Doctor′s Thesis

Systematic Analysis of Diverse Epitopes Recognized by TCR;
Identification of Mimicry Epitopes of CD4$^+$ T Cell Clones
Autoreactive to 65kDa Glutamic Acid Decarboxylase

（自己反応性 CD4 陽性 T 細胞クローンが認識するエピトープの多様性の解析；
グルタミン酸脱炭酸酵素自己反応性 T 細胞クローンを活性化する
微生物抗原の同定）

植 村　靖 史
Uemura Yasushi

指導教官

西村　泰治　教授

熊本大学大学院医学研究科免疫識別学

2003 年 3 月

Summary

Activation of autoreactive $CD4^+$ T cells is a crucial step in the development of T cell mediated autoimmunity and crossreactivity between microbial and self antigens, a phenomenon known as molecular mimicry, is one of the mechanisms that account for the link between infection and autoimmunity. Accumulating evidence indicates that recognition by TCRs is far more degenerate than formerly presumed. Therefore, elucidating the recognition nature of TCRs has a great significance for revelation of the disease process. For this purpose, we developed a new expression cloning system to identify $CD4^+$ T cell epitopes using an epitope presenting vector, pCI, a derivative of a human invariant chain (Ii) expression vector, in which the class II associated invariant chain peptide (CLIP, Ii p89-101) could be substituted with antigenic peptides. We inserted double-stranded oligo DNAs of randomized sequences into this vector and prepared an epitope-presenting library which loads randomized 13-mer peptides onto HLA class II molecules co-expressed in COS-7 cells. Utilizing this library, we isolated a cross-reactive epitope recognized by a glutamic acid decarboxylase (GAD) 65-autoreactive T cell clone established from a patient with type I diabetes. Although the newly identified epitope was far different from the original epitope, GAD65 p116-128, it did have the capacity to stimulate the T cell clone comparable to that of the original GAD epitope. Furthermore, we generated a DNA-based randomized epitope library based on the original GAD epitope using CLIP-substituted invariant chains and analyzed cross-recognition profiles of two GAD65-autoreactive $CD4^+$ T cell clones, established from independent type I diabetes patients. The epitope library was composed of seven sublibraries, in which three successive residues within the epitope were randomized simultaneously. Analysis of agonistic epitopes indicate that recognition by both TCR was significantly affected by combinations of amino acids in the antigenic peptide, although the degree of combinatorial effect differed between the two TCRs. Protein database searching based on the TCR recognition profile proved successful in identifying several microbial and self-protein-derived mimicry epitopes. Some of the identified mimicry epitopes were actually produced from recombinant microbial proteins by APCs to stimulate T cell clones. The epitope expression library system using CLIP-substituted Ii-genes provide an entirely new strategy not only for identification of cross-reactive epitopes for $CD4^+$ T cells of known specificity, but also for detection of epitopes stimulatory for $CD4^+$ T cells the epitopes of which are unknown. Importantly in analyzing molecular mimicry, this system deciphers overall conformations within epitopes on TCR recognition and also holds important prospects for providing a greater understanding of recognition mode in HLA-class II restricted TCR.

Publication list

Yasushi Uemura, Satoru Senju, Katsumi Maenaka, Leo Kei Iwai, Shinji Fujii, Hiroki Tabata, Hirotake Tsukamoto, Shinya Hirata**,** Yu-Zhen Chen, and Yasuharu Nishimura.
Systematic Analysis of the Combinatorial Nature of Epitopes Recognized by TCR Leads to Identification of Mimicry Epitopes for Glutamic Acid Decarboxylase 65-Specific TCRs. ***The Journal of Immunology, 170, 947-960, 2003***

Yasushi Uemura, Satoru Senju, Shinji Fujii, Leo Kei Iwai, Katsumi Maenaka, Hiroki Tabata, Takayuki Kanai, Yu-Zhen Chen, and Yasuharu Nishimura.
Specificity, Degeneracy, and Molecular Mimicry in Antigen Recognition by HLA-Class II Restricted T Cell Receptors; Implications for Clinical Medicine. ***Modern Rheumatology, in press.***

Satoru Senju, Shinya Hirata, Hidetake Matsuyoshi, Masako Masuda, Yasushi Uemura,
Kimi Araki, Ken-ichi Yamamura, and Yasuharu Nishimura.
Generation and genetic modification of dendritic cells derived from mouse embrionic stem cells. ***Blood, in press.***

Shinji Fujii*, Yasushi Uemura*, Leo Kei Iwai, Masayuki Ando, Satoru Senju, and Yasuharu Nishimura. (*equal contribution)
Establishment of an expression cloning system for $CD4^+$ T cell epitopes. ***Biochemical and Biophysical Research Communications 284, 1140-1147, 2001***

Yasushi Uemura, Yasuharu Nishimura.
Analysis of diverse TCR ligands recognized by autoreactive $CD4^+$ T cells using CLIP-substituted Ii gene library. ***Clinical Immunology 37 (2); 156-164, 2002 (Review in Japanese)***

Acknowledgments

These series of investigations were performed from 1998 to 2002, in the Division of Immunogenetics, Department of Neuroscience and Immunology, Kumamoto University Graduate School of Medical Sciences.

I wish to extend my warmest thanks to Professor Yasuharu Nishimura, professor of the department of the Division of Immunogenetics, Kumamoto University School of Medicine. He introduced me to this field and generously gave me advice and suggestions. I would also like to express my gratitude for his spending valuable time to weekly discussion and correcting my papers.

Likewise, I am grateful to Dr. Satoru Senju, Dr. Atsushi Irie, Dr. Tetsuya Nakatsura, and Dr. Yu-Zhen Chen, Division of Immunogenetics, Kumamoto University School of Medicine, and Dr. Shinji Fujii, the First Department of Internal Medicine, Kumamoto University School of Medicine, who provided me valuable time and advices.

I am grateful to Dr. Leo Kei Iwai, Laboratory of Immunology, Heart Institute (Incor), University of Sao Paulo Medical School, Brazil, whom it was a pleasure working with, and who generously gave me valuable time and advices during his stay in our laboratory as a research fellow supported by JICA.

I am grateful to Professor Sho Matsushita, Division of Immunology, Saitama Medical School, Dr. Katsumi Maenaka, Division of Structural Biology, Medical Institute of Bioregulation, Kyushu University and Dr. Naomi Sato, Division of Oral Microbiology, Niigata University School of Dentistry for helpful suggestions, and to Dr. Takaaki Akaike, and Dr. Teruo Akuta, Division of Microbiology, Kumamoto University School of Medicine for the *Legionella pneumophila* gene.

Finally, I would like to dedicate this work to; my wife, Yuki and my child, Sena. Especially, my wife, who always listened and was a constant source of moral support. She has helped to synthesize peptides used in these series of investigations and read all my manuscripts offering her considerable editorial and organizational skills. As well as my parents; my mother, who continuous encouragement throughout my education is deeply appreciated, and to my father, who supported me for my practical career.

Abbreviations

APC; antigen-presenting cell
cDNA; complementary DNA
CDR; complementary determining region
CLIP; class II-associated Ii chain peptide
DC; dendritic cell
DMEM; Dulbecco's Modified Eagle Medium
DNA; deoxyribonucleic acid
ELISA; enzyme-linked immunosorbent assay
FCS; fetal calf serum
GAD; glutamic acid decarboxylase
GM-CSF; granulocyte-macrophage colony stimulating factor
HLA; human histocompatibility leukocyte antigens
IFN; interferon
Ii; class II-associated invariant chain
IL; interleukin
MHC; major histocompatibility complex
mRNA; messenger RNA
PBMC; peripheral blood mononuclear cell
PS-SCLs; positional scanning synthetic combinatorial peptide libraries
RT-PCR; reverse transcriptase-polymerase chain reaction
TCR; T cell receptor

Introduction

1. Antigen-processing pathways for the MHC class II-restricted antigen presentation

The class II major histocompatibility complex (MHC) molecules, expressed in antigen presenting cells (APCs), present peptides originating from exogenous proteins, membrane proteins or intravesicular pathogens that enter the cell through the endocytic route to $CD4^+$ T cells (1). APCs include the dendritic cells initiating priming response of naive T cells, macrophages specialized to taken up particulate materials, and B cells that efficiently internalize specific antigen by receptor-mediated endocytosis of the antigen bound to their surface immunoglobulin (2, 3). As shown in Fig. 1, proteins incorporated into cells through endocytosis become enclosed in endosome, which become increasingly acidic as they progress into the interior of the cell, eventually fusing with lysosomes. The endosomes and lysosomes contain proteases, known as acid proteases, which are activated at low pH and eventually degrade the protein antigens contained in the vesicles. These acid proteases include the cysteine proteases cathepsins; B, D, and E, the last of which is the most active enzyme in this family. The biosynthetic pathway for MHC class II molecules starts with their translocation into the endoplasmic reticulum, and they must therefore be prevented from binding prematurely to peptides transported into the endoplasmic reticulum lumen or to the cell's own newly synthesized polypeptides. The binding is prevented by the assembly of newly synthesized MHC class II molecules with a protein known as the MHC class II-associated invariant chain (Ii) (4-7). The invariant chain have another function, a delivery of the MHC class II molecules to a low-pH endosomal compartment where peptide loading onto MHC class II molecules can occur. In such vesicles, proteases including cathepsins S and L cleave the invariant chain, leaving the CLIP peptide bound to the MHC class II molecules. Pathogens and their proteins are cleaved down into peptides within acidified endocytic vesicles, but these peptides cannot bind to MHC class II molecules that are occupied by CLIP. The class II like molecule, HLA-DM, binds to MHC class II/CLIP complexes, catalyzing the release of CLIP and the binding of antigenic peptides. HLA-DO is produced in thymic epitherial cells and B cells and acts as a negative regulator of HLA-DM. HLA-DO interacts with HLA-DM to inhibit both the HLA-DM-catalyzed release of CLIP from, and the binding of other peptide to, MHC class II molecules.

2. Structure of TCR-peptide/HLA-class II complex

The HLA-class II molecules are heterodimeric membrane glycoproteins consisting of α and β chains. DRα chains is monomorphic, in contrast DPα and DQα chains and β chains are highly polymorphic. The molecule has a peptide-binding groove on top of the molecule and binds antigenic peptides processed by antigen presenting cells (APC) such as dendritic cells (DCs) or B cells. Structural requirements for HLA-class II binding peptides have been analyzed in detail and peptide binding motifs specific for various human and mouse class II molecules have been reported (8). Three to five amino acid residues separated from each other by one to two intervening residue(s) acted as anchor residue(s) for binding to HLA-class II molecules (Fig. 2) (9-11). On the other hand, side chains of amino acid residues flanking anchor residues were the main recognition sites for TCR. This view was clearly established in crystallographic analyses of the DR molecules bound by either self- (4) or non-self (12) peptides. Sixty-five percent of the peptide surface made contact with the DR molecule and the remaining portion was accessible to solvents, thus being recognized by the TCR. Most pockets in the groove of the HLA-class II molecules are shaped by clusters of polymorphic residues, indicating the class II allelic variant has a major effect on differences in structures of peptides bound, and which determines individual difference in T cell responses to a given antigenic peptide (13).

TCR is composed of two membrane-anchored polypeptides, α and β chains, and each chain consists of one constant (C) and one variable (V) domain. The TCR Vα or Vβ regions are composed of V-Jα or V-D-Jβ productively rearranged gene products (14). The complementary determining regions (CDRs) are hypervariable loops at one end of the TCR that recognize the HLA molecule and the antigenic surface derived from the solvent accessible side chains of amino acid residues flanking HLA anchoring residues. The recent crystallographic studies of TCR–peptide/MHC

FIGURE 1

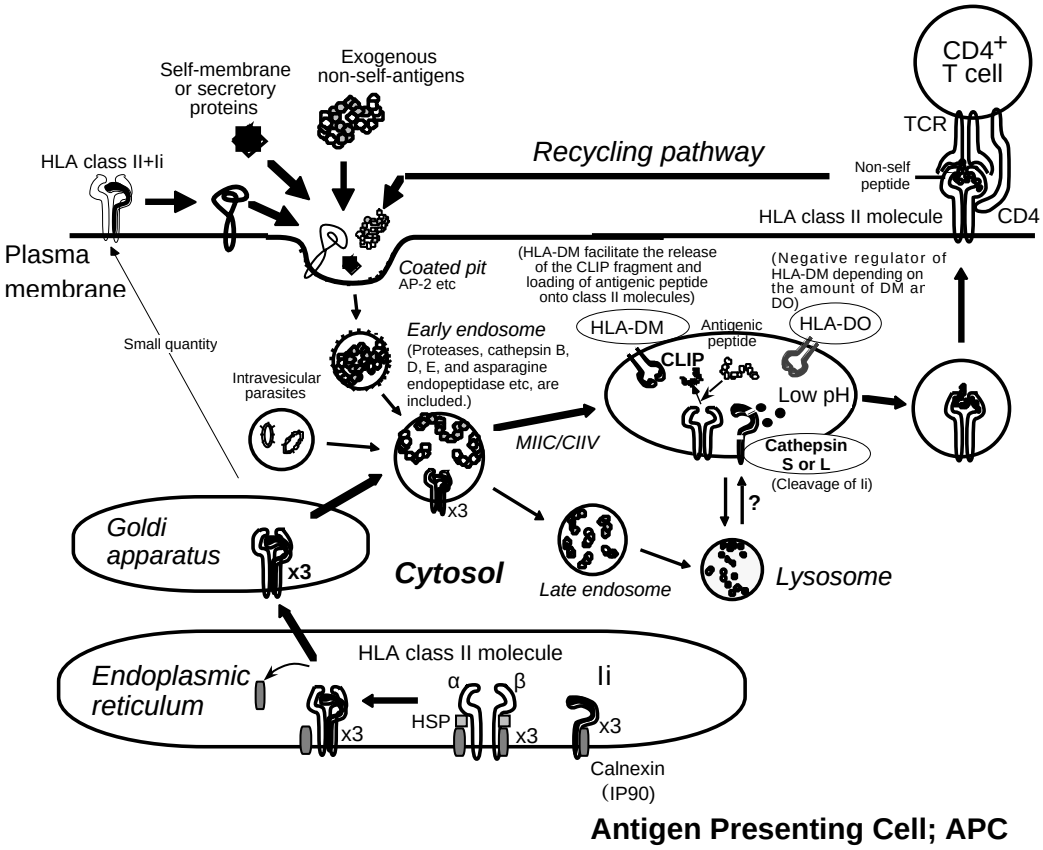


FIGURE 1.
Antigen-processing pathways for the MHC class II-restricted antigen presentation.

FIGURE 2

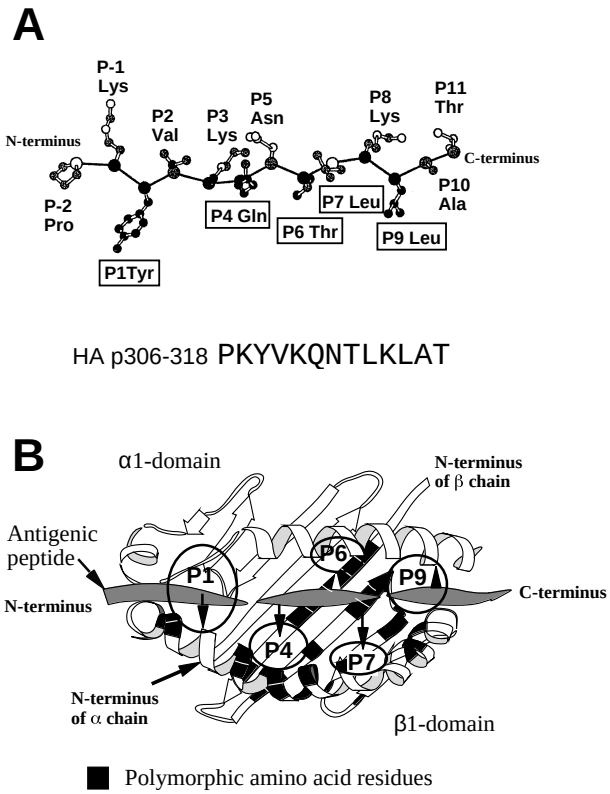


FIGURE 2.

Structure of a peptide-binding groove of a HLA-class II molecule and bound peptide. (ref. 12)

A. Conformation of HLA-DR1 bound HA p306-318 peptide. The HA peptide is bound to HLA-DR1 as a straight extended strand with the peptide N and C termini projecting out of the ends of the binding site. The peptide ha pronounced twist, and successive side chains project from the peptide backbone about every 130 degrees. Pepti atoms are shaded by degree of interaction with HLA-DR1: black, atoms completely buried in HLA-DR1/HA peptide complex; grey, atoms that contact HLA-DR1 and are also solvent-accessible in the complex; white, ator that do not contact HLA-DR1. Boxed residues indicate the HLA-DR anchoring residues.

B. Top view of the peptide-binding groove of a HLA-DR1 molecule. The top α1 and β1 domains create groove-like structure consisting of a β-sheet floor and two side walls made of two antiparallel α-helices. There are five pocket like structures indicated by circles in the groove and the side-chains of P1 (the most N-terminal 1st D anchor position), P4, P6, P7 and P9 anchor residues of the binding peptide are accommodated in each pock shown in this figure. Black colored residues are polymorphic in HLA-DR molecules.

complexes provide a structural basis for antigen recognition by αβTCRs (Fig. 3) (15). All αβTCRs represent a relatively flat surface, bound to the peptide/MHC complex, and represent a similar binding mode. The angle between peptide direction and the long axis of the class I-restricted TCR interface is between the 45~70 degrees (diagonal mode) (16). In contrast the class II-restricted TCR interface is between the 70~80 degrees (orthogonal mode) (Fig. 4) (17). Fig. 3 shows a model of a recent crystallographic structure of the HA1.7 TCR-HA p306-318/DR1 complex (18). The CDR1 loops of TCR V-regions contact both peptide and MHC molecules. On the other hand the CDR2 loops contact prominent α-helices of the MHC molecule. The long CDR3 loops of TCR V-regions extend down over the center of the antigenic peptide. TCR contacts span only nine residues (P-1~P8) of the antigenic peptide in both in human and murine TCR-peptide/MHC II complexes.

3. Structure and function of invariant chain

The invariant chain (Ii), a type II integral membrane glycoprotein, play a critical role in MHC class II-restricted antigen presentation pathway by stabilizing peptide-free class II αβ heterodimers in a nonameric $(\alpha\beta\ Ii)_3$ complex (Fig. 5) (5) and has the following two important functions with regard to antigen presentation by MHC class II molecules: 1) Ii associates with newly synthesized MHC class II α and β dimers in the endoplasmic reticulum to form a complex and directly prevents peptides in the endoplasmic reticulum from binding to MHC class II molecules, using a domain termed class II-associated Ii peptide (CLIP) that occupies the peptide-binding groove of class II αβ dimers (Fig. 5), 2) This αβ-Ii complex is composed of one Ii trimer and three αβ dimers and transported via the Golgi apparatus to the endosomal pathway where peptide loading of class II takes place, by the targeting signal; di-leucine motif [(D/E)XXX(L/M)(L/I/V)] (19, 20), in the cytoplasmic domain of Ii. In this pathway, Ii is proteolytically degraded from the lumental C-terminus and the class II αβ-CLIP complex is generated. In specialized endocytic organelles enriched in MHC class II molecules and designated MHC class II compartments (MIICs) or class II vesicles (CIIVs) (Fig. 1) (21-23), HLA-DM catalyzes the dissociation of CLIP from class II αβ dimer and binding of other peptides (24, 25). The CLIP region of Ii, roughly residues 81-104, is one of two segments shown to interact with class II HLA-DR molecules. The other segment, Ii 118-216 is C-terminal to CLIP, mediates trimerization of the ectodomain of Ii and interferes with DM/class II binding.

4. MHC class II-associated-susceptibility to autoimmunity triggered by infection

Particular class-II human histocompatibility leukocyte antigen (HLA-class II) alleles are associated with susceptibility to particular autoimmune diseases (26). $CD4^+$ T cells recognize 10-20 amino acid-long peptides in the context of class II molecules expressed on antigen-presenting cells through their T cell receptor (TCR). Autoreactive $CD4^+$ T cells are considered to have a central role in development of autoimmune diseases. Even in the presence of exogenous non-self antigens, the majority of HLA-class II molecules bind self-peptides processed mainly from self-membrane or secretory proteins. If the density of self-peptides/HLA-class II complexes expressed on the surface of cells is large enough to ensure high avidity engagement of TCR, most autoreactive $CD4^+$ T cells are deleted in the thymus or become anergic in the periphery. If the density of self-peptide/HLA-class II complexes is small enough not to activate T cells in the periphery, T cells do not need to acquire tolerance to such complexes and ignore them (27). Thereby, $CD4^+$ T cells do not respond to these self-peptides in the context of self HLA-class II molecules, except in autoimmune states.

Epidemiological studies indicated that a number of autoimmune diseases are either developed or are exacerbated after infections. Several possible mechanisms can account for the clinical association between microbial infection and clonal expansion of autoreactive T cells as a cause of autoimmune disease. One mechanism is activation and expansion of autoreactive T cells by antigen nonspecific inflammatory stimulus of the innate immune system (28).

FIGURE 3

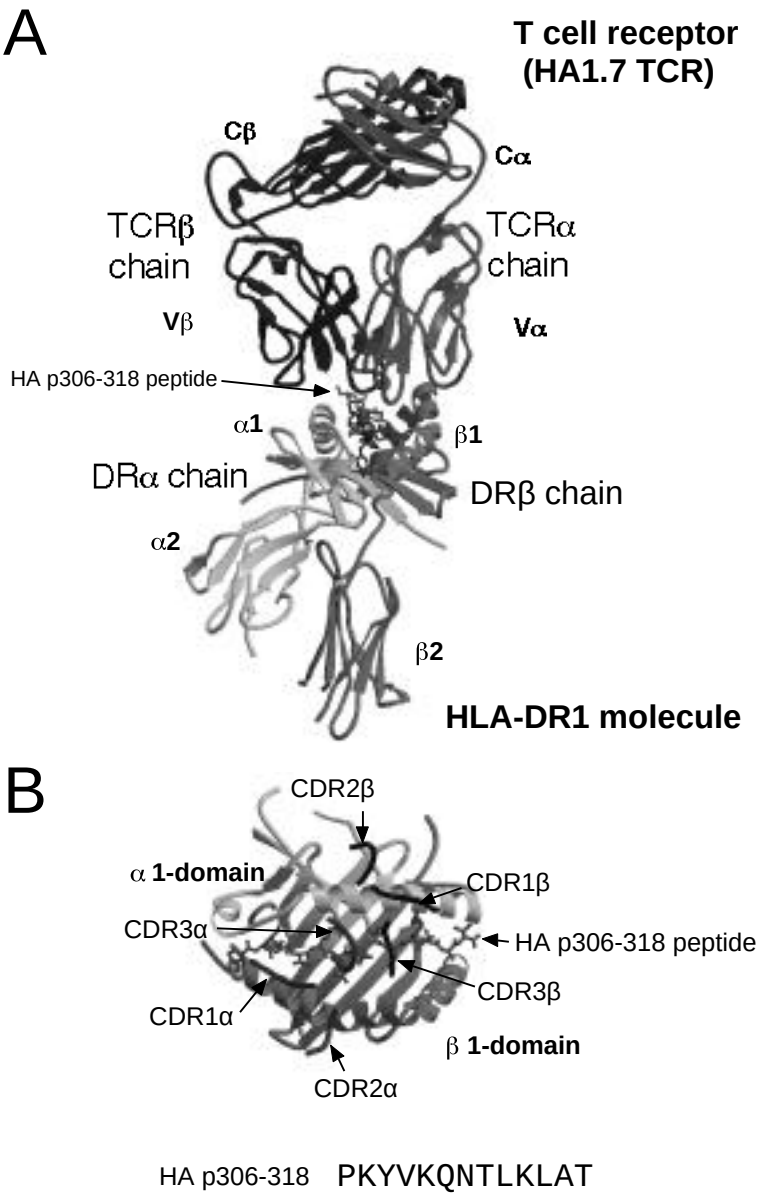


FIGURE 3. Structure of the HA1.7 TCR-HA/DR1 complex.

A. Structure of the HA1.7 TCR-HA/DR1 complex (18) with TCR at the top and DR1 at the bottom. The T cell clone HA1.7 is specific to HA p306-318 in the context of HLA-DR1. α1, α2, β1 and β2 indicate the extracellular domains of α and β chains of HLA class II molecules. Vα, Vβ, Cα and Cβ indicate the variable and constant-regions of T cell recepto α or β chains respectively.

B. The structure of HA p306-3018/HLA-DR1 complex and relative orientation of the CDR loops of HA1.7 TCR on top of HA/DR1 complex. CDR loops of TCR Vα and Vβ chains are displayed in tubes, respectively. HA p306-318 peptide shown in a ball-and-stick model. The top of α1 and β1 domains create a groove-like structure consisted of aβ-sheet floor and two side walls made of two anti-pararelα-helices. The peptide sequences of HA p306-318 are given in single lett amino acid code. This figure was produced by BOBSCRIPT (119).

FIGURE 4

FIGURE 4. Comparison of docking modes of TCRs on pMHC I versus p
ligands. This figure is cited from ref.17.
A. Stereo view of scD10 TCR (Vα Vβ) on Conalbumin/I-A^k. The MHC II molecule I-A^k is shown in molecular surface representation with the α chain in light green and the β chain in orange. The peptide is drawn in ball-and-stick format with NH_2- and COOH-termini at th left and right, respectively, in this view. The TCR V domains are shown as worm diagram, with Vα in green and Vβ in blue. The angle between peptide direction a the long axis of the TCR interface is 80 degrees, very close to the right angle. (orth mode)
B. Stereo view of 2C TCR (Vα Vβ) on dEV8/H-2K^b. The MHC I molecule, H-2K^b, is shown in yellow in molecular surface representation. The peptide is drawn in ball-and-stic format with NH_2- and COOH-termini oriented as in ***A***). (The TCR molecule is show brown as a backbone worm diagram. The angle between peptide direction and the long axi of the TCR interface is 45 degrees. (diagonal mode)

FIGURE 5

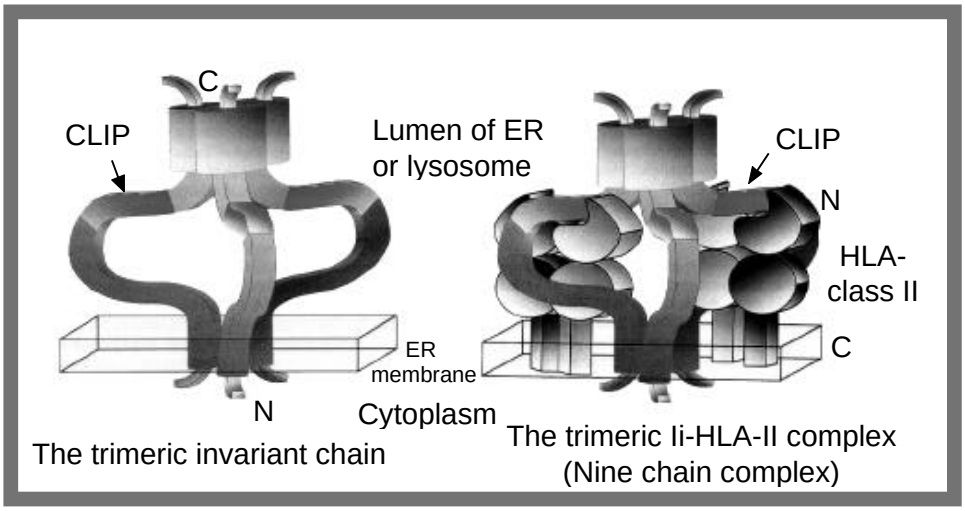


FIGURE 5.

Model of the trimeric invariant chain bound to MHC class II α:β heterodimers.

In the ER, the MHC class II α and β heterodimers associate with an invariant chain (Ii), to assemble into nonameric complexes comprising three α-, three β-, and three Ii-chain subunits. A lumenal domain of Ii, known as C interacts with peptide-binding groove of MHC class II, rending the groove inaccessible to peptides present in ER.

Microorganisms-induced activation of APCs leads release of inflammatory cytokine and/or chemokine, enhancing antigen-processing, up-regulation of peptide-MHC complexes and costimulatory molecules on the cell surface (microbial adjuvant effects) (29-33). Local infection causes tissue destruction and the release of sequestered antigen follows (34-35). The inflammatory state can also promote the expansion of memory T cell populations (by stander activation) (28, 36). Another mechanism is that microbial superantigens activate large numbers of T cells expressing particular Vβ gene segments, and a limited population can crossreact to a self-antigen (37-39). Cross-recognition by T cells between self antigens and infectious agents is other important mechanism (molecular mimicry theory) (40, 41). In any case, the tissue destruction would be expanded by epitope spreading. These concepts are useful to explain unsolved mechanisms for the etiological linkage between infection and autoimmunity.

5. Molecular mimicry and autoimmunity

One of the mechanisms that account for the break down in immune tolerance is cross-recognition by TCR between a pathogen-derived antigen and a host antigen (molecular mimicry theory) (40, 41). The concept of molecular mimicry was broadened by recent insights into T cell recognition. Molecular mimicry phenomena were noted in disease-associated autoreactive $CD4^+$ T cells in autoimmune disorders such as multiple scrlerosis (MS), Type I diabetes, and Lyme arthritis in humans (42-44). Lyme disease, a chronic inflammatory joint disease, is caused by infection with spirochete *Borrelia burgdorferi* (44, 45). Susceptibility to Lyme arthritis is associated with HLA-DR4 and HLA-DR1 alleles. It was reported that synovial fluid T cells from patients with treatment-resistant Lyme disease showed a strong response to outer surface proteins A (OspA) of *B. burgdorferi* and crossreacted to self LFA-1. The sequence homology between OspA p165-173 and αL-chain of hLFA-1 p332-340 was considered to be a basis for molecular mimicry in treatment-resistant Lyme disease (45). On the other hand, the association between microbial infection and autoimmune disease has been also investigated using a mice model (46-49). In a model of herpes simplex virus (HSV) type-I induced herpes stromal keratitis (HSK), a T cell mediated inflammatory disease of cornea, corneal antigen autoreactive T cells recognized HSV-1 UL-6 protein (46). However, an HSV-1 point mutant that contains a single amino acid exchange within the putative mimicry epitope impaired its capacity to induce HSK (47). In this system, two different pathogenic pathways, innate immune mechanisms and molecular mimicry, are involved. In that report, mimicry was considered to be essential for disease induction with a limited number of autoreactive T cells, while innate immune mechanisms are also important to provoke disease with high numbers of autoreactive T cells.

6. Degeneracy of antigen recognition by T cell receptor

For many years, antigen recognition by TCR was considered to be highly specific and the concept of molecular mimicry had been defined based on the level of primary sequence similarities between self and antigenic determinants of infectious microorganisms (50). Since the 1990s, studies using peptide analogues with single amino acid substitution (Fig. 6) (51) or positional scanning synthetic combinatorial peptide libraries (PS-SCLs) (Fig. 7) (52) demonstrated that antigen recognition by TCR is highly degenerate and many different peptides can activate an individual T cell. Wucherpfenning et al. reported that microbial peptides with a relatively limited sequence homology to myelin basic protein (MBP) could activate MBP autoreactive T cell clones (42). Using positional scanning synthetic combinatorial peptide libraries (PS-SCLs), Hemmer et al. noted differing recognition profiles of individual autoreactive T cell clones from patients with multiple sclerosis (MS) and predicted stimulatory ligands that showed no sequence homology with the known cognate peptide (53). Therefore, molecular mimicry may be a more frequent event than was generally assumed.

FIGURE 6

FIGURE 6. Application of single residue substituted peptide analogues for analysi of T cell epitopes.

This figure is cited from ref.51.

A. Substitutional analysis of myelin basic protein (MBP) Ac1-11 (AcASQKRPSQRSK). In this approach, each position of the epitope was substituted by all 20 naturally occurri amino acids. The amino acids allowed at each individual position of the epitope are to establish TCR recognition motif. I-A^u-restricted MBP Ac1-11 specific T cells tested for proliferation at a peptide concentration of 1μM. Stimulation index are shown i the figure. Dark boxes indicate SI > 15. Values in the top line represent the wild-type (w peptide; all other values correspond to single substitution analogues.

B. The defined motif resulting from the substitution analysis, i.e., the amino acids allowe at each individual position of the 11-mer epitope. Bracketed residues indicate the allov substitutions; X = all amino acids; braced residues represent all amino acids except in the brackets. The defined motifs were searched for protein database in pa search.

FIGURE 7

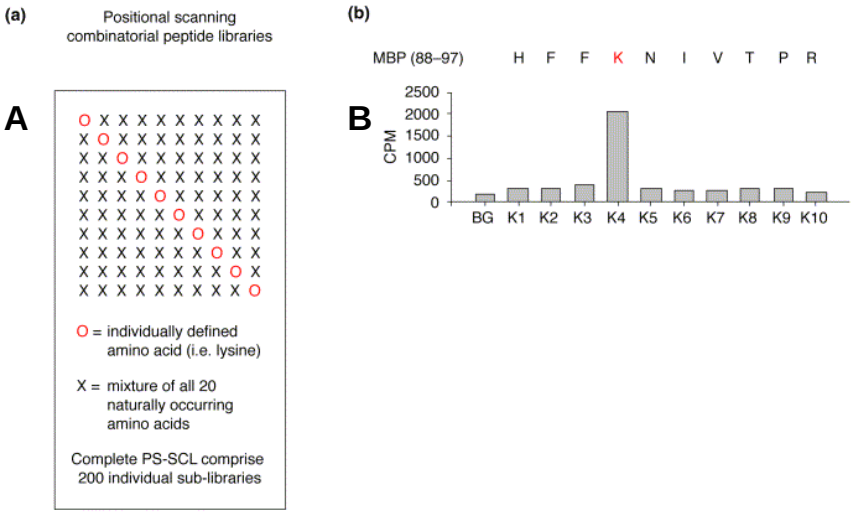


FIGURE 7. Application of positional scanning synthetic combinatorial peptide libraries (PS-SCLs) to the analysis of degeneracy in T-cell recognition of antigen. This figure is cited from ref.52.

A. The library was made up of 10 positional peptide libraries having N-termin and C-terminal amide groups. Each positional library was composed of 20 peptide mixtures, in which a single position is defined with one of the 20 natural L-amin (represented as O), and the remaining nine positions of the 10-residue sequen composed of mixtures (represented as X) of 19 amino acids (cystein omitted). Each positional library contains the same diversity of peptide sequences, the 10 positional libraries differ only in the location of their defined position. Each peptide mixture is made up of approximately 3 x 10^{11} (19^9) individual sequences for a library composed of approximately 6.4 x 10^{12} decapeptides. The advantage of the positional scanning metho is that it enables the identification of the relative importance of each amino acid for eve position of the peptide, thus providing an unbiased approach in comparison using individual substitution analogues.

B. The proliferative response of T-cell clones to the different positional libraries [sho as counts per minute (CPM)] is used to define the optimal amino acids for each posit For the GP3G6 T cell clone specific for myelin basic protein (MBP) peptide 88– lysine PS-SCL gives a positive response for position 4, matching the lysine in th sequence (displayed above the graph).

7. Differences in physiological outcomes of T cell responses stimulated by altered peptide ligands

Altered peptide ligands (APLs) represent a useful tool for studying the differential recognition by TCR. In days of old, it was considered that recognition and response of T cells were apparently an on/off phenomenon. However, findings in mice utilizing peptide analogues with single residue substitution revealed that T cell clones recognize these APLs and altered T cell responses occur. APLs induced T cell nonresponsiveness through TCR antagonism (54, 55) or induction of anergy as a consequence of partial activation (56, 57), and sometimes induced dissociation between proliferative response and cytokine production (58, 59). Some peptide analogues with antagonistic properties for TCR partially stimulated T cells to induce increases in cell size and expression levels of CD11a (LFA-1) and CD25 (IL-2R) on the T cell surface, but not proliferation (56). Analyses of physical interactions of purified TCR with MHC-APL complexes revealed differences in affinity and/or off-rate of receptor-ligand interaction these phenomena led to differences in signals transduced by the TCR, resulting in differences in functional outcomes (60). Our recent observations indicate that; 1) different physiological outcomes are induced in recognition of APLs in human $CD4^+$ T cell clones, as noted by other studies of murine T cells. 2) APLs with antagonistic properties were mainly observed by substitution of TCR contact sites, however substitution at the MHC anchor residues also could contribute to TCR antagonism. and 3) many APLs with substitution at HLA anchoring residues exhibit agonistic properties (61).

8. Slight alteration of an antigenic peptide or DR molecule even far from the recognition surface significantly affect the recognition by TCR

In our previous study, single amino acid polymorphism at residue 37 of the HLA-DRβ chain (DRβ37) between DRB1*0406 and 0403 markedly influences the responses to the altered peptide ligands (APLs) of T cell clone YN5-32 specifically recognizing a streptococcal M12 p54-68 in the context of DR4 (DRB1*0406) (62). In that study, it was suggested that; 1) single amino acid polymorphism (Ser-Tyr) at the DRβ37 residue induced conformational changes of peptides, which can be distinguished by TCR; and 2) these conformational changes were observed even in APLs with single residue substitutions at residues far from a putative DRβ37 contact site.

In recent studies, Kersh et al. described that TCRs can discriminate between the two APLs in which only a single $I\text{-}E^k$ P6 anchor residue was substituted for a chemically conserative one, which does not significantly alter the binding affinity to MHC (Fig. 8) (63, 64). Alterations of main chain conformation in P6-P8 and slight change in the angle of the P8 TCR contacted side chain were evident in a crystal analysis. As a result, the physiological response changed from full agonism to antagonism. This observation indicates that recognition by TCR is significantly affected by slight alterations far from the TCR recognition surface and its physiological reactivity can be markedly changed.

9. Degeneracy in antigen recognition by TCR is not predictable by an independent contribution model

Elucidation of the structural requirements for peptides to be cross-recognized by autoreactive T cell is a great importance for understanding disease processes. Heretofore, mimicry epitopes have been predicted and identified based on primary sequence homology (65, 66), the data obtained from single residue substituted peptide analogues (Fig. 6) (42, 51, 67, 68), or PS-SCLs (Fig. 7) (53, 69, 70, 71, 72). The latter, the majority of currently used means using synthetic peptide, are fundamentally based on the concept that the antigen recognition surface of TCR is relatively flat and each amino acid on each position of the peptide independently contributes to recognition by TCR (independent contribution model) (Fig. 9) (73). In these systems, putative mimicry peptides were searched using algorithms designed by combining the amino acides selected for for each position referring to tested data. However, combinations of amino acids used in these systems do not always function as expected. In fact, artificial peptides composed of the optimal residue for each position selected and based on analysis with PS-SCLs do not necessarily show agonistic activity, as we reported (74).

FIGURE 8

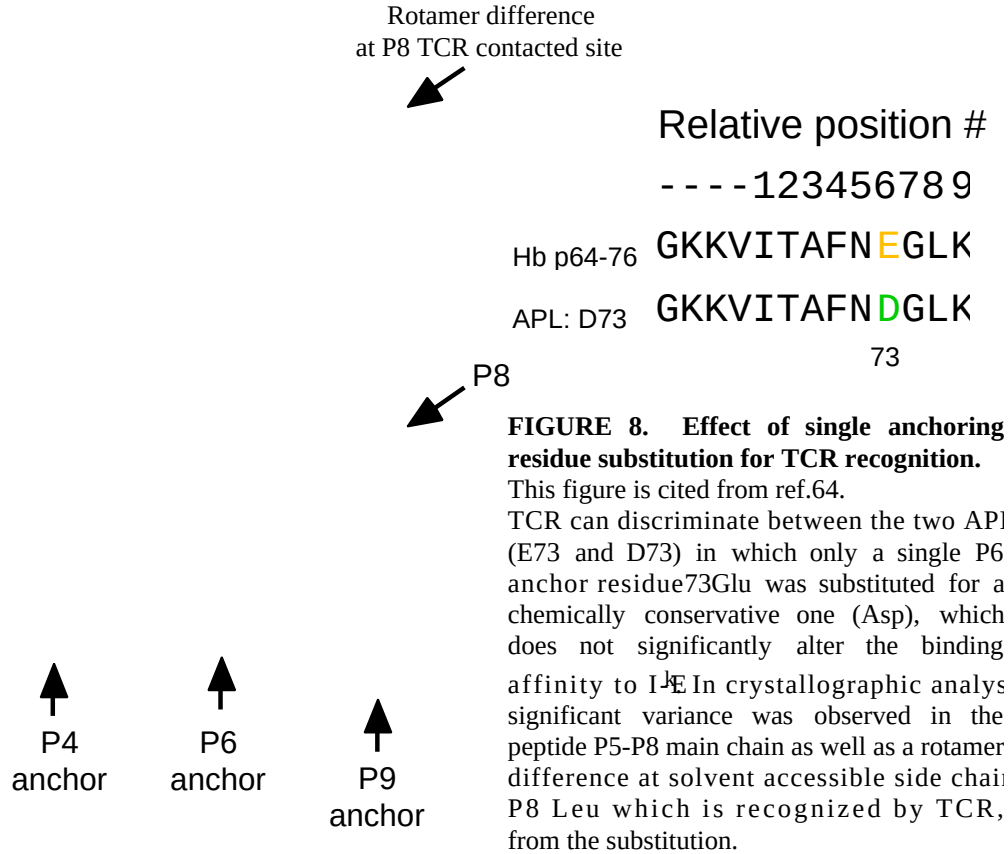


FIGURE 8. Effect of single anchoring residue substitution for TCR recognition. This figure is cited from ref.64.
TCR can discriminate between the two APL (E73 and D73) in which only a single P6 anchor residue73Glu was substituted for a chemically conservative one (Asp), which does not significantly alter the binding affinity to I-E^k. In crystallographic analys significant variance was observed in the peptide P5-P8 main chain as well as a rotamer difference at solvent accessible side chai P8 Leu which is recognized by TCR, from the substitution.

Significant changes in electron density between covalently bound Hb/I-E^k and Hb(D73)Hb/I-E^k are clustered around the side chain at position 73 (P6) and along the peptide mair between position 72 (P5) and 75 (P8). Models for the covalently linked Hb (y Hb(D73) (green) peptides have been superimposed and ori-ented such that the MHC-bindin groove is depicted below the peptides, and residues 72 (P5) and 75 (P8) point up anc from the MHC surface. In both models, the oxygen atoms are colored red and the n atoms are colored blue. Overlaid onto these models is a representative difference density map ($Fo_{Hb} - Fo_{,D73}$) α_{Hb} contoured at 4σ. The top panel shows positive dens (yellow), defined as density that is present in the Hb/I-E^k structure, but not in Hb(D73)/Hb/I-E^k. Conversely, the bottom panel displays negative density (green), present in the Hb(D73)/Hb/I-E^k structure, but not in HbHb^k/I-E. This difference map clearly show changes resulting from the Asp 73 substitution: the side chain of residue 73 (P6) has mov the main chain between residues 73 (P6) and 75 (P8) has shifted, and a water mole-cule migrated in the P6 pocket. This traveling water is colored yellow in the top panel and g in the bottom panel. All the density differences between the two peptides were localized t small region between residues 72 (P5) and 76 (P9). Similar analysis of difference density between the identical complexes yielded virtually featureless maps.

FIGURE 9

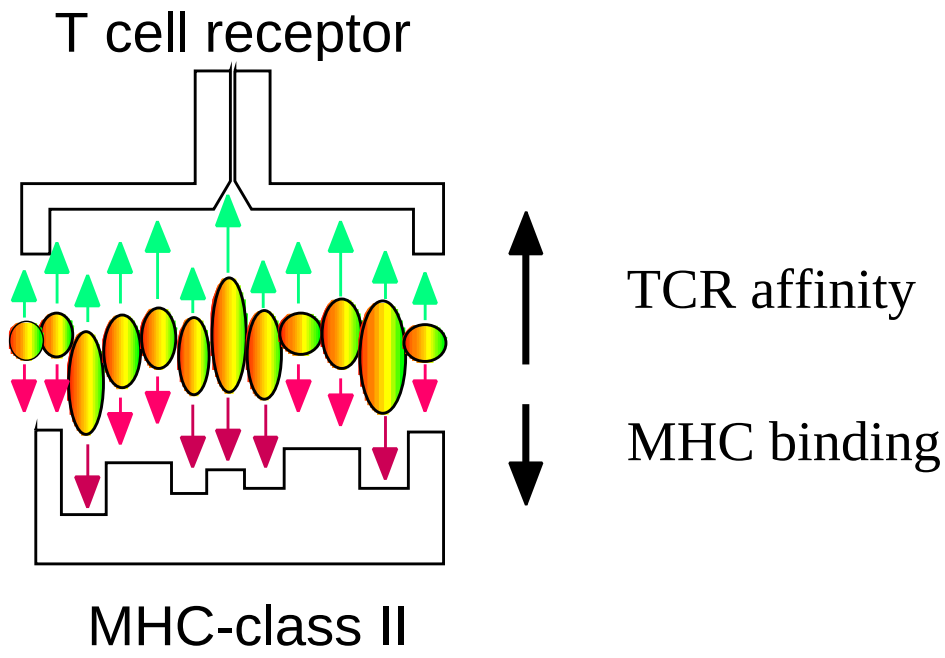


FIGURE 9 Model of independent contribution of each amino acid of antigeni peptide to the overall affinity of the MHC-peptide complex for the TCR.
This figure is cited from ref.73.
Each amino acid in the peptide antigen in most cases contributes independently tc interactions with both MHC and TCR. The sum of the effects introduced by each sing amino acid, which may be positive or negative, defines the final affinity and the e of T cell activation.

Although TCR shows a high degree of degeneracy in recognized peptides, slight alteration of an antigenic peptide or a DR molecule even far from the recognition surface can be discriminated by TCR, these showing exquisite specificity. It has been reported certain TCR recognition is affected by each amino acid adjacent or not adjacent to TCR contact residues or by each amino acid combinations in antigenic peptide (75-77). Therefore, the potency of peptide to stimulate certain T cells cannot be precisely predicted in approaches based on an independent contribution model.

10. Targeting antigens to HLA class II presentation pathway using invariant chain

The presentation of antigenic peptides by major histocompatibility complex (MHC) class II to $CD4^+$ T cells is crucial to initiate immune responses. Several groups including our group, developed a new system for delivery of an antigenic peptide to the MHC class II pathway, using the invariant chain (Ii) (78-80). As shouwn in Fig10, we designed a mutated human p33-form Ii, CLIP-substituted Ii, in which streptococcal M12p55-68 (RDLEQAYNELSGEA) was substituted for CLIP (class II associated invariant chain peptide) (78). We examined the peptide presenting function of this construct, in comparison with the previously reported C-terminal fused Ii, in which a cathepsin cleavage site and M12p54-68 was ligated to the C-terminus of Ii. Mouse L cell transfectants expressing either of these two mutated Ii along with HLA-DR4 could process and present M12p55-68 to the peptide specific and DR4-restricted $CD4^+$ T cell clone. CLIP-substituted Ii was much more efficient in antigen presentation than was the C-terminal fused Ii. Similar to the wild-type Ii, the CLIP-substituted Ii was associated intracellularly with DR4 molecules. These results indicate that the peptide substituted for CLIP of Ii p33 bound to the groove of DR molecules in the same manner as CLIP and it was preferentially presented to the $CD4^+$ T cell clone in the absence of HLA-DM molecules. This system may prove useful for immunotherapy with DNA vaccines or for construction of an antigen presenting cell library with diverse peptides.

11. 65kDa glutamic acid decarboxylase as a candidate autoantigen in type I diabetes

GAD65 is one of the important islet antigens implicated in autoimmunity of the NOD mouse and Type I diabetes in humans (81-83). We used two T cell clones established from Japanese patients with Type I diabetes and restricted by disease susceptible HLA-DR53 and they responded to GAD65 protein in the presence of APCs (84). The epitope (GAD65 p115-127) used in this study was also reported to be immunodominant in studies using HLA-DR4 transgenic mice (85, 86).

12. The aims of this study

As mentioned above, TCR shows a high degree of degeneracy in antigens recognition, therefore delineating the recognition nature is essential for revelation of the T cell mediated autoimmunity triggered by infection. The goal of this study is to establish a method to identify the epitopes for $CD4^+$ T cell clones, and on the basis of this method, to develop a strategy for systematic analysis of degeneracy of epitopes recognized $CD4^+$ T cell clones. By adopting this, we sought to identify candidate mimicry microbial epitopes for GAD65 autoreactive T cell clones established from type I diabetes patients.

FIGURE 10

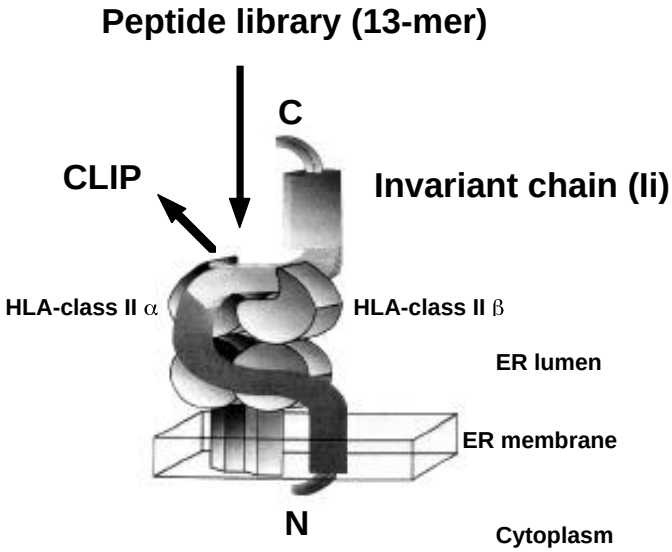


FIGURE 10. Strategy for targeting antigenic peptide to MHC class II pathway.
The use of an invariant chain with targeting signals to endosome is a pertinent strategy for antigen delivery to HLA class II restricted antigen presentation pathway The Ii chain with CLIP replacement exploit the natural assembly pathway of class II-Ii complex to obtain loading of randomized set of peptide library into peptide-bind groove of HLA class II molecules.

Materials and Methods

1. T cell clones and T cell proliferation assay

12-mer peptide analogues with single amino acid substitutions derived from GAD65 p116-127 were purchased from Chiron Mimotopes (Victoria, Australia) and 13-mer peptides were synthesized as described (61). Two human $CD4^+$ T cell clones, SA32.5 and MK20.2, recognizing GAD65 p116-127 (NILLQYVVKSFD) in the context of HLA-DR53 molecules (DRA*0101+ DRB4*0103) susceptible to type I diabetes were used throughout the study (84). T cells were fed weekly with 50 U/ml human rIL-2 and the irradiated DR53 matched allogenic PBMCs pre-pulsed with the GAD65 p111-131 (LQDVMNILLQYVVKSFDRSTK) in RPMI1640 medium supplemented with 10 % heat-inactivated human plasma, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. T cell proliferation assays were set up in 96-well flat-bottom culture plates (Falcon, Becton Dickinson, Lincoln Park,NJ) with $3x10^4$ T cells and irradiated (6000 cGy) DR53-positive $1.5x10^5$ PBMCs or $1.0x10^4$ dendritic cells per well in the presence of peptides or recombinant proteins at various concentrations. After 48 h of culture, [^{3}H]-thymidine was added (1 μCi/well), and after an additional 16 h cells were harvested onto glass fiber filters and radioactivity was counted on a β scintillation counter (Wallac, Gaithersburg, MD).

2. In vitro generation of dendritic cells (DCs)

DCs were generated from $CD14^+$ monocytes purified by positive immunoselection from HLA-DR53 positive allogenic PBMCs, using an anti-CD14 mAb coupled onto magnetic microbeads; CD14 microbeads (Miltenyl Biotec, Auburn, CA). The $CD14^+$ monocytes were cultured at 1 x 10^6 cells/ml in the presence of 100 ng/ml of GM-CSF and 100 U/ml of IL-4 (Ono Pharmaceutical Co., Ltd., Japan) in RPMI 1640 supplemented with 10% human plasma, 2 mM L-glutamine, and 100 μg/ml streptomycin. Cultures were fed on days 3 and 5 with fresh medium containing GM-CSF and IL-4. On day 5, DCs were treated with TNFα (20 ng/ml). On day 7, the non adherent cells were harvested and served as mature DCs. For the assessment of HLA class II-restricted presentation of recombinant microbial proteins, recombinant proteins were cultured for 14 h with mature DCs before used as APCs for proliferation assay.

3. 13-mer randomized peptide expression library

The procedure for construction of 13-mer randomized epitope presenting library is schematically depicted in Fig. 11 (87). Double-stranded DNAs encoding randomized peptides were prepared by PCR, using a 115-base single-stranded DNA including degenerate sequence, 5'-TCC CTC CTG GTG ACT CTG CTC CTC TTT AAA CCT GTG $(NNK)^{13}$ CTG CCT ATG GGA GCT CCA AGT CGG AAC AGC AGA TAA CAA T-3' as template and with 5'-biotinylated primers, 5'-TCC ATC CTG GTG ACT CTG CTC CTC-3' and 5'-ATT GTT ATC TGC TGT TCC GAC TTG-3'; where N means any nucleotide and K means G or T. PCR products were digested with *Dra* I and *Sac* I and biotinylated terminal fragments along with undigested and partially digested PCR products were removed with streptoavidin agarose. The purified DNA fragments containing degenerate regions were ligated to the modified pCI digested with *Sma* I and *Sac* I, to prepare the plasmid library. Subsequently, sub-libraries, each consisting of about 500 clones, were prepared for the primary screening.

4. GAD65-based randomized peptide expression library

Oligonucleotide fragments encoding degenerate-GAD65 p115-127 were synthesized and purified using polyacrylamide gel (Genemed Synthesis, Inc., South San Francisco, CA). These oligonucleotide fragments were amplified by PCR with 5'-biotinylated primers, 5'-TCC CTC CTG GTG ACT CTG CTC CTC-3' and 5'-ATT GTT ATC TGC TGT TCC GAC TTG-3'. The purified PCR products were digested with *Dra* I and *Sac* I, purified with streptavidin agarose and ligated to *Sma* I-*Sac* I-digested pCI, the CLIP-substituted epitope expression vector. The construct encodes Ii protein inserted with partially degenerate-fusion peptides based on GAD65 p115-127

FIGURE 11

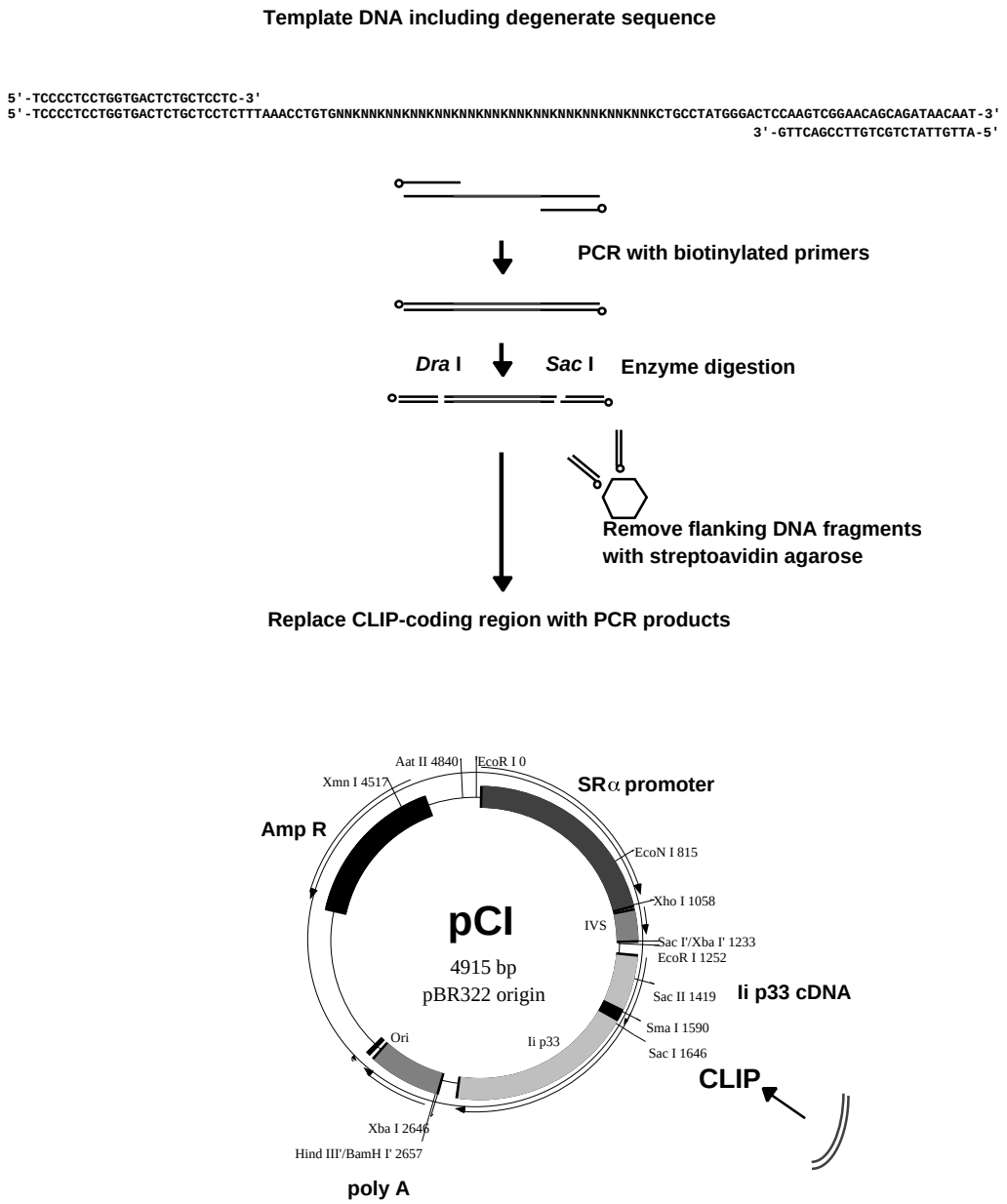


FIGURE 11. Construction of epitope library using CLIP-replacing epitope presenting vector, pCI.

Double-stranded DNAsincluding randomized sequences were prepared by PCR using a 115-base single-stranded DNA including degenerate sequence as template wi 5'-biotinylated PCR-primers. N means any nucleotide and K means Guanine or Thymine, and X means any amino acid. PCR products were digested with *Dra* I and *Sac* I and biotinylated terminal fragments were removed with streptoavidin The purified DNA fragments containing degenerate regions were ligated to pCI digested with *Sma* I and *Sac* I, to prepare plasmid libraries.

(MNILLQYVVKSFD) instead of Ii p89-101 (SKMRMATPLLMQA) within the CLIP sequence (88). *Escherichia coli (DH5α)* was transformed with the ligation mixture, and the transformants were divided into 96 well culture blocks (QIAGEN, Studio City, CA) to generate transformant pools of 30-50 clones and grown overnight in LB medium containing ampicillin (100 μg/ml). The amplified Plasmid DNA was purified using a QIAprep 96 Turbo Miniprep system. (QIAGEN, Studio City, CA). The complexity of each degenerate-GAD65 p115-127 expression sublibraries was approximately $1.5 \times 10^4 \sim 2.0 \times 10^4$.

5. Screening of the 13-mer randomized peptide expression library

The screeeing system of the 13-mer randomized peptide expression library is schematically depicted in Fig. 12. For transfection in each well, 33 ng each of library DNA and expression vectors for HLA-DRA*0101 and DRB4*0103 genes were mixed with 0.3 μg Transfectam (Promega) in 40 μl serum-free DMEM. The DNA/Transfectam mixture was then added to COS-7 cells (8×10^3 cells/well) in a 96 well-plate and incubated for 90 min at 37°C. After removal of the transfection medium, the COS-7 cells were incubated in 100 μl of DMEM supplemented with 10% FCS for 24 h. Subsequently, T cells (5×10^4/well) were added to the wells followed by incubation for 48 h in RPMI-1640 medium supplemented with 10% heat-inactivated human plasma, antibiotics, and L-glutamine. At the end of the culture, 50 μl of supernatants was collected from each well to quantify IFN-γ concentrations by ELISA. The library DNA pool, by which significant production of IFN-γ was induced, was transformed into bacteria to prepare sub-library DNA pools consisting of about 50 clones. Secondary screening was done as described above, using sub-libraries. Single plasmid clones were obtained by repeating the screening procedure for 3 times. DNA sequence of the purified plasmid clone was analyzed, using an automated sequence analyzer (Applied Biosystems).

6. Screening of GAD65-based peptide epitope expression library

Library DNA pools and expression vectors for HLA-DRA*0101 and DRB4*0103 were mixed with Transfectam Reagent (Promega, Madison, WI) in serum-free DMEM. The DNA/Transfectam mixtures were then added to the COS-7 cells (1×10^4 cells/well) in 96-well flat-bottom culture plates and incubated for 90 min at 37°C. After removal of the transfection medium, the COS-7 cells were incubated overnight in DMEM supplemented with 10% FCS. 24 h later, cells were washed twice with DMEM medium and T cells were added at a concentration of 3×10^4 /well in RPMI-1640 medium supplemented with 10% heat-inactivated human plasma, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. After 48 h incubation, the supernatant was collected and IFN-γ concentration was measured using a standard ELISA assay (ENDOGEN, Woburn, MA). The library of DNA pools, for which a significant production of IFN-γ was detected, was used to transform the bacteria to prepare a sublibrary of DNA pools consisting of about 10 clones. Secondary screening was done as described above, using sublibraries. Single plasmid clones were obtained after three rounds of screening. DNA sequences of the purified plasmid clones were analyzed using BigDye Terminator Cycle Sequencing Ready Reaction Kits and the ABI PRISM™310 Genetic Analyzer (Applied Biosystems, Foster City, CA). For construction of expression vectors encoding mimicry peptides or analogues of GAD65 epitope, both strands of oligonucleotide fragments encoding these peptides were synthesized (Espec Oligo Service, Tsukuba, Japan), annealed and ligated to *Sma* I-*Sac* I-digested pCI. The amplified plasmid DNAs were purified for transfection. The agonistic activity was assessed in an IFN-γ secretion assay at various dilutions with pCI (wild Ii: irrelevant DNA).

7. Analysis of TCR V-(D)-J junctional regions of GAD65 autoreactive T cell clones

Total RNA was extracted from T cells using the TRIZOL reagent (GIBCO BRL, Gaithersburg, MD) and first strand cDNA was synthesized using Superscript RNase H^- Reverse Transcriptase (GIBCO BRL, Gaithersburg, MD) and random hexamers. cDNA was subjected to PCR amplification for rearranged TCR α with 29 5'TCRAV family-specific oligonucleotides (Vα1-32) and a 3'TCRAC (Cα) constant primer, and for rearranged TCR β with 27

5'TCRBV family-specific oligonucleotides (Vβ1-25) and a 3'TCRBC (Cβ) constant primer (89). The amplified PCR products of the α-chain and of the β-chain were cloned into a plasmid vector, pGEM-T (Promega,Madison,WI), and sequenced. The resulting sequences were analyzed using IMGT: the international ImMunoGeneTics database (http://imgt.cnusc.fr: 8104/).

8. Northern blot analysis

Northern blot analysis was done as described (90). To prepare the probes, the MK20.2 cDNA was subjected to PCR amplification for 5'TCRVα15 or 5'TCRVα16 specific oligonucleotide and 3'TCRJα region oligonucleotide primers and each PCR product was TA cloned, digested and gel purified. The two cDNA fragments of the TCRAV gene were labeled with [α^{32}-P] dCTP. After hybridization and quantitative analysis of signal intensities, probes were stripped and a second hybridization was carried out using an β-actin probe. To assess crossreactivity of the Vα-specific probes, TCRAV cDNA fragments were arrayed onto two copies of nylon membrane filters and hybridized using TCRAV cDNA probes.

9. Generation of recombinant proteins

Genomic DNA of *Legionella pneumophila* (strain Philadelphia-1) was kindly provided by Dr. T. Akaike and Dr. T. Akuta (Kumamoto University School of Medicine, Japan). *Lactococcus lactis* (subspecies; lactis) was provided by the Institute of Physical and Chemical Research, Japan. Bacterial genomic DNA were purified using DNeasy Tissue Kits (QIAGEN). The O-succinylbenzoic acid-CoA ligase gene (coding for amino acid residues 101 - 201) / *Lactococcus lactis*, putative PTS system, lactose-specific component IIBC gene (coding for amino acid residues 426 - 546) / *Streptococcus pyogenes* (ATCC 19615), putative dihydrolipoamide dehydrogenase gene (coding for amino acid residues 101 - 205) / *Neisseria meningitidis*, glutamine amidotransferase, class I gene (coding for amino acid residues 1 - 104) / *Streptococcus pneumoniae* (ATCC49619), and the pilus assembly protein PilB gene (coding for amino acid residues 28 - 131) / *Legionella pneumophila* were PCR amplified and cloned into the plasmid vector (pGEM-T-easy vector system). Fusion proteins containing relatively small fragment (100-120 a.a.) of microbial proteins were generated, because larger recombinant proteins tend to become insoluble in bacteria and are difficult to purify. The inserted fragments were digested and ligated directionally into the prokaryotic expression vector pGEX-4T (Pharmacia) to produce glutathione-S-transferase (GST) fusion protein. The integrity of the constructs was confirmed by DNA sequencing. The procedure for protein induction and purification were described in our previous report (91). The purity and integrity of the fusion protein were confirmed by SDS/PAGE. The recombinant proteins were concentrated and separated from small peptide fragments with Centricon-30 (Amicon, MA, USA) and the buffer was replaced with culture medium.

Results

1. Determination of the sensitivity of screening for T cell epitope using the pCI expression vector.

We previously generated a vector, pCI, with which one can easily generate cultured cells expressing CLIP-substituted Ii, and the latter was efficient in loading an antigenic peptide onto HLA-DR4 molecule and stimulating $CD4^+$ T cells. Using this vector, we have now generated an epitope-presenting library and devised an expression cloning system to identify epitopes stimulating $CD4^+$ T cells. In the screening system (Fig. 12), we adapted transient transfection in COS-7 cells for expression of the epitope library, and quantification of IFN-γ by ELISA to detect the T cell response. Fig. 10 shows predicted trimolecular complex intra-cellularly generated in COS-7 cells composed of HLA class II α and β chains and mutated Ii bearing an antigenic peptide that replaces CLIP.

We first evaluated sensitivity of the screening, using a GAD65 p116-129 autoreactive T cell clone SA 32.5 restricted by disease susceptible HLA-DR53 and established from a patient with type I diabetes (84). The plasmid clone, pCIG, encoding a mutant Ii, in which human GAD65 p116-129 peptide (NILLQYVVKSFDRS) was substituted for CLIP (Ii p89-102) of Ii, was mixed with pCI, encoding wild type Ii, in various ratios. The plasmid mixtures (pCIG/pCI) together with HLA-DRA*0101 and HLA-DRB4*0103 genes, encoding HLA-DR53, were transfected into COS-7 cells. COS-7 cells expressing DR53 and CLIP-substituted Ii were cultured with SA 32.5 in a 96-well plate, and the IFN-γ produced by T cells was measured using ELISA. As shown in Fig. 13, we detected the target clone, pCIG, even when the plasmid was diluted with pCI at a molar ratio of 1:4000. Dilution experiments using pCIM, in which an epitope derived from streptococcal M12 protein was inserted into pCI (78), as an irrelevant plasmid gave similar results. These results suggest that this system can identify a positive clone in the presence of more than 1000 irrelevant clones in a single well of a 96-well plate, and that one can screen 10^5 clones using one 96-well plate. Sensitivity of the screening was considered to be sufficient to identify unknown epitopes.

2. Generation of a pCI-based epitope library encoding mutant Ii containing randomized 13-mer peptides substituted for CLIP.

Some $CD4^+$ T cell clones recognize 9-mer peptide as a minimal core epitope in the context of HLA class II molecules. But the T cell stimulating activities of the core peptides are usually lower than peptides containing flanking residues in addition to the core sequence, as reported (92). Thereby, we generated a pCI-based epitope library encoding mutant Ii containing 13-mer randomized peptides substituted for CLIP (Ii p89-101). The library was prepared by ligating double stranded oligo DNAs containing the degenerate sequence $(NNK)^{13}$ into pCI; where N stands for an equal mixture of the deoxynucleotides G, A, T and C, and K stands for an equal mixture of G and T. Thus, NNK represents an equal mixture of 32 triplets, including codons for all 20 amino acids along with the amber stop codon; and the insert fragment is expected to be an equal mixture of 32^{13} different nucleotide sequences (Fig. 14A). The degenerate sequence $(NNK)^{13}$ was replaced for the 89-101 residues of Ii, because these 13 residues in CLIP contain the binding site for HLA class II molecules.

3. Identification of a cross-reactive epitope recognized by the GAD65-autoreactive T cell clone.

Using the method described above, we searched for a novel epitope cross-recognized by SA 32.5. As a result of screening of a library containing 2×10^5 independent plasmid clones, we isolated a plasmid clone, designated as pCIGm1 and which stimulates SA 32.5. As shown in Fig. 14B, pCIGm1, when co-transfected with the DR53 expression vectors to COS-7 cells, stimulated SA 32.5 to produce IFN-γ in a dose-dependent manner. We determined if the epitope encoded by the inserted DNA in pCIGm1 was stimulatory for SA 32.5, when it was added as a synthetic peptide. We synthesized 4 peptides (Gm1.1-1.4) containing all or part of the 13-mer peptide (QLSNQWHVVGATF) substituted for CLIP (Ii p89-101) together with different flanking sequences derived from Ii, and examined them on the capacity to stimulate SA 32.5 (Fig. 15). T cell stimulatory activity of these peptides was tested by proliferation assay in which the T cell clone and DR53 positive, irradiated PBMC were co-cultured in the

FIGURE 12

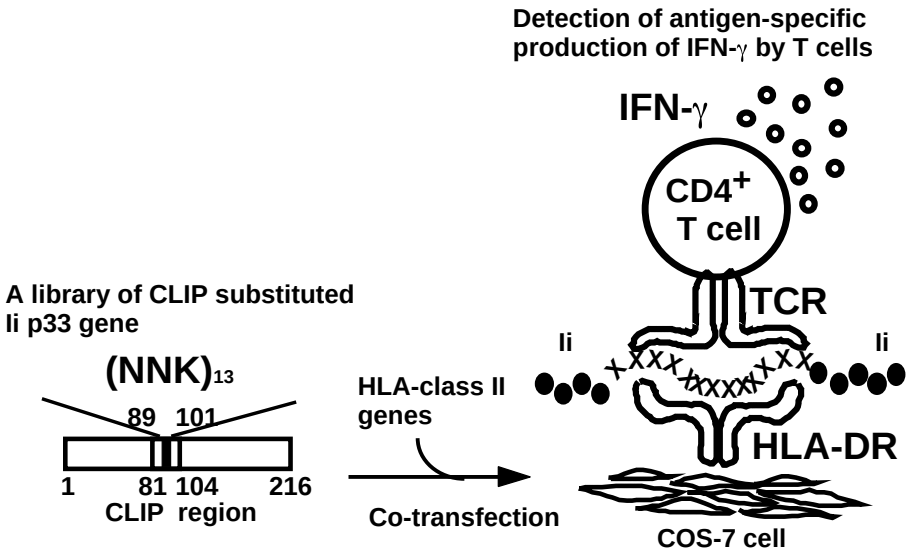


FIGURE 12. Schematic representation of the screening system to identify epitope from the epitope library.

Plasmid DNA of epitope library is introduced along with HLA class II expression vectors into COS-7 cells. Transiently trasfected COS-7 cells, which express diverse array of peptides in the context of class II molecules, are cocultured with CD4+T cells in 96-well culture plates. Response of T cells stimulated is detected by production of IFN-γ, if agonistic epitopes are expressed in the well.

FIGURE 13

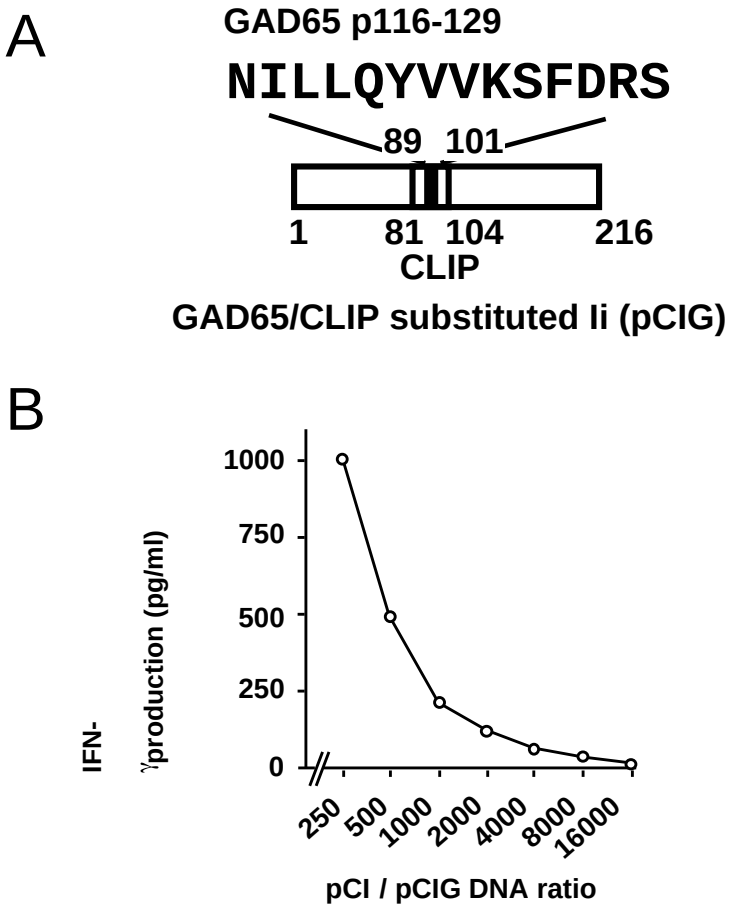


FIGURE 13. Determination of the sensitivity of screening for T cell epitopes, using the pCI expression vector.
A. Construct of pCIG; the GAD65-epitope-presenting vector in which human GAD65 p116-129 was inserted to pCI.
B. pCIG as relevant stimulative plasmid DNA was diluted with pCI as irrelevant plasmid at graded ratios. The mixtures of plasmids (pCI/pCIG) along with HLA-DRA*0101 and HLA-DRB4*0103 genes were transfected COS-7 cells in a well of a 96-well plate. After an overnight culture, 5×10^4 GAD65 autoreactive T cell clone SA 32.5 were added to COS-7 cells followed by 48 h culture. The amount of IFN-γ in the supernatants was measured, using ELISA. Results are expressed as means of triplicate determinations ± SD. The circle in this graph indicates IFN-γ produced in a well in which COS-7 cells were transfected with the pCI and DR expression vectors.

FIGURE 14

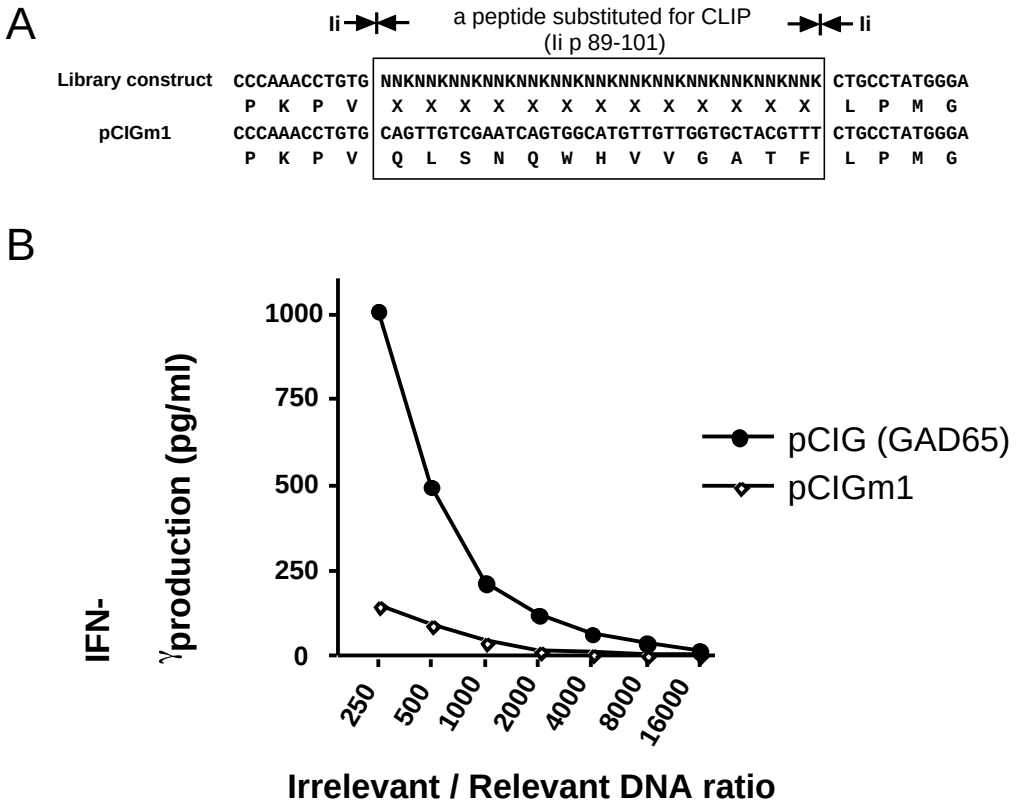


FIGURE 14. Identification of a cross-reactive epitope for a GAD-65 specific T cell clone SA 32.5.
A. The construct of CLIP-substituted peptide library and the peptide encoded by the insert DNA of the plasmid clone pCIGm1 identified by screening of the library. X indicates random amino acid encoded by nucleotide triplets NNK where N stands for an equal mixture of the deoxynucleotides Guanine, Adenine, Thymine and Cytosine, and K stands for an equal mixture of Guanine and Thymine.
B. Stimulation of T cell clone SA 32.5 by pCIGm1. pCIGm1, the plasmid encoding a novel cross-reactive epitope was diluted with pCI as an irrelevant plasmid at graded ratios(◇). The mixtures of plasmids (pCI/pCIGm1) along with HLA-DRA*0101 and HLA-DRB4*0103 expression vectors were transfected into COS-7 cells. Assay and quantification of IFN-γ produced by SA 32.5 was done as for Fig. 13B. Results are expressed as means of triplicate determinations ± SD.

FIGURE 15

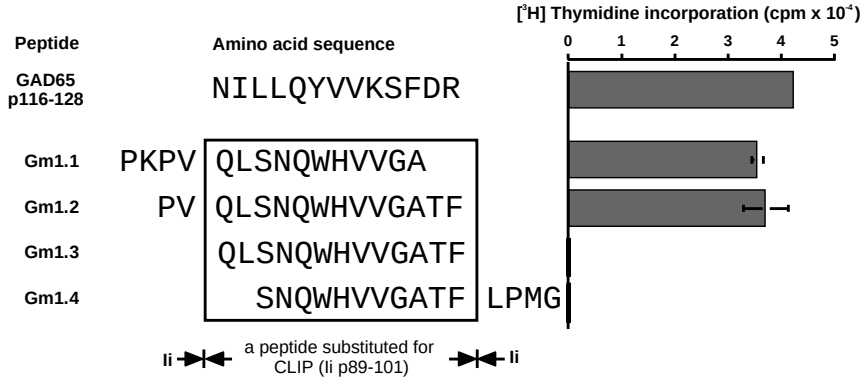


FIGURE 15. Determination of a novel cross-reactive epitope, using synthetic peptides.

As shown in Fig.14, the amino acid sequence encoded by the nucleotide sequence substituted for CLIP of the isolated plasmid clone pCIGm1 was QLSNQWHVVGATF. We synthesized 4 overlapping peptides containing all or part of this sequence together with Ii-derived flanking sequences. To investigate T cell stimulatory activities of these peptides, T cell proliferation assay was carried out. The T cell clone SA 32.5 (3×10^4 cells/well) was cultured in the presence of 50 μM of tested peptides and irradiated HLA-DR53 positive PBMC (1.5×10^5 cells/well) for 72h in wells of 96-well plate, and the proliferative responses was measured by counting ^{3}[H]-TdR incorporation. Results are expressed as means of triplicate determinations ± SD.

FIGURE 16

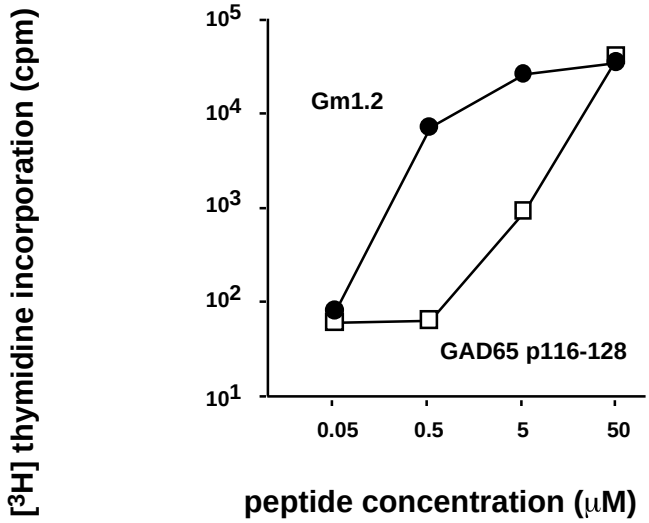


FIGURE 16. Comparison of the stimulatory activity of the identified peptide with the original GAD65 peptide.

The T cell clone and irradiated PBMC were co-cultured in the presence of indicat concentrations of Gm1.2 peptide or GAD65 p116-128 for 72 h. The proliferative response was measured by counting ^{3}[H]-TdR incorporation and results are expresse as means of duplicate determinations ± SD.

presence of 50 μM peptide. Of the 4 peptides, Gm1.1 (PKPVQLSNQWHVVGA) and Gm1.2 (PVQLSNQWHVVGATF) containing all or part of the insert sequence with two or four CLIP-flanking residues at the N-terminus stimulated SA 32.5, but peptide Gm1.3 with a deletion of two amino acids at the N-terminus of 13-mer insert did not do so (Fig. 15). To compare the stimulatory activity of the identified peptide Gm1.2 to the original peptide GAD65p116-128 (NILLQYVVKSFDR), we investigated proliferative responses of the T cell clone SA 32.5 cultured with different concentrations of these peptides in the presence of irradiated PBMC. As shown in Fig. 16, proliferative responses of SA 32.5 to the both peptides increased in a peptide dose-dependent manner, and the Gm1.2 peptide stimulated comparable responses to those induced by the GAD65 p116-128.

Several groups of researchers, including ours, have prepaired multiple-residue randomized peptide libraries from which agonistic epitopes could be directly isolated, using plasmid vectors expressing epitopes or bead-bound peptide library systems (93, 94). As described above, we identified crossreactive epitopes recognized by an autoreactive T cell clone using an expression cloning strategy by genetic exchange of CLIP with 13-mer randomized residues. Based on data obtained in these studies and in the study using combinatorial peptide libraries, one certain $CD4^+$ T cell clone was estimated to be able to recognize at least 10^6 different minimal peptide epitopes even at low nanomolar concentrations (93-96). Therefore, identification of all of the agonistic ligands for a single $CD4^+$ TCRs is practically impossible using currently available technologies. To analyze recognition profiles of TCRs, we planned to identify the majority of stimulatory ligands from peptide libraries in which randomized residues were narrowed down into three successive ones. The theoretical complexity of peptide mixtures containing the 3-mer randomized portion is 20^3 (=8000), and we considered it possible to make peptide libraries covering this scale of variation and to isolate agonistic epitopes. In the next study, we constructed seven libraries in which three successive residues were randomized on the basis of a native ligand, GAD65 p116-127, and consecutive libraries overlapped by 2 residues. We used two different GAD65-specific T cell clones, SA32.5 and MK20.2, expressing distinct TCR but recognizing the same epitope with the same restriction element, HLA-DR53 susceptible to type I diabetes in the Japanese population (84).

4. A novel strategy to analyze combinatorial effects of amino acids on the antigenic peptide in exhibition of molecular mimicry

The goal of this study was to develop new and comprehensive methods for analysis of the combinatorial effects of residues in antigenic peptides on recognition by HLA-class II restricted TCRs. We also tried to clarify how significant would be the effect of amino acid combination within antigenic peptide in recognition by TCR. By adopting the strategy, we sought to identify candidate mimicry epitopes for GAD65 autoreactive T cell clones established from Type I diabetes patients.

5. Specificity of GAD65 reactive T cell clones SA32.5 and MK20.2 analyzed using single amino acid residue substituted peptide analogues

To investigate structural features of peptides cross-recognized by autoantigen specific TCRs, we analyzed HLA-DR53-restricted SA32.5 and MK20.2 $CD4^+$ T cell clones reactive to GAD65 (p116-127). These T cell clones were established from two independent Type I diabetes patients in our previous study (84). At the beginning of this study, we verified that the two T cell clones expressed single TCRs, because it has been reported that a significant fraction of T cells in human peripheral blood expresses dual TCRs (97). The complementarity determining region (CDR) sequences of the antigen contact sites were defined. As shown in Table I, TCRα and TCRβ chains of SA32.5 T cell clone revealed functional TCRAV1S2 and TCRBV9S1 gene rearrangements as well as an out of frame rearrangement of AV27S1. TCRα and TCRβ chains of MK20.2 TCR revealed an in frame dual Vα rearrangement (TCRAV15S1 and TCRAV16S1 transcripts) and TCRBV3S1 rearrangement. In northern blot analysis (Fig. 17), T cell clone MK20.2 expressed TCRAV16S1 but not the TCRAV15S1 gene transcript. Therefore, TCRAV16S1

Table I.

TCR gene usage and TCR V-(D)-J junctional region sequences of α and β chains expressed in GAD65 (p116-127) autoreactive T cell clones SA32.5 and MK20.2

	TCRV	*FW*	*CDR3*	*FW*	*TCRJ*	CDR3 Length
SA32.5	***TCRAV1S2***	**CAV**	**SGQGAQKL**	**VFG**	***AJ 54*01***	8
	TCRAV27S1 (out of frame)	CAV	DSRVRNWSQZZADIWKRNNSECZT			
MK20.2	*TCRAV15S1*	CAD	SLLSPNSGSARQL	TFG	*AJ 22*01*	13
	TCRAV16S1	**CAA**	**WNNFNKF**	**Y**I	***AJ 21*01***	7
SA32.5 *(TCRBC2)*	***TCRBV9S1***	**CAS**	**SPTGQGAHTGEL**	**FFG**	***BJ 2-2*01***	12
MK20.2 *(TCRBC2)*	***TCRBV3S1***	**CAS**	**SSTGVSPGEL**	**FI**	***BJ 2-2*01***	10

Functional TCRs for each T cell clone are represented in boldface. The deduced amino acid sequence of the CDR3 loop is shown putatively supported by two framework branches (*FW*). Amino acids preceding "CA" of V region and those following the highly conserved "FG" of J region are not shown. Two T cell clones used distinct CDR3α and CDR3β sequences at the amino acid level and distinct CDR3 length. Both T cell clones used the same Jβ rearrangement.

FIGURE 17

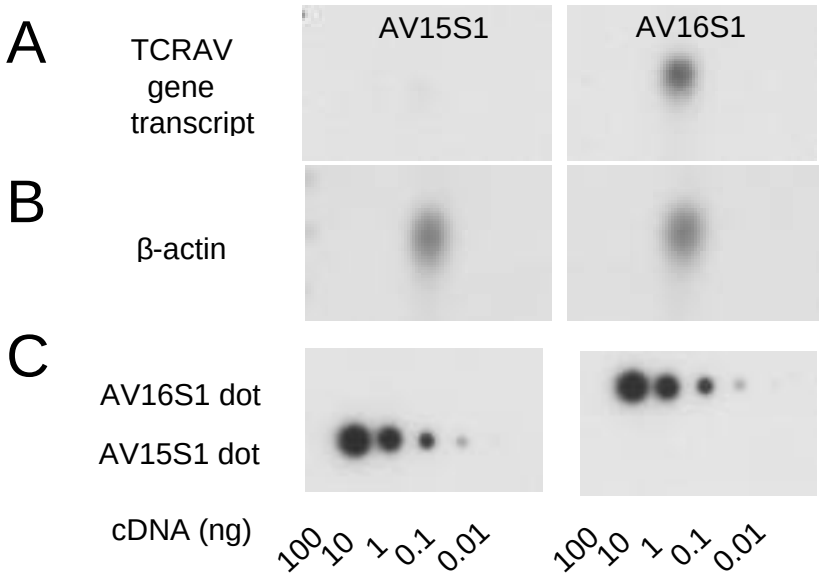


FIGURE 17. Identification of the TCRα chain expressed in T cell clone MK20.2 by Northern blot analysis.

A. Probes of TCRAV15S1 (left lane) and TCRAV16S1 (right lane) were hybridized parallel with replica filters.

B. Filters were probed with β-actin probe for control of amounts of RNA.

C. Cross-hybridization analysis of TCRAV16S1 and TCRAV15S1 cDNA probes. To assess the cross-hybridization of each probe, cDNA dot hybridization was done us replica filters. Replicate dot blots on which the indicated amounts of TCRAV16S1 (upper dots) and TCRAV15S1 (lower dots) were manually spotted and hybridized with cDN probes of TCRAV15S1 (left panel) or TCRAV16S1 (right panel). Cross-hybridization o each probe was not observed.

combined with TCRBV3S1 gene product mediates antigen recognition in MK20.2 TCR. These data indicated that reactivities of these T cell clones were determined by each single TCR. These T cell clones use distinct CDR3α and CDR3β sequences at the amino acid level and a distinct CDR3 length while both T cell clones use the same Jβ rearrangement.

Next we analyzed these T cell clones to understand the scheme of antigen recognition properties, using conventional means, in which we examined proliferative responses of these T cell clones to the 35 peptide analogues carrying chemically conservative or non-conservative single amino acid substitution in the native 12-mer epitope (GAD65 p116-127). As shown in Fig. 18, several amino acids with different chemical properties (K, D, N, S, and V) were tested for position 117 (I in the native ligand). MK20.2 tolerated all of these substitutions. In contrast, SA32.5 did not tolerate the negatively charged D. In the analysis using the same substitutions as position 117, these clones showed distinct specificity at position 118 (L in the native ligand) and at position 119 (L in the native ligand). With substitution at position 123 (V in the native ligand), MK20.2 responded to hydrophobic residues (L, I, A, and M), in several orders of magnitude. On the other hand, SA32.5 showed a significant response to only I among the tested substitutions at this position. SA32.5 tolerated Y to W substitution at position 121 and K to R substitution at position 124, albeit with a significantly weaker response. In contrast, MK20.2 did not respond to these conservative substitutions at these positions. These data indicated that these T cell clones differ in responses against peptide analogues with a single amino acid substitution. In particular, peptide analogues carrying replacement of Y121 and K124 with even conservative amino acids completely abrogated reactivity of MK20.2, suggesting that these residues are directly contacted by MK20.2 TCR.

6. Construction of the T cell epitope expression library using CLIP-substituted Ii-genes

We constructed a set of T cell epitope expression libraries by utilizing an Ii-based epitope presenting plasmid vector, pCI, in which the CLIP region of Ii was substituted for with MHC class II-restricted epitopes (Fig. 19A). In the libraries, sequences of peptides are derived from the GAD65 p115-127, and three successive residues within the sequence were totally randomized, the theoretical maximum complexity of each library being 20^3 (Fig. 19B). We defined the I117 of GAD65 p115-127 as the relative position 1, in this study. As shown in Fig. 19B, the library set (series CIR) is composed of seven sublibraries; CIR-1-2, CIR2-4, CIR3-5, CIR4-6, CIR5-7, CIR 6-8, and CIR7-9. (CIR-1-2 represents a library in which three successive residues from a relative position –1 to 2 in the T cell epitope GAD65 p115-127 are replaced by randomized amino acids.) The positions of inserted randomized amino acids are serially overlapped between individual sublibraries covering the core epitope GAD65 p116-125. Individual libraries contained about 14,400~19,200 DNA clones and were divided into sub-pools composed of 30-50 clones. Summation of all the complexity of peptides is estimated to be at least 120,000 species.

7. Two GAD65 p116-127 autoreactive and HLA-DR53-restricted TCRs responded differently to T cell epitope expression libraries; Crossreactivity scanning

The epitope recognition by the two GAD65 p116-127 reactive TCRs was further investigated using the T cell epitope expression system to identify the diverse peptide ligands of these T cell clones. The two T cell clones were examined in parallel with their production of IFN-γ in response to a set of T cell epitope expression libraries. Fig. 20 shows the frequency of pools stimulating T cell clones in the epitope libraries. The vector DNA encoding native GAD65 p115-127 mixed with a 100x excess amount of the wild type Ii gene was assigned to have an arbitrary activity of 1 unit, leading to determination of the relative activity of each separated pool. Thus, these TCRs responded to pools of each epitope library at different frequencies. MK20.2 responded to many of the pools of CIR-1-2 (99.7%) and CIR2-4 (66.0%) libraries with more than 0.5 units of response. In contrast, this TCR responded to a few of the pools in relatively C-terminus randomized libraries; CIR3-5 ~ CIR7-9 libraries (<7%). These results suggest that the

FIGURE 18

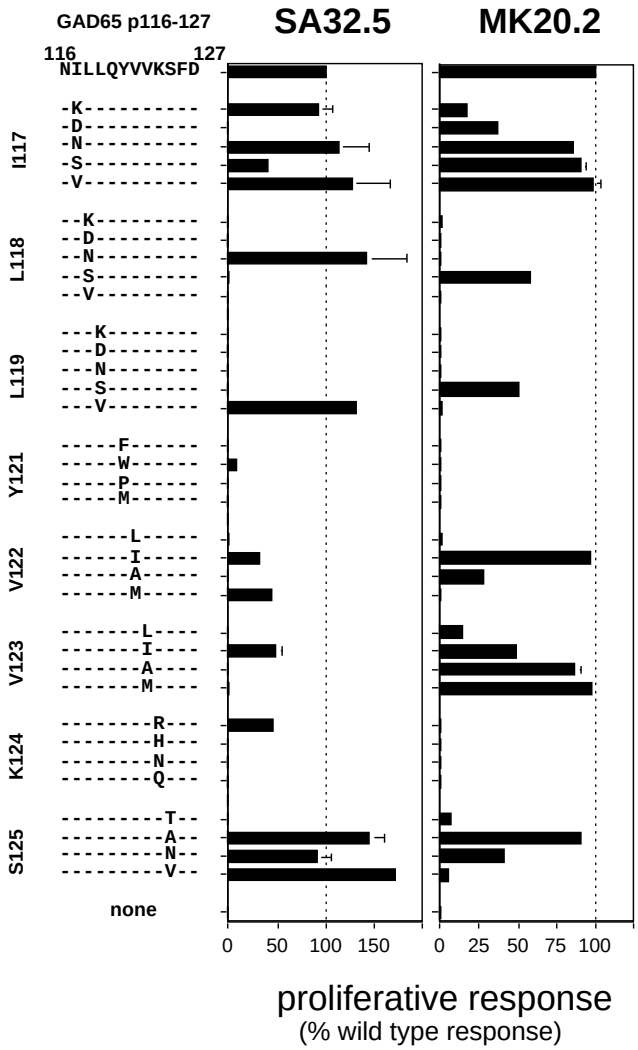


FIGURE 18. Proliferative response of GAD65 p116-127-autoreactive human T cell clones to peptide analogues carrying a single amino acid substitution in the GAD65 p116-127 peptide.

Data are given as % wild type response which were standardized by calculating the percentage to the response to wild-type peptide. The T cell responses to wt GAD65 p116-127 were 27,021 cpm for SA32.5 and 23,146 cpm for MK20.2. Medium control response without peptide was < 200 cpm. All data are expressed as the mean value of duplicate determinations ± SD.

FIGURE 19

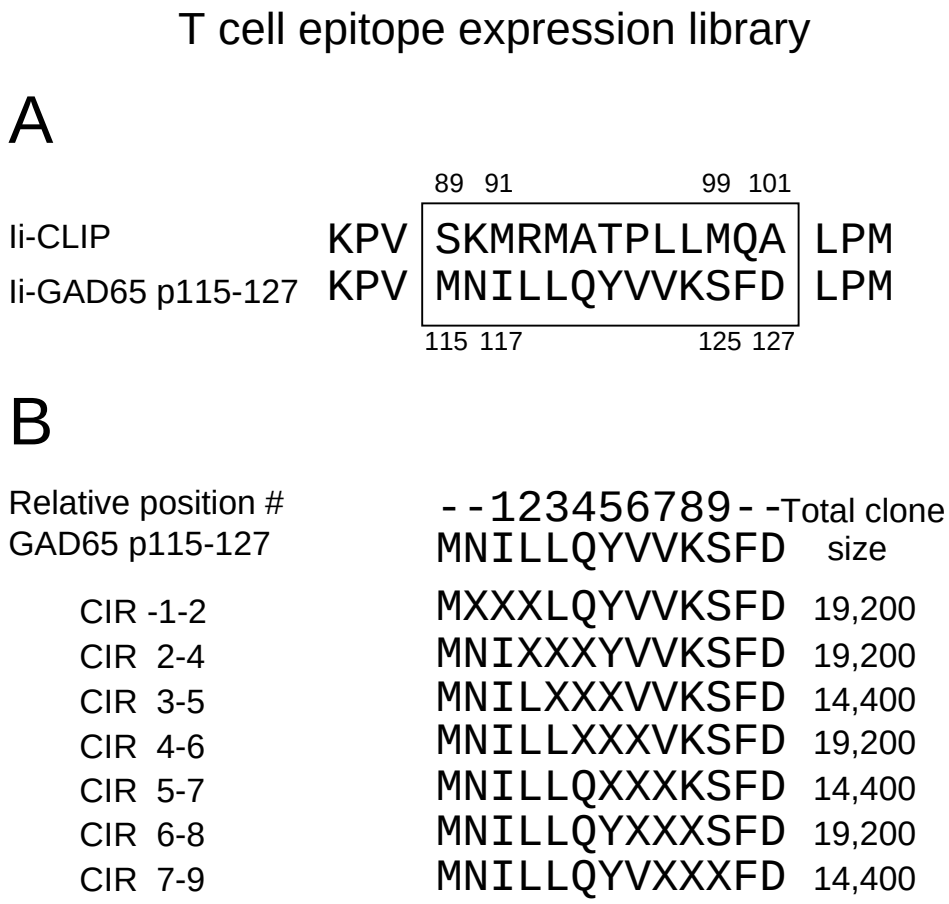


FIGURE 19. Design of a T cell epitope expression library using CLIP-substituted Ii-genes.
A. For construction of this library, the CLIP encoding region (Ii p89-101) was genetically replaced with GAD65 p115-127 to align I117 of GAD65 p115-127 to M91 of Ii p89-101, the first DR-anchoring residue of the CLIP.
B. The series of T cell epitope expression library was composed of seven sublibraries; CIR-1-2, CIR2-4, CIR3-5, CIR4-6, CIR5-7, CIR6-8, and CIR7-9. CIR-1-2 represents a sublibrary in which three successive residues from relative position –1 to 2 on the GAD65 p115-127 were replaced by randomized amino acids. Each sublibrary was aligned and is indicated in single letter amino acid code. X indicates a random amino acid encoded by nucleotide triplets NNK, where N stands for any nucleotide and K stands for G or T. In each of three randomized codons the third position was limited to Guanine or Thymine to minimize appearance of stop codons.

FIGURE 20

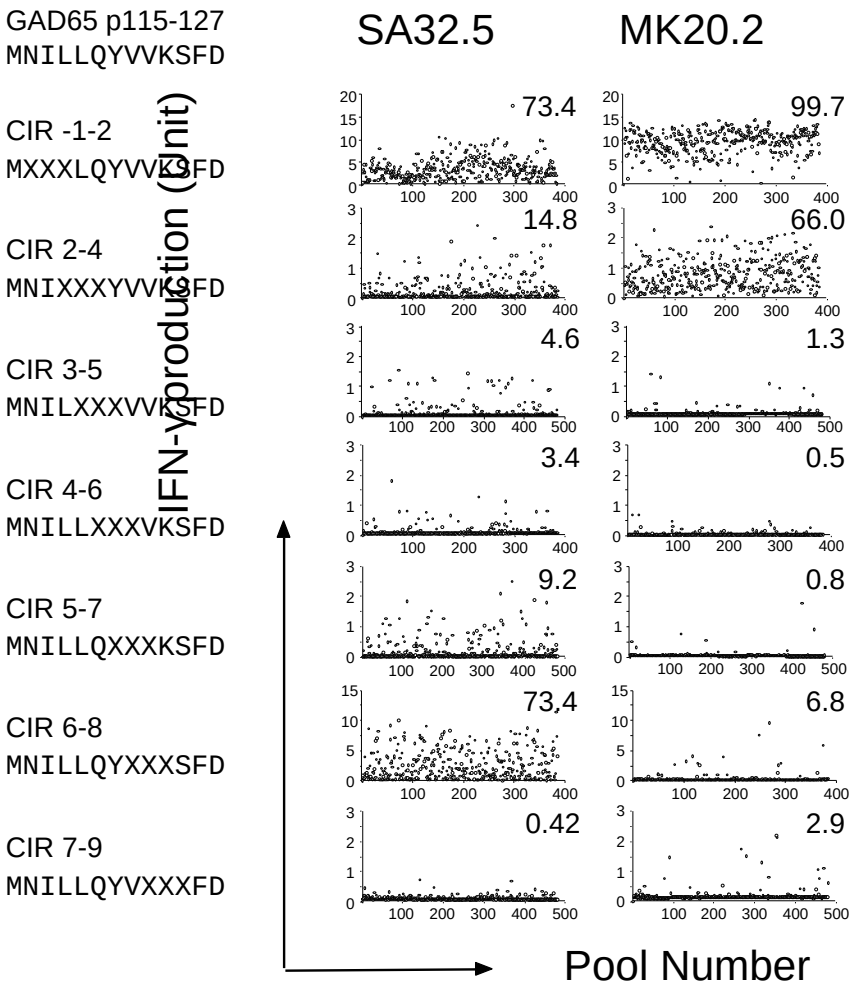


FIGURE 20. Response of GAD65 p116-127 autoreactive TCRs to pools of a peptide expression library.

Each pool was expected to contain about 30-50 distinct peptide ligands. IFN-γ production for each pool is expressed in terms of units. The response to pCIG (relevant DNA; GAD65 p115-127) 100 folds diluted with (irrelevant DNA; CLIP) was defined to be a relative value one unit IFN-γ production ranging from 232~506 pg/ml Percentages of positive pools for individual sublibraries are indicated. Positive pools which stimulated a significa IFN-γ production (relative value >0.5) were assigned. The negative control culture was stimulated by pCI and IFN-γ production stimulated by pCI ranged between 5.75~36.33 pg/ml. The experiment was repeated twice with reproducible results.

highest specificity of MK20.2 TCR exists at the relatively C-terminal side of GAD65 p115-127. In contrast SA32.5 showed a broader response profile against several libraries spanning the epitope functional core. Notably SA32.5 TCR showed a number of strong responses with library CIR6-8 (73.4%). These results collectively indicate that the spectrum of fine specificity in TCR recognition was clearly different between these two TCRs.

8. Isolation of diverse stimulatory peptides recognized by TCRs

The T cell epitope expression library was used to identify a series of agonistic peptide ligands for these TCRs. We screened 2,796 pools (total clone size was estimated to be 120,000) and after three rounds of screening, possible stimulatory peptides with higher antigenicity for SA32.5 (85 ligands) and MK20.2 (63 ligands) TCRs respectively were identified. The wt GAD65 p115-127 sequence was isolated from several libraries but not from library CIR-1-2, CIR3-5, and CIR6-8. For some peptide sequences identified, the same sequences were isolated more than twice, and in approximately 70% of such cases, peptides were encoded by distinct nucleotide sequences. The sequences of stimulatory peptides and their stimulatory activity are shown in Fig. 21 and 22. MK20.2 TCR responded to most of the pools (99.7%) of library CIR-1-2 in the first round of screening (Fig. 20). Therefore, agonistic peptide ligands for MK20.2 TCR were not isolated from this library and sequences isolated from library CIR-1-2 as stimulatory ligands for SA32.5 TCR were tested for their capacity to stimulate MK20.2 TCR (Fig. 21). With regard to library CIR2-4 for SA32.5 TCR (14.8%) and MK20.2 TCR (66.0%) and the library CIR6-8 for SA32.5 TCR (73.4%), stimulatory ligands were not exhaustively isolated because of the high frequency of positive pools.

9. Analysis of stimulatory ligands from library CIR-1-2 (MXXXLQYVVKSFD library*)*

The panel of stimulatory ligands isolated from library CIR-1-2 (Fig. 21) shows that both SA32.5 and MK20.2 tolerated F, M, L, I, V and C at relative position1 (I in the native ligand). These amino acids were compatible to the HLA-DR primary anchor residue that was restricted by the Val/Gly dimorphism at DRβ86 where Val is used in the HLA-DR53 molecule (98). For SA32.5 TCR, relative position 2 showed a strong preference for N and L. MK20.2 TCR tolerated most of the agonistic sequences identified with SA32.5 TCR with different stimulatory capacities. However MK20.2 TCR did not respond to peptides which incorporated N at relative position 2.

10. Analysis of stimulatory ligands for SA32.5 TCR; CIR2-4~CIR7-9 libraries

SA32.5 TCR had a preference for L and N at relative position 2 in screening of CIR-1-2 (Fig. 21) and CIR2-4 (Fig. 22), and M and H were also tolerated in CIR2-4. This TCR showed a strong preference for P with an imino-ring at relative position 3 in the screening of two separate libraries, CIR2-4 and CIR3-5. At this position, I sharing a chemically conservative side chain with native L was also tolerated. At relative position 4, L, M, V, and Q were tolerated almost equally in CIR2-4 and CIR3-5 libraries. In CIR3-5, I was also tolerated, however Q at relative position 4, the same amino acid as in the native ligand, was the most frequently observed (7/14) in CIR4-6. At this position, H, E, and S were also tolerated in CIR4-6. At relative position 5, located at the center of the epitope, this TCR did not tolerate chemically conservative single amino acid substitutions except for W in the analysis peptide analogues with single amino acid residue substitution (Fig. 18). However, ligands containing Y, F, and W, which share an aromatic ring and H with an imidazol ring at this position were isolated from the two libraries; CIR3-5 and CIR4-6. Neighboring residues may compensate for conformational mimicking.

Notably, W at relative position 5 was the most frequently observed amino acid (8/10) in stimulatory ligands isolated from CIR5-7. At relative position 6, this TCR has a preference for positively charged R and H in CIR4-6, CIR5-7, and CIR6-8. However, E, S, T, and V were also tolerated in CIR4-6. In addition, S, Q, V, and G were also tolerated in CIR5-7. Similarly, Q and V were also tolerated in CIR6-8. At relative position 7, this TCR showed a preference for hydrophobic residues (M, L, V, and P) in CIR5-7 and (L, M, V, and I) in CIR6-8. This preference in chemical character is not significantly changed in these two libraries. However, there is a drastic change

FIGURE 21

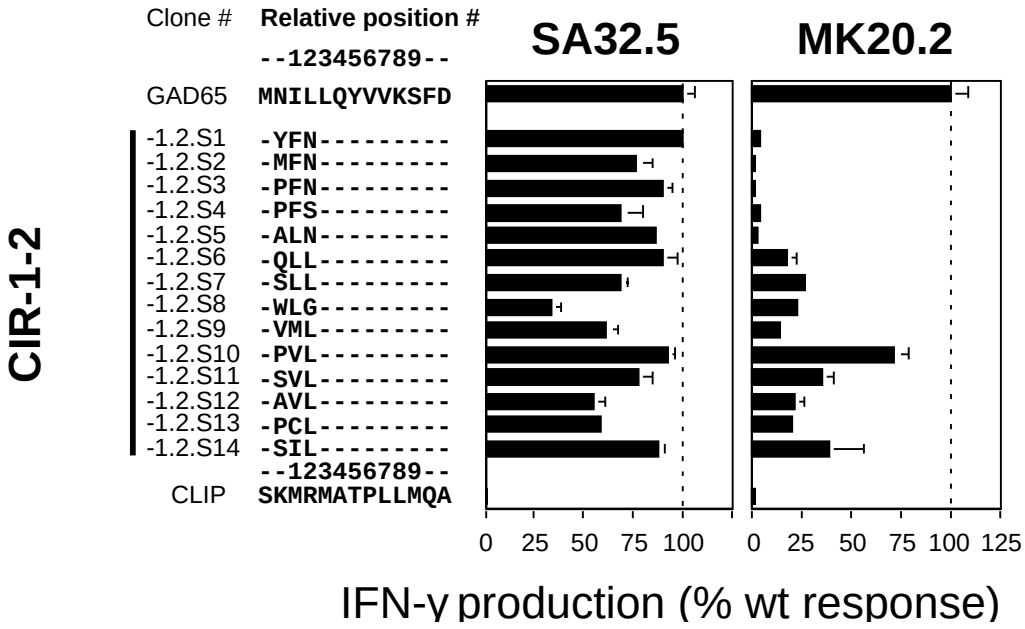


FIGURE 21. Identification of diverse crossreactive epitopes recognized by GAD65 autoreactive T cell clones using a T cell epitope expression cloning strategy.

Agonistic sequences isolated with SA32.5 TCR from library CIR-1-2 and their capacities to stimulate IFN-γ production by SA32.5 and MK20.2. The sequences of CLIP-substituted peptides and relative stimulatory activity of IFN-γ production measured in culture with a mixture of relevant: irrelevant DNA ratios at 1:50 are summarized. Data are given as % wild type response. All data are expressed as the mean value determinations ± SD.

FIGURE 22

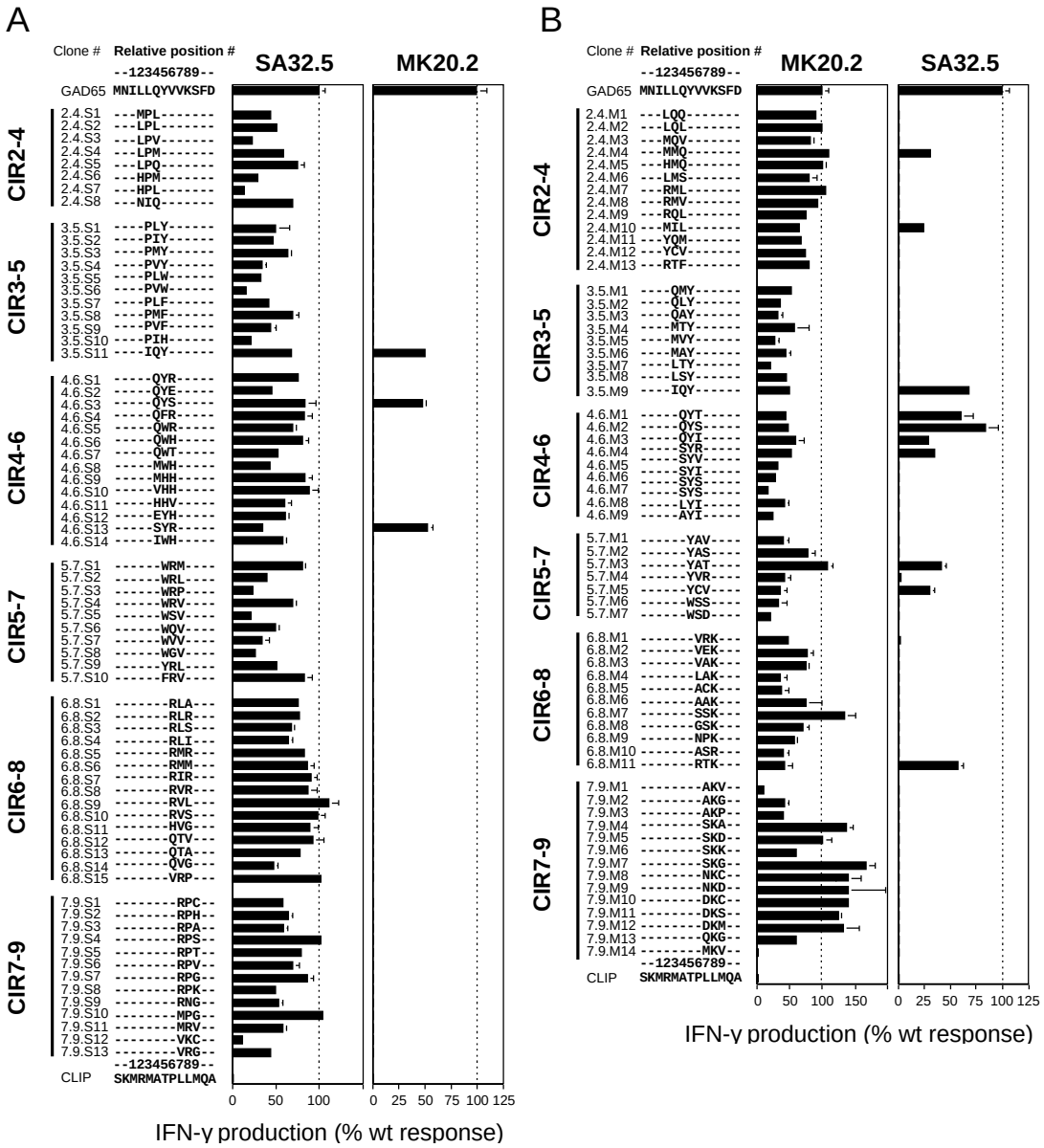


FIGURE 22. Identification of diverse crossreactive epitopes recognized by GAD65 autoreactive T cell clones using a T cell epitope expression cloning strategy.

A. Agonistic sequences isolated with SA32.5 TCR from CIR2-4, CIR3-5, CIR4-6, CIR5-7, CIR6-8, and CIR7-9 libraries and responses of SA32.5 and MK20.2.

B. Agonistic sequences isolated with MK20.2 TCR from CIR2-4, CIR3-5, CIR4-6, CIR5-7, CIR6-8, and CIR7-9 libraries and the response of SA32.5 and MK20.2.

The sequences of CLIP-substituted peptides and relative stimulatory activity of IFN-γ production measured in culture with a mixture of relevant: irrelevant DNA ratios at 1:50 are summarized. Data are given as % wild type response. All data are expressed as the mean value of duplicate determinations ± SD.

in the preference in CIR7-9 at this position in which the 3-mer randomized portion was moved to C-terminal side only by one amino acid from CIR6-8. This TCR has a preference for positively charged R at relative position 7. In addition, T and R in CIR6-8 and M and V in CIR7-9 were also permitted at this position. At relative position 8, several amino acids with different chemical properties (R, P, S, I, M, L, V, A, and G) were tolerated almost equally in CIR6-8. On the contrary, similar drastic changes in preference observed at position 7 were also observed at position 8 in CIR7-9, and this TCR has a strong preference for P at this position. Several amino acids with different chemical properties were tolerated at relative position 9.

11. Epitope expression library revealed the importance of linear combinations of residues in recognition by SA32.5 TCR

By observing the preference of residues in peptides in recognition by SA32.5 TCR, preferable residues at each position in the N-terminal side were relatively similar in each separated library. However, in the peptide C-terminal side (CIR5-7, CIR6-8, and CIR7-9 in Fig. 22A), there is a different preference at each position depending on which successive residues were randomized. It is conceivable that this phenomenon is affected by fixed residues flanking the randomized regions. Amino acids at relative position 6 in library CIR6-8 are not fixed, so that this position tolerates several amino acids with different chemical properties. In the isolated ligands from CIR6-8, if positively charged amino acids (R and H) located at relative position 6, hydrophobic amino acids (L, M, I, and V) were followed at relative position 7. Subsequently, R, S, or hydrophobic and aliphatic amino acids were followed at relative position 8 (see clones 6.8.S1~11 in Fig. 22B). At the same time, it shapes the most dominant motif in CIR6-8, and 11 of 15 agonistic clones (73%) were positive for this motif. Also if Q located at relative position 6, neutral or hydrophobic amino acids were followed at relative position 7 and aliphatic amino acids were followed at relative position 8 successively (6.8.S12~S14). In the case of V located at relative position 6, R at relative position 7 and P at relative position 8 were followed successively (6.8.S15 and 7.9.S1~S8). V at relative position 6 was fixed in the CIR7-9 library, so that it preferentially made up the most dominant V-R-P (relative position 6~8) motif (62%) and excluded other motifs. If M was located at relative position 7, P or R followed at relative position 8 (7.9.S10 and S11). In case of V at relative position 7, positively charged R or K followed at relative position 8 (7.9.S12 and S13). These observations indicate that fixed residues sometimes limit adjacent residues and exclude other recognition motifs. In this way, peptide libraries with three successive randomized residues and their overlapping system yielded different information related to TCR-recognition motifs depending on which successive ones are randomized.

In summary, SA32.5 exhibited a preference for P at relative position 3 of peptides in screening of two separate libraries; CIR2-4 and CIR3-5. This residue does not correspond to the native sequence. In addition, this TCR tolerated F, W, and H with similar structural side chains at relative position 5 when combined with some successive residues different from native ones. In the C-terminal side of peptide, three overlapping libraries; CIR5-7, CIR6-8, and CIR7-9, presented distinct recognition profiles. In this case, linear combinations of residues formed by the chemical properties of each were significantly influenced. With these promiscuous interactions, all of the TCR contacts may be altered by substitutions of other residues.

12. Analysis of stimulatory ligands for MK20.2 TCR; CIR2-4~CIR7-9 libraries

MK20.2 did not respond to most of the agonistic sequences among many peptides identified with SA32.5 (Fig. 22B). At relative position 2, MK20.2 TCR has a preference for M, L, and Y with hydrophobic side chains and for H and R with positively charged side chains in CIR2.4 (Fig. 22B). It seems that this position tolerates bulky side chains, as there is no relationship among chemical properties of the amino acid side chains identified. At relative position 3, this TCR indicates a strong preference for M and Q in two overlapped libraries; CIR2-4 and CIR3-5, which do not correspond to the native sequence. In addition, I, C and T in CIR2-4 and L and I in CIR3-5 are also tolerated. At relative position 4, this TCR has a preference for L, M, V, Q, and S in two overlapping libraries (CIR2-4

and CIR3-5). In addition, F in CIR2-4 and A and T in CIR3-5 were also tolerated. In contrast, with the screening of CIR4-6, this TCR has a preference for Q and S at this position, and L and A were also tolerated at this position. At relative position 5, this TCR showed almost exclusive specificity for Y in three consecutive libraries; CIR3-5, CIR4-6, and CIR5-7, which is the same amino acid as the native sequence. In addition, when S is at relative position 6, W was tolerated at position 5. At relative position 6, several amino acids with different chemical properties were tolerated. In CIR4-6, MK20.2 TCR tolerated S, T, R, V and I. In CIR5-7 and CIR6-8, small amino acids such as A, V, G, and S were preferred. At relative position 7, V, S, T, R, and D in CIR5-7 and V, C, S, and A, in CIR6-8 were permitted. However, this TCR has a preference for A, S, N, and D, at this position in CIR7-9. At relative position 8, MK20.2 TCR was highly specific to K in two overlapping libraries, CIR6-8 and CIR7-9, as in the native GAD65 p115-127 sequence. Several amino acids with different chemical properties were tolerated at relative position 9. SA32.5 responded to a limited fraction of the agonistic sequences identified with MK20.2.

In summary, MK20.2 TCR was specific for Y at relative position 5 and K at relative position 8 of the antigenic peptide, as judged by screening data from most of the separate libraries. However, several combinations of other residues were observed depending on which successive residues were randomized.

13. Combinatorial effects of multiple residues in exhibition of antigenicity

Several investigators reported that single amino acid modifications in a given peptide exert positive or negative effects when combined in one peptide species containing multiple substitutions (69, 99). These phenomena were also observed in a series of agonistic sequences we identified using the CLIP-substituted Ii library, as shown in left panels of Fig. 23 A-C. As shown in Fig. 23A and B, SA32.5 shows decreased or totally abrogated T cell reactivity against Y5W (standing for a peptide analogue having Y to W substitution at the relative position 5), Y5F, Y5H, V7R, V7M, K8P, and S9T. However, when these residues in each position were combined with other adjacent residues, these peptides had a completely restored T cell reactivity. For instance, when QWH (4.6.S6 clone in Fig. 22A, substituted residues are underlined), QFR (4.6.S4), MHH (4.6.S9), at relative positions 4-6, RPT (7.9.S5), and MPG (7.9.S10), at relative positions 7-9 were introduced into the peptide in combination, SA32.5 exhibited marked IFN-γ production and proliferation. Although the MK20.2 T cell clone exhibited strong specificity for K at relative position 8 (Fig. 22B), combinatorial effects were also observed for this TCR (Fig. 24A). For example, substitution of a single residue S9D decreased T cell reactivity but in combination with N (7.9.M9) or S (7.9.M5) at relative position 7 in combination exhibited an increased response. These data indicate that this strategy allows one to identify linear combinations of residues in antigenic peptide triggering TCRs.

To determine if combinations of two stimulatory sequences consisting of three successive amino acids would affect TCR recognition, we constructed CLIP substituted Ii expression vectors which encode peptides that incorporated two identified stimulatory sequences. These peptides did not always exhibit strong agonism for these TCRs (Fig. 23C and 24B). It seems that substitution of multiple residues have a substantial effect on the recognition of SA32.5 TCR. It is conceivable that the overall linear combination of residues in antigenic peptide significantly affect recognition by SA32.5 TCR.

We asked if the combinatorial effects we found in the analysis using the CLIP-substituted vector system were reproducible when epitopes were added as synthetic peptides. 13-mer peptides were synthesized and tested for proliferative response of T cell clones to the peptides at various doses. As shown in Fig. 23 A-C, 24 A and B right panels, the results of proliferation assay using synthetic peptides were almost in parallel with those obtained in experiments using epitope presenting vector and quantification of INF-γ produced by the T cell cones (Fig. 23, 24). Therefore, we could verify the combinatorial effect also in the experiments using synthetic peptides. Thereafter, 13-mer peptides incorporating multiple stimulatory three successive amino acids identified, MYFNIQYQTVGFD for SA32.5 and MPVRMLYVSKGFD for MK20.2, were synthesized. As shown in the right panels of Fig. 23C and 24B, these peptides with no resemblance to GAD65 115-127 activated T cell clones, respectively, at lower concentrations than did the native sequence, thereby indicating that simultaneous multiple residue modification deduced from the

FIGURE 23

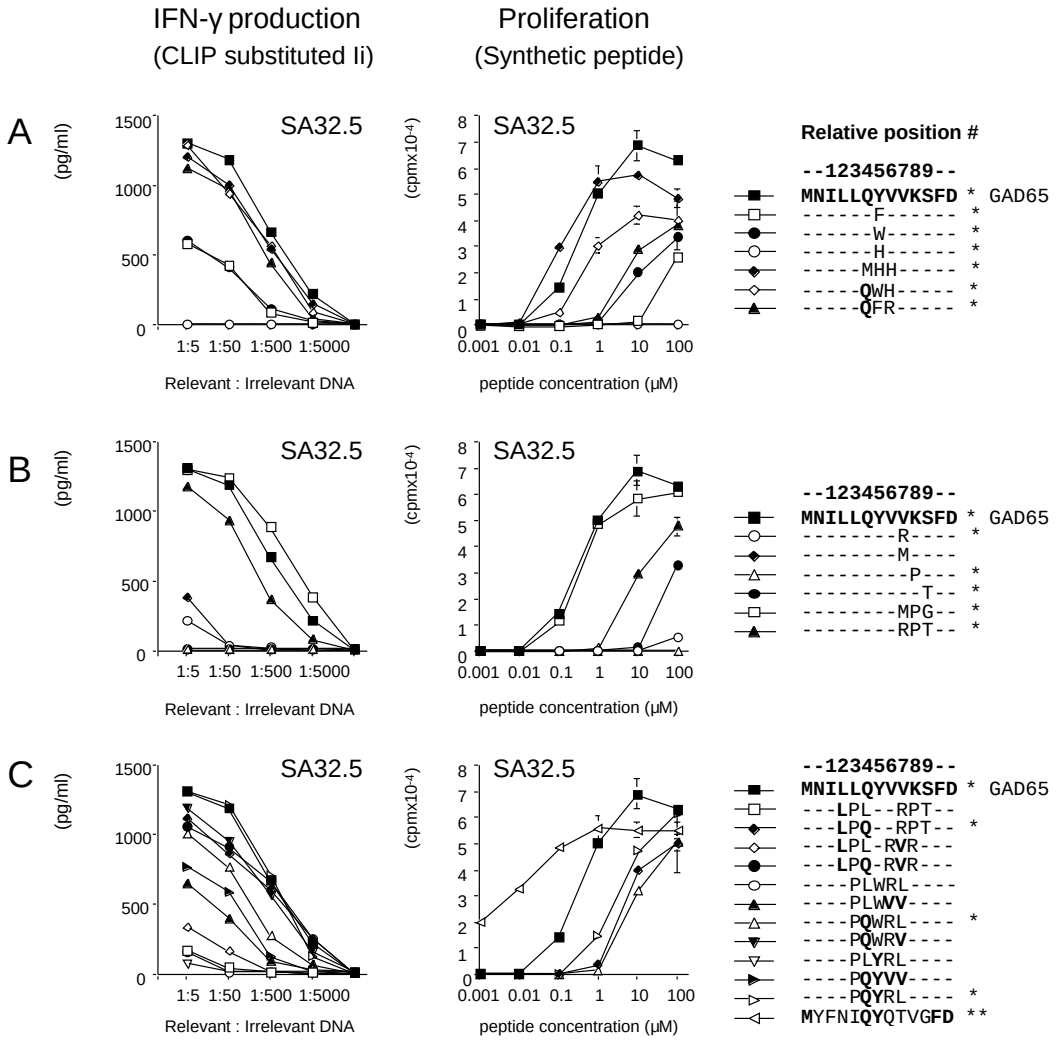


FIGURE 23. Combinatorial effects on TCR-recognition of multiple residues in antigenic peptide.
Left panels of A, B, and C: IFN-γ production stimulated by the CLIP substituted Ii-chain epi expression system in GAD65 p116-127 specific T cell clone SA32.5. All data are expresse mean value of duplicate determinations.
Right panels of A, B, and C: Proliferative responses of GAD65 p116-127 specific T cell clone SA32. to the synthetic peptides at indicated concentrations. Medium control response without peptide wer 200 cpm. All data are expressed as the mean value of triplicate determinations ± SD. Sequences CLIP-substituted peptides encoded for by mutated Ii-genes and synthetic peptides were indicated. Residues identical with GAD65 p115-127 are represented in boldface.
Single asterisks (*) indicate peptides tested in both transfection and synthetic peptide assay. I asterisks (**) indicate peptides tested only in proliferation assay to synthetic peptides.

FIGURE 24

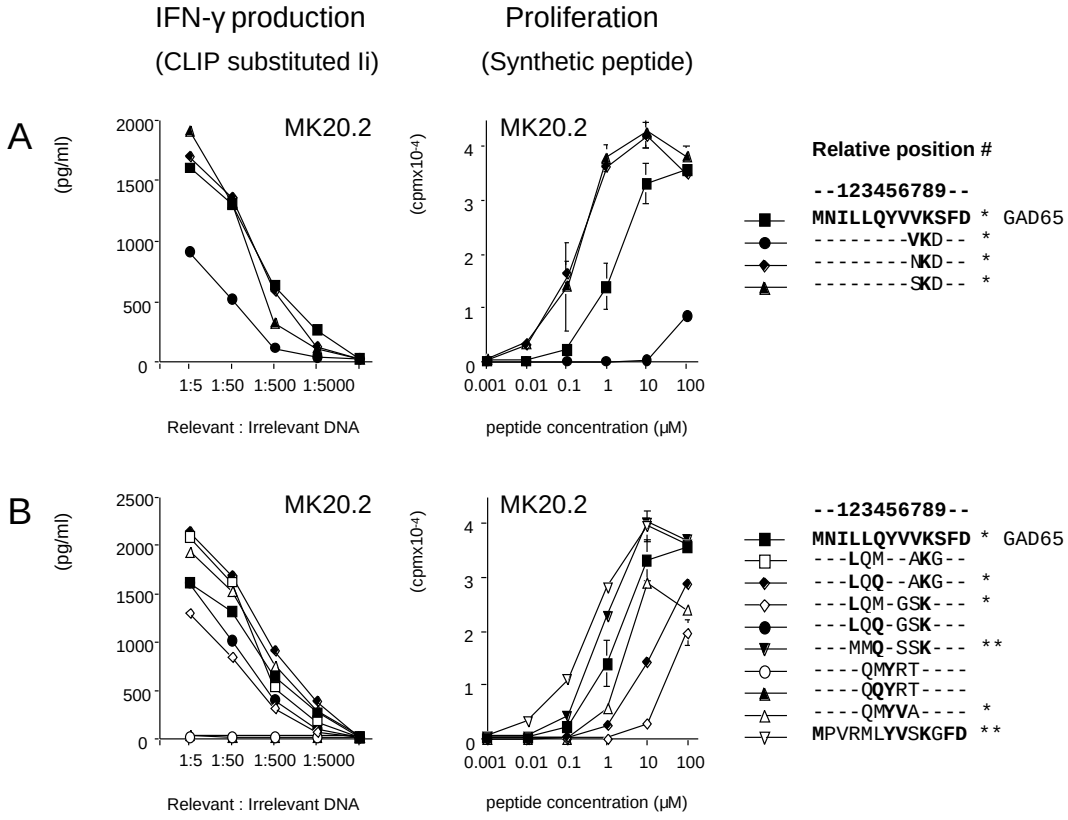


FIGURE 24. Combinatorial effects on TCR-recognition of multiple residues in antigenic peptide.

Left panels of A, and B: IFN-γ production stimulated by the CLIP substituted Ii-chain epitop expression system in GAD65 p116-127 specific T cell clone MK20.2. All data are expressed a the mean value of duplicate determinations.

Right panels of A, and B: Proliferative responses of GAD65 p116-127 specific T cell c MK20.2 to the synthetic peptides at indicated concentrations. Medium control response witho peptide were < 200 cpm. All data are expressed as the mean value of triplicate determinations SD. Sequences of CLIP-substituted peptides encoded for by mutated Ii-genes and sy peptides were indicated. Residues identical with GAD65 p115-127 are represented in boldface. Single asterisks (*) indicate peptides tested in both transfection and synthetic peptide Double asterisks (**) indicate peptides tested only in proliferation assay to synthetic peptides.

results obtained using epitope expression cloning strategy could generate superagonists.

These data strongly suggest that linear combinations of residues on antigenic peptides affect recognition by TCRs. Therefore, amino acid combinatorial effects should be considered when searching for crossreactive epitopes. We also suggest that T cell epitope expression cloning can provide the strategy for efficient identification of optimal sequences.

14. Identification of microbial and self mimics for GAD65 p115-127 autoreactive TCRs

The results obtained from T cell epitope expression library were used to establish recognition motifs for SA32.5 and MK20.2 TCRs. As shown in Fig. 25, amino acids with similarities in chemical characteristics and with successive stimulatory combination with residues obtained from library screening that may induce mimicry were considered to provide search criteria for peptide mimics of GAD65 (p115-127). As for the SA32.5 TCR, motifs consisting of amino acids L, I, and P at relative position 3, and Y, W, and F at relative position 5 were considered because these residues were critical for recognition by SA32.5 TCR (Fig. 22). With regard to the peptide C-terminal side (relative position 6-8), three kinds of search criteria were considered (Fig. 25A), because a successive combination of residues in the antigenic peptide may significantly affect the recognition by SA32.5 TCR. As for MK20.2 TCR, amino acids M, Q, I and L at relative position 3 with strong preference were considered. Amino acids Y at relative position 5 and K at relative position 8 were fixed on the basis of exquisite specificity of MK20.2 TCR (Fig. 25B).

To identify potential mimicry epitopes from natural proteins, a pattern match search for microbial and self proteins was done using SWISS-PROT (104559 protein entries) database and TrEMBL (560376 protein entries) database and ScanProsite program (http://www.expasy.ch/tools/scnpsit2.html; January 2002). Among all of the candidates conforming to the criteria, we selected 47 sequences with successive stimulatory combinations recognized by TCRs. In addition, with respect to the selection of microbial peptide, proteins derived from possible infectious pathogens were considered and listed (Table II). To determine whether these candidate peptides would activate T cell clones, we constructed CLIP substituted Ii expression vectors encoding these peptide candidates. These candidates were examined on their potential to stimulate production of IFN-γ by T cell clones using COS-7 cells expressing the CLIP-substituted Ii and HLA-DR53. Among the 47 candidates selected, SA32.5 responded to 7 candidates, *Lactococcus lactis*, *Streptococcus pyogenes*, *Neisseria meningitidis*, *Chlamydia pneumoniae*, *H. sapiens* / claudin-17, tafazzin, and tafazzin like protein-derived peptides. T cell clone MK20.2 responded to 5 candidates, *Legionella pneumophila*, *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Rickettsia prowazekii*, and Human herpesvirus 6 - derived peptides.

Based on the data obtained, we synthesized 12 candidate peptides and tested their capacity to stimulate T cell clones (Fig. 26). These peptides stimulated each T cell clone respectively and with different activity. Peptides derived from *Neisseria meningitidis*; putative dihydrolipoamide dehydrogenase (147-159) and self protein tafazzin (207-219) activated SA32.5 at concentrations of < 1nM despite a limited sequence homology to the native GAD65 sequence (Fig. 26A and B). Peptide mimics derived from *Legionella pneumophila*; pilus assembly protein PilB (39-51) and *Staphylococcus aureus*; hypothetical protein SAV0107 (227-239) activated MK20.2 at concentrations of < 100nM (Fig. 26C). In total, seven agonistic peptides derived from microbial or self protein (21.6% of the peptide mimic candidates conforming to the search criteria) were identified for SA32.5 and five agonistic peptides derived from microbial protein (25% of the peptide mimic candidates) were identified for MK20.2.

15. Crossreactivity of T cell clones SA32.5 and MK20.2 to naturally processed microbial antigens

To investigate whether these microbial peptide mimics identified by our library scanning can be naturally processed and presented, five recombinant proteins carrying mimicry epitopes were generated as GST-fusion proteins and tested for their capacity to induce proliferative responses of these T cell clones. As APCs we used dendritic cells generated from $CD14^+$ monocytes. As shown in Fig. 27, T cell clone SA32.5 responded to fusion proteins of putative

dihydrolipoamide dehydrogenase (101-205) derived from *Neisseria meningitidis* and O-succinylbenzoic acid-CoA ligase (101-201) derived from *Lactococcus lactis.* T cell clone MK20.2 responded to a fusion protein of glutamine amidotransferase, class I (1-104) derived from *Streptococcus pneumoniae*. However, fusion proteins derived from *Streptococcus pyogenes* and *Legionella pneumophila* did not activate either T cell clone. Furthermore, these responses were markedly inhibited by HLA-DR mAb L243 (data not shown). Crossreactivity of the SA32.5 T cell clone to fusion proteins carrying mimicry epitopes for MK20.2 were not observed, and vice versa. These data suggest that some of these microbial peptide mimics identified using our strategy can be naturally processed in DCs and presented to GAD65 specific $CD4^+$ T cell clones.

FIGURE 25

FIGURE 25. Summary of motifs recognized by TCRs for a database search of peptide mimics.

A. Motifs recognized by SA32.5 TCR for a database search.

B. Motifs recognized by MK20.2 TCR for a database search.

The search motifs were considered by amino acid preference in TCR recognition. Wi regard to SA32.5 TCR, three kinds of search motifs were considered depending on specific recognition properties. Search motifs are given in single letter amino acid co and X indicates all amino acids.

Table II.

1. Candidate selection for mimicry peptides

	Mimicry Candidates for T Cell Clone SA32.5			Mimicry Candidates for T Cell Clone MK20.2		
	No.	Sequence	Source	No.	Sequence	Source
		Relative position #			Relative position #	
		--123456789--			--123456789--	
		MNILLQYVVKSFD	*H. sapiens* / GAD65 115-127		**MNILLQYVVKSFD**	*H. sapiens* / GAD65 115-127
Motif 1	Y519	KNLLIQFVRNHYG	*Haemophilus influenzae*	M307	**KMSLLQYIVKNKI**	*Legionella pneumophila*
	Y520	DKNLIQFVVKGSN	*Borrelia burgdorferi*	M308	KTSLQQYGSKDQL	*Mycoplasma pneumoniae*
	Y521	TRLNPLWVRPVGS	Hepatitis G virus	M309	**DDIMMSYAAKGFV**	*Streptococcus pneumoniae*
Motif 2	Y531	LLSLPLYHVSGQG	*Haemophilus influenzae*	M310	**PYVLLAYIVKSVG**	*Staphylococcus aureus*
	Y532	LIVNPLYRLKGYG	*Bacillus subtilis*	M311	DAILQMYVSKFLF	*Mycoplasma capricolum*
	Y533	LATLLLWHVVGAT	*Mycobacterium avium*	M312	DTVLQMYGEKHAG	*Salmonella typhimurium*
	Y534	LGLMPLYHVVGFF	*Pseudomonas sp.*	M313	SIILMLYATKFFE	*Mycoplasma pulmonis*
	Y535	LLSLPLFHVSGQG	*Salmonella typhimurium*	M314	RKLLQAYGAKLVL	*Listeria innocua*
	Y536	**LVILPMFHVSGLS**	*Lactococcus lactis*	M315	**ELVLMSYRDKLVL**	*Rickettsia prowazekii*
	Y537	YNIMLQYRVKVES	*Vibrio cholerae*	M316	KYLRLVYGNKILS	*Prevotella ruminicola*
	Y538	AVLLPLYRLRQYA	*Bacillus halodurans*	M317	AQNRMSYSNKDYD	*Listeria monocytogenes*
	Y539	VQFNPQWQLALVA	*Pasteurella multocida*	M318	GESMMAYAVKGHR	*Pseudomonas aeruginosa*
	Y540	**REILPQYQLVILA**	*Streptococcus pyogenes*	M319	RDVMIAYATKAHV	*Streptococcus pneumoniae*
	Y541	KKNLLQWQTSADS	*Clostridium acetobutylicum*	M320	IAVRMAYSSKTPT	*Bacillus subtilis*
	Y542	QEFLISYRLKIVD	*Helicobacter pylori*	M321	TPMRLSYIEKKKG	*Salmonella typhimurium*
	Y543	**PVILPQWQSLGNR**	*Neisseria meningitidis*	M322	VNVMQTYTVKPGT	*Staphylococcus aureus*
	Y544	PKVLPQYQSLQNW	*Chlamydia pneumoniae*	M323	**KELLQSYVSKNNN**	Human herpesvirus 6
	Y545	**QAMNILYQTVQAF**	*Chlamydia pneumoniae*	M324	MRNMLQYVSKNLD	Orf virus
	Y546	WLMNPLFRLISKA	*Haemophilus influenzae*	M325	IADLQQYRNKLET	Human rotavirus
	Y547	SFVLPLFRVAALL	*Pseudomonas aeruginosa*	M326	GEVRQAYGAKGFS	*H. sapiens* / Glypican-6 precursor 31-43
	Y548	RPINPLFHILVET	*Streptococcus pneumoniae*			
	Y549	ILMLLQFRVLDRR	*Brucella melitensis*			
	Y550	YLINPMFRIIANT	*Brucella melitensis*			
	Y551	**TTLLPQWRVSAFV**	*H. sapiens* / Claudin-17 24-36			
	Y552	**PIILPLWHVGMND**	*H. sapiens* / Tafazzin 207-219			
	Y553	**PIILPLWHVGEPG**	*H. sapiens* / Tafazzin like protein 138-150			
Motif 3	Y554	QYVMLQFTVKERP	*Treponema paraluiscuniculi*			

Of the peptide mimicry candidates we obtained from a pattern match search, sequences with stimulatory combinations of three successive amino acids for recognition by TCRs were selected. Candidates for mimicry peptide sequences are given in a single letter amino acid code aligned with GAD65 p115-127. Sequences stimulating significant IFN-γ production (>200 pg/ml) determined using the CLIP-substituted epitope expression system are represented in boldface.

FIGURE 26

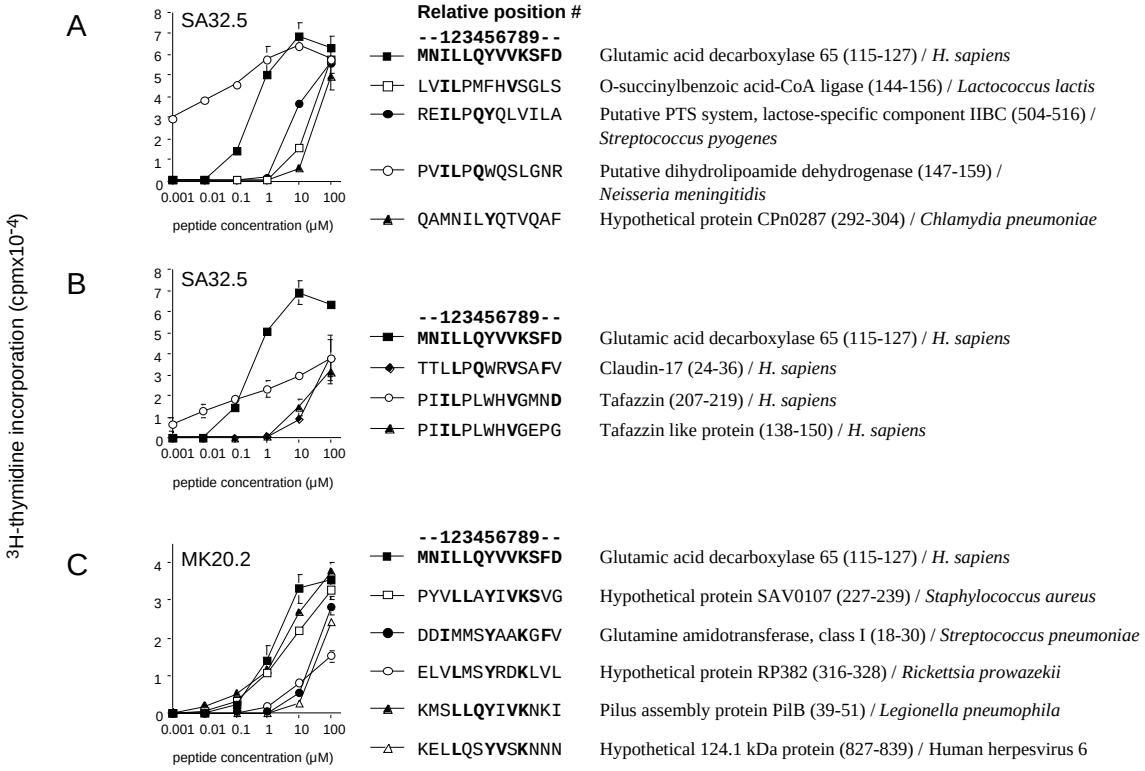


FIGURE 26. Proliferative response of GAD65 p116-127 specific T cell clones to the mimicry peptides in comparison with GAD65 p115-127.

A. Response of SA32.5 to microbial mimicry peptides.

B. Response of SA32.5 to self mimicry peptides.

C. Response of MK20.2 to microbial mimicry peptides.

Sequences of mimicry peptides are indicated and residues identical with GAD65 p115-127 are represente in boldface. Medium control responses without peptide were < 200 cpm. All data are expressed as the mea value of triplicate determinations ± SD.

FIGURE 27

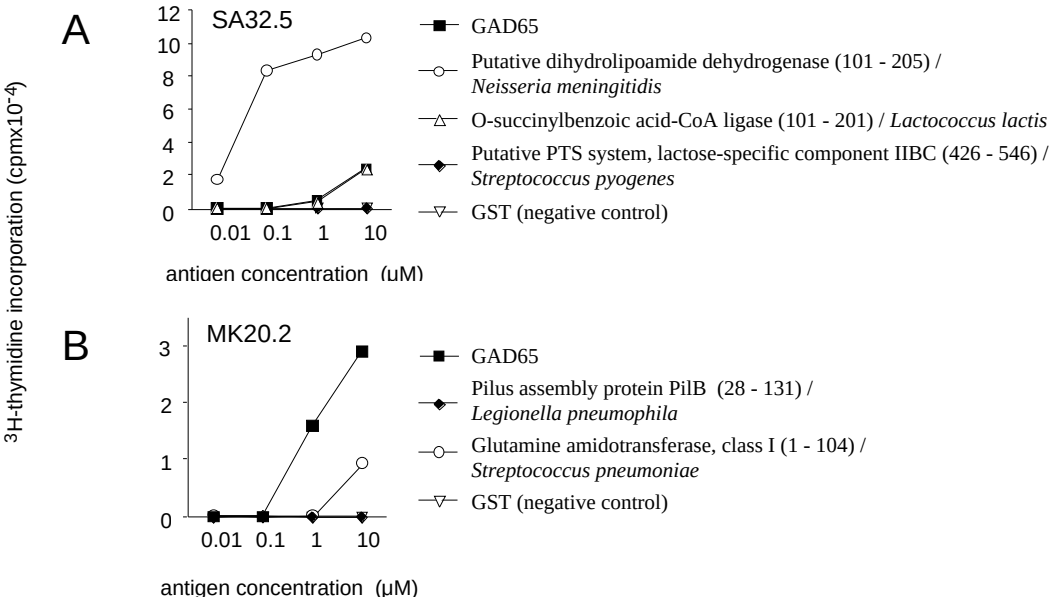


FIGURE 27. Proliferative response of GAD65 p116-127 specific T cell clones to recombinant proteins carrying the mimicry peptides in comparison with GAD65 protein.
A. Response of SA32.5 to microbial proteins: putative dihydrolipoamide dehydrogenase (101 - 205) *Neisseria meningitidis*, O-succinylbenzoic acid-CoA ligase (101 - 201) / *Lactococcus lactis*, and putative PTS system, lactose-specific component IIBC (426 - 546) / *Streptococcus pyogenes.*
B. Response of MK20.2 to microbial proteins: glutamine amidotransferase, class I (1 - 104) / *Streptococcus pneumoniae* and pilus assembly protein PilB (28 - 131) / *Legionella pneumophila*. Medium control responses without recombinant protein were < 150 cpm. Data are expressed as the mean value of duplicate determinations ± SD.

Discussion

1. Establishment of an expression cloning system for CD4$^+$ T cell epitopes.

In our first study, we established epitope expression cloning system for CD4$^+$ T cells. In the epitope library, randomized 13-mer peptides were produced and presented in the context of HLA class II molecules co-expressed in COS-7 cells. The use of an invariant chain with targeting signals to endosome is a pertinent strategy for antigen presentation to CD4$^+$ T cells (100). This approach by which multiple residues were simultaneously randomized has a great advantage to produce overall conformation of the peptide/HLA-class II complexs, and increases the possibility of finding degenerate sequences with agonistic properties. Plasmid clones encoding epitopes agonistic to CD4$^+$ T cells were identified by measuring IFN-γ produced by the stimulated T cells. The sensitivity of the screening is sufficient to detect one agonistic epitope from 500-1000 irrelevant sequences in one well of a 96-well culture plate. Using this system, we identified a cross-reactive epitope, which stimulates a Type I diabetes-related GAD-65-reactive human CD4$^+$ T cell clone.

Boen et al. reported a method similar to the present system, using a library of peptides covalently attached to the β chain of HLA-DR (101). From the library, they identified novel cross-reactive epitopes for an influenza hemagglutinin specific human CD4$^+$ T cell clone. Compared to their system, the library of peptides presented by recombinant Ii chains has some advantage in that, once produced, the library can be applicable to any TCRs whatever the restriction molecules. In addition, the present system is simpler in that T cell clones can be used for screening with sufficient sensitivity, while a T cell hybridoma introduced with a human-mouse-chimeric TCR and mouse ICAM-1 was used in their system (101).

We isolated the plasmid clone pCIGm1, which stimulated the CD4$^+$ T cell clone SA 32.5. The encoded amino acid sequence substituting for Ii p89-102 of CLIP in this plasmid clone was QLSNQWHVVGATF. The synthetic peptide of this sequence did not stimulate proliferative responses of the T cell clone, but the synthetic peptide, PVQLSNQWHVVGA, which included two CLIP-flanking residues at the N-terminus, was agonistic to the T cell clone. Because we have not definitely determined the MHC-anchor positions in the original GAD65 epitope and the newly identified epitope, we cannot precisely compare the structural similarity between these two epitopes. However, the newly identified epitope differs apparently from the original epitope NILLQYVVKSFDR, and is not deducible using currently available methods based on synthetic peptides.

2. Systematic analysis of the combinatorial nature of epitopes recognized by GAD65 specific TCRs.

In the succeeding study, we developed a novel strategy to analyze the combinatorial effects of residues in the antigenic peptides on recognition by TCRs. It allows for systematic separation and identification of diverse T cell epitopes from a mixture of randomized peptides. We showed that the degree of combinatorial effects differs depending on part of the antigenic peptide and structure of TCRs, even when the epitope and restriction molecule are identical, and the combinatorial effects can be classified by analyzing the epitope sequences with agonistic properties. We searched protein databases with the defined search criteria incorporating combinatorial effect, and we identified mimicry epitopes of microbial origin, which stimulate GAD65 autoreactive T cell clones established from Type I diabetes patients. The findings demonstrate the importance of combinatorial effects of each amino acid residue in the antigenic peptide on TCR recognition and propose a new direction for examining crossreactive epitopes of TCR in investigating autoimmunity.

Because of the importance of CD4$^+$ T cells in autoimmunity, much effort has been directed toward identifying cross-reactive epitopes of microbial antigens recognized by autoreactive CD4$^+$ T cells. So far, cross-reactive epitopes have been predicted and identified by primary sequence homology, the data obtained from single residue substituted peptide analogues, or PS-SCLs (42, 51, 67-72). The majority of these approaches using synthetic peptides have been fundamentally based on the concept that antigen-recognition surface of TCRs is relatively flat and each amino acid on each position of the peptide independently contributes to recognition by TCR (73). On the basis

of this assumption, mimicry epitopes have been searched in protein databases using pattern match searching with information on suitable amino acids for each position of the peptide. The use of PS-SCLs for analyses of T cell recognition has yielded the many useful concepts. However, some T cell clones did not respond to PS-SCLs (74) or responded to PS-SCLs ambiguously (70). In addition, artificial peptides composed of the optimal residue for each position selected based on analysis with PS-SCLs do not necessarily show agonistic activity, as we previously described (74). The major drawback of this method is that it identifies only the relative importance of each amino acid for each position of the peptide, and one could not directly analyze the agonistic activity induced by combinations of residues in antigenic peptides.

To address these problems, we used a totally different approach. The novelty of this system is that it enables one to directly identify sequences of T cell epitopes from plasmid-based epitope libraries composed of thousands of randomized sequences. Based on the accumulated information on sequences of agonistic peptides, one can determine the combinations of residues with agonistic properties. We used two different GAD65-specific T cell clones, SA32.5 and MK20.2, expressing distinct TCR but recognizing the same epitope with the same restriction element (84). At first, we verified that two T cell clones expressed single TCR, then we identified epitopes agonistic to each of these two TCRs from the epitope libraries and compared their sequences.

It is clear that TCRs of SA32.5 and MK20.2 represent distinct crossreactivity and different recognition profiles (Fig. 22A and B). It was observed that SA32.5 TCR tolerates structurally related amino acids at position 5 only when combined with specific adjacent residues (Fig. 22B and 23B). The patterns of the recognition profiles significantly differ depending on where randomized residues had been inserted. These findings were observed mainly on the peptide C-terminal side (Fig. 22A and 23B). Also, overall combinations of residues in the antigenic peptide affect the recognition by SA32.5 TCR more significantly than that by MK20.2 TCR (Fig 22B and 23A-C). Notably SA32.5 TCR permits the exchange of residues at all of the positions, as reported (102). In contrast, MK20.2 TCR permits specifically Y and K for relative positions 5 and 8, respectively, even when residues around them are randomly exchanged (Fig. 22B). These residues are also conserved in the original GAD65 p115-127. However, even in the case of this TCR, significant amino acid combinatorial effects were observed in some positions (Fig. 22A, 24A).

3. Structural aspect of GAD65 autoreactive TCRs

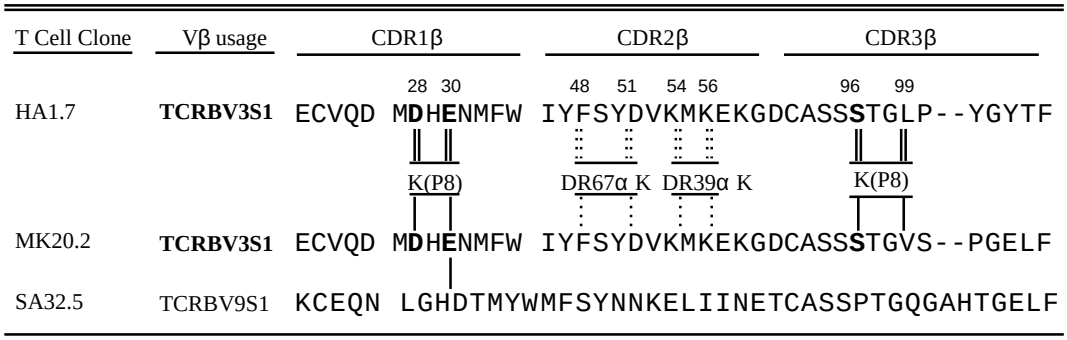
As shown in Table III, MK20.2 had the same Vβ3.1 usage as did HA1.7 and it was reported that Vβ3.1 was predominantly expressed in HA p306-318 specific TCRs which preferred P8 K of HA 306-318 (18). Molecular modeling (Fig. 28A) of the fit of MK20.2 TCR to the recent crystal structure of HA1.7 TCR-HA peptide/HLA-DR1 complex (18) was done. As shown in Fig. 28, the model predicted that the D28β and E30β of CDR1 loop of TCRVβ3.1 made a charged interaction with K at position 8 of HLA-DR53-bound GAD65 p115-127 peptide (Fig. 28B), thus implying the exclusive specificity for K at relative position 8. In contrast with BV3S1, BV9S1 of SA32.5 TCR uses the small amino acid G in 28β and the negatively charged amino acid of D in 30β (Table III), which would weaken electrostatic interactions toward K at position 8. This observation seems consistent with the finding that SA32.5 TCR did not have a strong preference for K at position 8 (Fig. 22A).

4. Peptide binding properties of HLA-DR53 molecule.

The HLA-DR53 binding motif has not yet been determined and only a few peptides with binding affinity to HLA-DR53 molecule have been reported. However, the data obtained in our study led us to speculate on the peptide binding properties to HLA-DR53 molecules. In a recent study on HLA-DR53 binding peptides, the molecule did not show any amino acid preference in P1 position (103). In fact, although HLA-DR53 possess pocket-1 consisting of V at DRβ86, which commonly accommodates aliphatic residues, it permits K and Y (103). These results are consistent with our finding that relative position 1 permits several residues with different chemical properties (Fig. 18). However, the peptide sequences isolated from the epitope expression library indicate that relative position 1 preferred aliphatic residues. A more comprehensive scan may yield the information of this issue. In celiac disease, the disease susceptible HLA-DR53 molecule selectively binds a large number of gliadin-derived peptides that are

Table III.

Comparison of CDR sequences of TCRβ chains of GAD65 p115-127 specific MK20.2, SA32.5 and HA p306-318 specific HA1.7



As determined in a crystallographic analysis (18), interactions between residues that provide TCR-DR binding sites between α-helical structure of DRα chain and CDR2 region of HA1.7 TCR are lined with dotted doublet lines. Electrostatic interactions between P8K of HA (p306-318) and CDR region of HA1.7 are indicated by solid doublet lines. Predicted interactions between residues of CDR region of MK20.2 or SA32.5 TCR and those of the GAD65 (p115-127)/DR53 complex are indicated and lined.

FIGURE 28

HA p306-318 PKYVKQNTLKLAT
GAD65 p115-127 MNILLQYVVKSFD

FIGURE 28. Structure of HA1.7 TCR-HA/DR1 complex (A) and molecular modeling of MK20.2 TCR-GAD65/DR53 complex (B).

A. Structure of the HA1.7 TCR-HA/DR1 complex (18) and relative orientation of the CDR lo HA1.7 TCR on top of HA/DR1 complex. CDR loops of TCRαVand Vβ chains are displayed in tube and colored in yellow and orange, respectively. HA p306-318 peptide is shown in a ball-and-stick mod and its TCR binding residues are in red and the others in gray. DRα-chain is in cyan and theβ-chain in blue.

B. Molecular modeling of predicted electrostatic interactions between P8 lysine of GAD65 p115-127 an acidic residues of MK20.2 TCR. Here the homology model of MK20.2 TCR-GAD65/DR53 complex was based on the above HA1.7 TCR-HA/DR1 complex. The residues on CDR1 and 3 of MK20.2 TCβ chain are in orange. The GAD65 peptide is shown in a ball-and-stick model and P8K is in green DR53 α-chain is displayed in cyan. Electrostatically active atoms are shown in red and blue (negative and positively charged respectively). Numbers represent the position of relevant side chain resi Hydrogen bonding and electrostatic interactions are shown as dotted yellow lines. This figure was produced by BOBSCRIPT (119) and Raster 3D (120). The peptide sequences of HA 306-308 and GAD65 115-127 are given in single letter amino acid code.

extremely rich in Q and P (104). Accordingly, it was suggested that one or more peptide binding pockets of HLA-DR53 molecules preferentially engaged the side chain of Q or P. In the TCR recognition motif of the T cell clone SA32.5, P at relative position 3 and Q at relative position 4 and 6 are the most important residues for TCR agonism (Fig. 22A). Although the two TCRs represented a distinct specificity at relative positions 3, 5, 7, or 8 of the peptides, SA32.5 TCR and MK20.2 TCR represented similar preferences for certain amino acids at some positions. For example, at relative position 1, both TCR tolerate F, M, L, I, V, and C, at relative position 4 they tolerate L, M, Q, and S, and at the relative position 6 neutrally or positively charged residues and small aliphatic amino acids are allowed (Fig. 22). These residues may be HLA-DR53 anchor residues, however this remains to be elucidated. It is conceivable that findings observed in this study also reflect peptide binding characteristics in which anchor combinations are important for HLA binding, as described (105). Not only requisition for direct TCR contact but also constraints of HLA-DR53 binding could explain the combinatorial effect observed on the peptide C-terminal side, i. e. a positively charged amino acid is required at 122-124 in combination with a bulky amino acid at 121 for the recognition by SA32.5 TCR.

5. Identification of candidate molecular mimics and it's epidemiologic correlations.

Several groups of investigators have reported epidemiologic correlations between infection and Type I diabetes (106, 107). Among the cross-reactive ligands identified in this study, epitopes derived from *N. meningitidis, S. pneumoniae,* and *L. lactis* can actually be produced from proteins by APCs to stimulate the T cells (Fig. 27). *N. meningitidis* is a Gram-negative and pathogenic bacterium. The outcome of meningococcal infection ranges from asymptomatic carriage to meningitis and fulminant meningococcaemia in children and young people (108). *S. pneumoniae* is a Gram-positive and pathogenic bacterium, which causes bacterial sepsis, pneumonia, meningitis, and otitis media in young children (109). *N. meningitidis* and *S. pneumoniae* that are also human respiratory commensals in common can spread in the bloodstream under an immune compromised state etc. It is conceivable that these normally residing as harmless commensals may spread and participate in priming of autoreactive T cells and increase memory pools in the periphery. Also, asymptomatic carriages may predispose genetically susceptible children and initiate the autoimmune process. *L. lactis* is a nonpathogenic Gram-positive bacterium, used to produce fermented foods and dairy goods. When orally taken with food, this bacterium is metabolically active in all compartments of the intestinal tract (110). It is presumed to be taken up by M cells in intestinal lymphoid organs and to sensitize T cells. Although its relationship to autoimmune process has yet to be reported, it is intriguing in consideration with a recent report that short-term breastfeeding and the early introduction of cow's milk-based infant formula can predispose genetically susceptible children to Type I diabetes (111).

6. Structural mimicry; it's implication to clinical medicine.

In the past few years, it has been considered that application of APLs to down regulate the responses of pathogenic T cells would be of therapeutic value in treating subjects with autoimmune disease (112-114). However, it is difficult to find APLs of self-antigenic peptides that serve no risk in activating autoreactive T cells. Recent clinical trials with an APL of MBP p85-99 for MS patients indicate that a high dose of an APL induced MBP reactive T cells, at a high frequency, resulting in exacerbation of MRI detectable lesions. In contrast, a low dose of an APL led to immune deviation toward increases in IL-4 secretion by MBP reactive T cells, with no progression in lesions (115, 116). This observation suggests that engagement of diverse TCRs with single APL derived from a self-antigen yield a variety of substantial cross-reactivities to a self-antigen. Although monoclonal or oligoclonal expansion of autoreactive T cells has proven to play a central role in the pathogenesis of autoimmunity, T cell populations which recognize certain self-antigens are relatively somewhat more diverse. Thus an absolutely different sequence that mimics self-antigen may be a key epitope that activates a limited fraction of autoreactive T cells. Our findings indicate that cross-reactivity of TCR can be triggered by the peptide with unexpectedly no resemblance to the epitope of autoantigen.

Recently, the mode of recognition by single autoreactive TCR of two independent peptides in the context

of two different DR2 molecules was structurally presented (117). The T cell clone established from an MS patient cross-recognized MBP p85-99 in the context of DRB1*1501 and EBV DNA polymerase p627-641 peptide in the context of DRB5*0101, both DRB alleles are in a strong linkage disequilibrium and are associated with susceptibility to MS. This finding not only provides importance for molecular mimicry involving antigenic peptides but also supports the structural mimicry generated by peptide-HLA-class II interactions.

Conclusion

Recent elucidation of three-dimensional structures of TCR-peptide/HLA-DR1 complex provide a structural basis for antigen recognition by HLA-class II restricted TCR. Flexibility in recognition by MHC-class I restricted TCR could also be structurally explained by large conformational changes of three CDR loops on binding to the ligand (118). Also similar changes in CDR loops of MHC-class II restricted TCR on binding can be expected. The use of synthetic peptides in analyses of TCR recognition has provided a large amount of useful concepts in T cell recognition. Using a set of peptide analogues, it has become clear that many modifications of the antigenic peptide are tolerated. Using an extensive PS-SCLs, a high degree of degeneracy in TCR recognition has become evident. Although degeneracy in antigen recognition by TCR becomes effective to protect against infection in a limited T cell repertoire and to maintain an immune system to adopt with a continuously changing antigenic environment, it eventually may cause autoimmunity through molecular mimicry. Heretofore, conformational changes in peptides on the groove of MHC class II molecules caused by binding could not have been predicted. This was also the case even if PS-SCLs were adapted. We clearly showed that our epitope expression library system using CLIP-substituted Ii-genes provides an entirely new strategy not only for identification of the crossreactive epitopes for $CD4^+$ T cells of known specificity, but also for detection of epitopes stimulatory for $CD4^+$ T cells, the epitopes of which are unknown. Also, we established a novel system to analyze the combinatorial effects of residues in the antigenic peptide on recognition by HLA-class II restricted TCR. Importantly in analyzing molecular mimicry, this system deciphers overall conformations within epitopes on TCR recognition. The system also holds important prospects for providing a greater understanding of recognition mode in HLA-class II restricted TCR that could lead to preventive and therapeutic approaches.

References

1. Germain RN. MHC-dependent antigen processing and peptide presentation: providing ligands for T lymphocyte activation. *Cell 1994; 76(2):287-99.*
2. Watts C. Capture and processing of exogenous antigens for presentation on MHC molecules. *Ann. Rev. Immunol. 1997; 15:821-50.*
3. Mellman I. Endocytosis and molecular sorting. *Ann. Rev. Cell Dev. Biol. 1996; 12:575-625.*
4. Ghosh P, Amaya M, Mellins E, Wiley DC. The structure of an intermediate in class II MHC maturation: CLIP bound to HLA-DR3. *Nature 1995; 378(6556):457-62.*
5. Cresswell P. Invariant chain structure and MHC class II function. *Cell 1996; 84(4):505-7.*
6. Cresswell P. Assembly, transport, and function of MHC class II molecules. *Ann. Rev. Immunol. 1994; 12:259-93.*
7. Roche PA, Marks MS, Cresswell P. Formation of a nine-subunit complex by HLA class II glycoproteins and the invariant chain. *Nature 1991; 354(6352):392-4.*
8. Rammensee HG, Friede T, Stevanoviic S. MHC ligands and peptide motifs: first listing. *Immunogenetics 1995; 41(4)):178-228.*
9. Hammer J, Valsasnini P, Tolba K, Bolin D, Higelin J, Takacs B, Sinigaglia F. Promiscuous and allele-specific anchors in HLA-DR-binding peptides. *Cell 1993; 74(1)):197-203.*
10. Hammer J, Takacs B, Sinigaglia F. Identification of a motif for HLA-DR1 binding peptides using M13 display libraries. *J. Exp. Med. 1992; 176(4):1007-13.*
11. Sturniolo T, Bono E, Ding J, Raddrizzani L, Tuereci O, Sahin U, Braxenthaler M, Gallazzi F, Protti MP, Sinigaglia F, Hammer J. Generation of tissue-specific and promiscuous HLA ligand databases using DNA microarrays and virtual HLA class II matrices. *Nat. Biotech. 1999; 17(6):555-61.*
12. Stern LJ, Brown JH, Jardetzky TS, Gorga JC, Urban RG, Strominger JL, Wiley DC. Crystal structure of the human class II MHC protein HLA-DR1 complexed with an influenza virus peptide. *Nature 1994; 368(6468)):215-21.*
13. Matsushita S, Takahashi K, Motoki M, Komoriya K, Ikagawa S, Nishimura Y. Allele specificity of structural requirement for peptides bound to HLA-DRB1*0405 and -DRB1*0406 complexes: implication for the HLA-associated susceptibility to methimazole-induced insulin autoimmune syndrome. *J. Exp. Med. 1994; 180(3)):873-83.*
14. Arden B, Clark SP, Kabelitz D, Mak TW. Human T-cell receptor variable gene segment families. *Immunogenetics 1995; 42(6):455-500.*
15. Hennecke J, Wiley DC. T cell receptor-MHC interactions up close. *Cell 2001; 104(1):1-4.*
16. Ding YH, Baker BM, Garboczi DN, Biddison WE, Wiley DC. Four A6-TCR/peptide/HLA-A2 structures that generate very different T cell signals are nearly identical. *Immunity 1999; 11(1):45-56.*
17. Reinherz EL, Tan K, Tang L, Kern P, Liu J, Xiong Y, Hussey RE, Smolyar A, Hare B, Zhang R, Joachimiak A, Chang HC, Wagner G, Wang J. The crystal structure of a T cell receptor in complex with peptide and MHC class II. *Science 1999; 286(5446):1913-21.*
18. Hennecke J, Carfi A, Wiley DC. Structure of a covalently stabilized complex of a human alphabeta T-cell receptor, influenza HA peptide and MHC class II molecule, HLA-DR1. *EMBO J. 2000; 19(21):5611-24.*
19. Bakke O, Dobberstein B. MHC class II-associated invariant chain contains a sorting signal for endosomal compartments. *Cell 1990; 63(4):707-16.*
20. Pond L, Kuhn LA, Teyton L, Schutze MP, Tainer JA, Jackson MR, Peterson PA. A role for acidic residues in di-leucine motif-based targeting to the endocytic pathway. *J. Biol. Chem. 1995; 270(34):19989-97.*
21. Amigorena S, Drake JR, Webster P, Mellman I. Transient accumulation of new class II MHC molecules in a novel endocytic compartment in B lymphocytes *Nature 1994; 369(6476):113-20.*
22. Peters PJ, Neefjes JJ, Oorschot V, Ploegh HL, Geuze HJ. Segregation of MHC class II molecules from MHC

class I molecules in the Golgi complex for transport to lysosomal compartments. *Nature 1991; 349(6311):669-76.*

23. Tulp A, Verwoerd D, Dobberstein B, Ploegh HL, Pieters J. Isolation and characterization of the intracellular MHC class II compartment. *Nature 1994; 369(6476):120-6.*
24. Denzin LK, Cresswell P. HLA-DM induces CLIP dissociation from MHC class II alpha beta dimers and facilitates peptide loading. *Cell 1995; 82(1):155-65.*
25. Sloan VS, Cameron P, Porter G, Gammon M, Amaya M, Mellins E, Zaller DM. Mediation by HLA-DM of dissociation of peptides from HLA-DR. *Nature 1995; 375(6534)):802-6.*
26. Todd JA, Acha-Orbea H, Bell JI, Chao N, Fronek Z, Jacob CO, McDermott M, Sinha AA, Timmerman L, Steinman L. A molecular basis for MHC class II--associated autoimmunity. *Science 1988; 240(4855):1003-9.*
27. Goldrath AW, Bevan MJ. Selecting and maintaining a diverse T-cell repertoire. *Nature 1999; 402(6759):255-62.*
28. Horwitz MS, Sarvetnick N. Viruses, host responses, and autoimmunity. *Immunol. Rev. 1999; 169:241-53.*
29. Kadowaki N, Ho S, Antonenko S, Malefyt RW, Kastelein RA, Bazan F, Liu YJ. Subsets of human dendritic cell precursors express different toll-like receptors and respond to different microbial antigens. *J. Exp. Med. 2001; 194(6):863-9.*
30. Mellman I, Steinman RM. Dendritic cells: specialized and regulated antigen processing machines. *Cell 2001; 106(3):255-8.*
31. Rescigno M, Borrow P. The host-pathogen interaction: new themes from dendritic cell biology. *Cell 2001; 106(3):267-70.*
32. Lanzavecchia A, Sallusto F. Regulation of T cell immunity by dendritic cells. *Cell 2001; 106(3):263-6.*
33. Liu YJ. Dendritic cell subsets and lineages, and their functions in innate and adaptive immunity. *Cell 2001; 106(3):259-62.*
34. Lehmann PV, Forsthuber T, Miller A, Sercarz EE. Spreading of T-cell autoimmunity to cryptic determinants of an autoantigen. *Nature 1992; 358(6382):155-7.*
35. Miller SD, Vanderlugt CL, Begolka WS, Pao W, Yauch RL, Neville KL, Katz-Levy Y, Carrizosa A, Kim BS. Persistent infection with Theiler's virus leads to CNS autoimmunity via epitope spreading. *Nat. Med. 1997; 3(10):1133-6.*
36. Horwitz MS, Bradley LM, Harbertson J, Krahl T, Lee J, Sarvetnick N. Diabetes induced by Coxsackie virus: initiation by bystander damage and not molecular mimicry. *Nat. Med. 1998; 4(7):781-5.*
37. Scherer MT, Ignatowicz L, Winslow GM, Kappler JW, Marrack P. Superantigens: bacterial and viral proteins that manipulate the immune system. *Ann. Rev. Cell Biol. 1993; 9:101-28.*
38. Brocke S, Gijbels K, Allegretta M, Ferber I, Piercy C, Blankenstein T, Martin R, Utz U, Karin N, Mitchell D, Veromaa T, Waisman A, Gaur A, Conlon P, Ling N, Fairchild PJ, Wraith DC, O'Garra A, Fathman CG, Steinman L. Treatment of experimental encephalomyelitis with a peptide analogue of myelin basic protein. *Nature 1996; 379(6563):343-6.*
39. Dalwadi H, Wei B, Kronenberg M, Sutton CL, Braun J. The Crohn's disease-associated bacterial protein I2 is a novel enteric t cell superantigen. *Immunity 2001; 15(1):149-58.*
40. Oldstone MB. Molecular mimicry and immune-mediated diseases. *FASEB J. 1998; 12(13):1255-65.*
41. Fujinami RS, Oldstone MB. Amino acid homology between the encephalitogenic site of myelin basic protein and virus: mechanism for autoimmunity. *Science 1985; 230(4729):1043-5.*
42. Wucherpfennig KW, Strominger JL. Molecular mimicry in T cell-mediated autoimmunity: viral peptides activate human T cell clones specific for myelin basic protein. *Cell 1995; 80(5)):695-705.*
43. Oldstone MB, Nerenberg M, Southern P, Price J, Lewicki H. Virus infection triggers insulin-dependent diabetes mellitus in a transgenic model: role of anti-self (virus) immune response. *Cell 1991; 65(2):319-31.*
44. Sigal LH. Immunologic mechanisms in Lyme neuroborreliosis: the potential role of autoimmunity and

molecular mimicry. *Sem. Neurol. 1997; 17(1):63-8.*

45. Gross DM, Forsthuber T, Tary-Lehmann M, Etling C, Ito K, Nagy ZA, Field JA, Steere AC, Huber BT. Identification of LFA-1 as a candidate autoantigen in treatment-resistant Lyme arthritis. *Science 1998; 281(5377):703-6.*
46. Zhao ZS, Granucci F, Yeh L, Schaffer PA, Cantor H. Molecular mimicry by herpes simplex virus-type 1: autoimmune disease after viral infection. *Science 1998; 279(5355):1344-7.*
47. Panoutsakopoulou V, Sanchirico ME, Huster KM, Jansson M, Granucci F, Shim DJ, Wucherpfennig KW, Cantor H. Analysis of the relationship between viral infection and autoimmune disease. *Immunity 2001; 15(1):137-47.*
48. Bachmaier K, Neu N, de la Maza LM, Pal S, Hessel A, Penninger JM. Chlamydia infections and heart disease linked through antigenic mimicry. *Science 1999; 283(5406):1335-9.*
49. Olson JK, Croxford JL, Calenoff MA, Dal Canto MC, Miller SD. A virus-induced molecular mimicry model of multiple sclerosis. *J. Clin. Invest. 2001; 108(2):311-8.*
50. Oldstone MB. Molecular mimicry and autoimmune disease. *Cell 1987; 50(6):819-20.*
51. Grogan JL, Kramer A, Nogai A, Dong L, Ohde M, Schneider-Mergener J, Kamradt T. Cross-reactivity of myelin basic protein-specific T cells with multiple microbial peptides: experimental autoimmune encephalomyelitis induction in TCR transgenic mice. *J. Immunol. 1999; 163(7):3764-70.*
52. Hemmer B, Vergelli M, Pinilla C, Houghten R, Martin R. Probing degeneracy in T-cell recognition using peptide combinatorial libraries. *Immunol. Today 1998; 19(4):163-8.*
53. Hemmer B, Fleckenstein BT, Vergelli M, Jung G, McFarland H, Martin R, Wiesmuller KH. Identification of high potency microbial and self ligands for a human autoreactive class II-restricted T cell clone. *J. Exp. Med. 1997; 185(9)):1651-9.*
54. De Magistris MT, Alexander J, Coggeshall M, Altman A, Gaeta FC, Grey HM, Sette A. Antigen analog-major histocompatibility complexes act as antagonists of the T cell receptor. *Cell 1992; 68(4):625-34.*
55. Racioppi L, Ronchese F, Matis LA, Germain RN. Peptide-major histocompatibility complex class II complexes with mixed agonist/antagonist properties provide evidence for ligand-related differences in T cell receptor-dependent intracellular signaling. *J. Exp. Med. 1993; 177(4):1047-60.*
56. Sloan-Lancaster J, Evavold BD, Allen PM. Induction of T-cell anergy by altered T-cell-receptor ligand on live antigen-presenting cells. *Nature 1993; 363(6425):156-9.*
57. Sloan-Lancaster J, Evavold BD, Allen PM. Th2 cell clonal anergy as a consequence of partial activation. *J. Exp. Med. 1994; 180(4):1195-205.*
58. Evavold BD, Allen PM. Separation of IL-4 production from Th cell proliferation by an altered T cell receptor ligand. *Science 1991; 252(5010):1308-10.*
59. Soloway P, Fish S, Passmore H, Gefter M, Coffee R, Manser T. Regulation of the immune response to peptide antigens: differential induction of immediate-type hypersensitivity and T cell proliferation due to changes in either peptide structure or major histocompatibility complex haplotype. *J. Exp. Med. 1991; 174(4):847-58.*
60. Kersh GJ, Kersh EN, Fremont DH, Allen PM. High- and low-potency ligands with similar affinities for the TCR: the importance of kinetics in TCR signaling. *Immunity 1998; 9(6):817-26.*
61. Chen YZ, Matsushita S, Nishimura Y. Response of a human T cell clone to a large panel of altered peptide ligands carrying single residue substitutions in an antigenic peptide: characterization and frequencies of TCR agonism and TCR antagonism with or without partial activation. *J. Immunol 1996; 157(9):3783-90.*
62. Chen YZ, Matsushita S, Nishimura Y. A single residue polymorphism at DR beta 37 affects recognition of peptides by T cells. *Hum. Immunol. 1997; 54(1):30-9.*
63. Kersh GJ, Allen PM. Structural basis for T cell recognition of altered peptide ligands: a single T cell receptor can productively recognize a large continuum of related ligands. *J. Exp. Med. 1996; 184(4):1259-68.*

64. Kersh GJ, Miley MJ, Nelson CA, Grakoui A, Horvath S, Donermeyer DL, Kappler J, Allen PM, Fremont DH. Structural and functional consequences of altering a peptide MHC anchor residue. *J. Immunol. 2001; 166(5):3345-54.*
65. Hiemstra HS, van Veelen PA, Geluk A, Schloot NC, de Vries RR, Ottenhoff TH, Roep BO, Drijfhout JW. Limitations of homology searching for identification of T-cell antigens with library derived mimicry epitopes. *Vaccine 1999; 18(3-4):204-8.*
66. Schloot NC, Roep BO, Wegmann DR, Yu L, Wang TB, Eisenbarth GS. T-cell reactivity to GAD65 peptide sequences shared with coxsackie virus protein in recent-onset IDDM, post-onset IDDM patients and control subjects. *Diabetologia 1997; 40(3):332-8.*
67. Bach JM, Otto H, Jung G, Cohen H, Boitard C, Bach JF, van Endert PM. Identification of mimicry peptides based on sequential motifs of epitopes derived from 65-kDa glutamic acid decarboxylase. *Eur. J. Immunol. 1998; 28(6):1902-10.*
68. Hausmann S, Martin M, Gauthier L, Wucherpfennig KW. Structural features of autoreactive TCR that determine the degree of degeneracy in peptide recognition. *J. Immunol. 1999; 162(1):338-44.*
69. Wilson DB, Pinilla C, Wilson DH, Schroder K, Boggiano C, Judkowski V, Kaye J, Hemmer B, Martin R, Houghten RA. Immunogenicity. I. Use of peptide libraries to identify epitopes that activate clonotypic $CD4^+$ T cells and induce T cell responses to native peptide ligands. *J. Immunol. 1999; 163(12):6424-34.*
70. Judkowski V, Pinilla C, Schroder K, Tucker L, Sarvetnick N, Wilson DB. Identification of MHC class II-restricted peptide ligands, including a glutamic acid decarboxylase 65 sequence, that stimulate diabetogenic T cells from transgenic BDC2.5 nonobese diabetic mice. *J. Immunol. 2001; 166(2):908-17.*
71. Zhao Y, Gran B, Pinilla C, Markovic-Plese S, Hemmer B, Tzou A, Whitney LW, Biddison WE, Martin R, Simon R. Combinatorial peptide libraries and biometric score matrices permit the quantitative analysis of specific and degenerate interactions between clonotypic TCR and MHC peptide ligands. *J. Immunol. 2001; 167(4):2130-41.*
72. Hemmer B, Gran B, Zhao Y, Marques A, Pascal J, Tzou A, Kondo T, Cortese I, Bielekova B, Straus SE, McFarland HF, Houghten R, Simon R, Pinilla C, Martin R. Identification of candidate T-cell epitopes and molecular mimics in chronic Lyme disease. *Nat. Med. 1999; 5(12):1375-82.*
73. Hemmer B, Pinilla C, Appel J, Pascal J, Houghten R, Martin R. The use of soluble synthetic peptide combinatorial libraries to determine antigen recognition of T cells. *J. Pept. Res. 1998; 52(5):338-45.*
74. Tanaka Y, Ohyama H, Ogawa M, Nishimura Y, Matsushita S. Identification of peptide superagonists for a self-K-ras-reactive $CD4^+$ T cell clone using combinatorial peptide libraries and mass spectrometry. *J. Immunol. 1999; 162(12):7155-61.*
75. Leggatt GR, Hosmalin A, Pendleton CD, Kumar A, Hoffman S, Berzofsky JA. The importance of pairwise interactions between peptide residues in the delineation of TCR specificity. *J. Immunol. 1998; 161(9):4728-35.*
76. Ausubel LJ, Kwan CK, Sette A, Kuchroo V, Hafler DA. Complementary mutations in an antigenic peptide allow for crossreactivity of autoreactive T-cell clones. *Proc. Natl. Acad. Sci. U.S.A. 1996; 93(26):15317-22.*
77. Hemmer B, Pinilla C, Gran B, Vergelli M, Ling N, Conlon P, McFarland HF, Houghten R, Martin R. Contribution of individual amino acids within MHC molecule or antigenic peptide to TCR ligand potency. *J. Immunol. 2000; 164(2):861-71.*
78. Fujii S, Senju S, Chen YZ, Ando M, Matsushita S, Nishimura Y. The CLIP-substituted invariant chain efficiently targets an antigenic peptide to HLA class II pathway in L cells. *Hum. Immunol. 1998; 59(10):607-14.*
79. van Bergen J, Schoenberger SP, Verreck F, Amons R, Offringa R, Koning F. Efficient loading of HLA-DR with a T helper epitope by genetic exchange of CLIP. *Proc. Natl. Acad. Sci. U.S.A.1997; 94(14):7499-502.*
80. Malcherek G, Wirblich C, Willcox N, Rammensee HG, Trowsdale J, Melms A. MHC class II-associated

invariant chain peptide replacement by T cell epitopes: engineered invariant chain as a vehicle for directed and enhanced MHC class II antigen processing and presentation. *Eur. J. Immunol. 1998; 28(5)):1524-33.*

81. Atkinson MA, Kaufman DL, Campbell L, Gibbs KA, Shah SC, Bu DF, Erlander MG, Tobin AJ, Maclaren NK. Response of peripheral-blood mononuclear cells to glutamate decarboxylase in insulin-dependent diabetes. *Lancet 1992; 339(8791):458-9.*
82. Kaufman DL, Clare-Salzler M, Tian J, Forsthuber T, Ting GS, Robinson P, Atkinson MA, Sercarz EE, Tobin AJ, Lehmann PV. Spontaneous loss of T-cell tolerance to glutamic acid decarboxylase in murine insulin-dependent diabetes. *Nature 1993; 366(6450):69-72.*
83. Tisch R, Yang XD, Singer SM, Liblau RS, Fugger L, McDevitt HO. Immune response to glutamic acid decarboxylase correlates with insulitis in non-obese diabetic mice. *Nature 1993; 366(6450):72-5.*
84. Tabata H, Kanai T, Yoshizumi H, Nishiyama S, Fujimoto S, Matsuda I, Yasukawa M, Matsushita S, Nishimura Y. Characterization of self-glutamic acid decarboxylase 65-reactive $CD4^+$ T-cell clones established from Japanese patients with insulin-dependent diabetes mellitus. *Hum. Immunol. 1998; 59(9):549-60.*
85. Wicker LS, Chen SL, Nepom GT, Elliott JF, Freed DC, Bansal A, Zheng S, Herman A, Lernmark A, Zaller DM, Peterson LB, Rothbard JB, Cummings R, Whiteley PJ. Naturally processed T cell epitopes from human glutamic acid decarboxylase identified using mice transgenic for the type 1 diabetes-associated human MHC class II allele, DRB1*0401. *J. Clin. Invest. 1996; 98(11):2597-603.*
86. Patel SD, Cope AP, Congia M, Chen TT, Kim E, Fugger L, Wherrett D, Sonderstrup-McDevitt G. Identification of immunodominant T cell epitopes of human glutamic acid decarboxylase 65 by using HLA-DR(alpha1*0101,beta1*0401) transgenic mice. *Proc. Natl. Acad. Sci. U.S.A. 1997; 94(15):8082-7.*
87. Fujii S, Uemura Y, Iwai LK, Ando M, Senju S, Nishimura Y. Establishment of an expression cloning system for $CD4^+$ T cell epitopes. *Biochem. Biophys. Res. Commun. 2001; 284(5):1140-7.*
88. Uemura Y, Senju S, Maenaka K, Iwai LK, Fujii S, Tabata H, Tsukamoto H, Hirata S, Chen YZ, and Nishimura Y. Systematic analysis of the combinatorial nature of epitopes recognized by TCR leads to identification of mimicry epitopes for glutamic acid decarboxylase 65-specific TCRs. *J. Immunol. in press*
89. Misko IS, Cross SM, Khanna R, Elliott SL, Schmidt C, Pye SJ, Silins SL. Crossreactive recognition of viral, self, and bacterial peptide ligands by human class I-restricted cytotoxic T lymphocyte clonotypes: implications for molecular mimicry in autoimmune disease. *Proc. Natl. Acad. Sci. U.S.A. 1999; 96(5):2279-84.*
90. Masuda M, Senju S, Fujii Si S, Terasaki Y, Takeya M, Hashimoto Si S, Matsushima K, Yumoto E, Nishimura Y. Identification and immunocytochemical analysis of DCNP1, a dendritic cell-associated nuclear protein. *Biochem. Biophys. Res. Commun. 2002; 290(3):1022-9.*
91. Fujita H, Senju S, Yokomizo H, Saya H, Ogawa M, Matsushita S, Nishimura Y. Evidence that HLA class II-restricted human $CD4^+$ T cells specific to p53 self peptides respond to p53 proteins of both wild and mutant forms. *Eur. J. Immunol. 1998; 28(1):305-16.*
92. Carson RT, Vignali KM, Woodland DL, Vignali DA. T cell receptor recognition of MHC class II-bound peptide flanking residues enhances immunogenicity and results in altered TCR V region usage. *Immunity 1997; 7(3):387-99.*
93. Hiemstra HS, van Veelen PA, Schloot NC, Geluk A, van Meijgaarden KE, Willemen SJ, Leunissen JA, Benckhuijsen WE, Amons R, de Vries RR, Roep BO, Ottenhoff TH, Drijfhout JW. Definition of natural T cell antigens with mimicry epitopes obtained from dedicated synthetic peptide libraries. *J. Immunol. 1998; 161(8):4078-82.*
94. Hiemstra HS, Duinkerken G, Benckhuijsen WE, Amons R, de Vries RR, Roep BO, Drijfhout JW. The identification of $CD4^+$ T cell epitopes with dedicated synthetic peptide libraries. *Proc. Natl. Acad. Sci. U.S.A. 1997; 94(19):10313-8.*
95. Mason D. A very high level of crossreactivity is an essential feature of the T-cell receptor. *Immunol. Today*

1998; 19(9):395-404.

96. Hiemstra HS, van Veelen PA, Willemen SJ, Benckhuijsen WE, Geluk A, de Vries RR, Roep BO, Drijfhout JW. Quantitative determination of TCR cross-reactivity using peptide libraries and protein databases. *Eur. J. Immunol. 1999; 29(8):2385-91.*
97. Padovan E, Casorati G, Dellabona P, Meyer S, Brockhaus M, Lanzavecchia A. Expression of two T cell receptor alpha chains: dual receptor T cells. *Science 1993; 262(5132):422-4.*
98. Busch R, Hill CM, Hayball JD, Lamb JR, Rothbard JB. Effect of natural polymorphism at residue 86 of the HLA-DR beta chain on peptide binding. *J. Immunol. 1991; 147(4):1292-8.*
99. Vergelli M, Hemmer B, Kalbus M, Vogt AB, Ling N, Conlon P, Coligan JE, McFarland H, Martin R. Modifications of peptide ligands enhancing T cell responsiveness imply large numbers of stimulatory ligands for autoreactive T cells. *J. Immunol. 1997; 158(8):3746-52.*
100. Koch N, van Driel IR, Gleeson PA. Hijacking a chaperone: manipulation of the MHC class II presentation pathway. *Immunol. Today 2000; 21(11):546-50.*
101. Boen E, Crownover AR, McIlhaney M, Korman AJ, Bill J. Identification of T cell ligands in a library of peptides covalently attached to HLA-DR4. *J. Immunol. 2000; 165(4):2040-7.*
102. Hemmer B, Vergelli M, Gran B, Ling N, Conlon P, Pinilla C, Houghten R, McFarland HF, Martin R. Predictable TCR antigen recognition based on peptide scans leads to the identification of agonist ligands with no sequence homology. *J. Immunol. 1998; 160(8):3631-6.*
103. Texier C, Pouvelle-Moratille S, Busson M, Charron D, Menez A, Maillere B. Complementarity and redundancy of the binding specificity of HLA-DRB1, -DRB3, -DRB4 and -DRB5 molecules. *Eur. J. Immunol. 2001; 31(6):1837-46.*
104. Clot F, Gianfrani C, Babron MC, Bouguerra F, Southwood S, Kagnoff MF, Troncone R, Percopo S, Eliaou JF, Clerget-Darpoux F, Sette A, Greco L. HLA-DR53 molecules are associated with susceptibility to celiac disease and selectively bind gliadin-derived peptides. *Immunogenetics 1999; 49(9):800-7.*
105. Geluk A, van Meijgaarden KE, Southwood S, Oseroff C, Drijfhout JW, de Vries RR, Ottenhoff TH, Sette A. HLA-DR3 molecules can bind peptides carrying two alternative specific submotifs. *J. Immunol. 1994; 152(12):5742-8.*
106. Baum H, Brusic V, Choudhuri K, Cunningham P, Vergani D, Peakman M. MHC molecular mimicry in diabetes. *Nat. Med. 1995; 1(5):388.*
107. Akerblom HK, Knip M. Putative environmental factors in Type 1 diabetes. *Diabetes Metabolism Rev. 1998; 14(1):31-67.*
108. Taha MK, Deghmane AE, Antignac A, Zarantonelli ML, Larribe M, Alonso JM. The duality of virulence and transmissibility in Neisseria meningitidis. *Trend. Microbiol. 2002; 10(8):376-82.*
109. Giebink GS. The prevention of pneumococcal disease in children. *N. Engl. J. Med. 2001; 345(16):1177-83.*
110. Steidler L, Hans W, Schotte L, Neirynck S, Obermeier F, Falk W, Fiers W, Remaut E. Treatment of murine colitis by Lactococcus lactis secreting interleukin-10. *Science 2000; 289(5483):1352-5.*
111. Kimpimaki T, Erkkola M, Korhonen S, Kupila A, Virtanen SM, Ilonen J, Simell O, Knip M. Short-term exclusive breastfeeding predisposes young children with increased genetic risk of Type I diabetes to progressive beta-cell autoimmunity. *Diabetologia 2001; 44(1):63-9.*
112. Anderton SM, Manickasingham SP, Burkhart C, Luckcuck TA, Holland SJ, Lamont AG, Wraith DC. Fine specificity of the myelin-reactive T cell repertoire: implications for TCR antagonism in autoimmunity. *J. Immunol. 1998; 161(7):3357-64.*
113. Windhagen A, Scholz C, Hollsberg P, Fukaura H, Sette A, Hafler DA. Modulation of cytokine patterns of human autoreactive T cell clones by a single amino acid substitution of their peptide ligand. *Immunity 1995; 2(4):373-80.*
114. Ausubel LJ, Krieger JI, Hafler DA. Changes in cytokine secretion induced by altered peptide ligands of

myelin basic protein peptide 85-99. *J. Immunol. 1997; 159(5):2502-12.*

115. Bielekova B, Goodwin B, Richert N, Cortese I, Kondo T, Afshar G, Gran B, Eaton J, Antel J, Frank JA, McFarland HF, Martin R. Encephalitogenic potential of the myelin basic protein peptide (amino acids 83-99) in multiple sclerosis: results of a phase II clinical trial with an altered peptide ligand. *Nat. Med. 2000; 6(10):1167-75.*
116. Kappos L, Comi G, Panitch H, Oger J, Antel J, Conlon P, Steinman L. Induction of a non-encephalitogenic type 2 T helper-cell autoimmune response in multiple sclerosis after administration of an altered peptide ligand in a placebo-controlled, randomized phase II trial. The Altered Peptide Ligand in Relapsing MS Study Group. *Nat. Med. 2000; 6(10):1176-82.*
117. Lang HL, Jacobsen H, Ikemizu S, Andersson C, Harlos K, Madsen L, Hjorth P, Sondergaard L, Svejgaard A, Wucherpfennig K, Stuart DI, Bell JI, Jones EY, Fugger L. A functional and structural basis for TCR cross-reactivity in multiple sclerosis. *Nat. Immunol. 2002; 3(10):940-3.*
118. Garcia KC, Degano M, Pease LR, Huang M, Peterson PA, Teyton L, Wilson IA. Structural basis of plasticity in T cell receptor recognition of a self peptide-MHC antigen. *Science 1998; 279(5354):1166-72.*
119. Esnouf, R. M. 1997. An extensively modified version of MolScript that includes greatly enhanced coloring capabilities. *J. Mol. Graph. Model. 15:132.*
120. Merritt, E. A., and M. E. P. Murphy. 1994. Raster3D version-2.0 - A program for photorealistic molecular graphics. *Acta. Cryst. D50: 869.*