Insight into the Structure and Function of Empty Class II Major Histocompatibility Complexes

by

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ABSTRACT

Class II major histocompatibility complex (MHC) proteins bind peptides and present them at the cell surface for interaction with $CD4^+$ T cells as part of the system by which the immune system surveys the body for signs of infection. Peptide binding is known to induce conformational changes in class II MHC proteins on the basis of a variety of hydrodynamic and spectroscopic approaches, but the changes have not been clearly localized within the overall class II MHC structure. Local structural changes were mapped for HLA-DR1, a common human class II MHC variant, using a series of monoclonal antibodies which recognize the beta subunit and are specific for the empty conformation. Additional structural information was obtained using side chain-specific chemical modification and identification of modified residues by in-gel tryptic digestion and mass spectrometric peptide mapping. Together, the chemical modification studies and the mapping results illuminate aspects of the structure of the empty forms and the nature of the peptide-induced conformational change.

Empty class II MHC proteins have been observed on the surface of immature dendritic cells in both humans and mice. Immature DC also secrete a protease activity that is capable of generating antigenic peptides from whole antigen. The protease activity secreted by dendritic cells is characterized and the role of empty MHC proteins in dendritic cell antigen presentation is discussed.

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Abbreviations used in this thesis:

AEBSF: aminoethyl-benzene sulfonyl fluoride

AMC: aminocoumarin

APC: antigen presenting cell

BSA: bovine serum albumin

CBZ: benzyloxycarbonyl

CK: chloromethylketone

CNS: central nervous system

CTL: cytotoxic T lymphocyte

DC: dendritic cell

DMEM: Dubelco's minimal essential medium

DR1: HLA-DR1

DTT: 1,4-dithiothreitol

E64: trans-epoxysuccinyl-L-leucylamido-(4-guanidino)butane

EDTA: ethylene-diamine-tetraacetic acid

ELISA: enzyme linked immunosorbent assay

FBS: fetal bovine serum

FITC: fluorescein isothiocyanate

GMCSF: granulocyte macrophage colony stimulating factor

HA: hemagglutinin

HEL: hen egg lysozyme

HLA: human leukocyte antigen

IAA: iodoacetamide

LPS: Lipopolysaccharide

mAb: monoclonal antibody

MHC: major histocompatibility complex

NHS: N-hydroxysuccinimide

PBMC: peripheral blood mononuclear cells

PBS: phosphate-buffered saline

PBST: phosphate-buffered saline containing 0.1% Triton-X 100

PMSF: phenylmethylsulfonyl fluoride

pNA: para-nitroanaline

PNS: post nuclear supernatant

SA: streptavidin

SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SPR: surface plasmon resonance

TCR: T cell receptor

TLCK: tosyl-lysylchloromethylketone

TPCK: tosyl-phenylchloromethylketone

I. Introduction: Major Histocompatibility Complexes

I. A. Introduction

Microorganisms such as bacteria and viruses are encountered on a regular basis during the activities of daily life. Most are detected and destroyed by the host without perception or prolonged incubation periods. This is because the body employs a multi-layered set of defenses to deal with such occurrences. The innate immune response consists of mechanisms which are present and employed at all times. Examples of the innate immune system include the skin and mucosa which exclude pathogens based on their extra-organismal location. Pathogens which overcome these barriers must then deal with cell-mediated aspects of the innate immunity including the activation of compliment and the engulfment of microorganisms by phagocytes. Innate responses occur rapidly without the need for clonal expansion or cell maturation.

If an infectious organism is able to breach these early lines of defense, an adaptive immune response will ensue, with the generation of antigen specific cells which specifically target a particular pathogen. These effector cells which eliminate pathogens and the memory cells which prevent reinfection make up the adaptive immune response. In most cases, an adaptive immune response confers lifelong protection against reinfection by the same antigen.

One of the most important aspects of the adaptive immune response is the processing and presentation of foreign material by specialized cells called antigen presenting cells (APCs). In this chapter, I will examine antigen presentation and the

structure of class II major histocompatibility complex (MHC) proteins and their involvement in the generation of antigen-specific immune responses.

I. B. The Antigen Presentation Pathway

I. B.1 Generation and trafficking of MHC molecules

Major histocompatibility complex (MHC) molecules are heterodimeric cell-surface proteins that play an important role in the initiation of antigen-specific immune responses (reviewed in (Watts, 1997)). Class I MHC molecules are expressed on all cell types and are responsible for binding and presenting antigenic peptides derived from intracellular antigens. Cytoplasmic proteins are degraded via the proteasome complex, ((Rock and Goldberg, 1999)), and the resultant peptides are transported into the ER by the TAP membrane transporter, loaded onto class I MHC proteins by specialized machinery, and finally presented on their cell surface for inspection by $CD8^+$ T cells (Pamer and Cresswell, 1998).

Membrane bound antigens and foreign antigens which are located in the extracellular milieu are recognized by a different class of MHC molecule. Class II MHC proteins bind peptides derived from extracellular, endosomal, and internalized cell-surface antigens, and present them at the cell surface for inspection by $CD4^+$ T cells. This is accomplished by a set of specialized cells called antigen presenting cells (APCs) made up of macrophages, B cells and dendritic cells (DCs).

Like other cell surface proteins, class II MHC molecules are synthesized in the endoplasmic reticulum (Figure I.1). The α and β chains are synthesized separately and immediately associate with the invariant chain chaperone (Ii) (Cresswell, 1996). The

invariant chain places an extended loop in the peptide binding site of the MHC which prevents binding of peptides or unfolded proteins present in the endoplasmic reticulum. The invariant chain also contains a targeting signal which directs the complex through the golgi to the endosomal compartments where peptide loading can occur (Brachet et al., 1997).

I. B.2 Antigen processing, loading and presentation

The antigen processing and presentation pathways utilized by B cells have been well characterized (reviewed in (Watts, 2001)). The pathways present in other APCs are less well understood. It is possible that alternative pathways exist in dendritic cells and macrophages (Guermonprez et al., 2002; Rovere et al., 1998).

Once the MHC-Ii complex reaches the endosome, the invariant chain chaperone is cleaved by proteases such as cathepsin S and dissociates away from the MHC molecules leaving a fragment of Ii, called CLIP (for class II associated invariant chain peptide) bound within the peptide binding site of the MHC At the same time, extracellular proteins are brought into the endosome through endocytosis and macropinocytosis. These antigens are degraded by endosomal resident proteases resulting in peptide fragments which can bind to class II MHC molecules (Villadangos et al., 1999).

A specialized MHC-like molecule, HLA-DM catalyzes the loading of MHC molecules with peptides (Figure I.1). HLA-DM binds to and stabilizes empty class II molecules which might otherwise aggregate. HLA-DM helps catalyze peptide binding and release through conformational effects on DR1 (Zarutskie et al., 2001) and several models of interaction have been proposed (Beeson and McConnell, 1994; Chou and

Sadegh-Nasseri, 2000; Dornmair et al., 1989; Sadegh-Nasseri and McConnell, 1989; Schmitt et al., 1999b; Zarutskie et al., 2001). Many of these models involve HLA-DM recognizing or promoting a structural change in HLA-DR which could facilitate peptide release.

Upon stable binding of a new peptide, MHC-peptide complexes then are delivered to the cell surface where their ultimate engagement by $CD4^+$ T cells induces a signaling cascade within the T cell thereby generating an immune response.

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I. C. Structure of class II MHC proteins

I. C.1 Structure of HLA-DR1 with bound peptide

The class II MHC is a ~60 kDa heterodimeric cell surface glycoprotein consisting of two noncovalent chains α and β. Three dimensional structures have been determined for peptide complexes of several polymorphic variants of both human and murine class II MHC molecules (reviewed in (McFarland and Beeson, 2002)). Both the MHC α and β chains contribute to the peptide binding site, which is made up of a beta sheet floor topped by two roughly parallel alpha helical regions. Each subunit contributes an immunoglobulin-like domain below the peptide binding site, as well as short transmembrane and cytoplasmic domains. Peptides bind in an extended conformation in the groove between the two helices, with approximately 10 residues able to interact with the MHC protein, and the peptide termini extending from the binding site. This conformation, similar to a polyproline type II helix, has a 2.7 residue repeat and appears to be dictated by a network of 12–15 hydrogen bonds involving the MHC main chain

(α51–53) and conserved MHC residues (α62, α69, α71, β61, β81, and β82) (Figure I.3) (Jardetzky et al., 1996). The conformation places 4-6 of the peptide side chains into pockets within the overall groove (Figure I.4). The residues lining these pockets vary between allelic variants, providing different peptide-sequence binding specificity. Overall the interaction buries approximately 70% of the peptide surface area in the central region of a bound peptide, leaving the remainder available for interaction with antigen receptors on T cells (Stern and Wiley, 1994).

I. C.2 MHC Conformational changes upon peptide binding

Although the canonical structure visualized by X-ray crystallography is relatively stereotyped, a number of studies have suggested that alternate conformations of class II MHC molecules can exist under certain conditions (Natarajan et al., 1999a; Natarajan et al., 1999b; Sato et al., 2000; Zarutskie et al., 1999). Kinetic studies of the peptide binding reaction indicate a multi-step binding pathway, suggesting that peptide binding is accompanied by a conformational change in the MHC. (Beeson and McConnell, 1994; Beeson et al., 1996; Mason et al., 1995; Mason and McConnell, 1994; Sadegh-Nasseri and McConnell, 1989; Sadegh-Nasseri et al., 1994). Conformational transitions between such isomers have been measured by NMR (Schmitt et al., 1999a). Other studies have shown that at low pH, MHC molecules shift their equilibrium from a "closed" to an "open" or "peptide-receptive" state (Dornmair et al., 1989; Jensen, 1990; Sadegh-Nasseri and Germain, 1991). These alternate conformations have been suggested to play an important role during the peptide binding reaction, and also in the interaction with the endosomal peptide exchange factor HLA-DM (Denzin and Cresswell, 1995; Kropshofer

et al., 1996; Natarajan et al., 1999a; Rabinowitz et al., 1998). The change can be induced by binding any of a large variety of peptides with very different sequences and affinities, including a capped dipeptide (Sato et al., 2000), and also by filling the P1 side chain binding pocket through mutagenesis (Natarajan et al., 1999b).

I. C.3 Empty class II MHC molecules

Little structural information is available for the empty form of the protein. Spectroscopic and hydrodynamic studies on HLA-DR1, a common human class II protein and the subject of this study, have shown that a distinct conformational change occurs upon peptide binding, suggesting that the conformation of the empty protein is different from that of the peptide-loaded form (Joshi et al., 2000; Zarutskie et al., 1999). In contrast to DR1-peptide complexes which migrate as a single heterodimeric band on SDS-PAGE, the empty DR1 subunits dissociate and migrate as monomeric α and β chains (Figure I.5) in the absence of peptide. The empty protein also has reduced thermal stability and reduced unfolding cooperativity relative to the peptide bound form consistent with a looser conformation for the empty protein. Additionally, the conformational change results in a decrease in hydrodynamic radius from 35 Å to 29 Å, together with a small increase in helical content as observed by circular dichroism (Zarutskie et al., 1999).

There are a number of possible changes in secondary structure which could account for the observed spectroscopic and hydrodynamic parameters. Partial local unfolding of certain regions in the absence of peptide might provide a likely explanation. The peptide binding domain makes a number of conserved hydrogen bonds with the

peptide backbone in the peptide bound form. Lack of a bound peptide would result in the loss of these hydrogen bond interactions creating a more open structure and allowing the alpha helical regions to relax or become flexible and move away from the center of the antigen binding. Helix packing upon peptide binding (and formation of hydrogen bonds) could prevent peptide release and provide a physical mechanism for a kinetic trap in order to maintain long enough peptide half-lives for the complex to traffic to the cell surface for inspection by $CD4^+$ T cells.

Another potential change includes a shift in the orientation of the immunoglobulin-like (Ig-like) domains relative to the peptide binding site or relative to each other. In class II MHC proteins, the lower domains are docked onto the peptide binding domain through interactions between a loop at the top of the domain and a depression beneath the P1 pocket (Murthy and Stern, 1997). Residues in the pocket could communicate changes in the peptide binding domain to the lower domains.

I. D. Placing the work of this thesis into context

I. D.1 Importance of empty MHCs in the immune system

Although most of the work to date has focused on *in vitro* characterization of empty MHCs, they may play an important role in the immune system *in vivo*. Empty class II MHC molecules are stable (relative to empty MHC class I molecules), and their presence has been observed on the surface of immature dendritic cells in both human and murine cells (Santambrogio, 1999b; Setterblad et al., 2003). Several mechanisms for a physiological role of the empty cell-surface class II MHC molecules can be envisioned. Empty MHC molecules could function as antigen-specific endocytic receptors similar to

previously identified dendritic cell receptors such as the mannose and Fc receptors (Sallusto et al., 1995; Sallusto and Lanzavecchia, 1994) and DEC-205 (Jiang et al., 1995).

In an alternative mechanism, empty cell-surface class II MHC molecules could bind peptide antigens at the cell surface. In this mode, empty MHC molecules could capture peptide antigens directly from the extracellular milieu and present them to T cells without internalization or further processing. This pathway could be extremely useful to the sentinel function of DC and would serve to broaden the peptide repertoire by preserving antigenic peptides that might otherwise be terminally degraded in the extremely proteolytic endosomal/lysosomal environment.

Empty MHCs may also play a role in positive selection of T cells during development as well as in the modulation of T cell responses by regulatory APCs, however the details of these potential functions has not yet been studied.

I. D.2 Overview of thesis

This thesis describes experiments designed to better understand the structure and function of empty class II MHC molecules and their contribution to adaptive immune responses.

Chapter II describes the generation and characterization of monoclonal antibodies (mAbs) which are specific for the empty conformation of HLA-DR1. These reagents were used to infer structural information about the empty conformation of DR1. The binding epitope of each mAb was mapped using a number of independent methods in order to determine the residues important for binding to empty MHCs. This chapter was published in the *Journal of Biological Chemistry* (Carven et al., 2004).

Chapter III describes characterization of the surface topology of the empty conformation of HLA-DR1 utilizing side chain-specific chemical modification of arginine, lysine, tyrosine, and tryptophan residues. Modifications were differentially incorporated into empty and peptide loaded DR1 and sites of modification were identified by digestion with trypsin and mass spectrometric mapping. This chapter has been prepared as a manuscript and submitted to *Biochemistry* for publication.

Chapter IV describes the presence of empty MHC molecules on the surface of dendritic cells. An alternate pathway of antigen processing and presentation in dendritic cells is proposed. As part of the proposed pathway, a novel secreted protease activity is observed and characterized. This work was part of a larger project done in collaboration with Aaron K. Sato (Ph.D. 1998) and Laura Santambrogio (currently at Albert Einstein College of Medicine). My role in this project was the characterization of a protease activity which is secreted by dendritic cells. The results of the work discussed in this chapter were included as part of a paper published in the *Proceedings of the National Academy of Sciences* (Santambrogio, 1999a).

Chapter V describes other work done characterizing the protease activity present on the surface of microglial exosomes. This work was part of a collaborative project with Illaria Portoliccio (Harvard University) and Laura Santambrogio in which we investigated the role of exosomes that are secreted by microglial cells. This work has been prepared as a manuscript and submitted to the *Journal of Biological Chemistry* for publication.

Chapter VI summarizes the conclusions of this thesis, particularly the physiological implications of the conformational change and the interaction of empty

class II MHC proteins with the peptide exchange factor, HLA-DM. The second part of the chapter discusses possible future directions of research based on the work presented here.

The Appendices include detailed protocols used or developed during my graduate career, tables of content for my research notebooks and a biographical note.

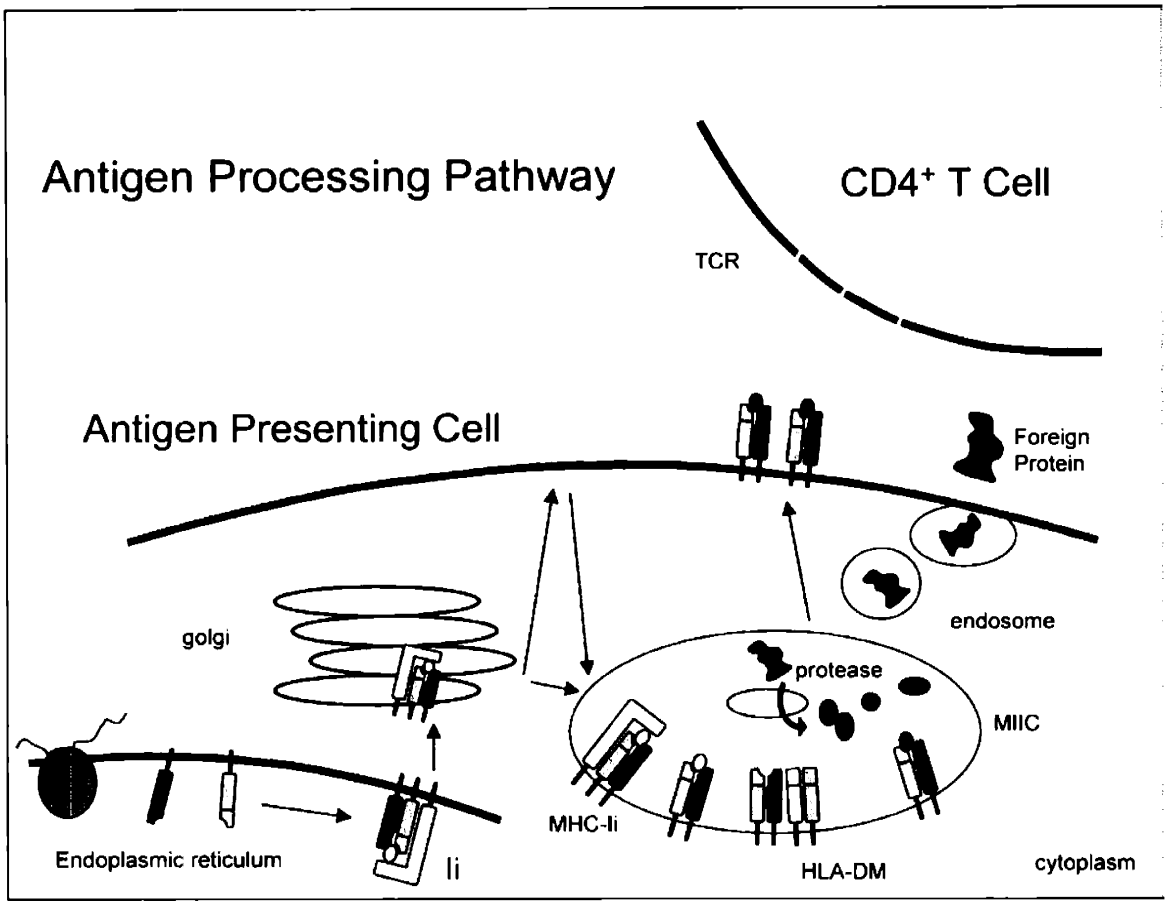


Figure I.1: Antigen Processing Pathway

Class II MHC subunits are synthesized in the endoplasmic reticulum and associate with the invariant chain. MHC-Ii complexes travel through the golgi complex and are targeted to late endosomal compartments calles MIIC. Extracellular proteins enter the cell through the endocytic pathway where they are degraded by proteases to form small peptides. MHC-Ii complexes. The proteases in the MIIC degrade the invariant chian, leaving only the CLIP peptide bound to the MHC. The peptide exchange factor HLA-DM binds to the MHC-CLIP complex and facilitates the exchange of peptide for a peptide derived from the endocytosed antigen. The new MHC-peptide complex travels to the surface of the antigem presenting cell and displays its antigen for inspection by CD4+ T cells via the T cell receptor (TCR).

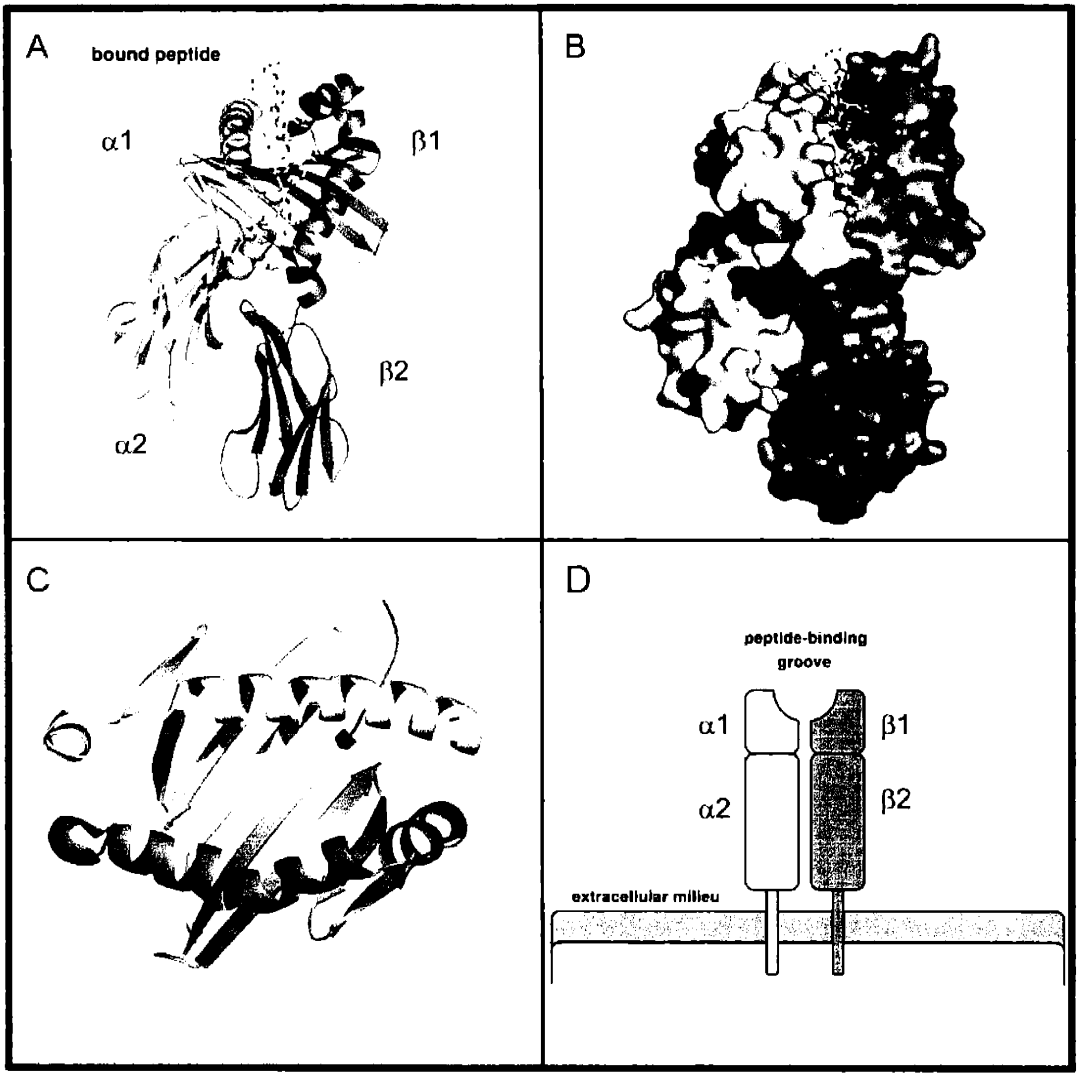


Figure I.2: Structures of class II MHC HLA-DR1

In all panels, the α subunit is colored light green, the β subunit blue, and the peptide yellow. A) HLA-DR1 cartoon structure with bound peptide. The binding cleft is formed by a β-sheet floor topped by two α-helices. Each subunit contributes an immunoglobulin-like domain below the peptide binding site, as well as short transmembrane and cytoplasmic domains. B) Surface view as in panel (A). C) Top-down view of the peptide binding site (peptide not shown). D) Organization of class II MHC protein domains on the cell surface.

Figure I.3: Sequence independent main chain interactions

Top down cartoon view of the HLA-DR1 peptide binding site. The α chain is shown in light green, β chain in blue. Bound Ha peptide is shown as stick model. Protein side chains make hydrogen bonds (indicated as dashed white lines) with peptide backbone atoms conferring sequence independent binding affinity.

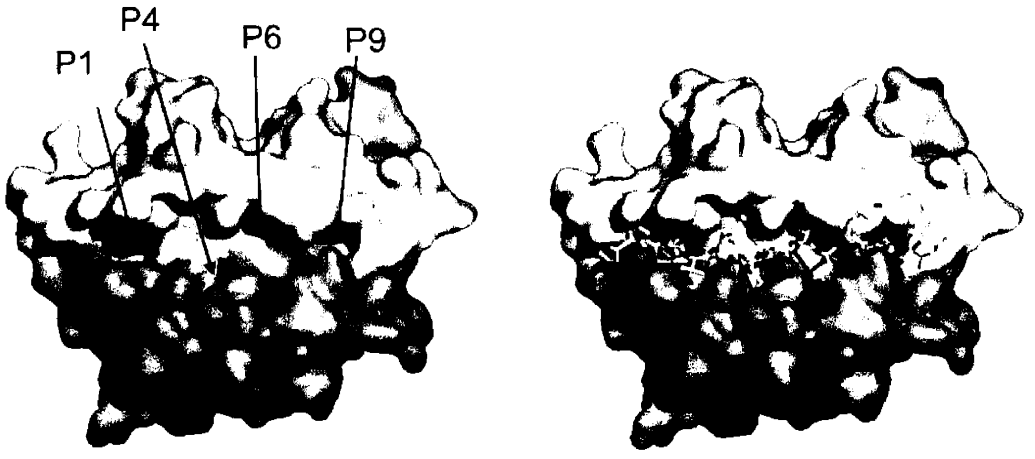


Figure I.4: Class II MHC Peptide Binding Site

Top down surface view of the HLA-DR1 peptide binding site. Alpha chain is shown in light green, beta chain in blue. Pockets are labeled (left, peptide not shown) from N- to C-terminal position relative to the bound peptide. The peptide places side chains into pockets within the overall groove (right). Pockets confer sequence specific binding affinity.

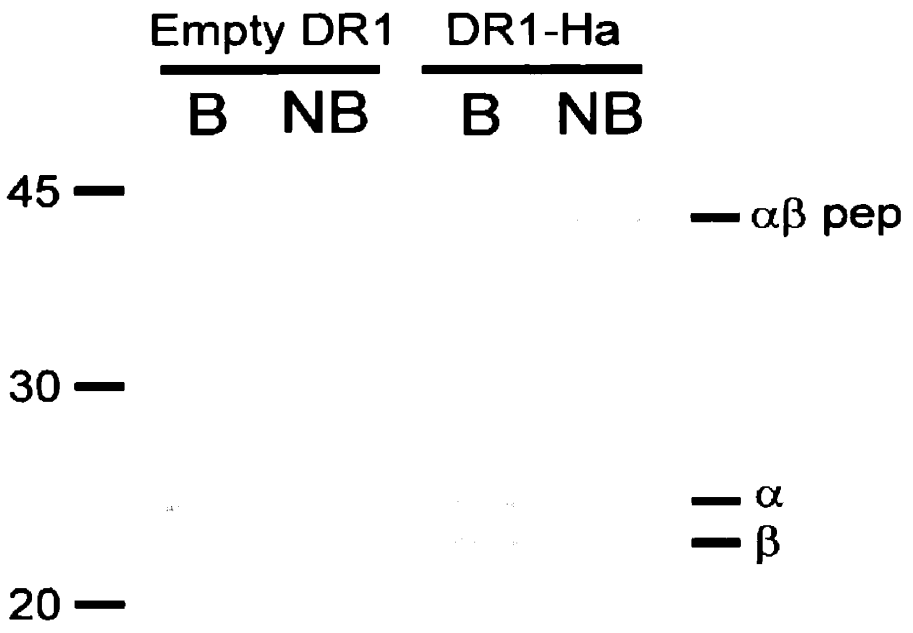


Figure I.5: Empty and peptide-loaded DR1

SDS-PAGE with samples boiled (B) or not boiled (NB) before loading. Empty DR1 produced by folding in vitro subunits produced in E. coli is sensitive to SDS-induced αβ dissocation (lanes 1 and 2), while peptide-bound DR1-Ha is resistant (lanes 3 and 4). Protein was detected with colloidal commassie (GelCode Blue).

II. Monoclonal Antibodies Specific for the Empty Conformation of HLA-DR1 Reveal Aspects of the Conformational Change Associated with Peptide Binding

SUMMARY

Class II MHC proteins bind peptides and present them at the cell surface for interaction with $CD4^+$ T cells as part of the system by which the immune system surveys the body for signs of infection. Peptide binding is known to induce conformational changes in class II MHC proteins on the basis of a variety of hydrodynamic and spectroscopic approaches, but the changes have not been clearly localized within the overall class II MHC structure. To map the peptide-induced conformational change for HLA-DR1, a common human class II MHC variant, we generated a series of monoclonal antibodies recognizing the beta subunit which are specific for the empty conformation. Each antibody reacted with the empty but not the peptide-loaded form, for both soluble recombinant protein and native protein expressed at the cell surface. Antibody binding epitopes were characterized using overlapping peptides and alanine scanning substitutions and were localized to two distinct regions of the protein. The pattern of key residues within the epitopes suggested that the two epitope regions undergo substantial conformational alteration during peptide binding. These results illuminate aspects of the structure of the empty forms and the nature of the peptide-induced conformational change.

II.A. Introduction

Conformation-sensitive monoclonal antibodies have long been used to investigate structural properties of proteins (Goldberg 1991). These antibodies are able to distinguish between two or more structural forms of a protein, and are useful as structural and biological probes of the molecular surface. Mapping the epitopes of such antibodies can provide information on the nature of the structural change and on the location of the regions involved (Fedorov, Friguet et al. 1992). In previous work, the happenstance cross-reactivity of an antibody raised against the murine MHC protein I-A^s was used to help map regions involved in the peptide induced conformational change in human HLA-DR1, which is only 66% identical with I-A^s.

In the present work, a set of monoclonal antibodies directed against the beta chain of HLA-DR1, raised by immunization with unfolded beta subunit, was screened for the ability to distinguish between empty and peptide-loaded conformations of the intact, folded protein. In an effort to gain site-specific structural information about the peptide-induced conformational change, the epitope of each of these antibodies was mapped. Two distinct regions of the MHC beta chain that are accessible in the empty but not peptide-loaded conformation were identified: one nearby but not coincident with the peptide binding site, and one across the molecule near the membrane-spanning region.

II. B. Materials and Methods

II. B. 1. Recombinant MHC Molecules

Empty and peptide-loaded DR1 were prepared by expression in *Escherichia coli* and folded *in vitro*, as previously described (Frayser, Sato et al. 1999). Briefly, HLA-DR1 extracellular domains were expressed individually as insoluble inclusion bodies, isolated by denaturing ion exchange chromatography, and refolded *in vitro* without peptide. Peptide complexes were prepared by incubating immunoaffinity-purified empty HLA-DR1 (1-5 uM) with at least 5 fold molar excess peptide for 3 days at 37°C in PBS. The resultant peptide-DR1 complexes (or HLA-DR1 which had not been loaded with peptide and had been stored at 4°C) were purified by gel filtration to remove aggregates and unbound peptide, and stored at 4°C. HLA-DR allelic variants other than HLA-DR1 (and HLA-DRB5*0101 (Li, Li et al. 2000)) have been difficult to prepare by this method, and so HLA-DR4 and HLA-DR52 (and control HLA-DR1) were produced instead using an insect cell expression system (Stern, Brown et al. 1994; Cameron, Norris et al. 2002). Briefly, HLA-DR extracellular domains were secreted by *Drosophila* S2 cells transfected with genes carrying endogenous signal sequences, and HLA-DR proteins were isolated from conditioned medium by immunoaffinity and loaded with peptide as described above for HLA-DR1 from *E. coli.*

II. B. 2. Peptide synthesis and purification

Peptides were synthesized using an Advanced ChemTech 490 synthesizer and standard Fmoc (N-(9-fluorenyl)methoxycarbonyl) chemistry. Biotinylated peptides were produced by addition of biotin-LC-LC-NHS ester (succinimdyl-6'-(biotinamido)-6-hexanamido hexanoate, Pierce, Rockford, IL) to the deprotected N-terminus of each peptide while still on the solid

support. The reaction was carried out overnight in dimethylformamide with a catalytic amount of diisopropylethylamine. Crude peptides carrying either free amino- or biotin-LC-LC termini were deprotected and cleaved with a solution of 83% trifluoroacetic acid, 5% phenol, 5% water, 5% DTT, and 2% triisopropyl silane, and then precipitated in ether, washed, and lyophilized. Peptides were purified by reverse phase HPLC using a gradient of 2% to 100% acetonitrile over 80 minutes. Purity was determined by analytical HPLC and mass spectrometry.

II. B. 3. Development of monoclonal antibodies

Mice (BALB/c x B10.A F1 hybrid) were immunized with purified, insoluble DR1 beta chain (DRB1*0101) expressed in *E. coli* inclusion bodies. Hybridomas were obtained by standard techniques, including fusion with Sp2/0 myeloma, selection in HAT medium and repeated cloning by limiting dilution. After primary ELISA screening of hybridoma supernatants for reactivity with the denatured DR1 beta subunit immunogen, the positive supernatants were screened for their ability to distinguish empty DR1 from HA-peptide loaded DR1 using ELISA (see below). After three rounds of subcloning and screening, four monoclonal hybridomas were selected for further study. Each of the four antibodies selected (MEM-264, MEM-265, MEM-266, and MEM-267) reacted with empty but not HA-peptide loaded DR1 as well as several other DR allelic variants (see below). For western blotting, purified DR proteins, or whole-cell lysates of B cells or transfectants, were separated by SDS-PAGE, transferred to nitrocellulose or Immobilon-P (Millipore), and blocked with 1% BSA before incubation with the monoclonal antibodies. Bound antibodies were detected using alkaline phosphatase-coupled, affinity-purified, goat anti-mouse IgG. For epitope mapping and

cellular studies, the monoclonal antibodies were produced either as ascites in mice or in hybridoma culture using serum-free medium (Hybridoma SFM, Gibco), and were purified by protein A affinity chromatography. The antibody class of each mAb was determined by enzyme immunoassay using a mouse-hybridoma subtyping kit (Roche).

II. B. 4. Sandwich ELISA

A sandwich ELISA was used for determination of the relative binding of antibodies to empty and peptide loaded HLA-DR1. Purified antibodies (1 μg/mL unless noted) were immobilized onto Dynatech Immobilon-4 polystryrene 96-well plates, by incubation for 4 hours at room temperature (or overnight at 4°C). Non-specific binding sites were blocked by incubation with 3% BSA in PBS overnight at 4°C. Antibodies present in ascites fluid or hybridoma culture supernatant were bound to goat anti-mouse IgG that had been immobilized as described above for purified antibodies. After antibody coating, the plates were washed three times with TBST (25 mM Tris, 0.15 M NaCl 0.05% Tween 20 pH=7.4). Dilutions of empty HLA-DR1, HA-peptide loaded HLA-DR1, or HLA-DR1 complexes with other peptides were prepared in PBS containing 0.1% Tween 20 and 0.3% BSA, and incubated with the immobilized antibodies for 2 hr at 25°C. After binding, the plates were washed three times with TBST, incubated for 1 hour at 25°C with rabbit anti-DR1 antiserum ("CHAMP") diluted 1/25,000 in PBST, and washed again. Finally, the plates were incubated with peroxidase-labeled goat anti-rabbit Fc (Roche) diluted 1/4000, washed, and developed using the colorimetric peroxidase substrate ABTS (Roche). Absorbance increase due to peroxidase activity (405nm) was measured in a microtiter plate reader (Wallac, Perkin Elmer Life Sciences, Boston, MA). For complexes of the very weakly-bound peptide YRAL, excess

peptide was included in the HLA-DR1 incubation. Apparent antibody binding affinities and optimum pH for each antibody was determined using this ELISA as well.

A variation of the sandwich ELISA was used to estimate antibody-peptide affinity. Plates were coated with MEM antibodies, blocked, and washed as above, and then incubated with varying concentrations of biotinylated peptide for 1 hour at 25°C. Plates were washed three times with TBST, and binding was detected with peroxidase-labeled streptavidin and the colorimetric peroxidase substrate ABTS, as above. The concentration of half-maximal binding is reported as a measure of relative binding affinity.

Another variation of the sandwich ELISA was used to confirm that the antibodies react with intact DR1 αβ heterodimers and not only isolated β subunits. Plates were coated with MEM antibodies, blocked, and washed as above, and then incubated for 1 hour at 25°C with various concentrations of empty DR1 that had been biotinylated on the a cysteine residue introduced at the C-terminus of the alpha chain(Cochran and Stern 2000). Plates were washed three times with TBST, and binding was detected with alkaline phosphatase-labeled streptavidin and the colorimetric substrate p-nitrophenylphosphate (both from KPL, Gaithersburg, MD).

II. B. 5. Epitope mapping- Direct binding ELISA

For epitope mapping, antibody-peptide interaction was measured using a direct ELISA with immobilized peptides and immunochemical detection of bound antibody. Ninety-six-well microtiter plates (as above) were coated with streptavidin (Prozyme, San Leandro, CA) at a concentration of 500 ng per well (overnight at 4°C, in PBS). Plates were then blocked with 3% BSA in PBS for 3h at 25°C to block nonspecific binding sites. After washing three times with

TBST, biotinylated peptides were added at a concentration of 10 μM in PBS containing 0.1% Tween 20 and 0.3% BSA and allowed to bind for 1 hour at 25°C. Plates were washed with TBST and test mAbs (100 uL of 1 ug/mL mAb) were added, followed by incubation for 1 hour at 25°C. Plates were again washed with TBST three times and binding was determined using a peroxidase labeled goat anti-mouse antibody, as above (Roche). Plates were developed using ABTS and absorbance was measured in a microtiter plate reader.

II. B. 6. Epitope mapping - Competition ELISA for peptide screening

Antibody-peptide interaction was also measured using a competition ELISA, in which soluble peptide and empty HLA-DR1 competed for binding to immobilized antibody, with immunochemical detection of bound HLA-DR1. Microtiter plates were coated with test mAbs (100 uL at a concentration of 1μg/mL) overnight at 4°C. Plates were then blocked and washed as above and incubated with or without peptides. After a 30 minute peptide incubation, 100 ng empty HLA-DR1 was added to each well (peptides were not washed away). DR1 binding was measured as above, using rabbit anti-DR1 antiserum (1/25,000) followed by peroxidase-labeled goat anti-rabbit Fc (1/4000) as the secondary and detection antibodies respectively. Plates were developed using ABTS and absorbance was measured in a microtiter plate reader.

II. B. 7. Epitope mapping - SPR SpotMatrix Analysis

SpotMatrix SPR analysis was performed using an Applied Biosystems 8500 Affinity Chip Analyzer, with immobilized peptides and surface plasmon resonance detection of bound antibody. Biotinylated peptides were spotted using a SMP10B pin (Telechem) on a MicroSys spotter (Cartesian Dispensing Systems, presently Genomic Solutions) at a concentration of 25

μg/mL (~10 mM) in 20 μL of either DMEM (Gibco) with 10% FCS or 4 mg/mL BSA in PBS. Each spot had an approximate diameter of 330-360 μm and a volume of 2.5 nL per spot. Multiple copies of each chip were made and tested. Following chip equilibration for approximately one hour in PBST at a flow rate of 0.5 mL/min, 5 mL of test monoclonal antibody at the indicated concentration in PBST (10 mM sodium phosphate, 138 mM NaCl, 2.7mM KCl, 0.05% Tween, pH 7.4) was flowed over the SpotMatrix at a flowrate of 0.5 mL/min for 8 minutes. Dissociation was monitored over a 10 minute time span. End-point binding was calculated after background subtraction of individual reference spots from the generated SpotMatrix affinity traces (RCU, resonance change units, as a function of time). One RCU corresponds to a one millidegree surface plasmon resonance (SPR) shift. The endpoint consisted of the averaged RCU values for the last minute of the association/equilibrium phase prior to the start of dissociation.

II. B. 8. Analysis of cell surface MHC on immature dendritic cells

Cell surface expression of empty and total HLA-DR1 on immature dendritic cells and control splenocytes was assessed by flow cytometry. Bone marrow derived dendritic cells were prepared as previously described by culture of bone marrow cells from class II-deficient mice transgenic for a chimeric DRB1*0401 molecule (a gift of B. Huber) or from normal non-transgenic C57BL/6 mice. Bone marrow cells depleted for red blood cells were cultured (1-2 x 10^6 cells/mL) in the presence of GMCSF and IL4 (10 ng/ml each, R&D systems) in RPMI medium supplemented with 10% FBS, HEPES (10 mM), glutamine and Penicillin/Streptomycin (100 units each/mL). The cultures were replenished with cytokines every third day before harvesting for staining with MEM antibodies. Cells were harvested

carefully by using cell lifters (COSTAR) to avoid unwanted cell damage/injury, washed in FACS buffer (PBS containing 1% BSA, 0.1% sodium azide, 1mM EDTA, pH 7.2) and suspended in the same buffer. Approximately $1x10^5$ cells were stained with saturating amounts of antibody for 45 min on ice, washed three times with FACS buffer and incubated with fluorescein-conjugated $(Fab')_2$ anti-mouse IgG (H+L) secondary antibody (Jackson ImmunoResearch) for 45 min. After incubation, the cells were washed three times, suspended in 300 μL of buffer and analyzed immediately using a FACScalibur flow cytometer (Becton Dickinson). Propidium iodide (BD Pharmingen) negative cells were gated for analysis. Splenocytes used as a control were obtained from the same mice and were used freshly after red blood cell lysis.

II. C. Results

II. C. 1. Generation of conformation-specific monoclonal antibodies

Both class I and class II MHC proteins undergo conformational changes upon their binding peptide ligands, as observed by spectroscopic, hydrodynamic, and immunological criteria (Fahnestock, Tamir et al. 1992; Bouvier and Wiley 1994; Boniface, Lyons et al. 1996; Runnels, Moore et al. 1996; Bouvier and Wiley 1998). The human class II MHC protein HLA-DR1 can be obtained in well-characterized empty and peptide-loaded forms by separately expressing the α and β subunits in *E. coli* and subsequently refolding the subunits together *in vitro* in the absence or presence of peptide ligand (Frayser, Sato et al. 1999). Previously, we have used these species to characterize the peptide-induced conformational change in HLA-DR1 (Zarutskie, Sato et al. 1999; Sato, Zarutskie et al. 2000; Zarutskie, Busch et al. 2001). Binding of any of a variety of peptide ligands induces a distinct conformational change,

characterized by a decrease in hydrodynamic radius, an increase in per-residue molar ellipticity, and changes in binding of an antibody recognizing a polymorphic epitope on the beta subunit. Similar changes have been shown for mouse homologues of HLA-DR1 (Boniface, Lyons et al. 1996; Runnels, Moore et al. 1996). In order to probe in more detail the structural changes involved in the ligand-induced conformational change in HLA-DR1, a panel of murine monoclonal antibodies which distinguish the empty and peptide-loaded conformations were generated. Antibodies were raised by immunization with purified, denatured beta subunit, and hybridomas were screened using a sandwich ELISA for their ability to preferentially bind to the empty but not the peptide-loaded form of HLA-DR1 (see Methods for details). Four antibodies (MEM-264, MEM-265, MEM-266 and MEM-267) were selected based upon these characteristics. Each of the antibodies specifically binds to the empty but not peptide-loaded form of HLA-DR1 (Figure II.1.A). Another antibody, LB3.1 (Gorga, Knudsen et al. 1986; Fu and Karr 1994), does not distinguish the two forms of the protein, and binds both forms (Figure II.1.B). Although the MEM epitopes are present on the β subunit, all MEM antibodies capture DR αβ heterodimers as efficiently as the αβ complex-specific antibody LB3.1 (Figure II.1.C).

Each of the antibodies was tested using a variety of different peptide complexes, to determine whether they were sensitive to the peptide-induced conformational change regardless of the peptide sequence, and to rule out the possibility that antibody binding might simply be blocked by particular features of the HA peptide used in the initial screen. In each case, the MEM antibody bound only to the empty complex, and not to any of the MHC-peptide complexes tested (Fig II.2). The set of peptides tested had completely different sequences with different MHC-interacting anchor residues (bold in Figure II.2.A), and included both high-affinity viral antigens and self-peptides, as well as designed variants of lower binding affinity

and decreased half-life (Sato, Zarutskie et al. 2000; Stratikos 2003). The MHC-peptide complex with CLIP, a fragment of the class II associated invariant chain chaperone, and an important intermediate in the class II biosynthetic pathway, was not recognized by the MEM antibodies. Importantly, even the minimal binding tetra-peptide YRAL, which occupies only the N-terminal half of the peptide binding site (Sato, Zarutskie et al. 2000), is able to completely abrogate the binding of the MEM antibodies. Thus, each of the MEM antibodies distinguishes empty and peptide-loaded forms of HLA-DR1, without regard for the detailed nature of the bound peptide.

The reactivity of the MEM antibodies was characterized in more detail. Antibodies MEM-264, MEM-265, and MEM-267 are of the IgG2b isotype, while MEM-266 is IgG2a, and all carry the kappa light chain. Each of the antibodies reacted strongly by western blotting with reduced and denatured DR1 beta subunits in whole cell lysates of B cells and transfectants expressing DR1, but not with SDS-solubilized (not boiled) samples from the same cells that contain native DR1αβ-peptide complexes (Stern and Wiley 1994). Each antibody reacted also with unidentified band of ~80 kDa present lysates of a variety of cells, and MEM-266 also reacted with several other unidentified bands. Other class II MHC proteins HLA-DQ1 and DP4 were not recognized in whole cell lysates of transfected cells by any of the antibodies. The sandwich ELISA was used to determine the optimum binding pH and the half maximal binding affinity for each antibody (Table II.1). MEM-264, MEM-265 and MEM-267 had pH optima between 5 and 6 while MEM-266 bound maximally at pH=7. At the appropriate pH, all four of the antibodies bound tightly to the empty form with apparent binding affinities below 100 nM.

II. C. 2. HLA-DR Allele specificity of MEM binding

The binding of the MEM antibodies to allelic variants of HLA-DR1, was analyzed in order to evaluate their sequence specificity, to provide initial epitope mapping information, and to evaluate their potential application to biological problems involving various alleles. Genes coding for the beta subunit of HLA-DR proteins are among the most polymorphic genes characterized in the human population, with over 400 alleles identified that differ by one to ~20 residues between protein variants (Robinson, Waller et al. 2003). (The alpha subunit of HLA-DR1 is conserved, with only 2 alleles currently identified characterized by a single amino acid polymorphism.) Of this large set of polymorphic genes, only two have been expressed as recombinant empty proteins in *E. coli,* HLADRB1*0101 (coding for the beta subunit of HLA-DR1) (Frayser, Sato et al. 1999) and the closely related HLADRB5*0101 (coding for the beta subunit of HLA-DR2a) (Li, Li et al. 2000). To investigate the specificity of the MEM antibodies to less closely-related variants, insect cells were used as an alternative expression system. Several mouse and human class II MHC proteins have been expressed in insect cells (Scheirle, Takacs et al. 1992; Stern and Wiley 1994; Hansen, Andersson et al. 1998). Peptide-free preparations can be obtained for many class II MHC proteins with careful control of culture conditions, although some alleles are unstable in the absence of peptide (Stockel, Meinl et al. 1994; Scott, Garcia et al. 1996; Scott, Garcia et al. 1998; Stratmann, Apostolopoulos et al. 2000), and sometimes the MHC proteins co-purify with mixtures of adventitiously bound endogenous insect or medium-derived peptides (Scheirle, Takacs et al. 1992; Stratmann, Apostolopoulos et al. 2000). Peptide-free preparations of HLA-DR1, HLA-DR4 (carrying the protein product of the HLA-DRB1*0401 gene) and HLA-DR52a (DRB3*0101 gene) were obtained using insect cell expression systems, and the corresponding peptide-loaded forms by

in vitro loading with appropriate peptides (HA for DR4, and PLG, an integrin variant for DR52a (Wu, Maslanka et al. 1997)). These proteins were tested for their ability to bind to the MEM antibodies using the sandwich ELISA (Figure II.3.A). None of the peptide-loaded forms bound to any of the antibodies. MEM-264, MEM-265, MEM-266, and MEM-267 all bound to the empty forms of DR1 and DR4, but none bound to the empty form of DR52a. Thus, the binding epitopes for the MEM antibodies are likely to include at least some of the eleven positions distributed throughout the sequences where DR1 and DR4 are identical but different from DR52a (Figure II.3.B). A more comprehensive mapping study was undertaken to better define the location of these epitopes (see below).

II. C. 3. Cellular binding

Previously, empty or "peptide-receptive" forms of the mouse class II MHC proteins I-A^s, I-A^k, and I-A^u have been observed on the surface of immature dendritic cells (Santambrogio, Sato et al. 1999). Most other murine cells that express class II MHC proteins, including B cells and macrophages, generally display only peptide complexes at their surface. Whether human MHC proteins behave similarly is not known. An important potential application of the MEM antibodies would be the detection and analysis of empty human HLA-DR molecules at the cell surface. To determine whether HLA-DR proteins could be expressed as empty molecules at the cell surface in a form recognized by the MEM antibodies, dendritic cells were prepared from HLA-DR4 transgenic mice, using a conventional *in vitro* differentiation procedure in which bone marrow-derived precursors develop into immature dendritic cells during culture in GM-CSF (Romani, Reider et al. 1996). Immature dendritic cells from $DR4^+$ transgenic mice exhibited substantial cell surface reactivity with the anti- DR

antibody LB3.1, and also with each of the MEM antibodies (Figure II.4). Splenocytes from these mice did not bind the MEM antibodies, although LB3.1 still bound (Figure II.4) showing that the lack of MEM antibody staining was not due to a lack of DR4 expression in these cells. None of the antibodies stained control cells which lacked the DR4 transgene. Immature dendritic cells from DR1-transgenic mice showed similar behavior (not shown). These results indicate that HLA-DR4, like I-A^s, can be expressed as an empty molecule in immature dendritic cells, and that the cell type-specific regulation of antigen processing pathways is similar for the DR transgenes as for endogenous mouse I-A proteins.

II. C. 4. Peptide mapping the MEM epitopes

The MEM epitopes were mapped using a series of peptides 20 amino acids in length, each overlapping by 10 residues, and spanning the entire sequence of the HLA-DR1 beta chain (Figure II.5.A). This peptide mapping strategy (Geysen, Rodda et al. 1987) assumes that each antibody epitope is contained within a short, contiguous stretch of polypeptide, and has been used successfully to map many antibody epitopes (Matsushita, Nisizawa et al. 1994; Maeda, Miyamoto et al. 2000; Gampfer, Samstag et al. 2002). Here, the antibodies had been raised using denatured DR1 beta subunit as an immunogen, which we did not expect to elicit antibodies recognizing conformational epitopes or epitopes including non-localized groups of residues. The series of overlapping peptides was tested initially in a direct binding immunoassay, using biotinylated peptides immobilized on streptavidin-coated wells (Figure II.5.B). For each antibody, a single peptide gave a clear binding signal. MEM-264, MEM-265 and MEM-267 each bound to DRβ-(50 – 69), whereas MEM-266 bound to the C-terminal peptide DRβ-(170 – 190). To confirm these results and to establish that the peptides bound to

the antigen-combining site of the antibody and not adventitiously at another location on the antibody, the series of overlapping peptides was retested using a competition assay, in which peptide and empty DR1 compete for binding to immobilized antibody (Figure II.5.C). The same mapping results were obtained. Finally, antibody binding to the series of peptides was evaluated using a new SpotMatrix-based SPR technology. With this technology, binding of fluid-phase protein to a set of immobilized ligands is detected in parallel by surface plasmon resonance. Biotinylated peptides were immobilized by spotting them onto a streptavidin-coated gold chip, and monitored binding for the MEM antibodies in PBS solution. Endpoint binding values for each peptide (Figure II.5.D) reflect the same binding specificity as observed in the direct binding and competition assays. The SpotMatrix SPR technology provides information on both association and dissociation phases of the binding interaction, in addition to the endpoint values. A comparison of apparent dissociation constants measured for each epitope and alanine scan peptides will be discussed elsewhere (manuscript in preparation).

II. C. 5. Fine Mapping of monoclonal antibody MEM-266

The MEM-266 epitope was defined more precisely using a submapping strategy. A series of 10-mer peptides overlapping by 7 residues, spanning the binding 20-mer peptide DRβ-(170 – 190), was tested, since typical linear epitopes are five to seven amino acids in length (Kabat 1970; Geysen, Rodda et al. 1987). Only the most C-terminal peptide DRβ-(182 – 190) bound (Figure II.6.A). An alanine scan of this peptide revealed a linear epitope with predominant contributions from Trp-188β and Arg-189β and a smaller contribution from Val-186β (Figure II.6.B, C). This epitope explains the observed allele specificity of MEM-266 (Fig II.3.B), as DR52a contains a substitution at position 189 (Arg to Ser).

II. C. 6. Fine Mapping of MEM-264, MEM-265 and MEM-267 Antibodies

As an aid to the fine mapping studies of the MEM-264, 265, and 267 epitopes, binding peptides shorter than the 20-mer DRβ-(50 – 69) peptide originally observed in the primary screen were sought. A submapping strategy similar to that described for MEM-266 was unsuccessful, as none of the antibodies bound to a series of four overlapping 10-mers spanning the DRβ-(50 – 69) sequence (not shown). We attempted to identify a minimal peptide by truncation analysis. Only 2-3 residues could be removed from either the N- or C-terminal end without abrogating binding (Figure II.7.A). The resultant peptides are much longer than typically found for linear epitopes (Kabat 1970; Geysen, Rodda et al. 1987) and if found in an extended conformation would be >50 Å, much longer than a typical antibody-combining site (~15-20 Å) (MacCallum, Martin et al. 1996). Only minor differences were observed between the binding behavior of MEM-264, MEM-265, and MEM-267 towards the truncation series. The 18-mer DRβ-(50 – 67) bound well to all of the antibodies and was selected for further mapping by alanine scanning. MEM-264, MEM-265 and MEM-267 each exhibited essentially the same pattern of sensitivity to alanine substitution, with important residues identified as Leu-53β, Asp-57β, Tyr-60β, Trp-61β, Ser-63β and Leu-67β (Figure II.7.B). These residues are arranged in an approximate 3.5 residue repeat, suggestive of a helical or other ordered structure in the peptide conformation recognized by the antibody. The epitope explains the reactivity of MEM-264, MEM-265, and MEM-267 with DR1 and DR4, which share these residues, and their lack of binding to DR52a, which contains two substitutions in this region (D57V and Y60S).

II. D. Discussion

The human class II MHC protein HLA-DR1 undergoes a distinct conformational change as it binds to its peptide ligand. Previous studies have shown that the empty protein has a larger, less compact structure than the peptide-bound complex (Zarutskie, Sato et al. 1999; Sato, Zarutskie et al. 2000), and that peptide binding induces conformational changes in the empty protein detectable in the ultraviolet circular dichrosim (Zarutskie, Sato et al. 1999) and NMR spectra (Zarixia Zavala-Ruiz, Harold Schwalbe, LJS, unpublished observations). In this study, regions of the protein that participate in the conformational change were identified. A panel of four conformation-sensitive monoclonal antibodies was generated which recognize the beta subunit and specifically bind to empty but not peptide-loaded forms of the protein. The antibodies also differentiate the empty and peptide-loaded forms of both purified recombinant protein and native protein expressed on the surface of antigen presenting dendritic cells. Two regions of the protein which change upon peptide binding were identified (Figure II.8). One site, DRβ-(53 – 67), shown in red in figure 8A, is on the beta subunit helical region near the peptide binding site, and overlaps with a similar site previously identified by an antibody raised against I-A^s (LaPan, Klapper et al. 1992; Zarutskie, Sato et al. 1999). A second site, DRβ-(186 – 189), shown in cyan in figure 8A, is remote from the peptide binding site at the bottom of the immunoglobulin-like domain.

The DRβ-(53 – 67) epitope is recognized by three of the antibodies, MEM-264, MEM-265 and MEM-267. The key residues for all of the antibodies are the same: Leu-53β, Asp-57β, Tyr-60β, Trp-61β, Ser-63β, and Leu-67β. These residues are spaced three to four residues apart in a pattern characteristic of a helical repeat. In an alpha helical model for this region, these residues would all fall in a contiguous region on one face of the helix. However, in

structures of DR-peptide complexes, this region includes two kinks, such that the key residues are not found in a contiguous region. The side chains of Leu-53β and Asp-57β are exposed on a different face of the protein than the other residues, and Ser-63β lies on the other side of the helix from Tyr-60β, Trp-61β, and Leu-67β (Figure II.8.B). This distributed, non-contiguous location of the key epitope residues in the structure of the peptide complex indicates that conformational changes in this region would be required to bring the key residues together in the empty protein and allow engagement by MEM-264, MEM-265, or MEM-267. Thus, these antibodies appear to be sensitive to conformational rearrangement(s) in this region, rather than to a simple steric block of key residues by peptide binding. This idea is supported by the loss of MEM recognition upon YRAL peptide binding – this peptide is likely to bind only in the P1-P4 region of the peptide binding site (Bolin, Swain et al. 2000), far away from the DRβ-(53 – 67) epitope, with a closest approach of approximately 9Å (from the peptide C-terminus to Trp-60). Some evidence for conformational lability in this region of HLA-DR1 can be seen in crystal structures of its peptide complexes, which exhibit significant peptide-to-peptide variation in this region, and relatively high thermal B-factors, particularly for residues 63-67. Based on the pattern of key residues within the epitope, the epitope region is probably recognized by the MEM antibodies in a simple helical conformation distinct from that observed in the crystal structures, as this would place the key residues in position to interact with the antibody combining site. Whether this new conformation is induced upon antibody binding or present in the empty MHC protein is not clear, although it should be noted that all of the antibodies that recognize this epitope bind to the empty protein much more tightly than to the free epitope peptides, consistent with the structure of the epitope region in the empty protein being similar to that recognized by the antibody.

The fourth antibody, MEM-266, shows the same conformational sensitivity as the other antibodies, but its epitope region is located at the C-terminal end of the DR1 extracellular domain, and includes the last five residues in the beta strand at the bottom of the immunoglobulin like domain. The key epitope residues, Trp-188β and Arg-189β (and to a lesser extent Val-186β) are located in a contiguous, linear epitope, and MEM-266 exhibits similar affinities for the empty protein and the corresponding epitope peptides. Thus, this epitope is most likely non-conformational, with differential antibody binding regulated by accessibility changes to this region of the protein, rather than by local rearrangements. These residues are solvent-accessible in the structures of HLA-DR1 peptide complexes, but located in a cleft between the domains, and antibody accessibility in the peptide complex might be limited by steric interactions with other overhanging regions. In this scenario, peptide binding would induce changes in the relative orientation of these domains, and expose Trp-188β and Arg-189β to MEM-266 binding. Rigid body shifts of the entire β2 domain, including rotations of up to 15 degrees, are routinely observed in comparisons of HLA-DR1 peptide complexes in different crystal forms (Brown, Jardetzky et al. 1988). These realign the β2 domain relative to the peptide binding domain, and could potentially couple peptide binding to domain rotation via interactions between loop residues at the top end of the β2 domain and the underside of the P1 pocket (Murthy and Stern 1997).

Thus, the conformational changes associated with peptide binding are distributed throughout the protein. In fact, the MEM-266 epitope is the region of the extracellular domain furthest from the peptide binding site. What could be a physiological role for such a large conformational change? One possibility might be that the conformational changes regulate interactions with HLA-DM, the peptide-exchange catalyst required for efficient intracellular

loading of peptides on class II MHC molecules. HLA-DM helps catalyze peptide binding and release through conformational effects on DR1 (Zarutskie, Busch et al. 2001) and several models of interaction have been proposed (Dornmair, Rothenhausler et al. 1989; Sadegh-Nasseri and McConnell 1989; Beeson and McConnell 1994; Schmitt, Kratz et al. 1999; Chou and Sadegh-Nasseri 2000; Zarutskie, Busch et al. 2001). Many of these models involve HLA-DM recognizing or promoting a structural change in HLA-DR which could facilitate peptide release. Recently, a screen for MHC mutants that disrupt DM activity has identified a number of putative DM-DR interaction sites, all on the same lateral face of the MHC proteins (Doebele, Busch et al. 2000). Surprisingly, this set included V186K, which is located well away from the peptide binding site (Doebele, Busch et al. 2000), and is part of the epitope region for MEM-266. Together, these results suggest that peptide-induced conformational changes propagate from the peptide binding site to the distal end of the beta subunit, and conversely, that interprotein interactions in this region can be transmitted to the peptide binding site to regulate peptide release and binding kinetics.

In summary, a small panel of monoclonal antibodies able to detect peptide binding to HLA-DR1 and related human class II MHC alleles was produced. These antibodies can be used to investigate peptide loading processes *in vitro* or *in vivo*, and have already provided important information on the location and extent of the peptide-induced conformational change in HLA-DR1.

II. E. Acknowledgments

I would like to thank Ivan Hilgert and Vaclav Horejsi for generation of the MEM antibodies, Mia Rushe for initial screening of antibody reactivity, Sriram Chitta for flow cytommetry, Rick Baggio and Jaime Arenas for SPR analysis, Julie Lau for performing antibody subtyping, and Bridgette Huber for the generous gift of the DR4 transgenic mice.

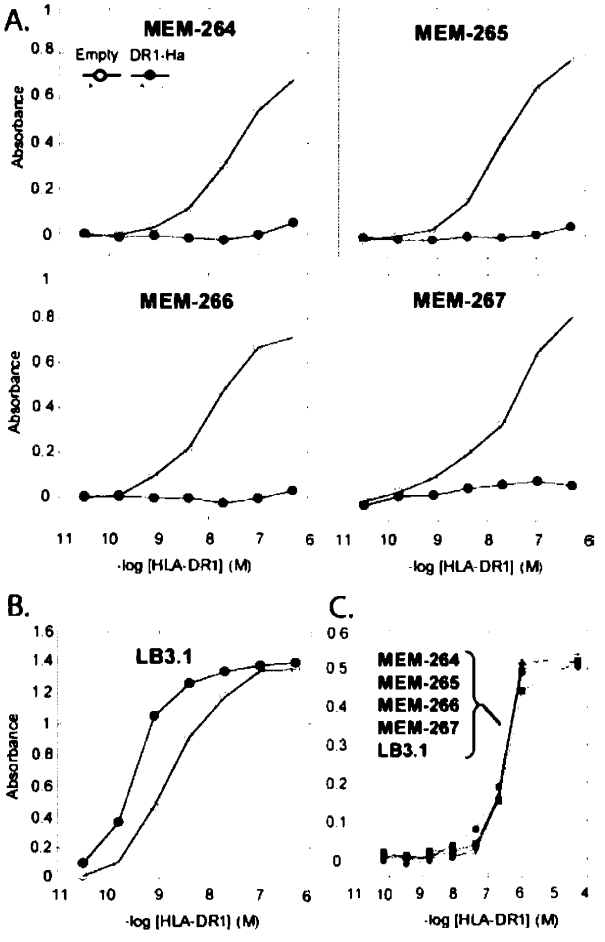


Figure II. 1. MEM Antibodies specifically recognize empty conformation of DR1.

(A) Sandwich ELISA using immobilized conformation-specific MEM antibodies MEM-264, MEM-265, MEM-266 and MEM-267 and rabbit anti-DR antiserum detection. Each MEM antibody binds empty DR1 (open circles) and does not bind DR1-Ha (closed circles). (B) Same as panel A except using the anti-DR1 monoclonal antibody LB3.1, which recognizes both empty DR1 and DR-Ha. (C) MEM antibodies recognize the DR1 αβ heterodimers. Sandwich ELISA using immobilized MEM antibodies (or LB3.1) for capture of DR1 carrying a C-terminal biotin label on the alpha chain, with streptavidin detection. (LB3.1, circles; MEM-264, squares; MEM-265, diamonds; MEM-266, triangles; MEM-267, plus). DR α chains are captured as effieiently by the MEM antibodies as by the αβ heterodimer-specific antibody LB3.1. (Inset to panel A, upper left) SDS-PAGE analysis of empty DR1 and DR1-Ha with samples in alternating lanes were boiled (+) or kept at room temperature (not boiled, -) before loading. DR1-Ha (but not empty DR1) is resistant to SDS-induced subunit dissociation, indicative of peptide loading.

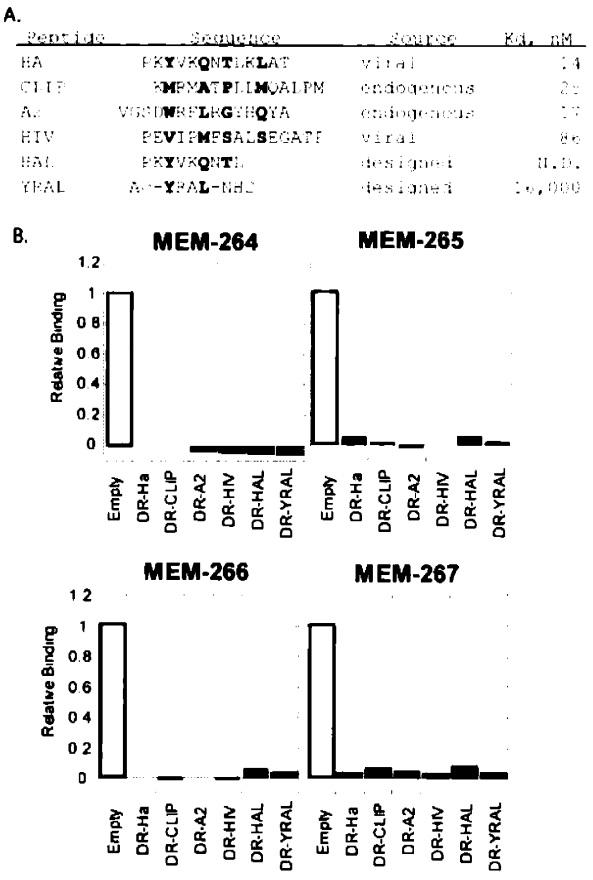


Figure II. 2. MEM antibody recognition of empty but not peptide-loaded DR1 does not depend on the nature of the bound peptide.

(A) Peptide sequences (B) MEM binding to DR1-peptide complexes as determined by sandwich ELISA as in Figure II.1. Excess peptide was included during the MHC-peptide incubation step for the very weakly-binding peptide Ac-YRAL-NH_2.

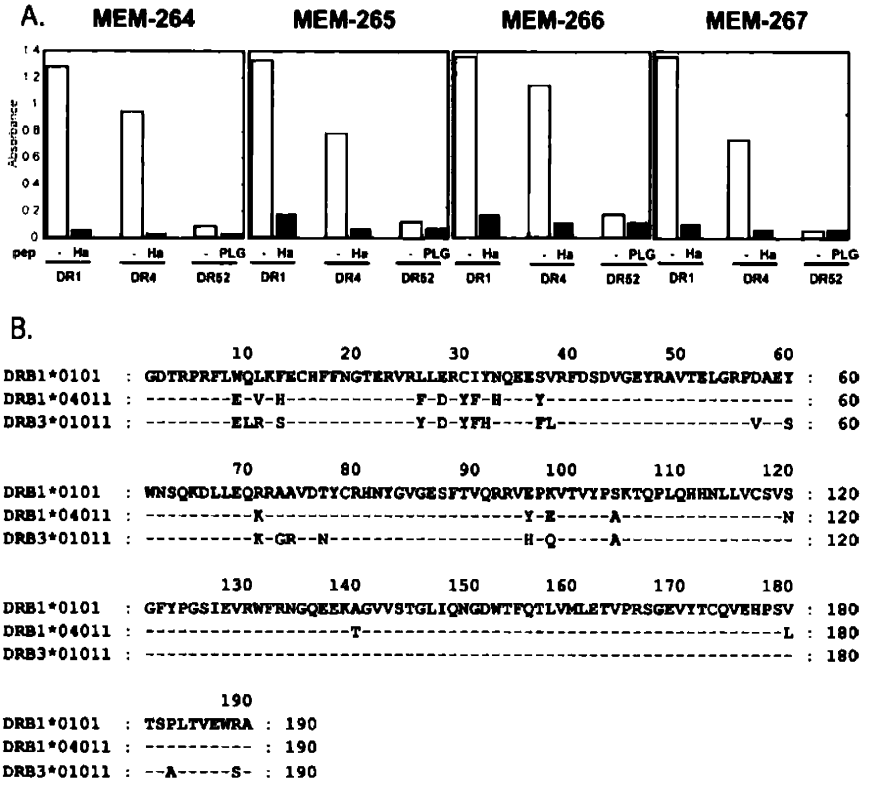


Figure II. 3. Allele Specificity of MEM Antibodies.

(A) All of the MEM antibodies bind to empty HLA-DR1 and DR4, but not to their peptide loaded forms. None bind to DR52a in either the empty or peptide-loaded forms. Open bars correspond to peptide-free, empty forms; closed bars correspond to peptide-loaded forms. Assay by sandwich ELISA as in Figure 1. (B) Sequences of the beta subunits of DR1, DR4.1 and DR52a proteins, shown with corresponding gene name, and identity with DR1 indicated by dashes.

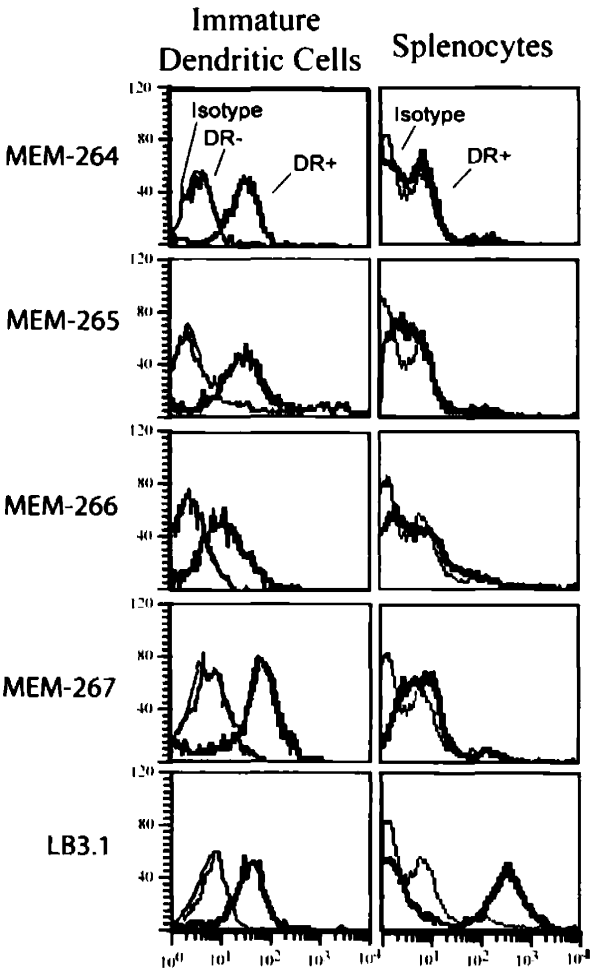


Figure II. 4. The MEM epitope can be detected at the cell surface.

MEM antibodies stained immature dendritic cells (left) which express empty cell surface MHC molecules, but not splenocytes (right) which express predominantly peptide loaded forms, while LB3.1 stained both cell types. Thick lines, antibody staining of cells from DR4+ transgenic mice; thin lines, isotype control. None of the antibodies stained control non-transgenic DR4- negative C57BL/6 immature dendritic cells (shaded).

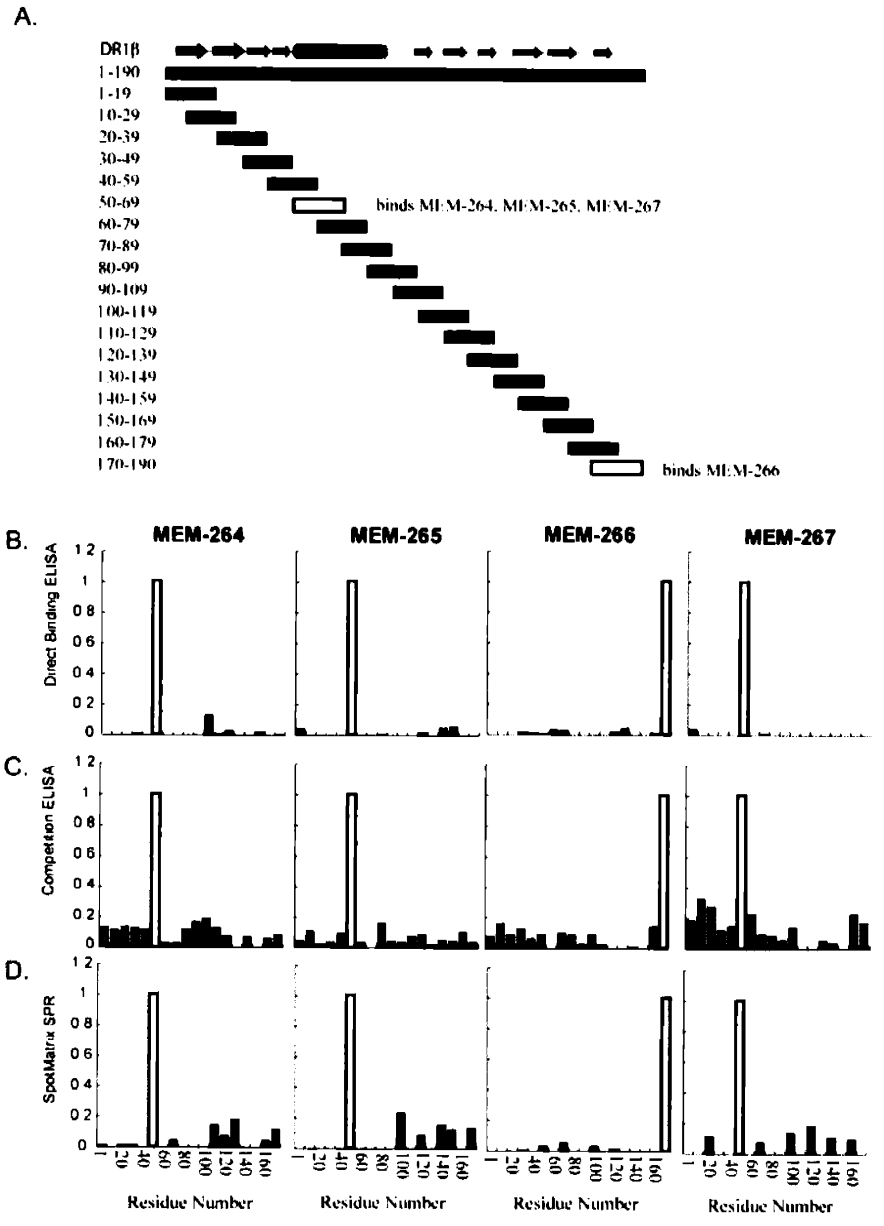


Figure II. 5. Epitope Mapping of MEM antibodies.

(A) Mapping strategy using overlapping 20-mer peptides (overlap of 10 amino acids) spanning the entire DR1 sequence. Each monoclonal antibody was tested by direct binding ELISA (B), by competition ELISA (C) and by SpotMatrix SPR analysis (D). For each antibody, a single peptide was observed to bind: MEM-264, MEM-265 and MEM-267 bind DRβ-(50 – 69), and MEM-266 binds DRβ-(170 – 190).

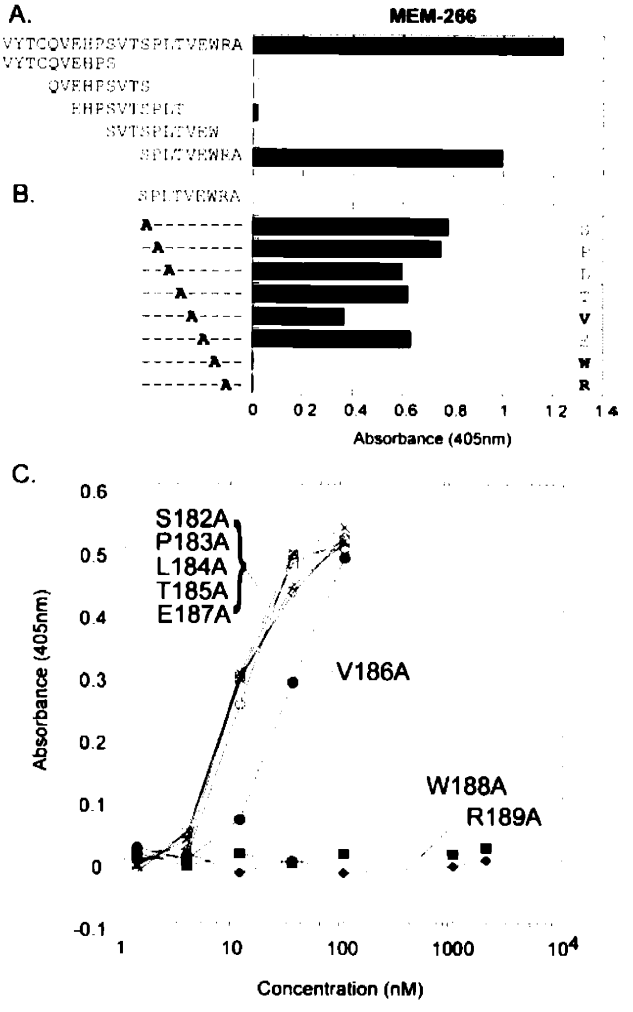


Figure II. 6. Fine Mapping of the MEM-266 epitope within the peptide DRβ-(170 – 190).

(A) Overlapping 10-mer peptides used to further define the MEM-266 epitope within the peptide DRβ-(170 - 190). In a direct binding ELISA, the antibody bound only to the C-terminal peptide DRβ-(182-190). (B, C) Alanine scan of DRβ-(182-190) to find the amino acid residues important for binding to MEM-266. Antibody binding was blocked by substitution of Trp-188β and Arg-189β and reduced by substitution of Val-186β.

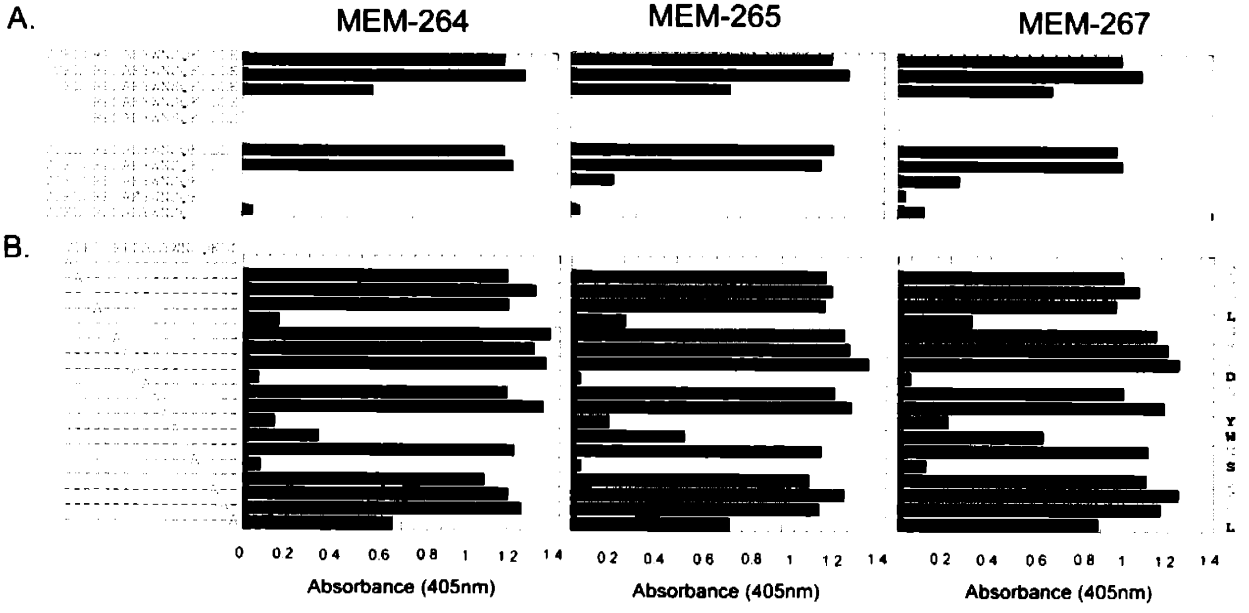


Figure II. 7. Fine mapping of MEM-264, 265, 267 epitopes within the peptide DRβ-(50 - 69).

(A) N-terminal and C-terminal deletions of the binding 20-mer peptide DRβ-(50 – 69) tested in an attempt to find a short minimal epitope for binding. Removal of more than two N-terminal or C-terminal residues abrogated binding activity, as observed using a direct-binding ELISA. (B) Alanine scan of the 18-mer peptide DRβ-(50 – 67) to find the amino acid residues important for binding to MEM-264. Antibody binding was blocked by substitution of residues Leu-53β, Asp-57β, Tyr-60β, Trp-61β, Ser-63β and Leu-67β.

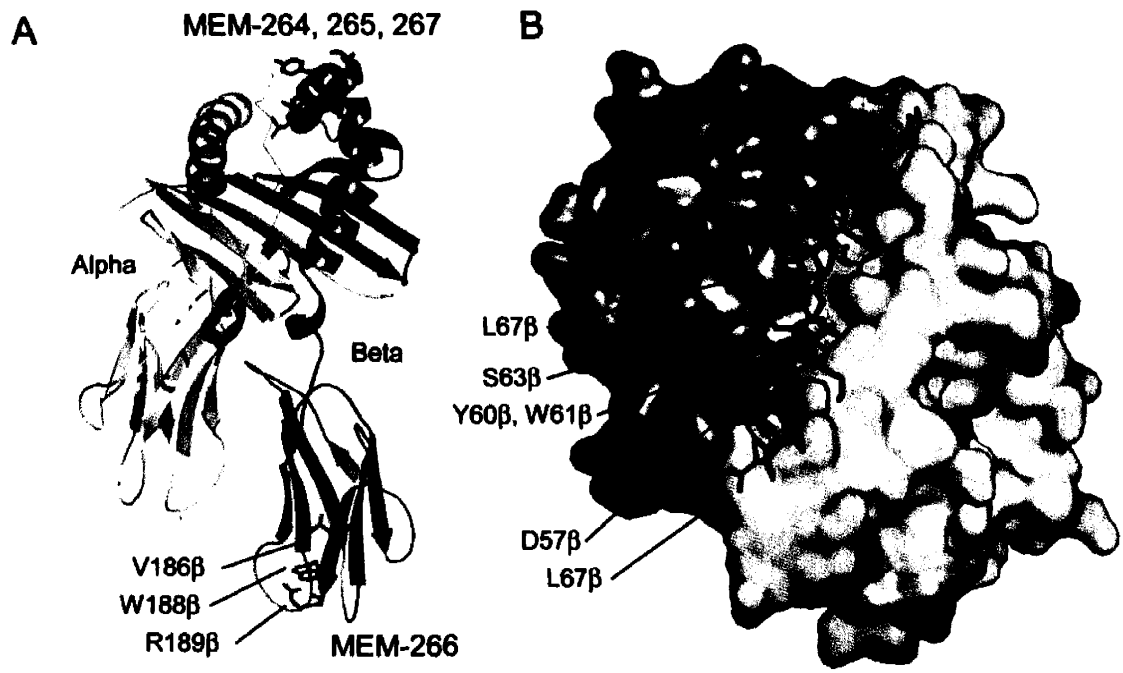


Figure II. 8. MEM Epitope Map onto DR1-Ha Structure

(A) Ribbon diagram of HLA-DR1 bound to HA-peptide. Important residues for binding of MEM-264, MEM-265 and MEM-267 are shown in red. Residues important for binding to MEM-266 are shown in cyan. (B) Close-up surface view of MEM-264, MEM-265 and MEM-267 epitopes, viewed from the opposite face as in panel (A). Note that the side chains of important residues are not located on a contiguous face of the protein. Leu-53β is located underneath the peptide binding groove and this region would appear to have to rearrange in order to bind to the MEM antibodies. Figures were generated using the program Pymol.

Antibody Characterization				
			Half Maximal Binding	
Antibody	Subtype	pH optimum	protein	Peptide
			nM	*nM*
MEM-264	IgG 2b	5-6	31	13400
MEM-265	IgG 2b	5-6	19	7400
MEM-266	IgG 2a	7	10	1
MEM-267	IgG 2b	5-6	50	6450

Table II. 1. Antibody Characterization

Protein binding and pH optima were determined by sandwich ELISA using immobilized MEM antibody, soluble DR1, and rabbit polyclonal anti-DR1 antibodies, with anti-rabbit detection. Binding to peptides was determined by ELISA using immobilized MEM antibodies, biotinylated peptide (bio-GGVTELGRPDAEYWNSQKDL for MEM-264, 265, and 267 and bio-GGSPLTVEWRA for MEM 266) followed by streptavidin detection.

III. Probing the Ligand-Induced Conformational Change in HLA-DR1 by Selective Chemical Modification and Mass Spectrometric Mapping

SUMMARY

Peptide binding induces conformational changes in class II MHC proteins that have been characterized using variety of hydrodynamic and spectroscopic approaches, but these changes have not been clearly localized within the overall class II MHC structure. In this study, empty and peptide-loaded complexes of HLA-DR1, a common class II MHC variant, were chemically modified using the side-chain specific chemical modifiers p-hydroxyphenylglyoxal (arginine), tetranitromethane (tyrosine), N-bromosuccinimide (tryptpophan), and NHS-biotin (lysine). Modified proteins were subjected to in-gel digestion with trypsin and subsequent analysis by MALDI/MS. Three arginine residues and two lysine residues were differentially reactive: modified in the empty form but not the peptide-loaded form of the protein, indicating that the solvent accessibility or chemical reactivity of these regions differs in the two conformations. Three of the differential modifications were located on a single lateral face of the protein indicating that this region is involved in the conformational change. Additionally a number of lysine and tyrosine modification sites were present in both protein conformations. Overall, the pattern of reactivity is inconsistent with the idea that empty MHC molecules exist as molten globules or other partially unfolded intermediates, and suggests that the peptide-induced conformational change is localized to only a few regions of the protein.

III.A. Introduction

Class I and class II MHC proteins undergo conformational changes upon binding peptide ligands. This phenomenon has been observed by spectroscopic, hydrodynamic, and immunological criteria (Boniface et al., 1996; Bouvier and Wiley, 1994; Bouvier and Wiley, 1998; Fahnestock et al., 1992; Runnels et al., 1996). For HLA-DR1, a well studied human class II MHC protein (Stern et al., 1994), binding of peptide ligands induces a distinct conformational change, characterized by a decrease in hydrodynamic radius, and an increase in per-residue molar ellipticity at 222 nm (Zarutskie et al., 1999). No high resolution structural information is available for the empty protein.

Recently, antibodies which are specific for the empty conformation of HLA-DR1 were used to identify a number of amino acid residues which change their accessibility upon peptide binding (Chapter II and (Carven et al., 2004)). These include residues both nearby to and distant from the peptide binding site. That study was limited by the fact that each of the antibodies was raised against the beta chain; therefore no data could be obtained pertaining to the structural rearrangement of the alpha chain. Thus, a more comprehensive study which can simultaneously analyze both the alpha and beta chains of DR1 was needed.

Side-chain specific chemical modification has often been used to elucidate the role of specific amino acids in enzyme active site reactivity (reviewed in (Anthony-Cahill and Magliery, 2002) and (Tyagi and Gupta, 1998)), protein-protein interactions (Hager-Braun and Tomer, 2002) and structural studies of protein conformation (Safarian et al., 2003). Mass spectrometric peptide mapping has been shown as an unequivocal method for the characterization of chemical modifications in proteins. The combination of

limited tertiary structure-selective chemical modification with mass spectrometric mapping allows molecular characterization of protein function when other methods are unreliable or unavailable (Suckau et al., 1992).

In this study we have probed the role of amino acid residues involved in the conformational change using a number of side chain-specific chemical modifiers. The arginine-specific modification reagent p-hydroxyphenylglyoxal (HPG) has been widely used in experiments to identify functional arginine residues (Hager-Braun and Tomer, 2002; Lin and Chang, 1992; Lin et al., 1996; Linder et al., 2002; Millett and Geren, 1991; Mukouyama et al., 1998). Tetranitromethane (TNM) has similarly been used to identify reactive tyrosine residues (Beckingham et al., 2001; Leite and Cascio, 2002), and N-hydroxysuccinimide esters (in this case NHS-biotin) have been used to label amine nitrogens of lysine with a number of different functional groups (Glocker et al., 1994; Zappacosta et al., 1997). Here, these chemical modification reagents were used to evaluate conformational differences between empty and peptide-loaded HLA-DR1 in an attempt to examine in more detail the structural changes involved in the ligand-induced conformational change in HLA-DR1.

III. B. Materials and Methods

IV. B. 1. Chemical Reagents

Proteomics grade trypsin, alpha-cyano-4-hydroxycinnamic acid, sinapinic acid (3,5-dimethoxy-4-hydroxycinnamic acid), NBS, TNM and trifluoroacetic acid were obtained from Sigma (St. Louis, MO). Hydroxyphenylglyoxal and NHS-biotin were obtained from Pierce (Rockford, IL).

III. B. 2. Recombinant MHC Molecules

Empty and peptide-loaded DR1 were prepared by expression in *Escherichia coli* and folded *in vitro*, as previously described (Frayser et al., 1999). Briefly, HLA-DR1 extracellular domains were expressed individually as insoluble inclusion bodies, isolated by denaturing ion exchange chromatography, and refolded *in vitro* without peptide. DR-Ha-peptide complexes were prepared by incubating immunoaffinity-purified empty HLA-DR1 (1-5 uM) with at least 5 fold molar excess peptide for 3 days at 37°C in PBS. The resultant peptide-DR1 complexes or unloaded HLA-DR1 that had been stored at 4°C were purified by gel filtration to remove aggregates and unbound peptide, and stored at 4°C.

III. B. 3. Chemical modifications

Arginine: Empty or peptide loaded DR1 samples were exchanged into 25mM ammonium bicarbonate pH=8.0 using a centrifugal filter device (Amicon Ultra 10,000 MWCO), and were incubated at a final protein concentration of 0.5 μg/μL (10 μM) with HPG ranging from 0 to 10 mM in 25 mM $NaHCO_3$ for 15 h at 25 °C in the dark.

Unmodified DR1 was prepared and treated similarly without incubation with HPG. After modification, each sample was exchanged into PBS to remove excess unreacted HPG.

Lysine: A 10 mM stock solution of N-hydroxysuccinimidobiotin ester was prepared in 100% dimethylsulfoxide (DMSO). Empty or peptide loaded DR1 samples (0.5 μg/μL) were mixed with 5 or 20 fold excess (modifier:lysine residue) NHS-biotin in PBS (final concentration of DMSO was <5%). The reaction was allowed to proceed for 3 h at room temperature. All reactions were stopped by addition of 10mM tris pH=7.8. Samples of unmodified DR1 were prepared under identical conditions as a negative control.

Tryptophan: Empty or peptide loaded DR1 samples (0.5 μg/μL) were mixed with 5 to 20 fold excess N-bromosuccinimide for one minute in PBS pH=7.0. The extent of reaction was measured in a spectrophotometer. The reaction was stopped by the addition of 1mM tryptophan and samples were exchanged into PBS using a centrifugal filter device.

Tyrosine: A solution of TNM was prepared in 6 mg/mL in 95% ethanol was prepared. Empty or peptide loaded DR1 samples (0.5 μg/μL) were mixed with 10-100 fold excess (modifier:tyrosine residue) TNM in PBS. The reaction was allowed to proceed for 1 h at room temperature. All reactions were stopped by addition of β-mercaptoethanol at a final concentration of 5% (v/v). Samples of unmodified DR1 were prepared under identical conditions as a negative control.

III. B. 4. SDS polyacrylamide gel electrophoresis (PAGE)

Aliquots of 150 pmol/protein of modified DR1 or unmodified DR1 as a control in were applied to a 12% polyacrylamide gel (Bio-Rad). SDS-PAGE was performed according to the instructions of the manufacturer. Subsequently, gels were stained with colloidal coomassie (GelCode Blue, Pierce) for 30 minutes and destained with deionized water until all background staining was removed.

III. B. 5. In-gel protein digetion.

Gel slices were excised and cut into small fragments, transferred into a 0.6-mL siliconized micro-centrifuge tube, and washed with 100 μL of deionizied water. After destaining with 50% acetonitrile/50 mM NH_4HCO_3, the gel was washed with 100 μL of 50 mM NH_4HCO_3 and then dried by vacuum centrifugation. The gel was rehydrated in buffer containing 25 mM NH_4HCO_3 and 12.5 ng/μL trypsin. Following digestion for 15 h at 37 °C, the peptides were concentrated and desalted using a C18 monolithic sorbent tip (OMIX, Varian) and cocrystallized on a stainless steel MALDI target in 4 μL of a saturated solution of 3,5-dimethoxy-4-hydroxycinnamic (in acetonitrile/deionized water/trifluoroacetic acid, 49/50/1, v/v) according to the dried-droplet method (Roepstorff, 1998).

IV. B. 6. Matrix-assisted laser desorption/ionization mass spectrometry (MALDI/MS).

Proteins and peptides were analyzed by MALDI/MS under positive ion conditions on a Micromass MALDI L/R (Waters, Milford, MA), equipped with a nitrogen UV laser (337nm). Peptides were analyzed in reflectron mode from m/z 675 to 5000. An external

calibration was used, and 50 shots were summed for each spectrum. Ten to twelve spectra were averaged for each sample. Relative abundances of peptides were calculated from their peak height (mean value of 3 measurements), assuming similar characteristics for the desorption/ionization of a specific ion in the samples for modified and unmodified DR1. All mass spectra were recorded in the Proteomics and Mass Spectrometry Core Facility at the University of Massachusetts Medical School.

III. C. Results

III. C. 1. Enzymatic digestion and mass spectrometry of unmodified DR1.

The human class II MHC protein HLA-DR1 can be obtained in well-characterized empty and peptide-loaded forms by separately expressing the α and β subunits in E. coli and subsequently refolding the subunits together *in vitro* in the absence or presence of peptide ligand (Frayser et al., 1999). The soluble alpha chain construct contains 182 residues and the beta chain contains 190 residues. These sequences correspond to the mature extracellular region of the protein not including the membrane-proximal "connecting-peptide" region.

For analysis by trypsin digestion and mass spectrometry, unmodified empty HLA-DR1 complexes were separated by SDS/PAGE under reducing conditions. Bands corresponding to alpha and beta chains were excised and digested with trypsin and the resulting peptides were analyzed by MALDI/MS. For the alpha chain, 14 of 15 expected peptides fragments were observed in the mass spectrum in the range *m/z* 675-5000 (Figure III.1, Table III.1). For the beta chain, twelve of the expected 14 peptide fragments were observed (Figure III.1, Table III.2). During electrophoresis, all cysteine

residues were observed to have been modified by acrylamide and the theoretical mass values were calculated accordingly. Generally, under the conditions used in this study, fragments containing fewer than 5 or greater than 35 amino acid residues were difficult to detect. Small peptides are hidden by ions resulting from the MALDI matrix. Larger peptides typically remain in the gel after digestion. Overall, the sequence coverage was greater than 75% for each chain.

III.C.2. Modification of DR1 with HPG and localization of derivitized residues by MALDI/MS.

The extracellular domains of DR1 contain 25 arginine residues on both the alpha and beta chains (8 on the alpha chain and 17 on the beta chain). Hydroxyphenylglyoxal was used to investigate the differential accessibility/reactivity of arginine residues in empty and peptide-loaded HLA-DR. HPG can react with the guanidino group of arginine via different mechanisms to yield different final products (Figure III. 2A) (Takahashi, 1968; Wood et al., 1998). At the mildly alkaline pH used in this study, equimolar amounts of HPG are incorporated. At high pH (>12), reaction yields incorporation of two HPG molecules per arginine residue.

Exposed arginine residues on empty and peptide-loaded DR were derivitized using 5 mM HPG. Modification was monitored using UV/visible absorption spectroscopy, and the number of modified arginine residues was calculated using the molar extinction coefficient of 18,300 (Yamasaki et al., 1980) (Figure III.3). This concentration of HPG was sufficient to differentially modify the two forms of the protein. Increasing the either the concentration of HPG or the time of reaction did not change the

extent of modification. Empty DR1 incorporated 1.5 HPG molecules per αβ heterodimer while peptide-loaded DR1 incorporated approximately 0.5 molecules per protein.

Modified empty and peptide-loaded HLA-DR1 complexes were separated by SDS/PAGE and digested with trypsin. Digestion by trypsin results in hydrolysis of the peptide backbone after arginine and lysine residues, and modification of either arginine or lysine disrupts the ability of trypsin to cut the peptide backbone and results in a different spectrum of tryptic peptides. This change allows easy identification of incorporation sites of the arginine modifier.

No differences were observed in the mass spectra of the unmodified sample and the peptide-loaded preparation which had been modified with HPG (Figures III.4, and III.5). This is consistent with the small amount of incorporation measured by absorbance: any modification of the peptide-loaded form that is present appears to be at too low a concentration to be measured by mass spectrometry, or possibly located on one or more of the tryptic peptides which were not observed in the experiment.

Empty DR1 was modified by HPG at three arginine residues indicating that the regions around these residues are likely to have a different accessibility to solvent and are therefore more reactive than the same residues in the peptide-loaded conformation.

On the alpha chain, two modifications to empty DR1 were identified (Table III.3). A mass peak of 2615.24 corresponding to a +132 Da shift for the HPG labeled peptide α-(45-67) (LEEFGR*FASFEAQGALANIVDK) was observed (Figure III.4, solid arrows). This longer peptide contains a modified arginine at position 50, and results from the inability of trypsin to cut the peptide backbone at this site. Additionally there was loss of the peak at 750.38 Da α-(45-50), and a marked decrease in the relative abundance of the

peak at 1751.90 Da α-(51-67), consistent with modification at Arg50α. A second modification site was observed at position Arg123 on the alpha chain. A mass peak of 3343.79 Da corresponding to a +132 Da shift for the peptide DRα-114-140 (FTPPVVNVTWLR*NGKPVTTGVSETVFLPR) was observed (Figure III.4, open arrow). This was not, however accompanied by a reduction in the abundance of the peptide containing unmodified Arg123 (mass peak of 816.40 Da for α-(141-146) or of the peptide generated by cutting the backbone at that position (mass peak of 1801.98 for α-(124-140)). This would indicate only a small degree of modification of empty DR1 at this position.

One site of modification was identified on the beta chain of empty DR1. A new peak at 2878.29 was observed (Figure III.5 solid arrows, Table III.3). This corresponds to the peptide β-(167-190) (SGEVYTCQVEHPSVTSPLTVEWR*A). This peptide includes a modified arginine at position 189 and is the result of a missed cleavage due to the modification). This arginine residue is located at the next to last position in the soluble DR1 construct. Modification resulted in the complete loss of the peak at 2675.28 Da (β-(167-189), the peptide which includes R189), indicating near complete labeling. The corresponding mass for the C-terminal alanine which would be generated by cleavage at this site in unmodified DR is not detectable by MALDI/MS in this system. Interestingly, Arg189 was one of the peptide residues to be identified as important to the conformational change as measured by a change in antibody reactivity (Carven et al., 2004).

In all, three arginine modifications were observed which were present only in the empty conformation of DR. Based on relative intensities of the unmodified tryptic

fragment peaks, Arg50α and Arg189β were modified to a large extent, while Arg123α was modified to a much smaller extent.

III. C. 3. Modification of HLA-DR1 with NHS biotin and localization of derivitized residues by MALDI/MS.

The charged amine group of lysine makes it a prime target for chemical modification. NHS esters can be used to add functional groups or other modifiers to lysine residues. In this case, NHS-biotin was used to label lysine residues in empty and peptide-loaded preparations of DR1 (Figure III.2.B). The soluble DR1 construct contains 14 lysine residues (nine on the alpha chain and five on the beta chain). These residues are located throughout the protein complex and are mainly solvent accessible.

Empty and peptide-loaded HLA-DR1 complexes were modified with NHS-biotin and separated by SDS/PAGE. Gel bands were excised and digested with trypsin. As with arginine, modification of lysine changes the pattern of tryptic peptides making identification of incorporated biotin moieties relatively easy. A concentration of 5 mM NHS biotin was sufficient to differentially modify the two forms of the protein. Empty DR was modified at four lysine residues while DR-Ha was modified at only two (Figures III.6, and III.7).

Both empty and peptide-loaded DR1 preparations were modified at position α126 (Figure III.6 open arrows, Table III.4). This modification was evident as a new peak observed at m/z 2027.64. This corresponds to the peptide DRα-(124-140) (NGK*PVTTGVSETFLPR) with a modified lysine at position Lys126. The longer peptide is observed regardless of modification because Lys126 is followed by proline and

is therefore unable to be cleaved by trypsin. A corresponding decrease in the intensity of the unmodified peptide peak at m/z 1801.91, was observed indicating approximately 80% modification.

In empty DR1, a second alpha chain modification was identified (Figure III.6, solid arrows). A new peak (m/z 2878.36) was observed which corresponds to the peptide DRα-(51-75) (FASFEAQGALANIAVDK*ANLEIMTK) and contains biotinylated lysine at postion Lys67. A reduction in the peaks which correspond to the unmodified tryptic peptides α-(51-67) and α-(68-75) is also noted. Both peaks are reduced by about half, indicating the the extent of modification at this site was approximately 50%.

The beta chain of DR1 was also differentially modified by NHS-biotin. In empty DR, modification of the beta chain occurred at position Lys98. This is indicated by a new mass peak at m/z 1472.72 (Figure III.7, solid arrow), and corresponds to the peptide β-(99-105) (VEPK*VTVYPSK). The extent of modification at this site is difficult to ascertain as both of the short tryptic peptides that make up the longer peptide in the modified sample were not observed. A new peak at 2944.36 was observed in digests of both empty and peptide-loaded DR1 (Figure III.7, open arrows; Table III.4). This corresponds to the peptide β-(49-71) (AVTELGRPDAEYWNSQK*DLLEQR) with a modified lysine at position 65. The modification of this residue occurred to approximately the same extent (about 60-80%) in both preparations.

Overall two modifications were observed which were present only in the empty conformation of DR (Lys67α and Lys98β). Modification at Lys126α and 65β was observed in preparations of both empty and peptide-loaded DR.

III. C. 4. Modification HLA-DR1 with TNM and localization of derivitized residues by MALDI/MS.

Under mild conditions, TNM is an efficient and specific reagent for the nitration of solvent-accessible tyrosine residues (Cuatrecasas et al., 1968; Sokolovsky et al., 1966) (Figure III. 2. C.). The soluble DR1 construct contains a total of 13 tyrosine residues (five on the alpha chain and eight on the beta chain) which are distributed throughout each of the three tertiary domains of the protein.

Empty and peptide-loaded DR1 were labeled with TNM and subjected to in-gel tryptic digestion and MALDI/MS. Labeling of tyrosine residues does not interfere with the trypsin digestion therefore, the spectrum of expected peaks is not complicated by missed cleavages. Surprisingly, for some modified peptides, in addition to the expected mass shift of +45 Da ($+NO_2$), mass shifts of +29 Da and +13 Da were also detected. Recently, similar losses of oxygen due to the prompt fragmentation of nitrated tyrosine species have been identified by MALDI-MS (Petersson et al., 2001). The authors believe that this fragmentation occurs upon laser excitation in the spectrometer. While the fragmentation actually reduces the abundance of signals that can be detected, the overall fragmentation pattern can be used to unequivocally assign mass peaks to peptides containing nitrotyrosine.

Using this approach, we identified five sites of incorporation (Table III.5). The extent of labeling appears to be small and the pattern of reactivity was similar for both chains in the location of nitrated residues as well as for the peak intensity of the observed peptides. Upon digestion of the alpha chain, three new peaks are observed. The mass peak at 2686.41 (Figure III.8, open arrow) corresponds to a +45 Da shift for the peptide

α-(77-100) (SNY*TPITNVPPEVTVLNSPVELR) due to nitration of Tyr79. There is not a significant decrease in the intensity of the peak at 2640.41 Da (corresponding to unmodified tyrosine at that position) therefore the extent of labeling is likely to be very low. Similarly, new peaks are observed for the nitration of tyrosine at positions α150 and α161. These residues are both located on the same peptide α-(148-164) (FHY*LPFLPSTEDVY*DCR). Mass shifts of +45 and +90 Da are observed corresponding to the nitration of one and two tyrosine residues respectively (Figure III.8, solid arrows). This sequence is also represented on peptide fragments which are generated by missed cleavage at position α147. Mass peaks corresponding to peptides α-(147-164) (KFHY*LPFLPSTEDVY*DCR) and their corresponding nitrated peptides are also observed (Figure III.8, gray arrows).

On the beta chain, two tyrosine residues become modified (Table III.5). Mass shifts of +45 Da and +29 Da are observed for the peptide β-(49-65) (AVTELGRPDAEY*WNSQK) indicating modification of Tyr60 (Figure III.9, open arrows). Additionally, a shift of +16 Da is observed for the peptide β-(81-93) (HNY*GVGESFTVQR) indicating modification of Tyr83 (Figure III.8, solid arrows).

Overall five tyrosine modifications were observed. These occurred at positions Tyr79α, 150α, 161α, 60β and 83β. Based on relative intensities of the unmodified tryptic fragment peaks, all of the reactive tyrosine residues were modified to a small extent.

III. C. 5. Modification HLA-DR1 with NBS

At neutral pH, N-bromosuccinimide can be used to selectively modify tryptophan residues (Burstein and Patchornik, 1972; Spande et al., 1970; Witkop and Ramachandran, 1964). Reaction with NBS can be used to probe the accessibility and reactivity of tryptophan residues in proteins. The indole side chain of tryptophan absorbs strongly at 280 nm and oxidation by NBS causes a shift in absorption maxima from 280 to 260 nm (Patchornik et al., 1958) thus, NBS incorporation can be monitored in a spectrophotometer.

Empty and Ha-loaded DR were treated with 1-15 molar equivalents NBS. Modification was monitored in a spectrophotometer and the number of tryptophan residues oxidized were calculated using the method of (Spande et al., 1970) (Figure III.10.A., B.). Addition of 15 molar equivalents of NBS resulted in two tryptophan residues in empty DR but only one residue in DR-Ha (Figure III.10.C., D.). It is not known whether all of the reactivity occurs at only a single tryptophan residue or whether more than one tryptophan is oxidized to a smaller degree.

Attempts to localize tryptophan oxidation by in-gel tryptic digestion and MALDI/MS were unsuccessful as the oxidation was reversible even under non-reducing conditions for SDS-PAGE. Solution digestion and LC/MS analysis was also unable to identify oxidized residues (not shown).

III. D. Discussion

The class II MHC protein HLA-DR1 undergoes a distinct conformational change as it binds to its peptide ligand. In previous studies, monoclonal antibodies specific for

the empty conformation identified two distinct regions on the beta chain which change upon binding peptide, however little localized structural information is available for the involvement of the alpha chain.

In this study, we probed the accessibilities of empty and peptide loaded protein to a variety of chemical modifiers. Differences in reactivity between the two conformations were observed for three of the modifiers used (Figure III.11). This information can be used to gain insight as to which regions are involved in overall conformational change. Modifications which were observed in both conformations give information as to regions which remain constant in the two conformations. Overall, these data can be used to generate a model for the conformational change.

The soluble DR construct used in this study contains 27 arginine residues (8 on the alpha chain and 17 on the beta chain). Of these, 100% of the arginine residues on the alpha chain and 13 of 17 arginine residues (76%) on the beta chain were observable by mass spectrometry. The arginine specific modifier HPG, differentially labeled empty and peptide loaded DR. Three sites of incorporation were identified in the empty conformation that were not present in Ha-loaded DR. Of the 27 arginine residues present in DR, all but two of them are involved in ion pairing or hydrogen bonding interactions in the crystal structure of DR bound to the Ha-peptide (Stern et al., 1994). Arg50α and Arg189β have no hydrogen bonding partner and are two of the three sites of HPG incorporation.

One of these sites, Arg50α, is located on the loop between the beta-sheet floor of the peptide binding site and the alpha chain helix. In the structure of DR bound to Ha peptide, the main chain atoms of nearby Ser53α and Phe51α make hydrogen bonds with

the N-terminal portion of the bound peptide holding it in an extended polyproline type II helix. It is likely that in the absence of peptide, this loop becomes more flexible and allows Arg50α to react with the HPG modifier.

The other, Arg189β, is located at the C-terminal end of the immunoglobulin-like domain of the beta chain. It is also one of the residues identified as important for binding to MEM-266, an antibody specific for the empty conformation of DR (Carven et al., 2004), confirming the involvement of this region in the conformational change. The residues in this region are solvent-accessible in the structures of HLA-DR1 peptide complexes, but are located in a cleft between the domains. The observed conformational differences in this region can be due to one of a number of possible structural transitions such as local unfolding or rigid body domain shifting. Either case would result in a change in the accessibility of the residues in this region to both antibodies and chemical modifiers. Given the general stability of the immunoglobulin fold it is unlikely that this region unfolds in the empty conformation. Rigid body shifts of the entire β2 domain, including rotations of up to 15 degrees, are routinely observed in comparisons of HLA-DR1 peptide complexes in different crystal forms (Brown et al., 1988). Such shifts realign the β2 domain relative to the peptide binding domain, and could potentially couple peptide binding to domain rotation via interactions between loop residues at the top end of the Ig domain and the beta sheet floor of the peptide binding domain.

The third arginine residue which was modified in this study was Arg123α. Peak intensity measurements indicate only a small degree of modification at this site. This residue is located on the α2 immunoglobulin-like domain and is exposed to solvent. In the crystal structure of DR1 bound to the Ha-peptide, this residue is involved in a

hydrogen bond with the side-chain hydroxyl oxygen of Thr130α. Its involvement in this hydrogen bond interaction could explain the low degree of reactivity at this position.

The soluble DR1 construct contains 14 lysine residues (nine on the alpha chain and five on the beta chain), nine of which are observable by mass spectrometry. In this study, four lysine modifications were observed in the empty protein but only two were observed for peptide loaded DR. Lys126α and Lys65β were found to be modified in both conformations. Analysis of the crystal structure of peptide-loaded DR indicates that these residues are completely solvent exposed and are not participating in any hydrogen bonds within the protein. Lys126α is located on the α2 Ig-like domain nearby to Arg123α and Tyr161α which were also chemically modified. Lys65β is located on the beta chain alpha helix near the peptide binding site. In the X-ray structure, this residue is oriented away from the peptide and does not appear to be involved in peptide binding. This region of the β chain helix is located within the binding epitope for three of the antibodies specific for empty DR identified by (Carven et al., 2004). Other evidence for conformational lability in this region can be seen in crystal structures of DR-peptide complexes, which exhibit significant peptide-to-peptide variation in this region, and relatively high thermal B-factors, particularly for residues 63-67. Whether the overall secondary or tertiary structure differs at this position in empty and peptide-loaded DR, the chemical susceptibility of Lys65β remains the same for both conformations.

Two lysine residues were differentially modified (modified in empty DR but not in peptide-loaded DR). One of these, Lys98β is located on the first beta strand of the beta chain Ig-like domain. It is situated at the interface between the α2 and β2 subunits and makes a hydrogen bond with Ser95α on the AB loop of the alpha chain Ig-like

domain. The involvement of Lys98β is consistent with a rigid body movement of the β2 domain relative to the α2 domain, opening up the region for modification at this site.

The other, Lys67α is located on the alpha chain helix and orientated away from the peptide binding site. In the structure of peptide-loaded DR, this residue is located at the interface of the alpha chain α-helix and the beta sheet floor of the peptide binding domain. It forms a hydrogen bond network with the side chains of Tyr13α, Asn15α and Gln18α from the beta sheet as well as Glu71α from the helix. In the empty protein, this residue is accessible for modification and must rearrange relative to its position in peptide-loaded DR. Nearby residues Asn62α, Asp66α, and Val65α make up the P6 binding pocket and other nearby residues (Asn62α, Asp66α, and Asn69α) make hydrogen bonds with the main chain atoms of bound peptide. The absence of peptide and lack of an MHC-peptide hydrogen bond may cause slight rearrangement of the helix relative to the beta sheet floor. Even a three to five degree rotation along the helical axis would cause Lys67α to disrupt the hydrogen bonds formed between the α-helix and the β-sheet.

The extracellular domain of DR contains nine tryptophan residues (four on the alpha chain and 5 on the beta chain). Four of these tryptophan residues are completely buried in the structure of DR-Ha. Two more are located in the peptide binding site and would be blocked by the addition of peptide. Though we did observe differential oxidation of tryptophan in empty and peptide-loaded DR (Figure III.10) (two oxidized residues in empty DR relative to one for peptide-loaded DR), we were unable to localize the site of modification by mass spectrometry.

Other evidence for the involvement of tryptophan in the conformational change has been shown in the literature. Using intrinsic fluorescence studies, Kropshofer and co-workers show that empty DR1, prepared by acid release of endogenous peptide, exposes two Trp residues which are normally buried in the peptide-loaded conformation (Kropshofer et al., 1991). Since there are two tryptophan residues are located in the peptide binding site, it is possible that in the absence of peptide, one or both of these residues becomes accessible. If the observed NBS oxidation of tryptophan in empty DR1 is the result of exposure of tryptophan residues in the peptide binding groove in the absence of peptide then the potential information that could be gained by identifying the site of NBS induced oxidation may not be very useful in understanding the conformational change.

TNM is a small aqueous reagent that specifically modifies tyrosine to o-nitrotyrosine. Under the mild conditions used in this study, labeling of empty and peptide-loaded DR with TNM did not result in differentially modified residues. There are 13 tyrosine residues present in the DR construct - seven of these are completely buried. Of the six solvent accessible tyrosine residues, nitration was observed at five. Modification occurred at the following positions: 79α, 150α, 161α, 60β, and 83β. The similar reactivity of the two protein conformations indicate that the local environments surrounding each tyrosine residue are likely the same in both conformations.

The structures of class I (Bouvier and Wiley, 1998) and class II (Boniface et al., 1996; Runnels et al., 1996) MHC molecules folded in the absence of peptide have previously been referred to as folding intermediates such as molten globules. Molten globules are characterized as containing secondary structure elements of the native state

in a less compact form with many of the interior packing interactions unformed. Also, loops and other elements of the surface structure remain largely unfolded with different conformations. A large unfolding event or a general loosening of the secondary structure elements such as those observed for molten globules or other folding intermediates is inconsistent with the pattern of reactivity seen in this study. If the empty protein resembled a molten globule, the expected pattern of reactivity with side-chain modifiers would be drastically different from that which was observed in this study. One would expect many more modifications in a molten globule than in the native form and for the observed modifications to be uniformly distributed around the protein. In empty DR, there were only 4 modifications which were observed that were not also observed in the peptide-loaded conformation. Additionally, three of those four modifications are located on the same lateral face of the protein (Figure III. 11).

Overall, it is clear from the chemical modification experiments that though the conformational changes are distributed throughout the protein, only a few localized regions are involved. Three of the modifications which were observed in the empty but not the peptide-loaded conformation are located on the same lateral face of the protein. Interestingly, this face has been proposed as the interaction face of DR with HLA-DM (Doebele et al., 2000). HLA-DM helps catalyze peptide binding and release through conformational effects on DR1 (Zarutskie et al., 2001), and several models of interaction have been proposed (Beeson and McConnell, 1994; Chou and Sadegh-Nasseri, 2000; Dornmair et al., 1989; Sadegh-Nasseri and McConnell, 1989; Schmitt et al., 1999; Zarutskie et al., 2001). Many of these models involve HLA-DM recognizing or

promoting a structural change in HLA-DR which could facilitate peptide release. The location of three modification sites on this face is consistent with such a model.

In summary, empty and peptide-loaded DR1 was modified using a number of side-chain specific chemical modifiers in order to probe the solvent accessible residues present in both conformations. Overall, the pattern of reactivity suggests that the conformational changes, while distributed throughout the protein, involve only a few localized regions. This data is inconsistent with the idea that empty MHC molecules exist as molten globules or other folding intermediates.

III. E. Acknowledgement

The authors would like to thank Karin Green and James Evans of the Proteomics and Mass Spectrometry Core Facility at the University of Massachusetts Medical School for assistance with mass spectrometry.

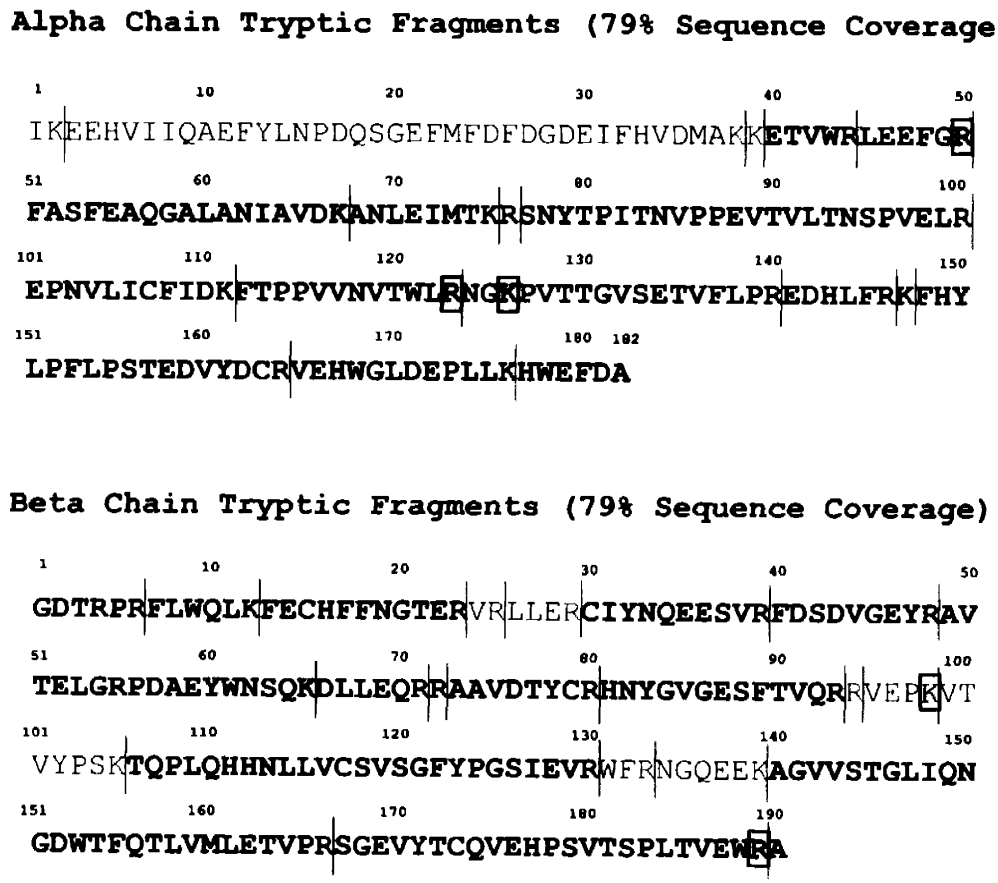


Figure III.1: Sequence coverage of DR1 tryptic digest.

Alpha chain (top) and beta chain (bottom). Trypsin cut sites are marked with a line. Peptides which were observed for unmodified DR1 are shown in bold. Sequence coverage for modified proteins was similar. All arginine, lysine and tyrosine residues are colored red. Modified residues are highlighted in gray. Modified residues which were observed in digests of empty, but not peptide-loaded DR1 are boxed.

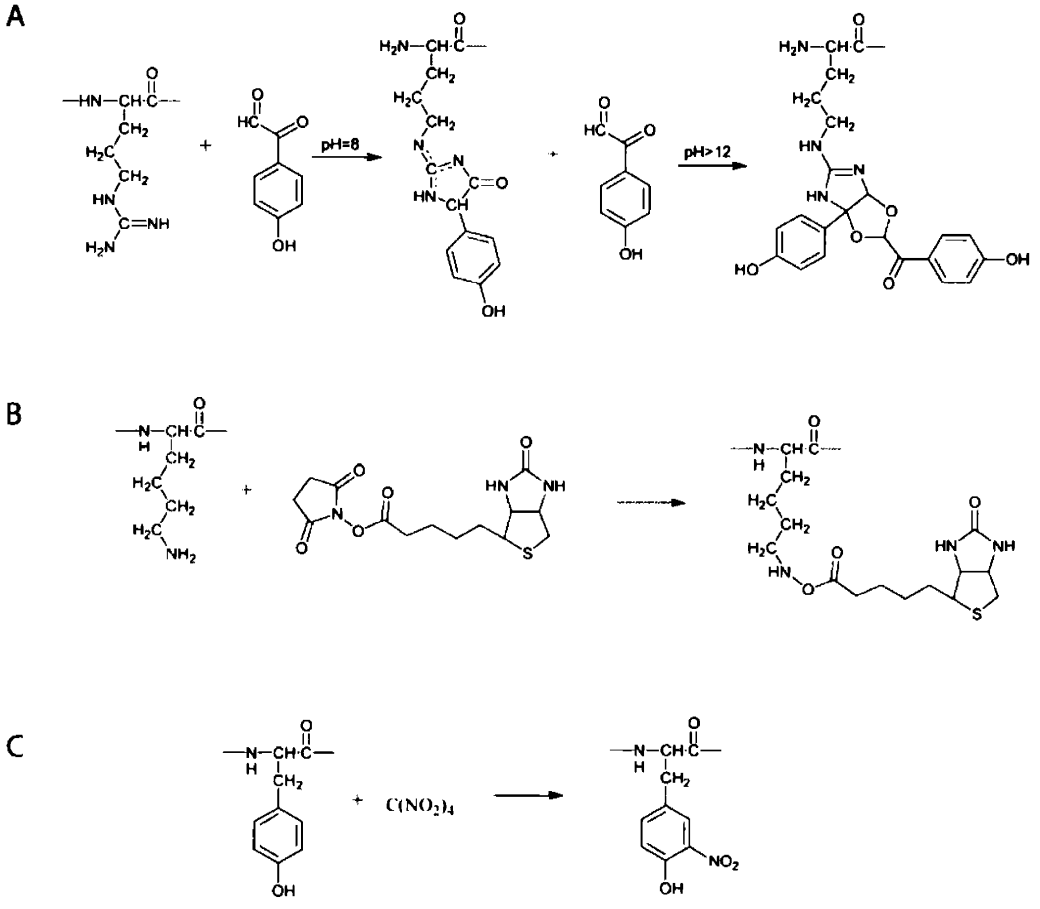


Figure III.2: Side chain-specific chemical modifications.

(A) Reaction of arginine with p-hydroxyphenylglyoxal. Reaction at pH=8 yields equimolar HPG incorporation as proposed by Wood et al. (1998); reaction above pH=12 incorporates two HPG molecules per residue. (B) Reaction of lysine with NHS-biotin. (C) Reaction of tyrosine with tetranitromethane.

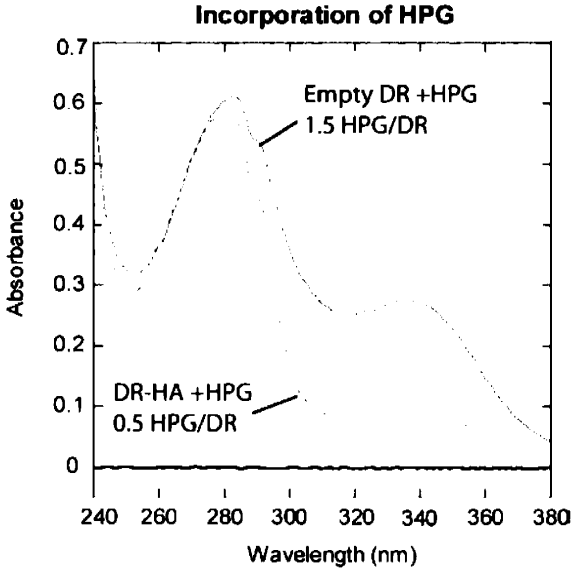


Figure III.3: Incorporation of HPG.

UV/Visible absorption spectra of empty and Ha-loaded DR modified by 5mM HPG. Empty DR incoprorates 1.5 molecules per protein complex. DR-Ha incorporates approximately 0.5 HPG molecules per protien.

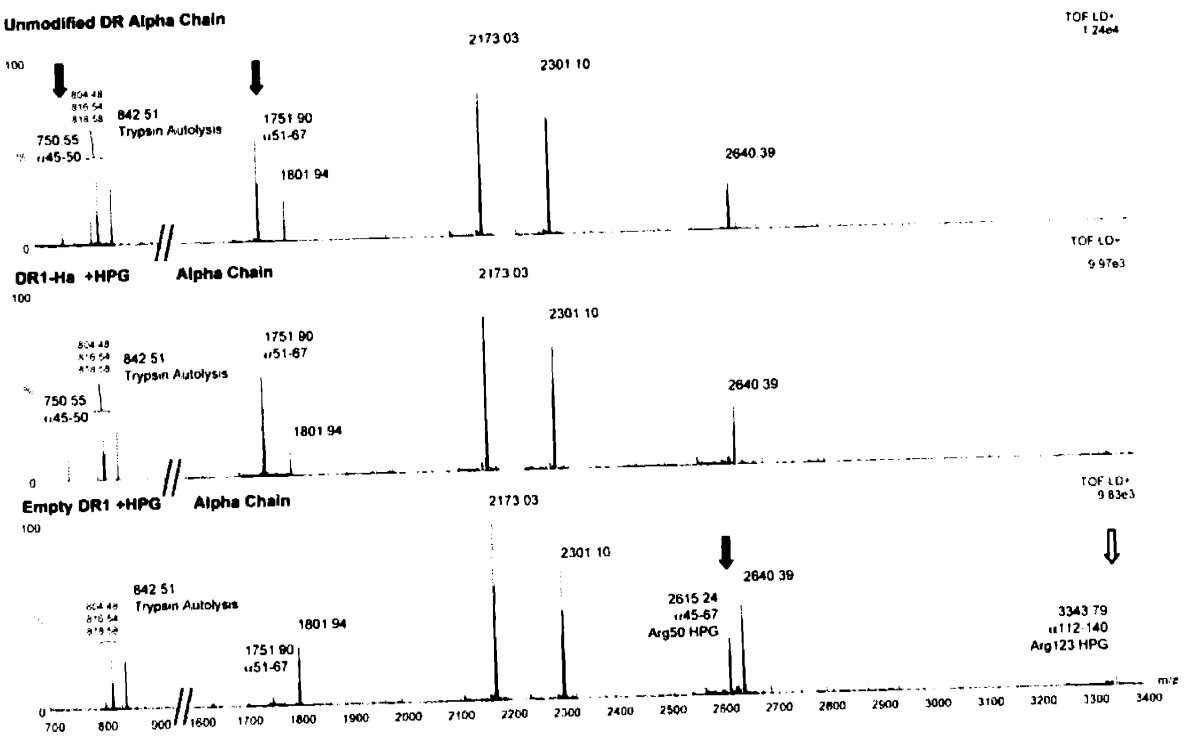


Figure III.4: MALDI mass spectra of alpha chain peptides after HPG modification and in-gel trypsin digestion.

Peptides derived from unmodified complex (top) or HPG modified DR1 (DR-Ha, middle; empty DR1, bottom) were analyzed by mass spectrometry. Modification at Arg50α in empty DR1 yields a new peak at m/z 2615.24 and a reduction in the intensities of peaks corresponding to unmodified peptides α45-55 and α51-67 (solid arrows). Modification at Arg123α yields a new peak at m/z 3343.79 (open arrow) without a reduction in peak intensity for corresponding unmodified peptides α112-123 and α124-140. No modification was observed for DR-Ha.

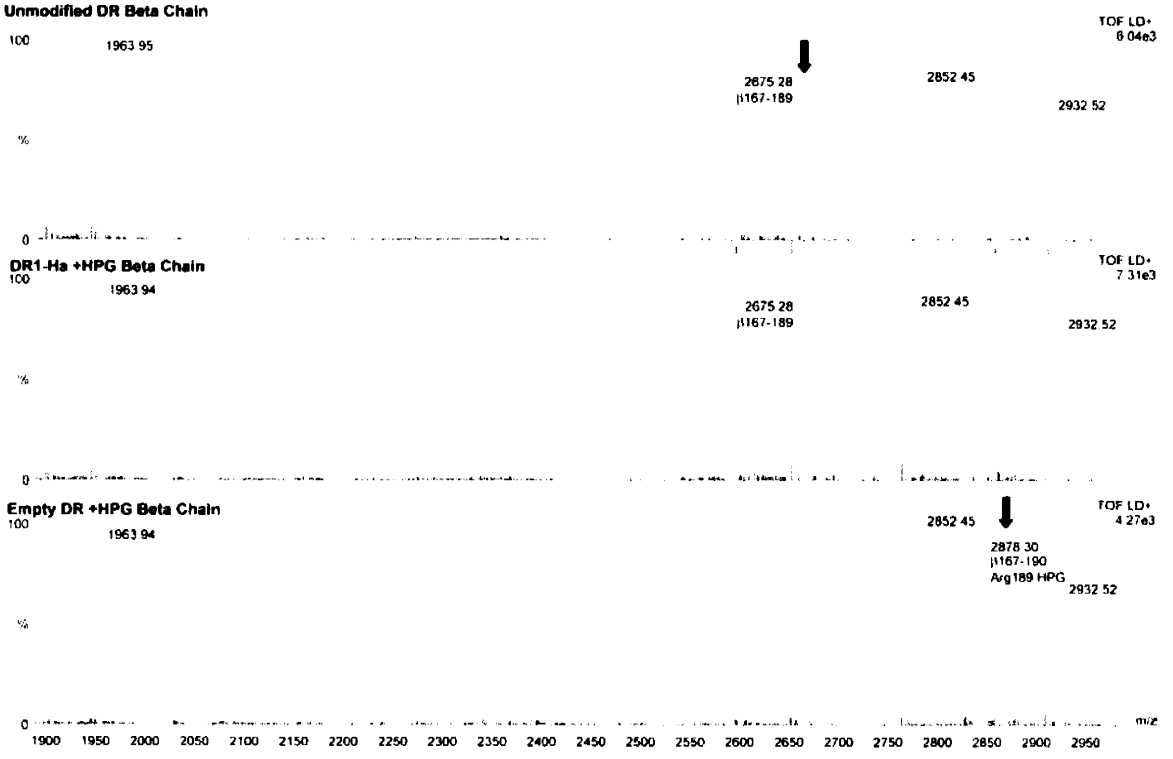


Figure III.5: MALDI mass spectra of beta chain peptides after HPG modification and in-gel trypsin digestion.

Peptides derived from unmodified complex (top) or HPG modified DR1 (DR-Ha, middle; empty DR1, bottom) were analyzed by mass spectrometry. For empty DR1, modification at Arg189β yields a new peak at m/z 2878.30 (β167-190) and a complete loss of intensity for the peak corresponding to unmodified peptide β167-189 (solid arrows). No modification was observed for DR-Ha.

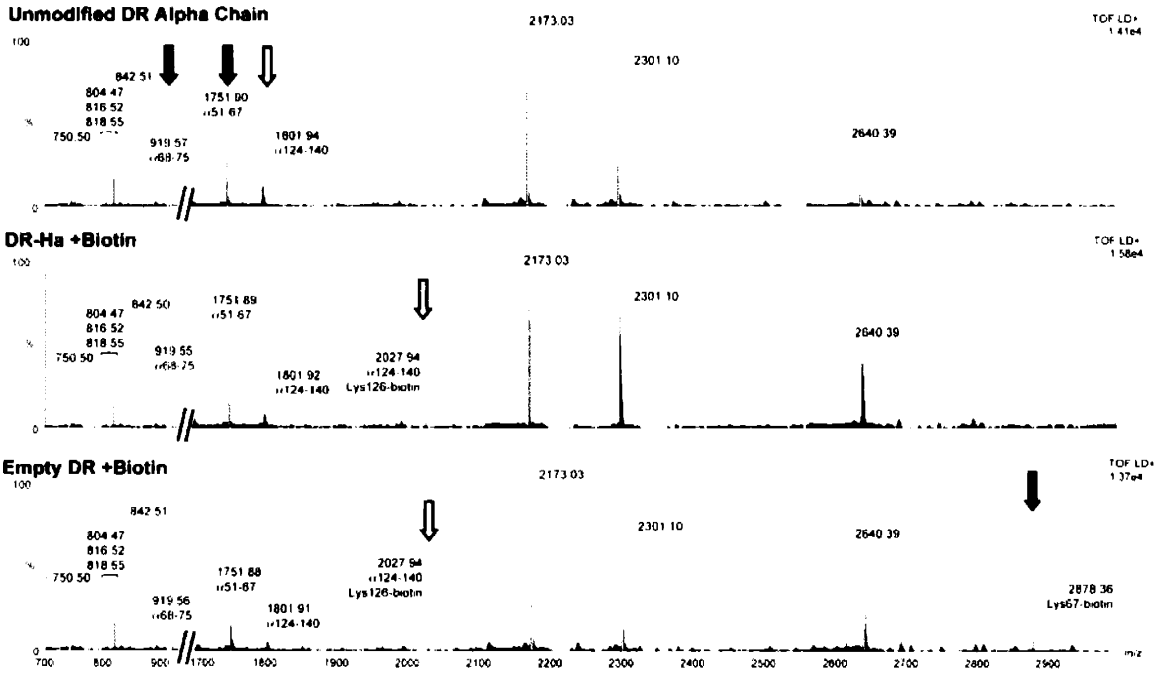


Figure III.6: MALDI mass spectra of alpha chain peptides after biotin incorporation and in-gel trypsin digestion.

Peptides derived from unmodified complex (top) or biotin modified DR1 (DR-Ha, middle; empty DR1, bottom) were analyzed by mass spectrometry. In empty DR1, modification at Lys67α yields a new peak at m/z 2878.36 and a reduction in the peak intensities for corresponding unmodified peptides α51-67 and α68-75 (solid arrows). Modification at Lys126α in both empty and peptide loaded DR yields a new peak at m/z 2027.94 (α124-140) and a reduction in the intensity of the peak corresponding to unmodified peptide α124-140 (open arrows).

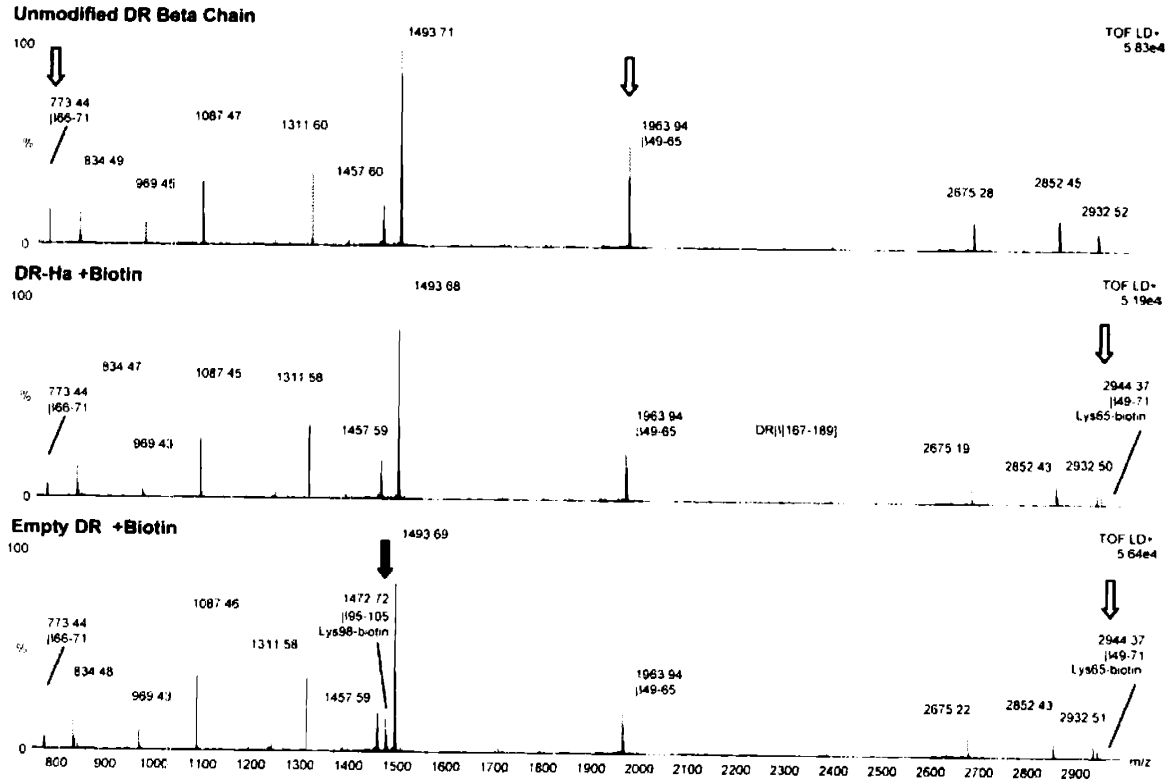


Figure III.7: MALDI mass spectra of beta chain peptides after biotin incorporation and in-gel trypsin digestion.

Peptides derived from unmodified complex (top) or biotin modified DR1 (DR-Ha, middle; empty DR1, bottom) were analyzed by mass spectrometry. In empty DR1, modification at Lys98β yields a new peak at m/z 1472.72 (solid arrow). The peaks corresponding to unmodified peptides β95-98 and β99-105 were not observed in the experiment. Modification at Lys65β in both empty and peptide loaded DR yields a new peak at m/z 2944.36 (β49-71) and a reduction in the intensities of the peaks corresponding to unmodified peptide β49-65 and β66-71 (open arrows).

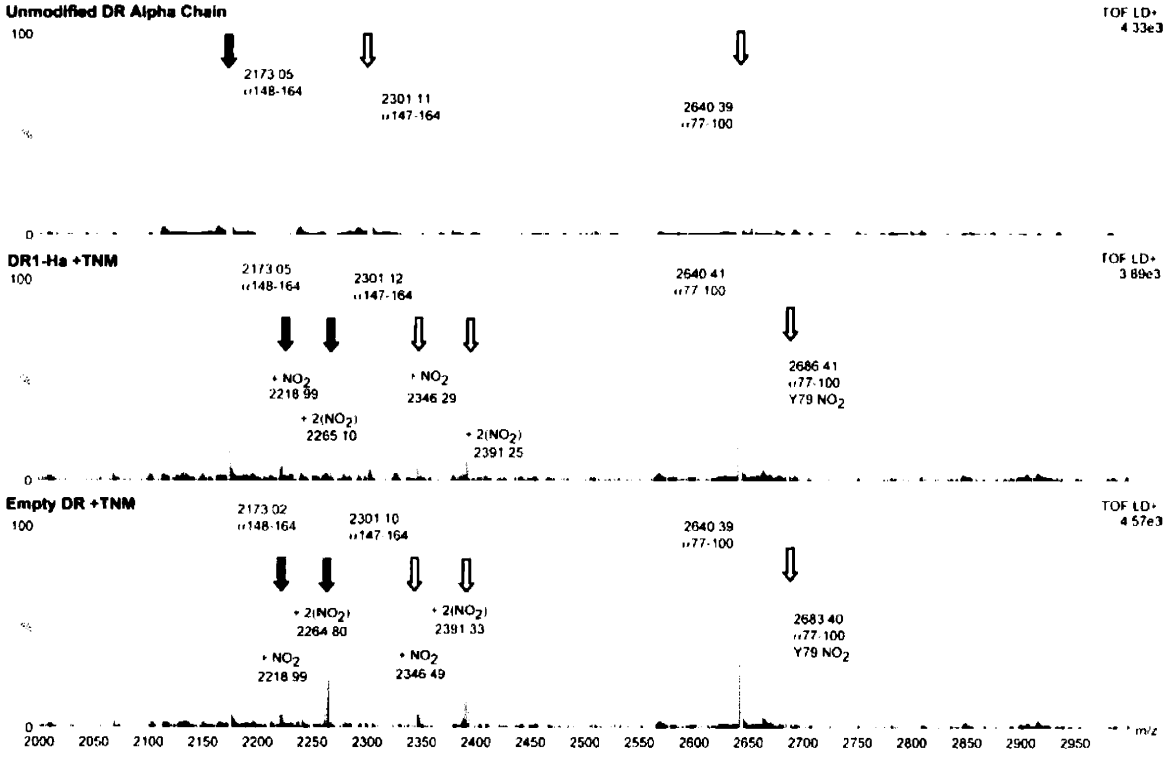


Figure III.8: MALDI mass spectra of alpha chain peptides after TNM modification and in-gel trypsin digestion.

Peptides derived from unmodified complex (top) or TNM modified DR1 (DR-Ha, middle; empty DR1, bottom) were analyzed by mass spectrometry. The residues Tyr150α and Tyr161α were observed on two tryptic peptides α148-164 (solid arrows) and α147-164 (gray arrows) due to a missed cleavage at position 147. Modification at these sites yield new peaks corresponding to mass shifts of +45 Da (one nitrotyrosine) and +90 Da (two nitrotyrosine residues). Modification at position Tyr79α yields a new peak at m/z 2683.40 (open arrows). Modification was similar in both empty and peptide-loaded DR.

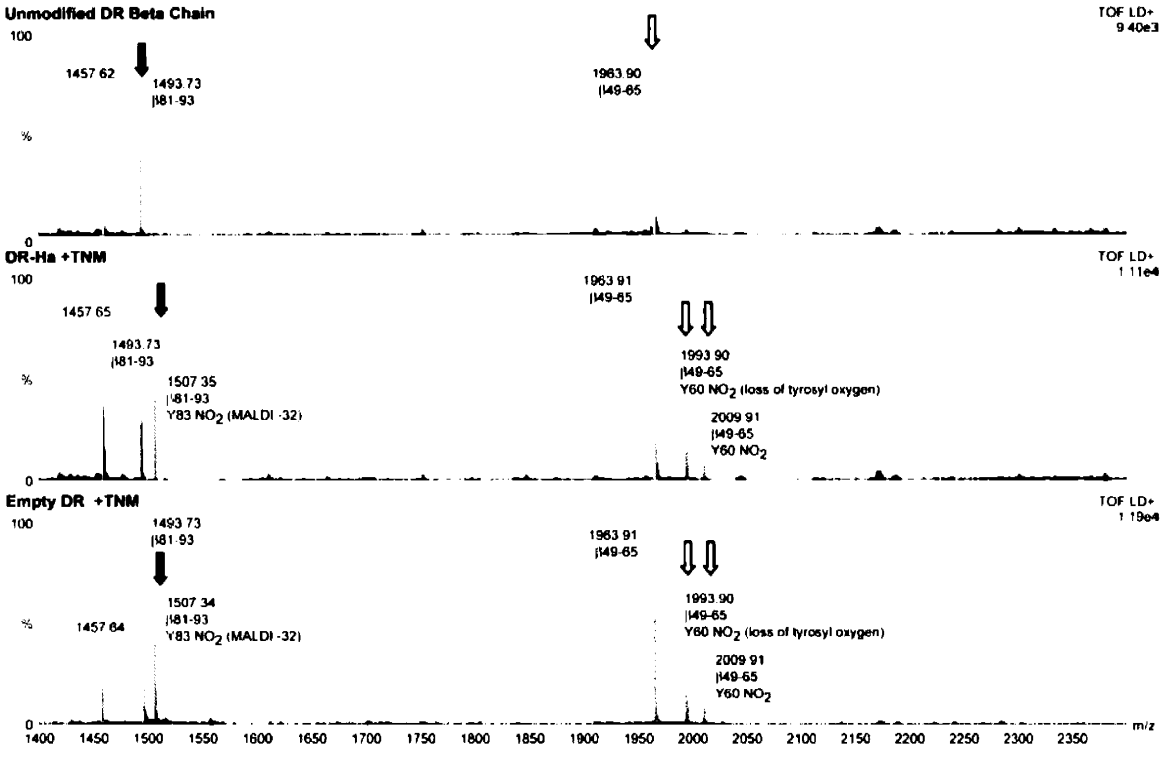


Figure III.9: MALDI mass spectra of beta chain peptides after TNM modification and in-gel trypsin digestion.

Peptides derived from unmodified complex (top) or TNM modified DR1 (DR-Ha, middle; empty DR1, bottom) were analyzed by mass spectrometry. Modification at position Tyr83β yields a new peak at m/z 1507.35 (solid arrows). Modification at position Tyr60β yields two new peaks corresponding to mass shifts of +29 Da and +45 Da (m/z 1993.90 and 2009.91) (open arrows). Modification was similar in both empty and peptide-loaded DR.

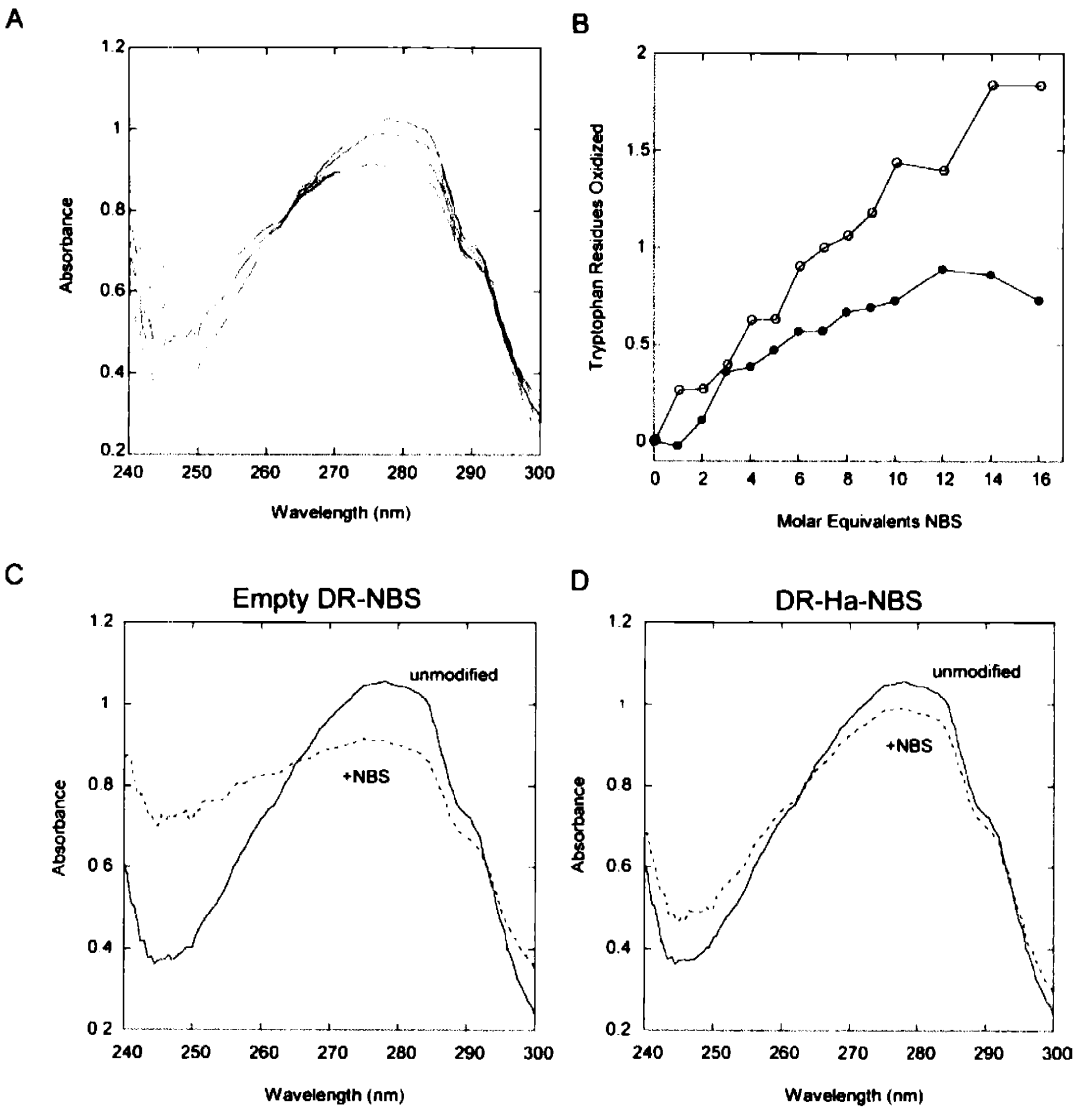


Figure III.10: Modification of DR1 by N-bromosuccinimide

(A) Addition of NBS to empty DR1 causes a decrease in the absorbance at 280 nm and an increase at 260 nm. (B) Oxidation of tryptophan residues in empty (open circles) and peptide-loaded (closed circles) DR by NBS. (C) Modification of empty DR with 15 molar equivalents NBS (1.8 tryptophan residues oxidized). (D) Modification of DR-Ha with 15 molar equivalents NBS (0.8 tryptophan residues oxidized).

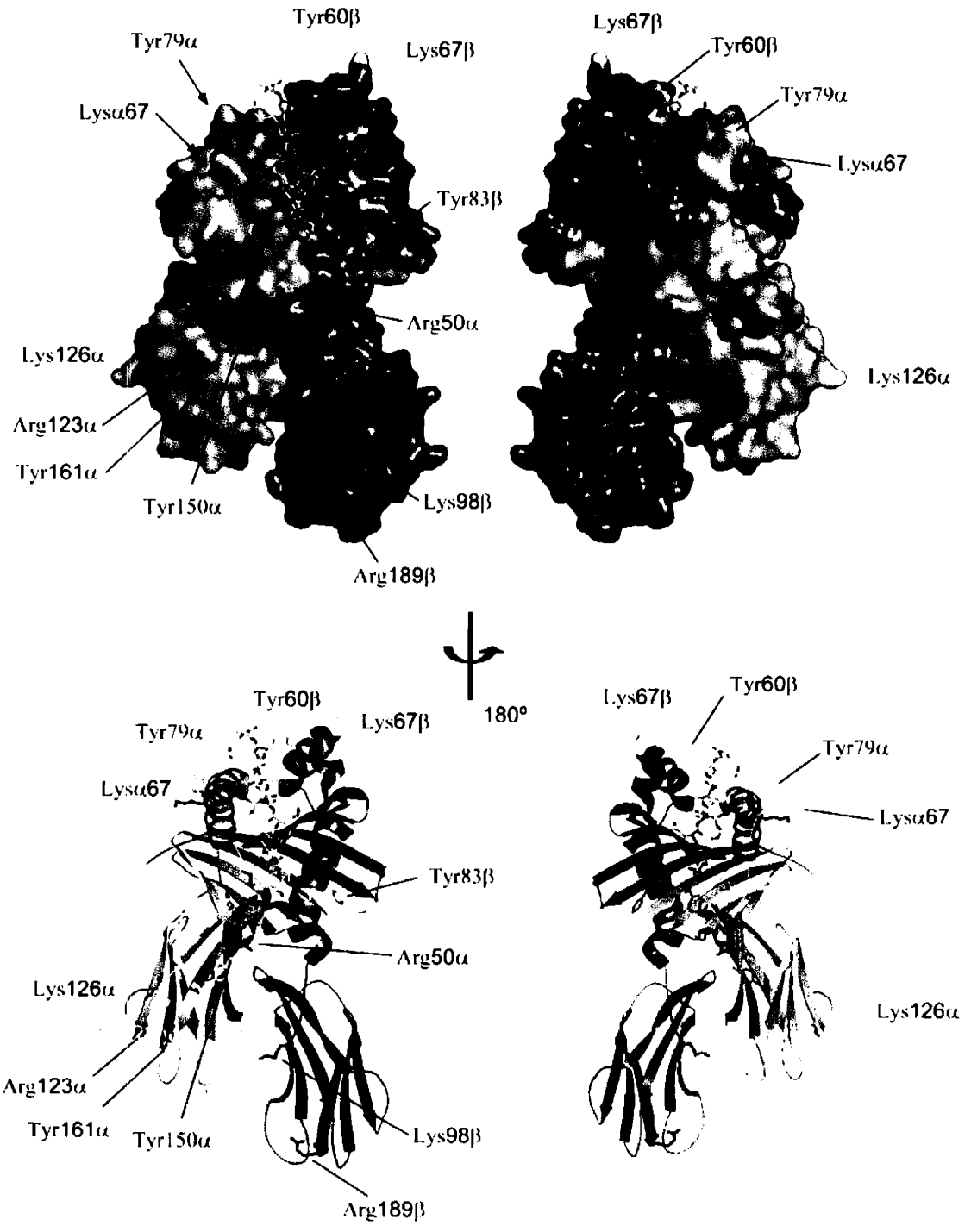


Figure III.11: Map of chemical modifications onto HLA-DR1 structure.

The α subunit is colored light green, the β subunit blue, and the peptide green. Modifications which occur only in empty DR1 shown in red. Modifications which occur in both empty and peptide-loaded DR1 are shown in yellow. Arg-α123, which is modified only to a small extent in empty DR1 (and not in DR-Ha) is shown in orange.

Table III.1 Alpha Chain Sequence Coverage

Theoretical Mass[a]	Peptide	MC[b]	Sequence	Observed Mass: Unmodified	Hydroxyphenylglyoxal: Empty DR	Hydroxyphenylglyoxal: DR-Ha	NHS-biotin: Empty DR	NHS-biotin: DR-Ha	Tetranitromethane: Empty DR	Tetranitromethane: DR-Ha
690.36	40-44	0	(K) ETVWR (L)	690.34	690.35	690.39	690.37	690.38	690.39	690.37
750.38	45-50	0	(R) LEEFGR (F)	750.36	n/o	750.63	750.50	750.50	750.63	750.61
804.33	177-182	0	(K) HWEFDA (-)	804.31	804.36	804.33	804.35	804.32	804.34	804.32
816.40	141-146	0	(R) EDHLFR (K)	816.44	816.44	816.45	816.42	816.42	816.45	816.43
818.45	39-44	1	(K) KETVWR (L)	818.43	818.43	818.43	818.43	818.43	818.43	818.43
919.49	68-75	0	(K) ANLEIMTK (R)	919.47	919.57	920.66	919.56	919.55	920.66	920.64
1361.71	101-111	0	(R)EPNVLICFIDK(F)	1361.83	1361.88	1361.85	1361.76	1361.74	1361.85	1361.83
1428.80	112-123	0	(K) FTPPVVNVTWLR (N)	1428.67	1428.75	1429.65	1429.75	1429.73	1429.65	1429.63
1435.76	165-176	0	(R) VEHWGLDEPLLK (H)	1435.74	1435.68	1435.80	1435.80	1435.80	1435.80	1435.80
1751.90	51-67	0	(R) FASFEAQGALANIAVDK (A)	1751.90	1751.90	1751.87	1751.88	1751.89	1751.43	1751.43
1801.98	124-140	0	(R) NGKPVTTGVSETVFLPR (E)	1801.94	1801.97	1801.92	1801.91	1801.92	1801.92	1801.92
2173.01	148-164	0	(K)FHYLPFLPSTEDVYDCR(V)	2173.03	2173.01	2173.01	2173.03	2173.03	2173.02	2173.05
2301.10	147-164	1	(R) KFHYLPFLPSTEDVYDCR(V)	2301.10	2301.10	2301.11	2301.10	2301.10	2301.10	2301.12
2640.39	77-100	0	(R) SNYTPITNVPPEVTVLTNSPVELR (E)	2640.39	2640.39	2640.39	2640.39	2640.39	2640.39	2640.39
4262.90	3-38	0	(K) EEHVIIQAEFYLNPDQSGEFMFDFDGDEIFHVDMAK (K)	n/o	n/o	n/o	n/o	n/o	n/o	n/o

[a]Monoisotopic Mass [MH]+ Mass window 675 to 5000 Da; [b]Missed Cleavage: n/a: Peak not in mass window; n/o: peak not observed

Table III.2 Beta Chain Sequence Coverage

Theoretical Mass[a]	Peptide	MC[a]	Sequence	Observed Mass: Unmodified	Hydroxyphenylglyoxal: Empty DR	Hydroxyphenylglyoxal: DR-Ha	NHS-bioitin: Empty DR	NHS-bioitin: DR-Ha	Tetranitromethane: Empty DR	Tetranitromethane: DR-Ha
701.3695	1-6	0	(-) GDTRPR (F)	701.22	701.38	701.37	701.33	701.32	701.35	701.33
704.3215	134-139	0	(R) NGQEEK (A)	n/o	n/o	n/o	n/o	n/o	n/o	n/o
773.4157	66-71	0	(K) DLLEQR (R)	773.44	773.38	773.39	773.41	773.40	773.38	773.37
793.4460	99-105	0	(K) VTVYPSK (T)	n/o	n/o	n/o	n/o	n/o	n/o	n/o
834.4878	7-12	0	(R) FLWQLK (F)	834.49	834.48	834.47	834.48	834.47	834.46	834.45
969.4464	73-80	0	(R)AAVDTYCR(H)	969.45	969.43	969.41	969.43	969.43	969.42	969.40
1087.4696	40-48	0	(R) FDSDVGEYR (A)	1087.47	1087.45	1087.44	1087.46	1087.45	1087.44	1087.43
1311.6003	30-39	0	(R)CIYNQEESVR(F)	1311.60	1311.59	1311.59	1311.58	1311.58	1311.56	1311.56
1457.6272	13-23	0	(K)FECHFFNGTER(V)	1457.60	1457.61	1457.60	1457.59	1457.59	1457.64	1457.65
1493.7137	81-93	0	(R) HNYGVGESFTVQR (R)	1493.71	1493.71	1493.70	1493.69	1493.68	1493.73	1493.73
1963.9514	49-65	0	(R) AVTELGRPDAEYWNSQK (D)	1963.95	1963.94	1963.94	1963.93	1963.93	1963.91	1963.91
2675.2775	167-189	0	(R)SGEVYTCQVEHPSVTSPLTVEWR(A)	2675.28	n/o	2675.28	2675.22	2675.19	2675.20	2675.25
2852.4518	106-130	0	(K)TQPLQHHNLLVCSVSGFYPGSIEVR(W)	2852.45	2852.45	2852.45	2852.43	2852.43	2852.42	2852.41
2932.5243	140-166	0	(K) AGVVSTGLIQNGDWTFQTLVMLETVPR (S)	2932.52	2932.52	2932.52	2932.50	2932.51	2932.50	2932.51

[a]Monoisotopic Mass [MH]+ Mass window 675 to 5000 Da; [b]Missed Cleavage; n/a: Peak not in mass window; n/o: peak not observed

Table III.3: Relevant HPG Modified Arginine Mass Peaks

DR Subunit	Peptide	Arginine	Observed Mass[a]	Calculated Mass[a]	MC[b]	Sequence	Modification	Relative Abundance[c]		
								Unmodified	Empty	DR-Ha
alpha	45-50	α50	750.55	750.38	0	(R) LEEFGR (F)	none	≡1	0[d]	0.95 ± 0.04
alpha	51-67	α50	1751.90	1751.90	0	(R) FASFEAQGALANIAVDK (A)	none	≡1	0.08 ± 0.02	1 ± 0.05
alpha	45-67	α50	2615.24	2615.28	1	(R) LEEFGR*FASFEAQGALANIAVDK (A)	Arg α50 HPG		≡1	0[d]
alpha	112-123	α123	1428.37	1428.80	0	(K) FTPPVVNVTWLR (N)	none	≡1	0.99 ± 0.04	0.97 ± 0.03
alpha	124-140	α123	1801.94	1801.98	0	(R) NGKPVTTGVSETVFLPR (E)	none	≡1	1.16 ± 0.08	0.73 ± 0.03
alpha	112-140	α123	3343.79	3343.78	1	(K) FTPPVVNVTWLR*NGKPVTTGVSETVFLPR (E)	Arg α123 HPG		≡1	0[d]
beta	167-189	β189	2675.28	2675.28	0	(R)SGEVYTCQVEHPSVTSPLTVEWR(A)	none	≡1	1.05 ± 0.05	0[d]
beta	190	β189	-	89.04	0	(R) A (-)	none	N/A[e]	N/A[e]	N/A[e]
beta	167-190	β189	2878.30	2878.30	1	(R)SGEVYTCQVEHPSVTSPLTVEWR*A (-)	Arg β189 HPG		≡1	0[d]

[a]Monoisotopic mass [MH]$^+$. [b]Missed cleavages. [c]Peak height relative to same peak in unmodified sample ± s.d.; s.d. for non-relevant peptides 0.05.
[d]Mass not present. [e]Not within mass window.
R*: Arginine-HPG

Table III.4: Relevant Biotinylated Lysine Mass Peaks

DR Subunit	Peptide	Lysine	Observed Mass[a]	Calculated Mass[a]	MC[b]	Sequence	Modification	Relative Abundance[c]		
								Unmodified	Empty	DR-Ha
alpha	51-67	α67	1751.88	1751.90	0	(R) FASFEAQGALANIAVDK (A)	none	≡1	0.50 ± 0.03	0.83 ± 0.04
alpha	68-75	α67	919.56	919.49	0	(K) ANLEIMTK (R)	none	≡1	0.40 ± 0.03	0.83 ± 0.05
alpha	51-75	α67	2878.36	2878.38	1	(R) FASFEAQGALANIAVDK*ANLEIMTK (R)	Lys α67-biotin		≡1	0[d]
alpha	124-140[f]	α126	1801.91	1801.98	0	(R) NGKPVTTGVSETVFLPR (E)	none	≡1	0.20 ± 0.03	0.50 ± 0.04
alpha	124-140[f]	α126	2027.96	2027.99	1	(R) NGK*PVTTGVSETVFLPR (E)	Lys α126-biotin		≡1	0.93 ± 0.06
beta	49-65	β65	1963.93	1963.95	0	(R) AVTELGRPDAEYWNSQK (D)	none	≡1	0.46 ± 0.03	0.41 ± 0.06
beta	66-71	β65	773.41	773.42	0	(K) DLLEQR (R)	none	≡1	0.24 ± 0.02	0.18 ± 0.04
beta	49-71	β65	2944.36	2944.36	1	(R) AVTELGRPDAEYWNSQK*DLLEQR (R)	Lys β65-biotin		≡1	0.98 ± 0.06
beta	95-98	β98	-	472.28	0	(R) VEPK (V)	none		N/A[e]	N/A[e]
beta	99-105	β98	-	793.45	0	(K) VTVYPSK (T)	none		N/O[g]	N/O[g]
beta	95-105	β98	1472.72	1472.72	1	(R) VEPK*VTVYPSK (T)	Lys β98-biotin		≡1	0[d]

[a]Monoisotopic mass [MH]$^+$. [b]Missed cleavages. [c]Peak height relative to same peak in unmodified sample ± s.d.; s.d. for non-relevant peptides 0.05.
[d]Mass not present. [e]Not within mass window. [f]Peptide contains sequence PK which is not cut by trypsin. [g]Peptide not observed.
K*: Lysine-biotin

Table III.5: Relevant Nitrotyrosine Mass Peaks

DR Subunit	Peptide	Tyrosine	Observed Mass[a]	Calculated Mass[a]	MC[b]	Sequence	Modification
alpha	77-100	α79	2640.39	2640.39	0	(R) SNYTPITNVPPEVTVLTNSPVELR (E)	none
alpha	77-100	α79	2686.41	2686.38	0	(R) SNY*TPITNVPPEVTVLTNSPVELR (E)	Tyr α77 NO2
alpha	147-164	α150,161	2301.11	2301.10	1	(R) KFHYLPFLPSTEDVYDCR(V)	none
alpha	147-164	α150,161	2346.26	2347.09	1	(R) KFHY*LPFLPSTEDVY*DCR(V)	Tyr α150 or 161 NO2
alpha	147-164	α150,161	2391.25	2393.09	1	(R) KFHY*LPFLPSTEDVY*DCR(V)	Tyr α150 and 161 NO2
alpha	148-164	α150,161	2173.05	2173.01	0	(K)FHYLPFLPSTEDVYDCR(V)	none
alpha	148-164	α150,161	2218.99	2219.00	0	(K)FHY*LPFLPSTEDVY*DCR(V)	Tyr α150 or 161 NO2
alpha	148-164	α150,161	2264.80	2264.99	0	(K)FHY*LPFLPSTEDVY*DCR(V)	Tyr α150 and 161 NO2
beta	49-65	β60	1963.90	1963.93	0	(R) AVTELGRPDAEYWNSQK (D)	none
beta	49-65	β60	1993.91	1993.9279	0	(R) AVTELGRPDAEYWNSQK (D)	Tyr β60 NO2 (-O)
beta	49-65	β60	2009.91	2009.9228	0	(R) AVTELGRPDAEYWNSQK (D)	Tyr β60 NO2
beta	81-93	β83	1493.73	1493.73	0	(R) HNYGVGESFTVQR (R)	none
beta	81-93	β83	1507.34	1507.7330	0	(R) HNY*GVGESFTVQR (R)	Tyr β83 NO2 (-2 O)

[a]Monoisotopic mass $[MH]^+$. [b]Missed cleavages.
Y*: Nitrotyrosine

IV. Empty Class II Molecules on the Surface of Dendritic Cells: Role of the Dendritic Cell Secreted Protease Activity

Note: This work was part of a large project done in collaboration with Aaron K. Sato (Ph.D. 1998) and Laura Santambrogio (currently at Albert Einstein College of Medicine). My role in this project was the characterization of a protease activity which is secreted by dendritic cells. The results of the work discussed in this chapter was included as part of a paper published in the *Proceedings of the National Academy of Sciences* (Santambrogio, 1999).

Summary: In antigen presentation to $CD4^+$ T cells, proteins are degraded to peptide fragments and loaded onto class II MHC molecules in a process involving the peptide exchange factors H-2M (murine) or HLA-DM (human). In many antigen-presenting cells these processes occur in intracellular endosomal compartments, where peptides are generated and loaded onto class II MHC proteins for subsequent transport to the surface and presentation to T cells. Here, we provide evidence for one aspect of an additional antigen-processing pathway in immature dendritic cells (DC). In other work, additional evidence for such a pathway was provided (Santambrogio, 1999). Immature DC express empty or peptide-receptive class II MHC molecules at the cell surface. Secreted DC proteases act extracellularly to process intact proteins into antigenic peptides. Peptides produced by such activity are efficiently loaded onto cell surface class II MHC molecules. Together these elements comprise an unusual extracellular presentation pathway in which antigen processing and peptide loading can occur entirely outside of the cell. In this report, the secreted protease activity is characterized and its role in the extracellular antigen presentation pathway is explored.

IV.A. Introduction

Recently, monoclonal antibodies specific for the empty conformation of class II MHC molecules revealed the presence of abundant empty molecules on the surface of spleen- and bone marrow-derived murine dendritic cells (Santambrogio et al., 1999b) and human PBMC ((Carven et al., 2004); Chitta, S. and Stern, L.J., unpublished observations). The empty class II MHC molecules are developmentally regulated and expressed predominantly on immature DC and DC precursors. The empty cell-surface class II MHC proteins are fully functional and bind peptides and present them to T cells without intracellular processing (Santambrogio et al., 1999a; Santambrogio et al., 1999b).

In conventional endosomal processing pathways, antigenic peptides are generated by the action of endosomal proteases (mainly cathepsins) which digest protein antigens brought into the cell by fluid-phase or receptor-mediated endocytosis (reviewed in (Watts, 2001)). Other antigen presenting cells, including non-professional APCs such as granulocytes, natural killer cells and cytoxic T cells, secrete granule proteases with a variety of specificities (Jenne and Tschopp, 1988). Many cell types express cell-surface proteases such as CD13, CD26 and matrix metalloproteases. Cathepsins have also been reported to be secreted (Katz and Taichman, 1999; Mort et al., 1984). Roles for these proteases outside of the endosome have been suggested (Accapezzato et al., 1998) but the relative efficiencies of such pathways are not known.

In this chapter, the ability of DC to secrete a protease activity is described and such activity is further characterized. The secreted protease activity is capable of processing intact proteins into peptide antigens which can be presented by empty cell-surface class II MHC molecules. Together, the presence of the empty MHC molecules

and other elements of antigen processing at the cell surface along with the secreted protease activity suggest an alternate (fully extracellular) pathway for the generation, processing and presentation of antigenic peptides to $CD4^+$ T cells.

IV. B. Materials and Methods

IV. B. 1. Cell Culture

Murine bone marrow DC were established by *ex vivo* differentiation of Thy1.2-, B220-, and GR1-negative precursor cells from SJL/J ($I\text{-}A^s$) or B.10.Br ($I\text{-}E^k$, $I\text{-}A^k$) mice, as described (Coligan, 1992). For immature DC, cells were used after 4 days in culture with 10 ng/ml granulocyte-macrophage colony-stimulating factor (GMCSF) in DMEM supplemented with 5% FBS, 2 mM glutamine, nonessential amino acids, 1 mM sodium pyruvate, and 20 mM Hepes buffer (5% complete DMEM). In some experiments, DC were further matured by subculture in media without GMCSF for 24-48 hr or by differentiation with 1 μg/ml lipolysaccharide (LPS) (Sigma) for 48 hr.

IV. B. 2. Generation of cell-free supernatants

Cell-free supernatants were prepared from 4-hr cultures of washed splenic cells (10^6 cells/ml) in complete DMEM, with or without 5% FCS as indicated, or in serum free defined culture media, by centrifugation at 4,000 × *g* for 12 min. Sodium azide (0.1%) was added to the cell-free supernatants, which were stored at 4°C. In some experiments, splenic DC supernatants were further fractionated by centrifugation at 100,000 × *g* for 60 min at 4°C. The high-speed pellet contained numerous 50- to 100-nm vesicular

exosomes (Zitvogel et al., 1998), and the high-speed supernatant contained soluble proteins.

IV. B. 3. Proteolysis of intact antigen

Bodipy-labeled casein, ovalbumin, and BSA were purchased from Molecular Probes, and Bodipy-labeled myelin basic protein was prepared as described (Jones et al., 1997). These reagents are heavily labeled with a fluorescent dye (3-10 Bodipy-FL per molecule), resulting in almost total quenching of the conjugate fluorescence. On proteolysis, Bodipy-labeled fluorescent peptides are released. Labeled proteins (5 μg/ml) were incubated at 22°C in 100 mM Tris·Cl, pH=8.0, with 10^6 DC, B cells, or macrophages, or with 10 μl of the corresponding 4,000 × *g* supernatants prepared as described. The net increase in fluorescence was measured by using 485-nm excitation and 535-nm emission wavelengths in a spectrofluorimeter.

IV. B. 4. Proteolysis of labeled peptides

Using a variety of peptide substrates carrying C-terminal fluorescent reporter groups, we determined that the secreted DC protease(s) efficiently cleaved the 7-amino-4-methylcoumarin (AMC)-labeled peptide Z-Gly-Pro-Arg-AMC (Bachem). Enzyme assays were performed at 37°C in 200 mM Tris·Cl with 0.1 mM peptide and 25 μl of cell-free supernatant. Fluorescence measurements were made by using excitation and emission wavelengths of 380 and 460 nm, respectively. The fluorescence was measured in increments of 10 sec for 5 or more min, and the initial velocity was calculated for each sample. For evaluation of protease inhibitors, cell-free supernatants were preincubated

before protease analysis for 30 min at 25°C with 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF, 1 mM), loxastatin (20 μg/ml), E64 [trans-epoxysuccinyl-L-leucylamido-(4-guanidino)butane, 5 μM], EDTA (5 mM), iodoacetamide (10 mM), leupeptin (50 μM), pepstatin (1 μM), PMSF (1 mM), *N*-tosyllysinechloromethylketone (100 μM), *N*-tosylphenylalaninechloromethylketone (100 μM), or α2-macroglobulin, or by boiling the supernatant for 5 min before assay, or by changing the pH of the assay from 8.0 to 5.1 followed by assay as above.

IV. B. 5. Chromatographic purification of protease activity

Serum-free cell supernatants were prepared as above and fractionated by ion exchange (HQ) chromatography. Fractions were tested using both the intact antigen and AMC-hydrolysis assays. Positive fractions were pooled and further purified by gel filtration using a Superdex S-200 gel filtration column (Pharmacia Biotech). Fractions containing protease activity were separated by SDS-PAGE and visualized by silver staining. Gel bands were cut, washed with acetonitrile, and submitted for sequencing and identification by in-gel tryptic digestion and mass spectrometric peptide mapping.

IV. B. 6. Affinity purification of protease activity

Serum-free cell supernatants were prepared as above and incubated with commercially available chloromethylketones containing a peptide linker and a biotin tag. Inhibited supernatants were tested for activity using the AMC hydrolysis assay as above. Biotinylated proteins were collected using streptavidin agarose beads. Beads were

washed with PBST and proteins were eluted from beads with SDS and separated by SDS-PAGE.

IV. C. Results

IV. C. 1. Dendritic cells secrete a protease activity

Secreted as well as membrane-bound proteases from a variety of cells are essential for the host defense and are part of the responsiveness to the environment of many APC (Cella et al., 1997). The finding of a protease activity associated with DC-secreted exosomes that can contribute to MHC class I-mediated presentation (Amoscato et al., 1998) suggested that DC themselves might express an extracellular proteolytic activity that could contribute to antigen processing.

Extracellular proteolytic activity was tested in DC cultures using casein, a generic proteolysis substrate, in a fluorescence assay that relies on relief of intermolecular quenching as fluorescent peptides are released from a highly labeled, internally quenched protein substrate (Jones et al., 1997). Both splenic and bone marrow-derived murine DC expressed substantial extracellular proteolytic activity, as compared to a much lower level for resting macrophages, B cells, or T cells (Figure IV.1.A). In addition to casein, proteolysis of other protein substrates by conditioned cell-free DC supernatants was observed, including myelin basic protein, ovalbumin, and BSA (Figure IV.1.B).

Using a conventional proteolysis assay based on hydrolysis of a short fluorescent peptide (CBZ-Pro-Gly-Arg-7-amino-4-methylcoumarin) that was efficiently cleaved by the supernatants (Figure IV.2.A), further characterizations of the proteolytic activity present in conditioned medium were performed. The DC activity was associated with

cell-free supernatants collected from 4-hr culture, as well as with intact cells treated with sodium azide (Fig. IV.2.B). The soluble proteolytic activity was not caused by cell lysis, as similar levels were observed in untreated cultures that maintained full viability during the culture period, including freshly sorted viable $CD11c^+$ cells. The activity was not associated with the exosomal fraction (Figure IV.2.B), which as reported (Zitvogel et al., 1999), consisted of numerous 50- to 100-nm unilamellar vesicles. The activity was completely blocked by boiling, by low pH or by the serine protease inhibitors PMSF and AEBSF, but not by inhibitors of cysteine proteases, metalloproteases, and acid proteases, or by other inhibitors of serine and cysteine proteases such as leupeptin, TLCK, TPCK or α2-macroglobulin (Figure IV.2.C). The pattern of inhibitor reactivity suggests that the DC secreted protease is of the serine protease family. The optimum pH for activity was 7.0 (not shown).

Incubation of intact HEL with cell-free supernatant resulted in the generation of an antigenic peptide containing the immunodominant HEL-(48-62) epitope in the culture supernatant from immature DC but not B cells, as detected by the T cell hybridoma 3A9 (not shown). Thus, the proteolytic activity secreted by DC can act to produce immunologically relevant antigenic peptides from intact proteins.

IV. C. 2. Purification of the DC secreted protease activity

Because the identification of the secreted protease activity required further characterization and isolation, two strategies for purification were employed. Using conventional chromatography, cell-free supernatants were fractionated using an ion-exchange column (Figure IV.3.A). Fractions were tested for the ability to digest both

whole protein and peptide using the protease assays described above. Positive fractions were pooled and further purified by gel filtration chromatography (Figure IV.3.B). The pattern of active fractions indicated that a large fraction of the total protease activity could reside within a single protease rather than a mixture of proteases.

Proteolytically active fractions were further separated by SDS-PAGE and visualized by silver staining (Figure IV.3.C). The positive fractions (7 and 8) from gel filtration chromatography each show one major band and some minor bands. The major band from each lane was cut from the gel and submitted for sequencing and identification by in-gel digestion and mass spectrometric peptide mapping.

The proteins identified in the two samples were found to be variants of bovine alpha-1-antiproteinase (present in fetal bovine serum) which co-purified with the protease activity. Unfortunately, the proteins identified by this analysis were not proteases therefore another method for identification was necessary.

A second strategy for isolation of the DC secreted protease involved the inhibition of the protease using a specific suicide inhibitor labeled with a biotin moiety. Chloromethylketones are irreversible inhibitors of serine proteases. These act by covalently modifying the active site nucleophile of the protease. Using the known substrate specificity for Gly-Pro-Arg-substrates, three similar, commercially available chloromethylkeotne-peptide substrates were chosen and tested for their ability to inhibit the DC protease (Figure VI.4). Each of the three inhibitors tested was able to inactivate the DC protease with approximately the same IC50 (not shown).

For purification, DC supernatant was incubated with biotinylated CK inihibitors and collected using streptavidin-agarose. The modified protease was then eluted from the

agarose beads and separated by SDS-PAGE (not shown). Biotinylated proteins were detected by Western blot analysis using peroxidase labeled streptavidin and chemiluminescent detection (not shown). Using this method, we were unable to identify the secreted protease due mainly to non-specific binding of BSA and related proteins present in culture medium (see discussion below).

IV. D. Discussion

IV. D. 1. Role of empty MHC proteins and the DC protease in antigen presentation

Dendritic cells act as the sentinels of the immune system. Immature DC in peripheral tissue constantly sample the environment for antigens, which are presented to T cells after DC maturation and migration to lymph notes. In current models proposed for DC maturation, immature DC express most or all of their class II MHC molecules in endosomal/lysosomal compartments (Pierre et al., 1997; Winzler et al., 1997) and not at the cell surface. Only upon DC maturation do endosomal class II MHC molecules become released from Ii and able to bind peptide fragments derived from endocytosed antigens; the resultant peptide complexes then are transported to the cell surface for presentation to T cells.

We find that a large fraction of the class II MHC molecules that are expressed on the surface of immature DC are present in the empty, peptide-receptive state. Thus, immature DC may use alternate pathways for trafficking and loading of class II MHC molecules, in addition to the endosomal retention pathway described above. Also, DC secrete a protease activity capable of cleaving a variety of protein antigens and generating antigenic peptide fragments for presentation on class II MHC molecules. Thus, DC can

utilize an alternate pathway for antigen processing and loading, which appears to act entirely at the cell surface. Such an extracellular mode of antigen presentation clearly would be distinct from current endosomal processing models. These data are consistent with some previously published data, including expression of HLA-DM on the surface of Langerhans cells (Andersson et al., 1998) and secretion of proteases by DC (Amoscato et al., 1998).

Two mechanisms can be envisioned for the production of empty class II MHC molecules at the DC surface. One possibility is that they are derived from newly synthesized class II MHC/Ii complexes that traffic to the cell surface en route to endosomal compartments. This pathway is minor in B cells (Pierre and Mellman, 1998), but has been reported to be an important trafficking route in DC (Saudrais et al., 1998). Cell-surface class II MHC/Ii complexes could interact with cell-surface H-2M or HLA-DM to release Ii, perhaps after cleavage of Ii by extracellular proteases. Ii is particularly sensitive to protease digestion (Park et al., 1995), and DC-conditioned medium has been observed to degrade Ii into small peptide fragments (G. J. Carven and L. J. Stern, unpublished data). Cell-surface class II MHC molecules released from Ii targeting signals by peptide exchange or by Ii processing would remain at the surface, whereas unprocessed class II MHC/Ii complexes would recycle into endosomes for processing and loading in the conventional pathway. Another possibility is that the empty cell-surface population is derived directly from class II MHC molecules that have not associated with invariant chain in the ER. Some allotypes of class II MHC molecules, including I-A^k (but not I-A^b), are able to fold correctly in the endoplasmic reticulum (ER) in the absence of invariant chain (Bikoff et al., 1995; Zhong et al., 1996). In B cells such molecules are

retained in the ER, but in DC they are expressed at the surface (Rovere et al., 1998). Ii-deficient mice express high levels of KL-304 reactive molecules on immature DC, indicating that empty class II MHC molecules can traffic to the surface without Ii. Cathepsin S-deficient mice express normal levels of class II MHC protein on the surface of DC, suggesting that an Ii-independent pathway could be active in DC (Shi et al., 1999).

The extracellular antigen presentation pathway described here could play an important role in the immunological function of immature DC. The ability of immature DC to sample their environment by producing and binding peptides directly in the extracellular space would serve to broaden the spectrum of antigens available for presentation to T cells. In DC, labile peptides could be protected from terminal degradation that might otherwise occur in the extremely proteolytic endosomal environment, by binding to empty class II MHC at the surface. Such class II MHC-mediated protection from proteolytic degradation has been observed for particular peptides in B cells (Sercarz et al., 1993). The problem of protecting labile peptides may be particularly acute for immature DC, as they do not appear to generate a continuous supply of class II MHC molecules in the endosomes until maturation, and potential antigens would need to survive in an internal compartment until generation of active class II MHC molecules. The empty class II MHC molecules also could act as antigen receptors, able to collect at the cell surface extracellular peptide antigens that might not be taken up efficiently by fluid phase uptake. Additionally, maintenance of class II MHC molecules at the surface in an empty, peptide-receptive state would allow binding and presentation of relatively low-affinity, weakly binding peptides that might not be able to compete efficiently for binding in the endosomes. This latter function may be more

important in maintenance of peripheral T cell tolerance and to thymic T cell selection than in presentation of foreign antigens at the lymph node.

IV. D. 2. Isolation and identification of the DC protease

Identification of the specific protease responsible for the observed activity was attempted using two different techniques. Using a standard chromatographic approach, the protease activity was fractionated by ion exchange and gel filtration columns in an attempt to remove components of the tissue culture supernatant. This strategy was able to purify the specific activity of the DC protease by more than 10-fold. Extrapolating from the range of specific activities known for very active proteases on similar substrates (~1000 U/umol), the total protease activity present in the gel filtration fractions corresponds to approximately 10-100 fmol (~0.5-5 ng for a protease with a MW=50,000 Da). While each step in the purification process yielded an increase in the specific activity of the protease due to the removal of contaminating proteins present in the preparation (about 11 fold), the total protease activity measured after each step decreased significantly (Figure IV.5.D). The overall activity decreased from 1910 pmol AMC released per minute to 284 pmol/min (a 6.7 fold decrease).

Using SDS-PAGE and in-gel tryptic digestion and mass spectrometric peptide mapping, we hoped to identify the source of the activity. Knowing that the protease was present at very low levels, the active fractions were separated on a gel and the gel was stained using silverstaining for its potentially high sensitivity. Unfortunately, the silverstain procedure which was used is poorly compatible with downstream MS. For this reason, the bands were sent to the Biopolymers facility at Harvard University, which

specializes in the identification of proteins at extremely low levels. The two excised bands were determined to be different isoforms of bovine alpha-1-antiproteinase, a contaminant from FBS present in the original culture supernatant. This protease inhibitor co-purified (perhaps specifically) with the protease activity in serum-free culture supernatants indicating that even substantial washing of DC prior to culture in serum-free medium is not sufficient to remove all serum components.

Using sensitive spectrofluorometric techniques, it is often possible to detect protease activity at protein levels that are far too low to purify. This is especially true in cases of complex protein mixtures such as serum and was apparently the case for the DC secreted protease. For this reason, it was determined that another method for identification of the protease was necessary.

In order to specifically label the DC secreted protease with an affinity tag, a second strategy was used. Chloromethylketones are potent, irreversible inhibitors of serine proteases. A number of peptide-chloromethylketone inhibitors are commercially available which contain a covalently linked biotin moiety specifically for the removal (and possible subsequent purification) of serine proteases from protein mixtures. Three chloromethylketones similar in sequence to the known substrate Gly-Pro-Arg were tested and shown to inhibit the protease activity. The biotinylated DFPR-CK was used to affinity label the DC protease in serum-free cell culture medium. Streptavidin-agarose beads were used to collect biotinylated proteins from the supernatant. The beads were washed and eluted by denaturing the streptavidin with SDS before loading onto a gel. Detection of biotinylated proteins could occur by Western blot analysis using labeled streptavadin as a probe.

Using this strategy, we were also unable to identify the secreted protease. The major limitation associated with the identification attempts that were undertaken was the inability to purify the activity away from high levels of protein content (relative to the small protease concentration) present in cell culture media. This resulted inextremely high background binding of media proteins to the streptavidin agarose beads. A number of commercially available "serum-free" defined media were used in an attempt to lower the amount of non-specific binding to the agarose beads. These media, however, contain high levels of other proteins (such as BSA) designed to simulate the presence of serum for the culture of cells and significant background bands corresponding to non-protease proteins were observed.

Recent developments in proteomics-based approaches have enabled the quantification and characterization of a diverse array of low and high abundance proteins found in complex mixtures (Griffin et al., 2001; Paweletz et al., 2001; Petricoin et al., 2002). A discussion of possible directions aimed at identifying the protease responsible for the observed activity is given in chapter VI.

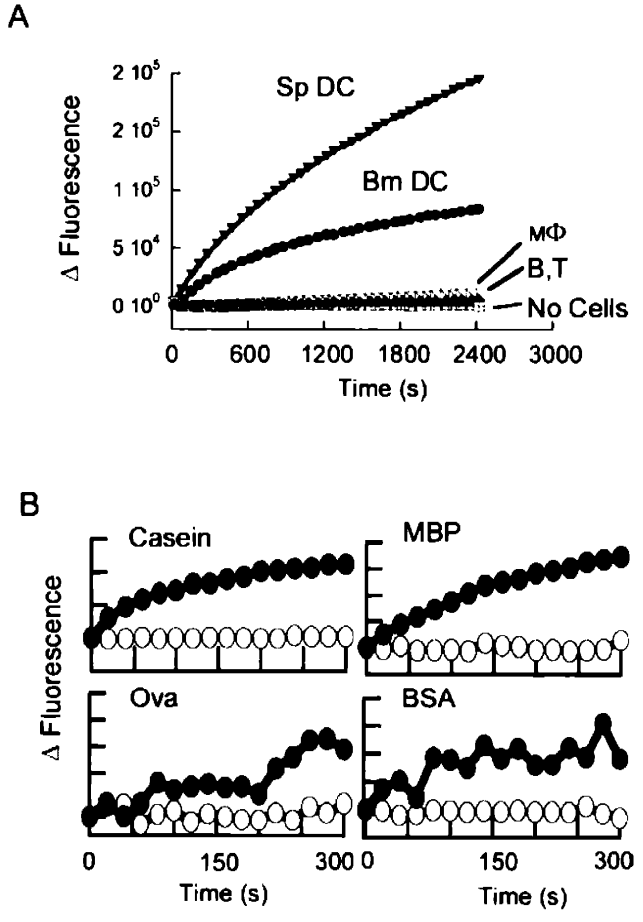


Figure IV.1: Dendritic Cells Produce a Protease Activity

(A) Proteolytic activity of splenic (Sp) DC, bone-marrow (Bm) DC, splenic macrophages (mΦ) or a mixture of splenic B and T cells (B,T) measured by release of quenched fluores on labeled β-casein in a fluorescence assay. (B) Conditioned medium from splenic DC has proteolytic activity which can degrade BODIPY labeled casein, myelin basic protein (MBP), ovalbumin (OVA) and serum albumin (BSA).

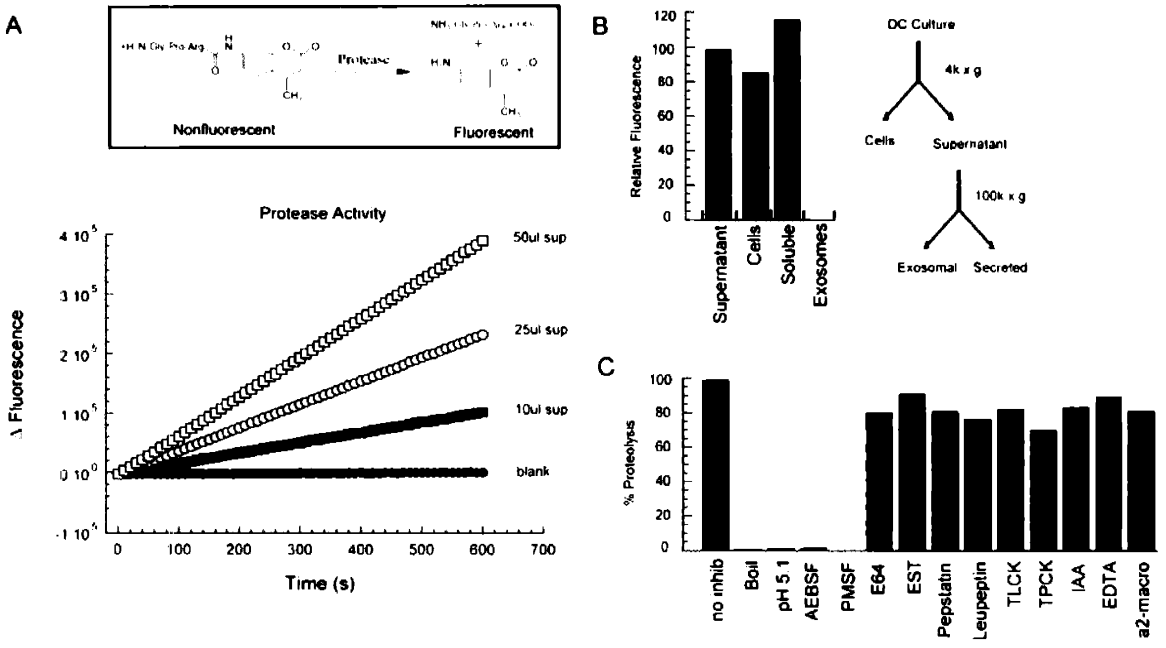
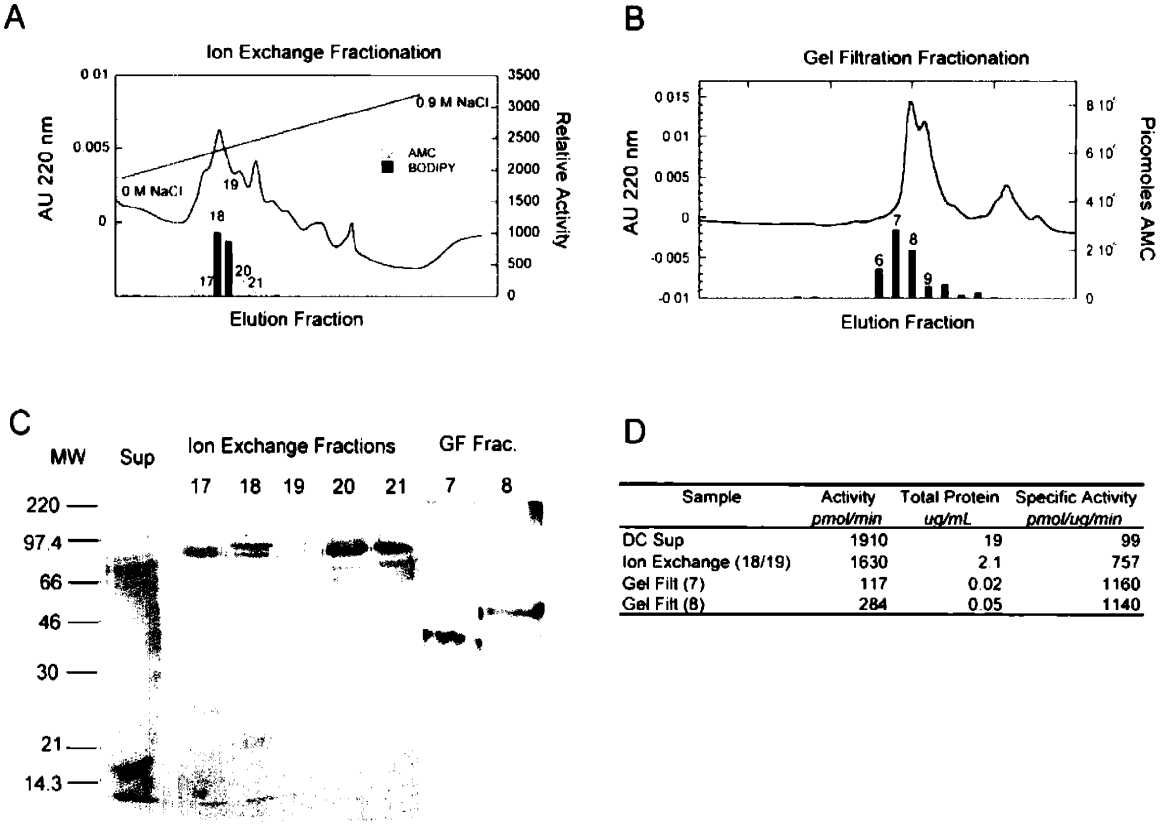


Figure IV.2: DC Protease Activity is Secreted

(A) Protease activity of DC conditioned medium measured using release of aminocoumarin from labeled peptide. (B) Subcellular fractionation. Protease activity for cells, culture supernatant, exosomes and high speed soluble fraction, prepared as shown. (C) Protease inhibitors. Conditioned serum-free medium can hydrolyze aminocoumarin labeled peptide substrate. Hydrolysis is blocked by boiling, low pH, and incubation with the general serine protease inhibitors 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF) and PMSF, but not inhibitors of other protease families of more specific serine protease inhibitors TLCK or TPCK.



Sample	Activity *pmol/min*	Total Protein *ug/mL*	Specific Activity *pmol/ug/min*
DC Sup	1910	19	99
Ion Exchange (18/19)	1630	2.1	757
Gel Filt (7)	117	0.02	1160
Gel Filt (8)	284	0.05	1140

Figure IV.3: Fractionation of DC Protease(s)

(A) HLPC anion-exchange chromatography of DC supernatant using HQ resin. Fractions were tested for peptide (hatched bars) and protein (solid bars) proteolysis activity. (B) Gel filtration chromatography using Superdex S-200, of pooled ion-exchange fractions. (C) SDS-PAGE of fractions, visualized by silver staining. (D) Purification table, with activity determined as GPR-AMC hydrolysis.

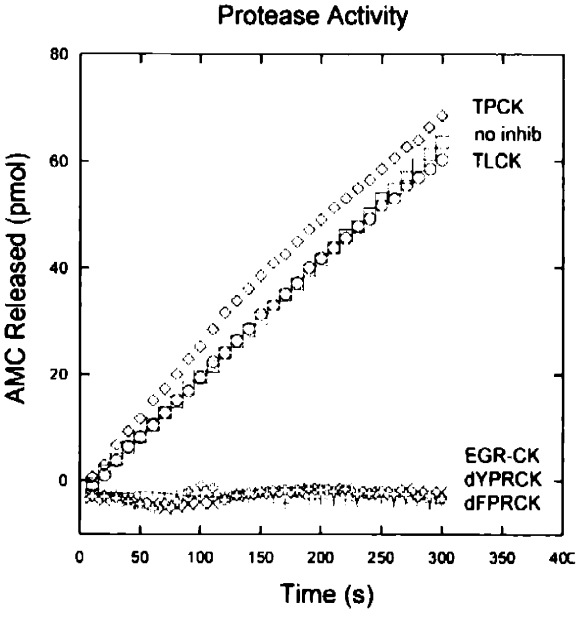


Figure IV.4: Inhibition of DC Protease Activity by specific Chloromethylketones

Hydrolysis of GPR-AMC by DC conditioned medium is inhibited by DTyr-Pro-Arg-CK (dYPR-CK), DPhe-Pro-Arg-CK (dFPR-CK), and Glu-Gly-Arg-CK (EGR-CK) but not by Tos-Phe-CK (TPCK) or Tos-Lys-CK (TLCK).

V. Characterization of the Activity of Aminopeptidase N (CD13) Activity on the Surface of Microglial Cell Derived Exosomes

Note: This chapter describes other work done characterizing the protease activity present on the surface of microglial exosomes. This work was part of a collaborative project with Illaria Portoliccio (Harvard University) and Laura Santambrogio in which we investigated the role of exosomes that are secreted by microglial cells. My role in this project was the characterization of a protease activity which present on the surface of microglial exosomes. This work, together with a proteomic characterization of exosomal protein content, has been prepared as a manuscript and submitted to the *Journal of Biological Chemistry* for publication.

Summary: Exosomes are small membrane vesicles that are secreted by a multitude of cell types as a consequence of fusion of multivesicular endosomes with the plasma membrane. Exosomal vesicles have been characterized in multiple cell systems including antigen presenting cells such as B lymphocytes and dendritic cells. In this study, a novel type of vesicle released by brain microglial cells was characterized. Proteomic analysis (discussed only briefly in this report) identified proteins known to be resident in exosomal preparations from dendritic cells and B cells including enzymes, chaperones, tetraspanins and membrane receptors. Other identified proteins, such as the aminopeptidase CD13 were not previously reported to be found in exosomal preparations and may be unique to microglial exosomes. In work described in detail in this chapter, the activity of exosomal CD13 was further characterized. Aminopeptidase activity was 20 times higher in exosomal preparations than on the cell surface in its ability to cleave enkephalin neuropeptides. The resultant cleaved neuropeptides were unable to bind to neuronal opioid receptors as assessed by cAMP levels. Microglial exosomal vesicles may represent an important, previously unrecognized delivery system in the brain, an organ where cell motility is highly restricted.

V. A. Introduction

Microglia are brain resident cells that are derived from bone marrow elements which infiltrate the brain early during the fetal and neonatal development periods (Carson et al., 1998; Ford et al., 1995; Frei et al., 1988; Hickey and Kimura, 1988). The role of microglial cells has best been characterized under degenerative and inflammatory conditions, where they function as antigen presenting cells. However, microglial function in normal brain physiology is largely unknown. Microglia may have a role in central nervous system (CNS) maintenance and homeostasis as suggested by their global gene expression profile which revealed mRNA transcripts for several growth factors, proteoglycans, and neuropeptides, as well as receptors for neurotransmitters, neuropeptides and endorphins (Re et al., 2002). The expression of surface receptors for neuropeptides and neurotransmitters on microglial cells suggest modulation of microglial functions by neuronal cells. In this respect, it has been previously shown that neuronal electric activity is a strong down-modulator of class II MHC protein expression (Neumann et al., 1996; Neumann et al., 1998). On the other hand, the expression of transcripts encoding proteoglycans and growth factors suggests a potential influence of microglial cells on neuronal growth and maintenance (Re et al., 2002). Altogether, these data underscore the existence of a bidirectional communication network between glial cells and neurons.

Cell-cell communication can rely on soluble mediators or direct membrane contact between the signaling and the targeting cells. Another mechanism has been recently described, which involves the release and uptake of exosomes, small vesicles, 40-100 nm in diameter secreted by different cell types. Exosomal vesicles have been

described in several bone marrow derived cell types (Pan et al., 1983), and are well characterized in antigen presenting cells (Raposo et al., 1996; Thery et al., 2002). Using exosomes from dendritic cells (DC), a comprehensive proteomic analysis has been performed (Thery et al., 2001). Analysis of exosomal protein composition reveals that they are small antigen loaded vesicles filled with class I and class II MHC proteins, heat shock proteins, adhesion molecules, T cell costimulatory molecules, and tetraspanins. Collectively, previous data suggest that exosomes are utilized by DC and B cell as "small packages of information" to be transferred to a target cell (Thery et al., 2002; Wubbolts et al., 2003).

To help evaluate a potential physiological role of microglial cells in neuronal homeostasis and communication between different brain resident cells, we investigated the production of exosomes by microglia. Microglial cells were found to produce exosomes, and exosomal protein content was analyzed by mass spectrometric peptide mapping, western blotting and enzymatic analysis. The analysis identified several proteins already reported in B cell and DC derived exosomes as well as exosomal proteins not previously described, including the aminopeptidase CD13. These results led us to test exosomal function in its ability to degrade neuropeptides *in vitro*. The ability of exosomes to carry out these processes supports the idea that microglial cells contribute to neuronal homeostasis, and underscore the importance of a novel exosomal delivery system in an organ where cell motility is highly restricted.

V. B. Materials and Methods

V. B. 1. CD13 Aminopeptidase activity measurements

Exosomes from N9 and LS102.9 cells were prepared as by Illaria Portoliccio (Harvard University) and provided to me for protease and enzyme characterization. Exosomes were preincubated in the presence or absence of the protease inhibitor phebestin (Sigma) at a final inhibitor concentration of 17 ug/ml. They were then mixed with 200-mmol leucine-p-nitroaniline (Sigma) in a 96 well plate (2-ug/ml total exosomal protein in 150 μL PBS per well). The release of p-nitroaniline at 10, 60, 120, 180, and 240 minutes was used to follow aminopeptidase activity by measuring the absorbance at 405 nm.

Cell membranes were obtained from post-nuclear supernatants (PNS) of N9 microglial cells. Briefly, 50 million cells were rinsed with ice-cold PBS and resuspended in 10 mM Tris (pH=8)/1 mM EDTA containing protease inhibitors. Cell suspensions were homogenized using a cell homogenizer. Cell debris and nuclei were pelleted at 1000 x *g* for 10 min and the PNS was saved. Cell membranes were pelleted by centrifugation at 75,000 x *g* for one hour and resuspended in PBS containing 1% NP40 and stored at 4°C until use. Aminopeptidase activity was measured as above using the p-nitroaniline release assay.

V. B. 2. Cleavage of opioid neuropeptides by CD13

Neuropeptides met-enkephalin (Bachem) and leu-enkephalin (Bachem) (at a final concentration of 100 uM of each peptide) were incubated in the presence or absence of exosomes from N9 microglial cells or LS102.9 B cells (3ug total protein) overnight in a

total volume of 0.5-mL PBS. Exosomes were separated from the reaction mixture by spinning in a microcon centrifugal concentration device (10,000 MWCO). (Millipore). The amount of remaining peptide was determined by reverse phase HPLC using a C18 column and a gradient of 2-80% acetonitrile in 60 minutes.

V. B. 3. cAMP immunoassay

The cAMP assay was performed by Laura Santambrogio and is included in this chapter for reference purposes. Three micrograms of N9 or LS102.9 exosomal preparations were incubated with or without 1 μM leu-Enkephalin for three hrs at room temperature in 100 μL of PBS. Samples, including a 1 μM leu-Enkephalin exosome-free, positive control were centrifuged through a centricon membrane centrifugal concentration device (10,000 MWCO). The exosome-free flow through for each condition was incubated, in triplicate, with the NIE 115 neuroblastoma cell line (CD13 negative) for 15 minutes at 37°C. At the end of the incubation the NIE-115 cells were lysed in 0.1M hydrochloric acid and the quantitation of acetylated cAMP present was determined by immunoassay with an anti rabbit polyclonal antibody (R&D systems).

V. C. Results

V. C. 1. Microglial cells secrete exosomes

Among the brain-resident cell types, microglial cells are unique in that they are derived from bone marrow precursors. Microglia function as antigen presenting cells in brain-mediated immune responses. Other antigen presenting cells have been shown to produce exosomes and the role of exosomes in ferrying MHC class II peptide complexes

and other proteins to target cells has already been described (Thery et al., 2002). We were interested in investigating whether microglial cells could also be a source of exosomes, and whether such exosomes could play a role in intracellular communication in the central nervous system.

A well-characterized murine microglial cell line, N9 (Corradin et al., 1993), was grown in serum free medium to avoid potential contamination from serum-derived vesicles. To avoid contamination with apoptotic bodies we collected microglia only when more than 95% of cells stained negatively for Annexin V and were viable by trypan-blue staining. Supernatants from two-day cultures were filtered (0.2 μm filter), concentrated 10-fold by ultrafiltration and exosomes were isolated by ultracentrifugation. Electron microscopy revealed the presence of numerous secreted vesicles varying in size from 20 to 120 nm (Figure V.1). These vesicles were similar in shape, structure and dimension to the previously described exosomes derived from dendritic cells, and B cells (Raposo et al., 1996; Thery et al., 2001).

V. C. 2. Molecular characterization of microglial exosomes

As part of this study, a full proteomic characterization of microglial derived exosomes has been performed and is discussed in more detail elsewhere ((Potolicchio et al., 2004)). Briefly, microglial exosomes were determined to be derived from endosomal multivesicular bodies similar to those previously reported in other antigen presenting cell types. This was determined by the presence of many known endosomal-specific proteins such as syntaxin 8 and vSNARE proteins Vti-1A and Vti-1B using Western blot analysis (not shown).

In antigen presenting cells, endosomal compartments are particularly enriched for MHC class II molecules, the MHC class II associated chaperone invariant chain (Ii), and several proteases important for Ii degradation and antigen processing (Amigorena et al., 1994; Tulp et al., 1994). Western blot analysis was performed on microglial exosomes and confirmed the presence of these proteins (not shown).

Further characterization was performed by SDS-PAGE followed by in-gel proteolytic digestion and MALDI-TOF mass spectrometric analysis. Many cytoskeleton and cytoskeleton associated proteins (actin, tubulin, cofilin) were identified (Table V.1). Also, several cytoplasmic proteins of the GTPase family, as well as tRNA binding protein could be detected. Several chaperone molecules, which have been previously identified in DC derived exosomes (Thery et al., 2001), were found in the microglial preparation including members of the HSP family such as HSC73 and HSP86 and three isoforms of 14-3-3 (β, ε, ζ), which are multifunctional proteins that bind and modulate the function of a wide array of cellular proteins (Fu et al., 2000). Several enzymes involved in glycolysis, and lipid metabolism were also identified.

Integrins, such as Mac1 and Mac2 antigens, and tetraspanin proteins, such as CD9 and CD81 were expressed in microglial exosomes. Both classes of proteins are involved in antigen presentation by promoting adhesion and membrane organization in microdomains. The pattern recognition receptor CD14, important for innate immunity, was also identified by MS/MS, as was the Fc receptor for IgE, and GP42, a membrane glycoprotein structurally related to the Fc receptor family (Guselnikov et al., 2002). Also notable, is the presence of NAP-22, a calmodulin associated protein previously described

on the external surface of the synaptic vesicle membrane and involved in vesicle cycling (Yamamoto et al., 1997).

V. C. 3. Role of the exosomal CD13 aminopeptidase

One protein not previously identified in B cell-derived or dendritic cell-derived exosomes was the aminopeptidase N or CD13 (Table V.1). CD13 is a cell-surface bound peptidase, which cleaves N-terminal amino acids other than proline and arginine. CD13 was detected by confocal microscopy and flow cytometry analysis on the surface of N9 cells but not the LS 102.9 B cell line (Figure V.2 A).

To assess the potential activity of CD13 in N9 derived exosomes, an exosomal preparation was incubated with leucine-pNA, which can be cleaved to form the bright yellow product p-nitroaniline in the presence of an aminopeptidase. Exosomes from N9 cells were determined to be proteolytically active while exosomes from LS102.9 cells were unable to cleave the chromogenic substrate (Figure V.2 B). This activity was completely inhibited by phebestin (Figure V.2 B), an inhibitor specific for aminopeptidase-N (CD13) (Nagai et al., 1997). The aminopeptidase activity of microglial exosomes, 41 pmol/min/ug, was 20 times higher than that of N9 cell surface membrane preparations, 2 pmol/min/ug (Figure V.2 C). Thus, microglial exosomes are enriched for CD13 over the cell surface.

CD13 has been implicated as an important enzyme responsible for the degradation of the neuropeptides methionine- and leucine-enkephalin (Lucius et al., 1995). To investigate whether exosomal CD13 was capable of degrading these neuropeptides, cleavage was monitored by reverse phase HPLC (Figure V.3 A). Met- and

Leu-enkephalin were completely degraded after overnight incubation with exosomes from N9 cells but remained intact after incubation with exosomes from LS102.9 cells. A shorter incubation (three hours) was sufficient to degrade more then 80% of 50 μM Leu-enkephalin (data not shown).

Enkephalins bind with high affinity to opioid receptors expressed on the surface of neuronal cells (Wilson et al., 2003). Activation of the opioid receptor leads to inhibition of adenylyl cyclase activity with a subsequent decrease in neuronal cAMP levels (Liu et al., 2001) (Figure V.3 B). To investigate whether enkephalin cleavage by exosomal CD13 could affect neuronal cAMP levels, exosomal preparations from N9 or LS102.9 cells were incubated with or without 1μM of Leu-Enk peptides for 3 hrs at room temperature. Samples were then centrifuged through a centricon 10 centrifugal filter device (to remove the exosomes) and the flow-through was incubated for 30 minutes at 37°C with the neuronal cell line NIE-115 (which expresses high levels of opioid receptors (Rao and Murphy, 1984) and is CD13 negative (data not shown)). Neuronal cells incubated with Leu-Enk showed a 50% decrease in the basal level of cAMP (Figure V.3 B). As predicted, exosomes alone did not alter the cAMP levels (Figure V.3 B). Leu-enk previously incubated with exosomes from LS102.9 was not cleaved and thus retained its ability to down-regulate cAMP. On the other hand, Leu-enk previously incubated with exosomes from N9 cells was cleaved and thus unable to signal through the opioid receptors as assessed by a statistically significant decrease in neuronal cAMP (Figure V.3 B).

Thus, the CD13 expressed by microglial exosomes is capable of neuropeptide degradation, and may have a distinct effect on the local neuropeptide activity in the brain.

V. D. Discussion

Exosomal vesicles have been described in several different cell types; most of which are derived from bone marrow. Here, we report that CNS microglia also produce and secrete exosomes. The analysis of proteins expressed in microglial exosomes identified quantitative and qualitative differences in protein content between microglial exosomes and exosomes previously described from in cell types (Thery et al., 2002). The expression of CD13 appears to be an important feature of microglial exosomes which distinguishes these vesicles from exosomes secreted in other hematopoietic cell types.

The role of brain microglia under normal physiological conditions is still poorly defined, even though evidence has been accumulating that microglial cells are an integral part of a communication network within the CNS. As further evidence of their postulated role in neuronal homeostasis, microglial exosomes also display enzymes important for protein degradation. CD13 is an aminopeptidase that functions as a membrane ectoenzyme in removing N-terminal amino acid residues from polypeptides (Hoffmann et al., 1993).

In this report, exosomal CD13 is shown to hydrolyze leucine- and methionine-enkephalins, thus regulating the ligand concentration available for opioid receptors and ultimately the neuronal levels of the second messenger cAMP. A number of different enzymes, including aminopeptidases (N and A), and endopeptidases (enkephalinase, dipeptidyl aminopeptidase and angiotensin II) are involved in the brain catabolism of enkephalins. However, the major mode of enkephalin inactivation *in vitro* and *in vivo* was shown to be the release of the amino-terminal tyrosine by an aminopeptidase (Hui et

al., 1998). More importantly exosomal aminopeptidase activity is 20 times higher than the plasma membrane surface activity.

The microanatomy of neuropeptide release differs from that of neurotransmitter release. Neuropeptides can be released from the neuronal soma, neuronal dendrites, and at the axonal level, whereas neurotransmitters are almost uniquely released at the axonal terminus. Neurons do express CD13 on the pre-synaptic membrane, which could control the catabolism of neuropeptides released at the synaptic level. On the other hand, glial and exosomal CD13 could control the catabolism of neuropeptides released at sites distant from the synaptic membranes.

In conclusion, proteomic and functional analyses of microglial exosomes indicate that exosomes may play a role in attending functions such as neuronal metabolic support and neuropeptide catabolism. Such functions are also provided by parental microglial cells; however, there are physiological advantages in releasing exosomes since they are mobile in an organ where cell mobility is highly restricted. Thus, similarly to neurotransmitter-filled vesicles exosomes could deliver their protein cargo where needed.

V. E. Acknowledgments

This work was done in collaboration with Illaria Potolicchio and Laura Santambrogio. I would also like to thank Richard Riese, S.H. Wong, and Christopher Stipp for their contributions.

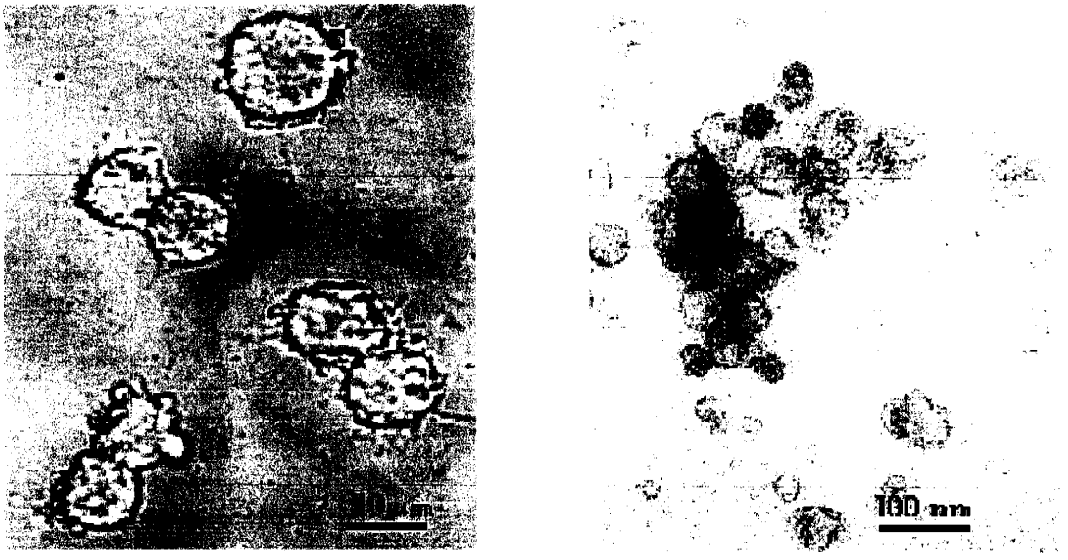
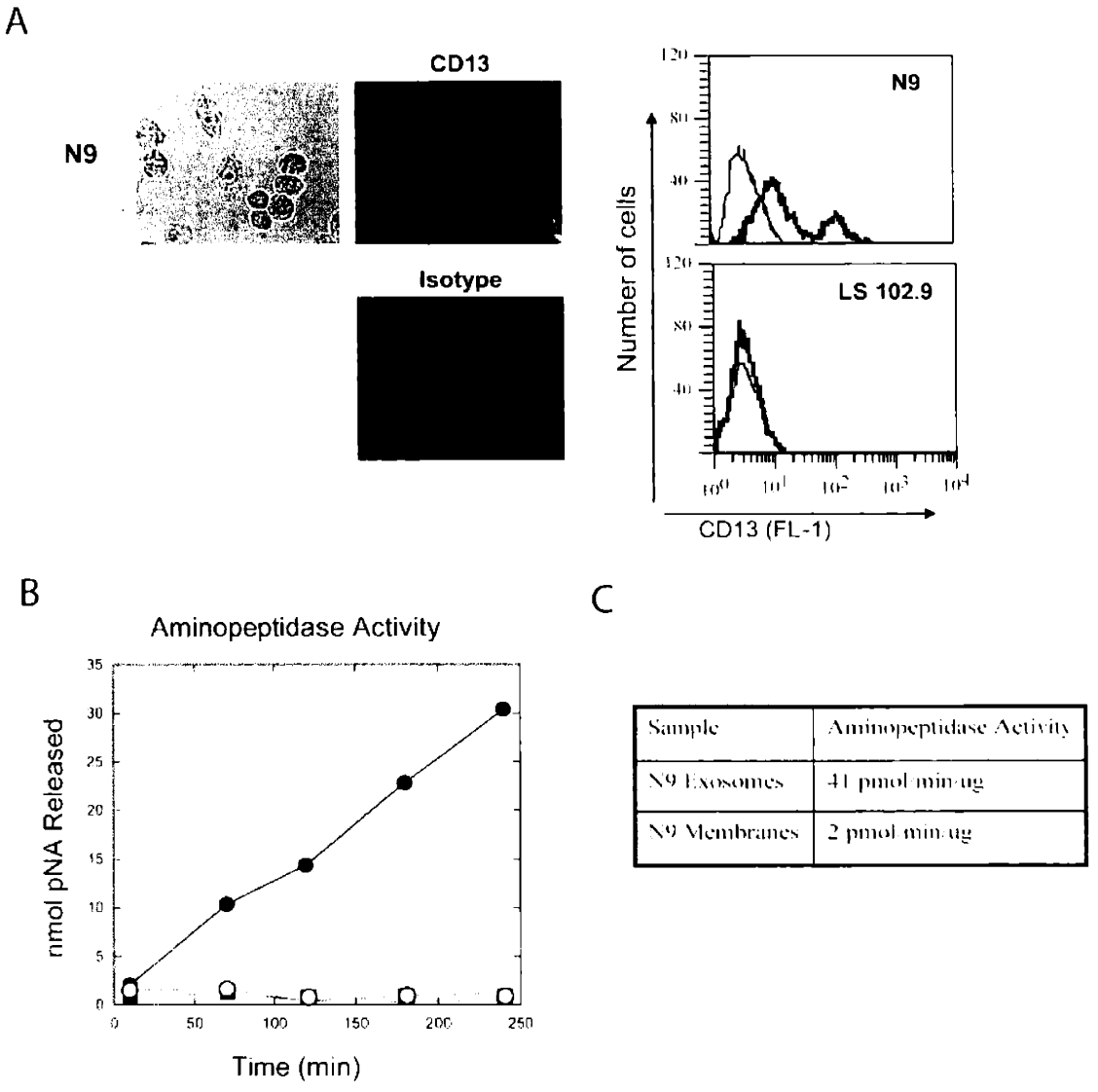


Figure V.1: Microglial cells produce exosomes.

(Left) Micrograph of the fetal murine microglial cell line N9. (Right) Electron micrograph of N9 derived exosomes. Electron microscopy was performed by Illaria Portoliccio (Harvard University).

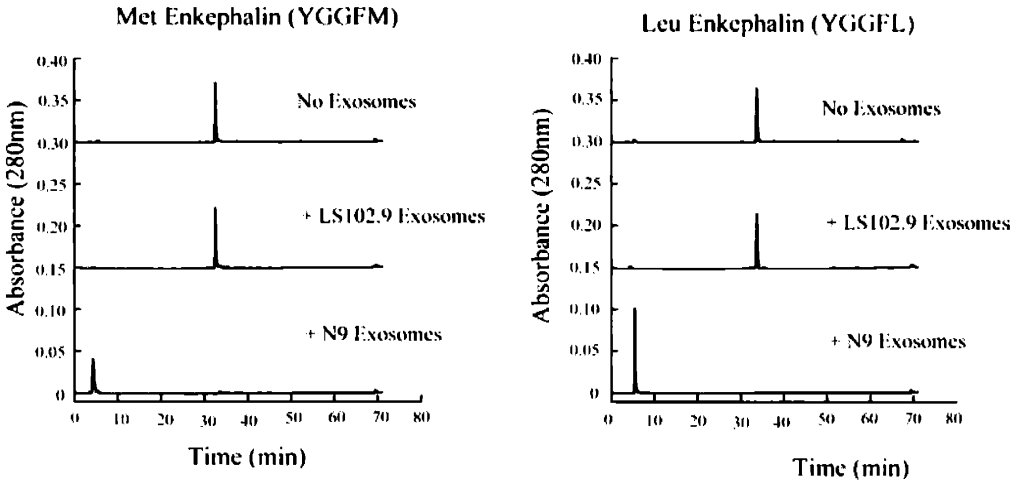


Sample	Aminopeptidase Activity
N9 Exosomes	41 pmol·min·ug
N9 Membranes	2 pmol·min·ug

Figure V.2: CD13 expression of microglial cells and aminopeptidase activity of microglial exosomes.

(A) CD13 is present at the cell surface of N9 microglial cells as shown by confocal microscopy (left panel) and flow cytometry (right panel). LS102.9 cells do not express CD13. (B) Microglial derived exosomes (N9, filled circles) and not B cell derived exosomes (LS102.9, filled squares) are able to cleave the chromogenic substrate leucine-p-nitroanaline. This activity is completely inhibited by the CD13 specific inhibitor, phebestin (open circles). (C) Comparison of aminopeptidase activity between exosomes and cell membrane after normalization for total protein content.

A



B

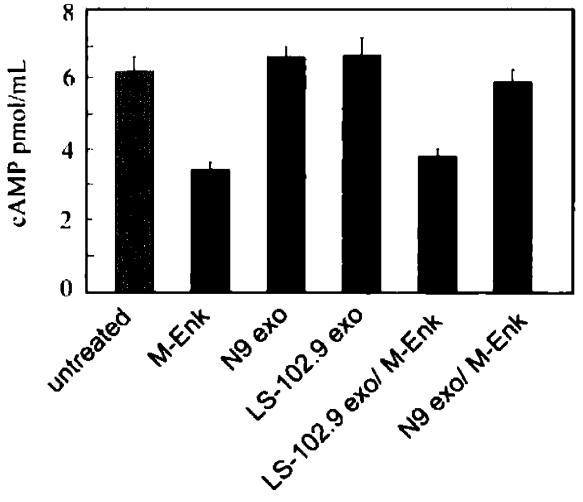


Figure V.3: Exosomal cleaved enkephalins decrease neuronal signaling as detected by cAMP levels.

(A) Exosomes from microglial cells (N9) and B cells (LS102.9) were incubated with neuropeptides met-enkephalin and leu-enkephalin. Cleavage of the neuropeptides was monitored by HPLC. (B) Met-Enkephalin (1μM) was incubated for 2 hrs with exosomes from microglia (N9) or B cells (LS102.9). Enkephalin cleavage/inactivation was determined by measuring the levels of the second messenger cAMP using the CD13 negative neuroblastoma cell line NIE 115 which express opiod receptors.

Cytoskeleton and associated membrane proteins

gi 1351867	Actin-β	i
gi 113255	Actin	i
gi 13097498	Actin filament	h
gi 10242168	α tubulin	j
gi 6680924	Cofilin 1, non muscle	c
gi 10440556	Myosin	c
gi 6981236	non-muscle myosin	c
gi 104222	Tropomyosin	f
gi 462608	Moesin	k
gi 13627751	L-plastin	j
gi 1705915	Clathrin heavy chain	l
gi 14010891	syntenin-1	g

Cytoplasmic proteins

gi 13591955	GTP-binding protein	i
gi 1519380	small GTP-bind prot.	a
gi 940395	Polyubiquitin	k
gi 578551	Ubiquitin	h
gi 119132/gi 119168	EF-1a S/EF-2	j
gi 4503531/	EIF4A-II	i
gi 4503519	EIF-3 epsilon	j
gi 5453695	CAP	j
gi 6677833/gi 6996913	Calpacitin I	b
gi 4633515	AIP1	k
gi 9625037	RhoG	d

chaperones

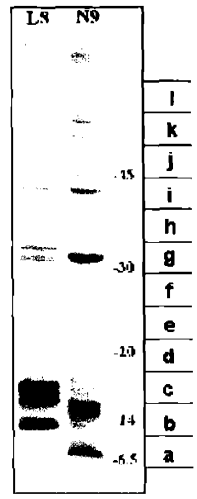
gi 13487931	14-3-3 protein zeta	f
gi 9055384	14-3-3 protein β	f
gi 13928824	14-3-3 protein epsilon	f
gi 13242237	Hsc 73	k
gi 194033	Hsp 86	k
gi 118098	Cyclophilin A	d

enzymes

gi 6679937	GAPDH	h
gi 2506796	Pyruvate kinase	k
gi 112128	Phosphog mutase 1	f
gi 6671539	Aldolase 1	i
gi 12963491	Enolase 1	j
gi 91176	Phosphog kinase	j
gi 1071864	Lipoprotein lipase	j
gi 227293	Cathepsin B	h
gi 11527245	Rho GTPase Cdc42	d
gi 4506787	IQ motif containing GTPase act. protein 1	l

Membrane proteins

gi 7304963	Chloride intr channel	f
gi 4502273	Na/K ATPase	e
gi 6680894	CD9	e
gi 126679	MAC-2	f
gi 6978639	CD81	f
gi 6753830	Fc receptor IgE	b
gi 13529377	CD13	k
gi 6753332	CD14	h
gi 3183523	CD18	k
gi 6680484	Cd11b/Mac-1	l
gi 631683	GP42	f
gi 11560135	NAP-22	e
gi 2652930	mP47 protein	i
gi 6677995	MCT 1	k



Nuclear protein

gi 10800138	H2B	b
gi 631691	H2A	b
gi 122097	H4	b

Unknown function

gi 11276938	DKFZp762H157.1	k
gi 12843863	putative	g
gi 12849385	putative	h
gi 12832783	putative	j

Table V.1: Proteomic analysis of microglial cells exosomes

One hundred micrograms of total protein lysate from N9 or LS.102.9 exosomes were separated by a 12% SDS-PAGE. Gel lanes were sliced in 12 fragments (a to l) according to their molecular weight. Excised bands were eluted, trypsin-digested and sequenced by microcapillary reverse-phase HPLC nano-electrospray tandem mass spectrometry on a Finnigan LCQDECA XP quadrupole ion trap mass spectrometer (LC-MS/MS). Sequence analysis was performed at the Harvard University Microchemistry Facility. Only proteins identified by three or more sequenced peptides are reported in the figure with the exception of * phosphoglycerate kinase. Gene bank accession number referred to as gi.

VI. Conclusions and Future Directions for Research

VI.A. Summary of thesis and overview of future directions for research

In chapters II and III, I describe experiments which illuminate aspects of the peptide induced conformational change in HLA-DR1. The conformational change was localized to a few regions both nearby to and distant from the peptide binding site. The conformational change involves a condensation or folding of the beta chain helix around the bound peptide and a rigid body shift of the α2 and β2 immunoglobulin-like domains relative to one another. Section VI.B discusses a potential role for the conformational change and its involvement in the interaction of class II MHC proteins with the peptide exchange factor HLA-DM.

Chapter IV describes the isolation and characterization of a protease activity which was observed to be secreted by dendritic cells. The secreted DC protease acts extracellularly to process intact proteins into antigenic peptides. Peptides produced by such activity are efficiently loaded onto cell surface class II MHC molecules. Together with the observation that immature DC express empty or peptide-receptive class II MHC molecules at the cell surface, we proposed an alternate presentation pathway in which antigen processing and peptide loading can occur entirely outside of the cell.

The protease responsible for the activity secreted by dendritic cells was never identified, however recent advances in proteomics technologies over the past five years gives hope that such identification may be possible. The development of methods and instrumentation for automated, data-dependent electrospray ionization (ESI) MS/MS, in conjunction with microcapillary liquid chromatography (μLC) and database searching,

has significantly increased the sensitivity and speed for the identification proteins directly from complex mixtures without gel electrophoretic separation (see below).

Chapter V describes the characterization of CD13, or aminopeptidease N which is present on the surface of exosomes that are secreted by brain microglia. Aminopeptidase activity was 20 times higher in exosomal preparations than on the cell surface and the activity is fully able to cleave enkephalin neuropeptides. Cell-cell communication can rely on soluble mediators or direct membrane contact between the signaling and the targeting cells. Secretion of exosomes has recently been identified as another mechanism for cellular communication. Microglial exosomal vesicles may represent an important, previously unrecognized delivery system in an organ where cell motility is highly restricted. The role of brain microglia under normal physiological conditions is still poorly defined. As part of this study, a full proteomic characterization of the proteins present on microglial exosomes was undertaken and many proteins were identified whose function in microglia has yet to be determined. Further examination of microglial exosomes will help elucidate the functions of these brain-resident antigen presenting cells.

VI.B. Implications of research: role of the conformational change and HLA-DM

The peptide exchange factor, HLA-DM, is required for cellular loading of antigenic peptide onto class II MHC proteins and in its absence, only MHC-CLIP complexes are found at the cell surface (Morris et al., 1994; Sette et al., 1992). Crystal structures of DM reveal a similar MHC-like fold, with a collapsed peptide binding groove which is not believed to bind peptides (Fremont et al., 1998; Kropshofer et al., 1997).

Many models for the mechanism of the peptide exchange factor HLA-DM involve the recognition or promotion of a structural change in HLA-DR which could facilitate peptide release.

There are currently no structures of DM in complex with any MHC proteins, however recent work has identified sites on HLA-DR and DM which are required for efficient peptide release (Doebele et al., 2000; Stratikos et al., 2002). Many of the sites identified my thesis work as differing in the empty and peptide-loaded forms of DR overlap with regions identified by the Mellins group as key residues which are important for peptide release (Figures VI.1 and VI.2). Presumably these residues are involved in the interaction with DM. All of the important residues are located on one lateral face of DR. Arginine 50α and Arg189β are located only one or two residues away from those residues which were identified by Doebele et al. (F51α and E187β). Valine 186β was identified in both studies. Interestingly, Lys98β, which was not identified in the DM interaction studies, lies on the proposed interaction face of DR with DM. The fact that residues which are important for the conformational change are located in regions which are important for interaction with DM supports a model in which DM recognizes or promotes a structural change in DR. It is likely that the conformation which is recognized by DM is similar to the empty form of the protein and that empty DR (in the alternate conformation) is an intermediate along the reaction pathway for peptide exchange. Future work aimed at further characterizing the DR conformational change may help elucidate aspects of this interaction.

VI.C. Future directions for research

VI.C.1. Surface topology mapping by hydrogen exchange and mass spectrometry

In this thesis, I have presented work aimed at understanding the peptide-induced conformational change in HLA-DR1 using non-standard biophysical techniques. Antibody binding experiments and differential incorporation of side-chain chemical labels has given us some "low-resolution" information as to which regions of the protein differ in the empty and peptide-loaded conformations. For many years, our lab has tried to use high resolution structural techniques to probe the conformation of empty DR with limited success. Problems arise due in part to the fact that empty DR1 is prone to aggregation at high concentrations such as those required for X-ray crystallography and NMR.

Mass spectrometry has provided a powerful method for monitoring hydrogen exchange of protein backbone amides with deuterium from solvent. In comparison to popular NMR approaches, mass spectrometry has the advantages of higher sensitivity, wider coverage of sequence, and the ability to analyze larger proteins. More importantly, in the case of DR1, experiments can be performed with small amounts of protein ad low concentration. Proteolytic fragmentation of proteins following the exchange reaction provides moderate structural resolution, in some cases enabling measurements from single amides. The technique has provided new insight into protein-protein and protein-ligand interfaces, as well as conformational changes each of which is relevant to structural studies of empty MHC molecules. Using this technique, it would be possible to probe not only differences between the empty and peptide-loaded conformations of DR,

but also interactions of DR with any number of peptide ligands as well as protein-protein interactions such as the peptide exchange factor HLA-DM or the MHC co-receptor CD4.

VI.C.2. Examination of inter-domain interactions involved in the conformational change

The results of this thesis work have shown that the conformational changes associated with peptide binding are distributed throughout the protein. In fact, the MEM-266 epitope (identified in chapter II) is the region of the extracellular domain furthest from the peptide binding site located at the C-terminal (or membrane-proximal) end of the β2 Ig-like domain. The results suggest that peptide-induced conformational changes propagate from the peptide binding site to the distal end of the beta subunit, and perhaps, that interprotein interactions in this region can be transmitted to the peptide binding site to regulate peptide release and binding kinetics.

Analysis of the crystal structure of DR1 shows that the β2 domain is docked onto the α1β1 peptide binding domain through an interaction between its top end and a depression formed underneath the very hydrophobic P1 pocket (Murthy and Stern, 1997). This interaction is dominated by Trp153β which extends from the β2 DE loop to interact with several of the α1 and β1 residues underneath the peptide binding domain and makes up part of the floor of this pocket. Previous work in our lab has shown that occupancy of the P1 pocket is the major trigger required for conversion from the empty to the peptide-loaded conformation (Sato et al., 2000). It is possible that Trp153β is one of the residues which is responsible for propagation of the conformational change from the peptide binding site to the Ig-like domains.

Other evidence for the involvement of tryptophan includes the side-chain specific oxidation by N-bromosuccinimide (discussed in chapter III). That study showed a difference in the number of modified trpytophan residues for empty and peptide-loaded DR1. One route to determine whether this residue is in fact involved in the conformational change is site directed mutagenesis of Trp153β. Primers for PCR mutagenesis have been designed and synthesized. Expression and folding studies will need to be done to determine whether this residue is important for the overall stability of the MHC complexes. Peptide binding studies and MEM-antibody binding assays can then be done to evaluate the importance of this residue in the conformational change and its role in propagating the conformational information between the peptide binding domain and the β2 Ig-like domain.

VI.C.3. Superantigen binding as a probe of conformational changes of the alpha chain

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). Recently, superantigens have been used successfully as an aid in the crystallization of HLA-DR-peptide complexes (Bolin et al., 2000; Sundberg et al., 2002). Our lab has used the affinity-matured 3B2 variant of staphylococcal enterotoxin SEC3 (Andersen et al., 1999) for such a purpose (Zavala-Ruiz et al., 2003). SEC3 interacts with HLA-DR1 outside the binding groove on the flanking helix from the α-subunit (Redpath et al., 1999) and DR residues which are important for recognition are easily identified by analysis of the crystal structure of DR-SEC3 complexes.

Though a comprehensive binding study has not taken place, several observations have led us to believe that SEC3 binds peptide-loaded DR1 much more tightly than to empty DR1. If this is indeed the case, then it will be possible to identify regions which change their conformation upon peptide binding similarly to those identified by mapping the MEM antibody interaction sites. Simple binding studies using native PAGE were inconclusive, but other binding studies such as surface plasmon resonance analysis can be done to determine binding affinities of SEC-DR complexes in the presence or absence of bound peptide.

VI.C.4 MHC epitope mapping using grating-coupled surface plasmon resonance

Identification pathogen-derived MHC-binding peptides is often used as a starting point for immunological research into the response against a particular pathogen. The basic parameters of MHC-peptide interaction have been elucidated through crystal structures of MHC-peptide complexes, characterization of endogenously bound peptides (Rotzschke et al. 1990; Van Bleek and Nathenson 1990; Hunt et al. 1992; Reich et al. 1994), site-specific mutagenesis (Hammer, Sturniolo et al. 1997), and library screening approaches (Chicz et al. 1992; Grey et al. 1995; Hammer et al. 1997; Southwood et al. 1998). These studies have resulted in several allele-specific binding motifs, currently known or predicted for about 100 different MHC proteins (Brusic et al. 1998). However, even in the best characterized cases the binding predictions are woefully inaccurate.

Experimental determination of MHC peptide binding activity is technically demanding. Often, this involves synthesis of peptides of interest, typically overlapping series of peptides spanning a particular protein antigen and incubating them for up to

three days with empty MHC molecules before analysis. ELISA based assays are usually performed as dilution series in duplicate or triplicate wells of a 96 well plate and are compared to peptides of known affinity. This analysis severely limits the total number of peptides which can be studied as only five to ten peptides can be thoroughly characterized on a single plate.

In collaboration with Rick Baggio and Jaime Arenas at Applied Biosystems, we have begun work aimed at studying MHC-peptide interactions using a high throughput SpotMatrix SPR Technology. This technology was used in the antibody epitope mapping experiments (described in chapter II) and can readily be applied to the identification of MHC binding peptides. With this technology, binding of fluid-phase protein to a set of immobilized peptides is detected in parallel by surface plasmon resonance. Up to 400 biotinylated peptides can be immobilized by spotting them onto a streptavidin-coated gold chip and assayed in a single experiment.

Preliminary experiments have shown that MHC binding peptides can be detected in a few hours rather than days and the SpotMatrix SPR technology provides information on both association and dissociation phases of the binding interaction, in addition to the endpoint values obtained by ELISA. Further development of this application will yield faster identification of MHC-binding peptides and allow for simultaneous kinetic characterization of the identified peptides.

VI.C.5. Cell-surface expressed empty MHC molecules

As part of this study we generated a panel of monoclonal antibodies recognizing the beta subunit which are specific for the empty conformation. These antibodies were useful in localizing differences in DR conformation, and they can be useful tools for evaluating the role of empty MHC proteins in the immune system.

The empty or peptide-receptive class II MHC conformation has been observed on the cell surface of immature dendritic cells in both the murine (Santambrogio et al., 1999) and human ((Carven et al., 2004); Sriram Chitta, Vaclav Horejsi, and Lawrence Stern, unpublished observations) systems using conformation-specific monoclonal antibodies. In the human system, the residues which are important for binding to each of the MEM antibodies have been mapped and are shared by many alleles in the DRB1*01, 03, 04, 14, DRB3*01 and DRB4*01 families (Table VI.1, Table VI.2). Other DR and DQ alleles with conservative substitutions in these regions may bind to the antibodies as well. *In vitro* binding studies have shown that each antibody binds empty HLA-DR1 and DR4 but none bind to the empty form of DR52a but a complete characterization of reactive alleles has not been performed.

Using these antibodies, it will be possible to evaluate expression of empty class II MHC molecules for several developmental cell types and MHC alleles. Dendritic cells can be prepared from peripheral blood mononuclear cells and matured to different extents along the developmental pathway. These cells can be HLA-typed and evaluated for their expression of peptide-receptive molecules. Expression analysis of cell types at different stages along the developmental pathway will help understand the role of empty MHCs in the antigen presentation.

VI.C.6. Identification of the DC secreted protease

The protease which is responsible for the activity associated with dendritic cells (discussed in chapter IV) has not yet been identified. The major limitation associated with the identification attempts that were undertaken was the inability to purify the activity away from high levels of protein content present in cell culture media. Recent developments in proteomics-based approaches have enabled the quantification and characterization of a diverse array of low and high abundance proteins found in complex mixtures (Griffin et al., 2001; Paweletz et al., 2001; Petricoin et al., 2002).

For identification of the secreted protease, the newly-developed isotope coated affinity tagging (ICAT) techniques can be used. This technique is designed to detect and quantify the relative abundance of proteins differentially present in similar samples. It has been successful in the identification and determination of relative concentrations of several hundreds of proteins over several orders of magnitude in concentration in a single experiment (Han et al., 2001). The method and is well suited to the identification of proteins that have been refractory identification using standard biochemical technologies.

The experimental system consists of three steps: (1) preparation of cellular supernatants from proteolytically active and inactive cells (immature DC and B cells); (2) covalent tagging of the proteins with isotope-coded affinity tag (ICAT) reagents followed by proteolysis of the combined labeled protein samples; and (3) isolation, identification, and quantification of the tagged peptides by multidimensional chromatography, automated tandem mass spectrometry.

The identity and sequence specificity of the DC secreted protease will be required to study the regulation of expression in various APCs. Once identified, its role in the extracellular antigen processing and presentation pathway can be further explored.

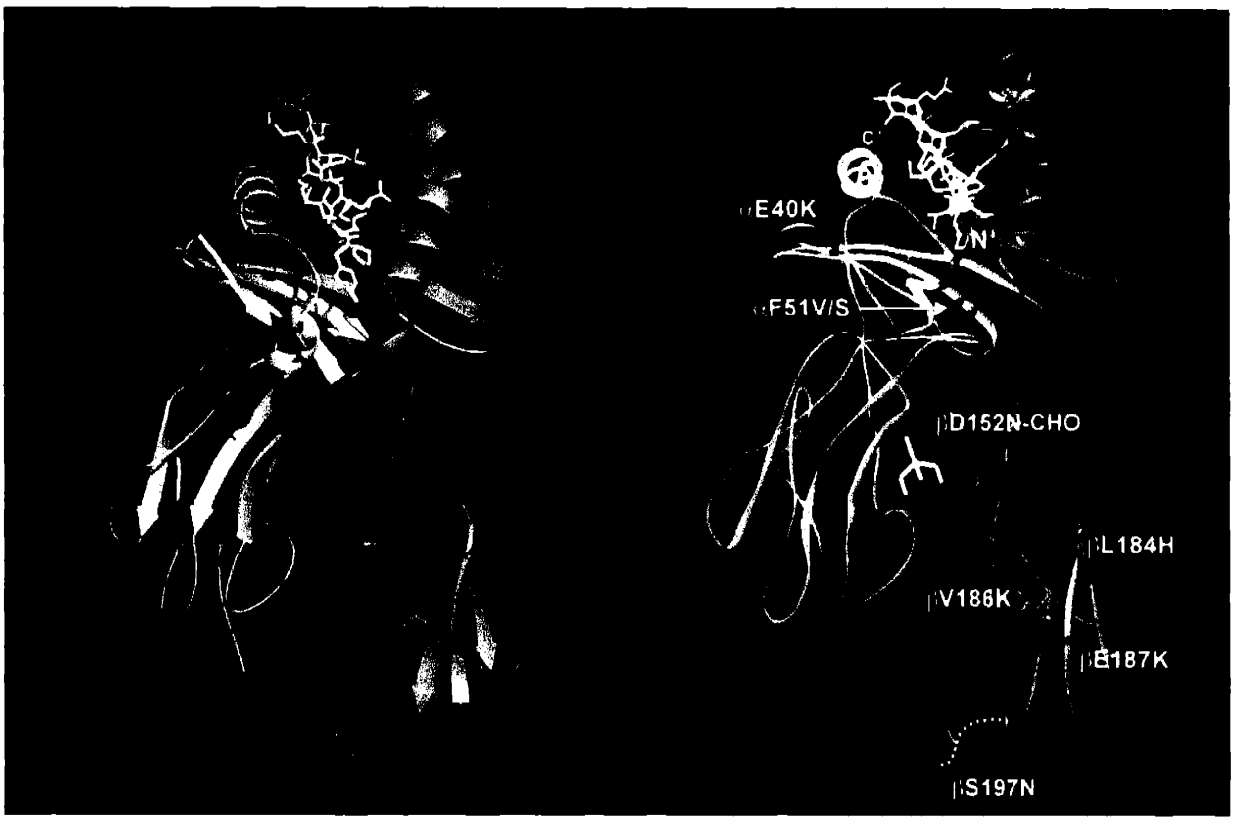


Figure VI.1: Differential Chemical Modifications and MEM-266 Antibody Epitope Map to Proposed HLA-DM Interaction Face

Residues which were modified in empty DR1 that were not modified in peptide-loaded DR1 (left panel, red) and residues identified in the MEM-266 epitope (left panel, purple) are in the same regions as residues identified by Doebele RC et al., (2000) as key residues which are required for efficient DM-mediated peptide release (presumably these residues are involved in the interaction with DM).

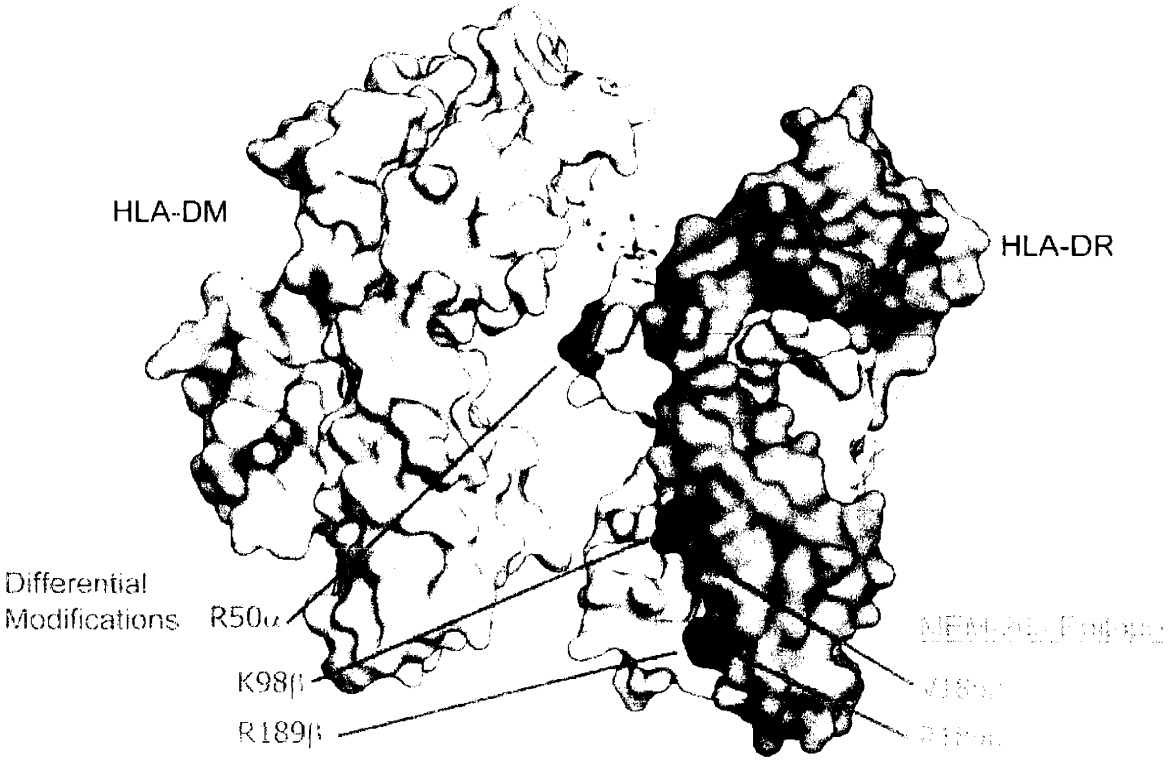


Figure VI.2: Differential Chemical Modifications and MEM-266 Antibody Epitope Map to Proposed HLA-DM Interaction Face

Residues which were modified in empty DR1 that were not modified in peptide-loaded DR1 (red) and residues identified in the MEM-266 epitope (purple) are located on the proposed face of interaction with HLA-DM. Model built by Stratikos et al (2002).

Table VI.1 HLA-DR Polymorphism MEM-264, 265, 267 Region

β 53	β 57	β 60	β 61	β 63	β 67	HLA-DRB* alleles
L	D	Y	W	S	L	*HLA-DRB1**: 0101-0102, 0104-0111, 0301-0309, 0311, 0314-0318, 0320-0325, 0401, 0403-0404, 0406-0408, 0413, 0416, 0419-0422, 0813, 1001, 1107-1108, 1113, 1126, 1134, 1136, 1142, 1301-1302 1306, 1308-1310, 1320, 1325, 1329, 1344, 1356, 1360, 1402-1403, 1405-1406, 1409, 1411-1412, 1414 1417-1421, 1423, 1429-1430, 1433, 1436, 1440-1444, 1446-1447, 1602, 1605, 1607 *HLA-DRB3**: 0107, 0201-0206, 0210, 0212-0215, 0218 *HLA-DRB4**: 0101-0106 *HLA-DRB5**: 0205
L	D	Y	W	S	I	*HLA-DRB1**: 0103, 0319, 0402, 0418, 1114, 1116, 1118-1121, 1145, 1148, 1204, 1209, 1315-1317 1319, 1322-1323, 1327, 1328, 1334-1337, 1340-1341, 1351-1353, 1361, 1424, 1437, 1445, 1501-1503 1505-1511, 1513-1514, 1605-1607 *HLA-DRB3**: 0211 *HLA-DRB5**: 0106-0107, 0111, 0202
L	D	Y	W	S	F	*HLA-DRB1**: 0415, 0802, 0804, 0809, 0820-0821, 0824, 0902, 1101-1106, 1109-1112, 1115, 1127-1130 1132-1133, 1135, 1137, 1141, 1143-1144, 1146-1147, 1149, 1305, 1307, 1311, 1314, 1318, 1324, 1326 1342, 1347, 1350, 1357, 1359, 1362, 1415, 1427, 1504, 1601, 1603-1604, 1608 *HLA-DRB3**: 0217 *HLA-DRB5**: 0101-0105, 0108-0110, 0113
L	A	Y	W	S	L	*HLA-DRB1**: 0446
L	A	Y	W	S	I	*HLA-DRB1**: 0706
L	A	Y	W	S	F	*HLA-DRB1**: 0811
L	A	H	W	S	L	*HLA-DRB1**: 0310, 1343, 1401, 1407, 1410, 1426, 1428, 1431-1432, 1435, 1438, 1439 *HLA-DRB3**: 0216
L	A	H	W	S	I	*HLA-DRB1**: 1345, 1416
L	A	H	W	S	F	*HLA-DRB1**: 0808, 1422, 1425
L	S	Y	W	S	L	*HLA-DRB1**: 0312, 0405, 0409-0411, 0417, 0424, 0428-0430, 0445, 0448, 1413 *HLA-DRB3**: 0208

Table VI.1 HLA-DR Polymorphism MEM-264, 265, 267 Region

β 53	β 57	β 60	β 61	β 63	β 67	HLA-DRB* alleles
L	S	Y	W	S	I	*HLA-DRB1**: 0412, 0803, 0810, 0812, 0814, 0818, 0823, 0827, 1303-1304, 1312-1313, 1330, 1332-1333 1338, 1358, 1512
L	S	Y	W	S	F	*HLA-DRB1**: 0801, 0805-0806, 0816-0817, 0822, 0826, 3121, 1349, 1355
L	V	S	W	S	L	*HLA-DRB1**: 1448 *HLA-DRB3**: 0101-0106, 0108-0111, 0209, 0301-0303
L	V	S	W	S	I	*HLA-DRB1**: 0701-0705, 0707-0708, 0825, 1201, 1203, 1205-1208
L	V	S	W	S	F	*HLA-DRB1**: 0901, 0903, 1202, 1354
L	V	Y	W	S	L	*HLA-DRB3**: 0207
L	V	Y	W	S	I	*HLA-DRB1**: 1331
L	V	Y	W	S	F	*HLA-DRB1**: 0807, 1346
L	I	Y	W	S	I	*HLA-DRB1**: 0819
L	D	S	W	S	L	*HLA-DRB1**: 0313
L	D	S	W	S	I	*HLA-DRB1**: 1339
L	D	S	W	S	F	*HLA-DRB5**: 0112
L	D	H	W	S	L	*HLA-DRB1**: 1434
L	D	H	W	S	I	*HLA-DRB1**: 0815, 1131

Table VI.2 HLA-DR Polymorphism MEM-266 Region

β 186	β 188	β 189	HLA-DRB* alleles
V	W	R	*HLA-DRB1**: 0101-0104, 0301-0302, 0310, 0401-0404, 0406, 0409, 0411, 0701, 0901, 1001, 1101-1104, 1201 1206, 1301-1302, 1401-01405, 1501-1502, 1601-1603, *HLA-DRB3**: 0101 *HLA-DRB5**: 0101-0102, 0202
V	W	S	*HLA-DRB1**: 0802 *HLA-DRB3**: 0201-0202, 0210-0211 *HLA-DRB4**: 0101-0103

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A.I Protocols

A.II Notebook tables of contents

A.I.1 Expression of class II MHC subunits in *E. coli*

Developed by M. M. Rushe, adapted for use by G. J. Carven

Autoclave:
10 L LB in fermentor
1 L flask with 500 ml LB
large funnel
500 ml bottle of LB
20% glucose
3 125 ml flasks

Make sure you have:
11 ml 50 mg/ml Ampicillin
15 ml 0.5M IPTG

Day 1: In the afternoon, streak bacterial glycerol stock on a LB agar plate containing 50 μg/ml ampicillin.

Day 2: put plate at 4 °C in the morning

In the early evening:
Pour 80 ml of LB into one of the flasks, 80 μl of 50 mg/ml ampicillin, and 0.8 ml 20% glucose.

Pipet 2 ml of this mixture into a 15 ml tube, pour 20 ml into a 50 ml tube, and 20 ml into each of the remaining flasks, leaving 20 ml in the flask. Remove 1 ml from one of the flasks, move to a plastic cuvette, add 0.02% azide, and cover with parafilm to use as a blank in the morning.

Pick one colony into the 2 ml tube and vortex extensively. Take 200 μl from this tube and add it to the 20 ml tube. Vortex again. Pipet 200 μl of this into flask #1 and swirl. Take 200 μl from flask #1 and add to flask #2. Swirl. Take 200 μl from flask #2 and add to flask #3. Grow flasks on shaker table at 37 °C overnight.

Day 3:

Set up the fermentor: Add 10 ml of 50 mg/ml, 10 ml of 35 mg/ml, 100 ml of 20% glucose, and 200 μl of antifoam A. Mix well. Remove 1 ml from the flasks, move to a plastic cuvette, add 0.02% azide, and cover with parafilm to use as a blank.

Take OD at 600 nm of the flasks that grew overnight, using the blank that was set aside. Pick the flask with the OD that is less then 1, and pour into the fermentor.

As the culture grows, check OD often. Grow until OD is 1-1.5. Add Antifoam as needed.

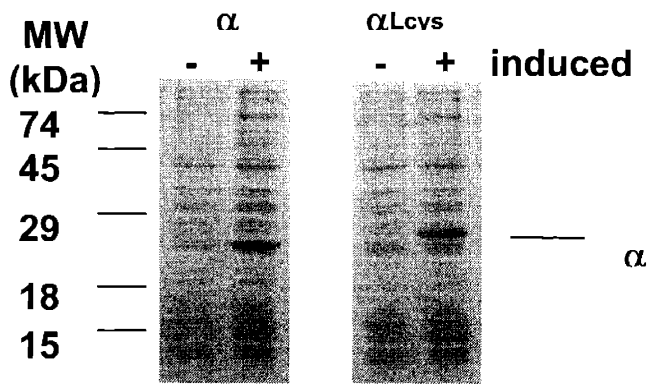
Induce between 1- 1.5 OD_{600} with 15 ml of 0.5 M IPTG. Be sure to save a non-induced sample: Take 1 ml, spin down in a microfuge, remove supernatant and freeze pellet.

For DR1 subunits in BL21 cells, harvest after 1 hour (minimum) post-induction.

Spin down culture in centrifuge bottles (5000 g for 10 minutes). Save 1ml of induced cells as before for a gel. Perform inclusion body prep to the end of step 3 and freeze.

To run the 12.5% SDS-gel (*picture courtesy of Jennifer Ogrodnick-Cochran*):

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.



A.I.2 Isolation of DR1 subunits from *E. coli* inclusion bodies

Developed by M. M. Rushe, adapted for use by G. J. Carven

Subunit Isolation:

Solutions:

DNAse solution (can be made and stored in freezer)

75 mM NaCl
50% glycerol
2 mg/ml DNAse (Sigma D-5025)

Sucrose Solution

50 mM tris pH 8.0
25% sucrose
1 mM EDTA
0.1% NaN_3
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% NaN_3
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% NaN_3
1 mM DTT (add just before use)

Tris Solution

50 mM tris pH 8.0
1 mM EDTA
0.1% NaN_3
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 $MgCl_2$
0.5 M EDTA pH 8.0

*Protocol for pellet of 10 liter fermentor culture

1. With rubber spatula, resuspend fresh bacteria with ~200 ml sucrose solution, using extra solution to rinse all containers and pipets. Combine in the homogenizer or polytron, not a sonicator.

2. Keep suspended bacteria in centrifuge bottles. If cells are not pLys^S add 1 mg dry lysozyme per ml bacterial suspension and stir for 10 minutes. If cells are pLys^S just stir for 10 minutes. Add ~500 ml deoxycholate/triton solution (2.5 ml/ml suspended bacteria). Solution will get very viscous due to cell lysis and the release of DNA.

3. Add ~ 1 ml 4 M $MgCl_2$ (to make 5 mM final) and 2 ml DNAse solution and stir until the solution becomes the viscosity of water. Freeze at –20 °C overnight or until ready to complete the prep.

4. Thaw solution thoroughly by swirling in warm water. Stir 10 minutes and allow the DNAse to work again. Add 4 mL 0.5 M EDTA. Centrifuge at 8000 RPM for 20 minutes. If the small rotor you will need for the spin in step 8 is cold, put it in the warm room at this time.

5. Discard supernatants and resuspend pellets in 300 ml or more of triton solution. Homogenize or "chop" in the polytron, just enough to resuspend the pellet. Keep everything on ice as much as possible for all the washes in step 5 and 6. Centrifuge at 8000 RPM for 20 minutes. Repeat this wash 3 times or more. I used to stop when the white pellet started to flake easily rather than just stick together as I poured off the supernatant.

6. Repeat this wash twice with tris solution. If using the same centrifuge for the final spin in step 8, it is advised that you turn the temperature of the centrifuge up to 20 °C during the final spin in this step.

7. Dissolve the pellet and homogenize in ~200 ml urea solution.

8. Centrifuge at 15,000 RPM for 30 minutes at 20 °C. You will need the small polycarbonate centrifuge tubes for this. Freeze at –70 °C until ready for use.

A.I.3. Refolding and purification of empty DR1

Developed by M. M. Rushe, adapted for use by G. J. Carven

Purification of Subunits by Urea HQ

Column: HQ resin: Poros 20 HQ
Buffer A: 8 M deionized urea, 20 mM Tris pH 8.0 or 9.0, 1 mM DTT.
Buffer B: 8 M deionized urea, 20 mM Tris pH 8.0 or 9.0, 1 M NaCl, 1 mM DTT.
pH α-subunits: 8.0
pH β-subunits: 9.0

Make each buffer fresh.
Filter Buffers A and Buffer B through 0.2 microns before use.

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.

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.

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.

Refolding of Subunits

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.

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 mix.

4. After all components are well mixed, cover tightly and store at 4 °C for at least 36 hours. Longer refolding times (1 week or more) yield more refolded empty complex.

Immunoaffinity purification of Empty DR1

1. Concentrate folding mixture in the Amicon spiral filter concentrator.

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. 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.

6. 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).

7. Wash column with at least 10 column volumes of PBS/0.02% NaN_3 and store in refrigerator.

8. 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.

9. 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% NaN_3

9. Pool the fractions containing DR1 based on their absorbance at 280 nm, concentrate and switch into a desired buffer (usually PBS).

10. Check the prep for peptide binding activity:

A.I.4. Loading of empty DR1 with peptides

Developed by M. M. Rushe, adapted for use by G. J. Carven

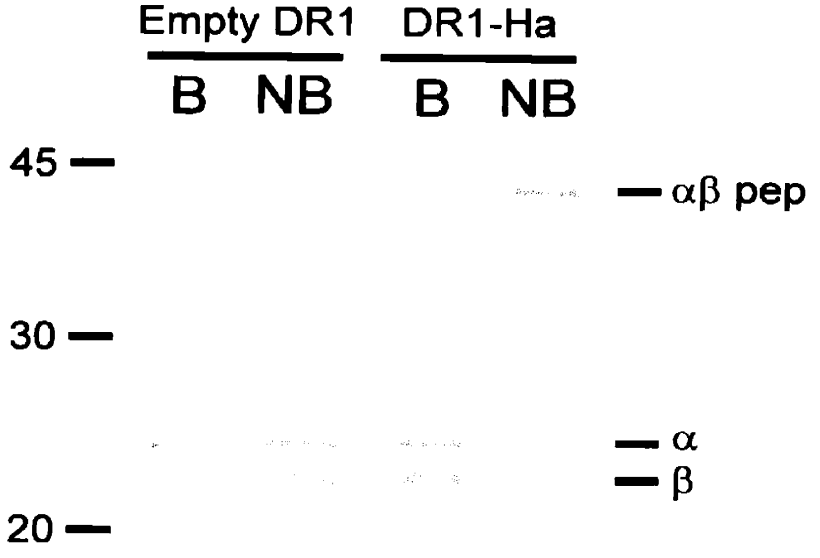
Incubate DR1 with 5-molar excess peptide at 37 °C for at least 72 hours.

Analyze by 12.5% SDS-PAGE: Run a boiled / not-boiled version of each sample

Ha-loaded class II should give a ~45,000 kDa band on an SDS gel that dissociates into α and β subunits when boiled (see below).

Empty class II is not SDS stable and will dissociate into α and β subunits, for the boiled and not-boiled samples.

DR-peptide complexes can be further purified by size exclusion chromatography. 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.I.5. ELISA for folded DR proteins

Developed by M. M. Rushe, adapted for use by G. J. Carven
Curve Fitting Equations by J. A. Zarutskie

Reagents:

TBST wash buffer (Tris-buffered saline + 0.1%Tween-20, pH 8.0)

Block solution:
PBS
3.0% BSA
0.02% NaN_3
store at 4 °C

Dilution solution:
PBS
0.3% BSA
0.1% Triton X100
filter and store at 4 °C

ABTS Solution:
Premade 1X ABTS works well, but does not store well for long periods of time. Alternately, ABTS tablets may be used as per instructions.

1. Coat Immulon IV (Dynex) plate with anti-DR1 monoclonal antibody LB3.1 or L243. Stock is usually around 1.8 mg/ml in PBS, 0.02% NaN_3. Dilute stock 1:1000 and put 100 µl in each well. Incubate plate for 2 hours at 37 °C or overnight at 4 °C.

2. Block the plate with 200 µL Block solution 3% BSA. Incubate at least 2 hours at 37 °C. Plates can be stored at 4 °C by covering them well with parafilm and wrapping in saran wrap to prevent them from drying out.

3. Wash wells 3 times with TBST.

4. Prepare standards in dilution solution from 2 ng/µl to 0.002 ng/µl by 2-fold dilutions. Use 100 µl of each per well to make a set of standards going from 100 ng/well to 0.1 ng/well. Include a blank. Run samples in duplicate

5. Prepare dilutions of unknown samples and add to wells in duplicate.

6. Incubate plate 37 °C for 1 hour, RT for 2 hours, or overnight at 4 °C.

7. Carefully wash each well 3 times with TBST, avoiding spillover from neighboring wells. The plate washer works well and saves time when using many plates, but for single plates, hand washing is adequate

8. Prepare 10 mL (per plate) detection antibody (rabbit CHAMP sera diluted 1:25,000 in dilution solution) Incubate plate 37 °C for 1 hour

9. Wash wells 3 times with TBST.

10. Prepare 10 mL (per plate) secondary antibody (goat anti-rabbit IgG-peroxidase (HRP) conjugate (Roche) diluted 1:4000 in dilution solution) Add 100 µl to each well. It is very important that no azide is present at this step- it inhibits the HRP. Incubate 0.5 to 1.5 hours at RT .

11. Wash each well 3 times with TBST.

12. Add 200 µl of ABTS solution to each well.

13. Read absorbance in ELISA plate reader at 405 nm.

14. If desired, stop plate from developing by adding 50 µl of stopping solution (20% SDS/50% DMF) to each well.

15. Use 4-parameter curve fit of standards to determine the MHC class II concentration of the samples.

Equations used to fit ELISA data:

ELISA fit-four

equation used to fit data:
line 1: ((m1-m2)/(1+ (m0/m3)^m4)) + m2;
inital 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)^m4)^(-2)*(m0/m3)^m4*ln(m0/m3)

inverse fit-four

c1=m3*((m1-m2)/(c0-m2)-1)^(1/m4)
an alternate equation for fitting ELISA data

A.I.6. MEM Antibody ELISA for empty HLA-DR1

Reagents:

TBST wash buffer (Tris-buffered saline + 0.1%Tween-20, pH 8.0)

Block solution:
PBS
3.0% BSA
0.02% NaN_3
store at 4 °C

Dilution solution:
PBS
0.3% BSA
0.1% Triton X100
filter and store at 4 °C

ABTS Solution:
Premade 1X ABTS works well, but does not store well for long periods of time. Alternately, ABTS tablets may be used as per instructions.

1. Coat Immulon IV (Dynex) plate with MEM monoclonal antibody. Stock is usually around 1 mg/ml in PBS, 0.02% NaN_3. Dilute stock 1:1000 and put 100 µl in each well. Incubate plate for 2 hours at 37 °C or overnight at 4 °C.

2. Block the plate with 200 µL Block solution 3% BSA. Incubate at least 2 hours at 37 °C. Plates can be stored at 4 °C by covering them well with parafilm and wrapping in saran wrap to prevent them from drying out.

3. Wash wells 3 times with TBST.

4. Prepare standards in dilution solution from 2 ng/µl to 0.002 ng/µl by 2-fold dilutions. For MEM-264, MEM-265 or MEM-267, use 100 µl of each per well to make a set of standards going from 200 ng/well to 0.1 ng/well. For MEM-266, use 100 µl of each per well to make a set of standards going from 100 ng/well to 0.1 ng/well. Include a blank. Run samples in duplicate

5. Prepare dilutions of unknown samples and add to wells in duplicate.

6. Incubate plate 37 °C for 1 hour, RT for 2 hours, or overnight at 4 °C.

7. Carefully wash each well 3 times with TBST, avoiding spillover from neighboring wells. The plate washer works well and saves time when using many plates, but for single plates, hand washing is adequate

8. Prepare 10 mL (per plate) detection antibody (rabbit CHAMP sera diluted 1:25,000 in dilution solution) Incubate plate 37 °C for 1 hour

9. Wash wells 3 times with TBST.

10. Prepare 10 mL (per plate) secondary antibody (goat anti-rabbit IgG-peroxidase (HRP) conjugate (Roche) diluted 1:4000 in dilution solution) Add 100 µl to each well. It is very important that no azide is present at this step- it inhibits the HRP. Incubate 0.5 to 1.5 hours at RT .

11. Wash each well 3 times with TBST.

12. Add 200 µl of ABTS solution to each well.

13. Read absorbance in ELISA plate reader at 405 nm.

14. If desired, stop plate from developing by adding 50 µl of stopping solution (20% SDS/50% DMF) to each well.

15. Use 4-parameter curve fit of standards to determine the MHC class II concentration of the samples.

MEM Competition ELISA

Follow steps 1-3 as above.

Incubate wells with competition peptide for 45 minutes at RT.

Do not wash away peptide.

Continue from step 4.

A.I.7. Epitope peptide mapping ELISA (SA direct binding)

Reagents:

TBST wash buffer (Tris-buffered saline + 0.1%Tween-20, pH 8.0)

Block solution:
PBS
3.0% BSA
0.02% NaN_3
store at 4 °C

Dilution solution:
PBS
0.3% BSA
0.1% Triton X100
filter and store at 4 °C

ABTS Solution:
Premade 1X ABTS works well, but does not store well for long periods of time. Alternately, ABTS tablets may be used as per instructions.

1. Coat Immulon IV (Dynex) plate with streptavidin (Prozyme) 500 ng in each well. Incubate plate for 2 hours at 37 °C or overnight at 4 °C.

1. Block the plate with 200 µL Block solution 3% BSA. Incubate at least 2 hours at 37 °C. Plates can be stored at 4 °C by covering them well with parafilm and wrapping in saran wrap to prevent them from drying out.

2. Wash wells 3 times with TBST.

3. Add epitope mapping peptides (25 µM in dilution solution) to each well

4. Incubate plate 37 °C for 1 hour, RT for 2 hours, or overnight at 4 °C.

5. Wash wells 3 times with TBST

6. Add 100 µL MEM monoclonal antibody (1 µg/mL) to each well.

7. Prepare 10 mL (per plate) labeled detection antibody (goat anti-mouse IgG-peroxidase (HRP) conjugate (Roche) diluted 1:4000 in dilution solution) Add 100 µl to each well. It is very important that no azide is present at this step- it inhibits

the HRP. Incubate 0.5 to 1.5 hours at RT .

8. Wash each well 3 times with TBST.

9. Add 200 µl of ABTS solution to each well.

10. Read absorbance in ELISA plate reader at 405 nm.

11. If desired, stop plate from developing by adding 50 µl of stopping solution (20% SDS/50% DMF) to each well.

A.I.8. Peptides used in this thesis

All peptides were synthesized on the Advanced ChemTech 490 Synthesizer unless otherwise noted. Purified peptides are >95% pure by HPLC. Crude peptides range from 35% to 80% indicated product.

Epitope Mapping Peptides

name	sequence	source	location & notes
bio1	bioGGMGDTRPRFLWQLKFECHFFN-NH2	ACT synthesizer	crude, S2-302 freezer; purified fridge
bio2	bioGGQLKFECHFFNGTERVRLLER-NH2	ACT synthesizer	crude, S2-302 freezer; purified fridge
bio3	bioGGGTERVRLLERCIYNQEESVR-NH2	ACT synthesizer	crude, S2-302 freezer; purified fridge
bio4	bioGGCIYNQEESVRFDSDVGEYRA-NH2	ACT synthesizer	crude, S2-302 freezer; purified fridge
bio5	bioGGFDSDVGEYRAVTELGRPDAE-NH2	ACT synthesizer	crude, S2-302 freezer; purified fridge
bio6	bioGGVTELGRPDAEYWNSQKDLLE-NH2	ACT synthesizer	crude, S2-302 freezer; purified fridge
bio7	bioGGYWNSQKDLLEQRRAAVDTYC-NH2	ACT synthesizer	crude, S2-302 freezer; purified fridge
bio8	bioGGQRRAAVDTYCRHNYGVGESF-NH2	ACT synthesizer	crude, S2-302 freezer; purified fridge
bio9	bioGGRHNYGVGESFTVQRRVEPKV-NH2	ACT synthesizer	crude, S2-302 freezer; purified fridge
bio10	bioGGTVQRRVEPKVTVYPSKTQPL-NH2	ACT synthesizer	crude, S2-302 freezer; purified fridge
bio11	bioGGTVYPSKTQPLQHHNLLVCSV-NH2	ACT synthesizer	crude, S2-302 freezer; purified fridge
bio12	bioGGQHHNLLVCSVSGFYPGSIEV-NH2	ACT synthesizer	crude, S2-302 freezer; purified fridge
bio13	bioGGSGFYPGSIEVRWFRNGQEEK-NH2	ACT synthesizer	crude, S2-302 freezer; purified fridge
bio14	bioGGRWFRNGQEEKAGVVSTGLIQ-NH2	ACT synthesizer	crude, S2-302 freezer; purified fridge
bio15	bioGGAGVVSTGLIQNGDWTFQTLV-NH2	ACT synthesizer	crude, S2-302 freezer; purified fridge
bio16	bioGGNGDWTFQTLVMLETVPRSGE-NH2	ACT synthesizer	crude, S2-302 freezer; purified fridge
bio17	bioGGMLETVPRSGEVYTCQVEHPS-NH2	ACT synthesizer	crude, S2-302 freezer; purified fridge
bio18	bioGGVYTCQVEHPSVTSPLTVEWRA-NH2	ACT synthesizer	crude, S2-302 freezer; purified fridge
pep1	MGDTRPRFLWQLKFECHFFN-NH2	ACT synthesizer	crude, S2-302 freezer
pep2	QLKFECHFFNGTERVRLLER-NH2	ACT synthesizer	crude, S2-302 freezer
pep3	GTERVRLLERCIYNQEESVR-NH2	ACT synthesizer	crude, S2-302 freezer
pep4	CIYNQEESVRFDSDVGEYRA-NH2	ACT synthesizer	crude, S2-302 freezer
pep5	FDSDVGEYRAVTELGRPDAE-NH2	ACT synthesizer	crude, S2-302 freezer
pep6	VTELGRPDAEYWNSQKDLLE-NH2	ACT synthesizer	crude, S2-302 freezer
pep7	YWNSQKDLLEQRRAAVDTYC-NH2	ACT synthesizer	crude, S2-302 freezer
pep8	QRRAAVDTYCRHNYGVGESF-NH2	ACT synthesizer	crude, S2-302 freezer
pep9	RHNYGVGESFTVQRRVEPKV-NH2	ACT synthesizer	crude, S2-302 freezer
pep10	TVQRRVEPKVTVYPSKTQPL-NH2	ACT synthesizer	crude, S2-302 freezer
pep11	TVYPSKTQPLQHHNLLVCSV-NH2	ACT synthesizer	crude, S2-302 freezer
pep12	QHHNLLVCSVSGFYPGSIEV-NH2	ACT synthesizer	crude, S2-302 freezer
pep13	SGFYPGSIEVRWFRNGQEEK-NH2	ACT synthesizer	crude, S2-302 freezer
pep14	RWFRNGQEEKAGVVSTGLIQ-NH2	ACT synthesizer	crude, S2-302 freezer
pep15	AGVVSTGLIQNGDWTFQTLV-NH2	ACT synthesizer	crude, S2-302 freezer
pep16	NGDWTFQTLVMLETVPRSGE-NH2	ACT synthesizer	crude, S2-302 freezer
pep17	MLETVPRSGEVYTCQVEHPS-NH2	ACT synthesizer	crude, S2-302 freezer
pep18	VYTCQVEHPSVTSPLTVEWRA-NH2	ACT synthesizer	crude, S2-302 freezer

Fine Mapping peptides

name	sequence	source	location & notes
6-18-A1	bioXX-ATELGRPDAEYWNSQKDL-NH2	ACT synthesizer	crude, S2-302 freezer; purified fridge
6-18-A2	bioXX-VAELGRPDAEYWNSQKDL-NH2	ACT synthesizer	crude, S2-302 freezer; purified fridge
6-18-A3	bioXX-VTALGRPDAEYWNSQKDL-NH2	ACT synthesizer	crude, S2-302 freezer; purified fridge
6-18-A4	bioXX-VTEAGRPDAEYWNSQKDL-NH2	ACT synthesizer	crude, S2-302 freezer; purified fridge
6-18-A5	bioXX-VTELARPDAEYWNSQKDL-NH2	ACT synthesizer	crude, S2-302 freezer; purified fridge
6-18-A6	bioXX-VTELGAPDAEYWNSQKDL-NH2	ACT synthesizer	crude, S2-302 freezer; purified fridge
6-18-A7	bioXX-VTELGRADAEYWNSQKDL-NH2	ACT synthesizer	crude, S2-302 freezer; purified fridge
6-18-A8	bioXX-VTELGRPAAEYWNSQKDL-NH2	ACT synthesizer	crude, S2-302 freezer; purified fridge
6-18-A9	bioXX-VTELGRPDAEYWNSQKDL-NH2	ACT synthesizer	crude, S2-302 freezer; purified fridge
6-18-A10	bioXX-VTELGRPDAAYWNSQKDL-NH2	ACT synthesizer	crude, S2-302 freezer; purified fridge
6-18-A11	bioXX-VTELGRPDAEAWNSQKDL-NH2	ACT synthesizer	crude, S2-302 freezer; purified fridge
6-18-A12	bioXX-VTELGRPDAEYANSQKDL-NH2	ACT synthesizer	crude, S2-302 freezer; purified fridge
6-18-A13	bioXX-VTELGRPDAEYWASQKDL-NH2	ACT synthesizer	crude, S2-302 freezer; purified fridge
6-18-A14	bioXX-VTELGRPDAEYWNAQKDL-NH2	ACT synthesizer	crude, S2-302 freezer; purified fridge
6-18-A15	bioXX-VTELGRPDAEYWNSAKDL-NH2	ACT synthesizer	crude, S2-302 freezer; purified fridge
6-18-A16	bioXX-VTELGRPDAEYWNSQADL-NH2	ACT synthesizer	crude, S2-302 freezer; purified fridge
6-18-A17	bioXX-VTELGRPDAEYWNSQKAL-NH2	ACT synthesizer	crude, S2-302 freezer; purified fridge
6-18-A18	bioXX-VTELGRPDAEYWNSQKDA-NH2	ACT synthesizer	crude, S2-302 freezer; purified fridge
bio18E	bioXX-GPLTVEWRA-NH2	ACT synthesizer	crude, S2-302 freezer; purified fridge
bio18EA1	bioXXAPLTVEWRA-NH2	ACT synthesizer	crude, S2-302 freezer; purified fridge
bio18EA2	bioXXALTVEWRA-NH2	ACT synthesizer	crude, S2-302 freezer; purified fridge
bio18EA3	bioXXPATVEWRA-NH2	ACT synthesizer	crude, S2-302 freezer; purified fridge
bio18EA4	bioXXPLAVEWRA-NH2	ACT synthesizer	crude, S2-302 freezer; purified fridge
bio18EA5	bioXXPLTAEWRA-NH2	ACT synthesizer	crude, S2-302 freezer; purified fridge
bio18EA6	bioXXPLTVAWRA-NH2	ACT synthesizer	crude, S2-302 freezer; purified fridge
bio18EA7	bioXXPLTVFARA-NH2	ACT synthesizer	crude, S2-302 freezer; purified fridge
bio18EA8	bioXXPLTVEWAA-NH2	ACT synthesizer	crude, S2-302 freezer; purified fridge

*XX-LCLC

DR Binding Peptides

name	sequence	source
HA	PKYVKQNTLKLAT-NH2	ACT synthesizer
CLIP	KMRMATPLLMQALMPM-NH2	ACT synthesizer
A2	VGSDWRFLRGYHQYA-NH2	ACT synthesizer
HIV-gag	PEVIPMFSALSEGATP-NH2	Rainin syntheizer
HAL	PKYVKQNTL	Rec'd from E. Stratikos
YRAL	Ac-YRAL-NH2	Rec'd from Z. Zavala Ruiz
PLG (6R)	AWRSDEALPLGS	Rec'd from D. DeOliviera

A.I.9. Expression of invariant chain exon 6B

Developed by M. M. Rushe, adapted for use by G. J. Carven

Protocol for Growing and Purifying 6B(His) in JM109 Cells

Bacterial Growth (For a 6L E. Coli prep):

Day1: Streak plate Exon 6B(His) on LB amp plate. 37°C overnight.

Day2: Put plate at 4°C.

Autoclave 4L LB in 4 2L flasks, 1L LB in 1L bottle, 3 250ml Erlenmeyer flasks, and some 20% Glucose stock.

Grow seed cultures in small flasks overnight.

Day3: Grow 6L of cells to an OD_{600} of 0.8 to 1.0. (Remove 1mL for non-induced sample) Induce with IPTG and grow 3 additional hours.

Harvest cells (6x1L bottles spin @ 5000 RPM for 15 mins). Pour off sup. Keep cells.

Resuspend cells into 1/50th culture volume sonication buffer (although I have used PBS and it worked fine):

50 mM NaPi pH 6.0
300 mM NaCl
1mM PMSF

Freeze cells to help break apart. Thaw and sonicate on power 2.5 for 30 sec on the 30 sec off for a total "on" time of 4 mins.

Spin at 15,000g for 30 mins. Keep sup and filter 0.2 μm.

A.I.10. Purification of invariant chain exon 6B

Developed by M. M. Rushe, adapted for use by G. J. Carven

NiNTA Purification

To 2L equivalent of lysate: raise the pH of the filtered sup to around 8.0 and add 5mL of NiNTA bead slurry. Place on neutator overnight.
Collect beads in a column and wash with 50 mL PBS.
Elute with 16 mL of each of the following buffers:

PBS + 10% glycerol pH=5.5
PBS + 10% glycerol pH=4.5
PBS + 10% glycerol pH=3.5
PBS + 10% glycerol pH=2.8

The elution profile is not the same every time so check all fractions by Tricine PAGE. Sometimes the protein elutes at pH=4, but very often it doesn't elute until a pH as low as 3.0.

See next page for gel

SepPak Purification

After NiNTA purification, all 6B containing fractions were pooled for buffer exchange and SepPak purification.

Wash one small (0.5 mL) SepPak column with 10 mL MeOH then 20 mL H2O.

Slowly drip the protein onto the column with a syringe.

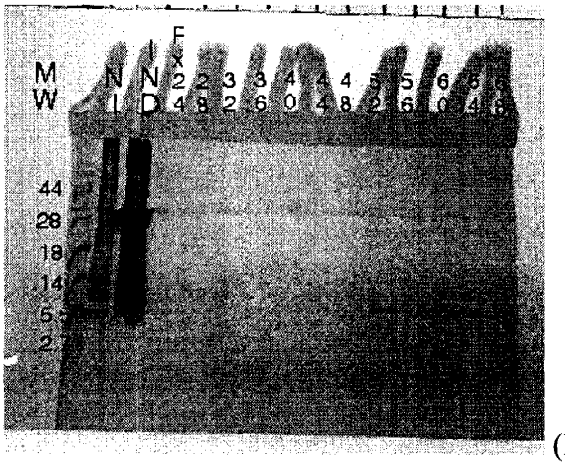
Wash column with 10 mL H2O.
Wash column with 10 mL 10% ACN.

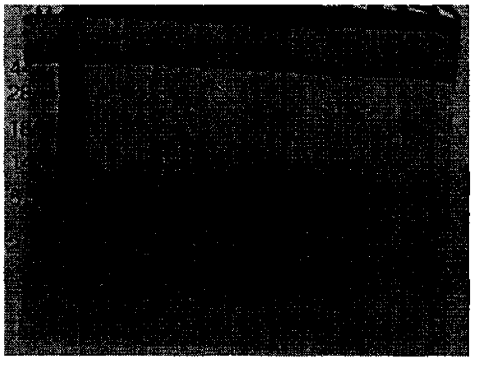
Elute with 4 x 1 mL 50% ACN.
Elute with 4 x 1 mL 70% ACN.
Elute with 4 x 1 mL 90% ACN.

Run each sample on a Tricine gel. Note: samples with high amount of ACN must be mixed with 2-3 times the amount of loading buffer (or add extra glycerol to the loading buffer).

Pool and lyophilize 6B-containing fractions

At this point the protein is most often pure enough to use for most purposes. Mia had further purified the protein by reverse phase (see her group meetings) for crystal trials. However, after SepPak separation, the Exon 6B band is often the only one seen on a gel. The added step of RP purification is likely unnecessary if you are using this for a cysteine protease inhibitor.

(A) 

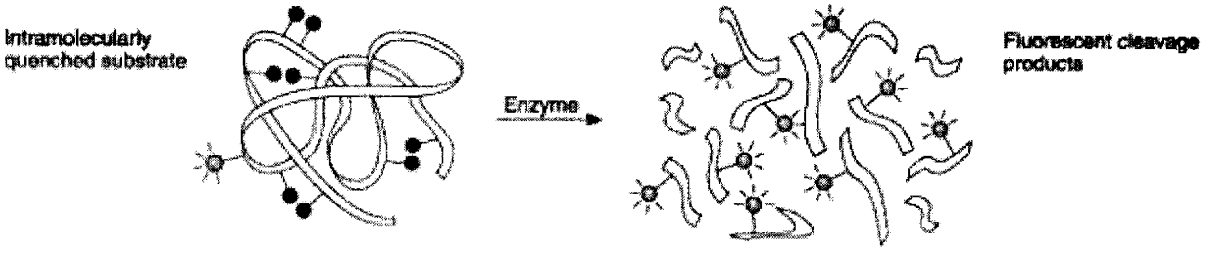
(B) 

Tricine gels of fractions after (a) NiNTA and (b) SepPak purification.

A.I.11. Quenched fluorescence protease activity assay

The quenched fluorescence assay developed here is was developed based on the EnzChek Protease Assay Kits (molecular probes)

This assay relies on the principle of enzyme detection via the disruption of intramolecular self-quenching. Enzyme-catalyzed hydrolysis of the heavily labeled and almost totally quenched substrate relieves the intramolecular self-quenching, yielding brightly fluorescent reaction products.



BODIPY-labeled Casein and Ovalbumin are available commercially, but I did not have any trouble labeling other proteins (such as) with BODIPY to the necessary extent of labeling.

1. Prepare a 1.0 mg/mL stock solution of the BODIPY substrate.

2. Prepare 1X digestion buffer. I use 200 mM Tris pH=8.0.

3. Prepare a 10 μg/mL working solution of the substrate. Add 0.2 mL of the stock solution prepared in step 1.1 to 19.8 mL of the 1X digestion buffer prepared in step 1.2.

4. To detect enzyme activity in a sample, dilute the sample to 1.0 mL (or 100 μL for microplate assays) in 1X digestion buffer prepared in 1.2. Add 1.0 mL (or 100 μL for microplate assays) of the BODIPY substrate working solution prepared in step 3.

For microplate analysis, incubate sample for one hour, protected from light. Read the

fluorescence in a fluorescence microplate reader. BODIPY FL

BODIPY labeled peptides have excitation/emission maxima of approximately 505/513 nm. Therefore standard fluorescein filters (e.g., excitation = 485 ± 12.5 nm, emission = 530 ± 15 nm) can be used

Alternately, you can measure the increase in fluorescence in real time in a fluorimeter with the same excitation/emission parameters.

A.I.12. Specific substrate protease activity assay

The carboxy terminus of single amino acids and short peptides can be conjugated to certain amine-containing fluorophores to create fluorogenic peptidase substrates. The dyes used to make these substrates are fluorescent at physiological pH; however, when the dyes are coupled in an amide linkage to peptides, their absorption maxima are usually shortened significantly. The resulting substrates are sometimes fluorescent but with relatively short-wavelength emission spectra. In an extreme case such as that of rhodamine 110–based substrates, detectable long-wavelength absorbance and fluorescence are completely eliminated by amide formation. Peptidase activity releases the fluorophore, restoring its free-dye fluorescence.

Rhodamine or aminocoumarin labeled peptides are commercially available (from Bachem and Molecular Probes). Most of the work done in this thesis used Gly-Pro-Arg-AMC.

Sample conditions were as follows

Ex: 380nm Em: 460 nm.
200 μM fluorescent substrate (5uL 20mM) in 200mM Tris pH=8.0
Total volume 0.5 mL.
Sample volume (of protease containing material 5μL).

Measure real time fluorescence increases in the fluorimeter for 5 minutes to 1 hour. Longer assay times can be used to measure lower protease concentrations.

Vary protease concentrations to generate a standard curve.
Include a blank to account for hydrolysis of the substrate.

A.I.13. Notes on JAWSII dendritic cell line

I obtained a cell line from ATCC that claimed to be an immature DC line. This cell line was derived from a **C57BL/6 mouse** and has the murine haplotype **I-A^{b}**. Because these cells are **I-A^{b}**, they will not react with KL304. KL295, even though it was raised against a peptide with the **I-A^{b,d}** sequence, does not stain these cells.

This cell line was originally made by the company Zymogenetics in Seattle WA. There are very few references to this cell line in the literature, as it has not been well characterized to date.

This cell line is patented and there is much information about the cells listed in the 2 patents (MacKay and Moore 1997; Moore 1998) as well as the paper (Butz and Bevan 1998).

This cell line was formed using p53 growth suppressor gene deficient animals (ie homozygous for a mutation in the growth suppressor gene p53 resulting in a lack of expression of a functional growth suppressor). It was not transformed with virus or other form of immortalization.

According to the patent, the preferred growth medium for these cells is alpha MEM without ribonucleosides, but containing 5-15% fetal calf serum , L-glutamine, sodium pyruvate, and GM-CSF.

I changed the medium slightly to cDMEM +5% FCS and 10 ng/mL GM-CSF.

My observations of these cells:
The JawsII DC grew very slowly (the patent stated this as well). They would double once every 2-3 weeks. They grow faster with more serum (ie 10%).

These cells were very adherent to plastic. I could easily wash these cells with very few of them coming off of the culture plate. Versene or cell scraping was required to remove them from the plates.

When the cells become overcrowded some of the cells begin to lift off of the plate. These confluent cells can be replated onto new plates/flasks and they adhere tightly.

These cells are also very big. They are 10-20 times bigger than the fresh DC that we get from a splenic DC prep. Visually they look like textbook immature DC. There are many dendrites and they have the characteristic veiled look.

A.I.14. In-gel tryptic digest

Using the Trypsin In-Gel Digestion Kit from Sigma (PP0100), I have had much success digesting DR1 into tryptic fragments that can be identified with mass spectrometry.

1. Reduce and alkylate the protein sample of interest prior to running on a gel. (*I actually skip this step and have not had any problems. Just be aware that cysteine can be modified by acrylamide and this might alter the MW of the expected tryptic fragments).*

2. Run the gel.

3. Stain the gel with a MS compatible dye (I stain with colloidal coomassie (Gel Code Blue from Pierce works well) or use a silverstain that is compatible with downstream MS (most silverstain protocols are NOT compatible)).

4. Carefully cut the band(s) of interest from the gel using a razor blade.

5. Place the gel band in a siliconized eppendorf tube. (A siliconized tube reduced binding of the peptides to the tube surface. You should also prewash the tube with 50% ACN to remove any chemicals which could leach into your tube.

6. Destain the band with 200uL Destaining solution. Incubate at 37 C for 30 mins. Remove and discard the solution.

7. Repeat step 6 one more time.

8. Dry the gel band in a speed vac for 15-20 mins. (Be careful not to let the gel band fly out of the tube…)

9. Add 20 uL of the prepared trypsin solution to the gel sample.

10. If you have a lot of protein in the gel band (a few micrograms or more) add 20-50 uL reaction buffer to the sample.

11. Confirm that the gel band is a the bottom of the tube and covered with liquid.

12. Incubate 4 hours to overnight at 37 C.

13. After the incubation, remove the liquid from the gel piece and transfer to a new labeled tube. This solution contains the extracted peptides. For MALDI analysis, acidification with TFA prior to matrix addition may be needed.

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Notebook 11: Chemical Modification and MS analysis (11/02-present)

A.III Curriculum vitae

Gregory J. Carven

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Maynard, MA 0754
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OBJECTIVE A research and development position in an industrial setting that takes advantage of my experience in protein chemistry and immunology.

HIGHLIGHTS

- Extensive experience in protein and peptide chemistry.
- Unique skill set of biophysical, immunological and cellular techniques.
- Research experience in the biotech industry.

EDUCATION

2004 Ph.D., Biological Chemistry. Massachusetts Institute of Technology, Cambridge, MA
Thesis: "Empty Class II MHCs: insight into their structure and function"
Adviser: Professor Lawrence J. Stern

1998 B.S., Honors Chemistry, University of Massachusetts, Amherst, MA, 1998
Magna Cum Laude, G.P.A. 3.64 / 4.0 overall; 3.81 / 4.0 in chemistry
Thesis: "Intrinsic Hydrogen Exchange Rates of Two Short Peptide Fragments of Cellular Retinoic Acid Binding Protein I."
Adviser: Professor Lila M. Gierasch

RESEARCH EXPERIENCE

1998 - present Graduate Research Assistant
Massachusetts Institute of Technology, Department of Chemistry
Mechanisms of Antigen Presentation and Response in the Immune System

- Produced MHC and related proteins in a number of expression systems.
- Characterized conformational change of HLA-DR1 upon peptide binding using side-chain chemical modification.
- Identified modified side chains using protein digestion and mass spectrometry.
- Mapped B-cell (antibody) epitopes of conformation specific monoclonal antibodies using synthetic peptides by ELISA as well as SPR arrays.
- Made point mutants of HLA-DR1 for use in folding and structural studies.
- Examined protease activities in dendritic cells, microglia and microglial exosomes.

1997-1998 Undergraduate Research Student
University of Massachusetts, Department of Chemistry
Biophysical Approaches to Protein Folding and Localization

- Expressed mutant versions of cellular retinoic acid binding protein I for folding studies.
- Probed secondary structure and stability using spectroscopic techniques including fluorescence spectroscopy and circular dichroism.
- Measured peptide HX rates and calculated protection factors for comparison to whole protein

Summer 1996, 1997, 1998 Summer Intern *Genetics Institute*, Cambridge, MA and Andover, MA

Dept. of Molecular Immunology; Receptor Driven Drug Discovery (1998)

- Developed and qualified ELISA immunoassays for antibodies directed against different potential drug products.
- Characterized in-house monoclonal antibodies directed against potential drug products.

Dept. of Bioanalytical Sciences (1997)

- Developed and qualified ELISA immunoassays for antibodies directed against high purity blood clotting factors.
- Optimized existing ELISAs for use with different blood matrices and detection methods.

Dept. of Biopharmaceutical Characterization and Analysis (1996),

- cGMP trained
- Developed a colorimetric method for the quantification of dextran sulfate in pre-clinic drug products.
- Performed quality control assays to find quantities of other drug product components by HPLC

TECHNIQUES AND SKILLS

Chemical/Biochemical – Protein expression and purification (E. coli, insect cells, B cell hybridomas), peptide synthesis (Fmoc solid phase), purification and characterization HPLC/FPLC (affinity column, reverse phase, ion exchange, gel filtration), mass spectrometry (MALDI, ESI, LC/MS), UV/Vis spectrometry.

Biophysical – Protein and peptide NMR, hydrogen exchange (by NMR), protein folding (by fluorescence and circular dichroism), protein conformation by chemical modification.

Immunological –ELISA (extensive experience in assay development), Flow cytommetry, ELISPOT, antibody epitope mapping, MHC binding affinity, T cell epitope determination using solid phase MHC-peptide complexes.

PUBLCATIONS AND PATENTS

1. Probing the Ligand Induced Conformational Change in HLA-DR1 by Selective Chemical Modification and Mass Spectrometric Mapping. **G.J. Carven** and L. J. Stern.
   Submitted to Biochemistry
2. Proteomics of Microglial-cell Derived Exosomes: Functions in Degradation of Opioid Peptides and Anaerobic Glucose Catabolism. I. Potolicchio, **G. J. Carven**, R. J. Riese, S. H. Wong, C. S. Stipp, J. L. Strominger, L. J. Stern, L. Santambrogio
   Submitted to J. Biol. Chem.
3. Monoclonal Antibodies Specific for the Empty Conformation of HLA-DR1 Reveal Aspects of the Peptide-Induced Conformational Change
   G. J. Carven, S. Chitta, I. Hilgert, M. Rushe, R. F. Baggio, M. Palmer, J. E. Arenas, J. L. Strominger, V. Horejsi, L. Santambrogio, L. J. Stern.
   J. Biol. Chem. (2004), **279**, 16561-16570.
4. Extracellular Antigen Processing and Presentation by Immature Dendritic Cells.
   L. Santambrogio, A.K. Sato, **G. J. Carven**, S.L. Belyanskaya, J. Strominger, L.J. Stern.
   Proc. Natl. Acad. Sci. (1999), **96**, 15050-15055.
5. **Carven, Gregory J.** "Intrinsic Hydrogen Exchange Rates of Two Short Peptide Fragments of Cellular Retinoic Acid Binding Protein I." Senior honors thesis, University of Massachusetts Honors Program, 1998.

Patent Application Submitted to USPTO. S/N 60/463,379
"Major Histocompatibility Complex Chips" L. J. Stern, **G. J. Carven**, S. Chitta, J. D. Stone, I Strug.