enlarge the scope of the search, which will require using more cells as well as adding improvements in mass spectrometry analysis of very complex peptide mixtures. But a serious drawback in the biochemical identification of natural peptides is the identification of epitopes present in low abundance or having fast dissociation kinetics from MHC molecules, or both. Some autoimmunom epitopes bind poorly to MHC molecules (28–30). Insulin is a key example: a dominant Ag (30–32) that we have failed to identify biochemically, although the NiCITTA did stimulate the insulin T cell hybridomas (data not shown). These weak-binding peptides may be difficult to identify using mass spectrometry due to the multiple steps required, from lysing cells in detergent to immuno-precipitating MHC molecules for extraction of natural peptides.

However, other alternative approaches may help identify low abundance peptides. We have recently developed an algorithm that successfully and accurately predicted peptides that would bind to I-A^g7 molecules (33). This computational approach could complement the biochemical approach and be applied to β cell-restricted genes to predict epitopes that are likely to be selected by I-A^7. Taken together, the biochemical isolation of natural peptides coupled with the predicted peptides from β cell proteome would give rise to a comprehensive “peptidome” that would be invaluable for dissecting the autoimmune T cell response in T1DM. Lastly, based on the similarities of natural peptides selected by the human DQ8 and NOD I-Ag7 molecules (8), there is a high probability that autoantigenic targets of β cells are shared between the two.

The approach also showed success in searching for diabetogenic T cells based on the identification of natural peptides. This was a reverse approach from the more conventional ones that tests unknown reactive T cells against known β cell Ags, or searches for T cells using known β cell proteins (i.e., the successful case of insulin in the NOD mouse). Despite the limited number of tissue-specific peptides identified in this trial, three of them stimulated PPLN T cells, an indication that the repertoire of autoreactive T cells will probably be quite extensive. Whether these autoreactive T cells are diabetogenic, i.e., capable of inducing β cell damage, needs now to be critically evaluated. In initial and limited transfer experiments, all three of the spontaneous T cell clones did not induce diabetes when transferred into NOD.scid recipients. Cogent analysis of the difficulties in testing spontaneous clones is given by Haskins in Refs. 34, 35. Regardless of the biological response, the finding does indicate the potentially broad repertoire of autoreactivity in the NOD mouse.

Among the peptides bound to I-Ag7 were some from proteins expressed in neuronal cells. Pancreatic β cells share some features with neuronal cells. Both endocrine cells and neuronal cells are secretory cells that release granule contents, either insulin or neurotransmitters, in response to appropriate physiological stimuli. Similar gene expression profiles have been noted between the two cell types (36–39). Studies in invertebrate species demonstrated that neurons are a primary source of insulin and that genetic ablation of these cells resulted in growth retardation and hyperglycemia (40, 41). In vitro insulin-secreting cells were generated from neuronal progenitor cells (42) and multipotent precursor cells that give rise to both neuronal and pancreatic lineages were recently identified (43). The early work in NOD and humans implicated glutamic acid decarboxylase, a neuronal protein, as an autoimmune target in islet β cells (20). NOD mice deficient in B7-2 develop spontaneous peripheral poly-neuropathy, but not diabetes (19). And, the peri-islet Schwann cells have been shown to be an early target in NOD diabetes and, more recently, an important role for pancreatic sensory neurons in islet inflammation has been uncovered (18, 22).

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References


