Tissue-Specific Self-Peptides Bound by Major Histocompatibility Complex Class I Molecules of a Human Pancreatic β-Cell Line

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The process of β-cell destruction in IDDM is mediated, in part, by CD8⁺ T-cells. Structural characterization of HLA-I–bound self-peptides presented by the human β-cell line HP-62 was performed to identify possible tissue-specific autoantigens in the context of CD8⁺ T-cell/HLA-I interactions. The sequences of the β-cell line HLA-I–bound peptides were compared with sequence databases. Six of the obtained sequences showed homology to known precursor proteins, three of which—GLUT2 receptor, phosphatidylinositol-glycan–specific phospholipase D, and 5-hydroxytryptamine-1F receptor—have a limited, tissue-specific expression. These HLA-bound self-peptides may be part of a pool of autoantigens recognized by β-cell reactive cytotoxic T-cells. *Diabetes* 45:1761–1765, 1996

IDDM is the end result of an autoimmune destruction of pancreatic islet β-cells (1). The fundamental role of T-cells in this process is supported by experimental data from the NOD murine model of IDDM (2). NOD strain mice have an inherited predisposition to develop an IDDM-like disease. However, β-cell destruction can be prevented by neonatal thymectomy or treatment with anti–T-cell antibodies (3). Conversely, β-cell destruction may be induced in athymic NOD mice by transfer of both CD4⁺ and CD8⁺ T-cells from diabetic NOD mice (4). Considered together, these data strongly suggest that β-cell destruction is to a large extent T-cell–mediated, although the target antigens remain unknown.

The process of β-cell destruction in newly diagnosed IDDM patients is characterized by insulinitis, a chronic inflammatory infiltrate of predominantly CD8⁺ cells and variable numbers of CD4⁺ cells, B-cells, natural killer cells, and macrophages (5,6). Susceptibility to IDDM is primarily linked with the expression of HLA-DQ2 and -DQ8 molecules (7); furthermore, HLA-II expression has been detected on β-cells (8). Hence, CD4⁺ T-cells are probably instrumental in the immunopathological process. At least initially, CD4⁺ T-cells with TCRs specific for peptides derived from islet β-cell proteins may themselves not only destroy β-cells (either by direct cytotoxicity or through release of various cytokines), but also recruit and help β-cell reactive cytotoxic CD8⁺ T-cells.

CD8⁺ T-cells appear to play a central role in the final destruction of β-cells, as evidenced by their prominence in insulinitis and the increased expression of HLA-I molecules by involved β-cells (9). HLA-I molecules present peptides derived from intracellular proteins for surveillance by the TCR of CD8⁺ T-cells. These as yet unidentified intracellular proteins, processed and presented as peptides by the HLA-I molecules on β-cells, are postulated to play a role in promoting the autoimmune process.

We have undertaken the structural characterization of the HLA-class–I bound self-peptides presented by the human islet cell line HP-62 to identify putative precursor proteins that might function as autoantigens in the context of CD8⁺ TCR/peptide/HLA-I interactions. The present study revealed peptides with restricted tissue distribution that might be targets of attack by CD8⁺ T-cells that infiltrate pancreatic tissue during insulinitis.

RESEARCH DESIGN AND METHODS

Cell lines and culture conditions. The human pancreatic β-cell line HP-62, transfected with a plasmid containing the early region of SV40 viral DNA, has been previously described (10). Cells were cultured in roller bottles using complete medium containing RPMI 1640 media supplemented with 10% fetal calf serum, 1% glutamine, and 0.5% gentamicin (all from Gibco, Grand Island, NY). The murine hybridomas producing the mAb W6/32, anti-HLA-A,B,C (obtained from American Type Culture Collection) was cultured in IgG-free media. The HLA-I phenotype of HP-62 was established by conventional serological methods (HLA-A2, B18, B7, C7).

Reagents. The mAb W6/32 was obtained from murine hybridoma culture supernatants and purified by protein A affinity chromatography with reagents supplied by Bio-Rad (Richmond, CA). Cyanogen bromide-activated Sepharose 4B, purified normal mouse IgG, aprotinin, tosylleucylchloroketone (TLCK), tosylprolylchloroketone (TPCK), and iodoacetamide were obtained from Sigma Co. (St. Louis, MO). Pepstatin A, leupeptin, and phenylmethylsulfonylfluoride (PMSF) were obtained from Boehringer Mannheim (San Diego, CA). All high-performance liquid chromatography (HPLC) solvents, trifluoroacetic acid (TFA), Nonidet-40, and the bicinchonnic acid (BCA) protein assay kit were obtained from Pierce (Rockford, IL). All other reagents were of the highest commercially available quality.

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Received for publication 26 April 1996 and accepted in revised form 26 July 1996.

BCA, bicinchonnic acid; CNS, central nervous system; GPI-PLD, phosphatidylinositol-glycan-specific phospholipase D; HPLC, high-performance liquid chromatography; 5-HT, 5-hydroxytryptamine; MHC, major histocompatibility complex; PMSF, phenylmethylsulfonylfluoride; PTH, phenolthiohydantoin; TCR, T-cell antigen receptor; TFA, trifluoroacetic acid; TLCK, tosylleucylchloroketone; TPCK, tosylprolylchloroketone.

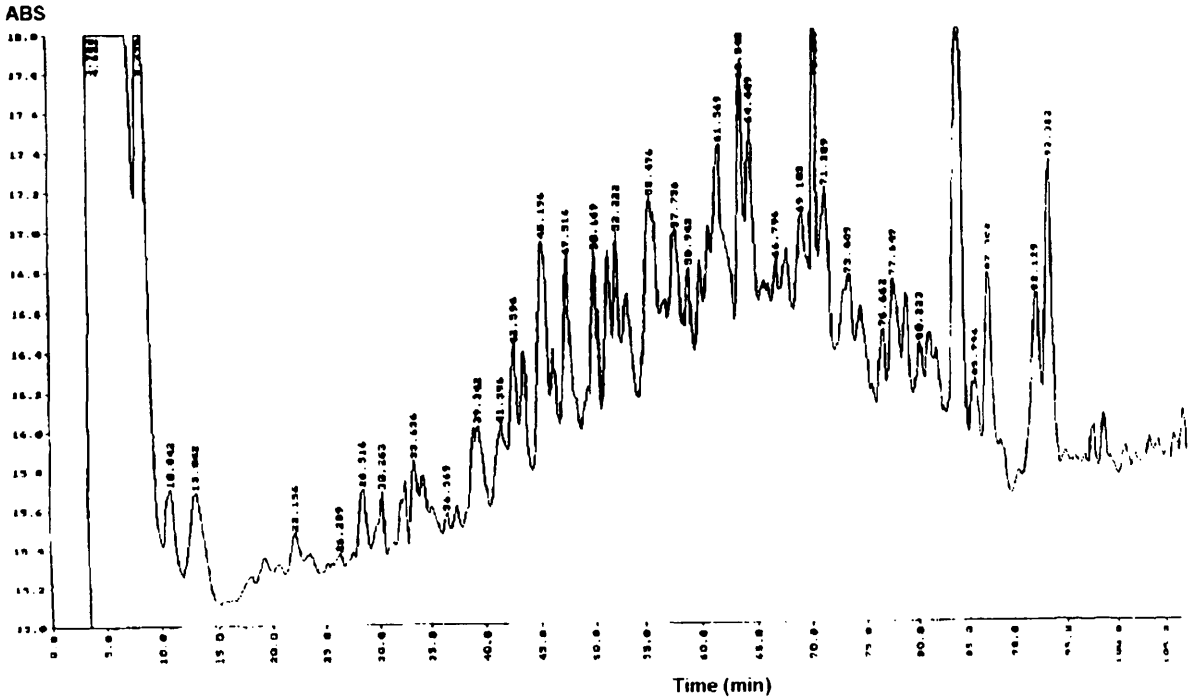

FIG. 1. HPLC signature of low molecular weight peptides eluted from HLA-I molecules purified from the β-cell line HP-62. The reverse-phase gradient was created using solution A of H_2O/0.1% trifluoroacetic acid (TFA) in combination with solution B of 80% acetonitrile/0.1% TFA at a flow rate of 0.200 ml/min. The low molecular weight fractions were reconstituted in solution A and applied to the column. One-minute fractions were collected from a linear 2-h gradient running from 2 to 80% solution B.

Immunoaffinity purification of class I molecules and elution of bound peptides. HLA-I molecules and bound peptides from 1×10^{10} HP-62 cells were purified by immunoprecipitation as previously described (11–14). Briefly, the cells were lysed in detergent buffer (10 mmol/l Tris HCl, pH 7.8, 150 mmol/l NaCl, 2 mmol/l EDTA, 1% vol/vol Nonidet P-40) containing 2 mmol/l PMSF, 20 μg/ml aprotinin, 1 μmol/l pepstatin A, 1 μmol/l leupeptin, 20 μg/ml TPCK, 20 μg/ml TLCK. The lysate was cleared by centrifugation followed by filtering (2 μm). The filtrate was first incubated with 10 ml ethanolamine-Sepharose-4B and then with 30 mg normal mouse IgG coupled to Sepharose-4B, for 2 h in each step. Immunoprecipitation was performed in the presence of 50 mg W6/32 bound to Sepharose-4B for 3 h. HLA-I molecules were eluted from mAb-Sepharose-4B in 0.1% aqueous TFA. The extract was lyophilized and then resuspended in 0.5 ml of 0.1% TFA. Protein concentration and purity were determined by BCA assay and SDS-PAGE, respectively. Approximately 221 μg of HLA-I protein was obtained. The eluted HLA-A, B, C molecules were incubated for 3 min at 100°C, then centrifuged through a 3000-Da MW cutoff ultrafiltration cartridge (Micron 3, Amicon, Danvers, MA). The low molecular weight fraction was stored at –70°C until use.

HPLC purification and sequencing of eluted peptides. The low molecular weight TFA eluates of HLA molecules were separated by reverse-phase HPLC using a C18 column (Vydac, 2.1 × 250 mm) and Waters Associates (Waltham, MA) equipment. The gradient was created using a solution of H_2O/0.1% TFA (solution A) in combination with a solution of 80% acetonitrile/0.1% TFA (solution B) at a flow rate of 0.200 ml/min. The low molecular weight fractions were reconstituted in solution A and applied to the column. One-minute fractions were collected from a linear 2-h gradient running from 2 to 80% solution B. From the absorbance trace at 215 nm of the eluate, the single fractions containing absorbance peaks were selected for microsequencing by N-terminal Edman degradation using a Hewlett-Packard G1000A protein sequencer (Hewlett-Packard, Palo Alto, CA).

RESULTS

Structure of HLA-I–bound peptides eluted from HP-62 cells. Low molecular weight material, eluted from HLA-I heterodimers, was released by acid-heat denaturation, purified by ultrafiltration, and fractionated by reverse-phase HPLC. The absorbance trace at 215 nm for the separation is shown in Fig. 1. To structurally characterize the HLA-I bound peptides of HP-62 cells, individual fractions were analyzed by sequential Edman degradation.

Although the material in the fractions was not homogeneous, we were able to identify, in many instances, the amino acid sequence of major peptide sequences present in each fraction. The major sequences were assignable on the basis of the picomolar yield of each phenolthiohydantoin (PTH) amino acid derivative in the cycle (Table 1). The assignment was corroborated by the repetitive yield of each sequence. The repetitive yields for all reported sequences ranged from 70 to 95%. Although sequences were assigned only when the yield of two or more PTH amino acid derivatives in a cycle differed by more than 35%, the possibility that some of the sequences are composites or contain errors cannot be excluded. In some fractions, it was not possible to assign major or minor sequences because of similar concentrations of differing peptides. Such fractions are indicated by "composite sequence" in Table 1. In certain instances PTH amino acid derivatives did not correspond to the PTH standards. Under these circumstances, the residue is indicated by X. The peptide sequences obtained generally conformed to the extended allele specific motifs published for HLA-A2 (15,16) or B18 (17), and the length of peptides in the pool isolated from the HLA-I molecules of HP-62 ranged from 8 to 11 residues.

Precursor proteins of MHC-I bound peptide. The sequences of major histocompatibility complex (MHC)-I–bound peptides, obtained from HP-62 cells, were compared with known sequences contained within the European Molecular Biology Laboratory (EMBL) and Swissprot databases. The database searches for peptide

TABLE 1
Peptide sequences from HLA class I of HP-62 cells

Fraction	Amino acid sequence											Homology (residues)
	–	2	–	–	–	–	–	8	9	10	–	
HLA-A2 group												
30	D	L	E	R	X	G	X	A	K	V	G	–
	W	R	A	N	A			L				
47	K	L	D	G	S	X	X	X	L	G		–
49	K	L	D	Q	G	V	R	X	A	X		–
51	V	L	K	G	Q	X	W	L	Y	A		–
54	N	L	R	D	K	L	R	P	L			Integrin VLA3 α chain
	G	F	D			D						(528–536)
60	X	L	A	E	R	S	X	X	X	I	X	–
61	D	L	D	E	N	Y	F	E	Y			–
70	K	L	A	P	X	X	X	K	Q	X		–
71	K	L	D	G	V	X	X	X	X	A		–
73	K	L	D	E	E	V	K	A	K			Glucose transporter 2 (249–257)
77	X	L	D	D	S	N	R	Y	G			–
78	G	L	D	D	L	X	V	M	K	L		–
83	A	L	Y	G	R	F	T	I	K	S		c-*src*
	V	R	D	S	Y		A					(434–443)
	S		N	P			W/N					
84	A	L	D	E	A	R	K	R	K	G	F	–
85	X	L	D	K	T	I	A	V	Y			–
		E	S	E	N		H		G			
87	Y	L	T	A	E	X	X	X	K	S		–
92	X	L	S	A	S	P	X	K	S			–
HLA-B18 group												
35	X	E	N	S	A	F	L	V	K	X	Y	–
		S	Q	L	E	N		G	A		F	
43	D	E	K	E	K	L	Q	L	V			Colligin (Hsp 47) (247–255)
44	K	E	K	X	S	N	X	S	X	S		–
48	K	E	V	S	D	L	E	R	S	K		–
56	K	E	K	E	R	N	Y	X	K	A	E	–
64	X	E	D	D	H	N	W	N	K			–
65	X	E	D	E	R	P	N	X	X	K		–
79	G	E	D	G	R	V	Y	V				PGI-PLD
				D	M	I		S				(128–135)
	D			E								
90	X	E	Y	A	R	K	X	T				5-HT 1F receptor (142–150)
Unassigned group												
36	K	P	G	X	S	G	X	S	G	S		–
	M					A						
	V											
28	X	F	Y	G	A	S	X	X	A	S		–
		R	S		E		W	E	K	E		
29	I	F	R	W	E	V	Q	F	A	S	F	–
		E		W	Y			S				
38	composite sequence											–
46	G	A	R	G	K	D	V	X	K			–
50	X	S	I	R	K	I	Q	X	X	K		–
53	composite sequence											–
74	composite sequence											–
82	composite sequence											–
98	S	R	G	S	A	Q	G	Y	X	A		–
100	composite sequence											–

Standard IUPAC amino acid abbreviations are used. X denotes an unidentifiable PTH-amino acid derivative. In some cycles, more than one PTH amino acid derivative was observed. In these instances, the derivatives are listed in decreasing order of yield.

sequence homology to known protein precursors revealed that six of the obtained sequences showed homology to known precursor proteins.

The peptide KLDEEVKAK from fraction 73 matched to the glucose transporter GLUT2 (residues 249–257) (18). Fraction 79 contained a peptide GEDGRVYV, corresponding to residues 128–135 of phosphatidylinositol-glycan-specific phospholipase D (GPI-PLD) (19). The peptide VEYARKXTP (fraction 90) matched at eight of nine residues to the 5-hydroxytryptamine (5-HT)-1F receptor (residues 142–150) (20).

The peptide ALYGRFTIKS from fraction 83 corresponded to the c-*src* proto-oncogene at residues 434–443 (21). In the sequences of peptides isolated from fractions 43 and 54, two additional matches were found. Fraction 43 contained the peptide DEKEKLQLV, which matched to residues 247–255 of the human collagen-binding protein colligin, a 47-kDa heat shock protein precursor (22). Fraction 54 contained a peptide NLRDKLRPL corresponding at eight out of nine residues to the VLA-3 α-chain (residues 528–536), a member of the integrin family of proteins (23).

DISCUSSION

The interaction between MHC, bound peptide, and specific TCR initiates nearly all adaptive immune responses. The association of specific MHC genes with susceptibility to IDDM suggests that similar processes are responsible for the development of this tissue-specific autoimmune disorder. Based on the postulated tissue specificity of MHC bound peptides, as first demonstrated by Marrack and Kappler (24), it has been proposed that IDDM is the result of the aberrant expression of MHC-bound, tissue-specific peptides by islet cells. Given that $CD8^+$ T-cells are believed to contribute to the destruction of islet cell tissue, we have partially characterized the repertoire of HLA-I bound peptides of a human β-cell line in an attempt to identify possible tissue-specific peptides that may be recognized by T-cells. The data presented identify six peptides, of which three are derived from protein precursors that have a limited, tissue-specific expression, and may be among the MHC-bound self antigens that are recognized by T-cells in IDDM.

The peptide KLDEEVKAK (fraction 73) matched with 100% homology to residues 249–257 of GLUT2 (18). GLUT2 is a facilitative glucose transporter with tissue specificity for the pancreatic islet β-cells, hepatocytes, and the basolateral membranes of small intestinal and renal epithelial cells. In the β-cells, GLUT2 is thought to comprise part of the glucose-sensing mechanism involved in the regulation of insulin secretion (25). This is the first demonstration of a tissue-specific peptide presented by the HLA-I molecules of human β–islet cells. GLUT protein expression has been shown to be altered in cell lines and by malignant transformation (26). However, the processing and presentation of GLUT2 peptide by HP-62 is unlikely to be merely a consequence of β-cell transformation. Additional evidence that this protein may function in the autoimmunity of IDDM is the presence of autoantibodies to GLUT2. In patients with IDDM of recent onset, 77% were positive for anti-GLUT2 IgG antibodies, whereas 94% of nondiabetic subjects were negative (27).

The peptide GEDGRVYV (fraction 79) corresponded with 100% homology to residues 128–135 of GPI-PLD (19). GPI-PLD, abundant in serum, is a GPI degrading enzyme whose principal tissue source and physiological function remain undefined. Bovine GPI-PLD differs from the human GPI-PLD at position 1 (E for G) of the peptide, excluding the culture medium as the source of the precursor protein. Expression of GPI-PLD has been observed in β–islet cells, brain, and myeloid cell lines, but is absent in numerous other cell types (28,29). GPI-PLD is localized mainly in vesicles, endoplasmic reticula, and Golgi and may thus be able to enter the MHC-I processing pathway.

Peptide VEYARKXTP (fraction 90) matched at eight of nine residues to the 5-HT-1F receptor (residues 142–150) (20). Position 7 (argenine) could not be identified during amino acid sequencing. 5-HT-1F receptor RNA has been detected in brain, and secretion of serotonin by islet cells suggests the possibility of 5-HT receptors in this locality (30,31).

In light of a neuroendocrine origin postulated for β-cells (32), the localization of GPI-PLD and possibly the 5-HT-1F receptor to both the pancreatic β-cell and central nervous system (CNS) is intriguing. The CNS is immunologically privileged, although cross-reactivity between β-cells and neurons to autoantibodies directed against glutamate decarboxylase have been shown in stiff-man syndrome (33).

In these studies, we were also able to characterize three other HLA-I–bound self-peptides whose protein precursors are widely distributed. Fraction 43 contained a peptide corresponding to residues 247–255 of the human collagen-binding protein colligin, a 47-kDa heat shock protein precursor (22). Heat shock proteins have been implicated in the pathogenesis of autoimmune diseases, but any possible role of colligin in IDDM remains to be determined. A second peptide was derived from the c-*src* proto-oncogene (residues 434–443) (21). Lastly, a peptide found in fraction 54, NLRDKLRPL, showed partial homology to residues 528–536 of the VLA-3 α-chain protein (34). The reported sequence of the VLA-3 α-chain (residues 528–536) is NLRDKLRPI and is mismatched at position 9 (isoleucine for leucine) from the HP-62 peptide. This mismatch may represent a point mutation. VLA-3 is a receptor for fibronectin, laminin, and collagen and part of a superfamily of molecules involved in adhesion and embryogenesis. Normally VLA-3 expression is limited to kidney glomeruli and the basal cells of epidermis and other epithelia. Aberrant expression of VLA-3, however, occurs in many cell lines and a variety of neoplasms (35,36).

We are currently studying the immunogenicity of the GLUT2-, GPI-PLD–, and 5-HT-1F–derived peptides using T-cells obtained from normal subjects and individuals with IDDM. Determination of the frequency of T-cell precursors reactive with these self-antigens should allow us to begin to define their role in the pathogenesis of IDDM.

ACKNOWLEDGMENTS

This work was supported by grants from the American Diabetes Association and Juvenile Diabetes Foundation.

We thank Dr. Marta Vives-Pi for her critical discussion of the present work.

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