Structural Studies of the Human Class II Major Histocompatibility Complex Protein HLA-DR1

By
Zarixia Zavala-Ruiz

B.S. Chemistry
University of Puerto Rico, Río Piedras, PR, 1999

Submitted to the Department of Chemistry in
Partial Fulfillment of the Requirements for the Degree of

DOCTOR OF PHILOSOPHY
In Biological Chemistry

at the

Massachusetts Institute of Technology
June 2004

© 2004 Massachusetts Institute of Technology
All rights reserved

Signature of Author

Department of Chemistry
April, 2004

Certified by

Lawrence J. Stern
Associate Professor
Thesis Supervisor

Accepted by

Robert W. Field
Chairman, Departmental Committee on Graduate Students
This doctoral thesis has been examined by a Committee of the Department of Chemistry as follows:

Professor Catherine L. Drennan
Committee Chairman

Professor Lawrence J. Stern
Research Supervisor

Professor Stuart S. Licht
Committee Member
Structural Studies of the Human Class II Major Histocompatibility Complex Protein HLA-DR1

By
Zarixia Zavala-Ruiz

Submitted to the Department of Chemistry in Partial Fulfillment of the Requirements for
the Degree of Doctor of Philosophy in Biological Chemistry, June 2004

ABSTRACT

Major Histocompatibility Complex (MHC) proteins are heterodimeric membrane
glycoproteins that bind antigens in the form of short peptides within the cell and present
them to the T cell receptors on the surface T cells. In this thesis work, the structural
aspects of the human class II MHC protein HLA-DR1 in complex with different peptides
and also in the peptide-free form were investigated. Biochemical, crystallographic, and
immunological analyses of an unusually long peptide antigen derived from HIV-gag
(p24) and its interaction with HLA-DR1 and a HIV-specific CD4+ T cell clone were
studied. The HIV-gag (p24) peptide binds in an unexpected conformation, with its C-
terminus region making a hairpin turn that bends back over the groove. The residues at
the C-terminus are critical for T-cell recognition, and disruption of the hairpin turn
abrogates the immune response. The results suggest a new mode of MHC-peptide-TCR
interaction. A set of viral peptide analogs designed to increase binding affinity for HLA-
DR while maintaining antigenic interactions with a virus-specific T cell receptor were
designed, tested and analyzed. Ultimately, a N-methyl substitution at position 7 is shown
to increase binding affinity by displacement of one of three water molecules bound
between the MHC and peptide. The results have implications for design of peptide-
mimetic vaccines, and are discussed in the broad context of other attempts to increase
protein-ligand interaction through displacement of tightly bound water molecules. The
role for the P10 shelf in peptide binding site was investigated. Crystallographic studies
confirm the formation of a P10 shelf that is lined with highly polymorphic residues.
Biochemical studies were conducted on a series of peptides different at the P10 position
on four HLA-DR1(P10) mutants showing that this shelf has some specificity and can be
involved in the discrimination of peptides that bind to class II MHC proteins. Studies of
the empty, peptide-free form of HLA-DR1 were conducted by NMR spectroscopy
showing that the conformation of this empty form is not in a molten globule-like state
and that in general is similar to that of the peptide-loaded form but with several
differences. Preliminary characterization of the peptide-receptive and peptide-averse
forms of the empty HLA-DR1 is described.

Thesis Supervisor: Lawrence J. Stern
Title: Associate Professor
ACKNOWLEDGEMENTS

Many people have contributed to my graduate career and made this thesis possible.

Larry Stern, my mentor and advisor, has been a great resource and teacher and has shown so much patience with me. I have learned so much from him!

I would also like to thank my thesis committee, Prof. Cathy Drennan and Prof. Stuart Licht, and my undergraduate advisors, Prof. Reginald Morales, Dr. Sharon Rounds and Dr. John C. Aster.

Thanks to all the members of the Stern lab, especially Mia for her help, advice, and for sharing with me her knowledge about science and life in general, and to Tom, Jen, Iwona, Tanyel, Dikran, Sasha, Greg, Sriram, Danny, Jenzy, and Liying for their advice and support during the past five years.

Quiero extender un agradecimiento muy especial a:
- Mis padres, Carmen y José, por su apoyo incondicional, por su paciencia y ayuda en los momentos más difíciles de mi carrera graduada y por el todo el amor que me brindan día a día. Los amo mucho!!!
- A mis hermanos, Junin y Fernando, por su apoyo y por siempre hacerme sentir parte de la familia a pesar de la gran distancia. Los quiero un millón!
- A Titi Elsa, gracias por toda tu ayuda y por confiar en mí.
- A el amor de mi vida, Eric R. Schreiter, por su dulzura, amor y por todo el apoyo que me ha brindado personal y profesionalmente.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overview</td>
<td>9</td>
</tr>
<tr>
<td>I. Introduction</td>
<td>13</td>
</tr>
<tr>
<td>A. The immune system</td>
<td>13</td>
</tr>
<tr>
<td>B. A brief introduction to antigen presenting cells and T-cells</td>
<td>13</td>
</tr>
<tr>
<td>C. MHC allelic polymorphism</td>
<td>14</td>
</tr>
<tr>
<td>D. Role of class II MHC proteins in the immune system</td>
<td>15</td>
</tr>
<tr>
<td>E. Molecular details of class II MHC proteins and peptide binding</td>
<td>18</td>
</tr>
<tr>
<td>F. The class II MHC-peptide-TCR complex</td>
<td>22</td>
</tr>
<tr>
<td>II. A hairpin turn in a HIV-Gag-derived peptide bound to HLA-DR1</td>
<td>25</td>
</tr>
<tr>
<td>orients peptide residues outside the binding Groove for T cell</td>
<td></td>
</tr>
<tr>
<td>recognition</td>
<td></td>
</tr>
<tr>
<td>A. Introduction</td>
<td>26</td>
</tr>
<tr>
<td>B. Materials and Methods</td>
<td>28</td>
</tr>
<tr>
<td>1. Peptide synthesis</td>
<td></td>
</tr>
<tr>
<td>2. Protein expression and purification</td>
<td></td>
</tr>
<tr>
<td>3. T-cell activation assay</td>
<td></td>
</tr>
<tr>
<td>4. Peptide binding assay</td>
<td></td>
</tr>
<tr>
<td>5. Crystallization</td>
<td></td>
</tr>
<tr>
<td>6. Data collection and processing</td>
<td></td>
</tr>
<tr>
<td>7. Structure determination</td>
<td></td>
</tr>
<tr>
<td>C. Results</td>
<td>33</td>
</tr>
<tr>
<td>1. Residues at the C-terminal end of a HIV-Gag-derived peptide are</td>
<td></td>
</tr>
<tr>
<td>required for T cell activation but not for MHC-peptide interaction</td>
<td></td>
</tr>
<tr>
<td>2. Crystal structure of HLA-DR1 in complex with Gag[PG13] and Gag[PP16] peptides</td>
<td></td>
</tr>
<tr>
<td>3. The C-terminal region of Gag[PP16] adopts a type-II beta turn</td>
<td></td>
</tr>
<tr>
<td>4. The C-terminal hairpin is required for T cell activation</td>
<td></td>
</tr>
<tr>
<td>D. Discussion</td>
<td>44</td>
</tr>
<tr>
<td>III. Exploration of the P6/P7 region of the peptide binding site of the</td>
<td>50</td>
</tr>
<tr>
<td>human class II major histocompatibility complex protein HLA-DR1</td>
<td></td>
</tr>
<tr>
<td>A. Introduction</td>
<td>51</td>
</tr>
<tr>
<td>B. Materials and Methods</td>
<td>54</td>
</tr>
<tr>
<td>1. Protein expression and purification</td>
<td></td>
</tr>
<tr>
<td>2. Peptide synthesis</td>
<td></td>
</tr>
<tr>
<td>3. IC\textsubscript{50} determination</td>
<td></td>
</tr>
<tr>
<td>4. HA1.7 T-cell hybridoma activation assay (IL2 production)</td>
<td></td>
</tr>
</tbody>
</table>
5. Crystallization
6. Data collection and processing
7. Structure determination

C. Results 58
1. D-amino acids in P6 and P7 position
2. N-methyl alanine and N-methyl glycine at P7
3. N-substituted glycine at P7
4. Crystal Structure of N-methyl alanine at P7
5. T-cell activation assay

D. Discussion 70

IV. Side chain specificity at the P10 position of the peptide binding site of a class II major histocompatibility protein

A. Introduction 78
B. Material and Methods 80
1. Peptide Synthesis
2. Protein expression and purification
3. Peptide binding assays
4. Crystallization
5. Data collection and processing
6. Structure determination
7. MHC-peptide database analysis
8. Construction and analysis of HLA-DR1 variants carrying single and double amino acid substitution in the P10 shelf

C. Results and Discussion 86
D. Significance 98

V. To what degree are MHC class II molecules unfolded or loosely folded in the absence of peptide? How much conformational alteration accompanies peptide binding?

A. Introduction 107
B. Materials and Methods 109
1. Protein expression and purification
2. Protease cleavage
3. NMR experiment
C. Results 111
1. Stability of the empty form of HLA-DR1
2. Peptide binding to $^2H,^{15}N$-labeled HLA-DR1
3. NMR experiments of HLA-DR1 with $^2H,^{15}N$-labeled $\alpha$-chain
4. NMR experiments of HLA-DR1 with $^2H,^{15}N$-labeled $\beta$-chain
5. NMR studies of HLA-DR1 in complex with Hay$_{308A}$
D. Discussion

VI. Characterization of the two empty forms of HLA-DR1 observed experimentally

A. Introduction

B. Materials and Methods
1. Production of peptide-loaded HLA-DR1 complexes
2. Peptide dissociation experiments
3. Inactivation experiments
4. General approach used to characterized the peptide-averse and peptide-receptive forms of HLA-DR
5. Gel Filtration
6. Circular Dichroism
7. KL295 ELISA

C. Results
1. Peptide-dissociation rates
2. Inactivation rates
3. Biophysical and Biochemical analysis for the characterization of the peptide-receptive and peptide-averse forms

D. Discussion

VII. Afterword

VIII. References

IX. Appendices

A.I. Crystallization trials of empty HLA-DR1 in complex with the superantigen SEB

A.II. Protocols
1. Expression of HLA-DR1 in E.coli (Fermentation)
2. Expression of HLA-DR1 in E.coli in Minimal Media
3. Isolation of class II MHC subunits from bacterial pellets
4. Refolding and Purification of HLA-DR1
5. Preparation of Ab-ProteinA Immunoaffinity Column
6. HLA-DR1 ELISA
7. SDS-PAGE and Native PAGE Electrophoresis
8. Streptavidin-Europium ELISA to determine Peptide-DR1 K_d
9. S2 Cell Culturing
10. Expression and purification of the superantigen SEC-3B2
11. Purification of zipper-stabilized, His-tagged sDM/sDR1
complexes (zDM/zDR) by Ni-NTA-agarose chromatography
12. DM/DO expression and purification
13. IgG-FC fusion cleavage on DM/DO protein
14. HLA-DM ELISA
15. Western Blot-Alkaline Phosphate Detection
16. Calculation of Apparent Molecular Weight from Gel Filtration

A.III. Characteristics of HLA-DR1/peptide/SEC3-3B2 crystals  206
Overview of this thesis

This thesis describes work directed at better understanding the peptide binding to the class II MHC protein, HLA-DR1. Chapter 1 provides a brief introduction on the immunology system and the role of MHC proteins in the T-cell mediated immune response. It is a summary of the molecular mechanisms involved in peptide binding to MHC molecules.

Chapter 2 describes the studies of an unusually long minimal antigenic peptide derived from the HIV protein Gag (p24) and its interaction with HLA-DR1 and a HIV-gag-specific CD4+ T cell clone. The HIV-gag (p24) peptide binds in an unexpected conformation, with its C-terminal region making a hairpin turn that bends back over the groove, a conformation never seen before on a peptide bound to a MHC protein. The results demonstrate that the residues located at the C-terminus are critical for T-cell recognition, and disruption of the hairpin turn abrogates the immune response. The studies demonstrate an alternative mode for MHC-peptide-TCR interaction, in which peptide residues outside the binding groove curl back over the remainder of the peptide and become accessible for TCR interaction. Iwona Strug, a post-doc in the Stern lab, Philip J. Norris and Bruce D. Walker from Massachusetts General Hospital contributed to this work. This chapter has been submitted for publication.

Chapter 3 describes the design, testing, and analysis of a set of viral peptide analogs designed to increase binding affinity for HLA-DR1, a human class II MHC protein. Briefly, a N-methyl substitution at position 7 is shown to increase binding affinity by displacement of one of three water molecules bound between the MHC and peptide that are usually seen in high-resolution class II MHC peptide structures. The work combines biochemical, crystallographic, and immunological analyses. The results
have implications for design of peptido-mimetic vaccines. Previous and current members of the Stern lab (Danny DeOliveira, Iat C. Chan, Jennifer Svendsen and Jennifer D. Stone) contributed to this work. Valuable reagents and advice were contributed to this work by Eric J. Sundberg and Roy A Mariuzza from University of Maryland Biotechnology Institute. This chapter was published in November 2003 as a regular article in the Journal of Biological Chemistry (Zavala-Ruiz et al., 2003).

Chapter 4 describes research conducted to explore the role of the P10 region of the peptide-binding site of the human class II MHC protein HLA-DR1. The position is usually neglected when investigating peptide binding to class II MHC proteins since the first crystallographic studies of class II MHC protein complexes had an alanine or a glycine from the peptide binding into the P10 region not contributing a lot to the binding. We noticed that peptides that only differ at the P10 position can bind with significantly different affinities to HLA-DR1. We used crystallographic and biochemical approaches to investigate in more detail the contribution of this shelf or pocket in peptide binding to HLA-DR1. Class II MHC molecules are highly polymorphic in the area that contacts the P10 residue form the peptide, so we made single and double mutations of HLA-DR1 for residues in that region and used then in our studies. Using two sets of peptides that differ only at the P10 position, we were able to identify some specificity related to the shelf or pocket but we can not really explain all the details of it. Iwona Strug contributed to this work. The chapter has been prepared in the form of a manuscript for submission in the near future.

Chapter 5 describes work on the empty, peptide-free form of HLA-DR1.

Previous studies on HLA-DR1 show that peptide binding induces a conformational
change of the protein. NMR spectroscopy was used to visualize the structural differences of the empty and peptide-loaded forms of the HLA-DR1. The spectra collected from different states of the protein confirm the conformational change as the chemical shift of some peaks change. The majority of the peaks are similar suggesting that the empty form of HLA-DR1 possesses a fold similar to that of the peptide-loaded form. This work was done in collaboration with Sergey Ilin, a member if the Harald Schwalbe group in the University of Frankfurt, Germany. This chapter was prepared as a manuscript for submission.

Chapter 6 describes work directed toward the physical characterization of the two empty class II MHC forms, the peptide-receptive and peptide-averse forms. This chapter describes the strategy that was used to isolate the forms and the experiments conducted to see if a significant difference exists between them in terms of hydrodynamic radius, secondary structure content, and reactivity to an antibody that recognize the empty form of HLA-DR1. No significant differences were observed as the protein converts from peptide-receptive to peptide-averse form.

The Appendices contain some crystallization experiments that were conducted on empty HLA-DR1. More details on each of the methods that have been used and developed during this thesis work as well as in other projects that were not addressed in this thesis but that might be helpful for other members of the lab in the future are included here also. In addition, a small section about some of the characteristics of HLA-DR1-peptide-SEC3-3B2 crystals was added.
Abbreviations used in this thesis:

ABTS: 2,2-azino-di-[3-ethylbenzathiazoline sulfonate]

AMCA: 7-amino-4-methylcoumarin-3-acetic acid

APC: Antigen Presenting Cell

CD: Circular Dichroism

ELISA: Enzyme-linked Immunosorbent Assay

ELISPOT: Enzyme Linked Immuno-Spot

FRET: Fluorescence Resonance Energy Transfer

HIV: Human Immunodeficiency Virus

HLA: Human Leukocyte antigen

IFN-γ: Interferon-gamma

IL-2: Interleukin-2

MALDI: Matrix-assisted Laser Desorption/Ionization Mass Spectrometry

MHC: Major Histocompatibility Complex

MW: Molecular Weight

PBMC: Peripheral Blood Mononuclear Cells

PBS: Phosphate-buffered Saline

PBST: Phosphate Buffered Saline containing 0.01% Tween-20 detergent

SA: Streptavidin

SDS-PAGE: Sodium Dodecyl sulfate-polyacrylamide Gel Electrophoresis

TCR: T-cell Receptor
Chapter I: Introduction

I.A. The adaptive immune system

The adaptive immune system is an adaptive defensive mechanism that vertebrates have developed to protect themselves from pathogenic microorganisms. A number of different cells and molecules that can recognize and eliminate an enormous variety of foreign molecules are generated by this system. The immune system has evolved in order to distinguish one foreign pathogen from another, and to discriminate between foreign molecules and our body's own cells and proteins.

In this chapter I will provide an overview of the T-cell mediated immune response and the role of major histocompatibility complex (MHC) proteins.

I.B. A brief introduction to antigen presenting cells and T cells

Antigen presenting cells (APCs) are cells that are able to express class I and/or class II MHC molecules on their surface, and therefore present peptide antigens on their surface. The job of antigen presenting cells can be divided into two categories, sampling intracellular material and sampling extracellular material.

In the intracellular sampling, cytoplasmic molecules and proteins are degraded in the proteasome machinery and the peptide fragments are transported to the endoplasmic reticulum (ER) by the TAP channel. In the ER, they are loaded onto class I MHC proteins which then travel to the cell surface to present the peptide antigen (process is reviewed in (Pamer and Cresswell, 1998)). The extracellular sampling is performed by specialized-professional APCs, which include B-cells, microphages and dendritic cells. These cells internalize antigens by either phagocytosis or endocytosis. The antigens are
degraded by proteases in the lysosomal compartments; the produced peptide fragments are loaded onto class II MHC proteins in the endosomes and then they are presented on the cell surface for examination by T-cells (reviewed in (Watts, 1997)).

T-cells are generated in the bone marrow and migrate to the thymus, where they mature. Each T-cell has only one variant of the T-cell receptor (TCR) gene, which is produced by genetic recombination events. This allows $\sim 10^{15}$ possible rearrangements per TCR, and thus our body generated an enormous repertoire of T-cells (reviewed in (Marrack and Kappler, 1988)). T-cells are divided into two groups that differentiate between class I and class II MHC molecules. T-cells that recognize class I MHC-peptide complexes are cytotoxic and contain a cell-surface glycoprotein that binds to class I MHC molecules, CD8 (CD8+ T-cells), whereas T-cells restricted by class II MHC-peptide complexes are called “helper T cells” because they secrete cytokines that induce other immune cells to express their effective function, they have the cell-surface glycoprotein CD4, which recognizes and binds to class II MHC molecules (CD4+ T-cells).

Binding of the TCR and the co-receptors CD8 or CD4 to the MHC-peptide complex induces clonal expansion and further differentiation of the T-cell.

I.C. MHC allelic polymorphism

In humans, the MHC alleles are carried on chromosome 6 and are called HLA (Human Leukocyte Antigen), while in mice they are termed H2 (for group II histocompatibility antigens). The human class I MHC gene contain the following loci, HLA-A, HLA-B, HLA-C, HLA-E and HLA-G, while the class II MHC gene complex
consist of the following loci, HLA-DR, HLA-DP, and HLA-DQ, HLA-DM and HLA-DO.

MHC genes are one of the most polymorphic genes in the human genome, and usually \( \sim 10 \) to 300 polymorphic variants of each subunit are found throughout the populations, with the exceptions of HLA-DM, HLA-DO, HLA-E and HLA-G, which are relatively non-polymorphic. For example, within the HLA-DR \( \beta \) subunit there are approximately 300 different alleles (the \( \alpha \)-subunit of DR is not polymorphic). Allowing for heterozygosity and multiple \( \alpha\beta \) pairs at some loci, and up to 20 different class II MHC proteins can be expressed in one person. That said, now we can imagine why tissue matching preceding organ transplantation is very challenging and difficult. The MHC types on the donor and receiver need to match to prevent the host’s immune system for rejecting the grafted tissue. However, some alleles are more common in one population than others, the allelic product of HLA-DRA*0101 and HLA-DRB1*0101 (commonly referred to as HLA-DR1) is carried by \( \sim 10\% \) of the human population and it is the MHC molecules studies in this thesis.

Certain autoimmune diseases, viral diseases, some neurological disorder and several allergies have been associated with particular MHC alleles.

I.D. Maturation of class II MHC proteins

Class II MHCs are heterodimeric cell surface glycoproteins of \( \sim 50 \) kDa that are found on professional antigen presenting cells; they are composed of two subunits, the \( \alpha \)- and \( \beta \)- subunits. The \( \alpha \)- and \( \beta \)- subunits are synthesized and then directed to the ER where they assemble with the invariant chain chaperone (\( \text{Ii} \)) (Cresswell, 1996). It has
been suggested the li places a loop in the class II MHC binding groove that helps prevent binding of self-peptides to the MHC before it is exposed to antigens. The li associates with the MHC molecule and travels to the Golgi complex (post-translational modification of the MHC occurs) and later to the specialized endocytic compartments, binding to the MHC to prevent the degradation and aggregation of the class II MHC molecule (Bakke and Dobberstein, 1990; Lotteau et al., 1990; Romagnoli et al., 1993). The endocytic compartments are acidic and it is there where the antigenic proteins that were obtained by phagocytosis, endocytosis, or pinocytosis, are proteolyzed into small fragments. Once the MHC molecules get to these compartments, the li is proteolyzed except for the fragment that is bound into the MHC peptide-binding site, this fragment is referred to as CLIP (class II-associated invariant chain peptide) (Riberdy et al., 1992; Roche and Cresswell, 1991). CLIP is released and exchanged for an antigenic peptide fragment through a mechanism that involves the protein HLA-DM. HLA-DM is a non-polymorphic heterodimer encoded in the class II region of the MHC gene; it contains the same general fold that conventional class II MHC molecules have but with the peptide binding site collapsed which is why peptides do not bind to it (Mosyak et al., 1998). Cells that lack HLA-DM express class II MHC molecules on the cell surface loaded with CLIP (Fling et al., 1994; Morris et al., 1994). Leaving HLA-DM in the endocytic compartments, class II MHC molecules with antigenic peptides bound travel to the cell surface of the antigen presenting cell. The formed MHC-peptide complex is presented to CD4+ T-cells (reviewed in (Germain and Margulies, 1993)). Figure 1 shows the pathway for class II MHC assembly and antigen loading.
Figure I.1. Representation of the class II MHC assembly and antigen presenting pathway. (1) Class II MHC α- and β- subunits are synthesized, and then fold together to form the heterodimer. Folded class II MHC molecules associate immediately with invariant chain (li) trimers. (2) MHC molecules in complex with li travel to the Golgi complex where glycosylation and post-translational modification occurs. (3) In the meantime, antigenic proteins and molecules enter the cell by endocytosis and are taken to the lysosome/endosome compartments. Once in the compartments, proteases degrade the antigen into peptide fragments. (4) MHC-li complexes are targeted to the endosomal compartments, where proteases cleave and degrade the li leaving only the peptide fragment bound in the MHC peptide binding site, CLIP. In these compartments, HLA-DM interacts with the class II MHC-CLIP complex facilitating the dissociation of CLIP and the association of antigenic peptides to the MHC. (5) The new MHC-peptide complex travels to the cell surface. This complex will be recognized by the T-cell receptor on the surface of CD4+ T-cells. (6) Cell surface class II MHC-peptide complexes can be recycled.

Figure courtesy of Jennifer A. Zarutskie, Ph.D.
The interaction of the class II MHC-peptide complex and the TCR, along with the proper co-stimulatory signals, induces a helper T-cell immune response. The activation stimulates the production of antibodies that will recognize the antigen.

I.E. Molecular details of class II MHC proteins and peptide binding

Many crystal structures of different human and murine class II MHC allelic variants in complex with peptides have been determined in the past decade (some of them are described in (Corper et al., 2000; Fremont et al., 2002; Ghosh et al., 1995; Lee et al., 2001; Li et al., 2000; Liu et al., 2002; Smith et al., 1998; Stern et al., 1994)). The fold of the protein is essentially identical in all the structures determined to-date. Each subunit (α and β) contributes to part of the β-sheet floor and one of the α-helices in the peptide binding site, they also contain an IgG-like domain (Figure 2). Peptides always bind into the MHC peptide binding groove in a polyproline type II-like conformation (Figure 3A). Multiple hydrogen bonds are found between conserved residues in the class II MHC protein and the peptide main chain carbonyl and amide groups (McFarland and Beeson, 2002) (Figure 3B), and enforce the polyproline type II-like conformation.
Figure 1.2. Structure of class II MHC. Cartoon representation of the class II MHC protein HLA-DR1 bound to an antigenic peptide. The peptide binding site is composed of a β-sheet floor and two α-helices. The IgG-like domains lie underneath the peptide-binding site. The α-subunit is colored purple and the β-subunit is blue. The peptide is in yellow. The figure was taken from Stern and Wiley, 1994 (Structure 15: 245).
Peptides bound to class II MHC proteins isolated from antigen presenting cells usually contain 15-20 residues, with longer peptides found occasionally (Chicz et al., 1992; Rudensky et al., 1991). The central region of the peptides interact directly with class II MHC proteins, with specific recognition of ~9-residues stretch (Stern et al., 1994). Within this binding region, strong side-chain preferences are found in certain positions with weaker preferences at others. The pattern of this side-chain specificity is
called the peptide binding motif, and it is consistent with available crystal structures, which reveals the presence of pockets in the peptide binding site. These pockets accommodate the side chains of peptide residues at the P1, P4, P6 and P9 positions with smaller pockets or shelves at the P3 and P7 position. These pockets correspond to positions where strong side-chain binding preferences are observed in studies of MHC peptide interaction (Hammer et al., 1992). Allelic differences in the residues lining the pockets determine the peptide binding specificity (or motif) of the various class II MHC alleles. For example in HLA-DR1 (DRB1*0101), the allele of study in this thesis, the P1 pocket shows a strong preference for large hydrophobic side-chains (Trp, Tyr, Phe, Leu, and Ile), the P6 pocket shows a strong preference for small residues (Gly, Ala, Ser, and Pro), and the P4 and P9 pockets have weaker affinities for residues with some aliphatic character (Hammer et al., 1992; Stern et al., 1994) (Figure 4). Characterization of the motif has shown the effect of positive and negative contributions of each amino acid at all positions within the binding frame, with the motifs described by full 20 (side chain) x 9 (position) matrices (Sturniolo et al., 1999).

The kinetics of peptide binding to class II MHC molecules has been studied by different research groups (Buus et al., 1986; Joshi et al., 2000; Mason and McConnell, 1994; Rudensky et al., 1992). Basically, the kinetic model includes an initial bimolecular binding step followed by a slow unimolecular conformational change that produces a stable MHC-peptide complex. In addition, a reversible inactivation of the empty MHC protein that competes with productive binding is observed.
I.F. The class II MHC-peptide-TCR complex

The important interactions that characterize this ternary complex have been investigated by mutagenesis studies (Sant'Angelo et al., 1996) and references therein), mapping experiments (Krieger et al., 1991; Sant'Angelo et al., 1996) and truncation studies (Norris et al., 2004; Norris et al., 2001). Detailed structural information on the MHC-TCR interaction is available only for three class II MHC-pep-TCR structures that include two class II MHC-restricted TCR: Ha1.7, which recognizes an influenza Ha peptide bound to HLA-DR1 or HLA-DR4 (Hennecke et al., 2000; Hennecke and Wiley,
2002), and D10, which recognizes a conalbumin peptide bound to the murine MHC II molecule IA\(^K\) (Reinherz et al., 1999). Difficulty producing sufficient quantities of TCRs either intact or as a single chain is one of the reasons for the limited high-resolution structures of these complexes.

In the MHC-pep-TCR interaction, the complementarity-determining regions (CDRs) from the V\(\alpha\) and V\(\beta\) domains of the TCR are lying across the MHC-peptide complex, with CDR3 loops of both domains extending down over the center of the peptide and the CDR1 and CDR2 loops contacting the alpha helices in the peptide binding site of the MHC protein (reviewed in (Hennecke and Wiley, 2001; Rudolph and Wilson, 2002)). TCRs contact from 6 to 7 residues of a span of 9 residues of the class II MHC bound peptides (Figure 5). Single amino acid substitutions in peptides, even in residues not directly contacted by the TCR, can convert a strong agonist MHC-peptide ligand into a weak agonist or even an antagonist.
**Figure I.5. MHC-peptide-TCR Interface.** HLA-DR1 and TCR are represented as green and light/dark brown cartoon, respectively. The peptide is shown as sticks with carbon atom in yellow; and nitrogen and oxygen atoms in blue and red, respectively. The β-chain of the class II MHC protein is towards the front, while the α-chain is in the back. Protein data bank code of complex is 1FYT.
Chapter II: A hairpin turn in a HIV-gag-derived peptide bound to HLA-DR1 orients peptide residues outside the binding groove for T cell recognition

Summary

T cells generally recognize peptide antigens bound to MHC proteins through contacts with residues found within or immediately flanking the seven-to-nine residue sequence accommodated in the MHC peptide-binding groove. However, some studies have identified T cells that require peptide residues outside this region for activation, the structural basis for which is unknown. Here we have investigated a HIV Gag-specific T cell clone that requires an unusually long peptide antigen for activation. The crystal structure of a minimally antigenic 16-mer bound to HLA-DR1 shows that the peptide C-terminal regions bends sharply into a hairpin turn as it exits the binding site, orienting peptide residues outside the MHC-binding region above the remainder of the peptide and in position to interact with a T cell receptor. Peptide truncation and substitution studies show that both the hairpin turn and the extreme C-terminal residues are required for T cell activation, and demonstrate a new mode of MHC-peptide-TCR interaction. How frequently such hairpin structures would be expected is discussed in light of intrinsic peptide conformational preferences and current structural and cellular studies of the MHC-TCR interaction.
II.A. Introduction

Class II Major Histocompatibility Complex (MHC) proteins are cell surface glycoproteins that bind antigens in the form of short peptides and present them for recognition to T cell receptors (TCR) on the cell surface of CD4+ T cells (Watts, 1997). Naturally-processed peptides isolated from class II MHC proteins found in antigen presenting cells usually contain 15-25 residues (Chicz et al., 1992; Rudensky et al., 1991). The central region of these peptides interacts directly with class II MHC proteins, typically with specific recognition of an approximately 9-residue stretch (Rudolph and Wilson, 2002). X-ray crystallography of human and murine class II MHC proteins has revealed that peptides bind to the protein in an extended polyproline type II conformation, with several peptide side chains bound into polymorphic pockets that line the peptide binding groove (Corper et al., 2000; Fremont et al., 2002; Ghosh et al., 1995; Lee et al., 2001; Li et al., 2000; Liu et al., 2002; Smith et al., 1998; Stern et al., 1994). A hydrogen bond network between the conserved residues on the class II MHC and the peptide main chain carbonyl and amide groups, independent of the sequence of the peptide, stabilizes the MHC-peptide complex (McFarland and Beeson, 2002), and enforces the polyproline conformation that directs some of the side chains into the MHC pockets and leaves the others accessible for TCR interactions (Jardetzky et al., 1996). Generally, pockets accommodate the side chains of peptide residues at the P1, P4, P6 and P9 positions, with smaller pockets or shelves in the binding site accommodating the P3 and P7 residues. Minor variations on this theme have been observed, for example in some complexes the P9 interactions are weak or absent (Corper et al., 2000; Li et al., 2000). In the canonical conformation, the side chains of residues at positions P-1, P2, P5, and P8 are solvent accessible and point toward the TCR, with portions of other side
chains and the peptide main chain also exposed for potential TCR interaction in the central region of the complex.

The interaction of TCR with MHC-peptide complexes also is relatively stereotyped, with complementarity-determining regions (CDRs) from the Vα and Vβ domain lying across the peptide-MHC complex, typically with CDR3 loops of both variable domains extending down over the center of the peptide and the CDR1 and CDR2 loops contacting the alpha-helices (Hennecke and Wiley, 2001; Rudolph and Wilson, 2002). Detailed structural information on the MHC-TCR interaction is available only for two class II MHC-restricted TCR: HA1.7, which recognizes an influenza HA peptide bound to HLA-DR1 or HLA-DR4 (Hennecke et al., 2000; Hennecke and Wiley, 2002), and D10, which recognizes a conalbumin peptide bound to IA^k (Reinherz et al., 1999). For both these systems, earlier mapping experiments had identified important T cell contacts as peptide positions where amino acid substitution abrogated T cell activation without disturbing MHC-peptide interaction (Krieger et al., 1991; Sant'Angelo et al., 1996). The class II MHC-peptide-TCR structures confirmed these predictions, and showed TCR contacting the MHC-bound peptides within the region circumscribed by MHC-peptide contacts, at the P-1, P2, P3, P5, and P8 positions. Mutagenesis studies have suggested that many other MHC-TCR pairs interact in this manner (ref. 19, and references therein). These structures provide no evidence for TCR contacts with peptide residues outside the peptide binding groove.

Peptide mapping experiments have suggested that for some peptides the flanking P11 residue also can be an important T cell contact (Arnold et al., 2002; Muller et al., 1996; Srinivasan et al., 1991; Vignali and Strominger, 1994). Although there is no direct structural evidence for an interaction of the P11 residue with TCR, where it has been observed in MHC-peptide complex structures the P11 residue is oriented away from the MHC binding site towards the TCR,
consistent with the approximate three residue repeat of the canonical polyproline-II conformation for a class II MHC-bound peptide.

Here we investigate a complex that suggests a different mode of class II MHC-peptide-TCR interaction. The peptide derives from the protein Gag (p24), which is the major capsid protein of HIV that surrounds the viral RNA-nucleoprotein complex in mature virions (Freed, 1998; Krausslich and Welker, 1996). A human Gag(p24)-specific T cell clone (Norris et al., 2004) requires an unusually long minimal peptide for activation, and MHC and TCR mapping studies indicate an important contact at the peptide P13 position, well outside the canonical interaction region. The crystal structure of the peptide bound to HLA-DR1 reveals an unusual conformation, in which a hairpin loop involving peptide residues P9-P12 orients the P13 side chain over the binding site in position to interact with the TCR.

II.B. Materials and Methods

II.B.1. Peptide Synthesis

Peptides were synthesized using solid-phase F-moc (N-(9-fluorenyl)methoxycarbonyl) chemistry on a Symphony instrument (Protein Technology Inc). All peptides were amidated at the C-terminus, to minimize effects of introduction of an ionizable group within the binding site. The Ha peptide PKYVKQNTLKLAT was N-biotinylated by coupling aminocaproyl-(LC)-biotin (Anaspec) to the N-terminus of the resin-bound, side-chain protected, peptide using standard amino acid coupling procedure. The peptides were deprotected and cleaved from the resin by a 2 hour treatment at room temperature with a mixture of trifluoroacetic acid/ H₂O/ thioanisole/phenol/dithiothreitol (82.5:5:5:5:2.5). The solution of peptides was precipitated with cold diethyl ether, and the crude peptides were filtered, washed with ether, and dried in vacuum. The crude peptides were purified by high performance liquid chromatography (Vydac-C18). The
purity and homogeneity of each peptide was checked by high performance liquid chromatography (Vydac-C18) and MALDI-TOF mass spectrometry. Peptide concentration was determined using amino acid analysis.

II.B.2. Protein Expression and Purification

For peptide binding experiments, the extracellular portion of HLA-DR1 (DRA*0101, DRB1*0101) was produced from recombinant baculovirus-infected SF9 insect cells as soluble empty αβ heterodimers, as described previously (Stern and Wiley, 1992). For x-ray crystallography, the extracellular portion of HLA-DR1 was produced by expression of isolated subunits in *Escherichia coli* inclusion bodies followed by refolding *in vitro* as described previously (Frayser et al., 1999). Refolded HLA-DR1 was purified by immunoaffinity chromatography using the conformation-specific monoclonal antibody LB3.1, followed by gel filtration chromatography in phosphate-buffered saline, pH 6.8. The protein concentration was measured by UV absorbance at 280 nm using ε_{280} of 54375 M^{-1} cm^{-1} for empty HLA-DR1. SEC-3B2 superantigen was expressed as a soluble protein in *E. coli* and isolated from the periplasmic fraction as described previously (Andersen et al., 1999).

II.B.3. T-cell activation assay

The CD4+ T cell clone AC25 and corresponding autologous B cell line were derived as previously described (Norris et al., 2001). Briefly, the clone was derived after stimulating PBMC with whole p24 antigen, resting for two weeks, then restimulating and plating at limiting dilution on a round-bottom 96-well plate. The AC-25 T cell clone was maintained by restimulation every
two weeks with IL-2 (100 U/ml), 12F6 (anti-CD3 antibody obtained from Johnson Wong, the Massachusetts General Hospital (0.1 μg/ml), and 10^6 feeder cells irradiated at 30 Gy (Norris et al., 2001) in R-10–RPMI 1640 (Sigma, St. Louis, MO), with penicillin-streptomycin (50 IU/ml, 50 μg/ml, Mediatech, Herdon, VA), HEPES (2.38 mg/ml, Mediatech), L-glutamine (Mediatech), plus 10% heat-inactivated human AB serum (Sigma). Proliferation and ELISPOT assays were performed in the same medium.

For proliferation assays, autologous B lymphoblastoid cell lines (B-LCL) were irradiated (120 Gy) and resuspended with the appropriate peptide antigen. AC-25 clones and B-LCL were plated in triplicate wells at 50,000 cells/well each, and after 48 hours, 1 μCi of ^3H-thymidine (Dupont NEN, Boston, MA) was added. Plates were harvested onto glass fiber filters after 18 hours. Results were expressed as net CPM, the difference between the counts in the presence of antigen and the counts without antigen present. Significant responses were considered to be net CPM greater than 1000 (Norris et al., 2001).

For IFN-γ ELISPOT assays, 96-well plates were coated overnight with anti-IFN-γ antibody (Mabtech, Mariemont, OH) and washed 6 times with PBS. B-LCL (5x10^5), peptide antigen (1 μg/ml) and AC-25 T cells (50 cells/well) were added, incubated overnight and then cells were discarded and plates were washed 6 times. Biotinylated anti- IFN-γ (Endogen) was added for 1.5 hours at 25°C. The plates were then washed again, streptavidin (Mabtech) (100 μl/well) was added for 45 minutes at 25°C, and after 6 more washes, NBT/BCIP (Bio-Rad) was added. Once dots appeared the coloring reagent was discarded and the wells were incubated with PBS plus 1% Tween for 10 minutes, then plates were washed in tap water thrice. Background responses to wells with no antigen or irrelevant antigen ranged from 0 to 2.5 spots.
per well. Responses greater than 5 spots per well and greater than 5 times maximum background were considered significant.

**II.B.4. Peptide binding assay**

A competition assay was used to determine binding affinities of peptides. Peptide-free HLA-DR1 produced in insect cells (25 nM) was mixed together with biotinylated Ha[306-318] peptide probe (Habio, 25 nM) and varying concentrations of unlabelled competitor peptide ($10^{-12}$ - $10^{-5}$M). The mixtures were incubated for 3 days at 37 °C in 100 mM sodium phosphate buffer at pH 5.5, containing 50 mM NaCl, 1 mg/ml PMSF, 37 μg/ml iodoacetamide, 10 mM EDTA, 0.02% NaN₃, 0.5 mg/ml octylglucoside, followed by detection of bound biotinylated peptide using an immunoassay that employed anti-DR1 capture antibody LB3.1 and alkaline phosphate labeled streptavidin. IC₅₀ values were obtained by fitting a binding curve to the plots of fluorescence versus concentration of competitor peptide, and apparent $K_D$ estimated using the formula $K_{D,app} = \frac{IC_{50}}{1+ [H_{bio}] / K_{D,Hbio}}$ together with the value for the $K_{D,Hbio}$ of the biotinylated Ha probe peptide measured in a direct binding assay, $K_{D,Hbio} \sim 10$nM.

**II.B.5. Crystallization**

HLA-DR1 peptide bound complexes were prepared by incubating purified empty HLA-DR1 (1-5 μM) with at least 5-fold molar excess peptide for 3 days at 37°C in phosphate-buffered saline with 0.02% sodium azide. The complex was purified by gel filtration to remove aggregates and free peptide. Each purified HLA-DR1/peptide complex was mixed in equimolar ratio with purified SEC3-3B2. Crystals of HLA-DR1/peptide/SEC-3B2 were grown by the vapor diffusion method in hanging drops. For the peptide PEVPMFSALSEG (Gag[PG13]), crystals of protein
complex grew at 4°C under the following conditions: 2-6% polyethylene glycol 4000, 5-10% ethylene glycol, 100mM sodium acetate pH 5.2-5.6, with 1 µL of precipitant solution mixed with 1µL of 10 mg/mL protein complex. For the peptide PEVIPMFSALSEGATP (Gag[PP16]), crystals of protein complex grew at 4°C under the following conditions: 2-6% polyethylene glycol 5000 monomethyl ether, 10% ethylene glycol, 100mM sodium acetate pH 5.2-5.6, with 1 µL of precipitant solution mixed with 1µL of 11 mg/mL protein complex. For x-ray diffraction experiments, the crystals were soaked for ~1 minute in a cryoprotectant solution consisting of 25% ethylene glycol in the mother liquor and then flashed cooled in liquid nitrogen.

II.B.6. Data collection and processing

A high resolution data set (2.25 Å) of the HLA-DR1/Gag[PG13]/SEC3-2B2 complex was collected on a single crystal (300 µm x 200 µm x 200 µm) on an R-AXIS IV image plate detector using CuKα radiation. For the complex HLA-DR1/Gag[PP16]/SEC3-3B2, a 2.45 Å data set was collected on a single crystal (80 µm x 60 µm x 60 µm) on a Mar180 image plate detector using CuKα radiation from a rotating anode source. Collected data were processed and scale using Denzo, Scalepack, and the CCP4 package (Collaborative Computational Project, 1994; Otwinowski and Minor, 1997).

II.B.7. Structure Determination

The structure of each complex was determined by molecular replacement. Coordinates for another complex of *E. coli*-derived HLA-DR1 and SEC3-3B2, carrying a N-methylated designed peptide (Protein Data Bank code 1PYW)(Zavala-Ruiz et al., 2003) were used as the search
model to find a molecular replacement solution. Waters and the peptide were removed from the search model before use. Refinement was carried out using CNS (Brunger et al., 1998) and manual inspection and rebuilding using XtalView (McRee, 1999). For HLA-DR1/Gag[PG13]/SEC3-2B2, after an initial rigid-body refinement, three rounds of refinement included minimization, B-factor, and minimization. For HLA-DR1/Gag[PP16]/SEC3-2B2, after an initial rigid-body refinement, six rounds of refinement included minimization, B-factor, and minimization. Waters were added to the refined molecule using CNS. The final model was verified for distortions on the secondary structure features using Procheck (Laskowski et al., 1993). Coordinates and structure factors for HLA-DR1/Gag[PP16]/SEC3-3B2 and HLA-DR1/Gag[PG13]/SEC3-3B2 have been deposited in the Protein Data Bank, with accession codes 1SJE and 1SJH respectively.

II.C. Results

II.C.1. Residues at the C-terminal end of a HIV-Gag-derived peptide are required for T cell activation but not for MHC-peptide interaction

A HIV Gag(p24)-specific, HLA-DR1 restricted CD4+ T cell clone (AC-25) isolated from an individual acutely infected with HIV-1 recognizes an antigen derived from the HIV Gag (p24) protein bound to the human class II MHC protein HLA-DR1. As previously reported, AC-25 requires an unusually long peptide for activation (Norris et al., 2004). Only three residues can be removed from either end of the Gag(p24)-derived 22-mer without affecting T cell activation (Figure 1A, and ref. 27). Removal of additional residues from either the N-terminus or C-terminus of the minimal gag-derived 16-mer PEVPMFSALSEGATP (Gag[PP16]) results in abrogation of T cell activation, as shown by a cellular proliferation assay (Figure 1A), and also
Figure II.1. A Gag(p24)-specific T cell clone requires an unusually long peptide for activation. A) T cell activation, measured using a proliferation assay and peptide-pulsed autologous antigen presenting cells. Open symbols and dashed lines indicated peptides that do not activate AC25 T cell proliferation at any concentration tested. B) MHC-peptide binding, measured using a HA-peptide competition binding assay and recombinant soluble HLA-DR1. Curves fits reflect IC₃₀ values of 29±1 nM (Gag[PP16]) and 28±3 nM (Gag[PG13]), which correspond to Kd values of approximately 6 nM and 9 nM assuming simple two-state binding. The Gag[PG13] peptide which is not able to activate the AC-25 T cell clone exhibits apparent peptide-binding affinity indistinguishable from that of the activating Gag[PP16] peptide.
abrogation of cytotoxicity and cytokine secretion functions (Norris et al., 2004).

For HLA-DR1, the most important determinants of peptide binding are a hydrophobic residue at position P1 and a small residue at position P6 (Hammer et al., 1992; O'Sullivan et al., 1991), with additional but weaker preferences at P4, P7, and P9. Within the Gag[PP16] peptide, the preferred binding frame is expected to be VIPMFSALS (Rammensee et al., 1999; Sturniolo et al., 1999). A shorter Gag-derived peptide containing this sequence, PEVIPMFSALSEG (Gag[PG13]), binds to HLA-DR1 with an apparent dissociation constant of <10 nM, essentially identical to that of the longer Gag[PP16] peptide (Figure 1B). In the predicted binding frame, the peptide Gag[PG13] would place Val into P1 and Ser into P9, allowing two residues on either side of the predicted frame (P-2, P-1, P10, P11) to interact with TCR. However, this peptide is inactive (Figure 1A, open squares). The inability of the Gag[PG13] peptide to induce activation responses in a T cell clone that recognizes the longer peptide is unexpected, and could indicate an unusual binding frame or mode of T cell interaction. These possibilities were addressed by structural studies of the HLA-DR1 complexes of Gag[PG13] and Gag[PP16], and by fine epitope mapping of the AC-25 T cell clone.

II.C.2. Crystal structure of HLA-DR1 in complex with Gag[PG13] and Gag[PP16] peptides

To determine the actual peptide binding frame of Gag[PP16] and to evaluate the importance of the C-terminal residues of Gag[PP16] not present in Gag[PG13], the crystal structure of HLA-DR1 bound to Gag[PP16] was determined in complex with the superantigen SEC3-3B2, using diffraction data to 2.45 Å (Table I). Superantigens are a class of disease-causing proteins of viral or bacterial origin that hyperactivate the immune system by cross-linking MHC proteins and T cell receptors (Balaban and Rasooly, 2000), and which previously
Figure II.2. Crystal structures of DR1/Gag[PP16]/SEC3 and DR1/Gag[PG13]/SEC3. A,B, Structure of the Gag[PP16] complex; C,D, structure of the Gag[PG13] complex. A) 2Fo-Fc electron density map contoured at 1σ using data in the resolution range of 20-2.45 Å. The Gag[PP16] peptide carbon atoms are in yellow, and nitrogen and oxygen atoms are blue and red, respectively. B) Side-view (same as A) of the surface of HLA-DR1 with the bound Gag[PP16] peptide. C) 2Fo-Fc electron density map contoured at 1σ using data in the resolution range of 50-2.25 Å. The Gag[PG13] peptide carbon atoms are in green, and nitrogen and oxygen atoms are blue and red, respectively. D) Side-view of surface of HLA-DR1 with bound Gag[PG13] peptide. Figures were generated using PyMol (DeLano, 2002).

have been used successfully as an aid to crystallization of HLA-DR-peptide complexes (Bolin et al., 2000; Sundberg et al., 2002; Zavala-Ruiz et al., 2003). Here we have used the affinity-matured 3B2 variant of staphylococcal enterotoxin SEC3 (Andersen et al., 1999), which interacts with HLA-DR1 outside the binding groove on the flanking helix from the α-subunit (Redpath et al., 1999). We used molecular replacement to obtain initial phases using as a search model another HLA-DR1/SEC3-3B2 complex (PDB:1PYW)(Zavala-Ruiz et al., 2003) with peptide coordinates removed, followed by a few rounds of manual rebuilding and refinement. Data collection and refinement statistics are shown in Table I. Electron density for HLA-DR1 and
superantigen was clear and continuous except for HLA-DR1 β109-111, a disordered loop away from the peptide binding site, and for SEC3-3B2 residues 99-105, a region located away from the interaction site with HLA-DR1. Clear electron density was observed for 14 amino acid residues of the peptide extending from the N-terminal proline up to the alanine (Figure 2A). Electron density for the penultimate threonine was observed but was not sufficiently clear to allow definitive placement of the side chain (see below). The terminal proline was not observed. The Gag[PP16] peptide binding frame can be unambiguously assigned using this and other electron density maps. The Gag[PP16] peptide binds in the expected frame, with Val (P1), Met (P4), Ser (P6), and Ser (P9) accommodated in pockets in the peptide binding groove (this pocket numbering system will be used throughout) (Figure 2A,B). The peptide adopts the usual polyproline conformation when the MHC peptide binding groove, but just outside the groove the peptide bends sharply and doubles back over itself.

The crystal structure of HLA-DR1 bound to Gag[PG13] was determined similarly to 2.25Å resolution, also in complex with SEC3-3B2 (Table I). Peptide electron density was interpretable from the N- to C-terminus (Figure 2B). The Gag[PG13] peptide binds to HLA-DR1 using the same binding frame as Gag[PP16], but without the C-terminal bend.

Crystals of the Gag[PP16] and Gag[PG13] peptide complexes were isomorphous, facilitating their comparison by difference Fourier analysis, which highlights differences between the two complexes. The only significant differences in a Gag[PP16] – Gag[PG13] difference Fourier map (|F_{Gag[PP16]} - F_{Gag[PG13]}| \exp(-i\alpha_{Gag[PP16]})) are found for the Gag[PP16] peptide C-terminus which is not present in Gag[PG13], and for the side chain of Gag peptide residue Leu (P8) (Figure 3). In Figure 3A, positive density, corresponding to regions present in the Gag[PP16] but not Gag[PG13] complex, is shown in yellow, and negative density,
Figure II.3. Difference Fourier analysis. A). Difference Fourier map \[ F_{\text{Gag[PP16]}} - F_{\text{Gag[PG13]}} \exp(-i\alpha_{\text{Gag[PP16]}}) \], contoured at 4\(\sigma\), with positive (Gag[PP16]) density in yellow, and negative (Gag[PG13]) density in green. B). Close-up view of the map in the region of significant differences, overlayed with the Gag[PP16] peptide, or C), overlayed with the Gag[PP16] peptide. Peptide coloring and viewpoint same as in Figure 2. Figures were generated using PyMol (DeLano, 2002).

corresponding to regions present in the Gag[PG13] but not Gag[PP16] complex, is shown in green. Positive and negative peaks (+/- 6.5\(\sigma\)) surround the side chain of Leu (P8), indicating a rotation of the side chain from chi1 = -175\(^\circ\) in Gag[PG13] to chi1 = -90\(^\circ\) in Gag[PP16] (Figure 3). A large positive peak above the peptide P9-P11 residues (8.4\(\sigma\)) together with a small negative peak beyond the Gag[PG13] C-terminus (7.5\(\sigma\)), indicate the deviation of the Gag[PP16] peptide from the usual extended conformation. The Gag[PP16] peptide turns sharply at P10.
bending back above the main peptide sequence (Figure 3B). The difference Fourier maps also help to place the Thr (P13) side chain (shown with gray carbon atoms), which was not clearly defined in the Gag[PP16] 2Fo-Fc map, within this region near the side chain of Leu (P8).

Overall, the Gag[PG13] and Gag[PP16] peptides bind to HLA-DR1 in essentially identical conformations from the N-terminus (P-1) through P7, with the Gag[PG13] peptide P8-P11 region extending conventionally out of the site, and the Gag[PP16] peptide P9-P12 region forming a hairpin turn. The turn places the Gag[PP16] peptide P13 (Thr) side chain near the P8 (Leu) side chain, and changes the orientation of Leu (P8) relative to that found in the Gag[PG13] peptide (Figure 3C).

II.C.3. The C-terminal region of Gag[PP16] adopts a type-II beta turn

Both Gag[PG13] and Gag[PP16] peptides bind with the usual main chain hydrogen bonding scheme involving HLA-DR1 residues Glnα9, Asnα62, Asnα69, Argα76, Aspβ57, Trpβ61, Argβ71, Hisβ81, and Asnβ82 (although with intervening waters for the Gag[PP16] interaction with Argα76 and Aspβ57) (Figure 4A). The Gag[PP16] peptide makes an additional intramolecular main chain hydrogen bond, involving the carbonyl oxygen of the P9 residue (Ser) and the amide nitrogen of P12 (Ala) (Figure 4B). This interaction is characteristic of a beta turn, a four-residue structural motif found frequently in proteins (Richardson, 1981). In Gag[PP16] the SEGA (P9-P12) sequence forms a β turn, with main chain backbone phi and psi angles characteristic of the type-II beta turn (Figure 4B). In type-II beta turns, the oxygen atom of the carbonyl of residue 2 (P10) crowds the C atom of residue 3, which is therefore usually glycine (Hutchinson and Thornton, 1994). The Gag[PP16] peptide has glycine at this position (P11).
Figure II.4. A type II β-turn at the C-terminal region of the Gag[PP16] peptide.
A) C-terminal region of the DR1-peptide binding site with bound Gag[PP16] peptide showing conserved MHC-peptide hydrogen bonds. Conserved MHC-peptide hydrogen bonds in this region are shown as dashed lines in gray, with the Gag[PP16] intraturn hydrogen bond between the nitrogen of Ala (P12) and the carbonyl oxygen of Ser (P9) as a dashed line in red. B) Close-up view of the Gag[PP16] hairpin, showing the intra-turn hydrogen bond and phi-psi values characteristic of a type II beta turn. C) Alignment of HLA-DR crystal structures exhibiting ordered peptide density beyond P10. Complexes were aligned by least-squares fitting of α1 and β1 domains. Peptides shown as Cα traces. DR1/TP1 shown in red, from 1KLU.pdb; DR2a/MBP, blue, 1FV1.pdb; DR3/CLIP, magenta, 1A6A.pdb; DR1/HA, cyan, 1DLH.pdb; DR2a/EBV, grey, 1H15.pdb; DR4/collagen, orange, 2SEB.pdb. D) Alignment of Gag[PP16] (yellow) and TPI23-37 (red) DR1-peptide crystal structures, both structures were crystallized in the presence of the superantigen SEC3-3B2. The Gag[PP16] peptide adopts a conformation not seen in any other HLA-DR peptide complexes. Figures were generated using PyMol (DeLano, 2002).
This bent conformation has not been observed previously among complexes of peptides bound to class II MHC molecules. An alignment of Gag[PP16] with all other crystal structures of HLA-DR1 complexes exhibiting ordered density beyond P10 is shown in Figure 4C. Where observed, peptide density extends straight out of the binding site, without any pronounced bends or turns. In particular, we compared the DR1/Gag[PP16] structure (Figure 4D,D, yellow) with that of DR1/TPI23-37 (PDB:1KLU(Sundberg et al., 2002) (Figure 4C,D, red), which also was crystallized in the presence of the superantigen SEC3-3B2, and which includes ordered peptide density to the P12 residue. The TPI peptide binds in the conventional orientation, without any evidence of a bent conformation as observed for Gag[PP16] bound to HLA-DR1.

II.C.4. The C-terminal hairpin is required for T cell activation

Alanine-scanning mutagenesis of the Gag[PP16] peptide was used to determine the importance of the C-terminal hairpin in T cell receptor interaction, and to confirm the physiological relevance of the peptide conformation observed in the crystal structure. Each peptide residue except for Ala (P7) and Ala (P12) was changed independently to alanine, and standard MHC-peptide binding and T cell proliferation assays were performed (Figure 5). In the MHC-peptide-binding assay (Figure 5B,C), no single alanine substitution abolished MHC-peptide interaction, but significant reductions in binding affinity were observed upon alanine substitution of Val (P1) and Met (P4), and to a lesser extent Ser (P9). These effects are consistent with the binding frame observed in the crystal structures. (Both Ser and Ala are preferred residues at P6 (Hammer et al., 1992), and no significant effect is expected or observed for this substitution).
Figure II.5. Alanine-scanning mutagenesis studies of Gag[PP16]. A) AC-25 T cell activation, measured using an ELISPOT assay. Peptide residues substituted by alanine are shown at the right of the figure. B) HLA-DR1 peptide binding, measured using a competition binding assay. Legend for panels A and B shown at bottom. C) Effect of alanine substitution on T cell activation and MHC-peptide binding affinity shown together on a logarithmic scale. For T cell activation, values represent the response induced by 1μg/ml peptide concentration (average of two independent experiments). For MHC-peptide binding, IC₅₀ values in nM are shown (average of two independent experiments). In both assays, shorter bars represent decreased activity. Values for alanine positions reflect the unmodified Gag[PP16] peptide. D) T-cell activation assay of additional substitutions at Gly(P11) and Ala(P12).
In the T cell activation assay, alanine substitutions of Glu (P-1), Ile (P2), Phe (P5), and Thr (P13) had dramatic effects, abolishing activation at all concentrations tested (Figure 5A,C). In the Gag[PP16] crystal structure, the side chains of residues at the P-1, P2, and P5 positions were observed to be oriented away from the peptide binding site, as they are in other class II MHC-peptide complexes, and the effects of alanine substitutions at these positions are consistent with conventional MHC-TCR interaction. In the Gag[PP16] crystal structure, the hairpin turn orientation of Thr (P13) above the bulk of the peptide, in the vicinity of the P8 side chain. The large effect of Thr (P13)-to-alanine substitution at this position on T cell activation but not MHC-peptide interactions suggests that this residue and the hairpin turn play an important role in TCR interaction.

To further establish the contribution of the residues at the Gag[PP16] C-terminus to T cell activation, additional substitutions were analyzed at Gly(P11) and Ala(P12) (Figure 5D). Ala(P12) was substituted by glycine, to evaluate a possible role of the alanine side chain in TCR recognition, which would not have been apparent in the initial alanine scan. This substitution was tolerated well by the TCR (closed squares). To evaluate the role of the reverse turn, Gly(P11) was substituted by proline, which (unlike alanine) cannot be accommodated in a type II turn. Gly (P11) to Pro substitution abrogated T cell activation (Figure 5D, open squares), again highlighting the importance of the hairpin turn in T cell activation.
II.D. Discussion

The crystal structure of the HIV Gag-derived peptide Gag[PP16] bound to HLA-DR1 shows that it binds in an unexpected bent conformation, with a hairpin turn at one end of the binding groove that orients its C-terminal region above the remainder of the peptide and in position to interact with TCR. Truncation analysis shows that this C-terminal region is important for recognition by a CD4+ T cell clone derived from a HIV infected person. The effects of alanine scanning and site-specific substitutions introduced into the peptide show that the Thr residue at position P13 has no effect on binding to HLA class II, but is crucial for TCR interaction, along with conventional T cell contacts at P-1, P2, and P5 (Figure 6). The hairpin turn is important in aligning the threonine for recognition by TCR, since introduction of a proline residue that interferes with turn formation blocked T cell activation.

The threonine at position P13 could have either a direct or indirect role in TCR interaction. In an indirect mechanism, substitution of the threonine residue would affect the conformation of another residue directly contacted by the TCR. For the Gag[PP16] peptide, the presence of Thr (P13) alters the conformation of the Leu (P8) side chain, as seen clearly in the Gag[PG13] vs. Gag[PP16] difference maps (Figure 3). However, this alteration may have only a minor effect on T cell interaction, since Leu-to-Ala substitution at P8 does not drastically reduce T-cell activation. Alternately, Thr (P13) could directly contact the TCR. While P13 is well outside the range of peptide residues generally considered to be seen by the TCR, comparison of the Gag[PP16] structure with previously determined class II MHC:TCR complexes (Hennecke et al., 2000; Hennecke and Wiley, 2002; Reinherz et al., 1999) suggests that a TCR in a normal orientation could contact Thr directly by interacting with the CDR3 and/or CDR2 loops on Vβ9 (see a model of the interaction in figure 7).
The Gag[PP16] C-terminal hairpin seems to represent a physiologically relevant conformation, rather than one induced only by crystallization or recombinant MHC-peptide production. Firstly, several beta turn prediction algorithms (Chou and Blinn, 1997; Chou and Fasman, 1974; Hutchinson and Thornton, 1994; Wilmot and Thornton, 1988) identify the SEGA (P9-P12) sequence as having strong type II-beta turn potential, with contributions from the Glu at position 2, in addition to the strongly preferred Gly at position 3. The Thr side chain is in van der Waals contact with Leu (P8), which also would tend to stabilize the turn and would be able to form an intrachain hydrogen bond between the Thr hydroxyl and the main chain carbonyl oxygen at position 1 of the type II-beta turn. Moreover, no crystal contacts are found in the vicinity of the hairpin. Secondly, AC-25 T cell recognition of the Gag[PP16] peptide appears to
be dependent on the C-terminal beta hairpin turn, as shown by the Gly(P11)-to-Pro substitution, which abrogates T cell activation, as well as by the pattern of important T cell contact residues (Figure 6). Finally, the AC-25 T cell clone specific for this conformation was isolated from an HIV infected subject who was treated with antiretroviral therapy during primary HIV infection. The clonotype presumably was elicited in response to viral infection, and was selected in vitro using peripheral blood mononuclear cells stimulated with intact Gag protein. This suggests that both naturally-infected and Gag protein-treated cells present on their surface an activating Gag-derived epitope that presumably includes the hairpin conformation. However, we note that the hairpin is not obligatory for T cell recognition of this region of Gag: the same Gag(24) epitope as studied here has been identified also at the target of an HLA-DR4-restricted T cell clone (Norris et al., 2004), and another HLA-DR1 restricted T cell clone (Boritz et al., 2003). Both of these clones appear to recognize a conventional 10-residue sequence EVIPMFSALS contained within the Gag[PG13] sequence.

How frequently might we expect such unconventional recognition of bent peptides? Except for the HLA-DR1-Gag[PP16] complex reported here, hairpin turn or other bent or bulged conformations have not been observed in the class II MHC crystal structures reported to date. However, several of the complexes crystallized do not contain residues outside of the P-1 to P10 region, and many complexes carry their peptides as covalent attachments to the MHC beta subunit, where the linker between the peptide C-terminus and the MHC beta N-terminus could interfere with potential turn formation (Corper et al., 2000; Fremont et al., 2002; Fremont et al., 1998; Kersh et al., 2001; Siebold et al., 2004; Zhu et al., 2003). Thus, at present we may not have a complete picture of the degree of potential conformational variability for peptides bound to class II MHC proteins. For class I MHC, this variability is quite large, and thought to be due
Figure II.7. Model of MHC-peptide-TCR ternary complex. The model was produced by superimposition of the common elements of the HLA-DR1-Gag[PP16]-SEC33B2 (PDB:1SJE) and HLA-DR1-Ha-HA1.7 (PDB:1FYT) structures. MHC is represented as a green cartoon with the peptide in sticks with carbon atoms in yellow, and oxygen and nitrogen atoms in red and blue, respectively. The TCR α-chain is represented in magenta cartoon with transparent surface while the β-chain is in blue.

to the lack of conserved MHC-peptide main chain contacts in the center of the peptide, which is bound mostly by main chain interactions and side-chain binding pockets near the peptide N- and C-termini (Stern and Wiley, 1994). For class II MHC proteins, the large number of main chain hydrogen bonds in the central region of the peptide constrain its conformation within the site (Figure 4), but outside of the P-1 to P10 region the peptide would appear to be unconstrained by the MHC. A peptide turn or bend would therefore have to be stabilized by interactions provided
by the peptide, as observed here for Gag[PP16]. In general, the conformational stability of structures formed by short peptides is thought to be low, but particular sequences are able to form stable conformations that potentially could be recognized by TCR. Larger regions from an MHC-bound peptide might also play a role: class II MHC proteins are known to be able to bind very large peptide and even loops of intact proteins, although the relevance of such complexes for T cell activation is not known.

On the other hand, TCR recognition of residues outside the MHC peptide binding groove has been observed in many cellular studies. As noted above, the flanking P11 residue of a class II MHC-bound peptide can affect T cell activation (Arnold et al., 2002; Muller et al., 1996; Srinivasan et al., 1991; Vignali and Strominger, 1994), and has been proposed to be a common feature of MHC-TCR interaction (Arnold et al., 2002; Sant'Angelo et al., 2002). However, the P11 side chain is oriented towards the TCR for a MHC-bound peptide canonical polyproline repeat (see for example DR1/TPI in Figure 4D), and does not require a peptide bend, bulge, or turn for recognition by TCR. There have been fewer reports of T cells that are sensitive to peptide residues lying outside of a reliably-predicted MHC binding region. In a murine IA\textsuperscript{b}-CLIP complex, a peptide-T cell contact residue outside the expected MHC interaction site, Met P12 (Naujokas et al., 1998; Zhu et al., 2003), follows a sequence with a high propensity for beta turn formation (PMSM)(Chou and Blinn, 1997). For a HLA-DR\textsubscript{1} restricted, measles specific T cell lines, and for IE\textsuperscript{k}-MCC-restricted clone, critical T cell contacts lie several residues N-terminal of the expected binding region (P-4 and P-2 respectively)(Carson et al., 1997; Muller et al., 1996). These clones may recognize their non-conventional peptide antigens by a mechanism similar to that observed here for Gag[PP16]. Such conformations should be considered more widely in prediction, testing, and design of putative T cell antigens.
### Table II.1. Data Collection and Refinement Statistics

<table>
<thead>
<tr>
<th>Crystal Parameters</th>
<th>Gag[PP16]*</th>
<th>Gag[PG13]*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Space Group</td>
<td>R3</td>
<td>R3</td>
</tr>
<tr>
<td>Cell dimensions</td>
<td>a, c (Å)</td>
<td>a, c (Å)</td>
</tr>
<tr>
<td></td>
<td>172.75, 121.40</td>
<td>172.46, 121.46</td>
</tr>
</tbody>
</table>

#### Data Collection

<table>
<thead>
<tr>
<th></th>
<th>Overall</th>
<th>Highest res. shell</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resolution limits (Å)</td>
<td>20.0-2.45</td>
<td>2.54-2.45</td>
</tr>
<tr>
<td>Unique reflections</td>
<td>49,701</td>
<td>4,962</td>
</tr>
<tr>
<td>Total reflections</td>
<td>286,608</td>
<td>26,633</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td>Mean I/σ(I)</td>
<td>11.3</td>
<td>3.4</td>
</tr>
<tr>
<td>R_{sym} (%)^b</td>
<td>8.4</td>
<td>41.3</td>
</tr>
</tbody>
</table>

#### Refinement

<table>
<thead>
<tr>
<th></th>
<th>Overall</th>
<th>Highest res. shell</th>
</tr>
</thead>
<tbody>
<tr>
<td>R_{free} (%)^c</td>
<td>22.3</td>
<td>31.2</td>
</tr>
<tr>
<td>R_{cryst} (%)</td>
<td>19.8</td>
<td>27.8</td>
</tr>
</tbody>
</table>

#### Model

<table>
<thead>
<tr>
<th></th>
<th>Average B-factor (Å²)</th>
<th># Residues (atoms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLA-DR1</td>
<td>39.9</td>
<td>369 (3035)</td>
</tr>
<tr>
<td>Peptide</td>
<td>34.7</td>
<td>15 (109)</td>
</tr>
<tr>
<td>SEC3-3B2</td>
<td>38.8</td>
<td>231 (1900)</td>
</tr>
<tr>
<td>Waters</td>
<td>42.8</td>
<td>264</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Average B-factor (Å²)</th>
<th># Residues (atoms)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>41.5</td>
<td>368 (3026)</td>
</tr>
<tr>
<td>Peptide</td>
<td>38.3</td>
<td>13 (97)</td>
</tr>
<tr>
<td>SEC3-3B2</td>
<td>40.9</td>
<td>229 (1883)</td>
</tr>
<tr>
<td>Waters</td>
<td>42.5</td>
<td>275</td>
</tr>
</tbody>
</table>

#### Ramachandran plot

<table>
<thead>
<tr>
<th></th>
<th>Core + Allowed (%)</th>
<th>99.3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Generous (%)</td>
<td>0.6</td>
<td>0.5</td>
</tr>
<tr>
<td>Disallowed (%)</td>
<td>0.2</td>
<td></td>
</tr>
</tbody>
</table>

^a Crystals contained HLA-DR1 bound to the indicated Gag(p24)-derived peptide and the superantigen SEC3-3B2.

^b R_{sym} = σ ||I - <I>/σ <I||, where I is the observed intensity and <I> is the average intensity of multiple observations of symmetry-related reflections.

^c R factor on structure factors for reflections omitted from the refinement and used as a test set (10% of total).
Chapter III: Exploration of the P6/P7 region of the peptide-binding site
of the human class II MHC protein HLA-DR1

Summary

Crystal structures of the class II MHC protein, HLA-DR1, generally show a tight fit between
MHC and bound peptide, except in the P6/P7 region of the peptide binding site. In this region
there is a shallow, water-filled pocket underneath the peptide, between the pockets that
accommodate the peptide’s P6 and P7 side chains. We investigated the properties of this pocket,
with the idea of engineering substitutions into the corresponding region of peptide antigens in
order to increase their binding affinity for HLA-DR1. We investigated D-amino acids and N-
alkyl modifications at both the P6 and P7 positions of the peptide, and found that binding of
peptides to HLA-DR1 could be increased by incorporating an N-methyl substitution at position 7
of the peptide. The crystal structure of HLA-DR1 bound to a peptide containing a P7 N-methyl
alanine was determined. The N-methyl group orients in the P6/P7 pocket, displacing one of the
waters usually bound in this pocket. The structure shows that the substitution does not alter the
conformation of the bound peptide, which adopts the usual polyproline type II helix. An
antigenic peptide carrying the N-methyl modification is taken up by antigen presenting cells and
loaded onto endogenous class II MHC molecules for presentation, and the resultant MHC-
peptide complexes activate antigen-specific T cells. These results suggest a possible strategy for
increasing the affinity of weakly-immunogenic peptides that might be applicable to development
of vaccines and diagnostic reagents.
III.A Introduction

As mentioned before, the binding of peptides to human and mouse class II MHC has been investigated by X-ray crystallography (Bolin et al., 2000; Brown et al., 1993; Fremont et al., 2002; Ghosh et al., 1995; Lang et al., 2002; Lee et al., 2001; Li et al., 2000; Liu et al., 2002; Mullen et al., 2002; Murthy and Stern, 1997; Stern et al., 1994). The structures reveal several conserved side-chain binding pockets within the overall peptide-binding groove (Figure 1A). These pockets accommodate the side chains of peptide residues at the P1, P4, P6 and P9 position, with smaller pockets or shelves in the binding site accommodating the P3 and P7 residues. (The pockets are numbered along the peptide relative to a large usually hydrophobic pocket near the peptide binding site).

Structures of HLA-DR peptide complexes generally show a tight fit between MHC and peptide. A prominent exception to this is a relatively large region underneath the peptide between the P6 and P7 pockets (Figure 1B,C). The conformation of peptides bound to class II MHC proteins is tightly restricted to a polyproline type II-like conformation, probably as a result of 12-15 conserved hydrogen bonds between the MHC and peptide main chain (Jardetzky et al., 1996), and a peptide side chain from a conventionally bound peptide is not likely to bind in this region. Crystal structures of HLA-DR1-peptide complexes reveal that several water molecules were tightly bound underneath the peptide between the P6 and P7 pockets. Four water sites in this region were observed to be variously occupied in lower resolution structures, 2.5-2.8 Å (Hennecke and Wiley, 2002; Murthy and Stern, 1997; Stern et al., 1994). At higher resolution, 1.93 Å, all four waters were observed (Sundberg et al., 2002).

As part of an effort to explore ways to increase the binding affinity of antigenic peptides, we attempted to modify known antigenic peptides to take advantage of the P6/P7 void by
displacing bound water molecules. We wanted to introduce relatively small alterations in the peptide, since the expected application of such modified peptides generally would require cross-reactivity with the native sequence, and T cell antigen receptors are very sensitive to structural changes in a MHC-bound peptide. Single amino acid substitutions at a T cell contact residue can abrogate T cell recognition (Krieger et al., 1991), or convert agonist peptides into antagonists (De Magistris et al., 1992). Modifications of the peptide backbone of class II MHC-binding antigens also have been reported, in an effort to increase bioavailability or serum half-life, and include alkylation, peptide-bond reduction, and incorporation of peptoid, azapeptide, dipeptide mimetic substitutions, and D-amino acids (Bolin et al., 2000; Cotton et al., 1998; de Haan et al., 2002; Hill et al., 1994; Howard et al., 1997; Maillere et al., 1995). In general, peptides carrying one or more of these modifications are less effective T-cell activators as compared to a peptide that does not include the modification (Cotton et al., 1998; Hart and Beeson, 2001; Maillere et al., 1995).

Using the crystal structures as a guide to design P6/P7 binding antigen analogues, we were able to develop a peptide modification strategy that increases MHC-peptide binding affinity by displacement of waters in the P6/P7 region. Crystal structure analysis of MHC-peptide complexes carrying the modification (P7 amide N-methylation) confirms that waters were displaced without significant conformational alteration, and T-cell activation studies show that the modification does not affect interaction with the T-cell receptor.
Figure III.1. Pockets in the peptide binding site of HLA-DR1. A) Surface of HLA-DR1-peptide binding site with the side chain binding pockets within the overall peptide binding site labeled according to the peptide side chain accommodated. B) Same view as in A with bound FVKQNA4AL (4=N-methyl alanine) peptide as CPK model. Carbon atoms are yellow; nitrogen atoms are blue; and oxygen atoms are red. C) Side view of peptide binding site, showing water molecules (white spheres) bound underneath the peptide in the P6/P7 region. The surface is shown as a green mesh, and the view is rotated 90° around the horizontal axis relative to that of B.
III.B. Materials and Methods

III.B.1. Protein expression and purification

For peptide binding experiments, the extracellular portion of HLA-DR1 was produced from a recombinant baculovirus-infected SF9 insect cells as soluble empty αβ heterodimers as described previously (Stern and Wiley, 1992). For x-ray crystallography experiments, extracellular portion of HLA-DR1 was produced by expression of isolated subunits in E. coli inclusion bodies followed by refolding in vitro in the presence of peptide as described previously (Frayser et al., 1999). Refolded HLA-DR1 protein was purified by immunoaffinity chromatography using the conformation-specific monoclonal antibody, LB3.1 and gel filtration in PBS (phosphate buffer saline) pH 6.8. The protein concentration was measured by UV absorbance at 280 nm using ε280 of 54375 M⁻¹ cm⁻¹ for empty DR1. SEC3-3B2 was expressed as a soluble protein in E. coli and isolated from the periplasmic fraction as described (Andersen et al., 1999). SEC3-3B2 is a variant of the SEC3 protein that binds with a higher affinity to the MHC class II molecule, HLA-DR1 (Andersen et al., 1999).

III.B.2. Peptide Synthesis

Peptides were synthesized using solid-phase F-moc (N-(9-fluorenyl)methoxycarbonyl) chemistry on an Advanced ChemTech 357 synthesizer. All peptides were synthesized containing an acetylated N-terminus and an amidated C-terminus to prevent possible effects of charged termini in the binding site, except for N-biotinylated peptides which were produced by coupling aminocaproyl-(LC)-biotin succinimide ester (Pierce Chemical) to the N-terminus of the resin-bound, side-chain protected peptide. The peptides were deprotected and cleaved from the resin by a 3 hour treatment at room temperature with a mixture of trifluoroacetic acid/ H₂O/
dithiothreitol/ triisopropylsilane (88:5:5:5:2.5). The solution of peptides was filtered into cold
diethyl ether, and the precipitated peptides were filtered, washed with ether, and dried in
vacuum. The crude peptides were purified by high performance liquid chromatography (Vydac-
C18). The purity and homogeneity of each peptide was checked by high performance liquid
chromatography (Vydac-C18) and MALDI-TOF mass spectrometry. Peptide concentration was
measured by absorbance using HPLC-UV detection at 280 and 254 nm for peptides containing
Tyr and Phe residues respectively. N-methyl amino acids were purchased as protected amino
acid precursors and coupled in the usual manner. For the other N-substitutions, the N-substituted
residue was generated piecemeal on the resin, with conventional Fmoc/DIC/HOBt chemistry
used to make the fore and aft parts of the peptide, following a “sub-monomer” strategy used
previously for generating N-substituted glycines (Figliozzi et al., 1996; Nguyen et al., 1998). At
the N-alkyl residue, the glycine backbone was first introduced using bromoacetic acid, coupled
twice with DIC, and then the N-substitution was added as an alkylamine (or Boc-
aminoalkylamine) by nucleophilic displacement for 2 hours at 37°C. The next amino acid was
coupled using a PyBrop/DIPEA activation strategy, with triple coupling for 120 minutes. The
remainder of the sequence was prepared normally. We attempted to similarly prepare N-
substituted alanines using bromo-propionic acid, but we were unable to efficiently couple the
subsequent residue. The N-alkyl peptides were deprotected and purified in the usual manner.

III.B.3. IC₅₀ Determination

A competition assay was used to determine binding affinities of peptides. Peptide-free
DR1 produced in insect cells (10nM) was mixed with biotinylated Ha[306-318] probe (10nM),
and varying concentrations of unlabelled competitor peptide (10⁻¹² - 10⁻⁵M). The mixtures were
incubated for 3 days at 37°C in PBS buffer containing 0.02% NaN₃, followed by detection of bound biotinylated peptide using an immunoassay that employed an anti-DR1 capture antibody LB3.1 and streptavidin-Eu detection (Tompkins et al., 1993). IC₅₀ values were obtained by fitting a binding curve to the plots of fluorescence versus concentration of competitor peptide. The Kᵩ of the biotinylated HA probe peptide is ~10nM (Roche and Cresswell, 1990; Zarutskie et al., 1999).

III.B.4. HA1.7 T-cell hybridoma activation assay (IL2 production)

We used a mouse T cell hybridoma transfected with a chimeric T cell receptor carrying human variable alpha and beta domains from the well-characterized HLA-DR1-restricted, Ha-peptide specific human T cell clone HA1.7 (Lamb and Fledmann, 1982) and murine constant domains. The T-cell hybridoma was developed as an indicator cell for productive MHC-TCR interactions, and produces IL-2 upon receptor engagement (Boen et al., 2000). In some experiments, HA1.7 hybridoma cells were incubated with purified MHC-peptide complexes immobilized on the polystyrene assay plate for 24 hours at 37°C. Culture supernatant was tested for IL2 production using a murine IL2 detection sandwich ELISA kit (PeproTech). In other experiments, antigen presenting cells pre-pulsed with peptide were used as stimulators. For antigen presenting cells, we used LG2, a DR₁⁺ homozygous EBV-transformed B cell line. Live or fixed (1% paraformaldehyde) LG2 cells (5.0 x10⁴ cells/ well) were pulsed with varying concentrations of peptide (10⁻⁹-10⁻⁵M) in serum-free medium in a round-bottom 96-well plate for 2 hours at 37°C. The peptide was washed away, and the LG2 cells were re-suspended in S-MEM medium (used for routine passage of HA1.7 hybridoma cells). HA1.7 hybridoma cells
were added to wells (10^5 cells/well) with peptide-pulsed LG2 cells and incubated for 24 hours at 37°C. Supernatant was tested for IL2 production as described above.

III.B.5. Crystallization

HLA-DR1-peptide bound complex was prepared by incubating purified empty HLA-DR1 (1-5 μM) with at least 5-fold molar excess peptide for 3 days at 37°C in PBS with 0.02% sodium azide. The complex was purified by gel filtration to remove aggregates and free peptide. Purified HLA-DR1/peptide complex was mixed in equimolar ratio with purified SEC3-3B2. Crystals of HLA-DR1/Ac-FVKQNAAL-NH2/SEC3-3B2 were grown by the vapor diffusion method in hanging drops. The crystals grew at 4°C under the following conditions: 2-6% PEG 4K, 10% ethylene glycol, 100mM sodium acetate, pH 5.2-5.6; 1μl of precipitant solution mixed with 1μl of 8mg/mL protein complex. For x-ray diffraction experiments, the crystals were soaked for ~1 minute in a cryo-protectant solution consisting of 25% ethylene glycol in the mother liquor, and then flash-cooled in liquid nitrogen.

III.B.6. Data collection and processing

A high resolution data set (2.1 Å) was collected for a single crystal (200 μm x 150 μm x 150 μm) on a Mar345 image plate detector using CuKα radiation from a rotating anode source. Collected data was processed and scaled using Denzo, Scalepack and the CCP4 package (Collaborative Computational Project, 1994; Otwinowski and Minor, 1997). An overall isotropic B factor was estimated to be 21.6 Å from a Wilson plot.

III.B.7. Structure Determination

57
The structure of the complex was determined by molecular replacement. HLA-DR1/TPI$_{23-37}$/SEC3-3B2 (1KLU, PDB) was used as the search model to find a molecular replacement solution (Sundberg et al., 2002). The peptide and waters were removed from the model. Refinement was carried out using CNS (Brunger et al., 1998); after initial rigid-body refinement, three rounds of refinement included minimization, B-factor, and minimization. After each round the protein was inspected using O and XtalView (Jones et al., 1991; McRee, 1999). Waters were added to the refined molecule using CNS. N-terminal acetyl group, C-terminal amide group and the N-methyl group at the P7 position were included in the peptide model using data generated by the Dundee PRODRG server (van Aalten et al., 1996). The final model was verified for distortions on the secondary structures features using Procheck (Laskowski et al., 1993).

III.C. Results

III.C.1. D-amino acids in P6 and P7 positions

In order to investigate the binding properties of the HLA-DR1 P6/P7 pocket, we initially substituted D-amino acids into the P6 and P7 positions of a known HLA-DR1 binding peptide, and measured the effects of the substitutions on peptide binding affinity using a competition assay. Naturally-occurring L-amino acid containing sequences orient the P6 and P7 residues into the side-chain binding pockets that flank this region (P6 and P7, Figure 1A,B), leaving the P6/P7 pocket open to be filled by several water molecules (Figure 1C). We reasoned that a peptide containing a D-amino acid at either position might be able to position a side chain into the pocket. The template peptide NH$_2$-FVKQNAAC$_1$H$_2$ was designed as a scaffold for the substitutions. The peptide is based on the central region of the viral peptide PKYVKQNTLKLAT (Ha306-318, (Hennecke and Wiley, 2002; Lamb and Fledmann, 1982;
Roche and Cresswell, 1990; Stern et al., 1994)), derived from the influenza virus hemagglutinin. The original peptide length was reduced from 13 residues, eleven of which are bound by HLA-DR1, to seven residues, in order to emphasize the interactions of interest in the P6/ P7 region while maintaining the original binding frame that places an aromatic residue in the P1 position. The five N-terminal residues FVKQN of the scaffold peptide correspond to the native Ha sequence, except that an original Y at position 1 was changed to F, to reduce the relative importance of the P1 pocket while maintaining the original binding frame. The Ala-Ala sequence at positions 6-7 was originally Thr-Leu; these were substituted to alanine to provide a rational basis for comparison of the D-amino acids. Finally, the N- and C-termini were acetylated and amidated, respectively, to prevent ionization of the termini that might disrupt the conserved hydrogen bonding arrangement that constrains the peptide in the binding site. Effects of introduction of peptide D-amino acids to the MHC peptide binding affinity have been investigated previously by other groups (Hill et al., 1994; Mailleire et al., 1995).

Thirty-eight D-amino acid substitutions were introduced at the P6 and P7 positions of the FVKQNA test peptide (Figure 2). Substitutions with D-amino acids were made at position 6 holding position 7 constant with L-alanine (Figure 2a), and conversely at position 7 holding position 6 constant with L-alanine (Figure 2b). Nineteen D-amino acids were tested at each position (D-analogs of all naturally-occurring amino acids except D-Ile and D-Cys were included in the library, along with D-ornithine). Of the 38 D-amino acid substitutions tested, only D-Pro at position 7 was found to increase the binding affinity of the base peptide. The peptide Ac-FVKQNA-P-NH$_2$ (where P=D-Proline), exhibited IC$_{50}$ of 8μM (Kd ~ 4 μM, see Methods), binding 20- to 100-fold more tightly than the other sequences.
Figure III.2. Effect of D-amino acids at position 6 or 7 of the Ha peptide analogue. A) Competitive binding analysis of peptide with D-amino acids at the P6 position. A fixed concentration of empty HLA-DR1 and biotinylated Ha peptide was incubated with increasing concentrations of Ac-FVKQNXA peptides having various D-amino acids residues at the P6 position (see “Materials and Methods”). B) Competitive binding analysis of peptides with D-amino acids at P7. Of 38 D-amino acid residues tested at P6 and P7, only D-Pro (P7) substantially increased the binding affinity of the base peptide.

We confirmed this result in the context of a different base peptide, YTALAAA (Figure 3). This peptide binds ~10^3-fold more tightly than FVKQNA, as it carries the residues most favorable for HLA-DR1 binding at the key P1, P4 and P6 positions (Hammer et al., 1992), as well as a threonine at position 2 which can make a favorable interaction with the MHC backbone (Fremont et al., 2002). P3 and P5, both alanine in this peptide, are primarily T cell contacts and
do not contribute significantly to the MHC peptide binding affinity (Hennecke and Wiley, 2002). YTALAAP binds tightly to HLA-DR1 relative to the already tightly-binding YTALAAA, indicating a positive contribution to the binding of D-Pro at position 7 in this peptide, similarly to that observed for FVKQNAAP (Figure 3). As similar effects were observed for D-Pro at position 7 in the context of two completely different sequences, we concluded that incorporation of an alkyl group at the nitrogen group of the P7 residue can increase peptide binding to HLA-DR1.

Surprisingly, incorporation of D-Pro at position 7 prevented productive C-terminal extension of the peptide. Peptides ending with either D-Pro or L-Pro at position 7 bind similarly, but longer peptides having a D-Pro at P7 bind 100-500-fold worse than the corresponding L-Pro containing peptides. This appears to be due to the inability of D-Pro containing peptides to make use of productive interactions in the P8-P10 region, probably because of alterations to the peptide backbone conformation induced by binding a side chain in the D conformation into a pocket adapted to L-amino acid residues. In general, C-terminal extensions of conventional L-amino acid containing peptides lead to substantial increases in peptide binding affinity. This is due to the contribution of MHC-peptide main-chain hydrogen bonds in the P8-P10 region, together with occupancy of the P9 pocket (specific for residues with aliphatic side chains). However, in both the FVKQA and YTALA series, C-terminal extension beyond the D-Pro at position 7 by the usually favorable Ala-Ala and Ala-Leu sequences led to a substantial decrease in binding affinity. Addition of AA or AL beyond a C-terminal L-Pro increased the binding affinity by 20- to 40-fold for the various peptides, whereas addition beyond D-Pro in the same sequences decreased the affinity by 2.5 to more than 50-fold (Figure 3).
<table>
<thead>
<tr>
<th>Peptide</th>
<th>IC_{50}(nM)</th>
<th>Peptide</th>
<th>IC_{50}(nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FVKQN</td>
<td>400,000</td>
<td>YTALA</td>
<td>8000</td>
</tr>
<tr>
<td>FVKQNAAA</td>
<td>15,000</td>
<td>YTALAAA</td>
<td>20</td>
</tr>
<tr>
<td>FVKQNAAAA</td>
<td>108</td>
<td>YTALAAAA</td>
<td>22</td>
</tr>
<tr>
<td>FVKQNAAL</td>
<td>90</td>
<td>YTALAAAAL</td>
<td>&lt;1</td>
</tr>
<tr>
<td>FVKQNAP</td>
<td>1430</td>
<td>YTALAAP</td>
<td>46</td>
</tr>
<tr>
<td>FVKQNAPAA</td>
<td>31</td>
<td>YTALAAP</td>
<td>2</td>
</tr>
<tr>
<td>FVKQNAPAL</td>
<td>34</td>
<td>YTALAAPAL</td>
<td>≤1</td>
</tr>
<tr>
<td>FVKQNAP</td>
<td>8000</td>
<td>YTALAAP</td>
<td>12</td>
</tr>
<tr>
<td>FVKQNAPAA</td>
<td>20,000</td>
<td>YTALAAP</td>
<td>700</td>
</tr>
<tr>
<td>FVKQNAPAL</td>
<td>20,000</td>
<td>YTALAAPAL</td>
<td>129</td>
</tr>
<tr>
<td>123456789</td>
<td>123456789</td>
<td>123456789</td>
<td></td>
</tr>
</tbody>
</table>

\[ P = D\text{-proline} \]

**Figure III.3. Effect of peptide extension beyond D-Pro at position 7.** IC_{50} values obtained from a competitive binding assay (as in Figure 2) for peptides that include extension beyond the P7 position.

The inability to productively extend D-Pro containing peptides could be due to the constraints of the Pro ring in the D-form, which might prevent the peptide C-terminal portions from adopting the canonical polyproline type II conformation (approximately three residues per turn with a right-handed twist) that has been observed in all class II MHC crystal structures determined to date. In this scenario, the peptide main chain of L-Pro containing peptides can adopt the polyproline type II conformation, thus maintaining key hydrogen-bonding contacts and occupation of the P9 pocket. Peptides that contain a D-Pro however, would direct the peptides' C-terminal main chain out of the binding groove, due to the opposite orientation of the constrained ring, and thus would lose binding affinity. Since none of the other D-amino acids at position 7 provides any substantial binding affinity, we tentatively ascribed the positive effects
on peptide binding at P7 to the N-alkyl portion of the D-Pro side chain, and the negative effects on productive peptide extension to the constraints introduced by the five-membered proline ring.

III.C.2. N-methyl alanine and N-methyl glycine at P7

In order to determine whether the N-alkyl portion is in fact responsible for the increase in binding affinity observed upon D-Pro substitution, we decided to mimic the δ-CH₂ moiety by incorporating amino acids that contain an N-methyl substituent at the P7 position. To this end, FVKQNAA peptides containing N-methyl alanine or N-methyl glycine (sarcosine) at P7 were synthesized and evaluated for HLA-DR1 binding. Peptides containing N-methyl alanine or N-methyl glycine bound 7.5-fold and 5.5-fold more tightly than those containing the corresponding natural amino acid residues (Figure 4). The 9-mer peptide FVKQNAA (A=N-methyl alanine; IC₅₀ = 2µM) had similar affinity as did the FVKQNAP sequence (P=D-Pro; IC₅₀ = 1.4µM), supporting the idea that the N-alkyl substituent is a key determinant of the affinity increase. Moreover, both N-methyl glycine and N-methyl alanine peptides could be extended with concomitant increases in binding affinity, suggesting that they bind productively in the normal conformation (Figure 4).

III.C.3. N-substituted glycine at P7

In order to determine whether N-substituted groups larger than methyl could be accommodated in the P6/P7 pocket, we synthesized peptides containing additional substituents on the amide nitrogen of the P7 residue, and evaluated their binding to HLA-DR1. The substituents included linear and branched aliphatic chains designed to identify whether the P6/P7 pocket would be able to accommodate groups larger than a methyl, and linear, aliphatic amines.
designed to be complementary to the generally acidic electrostatic character of the P6/P7 region. The N-substituted residue was generated in situ on the resin, using a sub-monomer strategy previously described for generation of N-substituted glycines (Figliozzi et al., 1996; Nguyen et al., 1998), with conventional Fmoc chemistry used to make the fore and aft parts of the peptide. We attempted but were unable to extend this methodology to generation of N-substituted alanines (see Methods). None of the N-alkyl substitutions (ethyl, isopropyl, aminoethyl, aminopropyl, aminobutyl) increased the binding affinity over that observed for the N-methyl substitution (Figure 5), suggesting that larger is not necessarily better in the P6/P7 pocket.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>IC$_{50}$(nM)</th>
<th>Peptide</th>
<th>IC$_{50}$(nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FVKQNA</td>
<td>15,000</td>
<td>FVKQNAG</td>
<td>278,000</td>
</tr>
<tr>
<td>FVKQNA</td>
<td>2,000</td>
<td>FVKQNAG</td>
<td>50,000</td>
</tr>
<tr>
<td>FVKQNAAAA</td>
<td>70</td>
<td>FVKQNAGAA</td>
<td>2000</td>
</tr>
<tr>
<td>FVKQNAAL</td>
<td>20</td>
<td>FVKQNAGAL</td>
<td>500</td>
</tr>
<tr>
<td>1 2 3 4 5 6 7 8 9</td>
<td></td>
<td>1 2 3 4 5 6 7 8 9</td>
<td></td>
</tr>
</tbody>
</table>

A = N-methyl alanine  
G = N-methyl glycine

Figure IV.4. Extension beyond and N-methyl residue at the position 7 of the peptide. IC$_{50}$ values obtained from a competitive binding assay for peptides carrying an N-methyl glycine and N-methyl alanine at P7.
III.C.4. Crystal structure of N-methyl alanine at P7

To evaluate whether the N-methyl substitution introduced any unexpected alteration of the peptide conformation, and to determine the nature of the constraint on the size of the N-alkyl moiety, the crystal structure of the peptide Ac-FVKQNA\textsubscript{A}AL-NH\textsubscript{2} (\textsubscript{A} = N-methyl alanine) bound to HLA-DR1 was determined at 2.1 Å. The complex was crystallized in the presence of the superantigen SEC3-3B2 (Figure 6). Superantigens are a class of disease-causing proteins of viral or bacterial origin that hyperactivate the immune system by crosslinking MHC proteins and T cell receptors (Balaban and Rasooly, 2000), and which have been used successfully as an aid
to crystallization of HLA-DR-peptide complexes (Bolin et al., 2000; Sundberg et al., 2002). Here we have used the affinity matured 3B2 variant of staphylococcal enterotoxin SEC3 (Andersen et al., 1999), which interacts with HLA-DR1 outside the binding groove on the flanking helix from the \( \alpha \)-subunit (Redpath et al., 1999). MHC-peptide-superaantigen crystals, grown under previously determined conditions (Sundberg et al., 2002) were used for X-ray data collection at \(-100^\circ\text{C}\). Diffraction data to 2.1 angstroms were collected using a Mar345 image plate detector and a Cu-K\( \alpha \) X-ray source (Table I). Initial phases were obtained by molecular replacement using as a search model another HLA-DR1/SEC3-3B2 complex (1KLU, PDB) (Sundberg et al., 2002) with peptide coordinates removed, followed by a few rounds of manual rebuilding and refinement (see Methods for details).

Figure III.6. HLA-DR1/ Ac-FVKQNAAL in complex with the superantigen SEC3-3B2. Left) Structure of the HAL-DR1/ Ac-FVKANAAAL/SEC3-3B2. Peptide is shown in sticks with carbon atoms in yellow, nitrogen in blue and oxygen in red. DR1 and SEC3-3B2 are shown as a cartoon representation (DR1\( \alpha \)-chain in cyan, DR1\( \beta \)-chain in blue, SEC3-3B2 in orange). Right) Crystal obtained with the complex grown in 2-6\% PEG 4K, 10\% ethylene glycol, 100mM sodium acetate, pH 5.2-5.6
Electron density for HLA-DR1 was interpretable and continuous throughout the molecule with the exception of residues 112-113, in a loop at the distant region of the β-chain and away from the peptide-binding site. In the case of SEC3-3B2, the electron density was clear and continuous except for residues 97-105, for which no interpretable density was seen. Clear density can be seen for all of the peptide, including the N-methyl group from the P7 residue (Figure 7). N-methyl density can be seen in a standard 2Fo-Fc composite omit map (Figure 7A), and particularly clearly in a Fo-Fc difference omit map, calculated with the N-Me group omitted (Figure 7B). In this case the N-Me peak is the most prominent feature of the map.

In the structure of a related complex carrying a normal, non-methylated peptide, DR1/TP123-37/SEC3-3B2 (Sundberg et al., 2002), four waters bind in the P6/P7 area of the peptide binding groove and participate in a network of hydrogen bonds (Figure 8B). These same waters are observed in other HLA-DR1 structures determined at lower resolution, although not every water is observed in each structure. One of these waters normally makes a hydrogen bond with the nitrogen of the peptide backbone at the P7 position (Figure 8B). This water is displaced by the introduction of the N-methyl group at the P7 position of the peptide (Figure 8A). The hydrogen bond network linking the other water molecules in the area adjusts slightly in response to the perturbation. Except for this change, no other significant structural alteration appears to result from the N-methyl substitution.

III.C.5. T-cell activation assay

To assess the effect of N-methylation of the P7 residue on recognition by T cells, the substitution was incorporated into the native antigenic peptide (Ha306-318) derived from
Figure III.7. Electron density maps for the P6/P7 region of HLA-DR1/Ac-FVKQNAAL/SEC3-3B2. A) Composite omit F_c-F_e electron density map contoured at 1σ using data in resolution range of 20-2.1 Å. HLA-DR1 carbon atoms are colored in green, Ac-FVKQNAAL peptide carbon atoms are colored in yellow, nitrogen and oxygen atoms are colored blue and red, respectively. Waters are represented as small red spheres. B) Difference F_o-F_c electron density map with the N-methyl group of the P7 position of peptide omitted from the map phase calculation contoured at 3σ. Figures were generated using PyMol (DeLano. 2002)
influenza hemagglutinin (Lamb and Fledmann, 1982). In the context of this peptide, which binds very tightly (Roche and Cresswell, 1990), no significant improvement of binding affinity to HLA-DR1 was observed as a result of the N-methyl introduction (Figure 9A). The corresponding MHC-peptide complexes were used to activate a T-cell hybridoma carrying a HLA-DR1 restricted, Ha306-318 specific, T cell receptor, with induction of IL-2 secretion used to monitor T cell activation (Bolin et al., 2000). HLA-DR1 complexes of both the native Ha peptide and Ha7 N-CH₃ peptide were able to similarly stimulate the T cell hybridoma, while an irrelevant peptide complex was inactive (Figure 9B). We tested the ability of the N-methylated peptide to be presented by cell-surface MHC molecules as normally expressed by antigen presenting cells. Native and modified peptides were added to antigen presenting cells (an EBV-transformed B-cell line) that express endogenous HLA-DR1, to allow cellular binding and presentation. Both native Ha peptide and Ha7 N-CH₃ were presented effectively to T cells by fixed (Figure 9C) or live (Figure 9D) antigen-presenting cells. These experiments indicate that the N-methyl introduction does not interfere with the ability of the Ha peptide to be presented by cellular MHC proteins or to activate T cells.

III. D. Discussion

Class II MHC proteins, together with their bound antigenic peptides, are the ligands for antigen receptors on CD4+ T cells. The interaction is an important part of the process that triggers inflammatory and antibody-mediated immune responses to foreign antigens in the body, and peptide antigens represent a possible target for design of immunotherapeutic agents. Approaches to peptide-based immunotherapeutic agents include attempts to design MHC-binding antigens with improved bioavailability (Bolin et al., 2000), altered T cell response
<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ha</td>
<td>PKYVKQNTKLAT</td>
<td>10</td>
</tr>
<tr>
<td>Ha 7 N-CH₃</td>
<td>PKYVKQNTKLAT</td>
<td>7</td>
</tr>
</tbody>
</table>

\( L = \text{N-Methyl leucine} \)

**Figure III.9. Peptide binding data and HA1.7 T-cell hybridoma assays for Ha and HA7 N-CH₃ peptides.**

A) Peptide sequences and IC<sub>50</sub> values from a competitive-binding assay for native and N-methyl P7 variant of the influenza virus hemagglutinin Ha peptide. B) Responses of HLA-DR1-restricted Ha-specific T-cell hybridoma to immobilized MHC-peptide complexes. The peptide A2 used as a negative control derives from the class I MHC protein HLA-A2 (Chicz et al., 1992). Immobilized anti-CD3 antibody was used as a positive control for T-cell activation. C) Response of the T cell hybridoma to non-fixed (dashed lines and filled symbols) and fixed (continuous lines and open symbols) HLA-DR1-expressing antigen presenting cells pulsed with Ha peptide (circles), Ha 7 N-CH₃ peptide (squares) or negative control A2 peptide (triangles).
(Sette et al., 1994), and stabilized MHC-peptide interaction (Vargas et al., 2003). The last approach is potentially important for development of MHC-peptide complexes able to tolerize auto-reactive T cells, which frequently recognize weakly-binding peptides (Brand et al., 2001; Kjellen et al., 1998; Stratmann et al., 2000).

As a step towards development of a strategy for stabilizing MHC-peptide interaction without abrogating T cell receptor recognition, we have investigated the P6/P7 region of the human class II MHC protein HLA-DR1, using as a model antigen the antigenic peptide Ha(308-316) derived from influenza heamagglutinin. This region, underneath the peptide and between the MHC pockets that bind the peptides's P6 and P7 side chains, is one of the few areas of shape non-complementarity in the overall binding site. In crystal structures of several HLA-DR1 peptide complexes, water molecules are found in this region, making hydrogen bonding interactions with each other and with MHC side chain and peptide main chain groups. We investigated the ability of D-amino acid side chains at P6 and P7 to bind into the P6/P7 pocket. Only D-Pro at position P7 increased the binding affinity over a P7 glycine residue. Structural constraints on the peptide conformation prevented P7 D-Pro-containing peptides from interacting productively with the MHC beyond the P7 position, but N-methylation of the P7 amide provided increased binding due to interactions in the P6/P7 region while at the same time allowing the native peptide polyproline conformation. Crystal structure analysis verified that the N-methyl substitution bound in the P6/P7 region by displacing one of the bound water molecules, without appreciable alteration of the peptide structure elsewhere. T cell activation studies confirmed that the native antigenic T cell receptor interaction was not altered by the modification.

Displacement of bound water molecules is thought to promote ligand binding through favorable entropic effects on the free energy. Improved inhibitors of HIV protease (Lam et al.,
1994), FK506 binding protein (Connelly et al., 1994), glycogen phosphorylase (Gregoriou et al., 1998), and cholera toxin (Fan et al., 2001) have been designed based on this principle. However, displacement of bound water molecules can have enthalpic consequences as well, including loss of favorable bridging interactions (Bhat et al., 1994; Chung et al., 1998), and alteration of protein ionization potentials (Dullweber et al., 2001). Moreover, displacement of bound water can result in protein or ligand conformational changes, with corresponding effects on the energetics of the interaction (Luque and Freire, 2002). These factors have been evaluated recently for HIV protease, a particular system for which detailed structural and thermodynamic data are available for several inhibitors that displace a highly-ordered water molecule. In this case, significant contributions were found for each of these factors described above, with large and partially offsetting enthalpic and entropic contributions, and with the overall result that release of a bound water only marginally contributed to the overall free energy of ligand binding (Li and Lazaridis, 2003; Luque and Freire, 2002).

For class II MHC proteins, detailed thermodynamic data on the energetics of peptide interaction are not available, because the elaborate kinetic pathway (Beeson and McConnell, 1994; Joshi et al., 2000; Rabinowitz et al., 1998; Sadegh-Nasser and McConnell, 1989) and presence of multiple slowly-converting MHC conformations (Rabinowitz et al., 1998; Schmitt et al., 1999; Zarutskie et al., 1999) have precluded equilibrium thermodynamic analysis. In the absence of detailed thermodynamic data, we can use existing information to help understand the role of water binding in P6/P7 region. Only one of the four water molecules bound in the P6/P7 region could be displaced productively. In addition to forming a hydrogen bond with the peptide P7 amide nitrogen, this water molecule usually forms hydrogen bonds to two other buried water molecules in the vicinity, and also to MHC Glu β28 (Figure 8B). The N-methyl substitution
displaces the water molecule, but the local hydrogen-bonding network rearranges slightly, so that the only net loss is the hydrogen bond to Glu β28, with the gain of new hydrogen bond to the peptide backbone P7 carbonyl oxygen (Figure 8A). (Both Glu β28 and the P7 carbonyl form additional hydrogen bonds not shown in Figure 8, with Arg β71 and Asn α69, respectively). Thus, the N-methyl substitution apparently did not result in significant loss of favorable hydrogen-bonding interactions. No significant conformational changes were observed as a result of the substitution, suggesting that both peptides are able to effect the ligand-induced conformational change in the empty MHC protein that helps to trap peptides in the binding site (Zarutskie et al., 1999). Finally, it is not likely that nearby side chains change their ionization state as a result of the N-methyl introduction, since the only ionizable groups in the vicinity are Gluβ28 and Argβ74, which form a buried salt bridge that would not be affected by the substitution. Overall, it appears that for the water molecule displaced by P7 N-methylation, the favorable entropic contribution resulting from displacement of the bound water is sufficient to overcome other potentially offsetting contributions resulting from the substitution. For the other water molecules bound in the P6/P7 pocket, this would not appear to be the case. We attempted to displace additional water molecules by enlarging the N-alkyl substitution (Figure 5). Modeling studies (data not shown) had indicated that the modifications introduced would be able to displace additional water molecules in low-energy conformers without large steric clashes. However, enlarging the N-alkyl substitution did not result in increased binding affinity. In displacement of additional water, then, other effects apparently oppose an increase in binding affinity. Likely candidates for such effects would include unsatisfied hydrogen bond donors or acceptors left behind by displacement of water molecules that bridge between the MHC and bound peptide.
The P7 N-methyl substitution described here might be of general use in improving weakly-binding peptides. Moreover, the change in backbone chemistry might also increase the resistance to proteolysis in vivo, which could be important in potential immunotherapy applications. Although the N-methyl substitution is completely buried beneath the peptide, in several cases buried substitutions have been shown to alter T cell activation, presumably as a result of subtle internal structural changes transmitted to the surface. For example, in the case of HLA-DR1, alterations of the completely buried P1 side chain can affect T cell recognition (Wu et al., 1996). In the context of another class II MHC protein I-E(k) substitution of the buried MHC residue β29 (β28 in HLA-DR1, see Figure 7) altered T cell recognition (Boehncke et al., 1993). The side chain of β29 forms one of the hydrogen bonds to the water displaced by the N-methyl substitution described here. The lack of such disruption of T cell activation observed here shows that the N-methyl substitution is well tolerated, and is consistent with the lack of detectable alteration in the crystal structure. In summary, we have shown that the binding affinity of peptides for the class II MHC protein HLA-DR1 can be increased by incorporating N-methyl amino acids at position 7 in the peptide, without alteration of the antigenic T cell interaction. The ability to increase the affinity of biologically-relevant peptides that bind weakly to HLA-DR1 could serve as tool for design of potential therapeutic synthetic peptides.
Table III.1. Data Collection and Refinement Statistics

<table>
<thead>
<tr>
<th>Crystal Parameters</th>
<th>Overall</th>
<th>Highest res. shell</th>
</tr>
</thead>
<tbody>
<tr>
<td>Space Group</td>
<td>R3</td>
<td></td>
</tr>
<tr>
<td>Cell dimensions</td>
<td></td>
<td></td>
</tr>
<tr>
<td>a(Å)</td>
<td>172.50</td>
<td></td>
</tr>
<tr>
<td>c(Å)</td>
<td>121.65</td>
<td></td>
</tr>
<tr>
<td>Data Collection</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Resolution limits (Å)</td>
<td>20.0-2.10</td>
<td>2.17-2.10</td>
</tr>
<tr>
<td>Unique reflections</td>
<td>79,802</td>
<td>7,968</td>
</tr>
<tr>
<td>Total reflections</td>
<td>366,342</td>
<td>32,165</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>100.0</td>
<td>99.9</td>
</tr>
<tr>
<td>Mean I/σ(I)</td>
<td>11.5</td>
<td>3.5</td>
</tr>
<tr>
<td>R_{sym} (%)(^{a})</td>
<td>7.5</td>
<td>39.4</td>
</tr>
<tr>
<td>Refinement</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R_{free} (^{b})</td>
<td>23.0</td>
<td>31.2</td>
</tr>
<tr>
<td>R_{crys} (^{b})</td>
<td>20.5</td>
<td>28.4</td>
</tr>
<tr>
<td>Model (^{c})</td>
<td></td>
<td></td>
</tr>
<tr>
<td># Residues (atoms)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HLA-DR1</td>
<td>368 (3026)</td>
<td>32.5</td>
</tr>
<tr>
<td>Peptide</td>
<td>7 (67)</td>
<td>22.9</td>
</tr>
<tr>
<td>SEC3-3B2</td>
<td>229 (1883)</td>
<td>31.0</td>
</tr>
<tr>
<td>Waters</td>
<td>414</td>
<td>34.9</td>
</tr>
<tr>
<td>Real Space</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R_{factor} (^d)</td>
<td>0.063</td>
<td>0.030</td>
</tr>
</tbody>
</table>

Ramachandran plot statistics

| Core + Allowed (%) | 99.6 |
| Generous (%)       | 0.4  |

\(^{a}\) R_{sym} = \sum |I - \langle I\rangle| / \sigma \langle I\rangle, where I is the observed intensity and \langle I\rangle is the average intensity of multiple symmetry-related reflections, and the summation taken over all multiply-recorded reflections (average redundancy 4.6).

\(^{b}\) Reciprocal space R factor = \sum |F_{obs} - F_{calc}| / \Sigma F_{obs}, with the summation taken over 10\% of reflections omitted from the refinement and used as a test set (R_{free}) or else the remaining 90\% of reflections used in the refinement (R_{crys}).

\(^{c}\) The atomic coordinates for the crystal structure of this protein are available in the Research Collaboratory for Structural Bioinformatics Protein Databank PDB # 1PYW.

\(^{d}\) Real space R factor = (\sum |\rho_{obs} - \rho_{calc}|) / (\Sigma |\rho_{obs} + \rho_{calc}|), with the summation taken over the space occupied by the molecule. The function describes the fit between the model and the electron density map.
Chapter IV: Side chain specificity at the P10 position of the peptide binding site of a Class II MHC Protein

Summary

Peptides bind to class II MHC proteins in an extended conformation, with pockets in the peptide-binding site spaced to accommodate peptide side chains at the P1, P4, P6, and P9 positions. Two peptides differing only at the P10 position have significantly different binding affinities for HLA-DR1. The structure of HLA-DR1 in complex with the tighter-binding peptide shows that the peptide binds in the usual, polyproline type II conformation, but with the P10 residue accommodated in a shallow shelf or pocket at the end of the binding groove. Two residues that line the P10 pocket, β57 and β60, are variable within the highly polymorphic set of human MHC class II proteins. HLA-DR variants with polymorphic residues at these positions were produced and found to exhibit different amino acid side chain specificity at the P10 position. These results define a new specificity position in HLA-DR proteins.
IV.A. Introduction

Major histocompatibility complex (MHC) proteins play an important role in the generation of immune responses by binding peptide antigens and presenting them at the cell surface for inspection by antigen receptors on T cells (Watts, 1997). T cell recognition of MHC-bound antigens is required for generation of adaptive immune responses against pathogenic viruses, bacteria, and fungi, and for selection and maintenance of the large repertoire of circulating T cells. Class II MHC proteins are found in macrophages, B cells, dendritic cells, and other “antigen-presenting cells” of the immune system, where they bind peptides produced by endosomal proteolysis. The endosomal antigen processing machinery results in a large variety of peptide antigens bound to class II MHC proteins, with an average length of 15-20 residues (Chicz et al., 1992; Rudensky et al., 1991). Peptides bind to class II MHC proteins in an extended conformation, with up to 12 peptide residues in direct contact with the MHC peptide-binding groove. Pockets in the peptide-binding groove are spaced to accommodate some of the peptide side chains, with the remainder held accessible for interaction with T cell receptors. In structures of human and mouse class II MHC proteins, pockets within the overall peptide-binding site are found at the P1, P4, P6, and P9 positions, with smaller pockets at P3 and P7 (Figure 1A) (Corper et al., 2000; Fremont et al., 2002; Ghosh et al., 1995; Lee et al., 2001; Li et al., 2000; Liu et al., 2002; Southwood et al., 1998; Stern et al., 1994). Pockets are numbered along the peptide relative to a large hydrophobic pocket near the peptide binding site. These positions are consistent with experimentally determined peptide binding motifs, which indicate peptide sequence preferences at these positions.
Class II MHC proteins are highly polymorphic in all species that express them. Over 400 alleles have been characterized for the beta subunit of HLA-DRB, the most highly-expressed of the three human class II MHC proteins. (The alpha subunit is non-polymorphic). Most of the polymorphisms can be found in positions that correspond to the residues lining the pockets within the peptide-binding site. The allelic differences influence the peptide binding specificity of the various class II MHC alleles, and thus determine allele-specific differences in the spectrum of peptides presented to the immune system. These differences have important consequences for susceptibility to autoimmune diseases, resistance to infection, vaccine efficacy, and rejection of transplanted organs and administered proteins. In the common allelic variant HLA-DR1 (DRB1*0101), for example, the P1 pocket shows strong preferences for large hydrophobic residues (like Tyr, Trp, Phe, Leu and Ile), the P6 pocket prefers smaller residues (Gly, Ala, Ser and Pro), and the P4 and P9 pockets tend to have weaker preferences for residues that have aliphatic character (Hammer et al., 1992). Algorithms designed to predict peptide binding affinities for HLA-DR1 and for other class II MHC allelic variants have been developed based on experimental peptide and phage display binding data together with the pattern of MHC-peptide side-chain contacts observed in the crystal structures. Generally, such approaches have considered only positions P1 through P9 or a subset of these positions, although one report analyzed specificity at positions P-1 through P10 (Fleckenstein et al., 1996; Rammensee et al., 1999).

A recent study of HLA-DR52a (HLA-DRB3*0101) bound to an integrin β3-derived peptide suggested a role for the P10 position in peptide binding, and a similar effect was observed in a systematic study of contributions of anchor and non-anchor
positions to the overall peptide binding affinity of HLA-DR1 (Yassai et al., 2002). Here, we have investigated the structural basis for specificity at the P10 position of peptides bound to HLA-DR1. A newly-described shallow pocket or shelf on the edge of the MHC peptide binding site interacts with peptide P10 side chains, and common MHC polymorphisms in this region alter the peptide side-chain specificity. These results suggest that the P10 position can play an important role in MHC peptide binding selectivity.

IV.B. Materials and Methods

IV.B.1. Peptide Synthesis

Peptides were synthesized using solid-phase F-moc (N-(9-fluorenyl)methoxycarbonyl) chemistry on a Symphony instrument (Protein Technology Inc). All peptides were amidated at the C-terminus. N-biotinylated peptides were prepared by coupling aminocaproyl-(LC)-biotin (Anaspec) to the N-terminus of the resin-bound, side-chain protected, peptide using standard amino acid coupling procedure. The peptides were deprotected and cleaved from the resin by a 2 hour treatment at room temperature with a mixture of trifluoroacetic acid/ H2O/ thioanisole/phenol/dithiothreitol (82.5:5:5:5:2.5). The solution of peptides was precipitated with cold diethyl ether, and the crude peptides were filtered, washed with ether, and dried in vacuum. The crude peptides were purified by high performance liquid chromatography (Vydac-C18). The purity and homogeneity of each peptide was checked by high performance liquid chromatography (Vydac-C18) and MALDI-TOF mass spectrometry. Peptide concentrations were measured by absorbance at 280nm (ε280nm = 1280 M⁻¹ cm⁻¹) in the case of peptides containing tyrosine.
or determined using amino acid analysis in the case of CLIP peptide.

IV.B.2. Protein expression and purification

For peptide binding experiments and x-ray crystallography, the extracellular portion of HLA-DR1 was produced from S2 (Schneider cell) insect cells as soluble empty αβ heterodimers, as described previously (Stern and Wiley, 1992). For x-ray crystallography of the MHC-peptide complex in the presence of the superantigen, the extracellular portion of HLA-DR1 was produced by expression of isolated subunits in *Escherichia coli* inclusion bodies followed by refolding *in vitro* as described previously (Frayser et al., 1999). Refolded HLA-DR1 was purified by immunoaffinity chromatography using the conformation-specific monoclonal antibody LB3.1, followed by gel filtration chromatography in phosphate-buffered saline, pH 6.8. The protein concentration was measured by UV absorbance at 280 nm using $\varepsilon_{280}$ of 54375 M$^{-1}$ cm$^{-1}$ for empty HLA-DR1. SEC-3B2 superantigen was expressed as a soluble protein in *E. coli* and isolated from the periplasmic fraction as described previously (Andersen et al., 1999)

IV.B.3. Peptide binding assays

A competition assay was used to determine binding affinities of peptides to HLA-DR1 wild type and mutants. Peptide-free wild type HLA-DR1 produced in insect cells or mutated form of HLA-DR1 produced in *E. coli* (25 nM) was mixed together with biotinylated Ha[306-318] peptide probe (Ha$_{bio}$, 25 nM) and varying concentrations of unlabelled competitor peptide ($10^{-12}$- $10^{-5}$M). The mixtures were incubated for 3 days at 37 °C in 100 mM sodium phosphate buffer at pH 5.5, containing 50 mM NaCl, 1 mg/ml
PMSF, 37 μg/ml iodoacetamide, 10 mM EDTA, 0.02% NaN₃, 0.5 mg/ml octylglucoside, followed by detection of bound biotinylated peptide using an immunoassay that employed anti-DR1 capture antibody LB3.1 and alkaline phosphate labeled streptavidin. IC₅₀ values were obtained by fitting a binding curve to the plots of fluorescence versus concentration of competitor peptide.

A direct binding assay was used to determine binding abilities of HLA-DR1 mutants and to confirm binding affinities of some peptides tested in the competition assay. The assay was performed for 3 days at 37°C in the same buffer as described above using constant concentration of the proteins (25nM) and the range of concentrations of biotinylated peptides depending on the binding affinity of the particular peptide (10⁻¹¹-10⁻⁶ for tight binders and 10⁻¹¹-10⁻² for weak binders). The incubation was followed by immunoassay described above.

**IV.B.5. Crystallization**

HLA-DR1 peptide bound complexes were prepared by incubating purified empty HLA-DR1 (1-5 μM) with at least 5-fold molar excess peptide for 3 days at 37°C in phosphate-buffered saline with 0.02% sodium azide. The complex was purified by gel filtration to remove aggregates and free peptide. Crystals of HLA-DR1/AAYSQATPLLSPR complex were grown at room temperature by the vapor diffusion method in 10% polyethylene glycol 6000, 100 mM glycine pH 4.0, with 1 μL of precipitant solution mixed with 1 μL of 10 mg/mL. For x-ray diffraction experiments, the crystal was soaked in mother liquor with 25% glycerol for 2 minutes and flashed cooled in liquid nitrogen.
For the HLA-DR1/ AAYSDQATPLLSSPR/SEC3-3B2 crystal structure, purified HLA-DR1/ AAYSDQATPLLSSPR complex was mixed in equimolar ratio with purified SEC3-3B2. Crystals of HLA-DR1/ AAYSDQATPLLSSPR /SEC-3B2 were grown by the vapor diffusion method in hanging drops at 4°C under the following conditions: 2-6% polyethylene glycol 4000, 5-10% ethylene glycol, 100mM sodium acetate pH 5.2-5.6, with 1 μL of precipitant solution mixed with 1μL of 8 mg/mL protein complex. For x-ray diffraction experiments, the crystals were soaked for ~1 minute in a cryoprotectant solution consisting of 25% ethylene glycol in the mother liquor and then flashed cooled in liquid nitrogen.

IV.B.6. Data collection and processing

Diffraction data for HLA-DR1/ AAYSDQATPLLSSPR was collected at the National Synchrotron Light Source (NSLS) using X25 beamline (λ=1.10) to a resolution of 2.4 Å.

A high resolution data set (2.40 Å) of the HLA-DR1/ AAYSDQATPLLSSPR/SEC3-2B2 complex was collected on a single crystal (300 μm x 200 μm x 200 μm) on an R-AXIS IV image plate detector using CuKα radiation. Collected data for both complexes were processed and scale using Denzo, Scalepack, and the CCP4 package (Collaborative Computational Project, 1994; Otwinowski and Minor, 1997).

IV.B.7. Structure determination

The structure of each complex was determined by molecular replacement. For the HLA-DR1/ AAYSDQATPLLSSPR structure, coordinates from another complex of
HLA-DR1 (Protein Data Bank code 1AQD) was used as the search model. After an initial rigid body refinement, five rounds of refinement included minimization, B-factor and minimization. A two-fold non-crystallographic symmetry (NCS) averaging improved the quality of the electron density map.

In the case of HLA-DR1/ AAYSDQATPLLLSPR /SEC3-2B2, coordinates for another complex of *E. coli*-derived HLA-DR1 and SEC3-3B2, carrying a N-methylated designed peptide (Protein Data Bank code 1PYW) (Zavala-Ruiz et al., 2003) was used as the search model to find a molecular replacement solution. Waters and the peptide were removed from the search model before use. After an initial rigid-body refinement, eight rounds of refinement included minimization, B-factor, and minimization.

For both structures, refinement was carried out using CNS (Brunger et al., 1998) and manual inspection and rebuilding using XtalView (McRee, 1999). Waters were added to the refined molecule using CNS. The final models were verified for distortions on the secondary structure features using Procheck (Laskowski et al., 1993).

**IV.B.8. MHC-peptide database analysis**

List of peptides known to bind to particular class II MHC alleles, and predicted allele-specific peptide binding motifs, have been collected in several databases (Blythe et al., 2002; Rammensee et al., 1999; Sturniolo et al., 1999). We scanned one database, SYFPEITHI, for sets of similar HLA-DR1 alleles that differ in the P10 pocket residues β57 and β60, and for which reliable peptide-binding motifs were available. DRB1*0401, DRB1*0404, and DRB1*0405 form the set that was used in our analysis. Peptides were aligned using the peptide-binding motif and the residues that bind into the
P10 pocket were identified. The residues were accommodated in different groups: aromatic (Trp, Tyr, Phe), hydrophobic (Ile, Leu, Val, Met), small (Ala, Pro, Gly), polar (Thr, Ser, Cys, Asn, Gln), and a charged group (His, Glu, Arg, Lys, Asp). The frequency of each residue group was calculated and plotted as a pie-graph to determine if the alleles show specificity for one or more groups.

IV.B.10. Construction and analysis of HLA-DR1 variants carrying single or double amino acid substitutions in the P10 shelf

We used in vitro site-directed mutagenesis to make single or double-point mutations on the β-chain of HLA-DR1. For each reaction, our vector (pLM1) with an insert of the β-chain of HLA-DR1 and two synthetic oligonucleotides primers, both containing the desire mutations, were used. The oligonucleotides, each complementary to opposite strands of the vector, were extended during temperature cycling by PfuUltra HF DNA polymerase (Stratagene), without primer displacement. After the temperature cycling, the product was treated with Dpn I endonuclease (Stratagene) to digest the parent DNA template and select for mutation-containing synthesized DNA. The vector DNA containing the desired mutations was transformed into XL1-Blue supercompetent cells (Stratagene). Sequencing of five randomly chosen clones reveal mutations desired. Plasmids were purified using Quigen Mini-prep Kit.

To make HLA-DR1-βD57A, the primers used were: 5’ GAC GGA GCT GGG GCG GCC TGC TGC CGA GTA TTG GAA C 3’ (sense) and 5’ GTT CCA ATA CTC GGC AGC AGG CCG CCC CAG CTC CGT 3’ (antisense). For HLA-DR1-βD57AY60H, the primers used were: 5’ GAG CTG GGG CGG CCT GCT GCC GAG
CAC TGG AAC AGC CAG AAG GAC 3' (sense) and 5'GTC CTT CTG GCT GTT
CCA GTG CTC GGC AGC AGG CCG CCC CAG CTC 3' (antisense). The primers for
HLA-DR1-βD57V were: 5'GAC GGA GCT GGG GCG GCC TGT GGC CGA GTA
TTG GAA C 3' (sense) and 5' GTC CCA ATA CTC GGC CAC AGG CCG CCC CAG
CTC CGT C 3' (antisense). For HLA-DR1-βD57VY60S, 5'GAG CTG GGG CGG CCT
GTG GCC GAG TCT TGG AAC AGC CAG AAG GAC 3' (sense) and 5'GTC CTT
CTG GCT GTT CCA AGA CTC GGC CAC AGG CCG CCC CAG CTC 3' (antisense).

IV.C. Results and Discussion

IV.C.1. The P10 position can influence binding of peptides to HLA-DR1

Two peptides with sequences differing only by a single amino acid predicted to
occupy the P10 pocket (AAYSDQATPLLSPR and AAYSDQATPLLGSPR) bind to
HLA-DR1 with 15-fold different apparent affinity as measured by a competition assay
(Figure 1B). The peptide sequences derive from the integrin β3 peptide previously
implicated in P10 effects in the context of HLA-DR52a, but with the “anchor” P1, P4,
P6 and P9 positions changed to the corresponding residues from the influenza
haemagglutin [306-318] peptide (HA), a well-characterized viral antigen that binds
tightly to HLA-DR1 (Kd ~10 nM). The difference in peptide binding affinity would
appear to be due to an interaction between the peptide P10 residue and HLA-DR1. As
noted above, a side-chain binding pocket at this position has not been described in HLA-
DR1 or any other class II MHC protein, and peptide-binding motifs do not include a side
chain preference at this position. It is possible however that these peptides bind in an
expected conformation, or that one of the peptides binds in an unexpected register, for
example the peptide might “bulge” as observed in complexes of peptides bound to class I MHC proteins, shifting the peptide side chains that are accommodated in the MHC pockets. To elucidate the structural basis for the apparent specificity at the P10 position, we crystallized HLA-DR1 in complex with the AAYSDQATPLL[L]SPR peptide and determined its structure by X-ray diffraction methods.

Figure IV.1. The P10 position of peptides can affect binding to HLA-DR1. A, Surface of the HLA-DR1 peptide binding site with bound AAYSDQATPLL[L]SPR peptide as sticks. In the peptide, carbon atoms are yellow; nitrogen atoms are blue; and oxygen atoms red. B, Competitive binding analysis of two peptides that differ only in the P10 position. A fixed concentration of peptide-free HLA-DR1 and biotinylated-Ha peptide was incubated with increasing concentrations of either AAYSDQATPLL[L]SPR or AAYSDQATPLL[G]SPR peptide. After binding for 3 days at 37°C, the amount of bio-Ha peptide/HLA-DR1 complexes was measured using HRP-labeled streptavidin.
IV.C.2. Crystal structure of HLA-DR1 reveals a P10 shallow pocket or shelf

The X-ray crystal structure of HLA-DR1 bound the AAYSDQATPLL\text{L}SPR peptide was determined in two crystal forms, one with two molecules in the asymmetric unit, and another for a complex with the bacterial superantigen SEC3-3B2 that has been used previously to facilitate crystallization of HLA-DR variants (Table 1). SEC3-3B2 makes contacts with the $\alpha$-chain of HLA-DR1 outside the binding site and does not contact the peptide. In each of the structures, clear and continuous density was observed for essentially all of the MHC protein, excluding a disordered loop away from the peptide binding site, and for the peptide from P-2 to P11, excluding the P8 Leu side chain, which is oriented towards the T cell receptor and does not contact the MHC. The peptide binds in the usual polyproline type-II conformation, using the expected anchor residues Tyr (P1), Gln (P4), Thr (P6) and Leu (P9) (Figure 2A). As commonly observed in class II MHC peptide structure, the peptide termini outside the binding site were disordered. The MHC and peptide portions of these structures were identical in the three views of the molecule provided by these crystals, except for some crystal contacts that vary between the crystals forms (Figure 2B). We were not able to obtain crystals of HLA-DR1 in complex with the other peptide (AAYSDQATPLL\text{G}SPR), possibly because it binds weakly to the MHC, and therefore the complex is not very stable.

The peptide P10 residue and surrounding the MHC region were well-resolved in each of the crystal forms HLA-DR1 residues located in close proximity to the P10 residue are Arg$\alpha$72, Pro$\beta$56, Asp$\beta$57 and Tyr$\beta$60 (Figure 2C). These residues form a shallow pocket or “shelf” that accommodates the P10 residue. (Figure 2D). The interaction buries $\sim$117 Å$^2$ of surface area from the P10 residue out of 1216 Å$^2$ that the
whole peptide buries, which is comparable to the surface area buried by the P6 residue (114 Å²). The interaction buries ~117 Å² of solvent accessible surface area from the Leu (P10) residue (of 1216 Å² that is buried from the entire peptide), and ~73 Å² of solvent accessible surface area from the MHC (of 850.4 Å² that is buried by interaction with the entire peptide). Essentially all of the MHC surface area buried by the P10 residue is hydrophobic. The fraction buried for the residue at the P10 is 0.64, which is comparable to the fraction buried for residues in the P3 (0.65), P4 (0.69), and P7 (0.77) pockets.

IV.C.3. Binding specificity to the P10 "shelf" on HLA-DR1

In order to explore peptide binding specificity for the P10 shelf, we synthesized a set of peptides that contained the AAYSDQATPLLXSPR sequence, where X is one of the 20 natural amino acids, and measured their apparent affinity for HLA-DR1 in a competition binding assay (Figure 3A). A second set of peptides also was synthesized and tested, AAYSDQATLLLXSPR, where the Pro (P7) was changed to the corresponding HA peptide residue Leu (P7) to eliminate possible backbone conformational and entropic effects unique to proline (Figure 3B). Peptide binding competition IC₅₀ values are shown in Table 2, and are plotted in Figure 3C and 3D relative to the average of all peptides in the series. There is clear peptide side chain specificity at the P10 position. Although the ranking differs somewhat in the two peptide series, perhaps due to interactions with the Pro (P7), the overall effects are similar in both series, for example with phenylalanine a strongly preferred residue and asparagine disfavored.
Figure IV.2. Crystal structure of HLA-DR1 bound to AAYSDQATPLLLSPR. A, 2Fo-Fc electron density map contoured at 1σ using data in the resolution range of 30-2.4 Å. The peptide carbon atoms are yellow, and nitrogen and oxygen atoms are blue and red, respectively. B, The AAYSDQATPLLLSPR peptide form the HLA-DR1/SEC3-3B2 complex (carbon atoms colored in yellow) was superimposed with the peptides present in the structure of HLA-DR1 solved without the superantigen with the carbon atoms colored in green or magenta. C, 2Fo-Fc electron density maps contoured at 1σ for the P10 region of HLA-DR1/ AAYSDQATPLLLSPR. The carbon atoms for the peptide are yellow, and the ones for HLA-DR1 are green. D, Surface of the HLA-DR1 P10 region with the residues that are located in that area are in sticks. Figures generated using PyMol ((DeLano, 2002)).
Figure IV.3. Effect of different amino acids at the P10 position of peptides in peptide-binding to HLA-DR1. A, MHC-peptide binding competition assay of a set of peptides that contained the AAYSDQATPLLXSPR sequence, where X is one of the 20 natural amino acids. B, Competition assay of a set of peptides that contained the sequence AAYSDQATLLLXSPR, where X is one of the 20 natural amino acids. C, Effect of each amino acid at the P10 position. Values represent the IC_{50} of the residue over the average IC_{50} of all the residues in the AAYSDQATPLLXSPR peptide series. IC_{50} values were calculated from the peptide binding competition assay. D, Effect of amino acids at the P10 position for the AAYSDQATLLLXSPR peptide.
Figure IV.4. The P10 “shelf” of HLA-DR1. The peptide’s carbon atoms are shown in yellow. The carbon atoms of HLA-DR1 are colored in green, except for the residues that are involve in the formation of the P10 pocket which are white. Conserved MHC-peptide hydrogen bonds in this region are shown as dashed white lines.

IV.C.4. Allelic polymorphism at the P10 region of HLA-DR

Inspection of the HLA-DR1 structure in the vicinity of the P10 shelf shows that the residues Ileα72, Argα76, Proβ56, Aspβ57, and Tyrβ60 play the major roles in formation of the shelf and interaction with the peptide (Figure 4). Arginine α76 is part of the non-polymorphic alpha subunit, and proline β56 is conserved within all the HLA-DR alleles, but aspartate β57 and tyrosine β60 are highly polymorphic (Table 3). In the context of other class II MHC proteins, polymorphism at aspartate β57 has been linked to human (HLA-DQ) and murine (I-A\(^8\)) diabetes.
Position-specific peptide binding preferences have not been investigated systematically for any HLA-DR alleles that have residues other than the most common Aspβ57 and Tyrβ60 combination (Table 3). Although a study that investigated the pocket profiles used alleles that were different in β57 and β60 to characterize the P9 pocket (Sturniolo et al., 1999). However, lists of peptides known to bind to particular MHC alleles, and predicted allele-specific peptide binding motifs, have been collected in several databases (Blythe et al., 2002; Rammensee et al., 1999). We scanned one such database, SYFPEITHI, for sets of similar HLA-DR1 alleles that differ in the P10 pocket residues β57 and β60, and for which reliable peptide-binding motifs were available. DRB1*0401, DRB1*0404, and DRB1*0405 form such a set. These alleles differ only at positions β57 (Asp or Ser), β70 (Arg or Lys), and β85 (Gly or Val). The pattern of substitutions at these positions allows the effects of the Asp/Ser polymorphism at β57 to be evaluated: preferences observed for DRB1*0405 that are not shared by DRB1*0401 and DRB1*0404 can be attributed to the presence of Ser β57. Moreover, β85 is found on the wall of the P1 pocket, and this polymorphism is expected to influence only the specificity at the P1 position (Newton-Nash and Eckels, 1993; Stern et al., 1994; Sturniolo et al., 1999), while the polymorphism at β71 is not expected to significantly influence peptide side chain specificity, as both Arg β71 and Lys β71 form a similar hydrogen bond with the peptide main chain carbonyl at position 5, between the P4 and P6 pockets (Ghosh et al., 1995; Stern et al., 1994). The peptide binding motif for HLA-DR4 has been studied extensively (Hammer et al., 1992; Sturniolo et al., 1999), allowing us to align a moderately-sized set of known binders comprising 40 DRB1*0401-binding peptides, 14 DRB1*0404-binding peptides, and 17 DRB1*0405 binding peptides,
according to their expected peptide binding frame (Table 4). While the databases do not contain quantitative binding data on these peptides, it is evident that the alleles have selected sets of peptides that differ in the residue predicted in the P10 position, with the HLA-DRB1*0405-binding set enriched in charged amino acid residues at the expense of hydrophobic and aromatic residues (Figure 5). Thus, the β57 polymorphism appears to influence the P10 peptide binding specificity.

![Pie charts showing peptide binding frequencies](image)

**Figure IV. 5. Analysis of peptides that bind to different MHC alleles.** The frequencies of different groups of amino acids at the P10 position was calculated using a list of peptides known to bind to DRB1*0401, DRB1*0404, DRB1*0405 from the SYFPEITHI database. The aromatic groups includes Trp, Tyr and Phe; the hydrophobic group includes, Ile, Leu, Val and Met; the small include, Ala, Pro and Gly; the polar group, Thr, Ser, Cys, Asn, Gln; and the charged group includes, His, Glu, Arg, Lys, Asp. Differences between the three alleles in the residues of the β-chain located in the peptide binding site of the protein are indicated.
IV.C.5. HLA-DR1 with polymorphic substitutions in the P10 region exhibit altered P10 specificity

To evaluate in a more quantitative way the influence of HLA-DR polymorphic residues on the P10 specificity, we constructed P10 pocket variants of HLA-DR1 (DRB1*0101) and evaluated their binding to the P10 peptide libraries as described above. Single and double amino acid replacements of polymorphic residues in the P10 shelf were introduced into HLA-DR1, and the resulting proteins (βD57A, βD57A Y60H, βD57V, and βD57V Y60S) were produced by expression in E. coli inclusion bodies and in vitro folding in the absence of peptide. The mutants were characterized to ensure that the substitutions did not result in gross alterations to the structure or to the peptide binding activity. First, each of the mutants exhibited a peptide dependent decrease in gel filtration elution volume (Figure 6A), which previously has been described for HLA-DR1. The change results from a conformational alteration that accompanies peptide as the binding site converts from a more open empty form to a more tightly packed closed form in the presence of peptide (Zarutskie et al, 1999). Second, each of the mutants bound the HA peptide (P10 Ala) with essentially identical apparent affinity (Figure 6B). Each of the mutants was also able to bind the CLIP peptide, a fragment of the class II-associated invariant chain that associates with class II MHC proteins during biosynthesis and intracellular trafficking. Apparent affinities for CLIP (P10 Gln) were similar but not identical. Thus, the mutations do not appear to have grossly altered the fold or activity of HLA-DR1.
Figure IV. 6. Characterization of HLA-DR1 mutants. A, Gel filtration profiles for the empty and peptide-loaded states of HLA-DR1 mutants. The peptide-loaded samples show the decrease in elution volume compared to empty that has been observed before for the wild type HLA-DR1 (Zarutskie et al, 1999). B, Direct peptide binding assays of the Ha peptide to HLA-DR1 wild type and mutants. Varying concentrations of biotinylated Ha-peptide were incubated with empty MHC protein for 3 days at 37°C. The amount of bio-Ha/HLA-DR1 complex was measured using HRP-labeled streptavidin. C, Direct binding assay of the CLIP peptide to HLA-DR1 wild type and mutants.
To assess the effects of introduction of polymorphic residues on the peptide binding affinity and specificity at the P10 position, peptide binding competition assays were done for each synthesized peptide and mutant. To facilitate comparison with the HLA-DR1 preferences, the bar graphs in Figure 7 are shown in the same order as in Figure 3D (See Tables 5 and 6 for IC$_{50}$ values). Clear differences were observed between the P10 side chain specificities of the HLA-DR1 variants (Figure 7). For example, asparagine at P10 position allows the peptide to bind to the βD57A/Y60H and βD57V/Y60S variants but significantly decreases binding on HLA-DR1 βD57A and βD57V variants. Some general preferences are apparent for the entire set, for example Phe, Leu, Trp and Met bind well, while Ile, and His bind poorly.

![Figure IV.7. Influence of different amino acids at the P10 position in peptide-binding to HLA-DR1 mutants. Values represent the IC$_{30}$ of the residue over the average IC$_{50}$ of all the residues in the AAYSDQATLLLXSPR peptide series for the particular HLA-DR1 mutant. IC$_{50}$ values were calculated from a peptide binding competition assay.](image-url)
IV.D. Significance

The MHC gene is one of the most polymorphic ones in the human genome, and the specificity of the alleles is very different. As a result of allelic variation, different individuals will react differently to a set of peptides from an antigen. The polymorphisms in class II MHC proteins have implications for vaccine design. Class II MHC molecules have the potential for different specificities, and if a vaccine needs to contain a unique peptide for each class II MHC allele possible it will need to contain thousands of peptides. Prediction programs can be used to identify epitopes for use in vaccine design. Available prediction programs use the specificity of residues in the P1, P4, P6 and P9 pockets of class II MHCs and do not take into account other factors that can be included in prediction, like for example specificity of the P10 shelf.
Table IV.1. Data Collection and Refinement Statistics

<table>
<thead>
<tr>
<th>HLA-DR1/AAYSDQATPLLLSPR</th>
<th>HLA-DR1/AAYSDQATPLLLSPR/SEC3-3B2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Crystal parameters</strong></td>
<td></td>
</tr>
<tr>
<td>Space Group</td>
<td>C222₁</td>
</tr>
<tr>
<td>Cell dimensions</td>
<td>a(Å) 96.55 b(Å) 112.65 c(Å) 172.75</td>
</tr>
<tr>
<td></td>
<td>R3</td>
</tr>
<tr>
<td></td>
<td>a(Å) 173.21 c(Å) 121.54</td>
</tr>
<tr>
<td><strong>Data Collection</strong></td>
<td>Overall Highest res. shell</td>
</tr>
<tr>
<td>Resolution limits (Å)</td>
<td>24.0-2.40 2.49-2.40</td>
</tr>
<tr>
<td>Unique reflections</td>
<td>44,505 4384</td>
</tr>
<tr>
<td>Total reflections</td>
<td>252,441 20,869</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>99.8 99.8</td>
</tr>
<tr>
<td>Mean I/σ(I)</td>
<td>11.0 3.5</td>
</tr>
<tr>
<td>R&lt;sub&gt;sym&lt;/sub&gt; (%)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.5 37.8</td>
</tr>
<tr>
<td></td>
<td>Overall Highest res. shell</td>
</tr>
<tr>
<td>Resolution limits (Å)</td>
<td>30.0-2.40 2.49-2.40</td>
</tr>
<tr>
<td>Unique reflections</td>
<td>53,191 5,392</td>
</tr>
<tr>
<td>Total reflections</td>
<td>319,169 30,576</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>99.9 100.0</td>
</tr>
<tr>
<td>Mean I/σ(I)</td>
<td>9.7 2.0</td>
</tr>
<tr>
<td>R&lt;sub&gt;sym&lt;/sub&gt; (%)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>11.4 49.7</td>
</tr>
<tr>
<td><strong>Refinement</strong></td>
<td></td>
</tr>
<tr>
<td>R&lt;sub&gt;free&lt;/sub&gt;&lt;sup&gt;b&lt;/sup&gt;</td>
<td>25.6 38.1</td>
</tr>
<tr>
<td>R&lt;sub&gt;cryst&lt;/sub&gt;</td>
<td>23.1 31.8</td>
</tr>
<tr>
<td></td>
<td>24.1 34.9</td>
</tr>
<tr>
<td></td>
<td>20.8 31.2</td>
</tr>
<tr>
<td><strong>Model</strong></td>
<td>Average B-factor # Residues Average B-factor # Residues</td>
</tr>
<tr>
<td></td>
<td>(Å&lt;sup&gt;2&lt;/sup&gt;) (atoms)</td>
</tr>
<tr>
<td>HLA-DR1</td>
<td>39.8 737 (6070)</td>
</tr>
<tr>
<td>Peptide</td>
<td>51.5 26 (188)</td>
</tr>
<tr>
<td>SEC3-3B2</td>
<td>N/A N/A</td>
</tr>
<tr>
<td>Waters</td>
<td>40.9 166</td>
</tr>
<tr>
<td><strong>Ramachandran plot</strong></td>
<td></td>
</tr>
<tr>
<td>Core + Allowed (%)</td>
<td>99.7</td>
</tr>
<tr>
<td>Generous (%)</td>
<td>0.0</td>
</tr>
<tr>
<td>Disallowed (%)</td>
<td>0.3</td>
</tr>
</tbody>
</table>

<sup>a</sup> R<sub>sym</sub> = σ|I - <I>|/ σ<I>, where I is the observed intensity and <I> is the average intensity of multiple observations of symmetry-related reflections.

<sup>b</sup> R factor on structure factors for reflection omitted from the refinement and used as a test set (10% of total).
Table IV.2. IC\textsubscript{50} values calculated from a peptide binding competition assay of the AAYSDQATPLLXSPR and AAYSDQATLLLXSPR peptide series to HLA-DR1.

<table>
<thead>
<tr>
<th>[\mu M]</th>
<th>AAYSDQATPLLXSPR</th>
<th>AAYSDQATLLLXSPR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC\textsubscript{50}</td>
<td>±SD</td>
</tr>
<tr>
<td>amino acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>none*</td>
<td>7.97</td>
<td>7.16</td>
</tr>
<tr>
<td>A</td>
<td>1.06</td>
<td>0.18</td>
</tr>
<tr>
<td>C</td>
<td>1.03</td>
<td>0.11</td>
</tr>
<tr>
<td>D</td>
<td>2.16</td>
<td>1.06</td>
</tr>
<tr>
<td>E</td>
<td>15.40</td>
<td>6.72</td>
</tr>
<tr>
<td>F</td>
<td>0.57</td>
<td>0.06</td>
</tr>
<tr>
<td>G</td>
<td>2.76</td>
<td>1.29</td>
</tr>
<tr>
<td>H</td>
<td>5.49</td>
<td>2.79</td>
</tr>
<tr>
<td>I</td>
<td>0.75</td>
<td>0.13</td>
</tr>
<tr>
<td>K</td>
<td>1.65</td>
<td>0.13</td>
</tr>
<tr>
<td>L</td>
<td>0.18</td>
<td>0.04</td>
</tr>
<tr>
<td>M</td>
<td>3.09</td>
<td>0.45</td>
</tr>
<tr>
<td>N</td>
<td>11.80</td>
<td>39.88</td>
</tr>
<tr>
<td>P</td>
<td>4.59</td>
<td>26.96</td>
</tr>
<tr>
<td>Q</td>
<td>2.92</td>
<td>2.16</td>
</tr>
<tr>
<td>R</td>
<td>4.35</td>
<td>0.38</td>
</tr>
<tr>
<td>S</td>
<td>1.53</td>
<td>0.29</td>
</tr>
<tr>
<td>T</td>
<td>3.91</td>
<td>4.81</td>
</tr>
<tr>
<td>V</td>
<td>6.32</td>
<td>1.74</td>
</tr>
<tr>
<td>W</td>
<td>0.02</td>
<td>0.00</td>
</tr>
<tr>
<td>Y</td>
<td>4.41</td>
<td>0.98</td>
</tr>
</tbody>
</table>

* truncation ending with amide at P9
Table IV.3. Polymorphic residues contacting the P10 residue of the peptide in the peptide-binding site.

<table>
<thead>
<tr>
<th>β57</th>
<th>β60</th>
<th>HLA-DRB* alleles</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td><strong>HLA-DRB3</strong>*: 0107, 0201-0206, 0210-0215, 0217-0218</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>HLA-DRB4</strong>*</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>HLA-DRB5</strong>*</td>
</tr>
<tr>
<td>A</td>
<td>Y</td>
<td><strong>HLA-DRB1</strong>*: 0446, 0706, 0811</td>
</tr>
<tr>
<td>A</td>
<td>H</td>
<td><strong>HLA-DRB1</strong>*: 0310, 0808, 1343, 1345, 1401, 1404, 1407, 1410, 1416, 1422, 1425-1426, 1428, 1431-1432, 1435, 1438-1439</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>HLA-DRB3</strong>*: 0216</td>
</tr>
<tr>
<td>V</td>
<td>Y</td>
<td><strong>HLA-DRB1</strong>*: 0807, 1331, 1346</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>HLA-DRB3</strong>*: 0207</td>
</tr>
<tr>
<td>V</td>
<td>S</td>
<td><strong>HLA-DRB1</strong>*: 0701-0705, 0707-0708, 0825, 0901, 0903, 1201-1203, 1205-1208, 1354, 1448</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>HLA-DRB3</strong>*: 0101-0106, 0108-0111, 0209, 0301-0303</td>
</tr>
<tr>
<td>S</td>
<td>Y</td>
<td><strong>HLA-DRB1</strong>*: 0312, 0405, 0409-0412, 0417, 0424, 0428-0430, 0445, 0448, 0801, 0803, 0805-0806, 0810, 0812, 0814, 0816-0818, 0822-0823, 0826-0827, 1303-1304, 1312-1313, 1321, 1330, 1332-1333, 1338, 1348-1349, 1355, 1358, 1413, 1512</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>HLA-DRB3</strong>*: 0208</td>
</tr>
<tr>
<td>I</td>
<td>Y</td>
<td><strong>HLA-DRB1</strong>*: 0819</td>
</tr>
<tr>
<td>D</td>
<td>S</td>
<td><strong>HLA-DRB1</strong>*: 0313, 1339</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>HLA-DRB5</strong>*: 0112</td>
</tr>
<tr>
<td>D</td>
<td>H</td>
<td><strong>HLA-DRB1</strong>*: 0815, 1131, 1434</td>
</tr>
</tbody>
</table>
Table IV.4. List of peptides used in the analysis of frequency of residues at the P10 position for DRB1*0401, DRB1*0404, DRB1*0405

<table>
<thead>
<tr>
<th>DRB1*</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>0401</td>
<td>FVRFDSDAASQRMR</td>
<td>APYEKEVPLSALTNILSAQL</td>
<td>DGKDYIALNEDLSS</td>
<td>LSSTAAADTAAQIT</td>
<td>IYFRNQKGSGLQPTGFL</td>
<td>DVAFVKDQTVIQNTD</td>
<td>YDHNFVKAINAIQKSW</td>
<td>HKVYACEVTHQGL</td>
<td>DGPFRIITVPAALDY</td>
<td>GSYLFYVYNITTNKYKAFLKQ</td>
</tr>
<tr>
<td></td>
<td>VYPEVTVPATK</td>
<td>PQQPRLILYDASNRATGIPA</td>
<td>LSSWTAADTAAQITQ</td>
<td>WNRQLYPEWTEAQRLD</td>
<td>DVPKwisIMTEERSVPH</td>
<td>WNRQLYPEWTEAQRLD</td>
<td>EFVVEFDLPGIKA</td>
<td>AKFYRDPTAFGS</td>
<td>FSWAMDLPKGA</td>
<td>LLESIQQLLLKAEKGN</td>
</tr>
<tr>
<td></td>
<td>QNLKKAEEKGKNAAR</td>
<td>AGFKGGEQGBPGEPE</td>
<td>QNILLSNAPLGQPFP</td>
<td>DYSYLQDSDPSFDQD</td>
<td>MNILLYVVKSFED</td>
<td>IAFTSEHSHSFLK</td>
<td>ATGFKQSSKALKQRPVAS</td>
<td>KHKVYACEVTHQGLSS</td>
<td>KVQKWVNDALQSGNS</td>
<td>FFRMVISNPAAHTQIDFILI</td>
</tr>
<tr>
<td></td>
<td>GYKVLYNPSVAAAT</td>
<td>FRKQNPDIVIQYMDDLYVG</td>
<td>LSRSFSWGAEGQRPGFGYG</td>
<td>PKYVQNTLKLATGMGNNV</td>
<td>EYLNIQNSLSTEWSPCSVT</td>
<td>GRHVVIDKSFGSPQVT</td>
<td>KVPIKWMALESILRRRF</td>
<td>RSTLATTLETGV</td>
<td>RNYRALMDKSLHVGTQCALTRR</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GSHSMRYFHTAMSRPGRGE</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>----</td>
<td>---------------------</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>YDNSLKIISNASCTTN</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>ANIAVDKANLEIMTKR</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>KDYIALNEDLRSWT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>TPDFIVPLTDLRIPS</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>FIVPLTDLRIPS</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>EIKILNIGVIKGFVEP</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>TLKYPIEHGIVTNWDD</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>VAPEEHPVLLTEAPLNPKA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>AWFPSIVGRORHQGVMV</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>SHSMTAMSRP</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>PGKAPKRLIYAA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>VVHFKNIVTPRTPPPSQGK</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

|    | RARYQWVRCPDNSNS    |
|    | REFKLSSKVWRDQH     |
|    | EDHYVVGAGORDA      |
|    | THYAVAVVKKDFTFK    |
|    | KELKIDIPNQPQER     |
|    | YLLYYTEFTPTEKD     |
|    | CAIHAKRVTIMPKDQLA  |
|    | GSTVFDNLPNPEIDGDYYGW|
|    | KPPQYIAVHVVPDQT    |
|    | VPIQRAVYQNVVVNNPXD |
|    | KPPQYIAVHVVPDQLM   |
|    | DPILYRPVAVALDTKGP  |
|    | LYYTEFTPTEKD       |
|    | KKVXVYQLKLDTAYD    |
|    | KPYNEAKTXFDKY      |
|    | IDGLEVDVPGIDPNA    |
|    | EQVAYKALPVLEN     |
Table IV.5. IC₅₀ values calculated from a peptide binding competition assay of the AAYSDQATPLLXXPR and AAYSDQATLLLXSPR peptide series to HLA-DR1 βDR57A and βD57A, Y60H mutants.

<table>
<thead>
<tr>
<th></th>
<th>HLA-DR1(βD57A)</th>
<th>HLA-DR1(βD57A, Y60H)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TPLLX</td>
<td>TLLLX</td>
</tr>
<tr>
<td>[μM]</td>
<td>IC₅₀ ±SD</td>
<td>IC₅₀ ±SD</td>
</tr>
<tr>
<td>none*</td>
<td>10.06 ± 15.98</td>
<td>8.15 ± 7.43</td>
</tr>
<tr>
<td>A</td>
<td>5.49 ± 5.64</td>
<td>34.20 ± 79.75</td>
</tr>
<tr>
<td>C</td>
<td>0.91 ± 0.07</td>
<td>18.12 ± 16.01</td>
</tr>
<tr>
<td>D</td>
<td>3.45 ± 0.20</td>
<td>4.72 ± 1.33</td>
</tr>
<tr>
<td>E</td>
<td>5.11 ± 1.41</td>
<td>2.28 ± 1.44</td>
</tr>
<tr>
<td>F</td>
<td>11.58 ± 2.41</td>
<td>2.37 ± 0.87</td>
</tr>
<tr>
<td>G</td>
<td>0.51 ± 0.28</td>
<td>11.00 ± 24.80</td>
</tr>
<tr>
<td>H</td>
<td>22.45 ± 58.21</td>
<td>29.64 ± 35.40</td>
</tr>
<tr>
<td>I</td>
<td>0.33 ± 0.06</td>
<td>49.16 ± 41.90</td>
</tr>
<tr>
<td>K</td>
<td>1.94 ± 0.55</td>
<td>3.67 ± 1.16</td>
</tr>
<tr>
<td>L</td>
<td>1.88 ± 0.52</td>
<td>10.16 ± 9.30</td>
</tr>
<tr>
<td>M</td>
<td>5.60 ± 1.65</td>
<td>4.92 ± 3.05</td>
</tr>
<tr>
<td>N</td>
<td>8.86 ± 30.57</td>
<td>16.80 ± 34.26</td>
</tr>
<tr>
<td>P</td>
<td>4.49 ± 2.71</td>
<td>2.33 ± 0.94</td>
</tr>
<tr>
<td>Q</td>
<td>9.67 ± 10.10</td>
<td>5.21 ± 1.53</td>
</tr>
<tr>
<td>R</td>
<td>7.33 ± 5.32</td>
<td>6.63 ± 12.63</td>
</tr>
<tr>
<td>S</td>
<td>4.05 ± 0.42</td>
<td>2.03 ± 1.22</td>
</tr>
<tr>
<td>T</td>
<td>20.76 ± 18.69</td>
<td>2.75 ± 1.07</td>
</tr>
<tr>
<td>V</td>
<td>65.13 ± 28.56</td>
<td>28.56 ± 23.44</td>
</tr>
<tr>
<td>W</td>
<td>0.49 ± 0.13</td>
<td>1.16 ± 1.31</td>
</tr>
<tr>
<td>Y</td>
<td>3.47 ± 16.50</td>
<td>3.06 ± 8.85</td>
</tr>
</tbody>
</table>

* truncation ending as amide at P9
Table IV.6. IC\textsubscript{50} values calculated from a peptide binding competition assay of the AAYSDQATPLLXSPR and AAYSDQATLLLLXSPR peptide series to HLA-DR1 βDR57V and βD57V, Y60S mutants.

<table>
<thead>
<tr>
<th></th>
<th>HLA-DR1(βD57V)</th>
<th></th>
<th>HLA-DR1(βD57V, Y60S)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TPLLX</td>
<td>IC\textsubscript{50} ±SD</td>
<td>TLLXL</td>
</tr>
<tr>
<td>[μM]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>none*</td>
<td>22.44</td>
<td>13.46</td>
<td>19.31</td>
</tr>
<tr>
<td>A</td>
<td>5.25</td>
<td>0.75</td>
<td>3.99</td>
</tr>
<tr>
<td>D</td>
<td>1.28</td>
<td>0.89</td>
<td>11.26</td>
</tr>
<tr>
<td>E</td>
<td>35.69</td>
<td>43.70</td>
<td>0.71</td>
</tr>
<tr>
<td>F</td>
<td>5.38</td>
<td>2.46</td>
<td>3.45</td>
</tr>
<tr>
<td>G</td>
<td>3.85</td>
<td>1.98</td>
<td>3.09</td>
</tr>
<tr>
<td>H</td>
<td>9.99</td>
<td>47.87</td>
<td>35.45</td>
</tr>
<tr>
<td>I</td>
<td>0.35</td>
<td>0.04</td>
<td>10.13</td>
</tr>
<tr>
<td>K</td>
<td>48.51</td>
<td>22.28</td>
<td>4.77</td>
</tr>
<tr>
<td>L</td>
<td>8.49</td>
<td>5.09</td>
<td>15.61</td>
</tr>
<tr>
<td>M</td>
<td>9.84</td>
<td>1.26</td>
<td>6.37</td>
</tr>
<tr>
<td>N</td>
<td>32.72</td>
<td>25.10</td>
<td>45.56</td>
</tr>
<tr>
<td>P</td>
<td>24.95</td>
<td>15.95</td>
<td>17.17</td>
</tr>
<tr>
<td>Q</td>
<td>27.24</td>
<td>22.80</td>
<td>9.14</td>
</tr>
<tr>
<td>R</td>
<td>17.98</td>
<td>7.74</td>
<td>41.42</td>
</tr>
<tr>
<td>S</td>
<td>13.27</td>
<td>13.96</td>
<td>17.02</td>
</tr>
<tr>
<td>T</td>
<td>15.77</td>
<td>19.41</td>
<td>2.75</td>
</tr>
<tr>
<td>V</td>
<td>21.32</td>
<td>18.59</td>
<td>11.16</td>
</tr>
<tr>
<td>W</td>
<td>1.12</td>
<td>0.35</td>
<td>8.44</td>
</tr>
<tr>
<td>Y</td>
<td>3.17</td>
<td>4.26</td>
<td>5.84</td>
</tr>
</tbody>
</table>

* truncation ending as amide at P9
Chapter V: To what degree are MHC class II molecules unfolded or loosely folded in the absence of peptide? How much conformational alteration accompanies peptide binding?

Summary

Previous studies in which the empty and peptide-loaded forms of the protein have been compared show that peptide binding induces a significant conformational change. Analysis of the hydrodynamic radius showed that the empty form of the human class II MHC protein HLA-DR1 has a larger, less compact structure than the peptide bound complexes. Kinetic studies have also indicated alternate conformational states of class II MHC proteins. It has been suggested that the empty form is partially unfolded. Proteolytic experiments were performed to address this idea and showed that the empty form of HLA-DR1 has the same proteolytic pattern to that of the peptide-loaded form suggesting a similar folded conformation. In order to identify the extent of the conformational change of class II MHC protein upon peptide binding NMR studies were performed on HLA-DR1 in the presence and absence of peptides. Our results show that overall architecture of the empty state of the protein is very similar to the peptide-loaded form and that it does not fit the description of a molten globule state and is unlikely to contain large stretches of unfolded sequences.
V.A. Introduction

Major histocompatibility complex (MHC) proteins are cell surface proteins that bind peptide antigens and present them to the T cell receptors of CD4+ T cells. In the crystal structures of class II MHC proteins (Corper et al., 2000; Fremont et al., 2002; Ghosh et al., 1995; Lee et al., 2001; Li et al., 2000; Liu et al., 2002; Smith et al., 1998; Stern et al., 1994), pockets within the peptide-binding site are observed. They are labeled according to the side chain of the peptide accommodated; major pockets within the overall peptide-binding site are P1, P4, P6 and P9.

The conformation of class II MHC proteins with bound peptides is essentially identical in all the crystals structures solved to date. However, different studies have suggested alternate conformations of the class II MHC proteins under certain conditions. A “floppy” conformation that contains reduced mobility in non-denaturing SDS-PAGE experiments has been observed for murine class II MHC molecules in vitro as an intermediate in the thermal denaturation and folding pathways (Dornmair and McConnell, 1990; Dornmair et al., 1989; Sergeev et al., 1988) and also in vivo in cells from mice lacking the Ii protein (Sadegh-Nasseri and Germain, 1991). The difference in electrophoretic mobility of these “floppy” species observed in vitro and in vivo can be due to absence of peptides bound to the protein, or the presence of peptides that bind weakly to the MHC.

Alternate conformational states of class II MHC proteins have also been suggested by kinetic studies of peptide binding. Peptide binding to class II MHC proteins shows a rather complicated kinetic behavior. The kinetic model includes an initial bimolecular binding step followed by a slow unimolecular conformational change. In
addition, a reversible inactivation of the empty MHC protein that competes with productive binding is observed (Joshi et al., 2000; Sadegh-Nasseri and McConnell, 1989; Sadegh-Nasseri et al., 1994). Even though these kinetic models cannot address the nature of a conformation change, the unimolecular step implies different conformations for the initial and final MHC-peptide complexes.

Biochemical and biophysical characterization of the empty and peptide-loaded forms of the human class II MHC protein HLA-DR1 show a conformational change of the protein induced by peptide binding (Zarutskie et al., 1999). In this study the conformational change was observed using a number of peptides with different sequences and affinities. Hydrodynamic measurements obtained using several methods show that the empty form has a larger, less compact structure that that of the peptide-loaded molecule. Additionally, the empty form has a reduced thermal stability and reduced unfolding cooperativity relative to the peptide-loaded form.

Antibodies have been used to study the structural properties of class II MHC proteins. The antibody KL304 interacts with β58-69 in the empty form of HLA-DR1 but not the peptide-loaded, suggesting a conformational change in that region of the MHC protein (Zarutskie et al., 1999). In a recent study, two regions on the β-chain of the MHC protein involved in the conformational change were mapped using a set of monoclonal antibodies directed against the β-chain of HLA-DR1 that recognize only the empty state of the protein (Carven et al., 2004).

Class II MHC folded molecules are composed of four domains. Each subunit (α and β) contributes to two domains. The membrane distant domains, α1 and β1, fold together to form a long groove in which the peptides bind to the protein. The peptide
binding groove might show unfolding in the absence of peptide. The remaining extracellular domains adopt an immunoglobulin-like domain structure. In class II MHC proteins, the lower immunoglobulin-like β2 domains is docked onto the α1 β1 peptide-binding domain through an interaction between its top end and a depression formed underneath the pocket (Murthy and Stern, 1997). Some of the residues in the peptide binding site contact the depression and also the P1 pocket, and can communicate peptide binding changes to the lower domains.

Attempts to produce crystals of empty HLA-DR1 for x-ray crystallography were performed without success. We believe a mayor barrier for crystallization is that at the concentration required for it, empty HLA-DR1 aggregates non-specifically. Nuclear magnetic resonance (NMR) can determine the structures of proteins that fail to crystallize. In an effort to determine the extent or global nature of the conformational change on HLA-DR1, we attempted to use NMR as a tool to obtain information of the empty and peptide-loaded forms of HLA-DR1.

V.B. Materials and Methods

V.B.1. Protein Expression and Purification

The extracellular portion of HLA-DR1 was produced by expression of isolated subunits in *Escherichia coli* inclusion bodies followed by refolding *in vitro* as described previously (Frayser et al., 1999). Uniform $^2$H,$^{15}$N-labeled β-subunit or α-subunit were expressed in 10L of minimal medium containing KH$_2$PO$_4$ (1.6 g/L), Na$_2$HPO$_4$ (10 g/L), sodium citrate (1 g/L), MgSO$_4$ (0.25 mg/L), thiamine (10 mg/L), (NH$_4$)SO$_4$ (0.7 g/L), ampicillin (50 μg/mL), 100% D$_2$O and trace metals, supplemented with glucose and
[\textsuperscript{15}N]-ammonium sulfate as sole carbon and nitrogen sources, respectively. The expressed $^2\text{H},\textsuperscript{15}N$ $\beta$-subunit of HLA-DR1 was refolded \textit{in vitro} with unlabeled $\alpha$-subunit in the presence or absence peptide. ($^2\text{H},\textsuperscript{15}N$ $\alpha$-subunit of HLA-DR1 was refolded \textit{in vitro} with unlabeled $\beta$-subunit). The folded protein was purified in an immunoaffinity column with the antibody LB3.1, which recognizes both the empty and peptide-loaded forms of HLA-DR1. The protein was then further purified by gel filtration in 10 mM pyrophosphate, 150 mM NaCl, 0.02% azide, 10% D$_2$O, pH 4.6 to remove protein aggregates. Protein concentration was measured by UV absorbance at 280 nm using $\varepsilon_{280}$ of 54375 M$^{-1}$ cm$^{-1}$ for HLA-DR1.

\textit{V.B.2. Protease cleavage}

HLA-DR1 samples at a protein concentration of 8 $\mu$M were digested at 37°C for 5 hours for endoproteinase Glu-C and papain and 1 hour for subtilisin. Thermolysin digestions were carried out at 37°C for 2 hours and then at 50°C for another set of 2 hours. For endoproteinase Glu-C digestion experiments, endoproteinase Glu-C (lyophilized powder, Sigma) was dissolved to 1 mg/ml in 0.1M ammonium bicarbonate. In the papain experiments, papain in suspension (Sigma) was used; 50 mM cysteine and 1mM EDTA were added to the reaction mixture. For subtilisin digestion reactions, subtilisin (Sigma) was dissolved to 1mg/ml in 0.1 M Tris, 0.1 M CaCl$_2$, pH 8.0. Thermolysin was dissolved to 1 mg/ml in 0.1 M ammonium bicarbonate, 2 mM CaCl$_2$, pH 7.8.

All the reactions were done at a ratio of 5:1, protein:protease. Reactions were stopped by boiling the mixtures for 10 minutes and then adding SDS loading buffer.
V.B.3. NMR experiments

Spectra were acquired at 25°C (empty HLA-DR1) and 35°C (peptide-loaded HLA-DR1) on Bruker DRX600 equipped with z-axis gradient \(^1\text{H}\{^{13}\text{C},^{15}\text{N}\}\) triple resonance probe. Spectra were processed with XWINNMR 2.1 (Bruker) or Felix 98 (Accelrys) and analyzed with Felix 98. All experiments utilized transverse relaxation-optimized spectroscopy (TROSY) pulse sequence to overcome size limitations of DR protein. In the spectra we observe a heteronuclear correlation between protons and directly (\(^1\text{J}\)) attached nitrogen atoms.

V.C. Results

V.C.1. Stability of the empty form of HLA-DR1

HLA-DR1-peptide complexes are resistant to proteolysis digestion when isolated from their native B-cell source (Gorga et al., 1987). Native fold of the empty form of HLA-DR1 was shown by circular dichroism and antibody experiments (Frayser et al. 1999; Stern and Wiley, 1992). However, it has been suggested based on experiments with IA and IE, murine homologs of DR, that at mildly acidic pH (4.5-6.5) class II molecules exhibit a molten globule-like state by its ability to bind the fluorescent probe ANS (8-amino-1-naphthalene-sulfonic acid) (Boniface et al., 1996; Runnels et al., 1996). Note that the acidic pH may be considered physiological, as in the cellular context class II MHC proteins bind peptides in endosomes and lysosomes, with pH 4-6.5 (Watts, 1997). The idea of a molten globule has been shown in studies of class I MHC proteins that are in the empty, peptide-free state; the empty proteins show an increased tendency
to aggregate, a low content of secondary structure, increased tendency to bind ANS, and
an increased susceptibility to proteolysis (Bouvier and Wiley, 1998). A molten globule is
a structure that has a compact conformation with native-like secondary structure but
disordered tertiary structure.

We decided to look at the protease sensitivity of empty and peptide-loaded HLA-DR1 more carefully by using different proteases in the presence and absence of peptide to
evaluate the possible presence of a molten globule protein and weather the
conformational change is not due to large unfolding of the empty state. Empty and
peptide-loaded HLA-DR1 were folded under standard conditions and digested with
papain, endoproteinase Glu-C, subtilisin, thermolysin (Figure 1). As controls, empty and
peptide-loaded HLA-DR1 without proteases but in the same conditions and buffers used
for each digestion were also analyzed to asses the stability of the protein.

Figure 1 shows that empty and peptide-loaded HLA-DR1 are both resistant to
papain and endoproteinase Glu-C digestion. For subtilisin, the protein is not stable in the
conditions used and some cleavage was observed for both states of the protein. In the
case of thermolysin, even when the protein is stable in the conditions used for digestion,
cleavage is observed for the empty and peptide-loaded form. When digestion is seen, it is
similar in both forms, both in the extent of digestion and the pattern of fragments.
**Figure V.1. Protease resistance of HLA-DR1.** Paptain (A), endoproteinase Glu-C (B), subtilisin (C) and thermolysin (D) were used to digest samples of HLA-DR1 in the absence and presence of the Ha peptide. All samples were boiled before reducing SDS-PAGE analysis.
V.C.2. Peptide binding to $^2H,^{15}N$-labeled HLA-DR1

HLA-DR1 is composed of two non-covalent subunits which are expressed separately in *E. coli* and refolded *in vitro* in the absence or presence of peptide. Empty forms of class II MHC proteins tend to aggregate in the absence of peptide. This aggregation can be reduced significantly in our refolding procedure by the presence of glycerol and by maintaining the protein at low temperature (Frayser et al., 1999). We investigated a number of other conditions including, 20% acetonitrile, 20% isopropanol, 4 M urea and 0.1% SDS in an effort to find conditions that stabilized the monomeric empty form against aggregation. Peptide binding to empty HLA-DR1 was not affected by any of the conditions suggesting that the protein retain is folded state; the extent of aggregation of the empty form did not change in the solvents tested compared to our usual observations in PBS solution (Zavala-Ruiz and Stern, unpublished results).

The peptide used in our studies is Ha, a tight-binding peptide derived from the influenza haemagglutinin virus. This peptide has been used in complex with HLA-DR1 in different studies conducted to understand general aspects peptide binding, MHC-peptide-TCR and MHC-peptide-superantigen interactions of class II MHC proteins (Hennecke et al., 2000; Jardetzky et al., 1994; Joshi et al., 2000; Sato et al., 2000; Stern et al., 1994; Zarutskie et al., 1999).

HLA-DR-peptide complexes are resistant to SDS-induced chain dissociation, and therefore this property is often used to determine peptide binding in the refolded mixture. To make sure that labeling of one of the subunits does not affect how the protein binds peptide, we checked the refolded protein using SDS-PAGE electrophoresis in the empty and peptide-loaded forms (Figure 2).
Figure V.2. Folding of HLA-DR1 in unlabeled and 2H,15N-labeled preparations. SDS-PAGE analysis of folded mixtures with and without Ha peptide; with samples non boiled (NB) and boiled (B) before gel analysis.

HLA-DR1 exhibits an SDS-resistant HLA-DR1-peptide complex band when folded in the presence of the peptide Ha. HLA-DR1 folded with 2H,15N labeled α-chain shows the same behavior (data not shown). These results indicate that growing and labeling HLA-DR1 in minimal medium does not affect the binding properties of protein, and thus can be used for NMR experiments.

V.C.3. NMR experiments of HLA-DR1 with 2H, 15N-labeled β-chain

Spectra of HLA-DR1 in the empty and peptide-bound forms were collected (Figure 3). The spectrum of HLA-DR1 in complex with the well-characterized Ha peptide (K_D ~ 14 nM) has 206 apparent 15N-1H peaks of the 226 total expected NH protons in this region (189 main-chain amides, 20 side-chains amides and 17 guanidiniums) showing that the spectrum can provide good information about the protein.
Figure V.3. NMR spectra of HLA-DR1 with $^2$H, $^{15}$N-labeled β-chain. A) HLA-DR1 bound to Ha measured at 35°C (14 mg/ml, 0.3 mM). B) Empty HLA-DR1 measured at 25°C (1 mg/ml, 22 μM).
Empty HLA-DR1 aggregates non-specifically; the aggregation is concentration-dependent (unpublished results, Lawrence J. Stern lab). To prevent aggregation, the protein was concentrated to 1mg/mL and a cryo-probe NMR 600 MHz instrument was used for the experiments. Clear peaks (129 apparent peaks) can be identified in the spectrum of the empty form of HLA-DR1 even though is not as resolved as the one collected for the peptide-loaded form. Since HLA-DR1 in its empty state tends to aggregate, the spectrum was collected at 25°C, while the peptide-loaded spectrum was collected at 35°C. Although the NMR chemical shifts are temperature-dependent, this effect would change all peaks in a similar manner. When the spectrum of HLA-DR1 in complex with Ha is collected at 25°C, the chemical shifts of peaks is not affected as compared to the spectrum at 35°C, but the intensity of some peaks is significantly lower, while others are not seen (approximately 6 peaks). Low dispersion of backbone amide $^1$H chemical shifts are a typical characteristic for unfolded or disordered proteins, with $^1$H chemical shifts spanning only 7.6-8.6 ppm (1.0 ppm) (Dyson and Wright, 2002). In the spectrum of empty HLA-DR1, backbone amide $^1$H chemical shifts spanning 6.5-10.5 ppm (4 ppm) are observed. We superimposed the spectra for both forms of the protein and could identify differences in the chemical shift of some of the peaks (Figure 4). Arrows in figure 4 indicate particularly prominent differences between both forms, although more shifts can be identified or expected. Clear chemical shifts in peaks are observed when the spectra are compare (arrows); in addition, some areas contain peaks that correspond only to the empty or the Ha-loaded sample.
Figure V.4. Overlay of HLA-DR1 empty (blue) and in complex with the Ha peptide (green). Red arrows show some of the regions in which look different in both spectra. The spectrum for empty HLA-DR1 is very noisy and does not contain all the peaks we were expecting.

V.C.3. NMR experiments of HLA-DR1 with $^2$H, $^{15}$N-labeled α-chain

Peptide-dependent changes in the α-chain of HLA-DR1 are expected but-to-date no direct evidence has been shown to confirm this. We performed NMR studies in the empty and peptide-loaded form of HLA-DR1 that was labeled only in the α-subunit to determine if differences in the chemical shifts of peaks are observed (Figure 5).
Figure V.5. NMR spectra of HLA-DR1 with $^2\text{H},^{15}\text{N}$-labeled $\alpha$-chain. A) HLA-DR1 bound to Ha measured at 35°C (8.5 mg/ml, 0.18 mM). B) Empty HLA-DR1 measured at 25°C (1 mg/ml, 22 $\mu$M).
The spectrum of HLA-DR1 in complexed with the Ha peptide has 160 apparent $^{15}$N-$^1$H peaks of the 209 total expected NH protons in this region (179 main-chain amides, 21 side-chains amides and 8 guanidiniums), while the spectrum for the empty form shows a total 143 apparent peaks with high dispersion of backbone amide $^1$H chemical shifts. Arrows in the figure 6 indicate some prominent differences between the empty and Ha-loaded form, although more differences are expected.

The spectrum collected for the empty form has a lower signal-to-noise ratio leading to poor resolution of peaks, but when compared to the spectrum of HLA-DR1 in complex with Ha obvious differences could be identified.

![Figure V.6. Overlay of empty (magenta) and Ha-loaded (blue) forms of α-chain labeled HLA-DR1. Red arrows show regions in the spectra that are different when comparing both states of the protein.](image-url)
V.C.5. NMR studies of HLA-DR1 in complex with Ha<sub>Y308A</sub>

In an effort to get some more information about the conformation change in HLA-DR1, we also prepare a sample of the MHC protein bound to an Ha analog-peptide, Ha<sub>Y308A</sub>. In this peptide one of the most important contacts with the protein was perturbed, the P1 pocket. Ha<sub>Y308A</sub> was used in a study where the role of the P1 pocket was carefully examined. The results of that study suggest that occupancy of the P1 pocket is responsible for partial conversion to the compact state in the peptide loaded form of HLA-DR1 (Sato et al., 2000).

The different experimental measurements that were conducted in Sato et al. have made us believed that the HLA-DR1-Ha<sub>Y308A</sub> complex behaves as an intermediate state of the empty and peptide loaded form of HLA-DR1. In an SDS-PAGE stability test, the HLA-DR1-Ha<sub>Y308A</sub> complex was not fully resistant to αβ chain dissociation compared to the Ha complex. When the apparent molecular weight is calculated from gel filtration elution volumes, the difference between the Ha and Ha<sub>Y308A</sub> complexes is within experimental error, but a significant difference is apparent for the empty form and that of the peptide complexes. The midpoint temperature (T<sub>m</sub>) calculated from the thermal denaturation profile was 76° for the Ha<sub>Y308A</sub> complex, 84° for the Ha complex and 67° for empty HLA-DR1. All the measurement and parameters reported in Sato et al. suggest that Ha<sub>Y308A</sub> can stabilize the compact form of HLA-DR1, but interestingly, it does not exhibit all the features of the CD spectrum characteristic of the peptide complex.
Figure V.7. NMR spectrum of HLA-DR1-HaY308A with $^2$H, $^{15}$N-labeled β-chain. Spectrum of HaY308A complex was collected at 35°C (3 mg/mL, 66 μM) A) HLA-DR1 bound to Ha (green) superimposed onto the HaY308A complex spectrum (blue) B) Empty HLA-DR1(blue) superimposed onto HLA-DR1-HaY308A (magenta).
Figure V.8. NMR spectrum of HLA-DR1-HaY308A with $^2$H, $^{15}$N-labeled α-chain. Spectrum of HaY308A complex was collected at 35°C (6 mg/mL, 0.13 mM) A) HLA-DR1 bound to Ha (blue) superimposed onto the HaY308A complex spectrum (red) B) Empty HLA-DR1(magenta) superimposed onto HLA-DR1-HaY308A (red).
Samples of HLA-DR1 with either the α- or β-chain labeled in complex with the peptide HαY308A were prepared and the spectra was collected and analyzed by comparing it to empty and Hα complex samples (Figures 7 and 8). In the β-labeled chain sample of HLA-DR1 in complex with HαY308A, the spectrum shows 179 apparent peaks; in the α-labeled chain sample 153 apparent peaks are observed. Some chemical shifts differences can be identified in the HαY308A complex spectra when compared to the Hα complex or the empty form when either the α- or the β-chain is labeled. Interestingly, some the regions that are different between the empty HLA-DR1 and HαY308A complex are similar between both peptide complexes, and vice versa (see grey arrows on figure 7 and 8). This suggests that the HαY308A complex shares some features of the empty state and some of the HLA-DR1-Hα complex

IV. D. Discussion

Different studies and experiments have shown that the human class II MHC protein HLA-DR1 goes through a conformational change upon peptide binding. Previous studies show that the thermal stability and the unfolding cooperativity of the empty form of HLA-DR1 were reduced compared to the peptide-loaded form. We used NMR as a tool to provide structural information about the empty state of the protein.

What is the extent of the conformational change that occurs on HLA-DR1 upon peptide binding? The empty state of HLA-DR1 is not unfolded as confirmed by our proteolysis digestion studies, showing that its stability and folding is very similar to that of the peptide-loaded form. In addition the chemical shifts themselves are highly similar in both states of the protein. This suggests that the majority of the main chains in the

124
empty form adopt a defined structure that is similar to the peptide-loaded protein. However, some of the regions in the empty HLA-DR1 spectra show difference in chemical shifts when compared to HLA-DR1 in complex with the Ha or Ha$_{Y308A}$ peptides (see arrows in the spectra), in addition, seems like some regions were not detected in the spectra of the empty form as the amount of apparent peaks is lower which could be due to conformational fluctuations or to partially-folded regions or because the peaks are below the signal to noise.

A molten globule state was suggested for apomyoglobin, but an NMR study of this apomyoglobin and holomyoglobin shows that the apomyoglobin structure possesses many of the characteristics of the native holomyoglobin and does not exhibit the behavior of a molten globule state since most of the chemical shifts were highly similar in both proteins (Eliezer and Wright, 1996).

The Ha$_{Y308A}$ peptide binds to HLA-DR1 with a weak binding affinity and even though it stabilizes the compact form of the protein it does not exhibit all the features that the peptide-loaded does on a circular dichroism experiments. As the NMR data shows, the spectrum of complex does not match the one from the peptide-loaded form exactly, but it does not match the spectrum from the empty state of the protein either. The HLA-DR1-Ha$_{Y308A}$ is possibly sampling between both conformations of the protein, or maybe some regions of the complex are in the peptide-loaded state and the others in the empty, peptide-free form.

If empty HLA-DR1 was in an unfolded or molten globule-like state, the backbone amide $^1$H chemical shift dispersion would have been low as observed and investigated before for unfolded or intrinsically disordered molecules (Dyson and Wright, 1998;
Dyson and Wright, 2002; Zhang et al., 1997). Extensive conformational heterogeneity and increased dynamic state of the proteins in a molten globule conformation would preclude observations and any meaningful interpretations of NMR experiments, and would also provide differences in the proteolytic footprint when compared to a protein in the native-state that is resistant to proteases. We propose that the empty, peptide-free form has many of the characteristics and features of the native globular peptide-loaded HLA-DR1 protein and does not exist in an unfolded state.
Chapter VI: Characterization of the two empty forms of HLA-DR1 observed experimentally

Summary

Hydrodynamic studies of class II MHC proteins have provided evidence for the apparent size heterogeneity of the empty HLA-DR1 preparations as compared to peptide-loaded forms, while kinetic studies have identified two forms of the empty protein. In the hydrodynamic studies, the empty form typically was composed of a mixture of the peptide-receptive and peptide-averse forms. In this chapter preliminary experiments and approaches that were used to try to differentiate and characterize both forms are described. Briefly, in order to isolate and analyze the freshly-dissociated, peptide-receptive from of HLA-DR1, the off-rate of a weakly bound peptide was measured and compared to the inactivation rate of the protein. Preliminary comparisons of the peptide-receptive form and the equilibrium of species that contains mainly peptide-averse from were made.
VI.A. Introduction

Alternate conformations of class II MHC proteins have been observed under some experimental conditions (Boniface et al., 1996; Joshi et al., 2000; Runnels et al., 1996). Using the results from kinetics studies, a model for the interaction of peptides with class II MHC proteins has been proposed (Joshi et al., 2000; Rabinowitz et al., 1998; Sadegh-Nasseri et al., 1994). The kinetic model includes an initial bimolecular binding step followed by a slow unimolecular conformational change to a tightly-bound form. In addition, a reversible inactivation of the empty MHC protein that competes with a productive binding is observed (Scheme 1). In the scheme, an inactive or peptide-averse form of the protein converts slowly to an active or peptide-receptive species.

Although conformational changes have been observed between empty and peptide-loaded HLA-DR1 (Zarutskie et al., 1999), virtually nothing is known about the conformational states of the two forms of the empty protein. In this chapter the strategy and experiments that were done in an attempt to determine whether or not these two states could be characterized will be described. The techniques used to characterize both forms of the empty protein are gel filtration, circular dichroism and an ELISA assay.

![Scheme VI. 1. Kinetic mechanism for peptide binding to the class II MHC proteins. MHC$_i$ and MHC$_a$ represent peptide-averse and peptide-receptive conformations of the empty protein, respectively. MHCpep' represents a transient peptide-bound species. Figure taken from Joshi et al, 2001 (Biochemistry 39, 3751).]
VI.A.1. Strategy of the study

Spectroscopic and biophysical methods were used previously in order to study the conformational change upon peptide binding. The techniques include circular dichroism, gel filtration and enzyme-linked immunosorbent assay (ELISA) using KL295, which is specific for the epitope β58-69 of HLA-DR1. As has been shown, this antibody recognizes empty HLA-DR1 but not peptide-loaded (Sato et al., 2000). In a recent study, some of the regions in the β-chain of HLA-DR1 involved in the conformational change were identified and mapped using a set of monoclonal antibodies directed against the β-chain that only recognizes the empty state of HLA-DR1 (Carven et al., 2004). In all these experiments the empty form used is expected to contain the mix of peptide-receptive and peptide-averse forms that was observed in the kinetic experiments.

To characterize the peptide-receptive (MHCa) and peptide-averse (MHCi) forms of HLA-DR1 we will generate MHCa by *in situ* dissociation, a technique previously developed in another lab (Rabinowitz et al., 1998). For the studies a complex of HLA-DR1 with a low-affinity peptide will have to be prepared. The complex should be maintained at 4°C to prevent peptide-dissociation during purification of the complex. There are several ways that can be used to disrupt the equilibrium to then obtain MHCa, for example, the protein sample can be diluted, the peptide can be removed, or the temperature can be changed. Dilution of the sample is a very easy method, but the concentration would be low for the experiments that we want to perform. Perhaps temperature jump is the simplest and is the one we will use. Once the complex is heated to 37°C, the peptide will start dissociating from the protein (Scheme 2). The assumption
that as the peptide dissociates, I have mainly peptide-receptive form, and that it will convert with time to the peptide-averse form will be made. At the beginning of the experiment, we will be looking at the peptide-receptive form of the protein, as times goes by the predominant form of the empty will be the peptide-averse.

\[
\begin{align*}
(MHC_{\text{pep}}) & \xrightleftharpoons{k_{-1} \sim 10^{-3} \text{s}^{-1}} \text{pep} + MHC_a \\
& \xrightarrow{k_{\text{act}} \sim 10^{-4} \text{s}^{-1}} MHC_i
\end{align*}
\]

**Scheme VI.2. Model of the kinetic mechanism for the *in situ* dissociation.** MHC_{pep} represents the complex of HLA-DR1 and the low-affinity peptide.

One difficulty of the experiment is to obtain MHC\textsubscript{a} since it is in constant equilibrium with MHC\textsubscript{i}. For these experiments, a peptide that dissociates fast should be used. In other words, the off-rate of the peptide needs to be faster that the inactivation rate step of the protein, this way the peptide-receptive form can be build-up and measurements can be taken before it converts to the peptide-averse form. In order to pursue this approach, the off-rate of weakly binding peptides will be measured and compared to the inactivation rate. This establishes conditions whereby the peptide-receptive form is predominantly present and preliminary comparisons can be made with the equilibrium mixture of species that contains mainly peptide-averse form.
IV.B. Materials and Methods

VI.B.1. Production of peptide-loaded HLA-DR1 complexes

HLA-DR1 was produced by expression of α and β subunits in E. coli inclusion bodies with folding in vitro followed by immunoaffinity purification to isolate properly folded HLA-DR1 as described previously (Frayser et al., 1999). The HLA-DR1 concentration was measured by absorbance at 280 nm ($\varepsilon_{280\text{nm}} = 54,375$).

For off-rate experiments of HLA-DR1-peptide complexes, purified empty protein (10 μM) was incubated with 2- to 3-fold molar excess AMCA-peptide in PBST (PBS + 0.01% Tween-20) with protease inhibitors (0.1 mM iodoacetamide, 5 mM EDTA, 0.1 mg/mL PMSF) at 37°C in the dark for 72 hrs, with subsequent isolation of peptide complexes by size exclusion chromatography. Concentration and fractional peptide loading were determined by absorbance at 280 nm using the appropriate $\varepsilon_{280\text{nm}}$ values for DR1 and AMCA (5300 M$^{-1}$ cm$^{-1}$) and $\varepsilon_{346\text{nm}}$ for AMCA. Purified DR1-peptide complexes were kept in the dark at 4°C.

VI.B.2. Peptide dissociation experiments

FRET experiments were used to measure dissociation kinetics. MHC (Trp) to peptide (AMCA) fluorescence resonance energy transfer (FRET) was measured using 285 nm excitation and 447 nm emission, which corresponds to peaks in the protein fluorescence excitation and AMCA emission spectra in an assay described previously (Joshi et al., 2000).

For measurement of dissociation kinetics, purified MHC-peptide complexes (100 nM) were incubated with 100-fold excess unlabelled peptide in CBST (20 mM citrate
buffer, 150 mM NaCl, 0.01% Tween 20, 0.02% azide, pH 5.0) at 37°C in the dark. At various times, fluorescence spectra were obtained by the FRET assay. Fluorescence values from the FRET spectra were normalized using the Raman spectra of a sample of deionized, distilled water to correct for day-to-day differences in the lamp intensity.

VI.B.2. Inactivation experiments

To measure the inactivation of empty HLA-DR1, samples enriched in the peptide-receptive form were prepared by dissociation of complexes of HLA-DR1 and a fast-dissociating peptide. The fast-dissociating complexes were prepared by reacting empty HLA-DR1 with a 50-fold M excess of peptide at 37°C for three days. The complexes were isolated by gel filtration in the cold to prevent dissociation. The purified HLA-DR1-peptide complex was diluted to 1 μM and allowed to sit at 37°C or 4°C. At various times, and equal volume of Ha peptide solution (800 nM final) was added and the initial rate of binding was plotted against the time of incubation. The decay of the initial rate was interpreted as the decay in the amount of peptide-receptive HLA-DR1.
VI.B.3. General approach used to characterize the peptide-averse and peptide-receptive forms of HLA-DR1 (Scheme 3)

Scheme VI.3. Strategy for obtaining peptide receptive of HLA-DR1 by release of a weakly binding peptide. MHC\textsubscript{a} represents the peptide-receptive form of HLA-DR1 and MHC\textsubscript{i} represents the peptide-averse form.

FRET-inactivation experiments will be done in parallel to check that the protein preparation used behaves as expected.

VI.B.4. Gel Filtration

A superdex 200 gel filtration column (Pharmacia), run at 0.3 ml/min in PBS, pH 6.8 was used to run all the samples.
VI.B.5. Circular dichroism

For CD analysis, purified empty and HLA-DR1 peptide complexes (at 0.5 mg/ml) were exchanged by dialysis into 10 mM phosphate buffer, pH 7.0, and filtered with a 0.22 μm filter. CD measurements were made at 4°C in 1 mm path length cell. The low-affinity peptide complex was heated to 37°C and different measurements were collected.

VI.B.6. KL295 ELISA

Antibody binding specificity was measured using a sandwich ELISA. Monoclonal antibody KL295 was used at 10 μg/ml to coat a 96-well polystyrene microtiter plate by incubation overnight at 4°C. The plate was blocked with 3% BSA in PBS plus 0.02% sodium azide and washed with PBS plus 0.05% Triton X-100 (PBST). Quadruplicate 2-fold dilutions of HLA-DR1 from 1 to 300 nM in PBST and empty, HLA-DR1-Ha and HLA-DR1 with the low affinity peptide were added, allowed to bind to the plate for 2 hours at 4°C and washed 3 times with PBST. The amount of bound HLA-DR1 was detected by sequential incubations with rabbit-anti-DR polyclonal antibody, goat-anti-rabbit peroxidase conjugate, and ABTS (405 nm) as described (Frayser et al., 1999).

VI.C. Results

VI.C.1 Peptide-dissociation rates

The off rates of three different peptides were measured with the intention of finding a peptide that can dissociate from HLA-DR1 quickly. HLA-DR1 was complexed
with excess AMCA-labeled peptide by incubation at room temperature. After 3 days, the complex was purified by gel filtration in the cold to remove excess peptide and protein aggregates. MHC-peptide purified complexes were incubated with 100-fold excess of unlabelled peptide and the fluorescence spectra was measured by FRET at different times. Table I contains the names and sequences of the three peptides that were tested in these experiments and figure 1 shows the dissociation rate traces obtained for each peptide.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>FRAL</td>
<td>FRAL-NH₂</td>
</tr>
<tr>
<td>HaY308G</td>
<td>Ac-PRGVKQNTLRALT</td>
</tr>
<tr>
<td>HaY308A</td>
<td>Ac-PRAVKQNTLRALT</td>
</tr>
</tbody>
</table>

Table I. Sequences of peptide used in off-rate experiments

The dissociation curves of the HLA-DR1-peptide complexes follow a single exponential fit. All these peptides have a fast dissociation rate. However, the dissociation rate of the peptide FRAL is very fast causing a concern about the stability of the complex once the excess peptide is removed by gel filtration so it was decided not to use it. The peptide HaY308A was selected to be used in the generation of the peptide-receptive form of HLA-DR1.
Figure VI.1. Peptide dissociation rates. Off rates experiments were done by the FRET experiment using excitation at 350 nm and monitoring emission with time at 447 nm.
VI.C.3. Inactivation rate

Inactivation experiments were performed at 37°C and 4°C to determine if the inactivation was highly-dependent on temperature (Figure 2). To measure the inactivation rate in isolation, we prepared samples enriched in MHC\textsubscript{a} by release in situ of a weakly bound peptide. The peptide HA\textsubscript{F308A} binds weakly to DR\textsubscript{1}, because key MHC-peptide interactions are abrogated by the alanine substitution (Sato et al., 2000). We isolated DR\textsubscript{1}-HA\textsubscript{F308A}, allowed the peptide to dissociate to form MHC\textsubscript{a}, and tracked the inactivation by the initial rate assay. Immediately after dissociation, the initial rate of reaction of HA (800 nM) with freshly dissociated DR\textsubscript{1} (500 nM total) was measured. The inactivation occurred with a tau (τ) of 13 minutes at 37°C (although it was slightly different in other experiments varying at 10 to 30 minutes). When the experiment was done at 4°C, the tau was of 12 hours. Since the inactivation was slowed considerably at 4°C, the preparation of freshly-dissociated HLA-DR\textsubscript{1} was done in the cold.

![Figure VI.2. Rate of conversion of peptide-receptive to peptide-averse of HLA-DR\textsubscript{1} versus time.](image)

The experiment was done at 37°C (A) and 4°C (B) in 20 mM citrate buffer pH 5.0, 150 mM NaCl, 0.01% Tween-20 and 0.02% azide.
VI.C.2. Biophysical and Biochemical analysis for the characterization of the peptide-receptive and peptide-averse forms.

The peptide Hα_{Y308A} will be used in the preparation of samples that contain peptide-receptive HLA-DR1 since it dissociated from the protein faster than the time it takes the protein to inactivate. After the temperature jump (~4-5 minutes at 37°C), the samples will be put back in the cold to prevent inactivation of the freshly-dissociated HLA-DR1.

Freshly isolated peptide-receptive HLA-DR1 isolated as described in scheme 3 was prepared and kept at 4°C during the concentration and quantitation process. For all the experiments that will be described, after taking an initial measurement of HLA-DR1-Hα_{Y308A} at 4°C, we allowed the sample to stand at 37°C and measurements were taken at various intervals. We measured the hydrodynamic properties of samples by gel filtration (Figure 3), and compared it to the profiles of empty and Ha-loaded HLA-DR1. From the gel filtration experiment looks as if the freshly-dissociated HLA-DR1 radius was larger than the Hα_{Y308A} complex. No further change was observed with continued incubation time, suggesting that the peptide-receptive species has hydrodynamic properties similar to the peptide-averse form; it could also suggest that we did not isolate successfully the peptide-receptive form.

CD experiments were performed to determine if the secondary structure changed as the samples converts from the peptide-receptive to the peptide-averse form (Figures 4); the secondary structure did not appear to convert after the sample was heated.

The antibody KL295, an antibody specific for β59-69, binds to the empty form of HLA-DR1. In an effort to determine if it can discriminate between the peptide-receptive
and peptide-averse forms we performed an ELISA experiment (Figure 5). The freshly-dissociated sample was incubated at 37°C for different times and the reactivity towards the antibody was measured. Purified HLA-DR1-HaY308A complex does not bind to the antibody KL295 showing as expected the same behavior as the Ha-loaded sample. Once dissociation of the peptide is allowed to happen, the protein starts reacting with the antibody but not to the same extent as the control empty binds regardless of how long is incubated at 37°C.
Figure VI.3. Gel filtration analysis of freshly-dissociated HLA-DR1. A) Empty and Ha-loaded HLA-DR1 show the expected conformational change in the gel filtration profile shown. B) Purified HLA-DR1-HA$_{Y308A}$ complex is compared to empty and Ha-loaded DR1. This complex does not behave like the Ha-loaded complex. C) Freshly dissociated HLA-DR1 has a profile similar but not identical to the HLA-DR1-HA$_{Y308A}$ complex.
Figure VI.4. Circular dichroism analysis of freshly-dissociated HLA-DR1. Freshly-dissociated HLA-DR1 has a spectrum similar to the HAY308A complex, but different than the one from the Haje-loaded of empty mix of peptide-receptive and peptide-averse forms of HLA-DR1. The length of time that the freshly dissociated protein was allowed to incubate at 37°C post-isolation was 5min, 120 min, and overnight.

Figure VI.5. KL296 reactivity assay. Freshly-dissociated HLA-DR1 binds to KL295 as does the mix of peptide-receptive and peptide-averse forms.
VI.D. Discussion

We did not find dramatic differences between the peptide-receptive and the empty form of HLA-DR1. It is possible that the conformational differences between the peptide-receptive and peptide-averse forms are subtle and not detectable in the experiments and techniques used, although there are clear differences between the empty and Ha-loaded. Empty HLA-DR1 is in an open conformation, while Ha-loaded HLA-DR1 is in a close conformation. The results from the gel filtration experiment suggest that freshly-dissociated HLA-DR1 has a similar conformation compared to the HaY308A complex.

VI.E. Evaluation of strategy and experiments

Nevertheless, after analysis of the experiments performed and evaluation of the strategy taken, we were concerned about the short amount of time in which the peptide-receptive form was present and the fact that were not only measuring peptide-receptive but a mixture that contained un-dissociated HLA-DR1-HaY308A complex, peptide-receptive and peptide-averse forms of the protein. We realized that changing our strategy to reduce the number of possible states of the protein was necessary to conduct the experiments. We designed a different approach in which the different possible states of the protein is reduced (Scheme 4). Basically, biotin-labeled HaY308A will be incubated with the protein for 3 days at 37°C. The complex will be purified by gel filtration in the cold to remove protein aggregates and free peptide. While still in the cold, streptavidin (SA)-beads will be added to the mixture and incubated for 30 minutes. After the incubation with the SA-beads is complete, heated PBS buffer will be added and incubated
at 37°C for no more than 5 minutes and the reaction will be transfer to the cold; this way dissociation of the peptide will begin but inactivation would be in the very early stages, leading to accumulation of peptide-receptive form. A sample will be taken and the beads will be removed by centrifugation from the mixture. This step will remove from solution any free peptide or MHC-peptide complex and will just leave the peptide-receptive form. A measurement can then be taken on the freshly-dissociated protein still in the cold to prevent inactivation. The sample can then be heated to 37°C, and another measurement can be taken after some time when the protein is mostly in the peptide-averse form.

![Scheme VI.4. New strategy for obtaining freshly-dissociated HLA-DR1. This procedure requires labeling of the low affinity peptide with biotin.](image-url)
Chapter VII: Afterword

VII. Overview of the results

In this thesis, I have shown biochemical, crystallographic and immunological data that adds to our current knowledge of peptide binding to class II MHC proteins. Peptides can bind to class II MHC proteins in unexpected conformations, such as forming a hairpin turn to orient residues outside the binding groove for interaction with T cell receptors on the cell surface of T-cells, like a peptide derived from the HIV-Gag-p24(34) protein does. In addition, new perspectives and ideas on how peptides can use the non-traditional pockets in the peptide binding site of class II MHC molecules to increase their affinities is shown. N-methylated residues at the P7 position of the peptide can increase their binding affinities to HLA-DR1 without disrupting a T cell activation response. The role of the P10 pocket of class II MHC molecules was also studied and we found that even though we could not provide a specific pattern of residues that are preferred at the P10 pocket of class II MHC proteins some specificity is observed. The specificity of preferred peptide residues changes with mutants of HLA-DR1 that are different in the residues that line the P10 pocket. Data that supports the conformational change of class II MHC proteins upon peptide binding are presented and demonstrate that the empty, peptide-free form of the protein is not unfolded and exhibits most of the native fold that the peptide-loaded form has.

VII. An alternate mode of MHC-peptide-TCR interaction

In class I MHC proteins, the peptides bind with both the N- and C-terminus pinned in pockets that are found at the ends of the binding groove. Peptides can bind to
class I MHC proteins forming a slight kink in the central region. Slightly different conformations can be observed in the peptides of class I MHC complexes. In class II MHC complexes the peptide binds in a polyproline type-II conformation. The ends of the peptide are not pinned, and can therefore extend from the ends of the groove. In the available crystals structures of class II MHC-peptide complexes, the peptides are bound in similar ways, with the N- and C-terminus extending outside the peptide binding site.

In chapter II, the details of a peptide derived from an HIV-Gag protein that binds to HLA-DR1 in an unusual conformation are shown. This peptide forms a beta type-II conformation at the C-terminus. This phenomenon has not been previously observed in any of the structures of class II MHC-peptide complexes. Our immunological and biochemical characterization of the complex demonstrate that this conformation is not the product of a trapped crystal conformation and that it is important for T cell recognition and activation. Our results provide evidence for a mode of MHC-peptide-TCR interaction that has not been suggested before. An important extension to this would be structural characterization of the MHC-peptide complex that we study here in the presence of the specific TCR to confirm the results and to identify the important interactions between the peptide and the TCR. There are only two structures of class II MHC-peptide-TCR available. From these structures and from most of the biochemical and immunological characterization of MHC-peptide-TCR complexes we would expect to always see the TCR binding to the MHC-peptide complex in a very similar orientation and contacting peptide residues that are accommodated between the P-1 and P10 position. However, some studies, including the one that we described in chapter II, have identified T-cell clones that are dependent on peptide residues that are located outside
this region. These results can not be explain or addressed with the current structural work available and suggest that not all the key structural principles of MHC-pep-TCR recognition have been gathered yet. In our case a structure of the ternary complex would remove any ambiguity resulting from only having a structure of the MHC-peptide complex and it would provide information on the important residues and regions from the TCR that contact the residues from the peptide that lie outside the traditional peptide binding site and that are critical for T-cell activation.

Stability of the TCR by itself and in complex with the MHC-peptide and is one of the reasons for the limited structural work on class II MHC-peptide-TCR complexes. We will use different strategies reported in both the class I and class II MHC-pep-TCR field to obtain the final ternary complex for crystallization studies.

VII.C. The class II MHC peptide binding site

The residues and interactions that dictate how and which peptides bind to a class II MHC protein are contained in the peptide binding site. Most of the polymorphic residues of class II MHC proteins are involved in interactions with the side chains of the peptide that bind into the pockets. Biochemical and crystallographic work have provided researchers with very good information about the pockets that can be found in a class II MHC protein.

Structures of HLA-DR peptide complexes generally show a tight fit between MHC and peptide, but some exceptions can be identified. We explored some of these regions in an effort to obtain more information of the peptide binding site of class II MHC proteins. A relatively large region underneath the peptide between the P6 and P7
pockets was investigated, as well as the P10 shelf or pocket. Our strategies and experiments allowed us to determine the kind of contributions that each region can offer in peptide binding. Development of strategies for stabilizing MHC-peptide interaction is potentially important for the development of MHC-peptide complexes that can induce immunological tolerance in autoreactive T-cells, which frequently recognize weakly bound peptides (Brand et al., 2001; Kjellen et al., 1998; Stratmann et al., 2000).

VII.D. The different conformational states of class II MHC molecules

Alternate conformations of class II MHC molecules have been observed under some experimental conditions and have been correlated to some of the different species that participate in the kinetic mechanisms for peptide binding to class II MHC proteins. In chapters 5 and 6 we describe some of the work done on the empty states of class II MHC proteins and also on a peptide-loaded complex that can be thought of as an intermediate in the peptide binding mechanism. Empty class II MHC proteins have been implicated in antigen presentation of dendritic cells (Santambrogio et al., 1999). Dendritic cells are professional antigen presenting cells; immature dendritic cells express empty class II MHC molecules on their cell surface where they can capture peptide antigens and present them to T-cells.

Information on the empty form and possible peptide-loaded intermediates of class II MHC proteins will be helpful in the estimation and calculation of binding affinities. A structure of the empty, peptide-receptive form of HLA-DR1 would help to achieve this goal but this seems to be a challenge and, therefore, new strategies should to be developed to trap this conformation. Without much success, we have performed
crystallization studies of empty HLA-DR1 in the presence of superantigen (for more
details see section I of appendices) and also in the presence of antibodies that bind
exclusively to empty HLA-DR1. Our experience tell us that empty preparations of the
protein usually contain small amounts of protein loaded with peptides, possibly from
contaminants, that can interfere when performing experiments like crystallization. A new
approach that could be explored is to purify empty HLA-DR1 in the presence of one of
the MEM antibodies; this way you remove any small amount of peptide-loaded protein,
and then crystallize the complex. MEM antibodies bind to the empty form of the protein
and were used in a recent study to identify some of the regions of the β-chain of HLA-
DR1 that are involved in the conformational change of class II MHC proteins induced by
peptide binding (Carven et al., 2004).

VII.E. Implications of this work

This thesis described work directed to better understand peptide binding to class II
MHC molecules and MHC-peptide-TCR interactions. Knowledge of all the possible
MHC-peptide and MHC-peptide-TCR modes and interactions will help in the future
prediction, testing and design of T-cell antigens. Basically, a complete understanding of
the antigen presentation processes is required to improve and accelerate efforts to design
therapeutic strategies to induce immunity or to alter an ongoing immune response, and to
understand the causes of autoimmune diseases.
Chapter VIII: References


are derived from MHC-related molecules and are heterogeneous in size. Nature 358, 764-768.


Chapter IX: Appendices

A.I. Crystallization trials of empty HLA-DR1 in complex with the superantigen SEB 164

A.II. Protocols 170

1. Expression of HLA-DR1 in E.coli (Fermentation) 170
2. Expression of HLA-DR1 in E.coli in Minimal Media 171
3. Isolation of class II MHC subunits from bacterial pellets 174
4. Refolding and Purification of HLA-DR1 177
5. Preparation of Ab-ProteinA Immunoaffinity Column 180
6. HLA-DR1 ELISA 182
7. SDS-PAGE and Native PAGE Electrophoresis 185
8. Streptavidin-Europium ELISA to determine Peptide-DR1 $K_D$ 187
9. S2 Cell Culturing 190
10. Expression and purification of the superantigen SEC-3B2 191
11. Purification of zipper-stabilized, His-tagged sDM/sDR1 complexes (zDM/zDR) by Ni-NTA-agarose chromatography 193
12. DM/DO expression and purification 196
13. IgG-FC fusion cleavage on DM/DO protein 199
14. HLA-DM ELISA 201
15. Western Blot-Alkaline Phosphate Detection 203
16. Calculation of Apparent Molecular Weight from Gel Filtration 205

A.III. Characteristics of HLA-DR1/peptide/SEC3-3B2 crystals 206
A.I. Crystallization trials of empty HLA-DR1 in complex with the superantigen SEB

Attempts to produce crystals of empty DR1 for X-ray studies have been performed without success. We believe a major barrier to crystallization is that at the concentrations required for it, empty HLA-DR1 aggregates nonspecifically. I will try to crystallize empty DR using the superantigen staphylococcal enterotoxin B (SEB) for stabilization. Superantigens are a class of disease-associated, immunostimulatory molecules that bind class II MHC molecules and stimulate large number of T-cells. It has the ability to bind to both MHC and TCR (T-cell receptor) molecules outside their normal antigen-specific sites. Several crystal structures have been solved on different superantigens bound to different MHC class II complexes as well as bound to TCR. From the structures you can see that some superantigens, like TSST-1, touch some of the residues in the peptide binding site. The one that I choose to work with was SEB which does not touch the peptide bound to the MHC protein.

Figure taken from the paper Immunity, Vol.4, 93-104, January, 2001. A) HLA-DR2a/SPE-C complex. B) HLA-DR1/SEB complex. C) HLA-DR1/TSST-1 complex
To test whether or not empty DR1 binds to SEB, a preliminary biacore experiment was performed. This was done on a quick inject mode in the instrument so $k_{on}$ and $k_{off}$ constant are difficult to extract from the graphs/ traces below. It has been reported that the $K_D$ for SEB and DR-Ha is 1.7 $\mu$M. SEB was immobilized on the chip, and empty DR1 (or DR-Ha) was run over it. DR-Ha was used as a control since it has been reported to bind SEB by this method. Empty HLA-DR1 seems to bind to SEB although from the curves obtained seems like the affinity to SEB is lower compared to that of the HLA-DR1 bound to Ha.

DR-Ha binding to immobilized SEB:
Empty DR binding to immobilized SEB:

At concentrations of 30 and 40 µM, the protein is probably aggregating, if we plot only the first three concentrations:
Extracellular portion of HLA-DR1 was produced by expression of isolated subunits in E.coli inclusion bodies followed by refolding in vitro and purified by immuno-affinity using an LB3.1 column. The monomeric form of the protein is collected using gel filtration. The buffer is then exchanged to 10 mM Tris pH 8.0. SEB (Sigma, lyophilized powder) is dissolved in that same buffer. A 1:1 mixture of DR1:SEB is incubated overnight at 4°C; the mixture is checked on a gel filtration column to assure monomeric state of the DR1.

Small crystals were obtained on different conditions, but were very small and did not diffract.

Figures (crystals): DR1/SEB = 2.4 mg/ml; Drop vol: 1 µL well buffer + 3 µL complex

10 mM NaOAc pH 4.7, 10 mM MgCl₂ 10% PEG 4K, 10% Ethylene Glycol (100 x 20 µm)

10 mM NaOAc pH 4.7, 10 mM MgCl₂ 12.5% PEG 4K, 10% Ethylene Glycol (120 x 20 µm)

100 mM Glycine pH 3.9, 10% PEG 4K 10% Ethylene Glycol (100x 20 µm)

100 mM Glycine pH 3.9, 12% PEG 4K 10% Ethylene Glycol
100 mM Glycine pH 4.2, 10% Glycerol, 10% Ethylene Glycol, 12.5% PEG 4K (120 x 20 μm)

100 mM Glycine pH 4.2, 1 mM CdSO₄, 10% Ethylene Glycol, 12.5% PEG 4K (20 x 20 x 40 μm)

100 mM Glycine pH 5.0, 12% PEG 4K, 10% Ethylene Glycol (300 x 50 μm)

100 mM Glycine pH 5.0, 14% PEG 4K, 10% Ethylene Glycol (300 x 30 μm)

100 mM Glycine pH 4.7, 12.5% PEG 4K, 10% Ethylene Glycol, 10% Glycerol (60 x 20 μm)

100 mM Glycine pH 4.7, 15% PEG 4K, 10% Ethylene Glycol (maybe d=100 μm)
Drop vol: 1 μL well buffer + 4μL DR/SEB
100 mM Glycine pH5.0, 16% PEG 4K
10% Ethylene Glycol
**Procedure:**

Day 1:
1) Streak an LB-amp (or kan if kanamycin resistant strain, chlor also if cells are pLys^3) plate in the evening with BL21 cells of interest from frozen stocks. Incubate at 37°C overnight.

Day 2:
1) Put streaked plate at 4°C in morning, sealed with parafilm.
2) Autoclave assembled fermentor filled with 10 L of 1 x LB broth, 1 L of distilled H₂O, 1 L of 1 x LB broth, at least 150 mL 20% glucose, and 3-125 mL Erlenmeyer flasks.
3) In the early evening, add 83 mL sterilized LB broth to one of the sterile flasks. Add 83 µL Ampicillin (50 mg/mL) and 1.2 mL 20% glucose. **IF the cells are kanamycin resistant, substitute 83 µL of a 50 mg/mL stock of kanamycin sulfate for ampicillin. If cells are pLys^3, add 83 µL chloramphenicol (35 mg/mL).**
4) Mix well and take a 1 mL blank sample for OD₆₀₀ measurements. You may want to add 1 µL 20% NaN₃ to prevent growth in the blank sample. Keep this blank in a plastic cuvette.
5) The remaining LB solution will be split into different containers. Pipet 2 mL into a sterile 15 mL conical tube, 20 mL into a 50 mL conical tube, and 20 mL into each of the remaining sterile Erlenmeyer flasks. Label the flasks 1, 2 and 3.
6) Pick one colony from your plate. Add it to your 15 mL tube and vortex vigorously. Take 200 µL of that tube and add it to the 50 mL conical tube and vortex vigorously. Take 200 µL of that tube and add it to Erlenmeyer flask #1 and swirl a lot. Take 200 µL from that flask and add it to Erlenmeyer flask #2 and swirl a lot. Take 200 µL of that flask and add it to Erlenmeyer flask #3.
7) Grow the 3 Erlenmeyer flasks on a shaker table at 37°C overnight.
Day 3:
1) Measure the absorbance at 600nm of each flask compared to the blank you saved. Pick the one that is less than 1.0 OD$_{600}$, but still has measurable growth in it. (0.7 is about ideal.)
2) Set up the fermentor: Add 10 mL of Ampicillin, 50 mg/mL, 100 mL 20 % glucose (sterile), and 200 µL antifoam A. **IF the cells are kanamycin resistant, substitute 10 mL of a 50 mg/mL stock of kanamycin sulfate for ampicillin. If the cells are pLysS, also add 10 mL 35 mg/mL chloramphenicol.** Mix well. Remove 1 mL as a blank and store in a plastic cuvette, possibly with 1 µL 20 % NaN$_3$.
3) Run the mixer on the fermentor at ~700 rpm, and run the air at 15 psi and 5 LPM. Watch for excessive foaming and add antifoam if it is necessary. **If you have never set the fermentor up before, ask for help!!
4) Seed the fermentor with 10 mL of the overnight flask you selected.
5) Take samples and check the OD$_{600}$ every hour until you get close to 1.0, and then check more frequently. Do not overgrow!
6) When the OD$_{600}$ reaches 1.0, take a 1 mL sample “not induced.” Spin down and re-suspend in 1x urea-SDS loading buffer. Freeze at -20°C.
7) Add IPTG to 0.75 mM final concentration. I use 10 mL of 0.75M IPTG stock (1000 x). Allow to grow for 2-5 hours with IPTG. (Longer for class I MHC subunits)
8) Take a 1 mL sample “induced.” Spin down and re-suspend in 1x urea-SDS loading buffer. Freeze at -20 °C until you are ready to check the “not induced” and “induced” samples on a gel by SDS-PAGE.
9) Spin down all the cells at 8000 x g and discard the supernatant. For inclusion bodies, you may freeze the pellet at -20°C, or proceed with the inclusion body prep through the DNAse step and freeze at -20°C.

To run the 12.5% SDS-gel:
Take the 1 ml uninduced/induced bacterial pellets and resuspend in 50 µl of water. Add 50 µl 2X Reduced SDS loading buffer. Boil for 2 minutes and spin down lysed cells. Load 10 µl of both samples onto the gel. Look for an induced band around 25,000 kDa.

<table>
<thead>
<tr>
<th>MW (kDa)</th>
<th>α</th>
<th>+</th>
<th>αLews</th>
<th>+</th>
<th>induced</th>
</tr>
</thead>
<tbody>
<tr>
<td>74</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>45</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>29</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>α</td>
</tr>
<tr>
<td>18</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
A.II.2. Expression of HLA-DR1 in E.Coli in Minimal Media 
(Fermentation)

The procedure for fermentation is the same as described in A.II.1, but the recipe for the media is below.

**Minimal Media Recipes:**

1) Glycerol Na+/K+ Phosphate
   10X stock:
   - Glycerol 172 mL/L
   - KH₂PO₄ 16 g/L
   - Na₂HPO₄ 100 g/L
   Autoclave
   If labeling the protein use ¹³C-labeled glycerol.

2) Na₃+ Citrate
   400X --- 400 g/L
   Autoclave

3) MgSO₄
   1000X --- 2M
   Autoclave

4) Thiamine
   1000X --- 5 mg/mL
   Filter Sterilize, store at -20°C

5) (NH₄)₂SO₄
   755X --- 4M
   Autoclave
   If labeling the protein use (¹⁵NH₄)₂SO₄

6) Trace Metals Solution
   667X
   ****1.5 mL/L of a solution that has
   CaCl₂ 2H₂O 0.5 g/L
FeCl₃ 6H₂O 16.7 g/L
ZnSO₄ 7H₂O 0.18 g/L
CuSO₄ 5H₂O 0.16 g/L
MnSO₄ H₂O 0.12 g/L
CoCl₂ 6H₂O 0.18 g/L

Autoclave

- The growth rate when using minimal media in 100% D₂O was very slow. Cell duplication took ~1 hour. If D₂O was not used, cell duplication was of 30 minutes.
- Induction time was of 3-4 hours (750μM IPTG was used for induction)

Subunits can be purified using the usual procedure (see A.II.3).

- Example of a test induction in minimal media for B1S:

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>111.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>79.6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>61.3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>49</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>36.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24.7</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>19.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9.3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

N.I. = non-induced, I = induced
A.II.3. Isolation of class II MHC subunits from bacterial pellets

Developed by Mia Rushe

Solutions:

**DNAse solution** (can be made and stored in freezer)
75 mM NaCl
50% glycerol
2 mg/ml DNAse (Sigma D-5025)

**Sucrose Solution** (if no DTT can be stored on bench; same for following three solutions)
50 mM tris pH 8.0
25% sucrose
1 mM EDTA
0.1% NaN₃
10 mM DTT (add just before use)

**Deoxycholate/Triton**
1% Na Deoxycholate
1% Triton x100
20 mM tris pH 7.5
100 mM NaCl
0.1% NaN₃
10 mM DTT (add just before use)

**Triton Solution**
0.5% Triton x100
50 mM tris pH 8.0
100 mM NaCl
1 mM EDTA
0.1% NaN₃
1 mM DTT (add just before use)

**Tris Solution**
50 mM tris pH 8.0
1 mM EDTA
0.1% NaN₃
1 mM DTT (add just before use)

**Urea Solution** (make up fresh each time)
8 M urea
20 mM tris, pH 8.0
0.5 mM EDTA
30 mM DTT

**4 M MgCl₂**
0.5 M EDTA pH 8.0
*Protocol for pellet of 10 liter fermentor culture*
Procedure (for pellet from 10 L culture):

1. Spin down cells at 5000 x g—collect supernatant into a container, sterilize with 1% Wescodyne for 10-20 minutes, and then dump down the sink.

2. With a rubber spatula, re-suspend fresh bacteria into a single plastic container with ~200 mL Sucrose Solution.

3. Chop the solution briefly in a homogenizer or polytron. **Do not sonicate!**

4. If cells are not pLys⁴, add 1 mg dry lysozyme per mL suspension (0.2 g) and stir for 10 minutes. If cells are pLys⁴, just stir for 10 minutes.

5. While stirring, add 500 mL Deoxycholate-Triton Solution. Solution will become very viscous due to cell lysis and DNA release.

6. Add 1 mL of 4 M MgCl₂ Solution to make 5 mM final concentration.

7. Add 2 mL DNAse Solution. Stir until the solution is the viscosity of water.

8. Freeze overnight at −20°C, or until ready to complete the prep.


10. Stir an additional 10 minutes after thawing to allow the DNAse to work again.

11. Spin down in 2 centrifuge bottles at 8000 x g for 20 minutes. Discard supernatant.

12. Re-suspend pellets in 300 mL or more each Triton Solution. Chop briefly, keeping the pellets on ice as much as possible. Spin down at 8000 x g for 20 minutes and discard supernatant.

13. Repeat step 12 three or more times.

14. Re-suspend pellets into 300 mL or more each Tris Solution. Chop briefly, keeping the pellets on ice as much as possible. Spin down at 8000 x g for 20 minutes and discard supernatant.

15. Repeat step 14 two or more times.

16. Re-suspend/dissolve the pellets and chop in ~200 mL Urea Solution.

17. Spin down at 20°C, 15,000 x g for 30 minutes. Filter through a 0.2 μm filter.

18. Freeze at −70°C until ready to purify by Urea HQ.
Subunit Purification:

Column resin: POROS 20 HQ (quaternized polyethyleneimine) (Boehr. Mannheim)
Buffers:     A: 8 M urea, 20 mM tris, 1 mM DTT
            B: 8 M urea, 20 mM tris, 1 M NaCl, 1 mM DTT
            For α-subunits: pH 8.0
            β-subunits: pH 9.0

Preparing inclusion bodies:

On a Perseptives HQ column, β-subunits bind best at pH 9.0. After urea solubilized inclusion bodies are thawed, adjust the pH of the β-subunit to 9.0.

1) Equilibrate the column with 1-5 column volumes of buffer A.

2) Inject or load protein.

3) Wash with at least 2-5 column volumes of buffer A. You'll know it's enough when the absorbance stabilizes again.

4) Run a gradient from 0% B to 30% B (0-300 mM NaCl) over 6 column volumes.

5) If there is more than one major peak eluting (as often is the case for β-subunits), analyze fractions by SDS-PAGE or possibly by setting up test refoldings if you’re really nervous about it. Personally, I simply pool those fractions which look like the majority of protein is β-subunit.

6) Pool peak fractions. For β-subunits adjust the pH back to 8.0. Add EDTA to a final concentration of 5 mM. Measure the concentration by UV absorbance scan against a urea (buffer A) blank. For α-subunits: 1.3 OD_{280} nm = 1 mg/ml, and for β-subunits: 1.7 OD_{280} nm = 1 mg/ml. Store in convenient aliquots at ~70 °C.

Notes:
A new HQ column has a capacity of about 5-10 mg protein per ml of resin. Components of the urea buffer (EDTA) seem to interfere with protein binding. Cleaning the column with 1 M NaCl/ 1M NaOH after every couple of runs helps to regenerate the column capacity. However, column capacity becomes significantly lower with use, despite complex regeneration.

The inclusion bodies give inconsistent, messy looking chromatograms, but this purification is necessary to get rid of an unknown component that affects protein folding.
A.II.4. Refolding and Purification of HLA-DR1
Developed by M. Rushe

Refolding of Subunits into Empty DR1:

Refolding Mix:
- 25% glycerol
- 20 mM tris, pH 8.5
- 1 mM EDTA
- 2 mM glutathione, reduced form
- 0.2 mM glutathione, oxidized form

(glutathione should be added just before refolding)

HQ purified alpha and beta subunits solubilized in 8 M urea

1) Chill refolding mix to 4 °C before adding glutathione. I used to do 10L per refolding. It takes at least overnight for this to happen. Refolding should be performed in a container close to the volume of refolding to minimize exposure to air. If desired, sparge the folding mix with argon for about 20 minutes.

2) Add glutathione and stir until just dissolved with a magnetic stirrer (about 30-45 minutes).

3) While rapidly stirring the folding mix, very slowly add HQ-purified subunits with a pipet. Add 2 mg of each subunit per 1 liter of refolding. If refolding peptide-loaded DR1, add 5M excess of peptide to the refolding mix assuming that 1mg/L is the concentration of protein.

4) After all components are well mixed, cover tightly and store at 4 °C for at least 36 hours.

Immunoaffinity purification of empty class II MHC from in-vitro folding reactions:

1) Concentrate folding mixture in the Amicon spiral filter concentrator. Save the flow-through in case the filter membrane breaks. If you routinely check the recovery with an ELISA, you will see if this happened by the presence of DR1 in the sample of flow-through you test. Once I got a feel for how much protein I got from a refolding,

2) Exchange the folding buffer by filling the concentrator vessel with PBS and concentrating. Repeat this step again. It is important to get rid of the glutathione in the folding mix or it will trash the immunoaffinity column!
3) Flow the concentrated folding mix over the immunoaffinity column by gravity. The column is composed of the anti-MHC class II antibodies LB3.1 or L243 covalently coupled to Protein A.

4) Wash with at least 10 column volumes of PBS.

5) Pre-elute the column with at least two column volumes 10 mM NaPi pH 7.

6) There are two ways to elute. Traditionally, the high pH method is used to elute class II from an immunoaffinity column.

   **High pH:** Elute the protein with 50 mM CAPS pH 11.5. Collect 1 ml fractions into tubes containing 300 μl of 300 mM NaPi pH 6 (to neutralize). About 5 column volumes should be sufficient. (Note: The lab experience is that the high pH method seems to yield more protein)

   **Low pH:** Elute the protein with 50 mM glycine pH 3. Collect 1 ml fractions into tubes containing 300 μl of 300 mM Tris pH 8 (to neutralize). About 5 column volumes should be sufficient.

   Prepare a “blank” (300 μl of 300 mM NaPi pH 6 plus 1 mL 50 mM CAPS pH 11.5, for example) to zero the spectrophotometer and also to check that the pH of the combined buffers is neutral.

7) Neutralize the column with 300 mM NaPi pH 6 (if high pH elution was used), or 300 mM Tris pH 8 (if low pH elution was used).

8) Wash column with at least 10 column volumes of PBS/0.02% NaN₃ and store in refrigerator.

9) Since the capacity of an immunoaffinity column varies greatly, you should repeat steps 1-7 with the “flow-through” until no more protein elutes from the column.

10) To clean the column after you are finished:
    5 CV 50 mM CAPS pH 11.5
    5 CV 50 mM glycine pH 3
    5 CV 50 mM CAPS pH 11.5
    5 CV 50 mM glycine pH 3
    10-20 CV PBS/0.02% NaN₃

11) Pool the fractions containing DR1 based on their absorbance at 280 nm, concentrate and switch into a desired buffer (usually PBS).
12) Check the prep for peptide binding activity:

Incubate 5-10 μg DR1 with and without 5-molar excess Ha peptide at 37 °C for at least 48 hours.

Example:
DR1 stock 1 mg/ml
+Ha sample: no peptide sample:
5 μl DR1
2 μl 0.25 mM Ha peptide
13 μl PBS/0.02% NaN₃
5 μl DR1
15 μl PBS/0.02% NaN₃

final DR1 concentration: 0.25 μg/ml = 5 μM
final peptide concentration: 25 μM

Analyze by 12.5% SDS-PAGE: Run a boiled / not-boiled version of each sample
Example:
Add 5 μl of 5X reducing SDS sample buffer to each tube. Load 12 μl into “non-boiled” lane.
Boil remaining 13 μl for 5 minutes and pulse in microfuge. Load 12 μl into “boiled” lane.

Ha-loaded class II should give a ~45,000 kDa band on an SDS gel that dissociates into α and β subunits when boiled. “Empty” class II is not SDS stable and will dissociate into α and β subunits, for the boiled and not-boiled samples. You can further purify the imunoaffinity eluted empty class II MHC by size exclusion chromatography to obtain monomeric DR1. There will be a lot of aggregated protein that elutes at a large molecular weight, but you should also see a peak at ~45,000 kDa corresponding to a class II αβ heterodimer. The oligomeric distribution varies from prep-to-prep; I usually saw ~25% monomer.
A.II.5. Preparation of Ab-ProteinA Immunoaffinity Column
Developed by Mia Rishe

Materials:
200 mM Borate: 1.24g/100 mL, titrate to pH 9.0 with NaOH
200 mM Ethanolamine: 1.2mL/100 mL, titrate to pH 8.0
Antibody solution, enough to have 10 mg/5mL matrix
Protein A-coupled matrix; I prefer IPA-400 Fast-Flow.
PBS + 0.02% NaN3
Dimethylpimelimidate (20 mM final, (MW=259), 5.18 mg/mL)
Advice: Use a fresh bottle (e.g. unopened) every time

Procedure:

1) Mix Antibody and ProteinA matrix in PBS, preferably with as small a volume as possible (ideally ~15 mL if using 5 mL matrix; can be scaled up accordingly). This can be done in a polypropylene centrifuge tube, or directly in the gravity column body (just make sure the ends are sealed) Rotate at room temperature 1 hour or longer. Remove 15 μL of suspension into tube labeled “TOTAL”.

2) Spin 2000 rpm 5 min, remove and save supernatant and pellet. Or, if using the column, let the solution drain out. Remove 15 μL of supe into tube labeled “NON-BOUND”.

3) Resuspend pellet in 50 mL borate solution, spin/drain, remove supernatant.

4) Resuspend pellet in 15 mL fresh borate solution. Remove 10 μL into tube labeled “BOUND”. Add 20 mM final dimethylpimelimidate as solid (77.7 mg for 15 mL) and dissolve. Rotate 30 minutes at room temperature (going longer will over-couple the matrix)

5) Spin/drain to remove supe and check that the pH of the supe is > 8.0.

6) Resuspend pellet in 50 mL ethanolamine, rotate 30 minutes room temperature or longer, spin/drain off supe.

7) Wash pellet with 50 mL ethanolamine, then 50 mL borate, then 50 mL PBS

8) Resuspend pellet in 15 mL PBS. Remove 15 μL of suspension into tube labeled “NON-COUPLED”. Store matrix at 4°C in PBS + NaN3.

Analysis:

Add 10 μL 2x Laemmlli buffer to each fraction, boil, and spin in microfuge.
Run 10% SDS-PAGE of MW markers, 1 μg mouse IgG, and 10 μL of each fraction taken. Gel should show heavy and light chains in TOTAL and BOUND, and nothing in
NON-BOUND and NON-COUPLED. If there is more protein in NON-BOUND than BOUND, try a different binding buffer (PBS works fine for LB3.1/L243). If there is any protein in NON-COUPLED, cycle the matrix through all binding and elution conditions that will be used in the affinity purification procedure, and next time use fresh pimelimidate!

Sample of analysis gel:

![Sample of analysis gel](image-url)
A.II.6. HLA-DR1 ELISA
Protocol from Mia Rushe

Materials:
1. Block Solution: 3% BSA in PBSZ
2. Dilution Solution: 0.3% BSA
   0.1% Triton X-100
   in PBS (filter and store at 4°C)
3. TBST: 25 mM Tris
   137 mM NaCl
   2.7 mM KCl
   0.05% Triton X-100
4. Alternatively you can PBST instead of TBST: PBS with 0.05% Triton X-100.
5. ABTS solution: 5 mL 10X ABTS buffer (from Roche-made and frozen at -20°C in 5 mL aliquots)
   45 mL ddH₂O
   1 ABTS tablet (from Roche also)
   *Unused portion can be stored in dark at 4°C for 2 weeks.
   Or you can use the ready-to-use-ABTS solution that Roche sells!!!

Protocol:

1) Coat 96-well plate with anti-DR monoclonal antibody LB3.1 (2 mg/mL stock in PBS + 0.02% azide). Dilute 1:1000 in PBSZ and put 100 μL in each well.
   Incubate at 37°C for 2 hours or 4°C overnight.

2) Block plates with block solution for at least 4 hours at room temperature. (It is okay to store the plates in the cold room at this stage and use them later, they are good for about 3 weeks.)
3) Put 50 µL of dilution solution in every well (It takes awhile to pipet samples and standards into wells to prevent them from drying out.)

4) Standards: Do 2-fold dilutions in duplicate starting with 100 ng/well: 100 ng, 50, 25, 12.5, 6.25, 3.125, 1.56, 0.78, 0.39, 0.19, 0.1. Dilute samples into dilution solution. (0.3% BSA, 0.1% triton X-100, in PBS)

5) Samples: Put appropriate amount of sample on. For tissue culture sup, put 50 and 5 µL (add and additional 45 µL total to each well that only 5µL of sample, so that you have 100 µL total in each well).

6) Incubate standards and samples at 37°C for 1 hour or at room temperature for 2 hours or overnight at 4°C.

7) Wash 3 times with PBST or TBST. (Be careful when washing the first 2 times, you have different samples in each well and you don’t want the wells to spill into one another.)

8) Add 100 µL/well of rabbit CHAMP diluted 1:25,000 in dilution solution. Incubate for 1 ½ hours at room temperature or 1 hour at 37°C or overnight at 4°C.

9) Wash wells 3 times with PBST or TBST.

10) Add 100 µL/well of goat anti-rabbit IgG-peroxidase diluted 1:4,000 in dilution solution. It is very important that no azide is present in this step, it inhibits the HRP. Incubate ½ to 2 hours at room temperature. (Background increases with if this step is left at 4°C overnight.)

11) Wash wells 3 times with PBST or TBST.

12) Add 200 µL of ABTS/ well and read absorbance at 405 nm.

13) Work up data.

**ELISA fit-four**

equation used to fit data:
line 1: \((m1-m2)/(1+ (m0/m3)^m4)) + m2;
initial guesses: m1=0.7; m2=0; m3=2; m4=-0.5

partial derivatives:
line 2: \(1/(1+(m0/m3)^m4)\)
line 3: \(1-(1/(1+(m0/m3)^m4))\)
line 4: \((m1-m2)*(1+(m0/m3)^m4)^(-2)*(m4*(m0/m3)^((m4-1)))*m0/m3^2\)
line 5: \[(m2-m1)*(1+(m0/m3)\cdot m4)^{-2} \cdot (m0/m3)^{m4} \cdot \ln(m0/m3)\]

inverse fit-four

c1=m3* ((m1-m2)/(c0-m2)-1)^{1/m4}

an alternate equation for fitting ELISA data

Example ELISA standard curve:
A.II.7. SDS-PAGE and Native PAGE Electrophoresis

**4X Lower Buffer**
181.7 g Tris base
4.0 g SDS
ddH₂O to 1L  pH 8.8

**4X Upper Buffer**
60.6 g Tris base
4.0 g SDS
ddH₂O to 1L  pH 6.8

**10X Running Buffer**
30.3 g Tris base
10.0 g SDS
144 g glycine
ddH₂O to 1L  pH 8.3

**Laemmli loading buffer 1X**
60 mM Tris-HCl pH 6.8
100 mM DTT
1% SDS
10% glycerol
0.001% bromophenol blue  make 1X to 6X
(for non-reduced loading buffer omit DTT)

*Note: for Native gel buffers, omit SDS from all solutions*

**Coomassie Blue stain**
0.25% CBBR-250
15.0% methanol
10.0% acetic acid
dissolve stain in methanol first, then add acetic acid and ddH₂O

**Destain**
15% methanol
10% acetic acid
Separating gel:

(it takes about 3.5 ml for a mini-gel)
For normal applications use 37.5:1 acrylamide:bis-acrylamide

<table>
<thead>
<tr>
<th></th>
<th>7.5%</th>
<th>10%</th>
<th>12.5%</th>
<th>15%</th>
</tr>
</thead>
<tbody>
<tr>
<td>4X lower buffer</td>
<td>1.5 ml</td>
<td>1.5 ml</td>
<td>1.5 ml</td>
<td>1.5 ml</td>
</tr>
<tr>
<td>30% acrylamide</td>
<td>1.5 ml</td>
<td>2.0 ml</td>
<td>2.5 ml</td>
<td>3.0 ml</td>
</tr>
<tr>
<td>water</td>
<td>3.0 ml</td>
<td>2.5 ml</td>
<td>2.0 ml</td>
<td>1.5 ml</td>
</tr>
<tr>
<td>temed</td>
<td></td>
<td>10 µl</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10% (w/v) ammonium persulfate (APS)</td>
<td></td>
<td></td>
<td>20 µl</td>
<td></td>
</tr>
</tbody>
</table>

Stacking gel:

(recipe makes 2 ml - enough for one mini-gel)

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>4X upper buffer</td>
<td>0.5 ml</td>
</tr>
<tr>
<td>30% acrylamide</td>
<td>0.3 ml</td>
</tr>
<tr>
<td>water</td>
<td>1.2 ml</td>
</tr>
<tr>
<td>temed</td>
<td>5 µl</td>
</tr>
<tr>
<td>APS</td>
<td>10 µl</td>
</tr>
</tbody>
</table>

Boiled/Not-boiled samples:

To run a "boiled" sample: Add loading buffer (reduced or not-reduced) to gel sample in an Eppendorf tube to a final concentration of 1X. Boil tube in a water bath for at least two minutes. Pulse in a microfuge to recover all of sample and then load onto gel.

For a not-boiled sample: Add loading buffer (reduced or not-reduced) to gel sample in an Eppendorf tube to a final concentration of 1X. Load sample onto gel.
A.II.8. Streptavidin-Europium ELISA to determine Peptide-DR1 $K_D$

Developed by Aaron Sato

Reagents:

TBST: 20 mM Tris, 0.2 M NaCl, 0.1% Tween-20 (pH 8)
Dilution solution: PBS, 0.05% Triton x-100, 0.1% BSA (pH 7)
Blocking solution: PBS, 0.02% NaN$_3$, 3% BSA (pH 7)
PBS + 0.02% NaN$_3$ (pH 7)
Wallac DELFIA Assay buffer (catalog# 1244-111)
Wallac DELFIA Enhancement solution (catalog# 1244-105)
Wallac Europium-labeled streptavidin (catalog# 1244-360)
Protein-A purified LB3.1
Affinity purified Sf9 or E. coli DR1, empty (no peptide)
Biotinylated Ha peptide, C18 purified, reconstituted in water
Binding Buffer: PBS, 0.05% Triton x-100, 1 mM PMSF, 0.5 mM Iodoacetamide, 2.5 mM EDTA, 0.02% NaN$_3$, 0.1% BSA

Setting up a competitive binding assay:
1) You should set up triplicates of all samples. Prepare all dilutions in this section with binding buffer.
2) Set up binding reactions in silanized polypropylene tubes. In one set of tubes on ice, prepare 10-fold dilutions of competitor peptide in binding buffer, starting with a concentration of ~1 mM and going down to below 1 nM. These stocks will be 2x the final assay concentration. I usually make ~250-300 μL of each.

3) In one 15 mL polypropylene tube on ice, prepare a solution of 5 nM of empty DR1 and 5 nM biotinylated-Ha peptide in binding buffer. 2 nM works well too; it’s up to you. Make 3 to 5 mL.

4) To each tube containing the competitor peptide, add an equal volume of the DR1/bio-Ha stock.

5) Incubate samples for at least 5 days at 37 °C.

**Setting up a direct binding assay:**

1) You should set up triplicates of all samples. Prepare all dilutions in this section with binding buffer.

2) Set up binding reactions in silanized polypropylene tubes. In one set of tubes on ice, prepare 10-fold dilutions of peptide in binding buffer, starting with a concentration of ~1 mM and going down to below 1 nM. These stocks will be 2x the final assay concentration. I usually make ~250-300 μL of each, double that if I’m going to run a no-DR1 control (necessary for some peptides that bind non-specifically at high concentration like bioHA-Y308A)

3) In one 15 mL polypropylene tube on ice, prepare a solution of 5 nM of empty DR1. Make 3-5 mL. If running a no-DR1 control, have an equal amount of binding buffer on hand.

4) To each tube containing the competitor peptide, add an equal volume of the DR1 stock or binding buffer for your control.

5) Incubate samples for at least 5 days at 37 °C.

**ELISA:**

1) Coat an Immunon-IV plate with 100 μL/well of 10 ng/μL LB3.1 antibody (1 μg/well final) in PBS + 0.02% NaN3. Incubate overnight at 4 °C or 37 °C for 1 hour.

2) Wash plate 3X with TBST. Block plate with blocking solution for 2 hours at room temperature.

3) Wash plate 4X with TBST.
4) Apply 150 μl of binding sample to each well. Incubate 1 hour at 37 °C.

6) Wash plate 5X with TBST. Add 100 μl of a 1:1000 dilution of streptavidin-Europium in DELFIA assay buffer. Incubate the plate at 37 °C for 30 minutes. In the mean-time, take the DELFIA enhancement solution out of the refrigerator and let it warm up to room temperature.

6) Wash plate 7X with TBST. Apply 200 μl/well of DELFIA enhancement solution and incubate at room temperature for 15-20 minutes. Read the plate using the standard Europium protocol on the Victor 1420 Multilabel Counter.

**Competitive Binding Equation:**

\[
\frac{m_1}{(1+10^{-(\log(m_0)-\log(m_2))})}; \quad m_1=55000; \quad m_2=20
\]

where \( m_1 \) is the value at which the curve saturates and \( m_2 \) is the IC50. You will need to have performed a baseline correction on the data. Values given are just examples.

**Direct Binding Quadratic:**

\[
m_1*((1+m_0+m_2)-\sqrt{(1+m_0+m_2)^2-(4*1*m_0))})/2; \quad m_1=2.5e5; \quad m_2=14
\]

where \( m_1 \) is the value at which the curve saturates and \( m_2 \) is the apparent \( K_D \). You will need to have performed a baseline correction on the data. Values given are just examples.
A.II.9. S2 CELL CULTURING
Protocol from Tom Cameron

S2 (Schneider cells) Cell Culturing

Materials:
1. Sf 900 SFM II (Gibco/Invitrogen)
2. G418 (also known as Geneticin) (Gibco/Invitrogen) (Final Concentration should be 1 mg/ml)
3. Antibiotic/ Antimycotic (PSF) (Gibco/Invitrogen) (Final Concentration: 1X)
4. Fetal Bovine Serum, heat inactivated (Sigma or Gibco/Invitrogen)

Notes:
- Cells are suspended in Sf900 SFM supplemented with G418, PSF, and 10% serum. Spin them down and save the media. Re-suspend them in less volume of media (~10-15 mL). If you don’t have fresh media, you can use the media in which they came (the one you just saved) until you get some.

- If you want to freeze some cells, freeze 6 million cells/mL in 1mL aliquots of 10% DMSO, 90% media with serum.

- Adaptation to serum free is very easy! I usually go from 10% to 5% to 3% to 2% to 1% to 0.5% to 0%.

- In Serum free media, they adhere pretty tight, but they can mostly be removed by pipetting.

- They seem to double every 48 hours. The cells like to be denser than 1 million/mL, plastic (flasks) or spinners. In spinners, they grow happily up to 6 million/mL.

- Induce with CuSO₄; I usually induce at 5 million cells/mL. Induce for 5 days. Harvest, take supernatant and add protease inhibitors, 5mM EDTA, 0.02% Sodium Azide, 1mM PMSF, 1mM Iodoacetamide.
A.II.10. Expression and purification of the superantigen SEC-3B2

Expression:
- I use 2xYT media to grow the superantigen.
  1) Several dilutions of 15 ml culture were grown overnight (2xYT media, 100 μg/ml ampicillin, 2% glucose) at 37°C.
  2) Fifteen ml were used to inoculate two 2L flask (each containing 500 ml of 2xYT media and 100 μg/ml ampicillin). Cells were grown for about 2-4 hours at 37°C until OD₆₀₀ was about 1 (you can induced when OD₆₀₀ is anywhere between 0.8-1.2)
  3) Cells were induced for about 5hrs (induction can be between 4-6 hrs).
  4) Cells were spin down at 5000 rpm for 15 minutes.
  5) The cells were resuspended in 10 mls of ice-cold TES buffer (100 mls of TES buffer: 3.15 g of Tris-HCl, 0.186 g of EDTA, 17.1 g of sucrose, pH 8.0). Suspension was left at 4°C overnight. Alternatively, it can be incubated on ice for 1 hour.
  6) Next day, 15 ml of ice-cold 0.2x TES buffer were added to the suspension and left on ice for 45 minutes (should not be left on ice for more than 1 hr).
  7) Cells were spin down for 10 minutes at 7000 rpm.
  8) The supernatant was transferred to a reusable tube and it was spin for 20 minutes at 12000 rpm.
  9) The yellowish liquid was dialyzed against 2L of 20mM Tris pH7.5 for ~8hrs at 4°C. Fresh buffer was used to dialyze overnight.

Red-A purification:
  1) Approximately 6 mls of Red-A agarose (Eric sent some of this agarose, since it is not commercially available anymore; Larry bought a similar one that works fine but the protein flows through it and about 80% of the contaminants bind to the column, either way works fine just remember to keep the flow through!) were transferred to a low-pressure column.
  2) It was washed with 2 CV of 0.5M NaOH, 6M Urea to remove excess dye (this step is only done when the resin is new).
  3) Column was equilibrated using 10 CV of 20mM Tris pH 7.5.
  4) Protein was added slowly, and the flow through was collected.
5) Column was washed with 50 ml of 20mM Tris pH 7.5.
6) The protein was eluted with 25 ml of 20mM Tris, 0.5M NaCl pH 7.5. (Fractions of 5 ml were collected)
7) The column was regenerated using 25 ml of 0.5M NaOH, 6M Urea.
8) Column was stored in water + 0.02% azide.
9) Fractions were checked on an SDS gel.
10) Protein buffer was exchanged to 20mM Tris pH 8.5 for ion-exchange purification.

**Ion-exchange-purification:**

1) HQ purification was performed using 20mM Tris pH 8.5 as buffer and eluting with 1M NaCl.
2) The protein comes out at about 30% NaCl. Fractions are checked, and the buffer is exchange to 10mM Tris pH 7.5 for crystallization.

Note: Is better to use a Mono-Q column instead of an HQ. You would think it doesn’t make a difference, but believe me it does!!! Using HQ, the protein will come out 85-90% pure, with Mono-Q is 95% pure. Purity is important for crystallographic purposes, trust me on this one!!!!!
A.II.11. Purification of zipper-stabilized, His-tagged sDM/sDR1 complexes (zDM/zDR) by Ni-NTA-agarose chromatography
Protocol by Robert Busch (Stanford University), small modifications by Zarixia Zavala-Ruiz

Introduction
This protocol describes the purification of sDM/sDR1 complexes stabilized by fusion of artificial AcidP1 and BaseP1 zippers with the sDM and sDR1 beta chains, respectively. Both beta chains have C-terminal His6 tags, allowing purification by Ni-NTA agarose. The protocol provided here is adapted from the Quiagen Ni purification handbook, with input from K. C. Garcia.

Materials
Insect cells transfected with neoR, sDMA, sDMB-AcidP1 and sDRA, and sDR1B-BaseP1 constructs

Schneider's Drosophila medium, supplemented with 10% FBS, glutamine, gentamycin (optional)

Pharmingen BaculoGold medium, supplemented with glutamine and gentamycin (optional)

PBS
Wash buffer: 50 mM sodium phosphate buffer, pH 8.0, 300 mM NaCl, 10 mM imidazole

Methods
Growing up cells
1) Maintain sterility throughout the tissue culture procedure.
2) Thaw out cells in T75 flasks and seed at 0.5-1 x 10^6 cells/ml in serum-containing medium. Cells can be slow to recover and start growing at this stage, so be patient with them. Culture at 27°C C with caps slightly open (you can use a humidified incubator, but this is not necessary if you allow only a little bit of gas exchange).
3) Once looking healthy and growing well, re-select for neoR cells using G418 (suggested starting concentration = 1 mg/ml active G418, but titrate this with your own G418 batches to find a concentration that kills untransfected S2 cells but spares transfectants). Transfected cells will slow down and enlarge under selection, but will still expand.
4) Expand into multiple T75 flasks and then successively into 100-ml, 250-ml, 500-ml and (optional) 1000-ml spinner flasks. While expanding into spinners, adapt gradually into serum-free medium (50, 75, 90, 100% serum-free after successive doublings). Keep cells upwards of 0.5 x 10^6 cells/ml throughout. With polyclonal populations (such as this one), I sometimes leave G418 in the culture during adaptation, but that’s expensive and probably dispensable.

5) Once final volume is reached, count every 2 days until limiting density is reached (should be upwards of 4 x 10^6 cells/ml). Induce expression of zDM/zDR genes by adding CuSO_4 to 1 mM from a sterile 100 mM stock in ddH_2O. Culture for another 7 days.

A protocol for single-cell cloning to isolate high expressors is available.

**Harvesting supernatant**

1) (At this point, there is no need to maintain sterility.) Spin down cells and large debris (the CuSO_4 may kill some of your cells during the induction period) at maximum speed in a benchtop centrifuge (I use 3300 rpm) for 20-30 minutes at 4°C. Supernatants may be frozen (-20°C or less) at this stage. After thawing frozen supernatants, spin again as above to remove protein precipitates (zDM/zDR complexes remain soluble during freeze/thaw).

2) Add 1 mM PMSF from 100 mM stock in dry (!) ethanol and (optional) 10 mM iodoacetamide from 1 M stock in H_2O. Azide may be added to 0.1% w/v if microbial contamination is a problem.

3) Remove particulates by vacuum filtration through 0.45 μm (or smaller) pore-size membrane filter (low protein binding).

**Buffer adjustments**

1) Exchange buffer into PBS (Any common PBS formulation should be fine; our recipe is above), using an Amicon pressure cell with a 30 kDa molecular weight cutoff. (Tangential flow ultrafiltration should work, too, and would be much faster.) This serves to remove chelating agents in the insect medium that interfere with Ni-NTA-agarose chromatography (K.C. Garcia, personal communication). I do three rounds of concentration and 5-10-fold dilution into PBS, for a 125- to 1000-fold dilution of chelating agents. Final volume can be severalfold more concentrated than starting material.

2) Spin out any precipitated matter in a benchtop centrifuge as above.

3) Adjust pH to 8.3 with 1 M NaOH, NaCl to 300 mM from 5 M stock, and imidazole to 10 mM from 1 M stock. (Alternatively, you could do the buffer exchange directly into wash buffer, rather than PBS, it works fine and it is very convenient.)

4) Filter through a 0.45 μm or smaller pore-size membrane filter. The filtrate will have a slight brownish hue, probably due to contaminating lipids. This is your crude protein extract.

**Chromatography**

1) Add 1 ml of 50% Ni-NTA-agarose slurry (0.5 ml packed column; Quiagen) to 50 ml of crude protein extract. Rotate overnight at 4°C.
2) Pour slurry into a column and drain off spent extract. (With larger volumes, you may wish to spin down the beads first).

3) Wash column extensively (100 column volumes) with wash buffer.

4) Elute column successively with wash buffer containing increasing concentrations of imidazole (I use 30 column volumes each, added in small aliquots, when working with small column volumes. Less is probably sufficient):
   - 20 mM imidazole (this fraction may contain some excess zDR chains)
   - 100 mM imidazole (this fraction should contain zDM and zDR chains in approximately equal amounts)
   - 250 mM imidazole (this fraction should contain little protein).

5) Keep column material for subsequent regeneration as per Quiagen protocol.

6) Analyze the fractions as described below. Initial capture from crude protein extract should yield material of modest purity, but zDM and zDR chains should be clearly visible in silver stained gels of the 100 mM imidazole eluate. Concentrate this fraction using Centriprep-30 ultrafiltration device.

7) Higher purity can be achieved by diluting this eluate 10fold in wash buffer without imidazole (final concentration = 10 mM imidazole) and capturing zDM/zDR complexes once more on Ni-NTA-agarose by repeating steps 1-5. A final polishing step by HPSEC can be added. Yield after 2 rounds of Ni-NTA-agarose chromatography is about 1 mg/l of supernatant.

**Analysis**

1) Determine A280 of each eluted fraction.

2) Determine purity and chain composition of eluted material by SDS-PAGE.

3) In addition, chain composition can be determined by Western blotting with anti-DM and anti-DR antisera (CHAMP), and peptide binding activity can be assessed by measuring association kinetics (I usually measure association of N-terminally biotinylated HA307-319 peptide by L243 capture ELISA with streptavidin-europium readout).
A.II.12. DM/DO expression and purification
Developed by Zarixia Zavala-Ruiz

**Cells:** *S2 insect cells*

**Expression:**
- Thaw cells (-80°C).
- Dilute thawed cells to 10 mls with media (Sf900 SFM II media + G418 + 10% Heat inactivated serum) and spin for 10 minutes at 1500 rpm.
- Discard sup and resuspend the cells in fresh media.
- As cells start growing, expand them. I usually expand them when cell density is about 4 million cells/mL, and bring them down to ~2 million cells/mL. (They doubled every 2-3 days on a shaker flask.)
- As I expand them, I also use less serum. I usually go from 10 to 7 to 5 to 3 to 1 to 0% serum.
- Induce when cell density is ~ 5-7 million cells/mL. Induce for 5 days with 1mM CuSO₄.
- Harvest cells by spinning down at 3500rpm for 25 minutes. Take the supernatant and add protease inhibitors (1mM PMSF, 1mM Iodoacetamide, 0.02% azide). Discard pellet, do not add wescodyne or bleach to centrifuge bottles, just rinse bottles with a lot of water and use the brush to scrape pellet.)

**Purification:**

*Note: If desired, take 1ml samples at every step during purification to do an ELISA and/or Western blot later.*

**Ni-NTA affinity chromatography:**
- Concentrate supernatant and exchange buffer (wash buffer) using the spiral concentrator. Wash buffer: 50mM NaH₂PO₄, 300mM NaCl, 10mM Imidazole, pH 8.0. (Once you are done make sure that the pH is 8.0 – readjust.)
- Spin down protein, 8000 rpm for 20 minutes.
- Filter protein through a 0.22μm pore-size membrane filter.
- Equilibrate Ni-NTA agarose beads (Qiagen) into the wash buffer. (I usually use 1mL of Ni-NTA per mL of starting supernatant.)
- Add the beads to the protein and let it rotate (nutator is ok) at room temperature overnight.
- Next day, elute flow through (FT) by pouring protein-beads mixture through a column. (I think is a good idea to collect and keep FT.)
- Wash column with wash buffer (~100 column volumes).
- Elute column successively with wash buffer containing increasing concentration of imidazole.
  1. 20mM Imidazole (10 CV, collect 2 fractions of 25 ml/each)
2. 100mM Imidazole (15 CV, collect 3 fractions of 25 ml/each, let
~25mL of buffer sit in the column for about 30-60 seconds and star
collecting with a slow flow rate)
3. 250mM Imidazole (10 CV, collect 2 fractions of 25 ml/each)
   - Keep beads for subsequent use.
   - Analyze fractions by Western blot using anti-αDM antibody and/ or DM ELISA.

*Gel filtration purification*
   - Gel filtration should be performed to further purified the complex (see trace
     below). A Biosep SEC3000 can be used.
**SDS-PAGE analysis of purified complex**

![SDS-PAGE Image]

**Dynamic light scattering trace of complex**

<table>
<thead>
<tr>
<th></th>
<th>Diff (μm²/s)</th>
<th>Radius (nm)</th>
<th>Mw (kDa)</th>
<th>Π (pN nm²/mg)</th>
<th>Π/poly</th>
<th>2Π/poly (nm²/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native Monomer</td>
<td>136.6</td>
<td>16.79</td>
<td>207.7</td>
<td>15.94</td>
<td>52.3</td>
<td>0.057</td>
</tr>
<tr>
<td>Fregul Peak 1</td>
<td>408.1</td>
<td>53.46</td>
<td>170.2</td>
<td>16.99</td>
<td>20.5</td>
<td>0.0422</td>
</tr>
<tr>
<td>Fregul Peak 2</td>
<td>56.76</td>
<td>41.03</td>
<td>2002.8x104</td>
<td>13.31</td>
<td>32.4</td>
<td>0.105</td>
</tr>
<tr>
<td>Fregul Peak 3</td>
<td>445.8</td>
<td>48.04</td>
<td>132.4</td>
<td>0.0694</td>
<td>16.1</td>
<td>0.0328</td>
</tr>
<tr>
<td>Fregul Peak 2</td>
<td>74.27</td>
<td>30.55</td>
<td>1107.7x104</td>
<td>9.424</td>
<td>30.8</td>
<td>0.0949</td>
</tr>
</tbody>
</table>

198
A.II.13. IgG-FC fusion cleavage on DM/DO protein

**Purpose:** Remove the Fc-fusion on DM/DO 227T. To make sure that papain can cleave Fc portion at the conditions that will be used (possibly) in the final assay I’ll use the LB3.1 antibody. Also to make sure that papain is not proteolysing protein non-specifically at the conditions that will be used in the final assay I’ll use BSA.

**Materials:**
1) LB3.1: 2mg/mL in 100mM Citrate, 50mM NaCl, 1mM EDTA, pH5.9
2) BSA: 2mg/mL in 100mM Citrate, 50mM NaCl, 1mM EDTA, pH5.9
3) Papain (dissolve solid material to 100 mg/mL in 100mM Citrate, 40mM Cysteine, 1mM EDTA, pH6.0, if the papain is in suspension also dilute with buffer, don’t forget to take into account that the units/ mg of solid are different for each papain, try to normalize by making same final dilutions)
   - Sigma (Cat #:P4762 or P4406)
   - ICN (Cat #: 0210092125 or 0210092425)
   - USB Corporation (Cat #: 19925 100MG)
4) E-64 (Stocks of 1mM in aqueous solution in -20°C)

**Protocol:**
- Turn on water bath to 37°C.
- Dissolve papain (at least the one from Sigma Aldrich) in buffer (see Materials) and activate for 10 minutes at 37°C (use within ½ hour).
- Incubate 20μg of protein (LB3.1 or BSA) that is 2mg/ml with 1μL of activated papain for x hr at 37°C (x = ½ or 3).
- After incubation is complete, add E-64 to a final concentration of ~10μM. (E-64 is a cysteine protease inhibitor, effective concentration: 1-10 μM; stock solutions of 1mM in water are stable at -20°C for months).

- Run a non reducing 12% SDS gel.
  - Don’t forget to run some controls (examples: LB3.1 by itself, BSA by itself, Papain by itself)
o Also don’t forget to do boiled and not-boiled. (There is a chance that boiling can increase activity of protease, although is no likely since I’ll be adding E-64 protease inhibitor... but just in case!)

- Stain gel.
- De-stain gel.
- Take a picture.
**A II.14. HLA-DM ELISA**
Protocol from Mia Rushe, modified by Zarxia Zavala-Ruiz

**Materials:**
1) Block Solution: 3% BSA in PBSZ
2) Dilution Solution: 0.3% BSA
   0.1% Triton X-100
   in PBS (filter and store at 4°C)
3) TBST: 25 mM Tris
   137 mM NaCl
   2.7 mM KCl
   0.05% Triton X-100
4) Alternatively you can PBST instead of TBST: PBS with 0.1% Triton X-100.

**Protocol:**
1) Coat 96-well plate with 300 ng/well of purified G2 antibody. Incubate for 2 hours at room temperature (or overnight in the cold room).
2) Block plates with block solution for at least 4 hours at room temperature. (It is okay to store the plates in the cold room at this stage and use them later, they are good for about 3 weeks.)
3) Standards: DO 2-fold dilutions in duplicate starting with 25 ng/well: 25 ng, 12.5, 6.25, 3.125, 1.56, 0.78, 0.39, 0.19, 0.1, 0.05, 0.025 and 0. Dilute samples into dilution solution. (0.3% BSA, 0.1% triton X-100, in PBS) Note: This ELISA is VERY sensitive, I recommend not going above 25 ng/well.
4) This ELISA does not have a big range, so make sure you do lots of dilutions of your samples, this way you can get a reading that you can use! Use dilution solution for dilutions.
5) Incubate standards and samples overnight at 4°C.
6) Wash 3 times with PBST or TBST. (Be careful when washing the first 2 times, you have different samples in each well and you don’t want the wells to spill into one another.)

7) Add 100 μL/well of rabbit anti-DM serum diluted 1:1000 in dilution solution. Incubate for 2 hours at room temperature.

8) Wash 3 times with PBST or TBST.

9) Add with 100 μL/well of goat anti-rabbit-HRP, 1:4000 dilution in PBST. Incubate for 1 hour at room temperature.

10) Wash 3 times with PBST or TBST.

11) Add 200 μL of ABTS/well and read absorbance at 405 nm.

12) Work up data.
A.II.15. WESTERN BLOT—ALKALINE PHOSPHATASE DETECTION
Procedure from L. Stern

**Note: This procedure will detect human DR1 proteins. If using another protein, the antibodies used will vary, but the procedure should still work.

Run an SDS gel using pre-stained molecular weight markers with two copies of each lane (including the markers). The duplicates should be arranged so you can cut the gel in half and get identical lanes on each half.

After running the gel, cut off part of the loading gel so that only nubs from the comb region remain.

Wet a gel-sized rectangle of transfer membrane with 70% EtOH.

Soak the membrane and 6 gel-sized rectangles of filter paper in transfer buffer.

Place the transfer membrane flat on the gel. Remove any bubbles gently.

Sandwich the transfer membrane and the gel between the filter paper rectangles soaked in Transfer Buffer—3 sheets on each side. Remember which side has the transfer membrane!

Place the sandwich flat on the blotter with the membrane on the cathode (+) side, and the gel on the anode (-) side. Once again, gently remove any bubbles.

Close the blotter and use screws to create even, light pressure between the plates. Do not over tighten screws.

Run the blotter at constant amperage: 400mamps for about ½ hour, or until most of the protein is transferred from the gel to the membrane (watch the pre-stained MW markers). When it is done, turn off the power source and open the blotter.

Remove the gel and the membrane from the blotter. Cut the transfer membrane into the 2 identical halves.

Place ½ of the membrane into Coomassie stain in a clean petri dish.

Place the other ½ of the membrane into Block Solution in a clean petri dish. Leave both on a shaker table at room temperature for 1 hour to overnight.

Place the membrane from the Coomassie into 50% methanol or isopropanol and 50% normal gel de-stain. Leave this membrane on the shaker table at room temperature, changing the de-stain mixture periodically, until it has de-stained properly. **This may take a long time!
Rinse the membrane in the Block Solution with TBST.

Add a 1:25,000 dilution of Champ in TBST to the petri dish and membrane. Leave on the shaker table for 1 hour at room temperature.

Wash WELL with TBST—4 times, 5 minutes each on the shaker table.

Add a 1:1000 dilution of anti-rabbit polyclonal antibody linked to alkaline phosphatase in TBST to the petri dish and membrane. Leave on the shaker table at room temperature for ½ hour.

Wash WELL with TBST—4 times, 5 minutes each on the shaker table.

Add 10 mL Alkaline Phosphatase Buffer with 70 μL NBT and 70 μL BCIP. The color should begin to develop on the membrane almost immediately. **Do not overdevelop!**

Stop the reaction by rinsing with ddH₂O.
A.II.16. Calculation of Apparent Molecular Weight from Gel Filtration

1) Run molecular weight standards (Bio-rad) containing thyroglobulin (670 kDa), γ-globulin (158 kDa), ovalbumin (44 kDa), and myoglobin (17 kDa) under the same conditions as you would your sample.

2) Run the samples of interest. Note: if you take the column off the HPLC for any reason, or introduce air in to the lines you cannot compare the samples or the standard curve and need to start over.

3) Determine the elution volume (ml) of the peak for both your standards and samples.

4) For the standards, make a plot of elution volume versus log MW.

5. Determine the log MW for the samples by linear regression, and convert to MW to obtain the $MW_{app}$.

Sample standard curve for BioRAD molecular weight markers (Figure courtesy of Jennifer Ogodnick-Cochran)
A.III. Characteristics HLA-DR1/peptide/SEC3-3B2 crystals

- Pictures of crystals characteristic of HLA-DR1/peptide/SEC3-3B2. I have noticed that the morphology of the crystals is very similar in all the complexes that I have crystallized. It takes about 3-4 weeks to get crystals with adequate size for data collection.

- The picture below shows a diffraction pattern characteristic of HLA-DR1/peptide/SEC3-3B2 crystals. This particular diffraction is from the crystal used to solve the structure with the Ac-FVKQNAAL peptide (Resolution limit 2.1Å). The details of this structure can be found in chapter 3.
- Space group of these crystals is R3 (although it is a good idea to double-check). Twinning is very common in this space group therefore, I recommend testing the data collected.

- The solvent content in these crystals is ~70%. The crystal packing characteristic of the structure is shown below.
Zarixia Zavala-Ruiz
zarixia@mit.edu
MIT Dept. of Chemistry
Cambridge, MA 02139
(508) 856-8804/ (617)251-6998

Education:
• Massachusetts Institute of Technology (MIT), Ph.D. in Biological Chemistry. June 2004
• University of Puerto Rico, B.S. magna cum laude in Chemistry. May 1999

Research Experience:
• Graduate Research Assistant 2000 to present, Department of Chemistry, MIT. Advisor: Lawrence J. Stern
  Investigating the mechanism of peptide ligand binding to human Class II Major Histocompatibility Complex Protein, HLA-DR1, an immune system protein that activates the T cell response

• Undergraduate Research Assistant June 1996-May 1999, Minority Access to Research Careers, Department of Chemistry, University of Puerto Rico, Rio Piedras, PR. Advisor: Reginald Morales. Study of heparin inhibition of phospholipase A2 as a probe to study lipids domains in the red cell membrane

• Undergraduate Research Assistant summer 1998, Summer Honors Undergraduate Research Program, Department of Pathology, Harvard Medical School, Boston, MA. Advisor: John Aster. Analysis of the folding of the first Lin12 module of human Notch-1.

• Undergraduate Research Assistant summer 1997, Leadership Alliance Early Identification Program, Brown University, Providence, RI. Advisor: Sharon Rounds. Investigated the role of protein tyrosine phosphatase in ATP/adenosine-induced apoptosis in cultures pulmonary artery endothelial cells.

Awards:
• NIH Graduate Fellowship, 2001 to present
• John Axtmeyer Award- May 1999
• American Chemical Society- Minority Undergraduate Fellowship, September 1996-May 1999
• Dean’s List, 1995-1999

Publications: