Second Class Minors: Molecular Identification of the Autosomal $H_{46}$ Histocompatibility Locus as a Peptide Presented by Major Histocompatibility Complex Class II Molecules

Hiroeki Sahara and Nilabh Shastri

Division of Immunology, Department of Molecular and Cell Biology, University of California, Berkeley, CA 94720

Abstract

CD4 T cells regulate immune responses that cause chronic graft rejection and graft versus host disease but their target antigens remain virtually unknown. We developed a new method to identify CD4 T cell–stimulating antigens. LacZ-inducible CD4 T cells were used as a probe to detect their cognate peptide/MHC II ligand generated in dendritic cells fed with *Escherichia coli* expressing a library of target cell genes. The murine $H_{46}$ locus on chromosome 7 was thus found to encode the interleukin 4–induced $IL_{4i1}$ gene. The $IL_{4i1}$ precursor contains the HAFVEAIPELQGHV peptide which is presented by $A_{b}$ major histocompatibility complex class II molecule via an endogenous pathway in professional antigen presenting cells. Both allelic peptides bind $A_{b}$ and a single alanine to methionine substitution at p2 defines nonself. These results reveal novel features of $H$ loci that regulate CD4 T cell responses as well as provide a general strategy for identifying elusive antigens that elicit CD4 T cell responses to tumors or self-tissues in autoimmunity.

Key words: transplantation • antigens • antigen processing • CD4 T cells • GVHD

Introduction

The histocompatibility $H$ loci are polymorphic genes that determine the outcome of transplanted tissue grafts as well as graft versus host disease reactions in both mouse and man (1, 2). Despite the complete match of MHC molecules between the donor and host strains natural polymorphisms within the $H$ loci result in the presentation of novel peptides by the MHC to T cells (3). For example, different $H$ loci located on the male Y chromosome encode peptides that are presented by both MHC I and MHC II molecules and elicit potent CD8 and CD4 responses respectively (4, 5). At least 60 $H$ loci, located largely on autosomal chromosomes are known to exist in mice and a similar number is likely to be present in the human as well (2). Determining the molecular identity of $H$ loci is critical not only because of their clinical relevance but also for understanding the fundamental mechanisms that make the $H$ antigens immunogenic within the context of highly complex mammalian cells.

In recent years several $H$ loci have been identified at the molecular level as the source of naturally processed peptides presented by MHC I molecules to CD8 T cells (6, 7, and references therein). In contrast, despite the appreciation of their critical role in regulating immune responses to tissue grafts and tumors (8, 9), the $H$ loci that encode antigenic peptides presented by MHC II molecules to CD4 T cells remain virtually unknown at the molecular level. For instance it is not known whether the genetically defined $H$ loci that regulate CD4 T cell responses do so because they encode the antigenic peptides or because they directly or indirectly regulate the MHC II antigen processing pathway as seen for one of the CD8 T cell defined human H antigen (10). It is also unclear whether professional APCs acquire the $H$ antigens from exogenous sources as are most antigens presented by MHC II molecules or whether processing of $H$ peptide/MHC II utilizes precursors synthesized endogenously within the professional APCs (11). The recently discovered Y chromosome encoded Dby $H$ antigen was presented by MHC II when professional dendritic cells (DCs)* acquired it from other cells expressing the Dby gene (5).

*Abbreviations used in this paper: BMDC, bone marrow–derived immature DC; DC, dendritic cell.
Whether this is a general paradigm for all CD4 T cell–defined H loci is unclear. Finally, to what extent the donor H antigens differ from self to elicit potent CD4 T cell responses remains to be determined.

The relative success in the identification of CD8 T cell–stimulating H loci is due to the availability of robust methods for expression cloning the antigenic precursor genes or for biochemical purification and identification of the processed antigenic peptides (12–14). By contrast, the identification of CD4 T cell–stimulating antigens has remained an extraordinarily difficult task. Unlike the homogenous peptides presented by MHC class I, the peptides presented by MHC class II are heterogeneous with varying N- and COOH termini flanking the core antigenic peptide (11, 15–17). Peptides eluted from MHC II therefore do not elute in a single peak when fractionated by HPLC and are thus difficult to purify. The peptide purification problem is further magnified by the generally low sensitivity of CD4 T cells to exogenous antigens, in the nano to micromolar concentration range. In comparison, CD8 T cells respond to exogenous antigens at femto to picomolar concentrations which makes it simpler to follow the purification of endogenously processed peptides from the target cells.

The expression cloning strategies for identifying CD4 T cell antigens are also challenging. The MHC I presentation pathway operates in almost all cell types, and it is usually sufficient to introduce an antigen encoding cDNA to obtain expression of the peptide/MHC I ligand (18). By contrast, the MHC class II processing pathway operates primarily in professional APCs (11). These APCs are specialized to present antigenic peptide/MHC II complexes from exogenous proteins but endogenously synthesized proteins are usually excluded. Thus, even if antigenic precursors were expressed in the professional APCs, their presentation as peptide/MHC II complexes may not occur. We and others circumvented this problem by targeting antigenic precursors directly into the MHC II processing compartment by expressing them as invariant chain fusion proteins, which significantly improved the efficiency with which peptide/MHC II complexes were generated (19, 20). Nevertheless, the limitations in transfection efficiency have so far precluded the use of this method in professional APCs. The invariant chain/fusion protein strategy has so far been successful only in specially engineered, readily transfectable cell lines allowing the identification of several CD4 T cell–stimulating tumor antigens (21, 22). In an elegant method that combined the expression of target antigen in transfecetable cell lines with the use of DCs as professional APCs, the Simpson group recently identified the Y-chromosome encoded Dby gene as the source of peptides presented by the A\(^b\) and E\(^\text{MHC}\) class II molecules to CD4 T cells (5). This method is presently limited to single candidate genes and has thus far not permitted the screening of complex cDNA libraries.

Here we developed a new method for identifying CD4 T cell stimulating antigens in mammalian cells containing thousands of potential precursors. The p/MHC II ligands were generated in professional APCs fed with pools of recombinant Escherichia coli expressing the target antigens. We took advantage of the high phagocytic and antigen presentation capacity of bone marrow–derived immature DCs to generate peptide/MHC class II complexes from proteins expressed in recombinant E. coli (23).

The peptide/MHC II complexes were probed with the exquisitely sensitive lacZ-inducible CD4 T cells to identify the bacteria expressing the recombinant antigen. We used this method to identify the elusive autosomal CD4 T cell–stimulating H loci. We report that the polymorphic H46 locus first discovered more than a decade ago (24), encodes the IL-4–induced IL4i1 gene that is located on chromosome 7. Furthermore, we have defined the antigenic peptide within the IL4i1 gene presented by the A\(^b\) MHC class II to CD4 T cells, the antigenic relationship between the donor and host strains as well as the antigen processing pathways used by the APCs.

**Materials and Methods**

**Mice, Cells, and Peptides.** Inbred mice were obtained from The Jackson Laboratory or bred within the OLAC facility at UC Berkeley. All procedures were performed in compliance with the institutional ACUC guidelines. Procedures for immunization, generation and maintenance of T cell lines and β-galactosidase (lacZ)-inducible T cell hybrids have been described (24, 25).

Briefly, the H46- specific CD4 T cell line, 2PB-TH3 was generated by immunizing the congenic B10.129-H46/H47\(^b\) (21M) with C57BL/10J [H46/H47\(^b\)] splenocytes and was provided by Dr. Derry Roopenian (The Jackson Laboratory, Bar Harbor, ME). The T cells were used to generate the lacZ-inducible CD4 T cell hybrid TH3Z by fusing them with the lacZ-inducible fusion partner, BWZ.36 (25).

Bone marrow cells obtained from the thigh bone of C57BL/6J (B6) or 129 P3/J (129/J mice) were cultured with RPMI 1640 supplemented with 5% fetal bovine serum (HyClone), 10 ng/ml GM-CSF (Biosource International), 2 mM glutamine, 50 μM 2-mercaptoethanol, 200 U/ml penicillin, 200 μg/ml streptomycin, and 20 μg/ml gentamicin. After 5 d the characteristic immature DCs in the CD11c\(^+\), CD86\(^-\), and A\(^b\) low cultures were greater than 65% by FACS\(^\text{®}\) analysis and are referred to as bone marrow–derived immature DCs (BMDCs) and used as APCs. Peritoneal macrophages were elicited by an intraperitoneal injection of aged thioglycolate (Difco). The mice were killed 4 d later, the macrophages harvested by peritoneal lavage with PBS, plated out in dishes in complete RPMI medium, and allowed to adhere at 37°C. After 2 h the nonadherent cells were washed away and the remaining cells were cultured overnight in complete RPMI medium supplemented with 100 U/ml IFN-γ (Genzyme). B cells were obtained from the spleen and purified by MACS\(^\text{®}\) system (Auburn, CA) with CD45R/B220-FITC conjugated mAb (BD Biosciences). Purified B cells were cultured with 1 μg/ml IL-4 (Biosource International) for 6 h. The peptides with the sequences shown in Fig. 5 and Fig. 6 were synthesized, purified by HPLC, and confirmed by mass spectrometry.

**T Cell Activation Assays.** LacZ-inducible T cell hybrids were cocultured overnight with APCs from mice, or cells that had ingested transformed bacteria, were transfected with cDNAs, or pulsed with exogenous peptides. In some experiments the APCs were preincubated with anti–H-2-D\(^b\) (B22.249) or anti-I–A\(^\text{M}^5\)/114.5.2 mAb for 1 h at 4°C before coculture with T cells. The
results shown are the lacZ activity measured as the absorbance at 595 nm (with 635 nm as the reference wavelength) of the chimeric product released after cleavage of the substrate chloroformol red β-pyranoside (25).

**cDNA Library and Recombinant Constructs.** PolyA+ mRNA from day 5 BMDCs was used for the construction of the cDNA library using the cDNA synthesis kit from Invitrogen. The cDNA fragments were inserted unidirectionally into the pLEX prokaryotic expression vector between the EcoRI/NotI sites and used to transform GI724 competent bacteria to yield ∼10^5 cfu with an average insert size of 0.5–3 kbp. The 5’ forward and 3’ reverse PCR primers for the DNA constructs used here are shown below. All primers included EcoRI and XbaI restriction sites except those used for amplification of IL4i1 gene which contained EcoRI and NotI sites to allow cloning into appropriate expression vectors (Table I).

First strand cDNA from polyA+ mRNA from day 5 BMDC from B6, 129/J, or 21M mice was used to obtain the full-length IL4i1 homologues. PCR was performed using the high fidelity Pfu Turbo polymerase system (Stratagene). The PCR products were purified by agarose gel, sequenced directly with gene-specific primers, and subcloned into pLEX, pcDNA3, or the MSCV vector using the BTX800 electroporator. IL4i1 genes from the B6, 129/J or the 224–26 fragment subcloned in the pcDNA3 vector using the Stratagene QuickChange Site-Directed Mutagenesis Kit.

**Expression Cloning.** Bacterial transformants were grown and induced to express the cDNA-encoded proteins according to instructions (PL Expression System; Invitrogen). Briefly, 2 × 10^8 transformants were plated out on drug selection agar plates and incubated overnight at 30°C. The colonies were harvested and resuspended in the culture medium. The transformant density was determined by OD_{595} (OD 1.0 = 2 × 10^8 cells/ml) and bacteria were plated out in 96-well round bottomed plates at 10–20 cfu/well in 100 μl medium. After 24–36 h at 30°C when the cultures had reached a 0.5 OD_{595}, 5 μl of each well was replica plated into a separate 96-well plate containing 100 μl medium/well and cultured for 1–2 h at 30°C to allow log growth. Induction medium containing 10 μg/ml L-tryptophan was then added and the incubation continued for 3 h at 37°C. The number of transformants was again estimated by OD_{595} and bacteria were transferred to 96-well plate to obtain a final concentration of 5 × 10^6 cfu/well and were used as the antigen source. The BMDCs from 129/J mice were resuspended in RPMI medium without fetal bovine serum and antibiotics and 10^6 cells/well were incubated with the induced bacteria for 1 h at 37°C to allow phagocytosis. These plates were centrifuged for 2 min at 1,500 rpm, the supernatant was removed, and 10^5 TH3Z hybridoma cells in medium containing 100 μg/ml gentamicin (to eliminate residual bacteria) were added to each well. T cell activation was measured as their lacZ response described above.

**A′ Competitive Peptide Binding Assay.** The ability of peptide analogs to bind A′ was determined by incubating A′-L cell transfectants with 10 μM Ea56–73 peptide (ASFEAQGLANIA-VDKRA) together with varying concentrations of the indicated competitor peptides. After overnight incubation, the cells were stained with Yae mAb specific for the Ea/A′ complex and PE-conjugated goat anti–mouse Ig and analyzed by flow cytometry. The mean fluorescence intensity values were used to calculate the percent inhibition. Both the Ea peptide and the Yae mAb supernatant were gifts of A. Rudensky (University of Washington).

**Transfections and Retroviral Transduction.** Simian COS7 (COS) cells were electroporated in 270 mM sucrose, 7 mM sodium phosphate at pH 7.4, and 1 M MgCl₂ with either full-length IL4i1 genes from the B6, 129/J or the 224–26 fragment subcloned in the pcDNA3 vector using the BTX800 electroporator. The electrode had a 1.9-mm gap-prong and was set to deliver 599 ms pulses at 700 V. 2 d later the cells were titrated and cocultured with the TH3Z T cells. Antigen-expressing retrovirus supernatants were prepared using 293T as packaging cells. The cells were transiently transfected with B6 or 129/J derived full-length IL4i1 gene in the MIG vector together with pGP-KV plasmid encoded Moloney murine leukemia virus structural genes, gag-pol (26), and VSV-G plasmids (27) by Superfect transfection as described (QIAGEN). After 24 h the supernatants were filtered through a 0.45 μm filter and 4 μg/ml polybrene was added before infection. Bone marrow cultures of 129/J mice plated out in a 24-well plate at 10^6 cells/well were infected with the retroviral supernatants after 2 d. The plate was centrifuged at 2,500 rpm for 2 h and the medium was replaced (28). After 2 d in culture ~8–10% CD11c+ cells were found to express the vector encoded GFP marker and used as APCs.

| Table I. Oligonucleotide Primers |
|-------------------------------|
| Construct | Forward (5’–3’) | Reverse (5’–3’) | Figure |
| ΔN1 | CCGGAATTCCATCGAGTCATGGAGACT | TGTAAGAAGGCAGCGAGCTGC | 5, A and B |
| ΔN2 | CCGGAATTCCATCGAGTCATGGAGACT | TGTAAGAAGGCAGCGAGCTGC | 5, A and B |
| ΔN3 | CCGGAATTCCATCGAGTCATGGAGACT | TGTAAGAAGGCAGCGAGCTGC | 5, A and B |
| ΔC1 | GGTGAGCCCTTTAAAATTAAGGC | GACTCTAGACGTTTCCACCAAGAAGCTGC | 5, A and B |
| ΔC2 | GGTGAGCCCTTTAAAATTAAGGC | GACTCTAGACGTTTCCACCAAGAAGCTGC | 5, A and B |
| ΔC3 | GGTGAGCCCTTTAAAATTAAGGC | GACTCTAGACGTTTCCACCAAGAAGCTGC | 5, A and B |
| H46b | CCGGAATTCCATCGAGTCATGGAGACT | GACTCTAGACGTTTCCACCAAGAAGCTGC | 6, A–C |
| H46b | CCGGAATTCCATCGAGTCATGGAGACT | GACTCTAGACGTTTCCACCAAGAAGCTGC | 6, A–C |
| P>L | CAGATGAAACGAGAGCTCTTCTCACAACACAT | GGTGTTTGTGAGAGCTCTTCTCACAACAC | 6, B and C |
| A>M | CAGATGAAACGAGAGCTCTTCTCACAACACAT | GGTGTTTGTGAGAGCTCTTCTCACAACAC | 6, B and C |
| IL4i1 | CCGGAATTCCATCGAGTCATGGAGACT | GACTCTAGACGTTTCCACCAAGAAGCTGC | 7, B, C, and D |
Results and Discussion

CD4+ TH3Z Recognize a Polymorphic Antigen Presented by the Aα MHC II Molecule. As the first step toward the identification of CD4 T cell antigens, we generated a lacZ-inducible CD4+ T cell hybridoma TH3Z (25). We used the CD4 T cell clone 2PB-TH3 that was specific for the mouse H46 antigen based upon its reactivity to a panel of congenic recombinant mice (24). This genetic analysis of the H46 locus had indicated that it was located within or was regulated by a gene within the H4 complex on chromosome 7 of C57BL/6 (B6) mice. In contrast to the H47 locus, located in the same H4 complex that elicited a CD8 T cell response, the closely linked H46 locus elicited a CD4 T cell response when 129/J-derived congenic B10.129/J-H46b,H47b (21M) mice were immunized with B10 or B6 spleen cells (6, 24). Accordingly, the lacZ activity was stimulated only when the TH3Z T cell hybridoma was cultured with donor B6-derived splenocytes, macrophages, or bone marrow cultures enriched for dendritic cells (BMDCs), but not with the same cell types from the MHC-matched 129/J strain (Fig. 1 A). Furthermore, the TH3Z response required expression of the Aα MHC II molecule because mAb specific for Aβ, but not Dβ MHC class I molecules inhibited its response to the APC (Fig. 1 B). The TH3Z hybridoma was therefore specific for a polymorphic antigen that was presented by the Aα MHC II molecule in the B6 background.

Interestingly, BMDCs derived from B6 mice were by far the most potent APCs in comparison to splenocytes or peritoneal macrophages. To identify the optimal source of the antigen we tested the APC function of B6 bone marrow cultures after different days in culture. On a per cell basis the TH3Z stimulating activity in bone marrow cells was low on day 0, but increased dramatically during the next 2 d, reached its peak on day 3, and remained at this level for up to 6 d of culture (Fig. 1 C). The expression of the TH3Z stimulating activity correlated well with a concomitant increase in the CD11c+ cells in the bone marrow cultures strongly suggesting that the antigen gene was expressed in immature DCs (unpublished data). We therefore chose day 5 BMDCs as the optimal source of mRNA to screen for the TH3Z stimulating antigen gene.

Generation of CD4 T Cell–stimulating Peptide/MHC Class II Ligands from Precursors Expressed in Recombinant Bacteria. We had previously shown that recombinant E. coli expressing heterologous proteins can serve as an antigen source for generating peptide/MHC class II complexes in activated macrophages (29, 30). The observed efficiency was adequate for screening small prokaryotic genomes such as that of Listeria monocytogenes for antigens recognized by lacZ-inducible CD4 T cells. To determine if the efficiency of introducing exogenous proteins into the MHC class II pathway could be further improved we compared peritoneal macrophages and BMDCs as APCs for processing exogenous ovalbumin to OTIIZ T cells. Both macrophages and immature BMDCs generated the OVA peptide/Aα complex from recombinant E. coli expressing OVA (Fig. 2 A).

The BMDCs were, however, consistently superior APCs in their requirement for lower number of E. coli to obtain a detectable T cell response as well as in stimulating a higher magnitude of the OTIIZ response. Similar results were also observed with the OVA/Aβ and HEL/Aβ-specific T cell hybridomas (unpublished data). The approximately threefold increase in efficiency encouraged us to undertake the task of screening cDNA libraries from mammalian cells that contain tens of thousands of different proteins as potential precursors for the antigenic peptide.

Based upon these results we devised a strategy schematically outlined in Fig. 2 B to search for CD4 T cell stimulating antigens using recombinant E. coli as the antigen source and BMDCs as recipient APCs. We planned first to construct a cDNA library in a prokaryotic expression vector which would allow expression of the antigenic precursor proteins in transformed bacteria. We chose to use the commercially available pLEX vector because it allows regulated transcriptional control in the presence of tryptophan (Trp) thereby reducing the chance that potentially toxic proteins would be excluded from the bacterial pools. Second, we planned to screen the potential precursor pools by feeding the bacteria to BMDCs and to probe them with
the TH3Z cells for the presence of their cognate peptide/Aβ ligand. If precursor pools were detected individual bacteria harboring the antigenic cDNA could be identified by cloning and rescreening those in the original well of the master plate.

Isolation of the TH3Z-stimulating Antigen Gene. A cDNA library was prepared from polyA+ mRNA isolated from day 5 B6 BMDCs because they were by far the most potent stimulators of TH3Z T cell response (Fig. 1, A and C). The cDNA library was screened in pools of ~10 cfu/well using immature BMDCs from the host 129/J strain as APCs because they did not express the cognate antigen (Fig. 1 A). The TH3Z response to pool #224, one of the approximately 1,000 different pools screened, showed a response that was above that of the other pools (Fig. 3 A). Pool 224 was fractionated into individual colonies several of which strongly stimulated the TH3Z response in the rescreen (Fig. 3 B). One of these bacterial colonies was designated 224–26 and selected for further analysis. A second clone 296–76 was also identified in the screen which after sequence analysis was found to be identical to clone 224–26.

The TH3Z response to 224–26 was antigen-specific and restricted by the Aβ MHC II molecule. The 224–26 bacteria failed to stimulate the OVA/Aβ-specific OTIIZ T cells (Fig. 3 C). The TH3Z response to 224–26 bacteria was enhanced over 100-fold when the bacteria were cultured with tryptophan (Trp). The bacterial pools containing the putative antigen are fed to host day 5 BMDCs which phagocytose the bacteria, process and present the antigen/MHC II complexes. The presence of these CD4 T cell ligands is detected by the lacZ response of the CD4 T cell hybrid. The individual bacterial clone is then identified by subcloning the bacteria in the replicate well from the “Master” plate and repeating the screen. See Materials and Methods for details.
whether the 224–26 cDNA encoding the antigenic precursor mapped to the same chromosomal location or was regulated by another gene located on chromosome 7, we searched the mouse genome database (http://www.informatics.jax.org/). Most significantly, the \( \text{IL4i1} \) gene was located on chromosome 7 within 3 centi-morgan (cM) of the previously suggested location of the \( \text{H46} \) locus. Based upon the current map of chromosome 7 both CD4 and CD8 T cell defined \( \text{H46} \) and \( \text{H47} \) polymorphic loci are located within 5.4 cM and close to the pink-eyed dilute locus \( p \) (Fig. 4 C). These data are in complete agreement with the previous genetic analysis and confirm that the \( \text{H46} \) locus is physically located on chromosome 7. Note that the close linkage seen here between the CD8 (\( \text{H47} \)) and CD4 (\( \text{H46} \)) T cell defined autosomal loci was also seen earlier for the Y-chromosome encoded CD8 (\( \text{Smcy} \), \( \text{Uty} \)) and CD4 (\( \text{Dby} \)) T cell defined loci (5). Thus, the discovery of the tight linkage between CD4 and CD8 T cell defined loci on the sex as well as autosomal chromosomes is in complete agreement with the original Roopenian hypothesis that the CD8 and CD4 T cell–stimulating \( H \) loci must cosegregate (33).

Identification of the Antigenic Peptide Encoded by 224–26 cDNA. To understand the molecular basis for the antigenicity of the \( \text{H46} \) locus we defined the putative antigenic peptide within the 224–26 encoded polypeptide. We generated a series of deletions at the 5\( ^{\prime} \) and 3\( ^{\prime} \) ends of the 224–26 cDNA (Fig. 5 A). The deleted fragments were recloned in the original pLEX vector and recombinant \( E. \ coli \) expressing the truncated polypeptides were tested for antigenic activity after feeding BMDC from 129/J mice (Fig. 5 B). All deletions of 106 or more nucleotides from the 5\( ^{\prime} \) end (\( \text{N1} \)) or 309 nucleotides from the 3\( ^{\prime} \) end (\( \text{C1} \)) caused complete loss of antigenic activity. Based upon the full antigenic activity of the \( \text{N2} \) and the loss of antigenic activity of \( \text{N1} \) and \( \text{C1} \) constructs we inferred that the antigenic activity within the 224–26 cDNA was dependent upon the presence of nucleotides 52–106 encoding aa18–36 (Fig. 4 A).

To directly establish the validity of this assignment as well as to determine whether the antigenic activity was contained within the peptide itself or was somehow induced in the recipient APC by the 224–26 polypeptide we tested a panel of overlapping synthetic peptides using 129/J BMDC as APC (Fig. 5, C and D). Peptide 24–37, but not peptides

![Image](image-url)
18–32 or 28–42 stimulated a strong TH3Z response at submicromolar concentrations. The antigenic activity was therefore contained within the 14 amino acids of peptide 24–37. Additional analogs of peptide 24–37 were prepared to define the N- and COOH-terminal boundaries of the antigenic epitope. The antigenic activity of all the analogs with up to three additional amino acids at the NH2 terminus and three fewer amino acids at the COOH terminus (p21–34) was comparable to that of p24–37. From these data we infer that the 11-mer sequence HAFVEAIPELQ (aa 24–34) within the 224–26 cDNA defines the core antigenic epitope that is presented by the Ab MHC II molecule.

Consensus sequence motifs for peptides presented by MHC II have been difficult to define. Nevertheless systematic mutagenesis of single amino acids has suggested that the Ab bound peptides share an aromatic tyrosine or phenylalanine residue at the $i_{1}$ anchor position, an uncharged amino acid at the $i_{5}/H_1$ position, with no positively charged residues at the $i_{8}/H_2$ position (34). The H46a peptide defined by the sequence HAFVEAIPELQ shares each of these features as well. Assuming that the phenylalanine (F) occupies the $i_{1}$ position, the $i_{5}/H_1$ and $i_{8}/H_2$ residues are uncharged proline (P) and a nonpositively charged glutamine (Q) residues. However, systematic amino acid substitutions are required for antigenic activity.
required to confirm the importance of these positions to the A<sup>b</sup> binding characteristics of the H46<sup>a</sup> peptide.

**A Single Amino Acid Polymorphism Defines the Antigenicity of H46 Alleles.** To determine the relationship between the donor and host strains that define the polymorphism at the H46 locus we first analyzed BMDC mRNA in the B6, the congenic B10.129/J-H46<sup>b</sup>-H47<sup>b</sup> (21M) and the parental 129/J strains by Northern blot. Transcripts hybridizing with the 224–26 probe were expressed in each strain (unpublished data). To determine if there were any sequence polymorphisms we generated by RT-PCR the 21M and 129/J-derived fragments corresponding to the 224–26 cDNA from B6 mice (Fig. 6 A). The nucleotide sequences of these fragments were identical to that expressed in the B6 strain except for three nucleotide substitutions C23/H11022 T, G73/H11022 A, and C74/H11022 T. These changes caused the Pro8/H11022 Leu and Ala25/H11022 Met substitutions in the predicted amino acid sequence of the H46<sup>b</sup> allele in the parental 129/J as well as its congenic derivative 21M strains.

Both amino acid substitutions were located in the proximity of the H46<sup>a</sup> antigenic peptide. The P8>L substitution was located 18 residues upstream while the A25>M substitution was contained within the antigenic peptide. To determine which one or both of these substitutions affected the antigenicity of the H46<sup>a</sup> (P-A) versus the H46<sup>b</sup> allele (L-M), we prepared mutant constructs in the pLEX bacterial expression vector that would yield precursors with only a single (P>L or A>M) substitution (Fig. 6 B). Recombinant E. coli expressing the H46<sup>a</sup>, H46<sup>b</sup> as well as the mutant constructs were fed to 129/J BMDCs and tested for their ability to stimulate the TH3Z T cells (Fig. 6 C). Clearly the B6-derived H46<sup>a</sup> and the mutant H46<sup>a</sup> P>L precursors were active in stimulating the TH3Z response while the allelic 129/J-derived H46<sup>b</sup> and the H46<sup>a</sup> A>M precursors were completely inactive at all concentrations tested. Thus, the antigenicity of the H46<sup>a</sup> allele was dependent upon the single A>M substitution within the antigenic peptide and was not affected by the upstream P>L substitution.

To directly establish that the difference in antigenicity was due to the A>M substitution we also synthesized the 14 amino acid H46<sup>b</sup> (residues 24–37) homologue of the H46<sup>a</sup> antigenic peptide (Fig. 6 D). In an exogenous pre-
sentation assay only the H46<sup>a</sup> peptide was active, and the H46<sup>b</sup> peptide with a single A<sub>11022</sub>→M substitution was completely inactive even at 100-fold higher concentration (Fig. 6E). Thus, the difference in antigenicity of the two H46 alleles could be directly attributed to the single alanine to methionine amino acid substitution within the core antigenic sequence.

The lack of TH3Z response to the H46<sup>b</sup> peptide could have been due to inability of this peptide to bind the A<sup>b</sup> MHC II molecule. We compared the two H46 analogs for their ability to compete with the Ea peptide for binding to A<sup>b</sup> MHC on the surface of A<sup>b</sup> expressing L cells. Differences in the level of the Ea/A<sup>b</sup> complex were monitored by flow cytometry using the Yae mAb (35). In contrast to A<sup>b</sup> binding HEL34–45 and OVA247–265 peptides which competed poorly, both H46<sup>a</sup> and H46<sup>b</sup> peptides were comparable in their ability to compete with the Ea peptide for binding A<sup>b</sup> (Fig. 6F). Thus, the failure of TH3Z T cells to recognize the H46<sup>b</sup> peptide is likely due to changes in the conformation of the peptide and/or the A<sup>b</sup> surface caused by the single Ala<sub>11022</sub>→Met substitution which may interfere with TCR recognition, rather than a simple failure to bind A<sup>b</sup> MHC. It would be interesting to determine whether the H46<sup>b</sup> peptide, which is predicted to be presented by DCs in the 129/J strain, is immunogenic in the H46<sup>a</sup> B6 strain.

**The H46<sup>a</sup> Peptide/A<sup>b</sup> Complex Is Generated via an Endogenous Processing Pathway.** MHC II molecules acquire their peptides primarily from exogenous sources but can also present some endogenously synthesized proteins (11). The IL<sub>4i1</sub> gene was originally identified as a transcript induced in IL-4–treated B cells, but is now recognized to be constitutively expressed in many tissues (reference 32; and http://www.ncbi.nlm.nih.gov/UniGene/). The intracellular location of the IL<sub>4i1</sub> protein is not known, and the presence of an ER translocation signal raised the possibility that IL<sub>4i1</sub> precursor may be secreted by other cells and acquired by professional APC as an exogenous antigen. Alternatively, because DCs appear to constitutively express the IL<sub>4i1</sub> gene as judged by Northern blots and direct APC function (Fig. 1, A and C), it is possible that the H46<sup>a</sup>/A<sup>b</sup> epitope is generated via an endogenous processing pathway.

To distinguish between these two possibilities, we first isolated the full-length cDNAs for the IL<sub>4i1</sub> gene from the B6 (H46<sup>a</sup>) and 129/J (H46<sup>b</sup>) strains. Similar to the COOH-terminal fragments represented by the 224–26 cDNA the full-length cDNA could also serve as antigenic precursors for the TH3Z epitope whether they were first expressed in E. coli and fed to 129/J BMDC (unpublished data) or expressed directly in the 129/J DCs via retroviral transduction (Fig. 7A). Likewise transient transfection of the B6 but not 129/J-derived full-length IL<sub>4i1</sub> cDNA together

### Figure 7.

The H46<sup>a</sup>/A<sup>b</sup> complex is generated from the full-length IL<sub>4i1</sub> gene via an endogenous presentation pathway. (A) 129/J BMDCs were transduced with retroviruses expressing either vector alone or the B6 or 129/J-derived full-length IL<sub>4i1</sub> cDNAs as described in Materials and Methods. 2 d later the transduced cells were titrated and used as APC for TH3Z T cells. About 8–10% cells expressed the retrovirus as indicated by the presence of CD11c<sup>GFP</sup> cells. (B) COS cells were transiently transfected with the indicated cDNA constructs representing the A<sup>b</sup> MHC II subunits A<sub>a</sub> and A<sub>b</sub> together with the B6 or 129/J derived full-length IL<sub>4i1</sub> genes or the 224–26 fragment. After 48 h, indicated number of transfected cells were cocultured with TH3Z T cells and their lacZ response was obtained after overnight incubation. (C and D) The H46<sup>a</sup>/A<sup>b</sup> epitope is not acquired by BMDC from other cells expressing the precursor gene. COS cells were transfected with the B6-derived full-length IL<sub>4i1</sub> cDNA with or without the A<sup>b</sup> subunits. 2 d later indicated numbers of transfected cells were cocultured with TH3Z T cells and their lacZ response was measured after overnight incubation. (C) and (D) indicate numbers of transfected COS cells (C) or BMDCs from B6 (H46<sup>a</sup>, A<sup>b</sup>) or B10.D2 (H46<sup>b</sup>, A<sup>b</sup>) were added to 129/J BMDC (H46<sup>a</sup>, A<sup>b</sup>). The lacZ response of TH3Z T cells was obtained only when the H46<sup>a</sup> precursor and the A<sup>b</sup> MHC II is expressed in the same cells.
with Aβα and Aββ cDNAs in COS cells resulted in the expression of the TH3Z stimulating epitope and was completely dependent upon the presence of the Aβ MHC II (Fig. 7 B). Interestingly, the cytoplasmic 224–26 fragment was insufficient for generating the TH3Z epitope in COS cells, suggesting that other features of the full-length protein are required for its entry into and/or processing within the MHC II processing compartments. These features remain to be identified.

The processing of the full-length precursor occurred via an endogenous processing pathway. A mixture of 129/J BMDCs and COS cells transiently transfected only with B6-derived IL4i1 cDNA failed to generate the TH3Z ligand while COS cells expressing both the IL4i1 precursor and Aβ stimulated TH3Z cells strongly (Fig. 7 D). Similarly, mixture of BMDCs from the H46α expressing B10.D2, that lack the restricting Ab MHC II and 129/J (H46β, H2β) derived BMDCs also failed to generate the TH3Z stimulating ligand (Fig. 7 E). In contrast, mixture of H46α and Aβ expressing BMDCs from B6 and 129/J mice were strongly stimulatory. Thus, whether the IL4i1 precursor was expressed in COS cells or BMDCs, it could not be used as an exogenous source by host 129/J-derived professional APCs. We therefore conclude that the generation of the H46α/Aβ epitope occurred via an endogenous MHC class II processing pathway within the APCs. How the IL4i1 is targeted to the MHC II processing pathway remains to be determined.

In conclusion, we have established a generally applicable method for the identification of CD4 T cell stimulating antigens. This method allowed us to screen large cDNA libraries from the target tissue in professional APCs that were generated from the host strain. Note that the ability to use professional APCs as recipients for the screen is a significant advantage because it obviates the necessity of generating designer recipient cell lines expressing the appropriate MHC II molecule, invariant chain, and DM (21). In fact, not only are the cDNAs of the appropriate MHC II subunits unnecessary even the knowledge of which particular MHC II presents the antigenic peptide is not essential. This could be very important in human applications where mul-


tigens. This method allowed us to screen large cDNA li-



mains to be determined.


ty of the murine H47 locus is determined by a single


ticevic, and E. Simpson. 2000. The HA-2 minor histocompat-



References

1. Goulmy, E., R. Schipper, J. Pool, E. Blokland, J.H.F. Falkenbur, J. Vossen, A. Gratwohl, G.B. Vogelsang, H.C. Van Houwelingen, and J.J. Van Rood. 1996. Mismatches of minor histocompatibility antigens between HLA-identical donors and recipients and the development of graft-versus-host disease after bone marrow transplantation. N. Engl. J. Med. 334:281–285.

2. Roopenian, D.C., and E. Simpson. 2000. Minor histocompatibility antigens: from the laboratory to the clinic. Landes Bioscience, Georgetown, TX.

3. Wallny, H.J., and H.G. Rammensee. 1990. Identification of classical minor histocompatibility antigen as cell-derived peptide. Nature. 343:275–278.

4. Simpson, E., D. Scott, and P. Chandler. 1997. The male-specific histocompatibility antigen, H-Y: A history of transplantation, immune response genes, sex determination and expression cloning. Annu. Rev. Immunol. 15:39–61.

5. Scott, D., C. Addey, P. Ellis, E. James, M. Mitchell, S. Jurcevic, and E. Simpson. 2000. Dendritic cells permit identification of genes encoding MHC class II-restricted epitopes of transplantation antigens. Immunity. 12:711–720.

6. Mendoza, L., G. Villaflor, P. Eden, D.C. Roopenian, and N. Shastri. 2001. Distinguishing self from nonself. Immunogenicity of the murine H47 locus is determined by a single amino acid substitution in an unusual peptide. J. Immunol. 166:4438–4445.

7. Pierce, R.A., E.D. Field, T. Mutis, T.N. Golovina, C. Von Kap-Herr, M. Wilke, J. Pool, J. Shabanowitz, M.J. Pettentati, L.C. Eisenlohr, et al. 2001. The HA-2 minor histocompatibility antigen is derived from a diallelic gene encoding a novel human class I myosin protein. J. Immunol. 167:3223–3230.

8. Hung, K., R. Hayashi, A. Lafond-Walker, C. Lowenstein,
D. Pardoll, and H. Levitsky. 1998. The central role of CD4(+) T cells in the antitumor immune response. *J. Exp. Med.* 188:2357–2368.

9. Zelenika, D., E. Adams, A. Mellor, E. Simpson, P. Chandler, B. Stockinger, H. Waldmann, and S.P. Cobbold. 1998. Rejection of H-Y disparate skin grafts by monospecific CD4+ Th1 and Th2 cells: no requirement for CD8+ T cells or B cells. *J. Immunol.* 161:1868–1874.

10. Brickner, A.G., E.H. Warren, J.A. Caldwell, Y. Akatsuka, T.N. Golovina, A.L. Zarling, J. Shabanowitiz, L.C. Eisenlohr, D.F. Hunt, V.H. Engelhard, and S.R. Riddell. 2001. The immunogenicity of a new mouse minor histocompatibility antigen results from differential antigen processing. *J. Exp. Med.* 193:195–206.

11. Unanue, E.R. 2002. Perspective on antigen processing and presentation. *Immunol. Rev.* 185:86–102.

12. Scott, D.M., I.E. Ehrmann, P.S. Ellis, C.E. Bishop, A.I. Agulnik, E. Simpson, and M.J. Mitchell. 1995. Identification of a mouse male-specific transplantation antigen, H-Y. *Nature.* 376:695–698.

13. Wang, W., L.R. Meadows, J.M.M. den Haan, N.E. Sherman, Y. Chen, E. Blokland, J. Shabanowitiz, A.I. Agulnik, R.C. Hendrickson, C.E. Bishop, et al. 1995. Human H-Y: A male-specific histocompatibility antigen derived from the SMGY protein. *Science.* 269:1588–1590.

14. Shastri, N. 1996. Needles in haystacks: Identifying specific peptide antigens for T-cells. *Curr. Opin. Immunol.* 8:271–277.

15. Rudensky, A.Y., P. Preston-Hurlburt, S.-C. Hong, A. Barlow, and C.A.J. Janeway. 1991. Sequence analysis of peptides bound to MHC class II molecules. *Nature.* 353:622–627.

16. Engelhard, V.H. 1994. Structure of peptides associated with class I and class II molecules. *Annu. Rev. Immunol.* 12:181–207.

17. Rammensee, H.G., J. Bachmann, and S. Stevanovic. 1997. MHC ligands and peptide motifs. Landes Bioscience, Austin, TX. 457 pp.

18. Shastri, N., S. Schwab, and T. Serwold. 2002. Producing nature’s gene-chips: The generation of peptides for display by MHC class I molecules. *Annu. Rev. Immunol.* 20:463–493.

19. Sanderson, S., K. Frauwirth, and N. Shastri. 1995. Expression of endogenous peptide-major histocompatibility complex class II complexes derived from invariant chain-antigen fusion proteins. *Proc. Natl. Acad. Sci. USA.* 92:7217–7221.

20. Bodmer, H., S. Viville, C. Benoist, and D. Mathis. 1994. Diversity of endogenous epitopes bound to MHC class II molecules limited by invariant chain. *Science.* 263:1284–1286.

21. Wang, R.F., X. Wang, A.C. Atwood, S.L. Topalian, and S.A. Rosenberg. 1999. Cloning genes encoding MHc class II-restricted antigens: mutated CDC27 as a tumor antigen. *Science.* 284:1351–1354.

22. Wang, H.Y., J. Zhou, K. Zhu, A.I. Riker, F.M. Marincola, and R.F. Wang. 2002. Identification of a mutated fibronectin as a tumor antigen recognized by CD4+ T cells: its role in extracellular matrix formation and tumor metastasis. *J. Exp. Med.* 195:1397–1406.

23. Mellman, I., and R.M. Steinman. 2001. Dendritic cells: Specialized and regulated antigen processing machines. *Cell.* 106:255–258.

24. Davis, A.P., and D.C. Roopenian. 1990. Complexity at the mouse minor histocompatibility locus H-4. *Immunogenetics.* 31:7–12.

25. Sanderson, S., and N. Shastri. 1994. LacZ inducible peptide/MHC specific T-hybrids. *Int. Immunol.* 6:369–376.

26. Takahara, Y., K. Hamada, and D.E. Housman. 1992. A new retrovirus packaging cell for gene transfer constructed from amplified long terminal repeat-free chimeric proviral genes. *J. Virol.* 66:3725–3732.

27. Burns, J.C., T. Friedmann, W. Drierer, M. Burrascano, and J.K. Yee. 1993. Vesicular stomatitis virus G glycoprotein pseudotyped retroviral vectors: concentration to very high titer and efficient gene transfer into mammalian and nonmammalian cells. *Proc. Natl. Acad. Sci. USA.* 90:8033–8037.

28. Bahnson, A.B., J.T. Dunigan, B.E. Baysal, T. Mohney, R.W. Atchison, M.T. Nimgaonkar, E.D. Ball, and J.A. Barranger. 1995. Centrifugal enhancement of retroviral mediated gene transfer. *J. Virol. Methods.* 54:131–143.

29. Sanderson, S., D.J. Campbell, and N. Shastri. 1995. Identification of a CD4+ T cell-stimulating antigen of pathogenic bacteria by expression cloning. *J. Exp. Med.* 182:1751–1757.

30. Campbell, D., and N. Shastri. 1998. Bacterial surface proteins recognized by CD4+ T cells during murine infection with Listeria monocytogenes. *J. Immunol.* 161:2339–2347.

31. Chu, C.C., and W.E. Paul. 1997. Fig1, an interleukin 4-induced mouse B cell gene isolated by cDNA representation analysis. *Proc. Natl. Acad. Sci. USA.* 94:2507–2512.

32. Chavan, S.S., W. Tian, K. Hseuh, D. Jawaeer, P.K. Gregersen, and C.C. Chu. 2002. Characterization of the human homolog of the IL-4 induced gene–1 (Fig1). *Biochim. Biophys. Acta.* 1576:70–80.

33. Roopenian, D.C. 1992. What are minor histocompatibility loci? A new look at an old question. *Immunol. Today.* 13:7–10.

34. Wall, K.A., J.Y. Hu, P. Carrier, S. Southwood, A. Sette, and A.J. Infante. 1994. A disease-related epitope of Torpedo aectyolcholine receptor. Residues involved in I-Ab binding, self-nonsel discrimination, and TCR antagonism. *J. Immunol.* 152:4526–4536.

35. Rudensky, A., S. Rath, P. Preston-Hurlburt, D.B. Murphy, and C.A. Janeway, Jr. 1991. On the complexity of self. *Nature.* 353:660–662.

36. Mendoza, L., P. Paz, A.R. Zuberi, G. Christianson, D.C. Roopenian, and N. Shastri. 1997. Minors held by majors. The H13 minor histocompatibility locus defined as a peptide/MHC class I complex. *Immunity.* 7:461–472.

37. Vogt, M.H., J.W. van den Muijsenberg, E. Goulmy, E. Spierings, P. Kluck, M.G. Kester, R.A. van Soest, J.W. Drijfhout, R. Willemze, and J.H. Falkenburg. 2002. The DBY gene codes for an HLA-DQ5-restricted human male-specific minor histocompatibility antigen involved in graft-versus-host disease. *Blood.* 99:3027–3032.