MHC Class I/peptide interactions: Binding specificity and kinetics

ARTICLE *in* JOURNAL OF MOLECULAR RECOGNITION · JUNE 1993

Impact Factor: 2.15 · DOI: 10.1002/jmr.300060204 · Source: PubMed

4 AUTHORS, INCLUDING:

David H Margulies
National Institute of Allergy and Infectious …
180 PUBLICATIONS **8,398** CITATIONS
SEE PROFILE

Lisa Boyd
U.S. Department of Health and Human Ser…
42 PUBLICATIONS **1,772** CITATIONS
SEE PROFILE

JOURNAL OF MOLECULAR RECOGNITION, VOL. 6, 59–69 (1993)

REVIEW PAPER

MHC Class I/Peptide Interactions: Binding Specificity and Kinetics

David H. Margulies,* Maripat Corr,† Lisa F. Boyd and Sergei N. Khilko

Molecular Biology Section, Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892, USA

Recent developments in the preparation of soluble analogues of the major histocompatibility complex (MHC) class I molecules as well as in the application of real time biosensor technology have permitted the direct analysis of the binding of MHC class I molecules to antigenic peptides. Using synthetic peptide analogues with cysteine substitutions at appropriate positions, peptides can be immobilized on a dextran-modified gold biosensor surface with a specific spatial orientation. A full set of such substituted peptides (known as 'pepsicles', as they are peptides on a stick) representing antigenic or self peptides can be used in the functional mapping of the MHC class I peptide binding site. Scans of sets of peptide analogues reveal that some amino acid side chains of the peptide are critical to stable binding to the MHC molecule, while others are not. This is consistent with functional experiments using substituted peptides and three-dimensional molecular models of MHC/peptide complexes. Detailed analysis of the kinetic dissociation rates (k_d) of the MHC molecules from the specifically coupled solid phase peptides reveals that the stability of the complex is a function of the particular peptide, its coupling position, and the MHC molecule. Measured k_d values for antigenic peptide/class I interactions at 25 °C are in the range of ca 10^{-4}–10^{-6}/s. Biosensor methodology for the analysis of the binding of MHC class I molecules to solid-phase peptides using real time surface plasmon resonance offers a rational approach to the general analysis of protein/peptide interactions.

INTRODUCTION

The major histocompatibility complex (MHC) class I molecules, cell surface glycoproteins found on virtually all types of mammalian somatic cells, form trimeric complexes of the MHC heavy chain, the MHC light chain (also known as β2-microglobulin, β2-m) and peptides acquired from the cell of origin during biosynthesis (Germain and Margulies, 1993). These tripartite intrinsic membrane proteins expressed on antigen presenting cells (APCs) then interact with T cell receptors (TcRs), clonally expressed as cell surface heterodimers of α and β chains in association with a signalling structure, the CD3 complex. The T cell responses initiated by the MHC/peptide and TcR interaction lead to lymphokine production and/or cytolytic activity. The crucial interactions that are involved in the sequence leading to such T cell responses can be viewed in molecular terms sequentially as the biosynthesis of the MHC class I heavy and light chains and the cotranslational transport of the chains into the lumen of the endoplasmic reticulum (ER); the generation and delivery of a set of self, viral or cellular parasitic peptides into the ER; the assembly and folding of the trimeric (MHC heavy, light and peptide) structures in the ER; the transport of the assembled intrinsic membrane molecule through the *cis, medial* and *trans* Golgi to the cell surface; and the binding of the cell surface displayed array of MHC/peptide complexes to cell surface TcRs and the subsequent activation of the T cells.

The past few years have been particularly exciting with respect to our understanding of each of these steps as structural, biochemical, molecular biological and cellular immunological approaches have contributed to a broad view of their details and interplay. Of particular interest are the specific parameters that influence the interaction of MHC class I molecules with specific peptides either in the lumen of the ER or at the cell surface. Thus, there has been considerable investigational activity in determining the amino acid sequences and motifs of peptides that are capable of binding to MHC class I molecules (Falk *et al.*, 1990; Van Bleek and Nathenson, 1990; Falk *et al.*, 1991; Corr *et al.*, 1992; Dibrino *et al.*, 1993), and in devising and refining methods for evaluating quantitatively the strength of this binding (Bouillot *et al.*, 1989; Chen and Parham, 1989; Townsend *et al.*, 1989; Chen *et al.*, 1990; Choppin *et al.*, 1990; Frelinger *et al.*, 1990; Townsend *et al.*, 1990; Boyd *et al.*, 1992; Ojcius *et al.*, 1992; Parker *et al.*, 1992). The focus of this review is to provide some background to the major biological questions that are of concern to the immunologist and to tie these to the physical chemical questions of the affinity and kinetics of MHC/peptide interactions. Some of the technical aspects of measuring MHC/peptide interactions will be considered and some recent developments both in genetic engineering and in real-time assays that offer valuable experimental approaches will be presented. Although the principle focus of this review is the MHC

* Author to whom correspondence should be addressed.
† Present address: Department of Medicine, UCSD Medical Center, 225 Dickinson St., San Diego, CA 92103-8417, USA.

Abbreviations used: MHC, major histocompatibility complex; β2-m, β2-microglobulin; APC, antigen presenting cell; TcR, T cell receptor; ER, endoplasmic reticulum; SDS, sodium dodecyl sulfate; SPR, surface plasmon resonance.

0952–3499/93/020059–11 $10.50

Received 6 August 1993
Accepted 10 August 1993

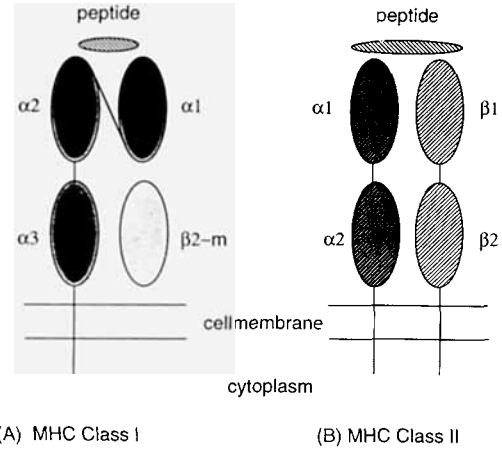

Figure 1. Schematic representation of the MHC class I and class II molecules. (A) The MHC class I molecule. α1, α2, and α3 refer to the respective domains of ca 90 amino acid residues that form the extracellular portions of the class I heavy chain. β2-m is the light chain, β2-microglobulin. The peptide is a self or foreign peptide usually of 8–10 residues in length. (B) The MHC class II molecule. α1 and α2 refer to the domains of the 33 kDa class II heavy chain. β1 and β2 refer to those regions of the 29 kDa light chain. For both class I and class II carbohydrate moieties are not indicated.

class I molecule, its structural and functional sibling, the MHC class II molecule, provides both similarity and contrast and will be discussed when appropriate.

MHC STRUCTURES

Any current discussion of MHC molecule function must begin with a description of the structure of the glycoprotein. The MHC class I molecule, as illustrated in Fig. 1(A), consists of a 46 kDa heavy chain, a polymorphic intrinsic membrane protein, non-covalently associated with the non-glycosylated light chain, β2-m, of 12 kDa (Bjorkman and Parham, 1990). Although the peptide portion is heterogeneous in preparations isolated from somatic cells, it is a critical component of the complex and is also depicted here. For comparison a class II molecule, consisting of a 33 kDa α chain and a 29 kDa β chain is also illustrated (Fig. 1(B)] (Kaufman *et al.*, 1984). The MHC class I molecule is characterized structurally by two membrane proximal immunoglobulin-like domains, one the α3 domain of the heavy chain, the other the single domain β2-m. The amino terminal portion (the α1 and α2 domains) of the molecule forms a peptide binding groove, and it is this part of the molecule with which specific T cells interact (Margulies and McCluskey, 1985).

The determination of the three-dimensional x-ray crystallographic structure of MHC class I molecules complexed either with heterogeneous self peptides (Bjorkman *et al.*, 1987; Madden *et al.*, 1991; Saper *et al.*, 1991; Madden *et al.*, 1992) or with homogeneous synthetic antigenic peptides (Fremont *et al.*, 1992; Guo *et al.*, 1992; Matsumura *et al.*, 1992; Silver *et al.*, 1992; Zhang *et al.*, 1992) has solidified our concept of these molecules. The peptide binding site is now pictured as a groove formed by the α helices of the α1 and α2 domains and a floor of β-sheet. A comparison of structures of the human class I MHC molecules HLA-A2 (Bjorkman *et al.*, 1987) and HLA-Aw68 led to the proposal of peptide binding pockets (Garrett *et al.*, 1989), now indicated by letters A to F (Saper *et al.*, 1991), that accomodate particular termini and/or side chains of the bound peptides. The determination of other class I crystallographic structures has confirmed this general view (Fremont *et al.*, 1992; Madden *et al.*, 1992; Zhang *et al.*, 1992), though for different MHC/peptide pairs different pockets may be important (Matsumura *et al.*, 1992). Molecular models of other MHC/peptide complexes have been built (Corr *et al.*, 1992, submitted). Figure 2(A) depicts a molecular model of the murine H-2L^d molecule (ribbon diagram) complexed with a viral antigenic peptide (van der Waals spheres). This emphasizes the location of the peptide binding site between the two α-helices, and the tight interaction of β-2m with the floor of the binding groove.

The recent description of the three-dimensional structure of the human MHC class II molecule, HLA-DR1, illustrates both similarities and differences to the class I molecules (Brown *et al.*, 1993) [Fig. 2(B)]. The class II molecule is similar to the class I in that the two membrane proximal domains, one in the α chain and one in the β chain, are immunoglobulin-like; the peptide binding groove is formed by eight strands of β-sheet and two anti-parallel α helices derived from both the α1 and β1 domains. The ribbon diagrams are viewed from a similar perspective to emphasize the domain similarities of the two molecules. The class II illustration does not contain bound peptide so as to allow visualization of the binding site.

Class II molecules can bind peptides of length significantly greater than that which is optimal for class I (Rudensky *et al.*, 1991; Chicz *et al.*, 1992). The three-dimensional structures offer a plausible explanation. While the class I molecule takes advantage of the amino and carboxyl termini of the peptide to anchor it by hydrogen bonds and salt bridges (Fremont *et al.*, 1992; Madden *et al.*, 1992), the class II binding site is open at both ends (Brown *et al.*, 1993). Rather, it takes advantage of interactions with a few peptide side chains and with the main chain as well. Thus, the class I peptide binding groove has closed ends that restrict the permissible length that can extend beyond the site itself. Class II, on the other hand, holds its peptide more loosely with the termini extending past the binding groove itself.

A critical observation made by the crystallographers is that the peptide makes deep contact with a large surface of the MHC class I, and is partially buried by MHC residues. In particular, it is not possible to move the peptide into and out of the binding cleft without encountering significant steric hindrance from the MHC, and thus, it is apparent that the MHC must undergo some conformational change to permit peptide binding. The contribution of the β2-m light chain to the stability of peptide binding has been the subject of considerable speculation and experiment (Townsend *et al.*, 1989; Rock *et al.*, 1990a,b; Vitiello *et al.*, 1990; Kozlowski *et al.*, 1991; Boyd *et al.*, 1992). In general, it seems that β2-m is a necessary component of the complex, and that β2-m and peptide mutually affect its stability. A remarkable feature of class I/peptide interactions is that a number of monoclonal antibodies that

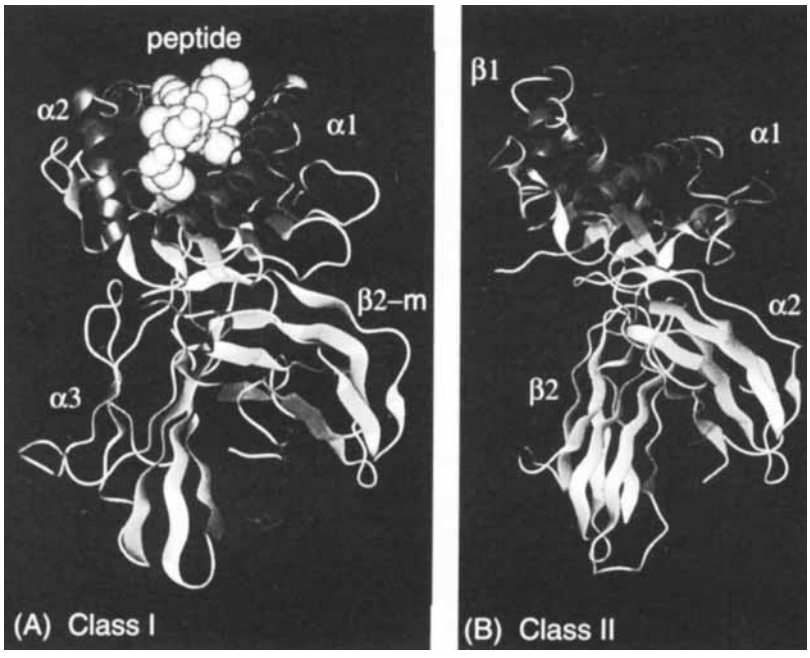

Figure 2. Three-dimensional models of MHC molecules. (A) Ribbon model of the H-2L^d_s molecule complexed with pMCMV (shown as van der Waals spheres) (Corr *et al.*, 1992). (B) Ribbon model of HLA-DR1 (Brown *et al.*, 1993). No peptide is shown in the HLA-DR1 model. Graphics models were constructed with QUANTA 3.3 on a Silicon Graphic Workstation.

recognize MHC structures of the α1 and/or α2 domains are dependent on peptide for their binding (Abastado *et al.*, 1989; Bluestone *et al.*, 1992; Catipovic' *et al.*, 1992; Uchanska-Ziegler *et al.*, 1993). Physical evidence that peptide interaction with the MHC changes its conformation has also been obtained (Fahnestock *et al.*, 1992). Evidence for peptide-dependent conformational change in the class II system derives from studies of the stability of the class II heterodimer on exposure to sodium dodecyl sulfate (SDS) at room temperature as assessed by gel electrophoresis. Remarkably, in the presence of the appropriate peptide, the class II heterodimer is stable in SDS, while it falls apart into its component chains in the absence of peptide (Dornmair *et al.*, 1989; Mellins *et al.*, 1990; Sadegh-Nasseri and Germain, 1991).

MHC CLASS I MOLECULES AS PEPTIDE BINDING CELL SURFACE RECEPTORS

The immunological function of both classes of MHC molecules is to bind antigenic peptides of limited size and sequence, and to display them at the cell surface in a multivalent array for recognition by TcRs. Since TcRs are expressed clonotypically, as a single αβ pair, individual receptors have specificity for only a single MHC/peptide combination. Thus, a T cell clone expressing a specific αβ heterodimer is activated by binding of a particular MHC/peptide complex. If the MHC is not the correct one or if the bound peptide is inappropriate, the TcR is not bound and the clone is not stimulated. This process, known as 'MHC-restricted antigen specific T cell recognition' is a central and initiating theme in T cell immunity.

Class I MHC-restricted recognition is the basis for the identification by T lymphocytes of cells altered by viral or parasitic infection, by neoplastic transformation, or of cells that are 'histoincompatible', that is, derived from genetically disparate sources. Such T cells usually express the CD8 accessory molecule, which interacts with a conserved region on the class I α3 domain (Salter *et al.*, 1989). CD8 + m, class I-restricted cells are often, but not exclusively cytolytic. The MHC class II system offers appropriate T cells (those of the CD4-expressing set) a means for identifying cells that have ingested foreign proteins from the extracellular milieu. Such foreign proteins enter the endosomal pathway of the APC where they are degraded and where their fragments encounter MHC class II molecules that have been directed there by their association with a third component of the class II molecule, the invariant chain (Jones *et al.*, 1979; Neefjes *et al.*, 1990).

The natural and predominant pathways of antigen presentation are 'inside out' for class I and 'outside in' for class II (Germain, 1986), but these can be circumvented by incubating the APC with peptides of defined length and sequence. Under such conditions, the peptide can still bind to apparently 'empty' cell surface MHC molecules, even on chemically fixed cells, and the complex can bind TcR and activate T cells. In addition to the cell dependent antigen presentation systems using fixed APC and defined peptides, purified MHC class I and class II proteins can provide the same stimulatory function when multivalent arrays of them are made, and then pulsed with peptide (Watts *et al.*, 1984; Fox *et al.*, 1987; Kane *et al.*, 1989; Kozlowski *et al.*, 1991; T. Takeshita *et al.*, in preparation). Such cell free systems have not only allowed the detailed evaluation of the peptide/MHC specificity of binding, but have also permitted kinetic analyses of the formation of the peptide/MHC complexes. In addition, such systems have revealed the role of proteases in the extracellular processing of defined peptides. Of particular interest in this regard is that some longer peptides that are not

capable of binding their appropriate MHC restricting element can in fact be activated to do so by specific proteolytic cleavage. One serum enzyme that contributes to this extracellular processing is the carboxy dipeptidase, angiotension converting enzyme (Kozlowski *et al.*, 1992; Sherman *et al.*, 1992). However, for non-optimal peptides, it is now clear that several different amino and carboxy peptidases contribute to this 'extracellular processing', creating a race between the generation and the degradation of the most active peptide (Kozlowski *et al.*, 1993).

THE ESSENTIAL PARADOX OF MHC-RESTRICTED T CELL RECOGNITION

MHC molecules possess a paradoxical characteristic, namely they bind particular antigens with exquisite specificity, discriminating between those that differ at single amino acid residues, and yet preserve profound degeneracy in the ability of each MHC molecule to bind a host of peptidic structures. Specificity denotes a relative situation, that is, the ability to bind some peptides amongst a large population of others. The degeneracy of the MHC/peptide binding is that any given MHC molecule can bind a very large number of peptides (Schumacher *et al.*, 1992).

The general outline of the resolution of this apparent paradox derives both from structural studies and from the characterization of peptides that copurify with MHC molecules. The structural studies, outlined above, indicated that particular parts of bound peptides make deep and intimate contact with the MHC molecule. This led to the view of peptide binding pockets. The characterization of self and viral peptides that copurify with MHC molecules, described below, led to the identification of peptide 'motifs' characteristic of those peptides that bound particular MHC molecules.

A clue to the role of peptide in contributing to the mature three-dimensional form of the MHC molecules was provided by the additional electron density that could not be accounted for by the primary amino acid sequence of the HLA molecule in the first x-ray crystallographic studies of class I (Bjorkman *et al.*, 1987). The realization that this electron density corresponded to bound self peptides led the way not only for the solution of the structure, but also to the identification of such self peptides in class I (Falk *et al.*, 1991) and in class II (Jardetzky *et al.*, 1991; Rudensky *et al.*, 1991). It soon became clear that 'motifs' of self peptides, characteristic of the MHC molecule with which they copurified, could usually be identified. For instance, for the mouse H-2K^b class I molecule, the motif is a phenylalanine or tyrosine at position 5 and leucine at the carboxyl terminal position 8 (or 9) of the peptide (Falk *et al.*, 1991). A number of motifs of different human and mouse class I molecules have been determined in the past few years and this subject has recently been reviewed (Rammensee *et al.*, 1993). The emerging solution to the specificity/degeneracy paradox seems to be that for peptides to be bound by a particular MHC molecule, they must have particular residues at particular positions, but in other positions considerable variability is tolerated.

A final solution of the degeneracy/specificity paradox of course demands a detailed appreciation of the contributions of individual amino acids of a wide range of antigenic peptides to MHC binding. The rest of this review will deal with the question of measuring MHC peptide interactions, and with the development of powerful new technologies to confront this difficult problem.

MHC/PEPTIDE BINDING

A class II review

Although the notion that MHC molecules bound antigenic peptides and presented them to the receptors on clones of T cells developed relatively early in the immunologist's interest in the phenomenon of antigen presentation, direct demonstration of the binding of specific synthetic peptides to purified MHC molecules lagged, and was first clearly demonstrated for purified detergent-solubilized class II molecules (Babbitt *et al.*, 1985; Buus *et al.*, 1986). In these experiments either fluoresceinated or radiolabelled MHC class II-restricted peptides were incubated with immunoaffinity purified class II molecules. Then free and bound tagged peptide were separated by either equilibrium dialysis or by gel filtration. Equilibrium analysis indicated that the K_d (equilibrium dissociation constant) for the interaction of the MHC class II and the peptide was of the order of 2×10^{-6} M. For a characteristic diffusion-limited situation, assuming a Langmuirian model for a bimolecular reaction, one might expect a value of the association rate constant, k_a, of 10^6/M/s (which is characteristic of antibody/antigen interactions) (Berzofsky *et al.*, 1989) or even as high as 10^9! Since $K_d = k_d/k_a$, this would suggest that the dissociation rate constant, k_d, would be of the order of 1/s or less. This would be too fast a dissociation to permit separation of free and bound ligand by conventional gel filtration chromatography, since the separation time is large relative to the off rate. However, when the dissociation rate of a radiolabelled peptide from a class II peptide complex was experimentally determined, surprisingly, a very slow kinetic off rate was observed, with a measured $t_{1/2}$ being on the order of 30 h at room temperature (Buus *et al.*, 1986). This corresponds to a kinetic dissociation rate constant of ca 6.4×10^{-6}/s. In addition, the apparent kinetic association rate constant was slow ($\sim$1/M/s). Thus, the MHC class II molecules had several peculiar binding characteristics: they interacted with peptides at moderately low affinity, they had extremely long dissociation times, and they had very slow apparent association rates. The long dissociation times seem to be generally characteristic for MHC/peptide interactions for tight binding peptides. The slow association rates may reflect a number of parameters: most MHC preparations prepared from cells consist largely of molecules that have bound self peptides, thus the number of available sites is not accurately reflected in the concentration of the purified protein; a two-stage binding process in which loose complexes form rapidly, and long-lived complexes are formed more slowly (Sadegh-Nasseri and McConnell, 1989); the class II molecule physiologically binds its peptide in an acidic cellular compartment, where on

rates are much faster, and then displays the complex at the cell surface at neutral pH—binding experiments rarely are designed to consider this change in pH; additional conformationally slow steps may be involved in tight peptide binding, including the possible formation of dimers of the class II αβ heterodimer as suggested by the quaternary crystal structure (Brown *et al.*, 1993).

The class I story

Initial attempts to evaluate the binding of antigenic peptides to purified class I MHC molecules were of mixed success, in contrast to the clear quantitative data obtained in several laboratories with class II molecules. In one early report, Chen and Parham 1989) detected binding of a radiolabelled influenza peptide quinquedecamer to immunoaffinity purified HLA molecules by gel filtration chromatography and immunoassay of the eluted fractions. They estimated that $<0.3\%$ of the MHC class I molecules were capable of binding peptide. In this assay, it was difficult to demonstrate the specificity of binding that had been determined in functional T cell assays. Shortly thereafter, Bouillot *et al.*, (1989) introduced a solid-phase assay in which synthetic peptides were adsorbed or coupled to plastic surfaces to which radiolabelled detergent-solubilized MHC class I preparations were added. Little specificity of binding was observed with this assay as well (Chen *et al.*, 1990; Choppin *et al.*, 1990; Frelinger *et al.*, 1990).

Within the next few years, several lines of evidence converged, offering explanations for the apparent imprecision of the solution and solid-phase assays previously described, and providing a basis for the development of more precise methods. Townsend and his colleagues (Townsend *et al.*, 1989) made the striking observation that the expression of cell surface class I MHC molecules on a tumor cell defective in this function could be markedly 'induced' by incubating the cells with peptides known to functionally interact with the MHC type of the cell. Further analysis of these 'peptide induced' molecules indicated that they had a much more stable association of the MHC class I heavy chain and β2-m following peptide exposure. Subsequent studies have demonstrated that the phenotype of this cell, and others like it (Otten *et al.*, 1992), resulted from a lesion in the system that delivers peptides to the ER. Such defective cell lines, in conjunction with a large battery of monoclonal antibodies directed against specific MHC class I molecules, have become the basis for an indirect peptide binding assay in which the effect of the interaction of the peptide is assessed by immunofluorescence analysis of the peptide stabilized class I epitopes. These assays revealed the known specificity of particular peptides for particular MHC class I molecules. One of the difficulties with many of the early biochemical assays, either solution or solid phase, was that the purified MHC molecules behaved as though they were already loaded with self peptides.

Other evidence, which in retrospect explains the difficulties with the early MHC class I peptide binding assays, was derived from the analysis of the amino acid sequences of peptides that copurified with MHC class I molecules (Falk *et al.*, 1991; Hunt *et al.*, 1992). The most remarkable feature was that the amino acid sequences were no longer than eight, nine or ten residues. It was clear, then, that class I bound peptides were likely to be smaller than the synthetic peptides used in many binding studies and in functional assays.

Studies from our laboratory (Boyd *et al.*, 1992) employed a molecularly engineered soluble analogue of the murine class I molecule H-2L^d and a synthetic peptide corresponding to residues 168 to 176 of the pp89 murine cytomegalovirus early regulatory protein, YPHFMPTNL (known as pMCMV) (Reddehase *et al.*, 1989). The soluble MHC analogue was generated from a chimeric gene derived from the 5' sequences encoding the amino terminal α1 and α2 domains of the mouse class I molecule, H-2L$_d$, and the 3' sequences encoding the α3 domain and the unique carboxyl terminus of the obligately soluble mouse class I-like molecule, Q10^b (Margulies *et al.*, 1986, 1990). When genes of this sort are transfected into an appropriate tissue culture cell line such as a L cell, the encoded protein assembles with endogenous peptides and the β2-m of the cell, is secreted, and accumulates in the culture supernatant. Our initial choice of soluble H-2L^d (known as H-2L^d_s) and of pMCMV was the result of some trial and error with other engineered soluble class I molecules and their cognate peptides. The peptide pMCMV could easily be radiolabelled at its amino terminal tyrosine with ^{125}I, and when incubated with H-2L^d_s, could bind, detected either by spin column gel filtration or by immunoprecipitation of the peptide/MHC complex. Both equilibrium and kinetic measurements were made, revealing characteristics similar to those described previously for MHC class II molecules. A careful analysis of the equilibrium binding data suggested that H-2L^d_s/peptide interactions could be described by two classes of binding sites of differing affinity, suggesting that molecular heterogeneity of the MHC molecule, perhaps related to the β2m, played a role. One model was that heavy chains with tight β2-m were high affinity sites, while those with no β2-m or with loose β2-m had low affinity sites. The K_d for the high affinity sites was ca 3.7×10^{-7} M. In addition, kinetic parameters were measured at 37 °C: a kinetic on rate of ca 10/M/s was determined, as well as a k_d of ca 10^{-4}/s. These values were of course not fully compatible with the measured K_d, and a variety of technical and practical concerns were discussed. These values were, however, comparable to those obtained by other groups using radiolabelled peptide and cell surface molecules deficient in self peptides: k_a of ca 700/M/s and k_d of 3.8×10^{-4}/s for a 17-mer peptide and H-2D^b (Christinck *et al.*, 1991). In addition, Cerundolo *et al.* (1991) used a similar crude lysate to examine equilibrium and kinetic binding with nonamer peptides and H-2D^b and obtained similar results (i.e., K_a (equilibrium association constant) ca 3×10^5 to ca 3×10^7/M; and $t_{1/2}$ for dissociation ranging from 39 h at 22 °C to 3 h at 37 °C for the nonamer.

Despite the relative success of the H-2L^d_s/pMCMV system using radiolabelled peptide, soluble purified MHC class I, and spin column gel filtration for the rapid separation of free from bound ligand, we were generally dissatisfied not only because of discrepancies in measured kinetic rates as compared with equilibrium values, but also because of the difficulty in identifying peptides that could be labelled and would retain binding activity for other purified class I molecules. Ojeius *et al.* (1992, 1993) have used a direct fluorescence enhancement technique to examine the binding of a

dansylated peptide to the MHC class I molecule, H-2K^d. This is a rapid and sensitive method, but requires that the peptide of interest be able to be derivatized without adverse affect on its binding. Though this may be the case for this particular MHC/peptide system, we were concerned that this may not be a generally applicable technique.

A REAL-TIME BIOSENSOR ASSAY FOR MEASURING MHC/PEPTIDE BINDING

Because of the obvious limitations of methods that require tagging of probe peptides for binding studies, we sought an approach that would permit rapid, reproducible and reliable measurements of protein/peptide interactions. Recently, surface plasmon resonance (SPR) has been expanded in a robotically controlled system to permit the evaluation in real time of a variety of macromolecular interactions (Jönsson *et al.*, 1991), including antibody/antigen interactions (Fägerstam *et al.*, 1990; Karlsson *et al.*, 1991; Altschuh *et al.*, 1992), receptor/ligand interactions (Brigham-Burke *et al.*, 1992) and protein/DNA interactions (Jost *et al.*, 1991). SPR is an optical phenomenon in which refractive index changes due to the interaction of macromolecules with other ligands coupled to a dextran-modified gold surface are detected by changes in the angle of complete internal reflectance of polarized light incident on the opposite surface of the gold film. Using SPR, measurements of such interactions can be made almost continuously. We have applied this methodology to the analysis of the binding of soluble MHC class I analogues to specifically coupled peptides (Khilko *et al.*, 1993).

Since the intensity of the SPR signal is proportional to the mass of the material that binds the gold surface, there is a significant advantage in using high molecular weight molecules as the fluid phase ligand. Therefore, we explored methods of coupling known antigenic peptides to the biosensor surface in the hope of visualizing direct binding of soluble MHC molecules. Our initial attempts employed the pMCMV peptide nonamer, YPHFMPTNL, coupled to the dextran/gold surface through the amino terminal amine. Under these conditions, very little binding of several different preparations of H-2L^d_s was detected. In an attempt to minimize steric effects, we used a derivative of this peptide in which the amino terminal amine was coupled through a long chain spacer to biotin. This biotinylated peptide was bound non-covalently to a surface that had been covalently coupled to streptavidin. Again, little binding was detected. Because the crystallographic structure of several MHC class I molecules had indicated that the amino terminal amine played an important role in the binding of the peptide to the MHC, we suspected that this poor binding was due to effects of coupling through the amino terminal amine. In addition, other functional and solution binding studies of the H-2L^d_s/pMCMV interaction performed in our laboratory (L.F. Boyd, unpublished observations) and by others (Reddehase and Koszinowski, 1991), as well as an evaluation of molecular models of H-2L^d_s/self peptide complexes (Corr *et al.*, 1992), had suggested that position 4 of the pMCMV peptide was likely to be exposed to solvent and not critical for binding to the MHC. Therefore, a peptide analogue with cysteine substituted for phenylalanine at position 4 was synthesized, and used to couple to the biosensor surface either indirectly through the biotin/avidin linkage, or directly through a bifunctional cross-linker. Good binding was observed with the biotinylated peptide coupled to the surface through streptavidin, but an even better signal was observed with the direct coupling method.

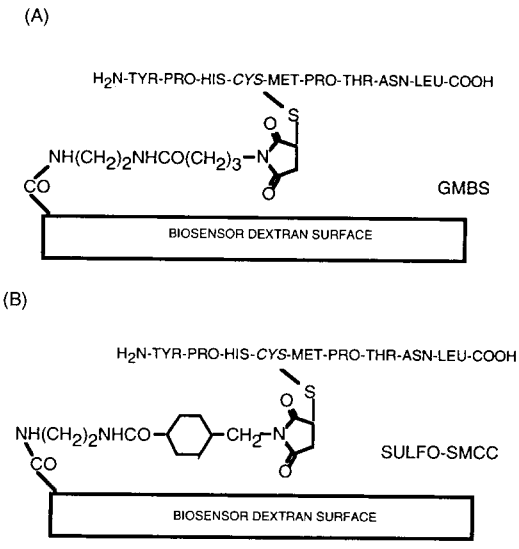

Figure 3. Schematic representation of the coupling chemistry of peptides to a biosensor surface. The peptide modified surfaces shown were synthesized by sequentially elongating the carboxy-terminated dextran with ethylene diamine and the indicated heterobifunctional cross-linkers, GMBS is *N*-γ-maleimidobutyryloxy-succinimide and sulfo-SMCC is sulfosuccinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate.

The chemical linkages that we have employed successfully are shown in Fig. 3. Figure 3(A) shows the coupling similar to that used by O'Shannessy *et al.* 1992) that we employed (Khilko *et al.*, 1993), which uses a *N*-γ-maleidimidobutyryloxy-succinimide coupling between amino groups generated on the biosensor dextran surface, and the free sulfhydryl of the cysteine substituted peptide. Figure 3(B) describes a somewhat more convenient chemistry, exploiting the now commercially available compound sulfosuccinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (S. N. Khilko, unpublished observations).

Using such vectorially coupled peptides, known colloquially as 'pepsicles', we then examined the ability of particular MHC class I molecules to bind to such solid phase peptides (Khilko *et al.*, 1993). A plasmon resonance sensorgram that illustrates the binding of solution phase H-2L^d_s to a surface coupled with the pMCMV-C4 analogue is shown in Fig. 4. Here, following the injection of the ligand, steady binding is observed. When the buffer wash-out is begun at $t=0$, dissociation of the MHC from the peptide surface is also observed.

MAPPING THE MHC BINDING SITE QUALITATIVELY USING SPR

To expand upon the observation that binding of MHC molecules to solid phase peptides could be readily detected with SPR, a full set of cysteine-substituted

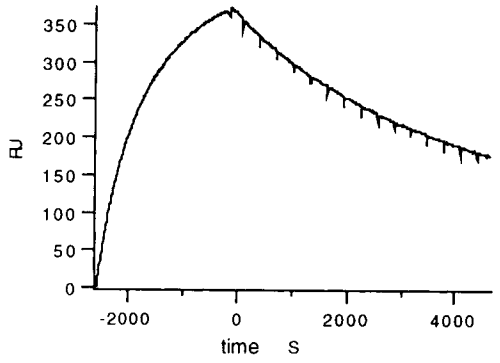

Figure 4. Kinetic sensorgram of binding of H-2L$_s^d$ to pMCMV-C4 modified biosensor surface. pMCMV-C4 was coupled to the surface as described by Khilko *et al.* (1993). H-2L$_s^d$ was offered as a 0.8 μM solution at a flow rate of 1 μL/min for 40 min. The dissociation phase was accomplished with buffer (10 mM HEPES, pH 7.1; 3.4 mM EDTA; 150 mM NaCl; 0.005% Tween 20) at a flow rate of 100 μL/min for 80 min.

pMCMV analogues was investigated (Khilko *et al.*, 1993). As expected, peptides coupled through amino acid side chains that were thought to play a crucial role in the interaction with the MHC could not bind very well, while those coupled with the same chemistry through residues exposed or partially exposed to the surface of the molecular complex were permissive for MHC binding. Specifically, peptides coupled through pMCMV positions, 2, 3 or 9, positions thought to be crucial for MHC binding, could not stably interact with the MHC by this assay, but peptides coupled through positions 4, 5, 6, 7 or 8 provided a fully accessible solid phase ligand for MHC binding. A position 1 analogue, when used for coupling, allowed some MHC binding, but the off rate was rather fast. The use of such a peptide scan for mapping an MHC binding site is illustrated in Fig. 5. Figure 5(A) shows that if side chains available to solvent in the bound state are used for coupling, then the MHC can interact well. Figure 5(b) illustrates the behavior of a peptide coupled through a crucial side chain, thus not permitting binding of the MHC. By systematic evaluation of a full set of peptides, the contribution of individual side chains to the binding can be assessed. This kind of scan has also been applied to H-2K$_s^b$/ovalbumin peptide, H-2D$_s^d$/gp 160 peptide, and HLA-A2$_s$/influenza peptide interactions (S. N. Khilko, unpublished data).

In addition to the direct binding assay illustrated here, competition assays can also be performed. Using a single tight binding solid phase peptide, one can easily assess the competition in solution phase of the same peptide uncoupled or of a large battery of related and unrelated peptides over a wide range of concentration. It is straightforward, then, to rank order the binding of a set of peptides in their competitive capacity for the solid phase.

KINETIC ANALYSIS OF MHC/PEPTIDE STABILITY

Dissociation rates

To examine the stability of different peptide/MHC complexes, we have first measured the dissociation rates of bound MHC molecules from peptide surfaces. Since dissociation is a first-order event, the values that we obtain by curve-fitting to the observed data should be independent of MHC concentration. In addition, as argued by others (Cerundolo *et al.*, 1991), the biological importance of MHC/peptide complexes is related to their stability at the cell surface, where they are available for TcR interactions. An example of the dissociation portion of a sensorgram for the MHC H-2L$_s^d$ molecule bound to a solid phase pMCMV-C4 is shown in Fig. 6. Because of the relatively stable complex, the

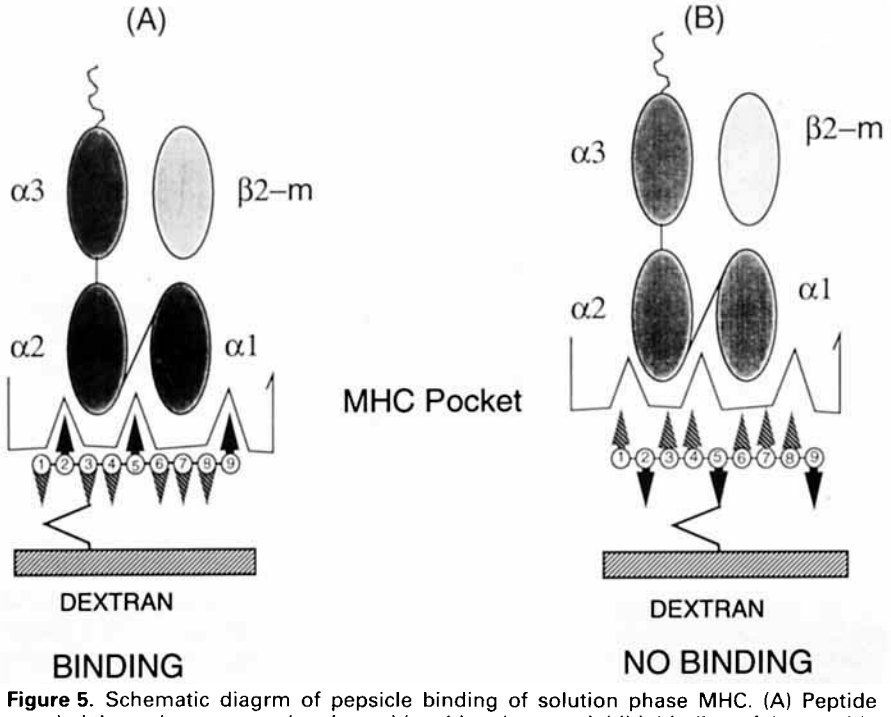

Figure 5. Schematic diagrm of pepsicle binding of solution phase MHC. (A) Peptide coupled through an exposed amino acid residue does not inhibit binding of the peptide of the MHC. (B) Peptide coupled through an amino acid position critical to MHC binding prevents interaction with the MHC.

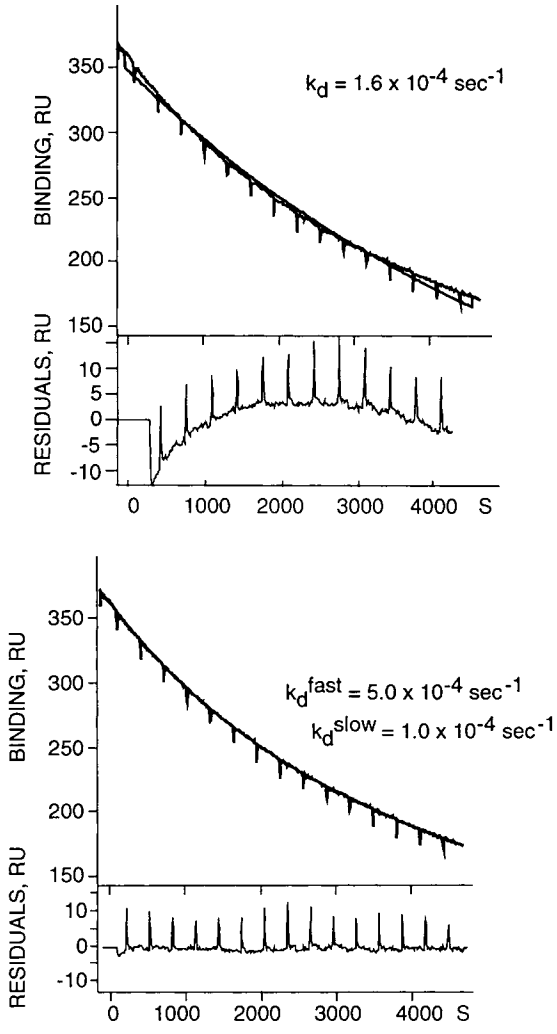

Figure 6. Kinetic dissociation sensorgram. The dissociation region from Fig. 4 is enlarged. (A) Curve fit to the single exponential equation: $B_t = B_0\exp(-k_d t)$, where B_t is the binding (RU) at time t, and B_0 is the binding at the beginning of the wash-out. $B_0 = 352$; $k_d = 1.6 \times 10^{-4}$ s. (B) Curve fit of the same data to the double exponential equation: $B_t = B_0^{fast}\exp(-k_d^{fast} t) + B_0^{slow}\exp(-k^d_{slow}\, t)$. $B_0^{fast} = 99$; $B_0^{slow} = 263$; $k_d^{fast} = 5.0 \times 10^{-4}$ s; $k_d^{slow} = 1.0 = 10^{-4}$ s.

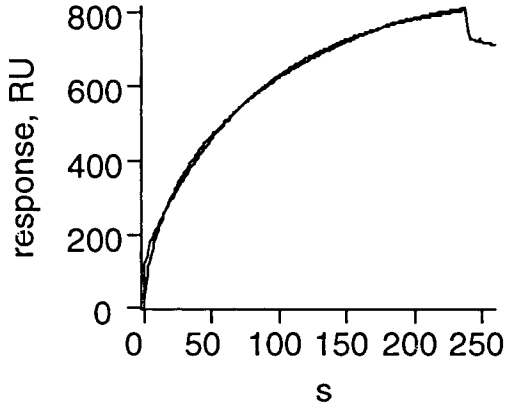

Figure 7. Kinetic association sensorgram. Association phase from the binding of H-2L^d_s to pMCMV-C5. H-2L^d_s (10 μM) was injected over a pMCMV-C5 surface prepared as described in the legend to Fig. 4, at a flow rate of 10 μL/min for 4 min. Curve fit was to a single exponential equation of the form: $B_t = B_{max}[1\text{-}\exp(-k_a ct)]$, where B_{max} is the expected value for maximal binding and c is the concentration of the soluble H-2L^d_s. $B_{max} = 848$; $k_a = 115$/M/s.

dissociation wash-out was performed over a long time period, and artifacts due to refilling of the injection syringe are evident. Data analysis was performed with the non-linear curve-fitting program IGOR, as suggested by O'Shannessy (O'Shannessy *et al.* 1993). The data points [Fig. 6(A)] reveal that a first-order exponential curve fit is relatively good, giving a k_d of 1.6×10^{-4}/s for this experiment peformed at 25 °C. However, as illustrated in Fig. 6(B), curve fitting to a biexponential decay, in which two species with different dissociation rates are assumed, clearly gives a much better fit. In this case for the fast component, k_d is 5.0×10^{-4}/s, and for the slow component, k_d is 1.0×10^{-4}/s. This apparent biphasic dissociation, consistently observed in the analysis of some 40 different peptide/MHC interactions, may reflect heterogeneity in the way the same solid phase peptide is bound by different populations of the MHC molecule. Whether this indeed is of biological importance and is not an artifact of the protein preparations or the coupled biosensor surfaces, remains to be determined.

Association rates

Analysis of the association rates of solution phase MHC class I molecules for solid phase peptide will be the next phase of our studies. At the present time, we have not concluded such analyses for several reasons. The most important is that an appropriate association rate analysis demands knowledge of the concentration of the active species of the fluid phase ligand. Usually for proteins that are readily purified this is assumed to be the same as the concentration of the protein. Often this is based on accurate equilibrium measurements that permit the measurement of available sites. For the MHC class I molecules, however, several problems complicate the issue. First, the class I molecules purified from cellular sources almost always copurify with a heterogeneous population of self peptides (see earlier). Second, MHC class I molecules that are artificially emptied of self peptides by treatments such as extremes of pH, are, particularly at low concentration, relatively unstable and are susceptible to proteolysis and denaturation. Third, the structure of the MHC molecule as a heterodimer of its heavy chain and β2-m, in which the stability of the complex is related to peptide binding, makes it likely that the kinetic association of purified MHC molecules is in fact a complex phenomenon in which co-operativity and occupied binding sites make the situation difficult to fully control.

One approach to simplifying this system is to use engineered single chain class I molecules (Mottez *et al.*, 1991; Godeau *et al.*, 1992; Mage *et al.*, 1992), which purify without the complication of the heterogeneity of tissue culture medium β2-m, and which do not have the potential difficulty of heterogeneous β2-m. However, when produced in tissue culture systems, these molecules contain self peptides (M. Corr *et al.*, unpublished observations). Efforts are now underway in our laboratory to develop quick and reliable methods for emptying preparations of such molecules of bound peptides, retaining their activity and analyzing their binding properties.

Nevertheless, attempts can be made to perform kinetic association analyses of purified MHC molecules to

solid phase peptides, and to take advantage of the non-linear curve-fitting approach described above. As illustrated in Fig. 7, one can obtain reasonable data and respectable curve fits. There is some difficulty in accurately fitting the association rate curves to simple mass action models. This may reflect complexities such as the biphasic kinetics observed in the dissociation phase. Even if one assumes that 100% of the molecules are fully active, the kinetic association rate constants are 10^3–10^4/M/s, a low value that cannot be explained trivially. The most reasonable explanation is that a significant conformational change must take place before the binding to peptide is permitted, and that either this conformation itself is relatively transient or that it takes a relatively long time until it occurs.

CONCLUSIONS

The analysis of biomolecular interactions is a critical enterprise for our understanding not only of molecular physiology but also in our comprehension of the cellular and organismic biology that relies on them. The activity of the immune system is now viewed not only in terms of antibodies and their antigens, but as the complex interactions of a host of cell surface and soluble mediators that influence each other profoundly and subtly in a symphonic dance. Our goals are to understand not only the system when not in harmony, as exemplified by autoimmunity and immunodeficiency, but also to fully appreciate the careful interplay of its components. Understanding several key molecular interactions in detail, such as the binding of peptides by MHC molecules, should allow us to speculate as to the causes of immune dysregulation and to propose molecular therapies to counter them.

Acknowledgements

We thank our colleagues S. Kozlowski and C. Hoes for their comments and help, and D. O'Shannessy for providing the details of his curve-fitting method prior to publication. We thank L. Stern for providing coordinates of the HLA-DR1 structure.

REFERENCES

Abastado, J., Darche, S., Jouin, H., Delarbre, C., Gachelin, G. and Kourilsky, P. (1969). A monoclonal antibody recognizes a subset of the H-2Dd mouse major class I antigens. *Res. Immunol.* **140**, 581–594.

Altschuh, D., Dubs, M. C., Weiss, E., Zeder, L. G. and van Regenmortel, M. H. (1992). Determination of kinetic constants for the interaction between a monoclonal antibody and peptides using surface plasmon resonance. *Biochemistry* **31**, 6298–6304.

Babbitt, B., Allen, P., Matsueda, G., Haber, E. and Unanue, E. (1985). Binding of immunogenic peptides to Ia histocompatibility molecules. *Nature* **317**, 359–361.

Berzofsky, J. A., Epstein, S. L. and Berkower, I. (1989). *Antigen–Antibody Interactions and Monoclonal Antibodies.* Raven Press Ltd, New York.

Bjorkman, P. and Parham, P. (1990). Structure, function, and diversity of class I major histocompatibility complex molecules. *Annu. Rev. Biochem.* **59**, 253–288.

Bjorkman, P., Saper, M., Samraoui, B., Bennett, W., Strominger, J. and Wiley, D. (1987). Structure of the human class I histocompatibility antigen, HLA-A2. *Nature* **329**, 506–512.

Bluestone, J. A., Jameson, S., Miller, S. and Dick, R. (1992). Peptide-induced conformational changes in class-I heavy chains alter major histocompatibility complex recognition. *J. Exp. Med.* **176**, 1757–1761.

Bouillot, M., Choppin, J., Cornille, F., Martinon, F., Papo, T., Gomard, E., Fournie-Zaluski, M. and Lévy, J. P. (1989). Physical association between MHC class I molecules and immunogenic peptides. *Nature* **339**, 473–475.

Boyd, L. F., Kozlowski, S. and Margulies, D. H. (1992). Solution binding of an antigenic peptide to a major histocompatibility complex class I molecule and the role of β-2 microglobulin. *Proc. Natl Acad. Sci. USA* **89**, 2242–2246.

Brigham-Burke, M., Edwards, J. and O'Shannessy, D. (1992). Detection of receptor–ligand interactions using surface plasmon resonance: model studies employing the HIV-1 gp120/CD4 interaction. *Analyt. Biohem.* **205**, 125–131.

Brown, J. H., Jardetsky, T. S., Gorga, J. C., Stern, L. J., Urban, R. G., Strominger, J. L. and Wiley, D. C. (1993). Three-dimensional structure of the human class II histocompatibility antigen HLA-DR1. *Nature* **364**, 33–39.

Buus, S., Sette, A., Colon, S., Jenis, D. and Grey, H. (1986). Isolation and characterization of antigen-Ia complexes involved in T cell recognition. *Cell* **47**, 1071–1077.

Catipovic', B., Dal Porto, J., Mage, M., Johansen, T. and Schneck, J. (1992). Major histocompatibility complex conformational epitopes are peptide specific. *J. Exp. Med.* **176**, 1611–1618.

Cerundolo, V., Elliott, T., Elvin, J., Bastin, J., Rammensee, H. and Townsend, A. (1991). The binding affinity and dissociation rates of peptides for class I major histocompatibility complex molecules. *Eur. J. Immunol.* **21**, 2069–2075.

Chen, B. and Parham, P. (1989). Direct binding of influenza peptides to class I HLA molecules. *Nature* **337**, 743–745.

Chen, B., Rothbard, J. and Parham, P. (1990). Apparent lack of MHC restriction in binding of class I HLA molecules to solid-phase peptides. *J. Exp. Med.* **172**, 931–936.

Chicz, R., Urban, R., Lane, W., Gorga, J., Stern, L., Vignali, D. and Strominger, J. (1992). Pedominant naturally processed peptides bound to HLA-DR1 are derived from MHC-related molecules and are heterogeneous in size. *Nature* **358**, 764–768.

Choppin, J., Martinon, F., Gomard, E., Bahraoui, E., Connan, F., Bouillot, M. and Lévy, J. (1990). Analysis of physical interactions between peptides and HLA molecules and application to the detection of human immunodeficiency virus 1 antigenic peptides. *J. Exp. Med.* **172**, 889–899.

Christinck, E., Luscher, M., Barber, B. and Williams, D. (1991). Peptide binding to class I MHC on living cells and quantitation of complexes required for CTL lysis. *Nature* **352**, 62–70.

Corr, M., Boyd, L. F., Frankel, S. R., Kozlowski, S., Padlan, E. A. and Margulies, D. H. (1992). Endogenous peptides of a soluble major histocompatibility complex class-I molecule, H-2L(d)(s)—sequence motif, quantitative binding, and molecular modeling of the complex. *J. Exp. Med.* **176**, 1681–1692.

Dibrino, M., Parker, K. C., Shiloach, J., Knierman, M., Lukszo, J., Turner, R. V., Biddison, W. E. and Coligan, J. E. (1993). Endogenous peptides bound to HLA-A3 possess a specific combination of anchor residues that permit identification of potential antigenic peptides. *Proc. Natl Acad. Sci. USA* **90**, 1508–1512.

Dornmair, K., Rothenhäusler, B. and McConnell, H. (1989). Structural intermediates in the reactions of antigenic peptides with MHC molecules. *Cold Spring Harb. Symp. Quant. Biol.* **54**, 409–416.

Fägerstam, L., Frostell, A., Karlsson, R., Kullman, M., Larsson, A., Malmqvist, M. and Butt, H. (1990). Detection of antigen–antibody interactions by surface plasmon resonance. Application to epitope mapping. *J. Mol. Recogn.* **3**, 208–214.

Fahnestock, M. L., Tamir, I., Narhi, L. and Bjorkman, P. J. (1992). Thermal stability comparison of purified empty and peptide-filled forms of a class-I MHC molecule. *Science* **258**, 1658–1662.

Falk, K., Rötzschke, O. and Rammensee, H. (1990). Cellular

peptide composition governed by major histocompatibility complex class I molecules. *Nature* **348**, 248–251.

Falk, K., Rötzschke, O., Stevanovič, S., Jung, G. and Rammensee, H. (1991). Allele-specific motifs revealed by sequencing of self-peptides eluted from MHC molecules. *Nature* **351**, 290–296.

Fox, B., Quill, H., Carlson, L. and Schwartz, R. (1987). Quantitative analysis of the T cell response to antigen and planar membranes containing purified Ia molecules. *J. Immunol.* **138**, 3367–3374.

Frelinger, J., Gotch, F., Zweerink, H., Wain, E. and McMichael, A. (1990). Evidence of widespread binding of HLA class I molecules to peptides. *J. Exp. Med.* **172**, 827–834.

Fremont, D., Matsumura, M., Stura, E., Peterson, P. and Wilson, I. (1992). Crystal structures of two viral peptides in complex with murine MHC class I H-2Kb. *Science* **257**, 919–927.

Garrett, T., Saper, M., Bjorkman, P., Strominger, J. and Wiley, D. (1989). Specificity pockets for the side chains of peptide antigens in HLA-Aw68. *Nature* **342**, 692–696.

Germain, R. N. (1986). Immunology. The ins and outs of antigen processing and presentation *Nature* **322**, 687–689.

Germain, R. N. and Margulies, D. H. (1993). The biochemistry and cell biology of antigen processing and presentation. *Annu. Rev. Immunol.* **11**, 403–450.

Godeau, F., Luescher, I., Ojcius, D., Saucier, C., Mottez, E., Cabanie, L. and Kourilsky, P. (1992). Purification and ligand binding of a soluble class I major histocompatibility complex molecule consisting of the first three domains of H-2Kd fused to 2-microglobulin expressed in the vaculovirus-insect cell system. *J. Biol. Chem.* **267**, 24 223–24 229.

Guo, H. C., Jardetzky, T. S., Garrett, T. P. J., Lane, W. S., Strominger, J. L. and Wiley, D. C. (1992). Different length peptides bind to HLA-Aw68 similarly at their ends but bulge out in the middle. *Nature* **360**, 364–366.

Hunt, D., Henderson, R., Shabanowitz, J., Sakaguchi, K., Michel, H., Sevilir, N., Cox, A. *et al.* (1992). Characterization of peptides bound to the class I MHC molecule HLA-A2.1 by mass spectrometry. *Science* **255**, 1261–1263.

Jardetzky, T., Lane, W., Robinson, R., Madden, D. and Wiley, D. (1991). Identification of self peptides bound to purified HLA-B27. *Nature* **353**, 326–329.

Jones, P. P., Murphy, D. B., Hewgill, D. and McDevitt, H. O. (1979). Detection of a common polypeptide chain in I-A and I-E sub-region immunoprecipitates. *Mol. Immunol* **16**, 51–60.

Jönsson, U., Fägerstam, L., Ivarsson, B., Johnsson, B., Karlsson, R., Lundh, K., Löfås, S *et al.*, (1992). Real-time biospecific interaction analysis using surface plasmon resonance and a sensor chip technology. *Biotechniques* **11**, 620–627.

Jost, J., Munch, O. and Andersson, T. (1991). Study of protein–DNA interactions by surface plasmon resonance (real time kinetics). *Nucleic Acids Res.* **19**, 2788.

Kane, K., Sherman, L. and Mescher, M. (1989). Molecular interactions required for triggering alloantigen-specific cytolytic T lymphocytes. *J. Immunol.* **142**, 4153–4160.

Karlsson, R., Michaelsson, A. and Mattsson, L. (1991). Kinetic analysis of monoclonal antibody–antigen interactions with a new biosensor analytical system. *J. Immunol. Methods* **145**, 229–240.

Kaufman, J. F., Auffray, C., Korman, A. J., Shackelford, D. A. and Strominger, J. L. (1984). The class II molecules of the human and murine major histocompatibility complex. *Cell* **36**, 1–13.

Khilko, S., Corr, M., Boyd, L., Lees, A., Inman, J. and Margulies, D. (1993). Direct measurement of MHC/peptide interactions using surface plasmon resonance. I. Immobilization chemistry and characterization of specificity. *J. Biol. Chem.* **268**, 15425–15434.

Kozlowski, S., Corr, M., Shirai, M., Boyd, L. F., Pendleton, C. D., Berzofsky, J. A. and Margulies, D. H. (1993). Multiple pathways are involved in the extracellular processing of major histocompatibility complex class I-restricted peptides. *J. Immunol.* **151**, 1–12.

Kozlowski, S., Corr, M., Takeshita, T., Boyd, L., Pendleton, C., Germain, R., Berzofsky, J. *et al.* (1992). Serum angiotensin-1 converting enzyme activity processes a human immunodeficiency virus 1 gp160 peptide for presentation by major histocompatibility complex class I molecules. *J. Exp. Med.* **175**, 1417–1422.

Kozlowski, S., Takeshita, T., Boehncke, W., Takahashi, H., Boyd, L., Germain, R., Berzofsky, J. *et al.*, (1991). Excess β2 microglobulin promoting functional peptide association with purified soluble class I MHC molecules. *Nature* **349**, 74–77.

Madden, D., Gorga, J., Strominger, J. and Wiley, D. (1991). The structure of HLA-B27 reveals nonamer self-peptides bound in an extended conformation. *Nature* **353**, 321–325.

Madden, G., Gorga, J., Strominger, J. and Wiley, D. (1992). The three-dimensional structure of HLA-B27 at 2.1′ resolution suggests a general mechanism for tight peptide binding to MHC. *Cell* **70**, 1035–1048.

Mage, M., Lee, L., Ribaudo, R., Corr, M., Kozlowski, S., McHugh, L. and Margulies, D. (1992). A recombinant, soluble, single-chain class I major histocompatibility complex molecule with biological activity. *Proc. Natl Acad. Sci. USA* **89**, 10 658–10 662.

Margulies, D., Boyd, L., Kozlowski, S., Kjer-Nielsen, L., Lopez, R., Schneck, J. and Hunziker, R. (1990). Multivalent Requirement for the Stimulation of Alloreactive T Cells: Studies with Engineered Soluble MHC Class I Proteins In Vitro and In Vivo. In *Transgenic Mice and Mutants in MHC Research*, ed. by I. K. Egorov and C. S. David, Springer-Verlag, Berlin.

Margulies, D. and McCluskey, J. (1985). Exon shuffling: new genes from old. *Surv. Immunol. Res.* **4**, 146–159.

Margulies, D., Ramsey, A., Boyd, L. and McCluskey, J. (1986). Genetic engineering of an H-2Dd/Q10b chimeric histocompatibility antigen: purification of soluble protein from transformant cell supernatants. *Proc. Natl Acad. Sci. USA* **83**, 5252–5256.

Matsumura, M., Fremont, D., Peterson, P. and Wilson, I. (1992). Emerging principles for the recognition of peptide antigens by MHC class I molecules. *Science* **257**, 927–934.

Mellins, E., Smith, L., Arp, B., Cotner, T., Celis, E. and Pious, D. (1990). Defective processing and presentation of exogenous antigens in mutants with normal HLA class II genes. *Nature* **343**, 71–74.

Mottez, E., Jaulin, C., Godeau, F., Choppin, J., Levy, J. and Kourilsky, P. (1991). A single-chain murine class I major transplantation antigen. *Eur. J. Immunol.* **21**, 467–471.

Neefjes, J., Stollorz, V., Peters, P., Geuze, H. and Ploegh, H. (1990). The biosynthetic pathway of MHC class II but not class I molecules intersects the endocytic route. *Cell* **61**, 171–183.

Ojcius, D. M., Abastado, J. P., Godeau, F. and Kourilsky, P. (1992). Peptide binding to MHC class-I proteins measured with a novel fluorescent technique. *CR Acad. Sci.* **315**, 337–341.

Ojcius, D. M., Godeau, F., Abastado, J. P., Casanova, J. L. and Kourilsky, P. (1993). Real-time measurement of antigenic peptide binding to empty and preloaded single-chain major histocompatibility complex class-I molecules. *Eur. J. Immunol.* **23**, 1118–1124.

O'Shannessy, D. J., Brigham-Burke, M. and Peck, K. (1992). Immobilization chemistries suitable for use in the BIAcore surface plasmon resonance detector. *Anal. Biochem.* **205**, 132–136.

O'Shannessy, D. J., Brigham-Burke, M., Soneson, K. K., Hensley, P. and Brooks, I. (1993). Determination of rate and equilibrium binding constants for macromolecular interactions using surface plasmon resonance—use of nonlinear least-squares analysis methods. *Anal. Biochem.* **212**, 457–468.

Otten, G., Bikoff, E., Ribaudo, R., Kozlowski, S., Margulies, D. and Gemain, R. (1992). Peptide and β 2-microglobulin regulation of cell surface MHC class I conformation and expression. *J. Immunol.* **148**, 3723–3732.

Parker, K., DiBrino, M., Hull, L. and Coligan, J. (1992). The β2-microglobulin dissociation rate is an accurate measure of the stability of MHC class I heterotrimers and depends on which peptide is bound. *J. Immunol.* **149**, 1896–1904.

Rammensee, H. G., Falk, K. and Rotzschke, O. (1993). Peptides naturally presented by MHC class-I molecules. *Annu. Rev. Immunol.* **11**, 213–244.

Reddehase, M. and Koszinowski, U. (1991). Redistribution of critical major histocompatibility complex and T cell receptor binding functions of residues in an antigenic sequence after biterminal substitution. *Eur. J. Immunol.* **21**, 1697–1701.

Reddehase, M., Rothbard, J. and Koszinowski, U. (1989). A pentapeptide as minimal antigenic determinant for MHC class I-restricted T lymphocytes. *Nature* **337**, 651–653.

Rock, K., Gamble, S. and Rothstein, L. (1990a). Presentation of

exogenous antigen with class I major histocompatibility complex molecules. *Science* **249**, 918–921.

Rock, K., Rothstein, L., Gamble, S. and Benacerraf, B. (1990b). Reassociation with β2-microglobulin is necessary for Kb class I major histocompatibility complex binding of exogenous peptides. *Proc. Natl Acad. Sci. USA* **87**, 7517–7521.

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

Sadegh-Nasseri, S. and Germain, R. (1991). A role of peptide in determining MHC class II structure. *Nature* **353**, 167–170.

Sadegh-Nasseri, S. and McConnell, H. (1989). A kinetic intermediate in the reaction of an antigenic peptide and I-Ek. *Nature* **337**, 274–276.

Salter, R., Norment, A., Chen, B., Clayberger, C., Krensky, A., Littman, D. and Parham, P. (1989). Polymorphism in the α 3 domain of HLA-A molecules affects binding to CD8. *Nature* **338**, 345–347.

Saper, M., Bjorkman, P. and Wiley, D. (1991). Refined structure of the human histocompatibility antigen HLA-A2 at 2.6 Å resolution. *J. Mol. Biol.* **219**, 277–319.

Schumacher, T. N., Van, B. G., Heemels, M. T., Deres, K., Li, K. W., Imarai, M., Vernie, L. N. *et al.*, (1992). Synthetic peptide libraries in the determination of T cell epitopes and peptide binding specificity of class I molecules. *Eur. J. Immunol.* **22**, 1405–1412.

Sherman, L., Burke, T. and Biggs, J. (1992). Extracellular processing of peptide antigens that bind class I major histocompatibility molecules. *J. Exp. Med.* **175**, 1221–1226.

Silver, M. L., Guo, H. C., Strominger, J. L. and Wiley, D. C. (1992). Atomic structure of a human MHC molecule and presenting an influenza virus peptide. *Nature* **360**, 367–369.

Townsend, A., Elliott, T., Cerundolo, V., Foster, L., Barber, B. and Tse, A. (1990). Assembly of MHC class I molecules analyzed *in vitro* (published erratum appears in *Cell* 1990 **62**, 1233). *Cell* **62**, 285–295.

Townsend, A., Ohlën, C., Bastin, J., Ljunggren, H., Foster, L. and Kärre, K. (1989). Association of class I major histocompatibility heavy and light chains induced by viral peptides. *Nature* **340**, 443–448.

Uchanska-Ziegler, B., Nößner, E., Schenk, A., Ziegler, A. and Schendel, D. J. (1993). Soluble T cell receptor-like properties of an HLA-B35-specific monoclonal antibody (Tü165). *Eur. J. Immunol.* **23**, 734–738.

Van Bleek, G. and Nathenson, S. (1990). Isolation of an endogenously processed immunodominant viral peptide from the class I H-2Kb molecule. *Nature* **348**, 213–216.

Vitiello, A., Potter, T. and Sherman, L. (1990). The role of β2-microglobulin in peptide binding by class I molecules. *Science* **250**, 1423–1426.

Watts, T., Brian, A., Kappler, J., Marrack, P. and McConnell, H. (1984). Antigen presentation by supported planar membranes containing affinity-purified I-Ad. *Proc. Natl Acad. Sci. USA* **81**, 7564–7568.

Zhang, W., Young, A., Imarai, M., Nathenson, S. and Sacchettini, J. (1992). Crystal structure of the major histocompatibility complex class I H-2Kb molecule containing a single viral peptide: implications for peptide binding and T-cell receptor recognition. *Proc. Natl Acad. Sci. USA* **89**, 8403–8407.