Novel Approaches to Generating
Cytotoxic T Lymphocyte Target Structures

By

Robert A. Uger

A thesis submitted in conformity with the requirements
For the degree of Doctor of Philosophy
Graduate Department of Immunology
University of Toronto

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ABSTRACT

Novel Approaches to Generating Cytotoxic T lymphocyte Target Structures

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Cytotoxic T lymphocytes (CTLs) are important effectors in the immune response against viral infections and tumors. CTL function is critically dependent upon the recognition of a specific molecular target: a class I major histocompatibility complex (MHC) molecule with bound peptide antigen. Considerable effort has been focused on elucidating the structure, biochemistry, and cell biology of class I molecules. This information has provided a solid foundation for understanding antigen presentation, a basic process central to cellular immune recognition. In addition, an intimate knowledge of CTL target structure formation is proving useful for the development of new vaccine and immunotherapy strategies. This thesis describes two novel approaches for generating class I MHC/peptide complexes, which share the common feature of coupling MHC and peptide antigen. They were undertaken within the framework of developing new tools for probing class I antigen presentation and devising unique strategies for consideration in disease prevention and treatment.

In the first approach, the biosynthesis and endoplasmic reticulum (ER) translocation of MHC and peptide were transiently coupled by inserting a CTL epitope into the signal sequence of a class I MHC heavy chain. It was speculated that such an integrated peptide would be liberated by ER enzymes and preferentially form CTL target structures. Although the signal sequence-incorporated epitope could be presented by its restricting class I molecule, this
presentation was unexpectedly observed to require TAP (transporter-associated with antigen processing) transport. This result indicated a cytosolic origin for the signal sequence peptide, and suggested a lack of ER processing.

The second strategy achieved a more permanent peptide-MHC linkage by covalently coupling CTL epitopes to the class I light chain, beta 2-microglobulin (β2m). Such peptide-β2m proteins could efficiently form CTL target structures when expressed endogenously or added to cells exogenously. Importantly, covalent linkage to β2m enhanced the MHC stability and antigenicity of suboptimal class I epitopes. This strategy, therefore, offers the potential to generate high levels of stable, defined class I MHC/peptide complexes, which could be valuable in vaccine and immunotherapy design.
ACKNOWLEDGMENTS

To begin, I gratefully acknowledge the enormous contributions made by my supervisor, Dr. Brian Barber. Through his scientific insight and enthusiasm, Brian has provided an outstanding intellectual environment that I have been fortunate to be a part of. He has demonstrated to me that science can be accomplished thoughtfully and critically, and most importantly, with passion and humor. In every respect, Brian has been an inspiration and a mentor.

I also thank the members of my supervisory committee, Dr. David Williams, Dr. Jim Rini, and Dr. John Chamberlain, for all of their advice and encouragement. I am also grateful to Cheryl Smith for her expert assistance with the flow cytometry analysis, and to Steven Chan for his contributions towards the generation and purification of the peptide-linked \( \beta_2m \) constructs described in Chapter 4.

To all members of the Barber and Watts labs, both past and present, I offer a collective thank you. By providing a remarkably friendly working environment, they have made my graduate career a highly enjoyable experience. Our frequent discussions and spontaneous debates have been a wonderful distraction, and are greatly appreciated.

I wish to also express my gratitude to all my other friends and family, recognizing that such a gesture is wholly inadequate. They have responded to my frequent absences and difficult moods with boundless patience and encouragement. In particular, I am deeply indebted to my parents, who have from the outset approached my graduate studies with an almost frightening enthusiasm. Such support is truly appreciated. In addition, I owe a personal acknowledgment to Nicole Cantkier, who demonstrated that the bonds of friendship can endure the travail of nearly a decade of post-secondary education.

And finally, to Marni I will say only one word: thanks.
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<tr>
<td>Ab</td>
<td>antibody</td>
</tr>
<tr>
<td>AIDS</td>
<td>acquired immunodeficiency syndrome</td>
</tr>
<tr>
<td>APC</td>
<td>antigen presenting cell</td>
</tr>
<tr>
<td>B22</td>
<td>B22-249.R1</td>
</tr>
<tr>
<td>$\beta_2$m</td>
<td>beta 2-microglobulin</td>
</tr>
<tr>
<td>BFA</td>
<td>brefeldin A</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CBP</td>
<td>calmodulin binding protein</td>
</tr>
<tr>
<td>CDR3</td>
<td>complementarity determining region-3</td>
</tr>
<tr>
<td>cim</td>
<td>class I modifier</td>
</tr>
<tr>
<td>CLIP</td>
<td>class II-associated invariant chain peptide</td>
</tr>
<tr>
<td>CMV</td>
<td>cytomegalovirus</td>
</tr>
<tr>
<td>CTL</td>
<td>cytotoxic T lymphocyte</td>
</tr>
<tr>
<td>DRiPs</td>
<td>defective ribosomal products</td>
</tr>
<tr>
<td>EBV</td>
<td>Epstein-Barr virus</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EK</td>
<td>enterokinase</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
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<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
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<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>Glc</td>
<td>glucose</td>
</tr>
<tr>
<td>GlcNAc</td>
<td>N-acetylglucosamine</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>granulocyte-macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>h$\beta_2$m</td>
<td>human beta 2-microglobulin</td>
</tr>
<tr>
<td>HIV</td>
<td>human immunodeficiency virus</td>
</tr>
<tr>
<td>HLA</td>
<td>human leukocyte antigen</td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
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<tr>
<td>HSP</td>
<td>heat shock protein</td>
</tr>
<tr>
<td>HSV</td>
<td>herpes simplex virus</td>
</tr>
<tr>
<td>IFA</td>
<td>incomplete Freund’s adjuvant</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>interferon gamma</td>
</tr>
<tr>
<td>li</td>
<td>invariant chain</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
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<tr>
<td>IPTG</td>
<td>isopropyl $\beta$-D-thiogalactopyranoside</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>LCMV</td>
<td>lymphocytic choriomeningitis virus</td>
</tr>
<tr>
<td>LDA</td>
<td>limiting dilution assay</td>
</tr>
<tr>
<td>LDH</td>
<td>lactate dehydrogenase</td>
</tr>
<tr>
<td>Man</td>
<td>mannose</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>MIIIC</td>
<td>MHC class II compartment</td>
</tr>
<tr>
<td>NP</td>
<td>nucleoprotein</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>NP-40</td>
<td>nonidet P-40</td>
</tr>
<tr>
<td>Ntn</td>
<td>N-terminal nucleophile</td>
</tr>
<tr>
<td>PBMC</td>
<td>peripheral blood mononuclear cell</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>pol</td>
<td>polymerase</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcriptase-polymerase chain reaction</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SIV</td>
<td>simian immunodeficiency virus</td>
</tr>
<tr>
<td>STI</td>
<td>soybean trypsin inhibitor</td>
</tr>
<tr>
<td>SV40</td>
<td>simian virus-40</td>
</tr>
<tr>
<td>TAP</td>
<td>transporter associated with antigen presentation</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TIL</td>
<td>tumor infiltrating lymphocyte</td>
</tr>
<tr>
<td>wt</td>
<td>wild type</td>
</tr>
<tr>
<td>2-ME</td>
<td>2-mercaptoethanol</td>
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Chapter 1

Introduction
A. CTLs and the Immune Response to Viruses and Tumors

Cytotoxic T lymphocytes (CTLs) are regarded as crucial components in the host response to viral infection. This belief is convincingly supported by a vast number of animal studies, using pathogens such as influenza virus (1, 2), lymphocytic choriomeningitis virus (3, 4), herpes simplex virus (5, 6), cytomegalovirus (7), and respiratory syncytial virus (8). In humans, the impediments to performing controlled virus infection experiments have made it more difficult to directly assess the protective role of CTLs. Nevertheless, CTLs appear important for the control of viruses such as influenza (9) and hepatitis B (10), and studies of bone marrow transplant patients have demonstrated a role for CTLs in cytomegalovirus immunity (11, 12). There is also an increasing appreciation for the role of CTLs in immunity to human immunodeficiency virus (HIV) infection. CTLs appear to control the initial HIV viremia (13, 14), and are correlated with long term non-progressor status (15), while the development of CTL escape mutants correlate with progression to AIDS (16, 17). Recently, an inverse relationship between high levels of anti-HIV CTL and plasma viral RNA levels has been conclusively demonstrated (18). Thus, CTLs are believed to constitute an important effector arm of the antiviral immune response, and are major consideration in the design of viral vaccines (19).

Within the realm of tumor immunology, CTLs are also considered to be important effectors. Animal models, adoptive transfer experiments (20, 21) and analysis of tumor escape variants (22, 23) have demonstrated the importance of CTLs in the in vivo control of cancer. Additionally, a multitude of experimental vaccine and immunotherapies have been developed which exploit the protective capacity of tumor-specific CTLs (22-28). As in the case of viral diseases, the obviously limited experimental data from human sources has made it more difficult to definitively demonstrate the anti-tumor efficacy of CTLs. Several lines of evidence,
however, suggest that CTLs may be important in the anti-cancer response. First, cytotoxic T lymphocytes can be isolated from the blood (29), lymph nodes (30) and tumor infiltrates (31) of cancer patients. Second, a multitude of different tumor antigens recognized by CTLs from patients have been identified (32). Lastly, emerging data from a number of clinical studies have correlated tumor-specific CTL responses with positive clinical outcome (33, 34).

Cytotoxic T lymphocytes, therefore, constitute a crucial effector mechanism in the defense against viral pathogens and tumors. CTL activity is dependent upon the recognition of an appropriate CTL target structure: a class I MHC molecule complexed with peptide antigen. Understanding the mechanisms responsible for generating this molecular complex would not only provide insight into a basic immunological process, but would be invaluable for designing strategies to mobilize effective, antigen-specific CTL responses. From this perspective, two strategies for generating defined class I complexes through coupling MHC and peptide antigen were explored. One approach transiently links MHC and peptide by coupling their biosynthesis and ER translocation, while the second forges a permanent union through covalent linkage. To place this work in proper context, I will describe in this Introductory chapter the basic features of antigen presentation, and how this information is being applied to vaccine design. To begin, the structure of class I MHC molecules is reviewed.

B. The Structure and Conventional Assembly of the CTL Target Structure

1. The Structure of Class I MHC Molecules

The three-dimensional structure of numerous class I MHC proteins has been determined by X-ray crystallography, including the human molecules HLA-A2 (35-38), A68 (39-41), B27 (42, 43), B35 (44), B53 (45) and the mouse molecules K\(^b\) (46-48), D\(^b\) (49), L\(^d\) (50, 51) and
Dd (52). From this structural data, a detailed picture of MHC conformation and peptide binding has emerged, the highlights of which will be summarized below. Since the bulk of the work detailed in this thesis is based upon the D\textsuperscript{b} molecule and the influenza virus nucleoprotein peptide NP366-374, whose crystal structure has been solved, I will illustrate the general principles of class I structure by referring specifically to this complex.

Class I MHC molecules consist of a membrane bound polymorphic heavy chain (with three extracellular domains, designated \(\alpha_1\), \(\alpha_2\), and \(\alpha_3\)), an invariant, soluble light chain (\(\beta_2\text{m}\)) and peptide epitope (see Figure 1). The \(\alpha_1\) and \(\alpha_2\) domains fold into a single, membrane-distal peptide binding domain, which consists of an eight-stranded anti-parallel \(\beta\)-sheet, with two long \(\alpha\) helices on either side, separated by a groove. The groove is approximately 25-30 Å long, 12 Å wide, and 11 Å deep, with narrow (5 Å) ends which are blocked by bulky amino acid residues. The majority of polymorphic residues are localized to the \(\alpha_1/\alpha_2\) domain, and are generally positioned pointing into the groove, for peptide contact, or out from the helices, to contact the T cell receptor. The membrane proximal \(\alpha_3\) domain and \(\beta_2\text{m}\) subunit adopt standard immunoglobulin folds, consisting of a three- and four-stranded anti-parallel \(\beta\)-sheet sandwich. The position and interaction of the \(\beta_2\text{m}\) subunit relative to the heavy chain varies between different class I molecules, as does the position of the \(\alpha_3\) domain, which can even be removed proteolytically without causing any major changes to MHC conformation (53). The CD8 coreceptor binds to the \(\alpha_3\) domain, principally at a solvent exposed flexible loop (residues 223-229), although binding to the \(\alpha_2\) and \(\beta_2\text{m}\) domains also occurs (54).

Peptides (usually nonamers) bind within the \(\alpha_1/\alpha_2\) groove in an extended conformation, with a defined N- and C-terminal orientation. There are three essential interactions which control the binding of peptide to MHC. First, the free amino and carboxyl termini of the peptide form hydrogen bonds with conserved residues at the narrow ends of the peptide binding cleft. In D\textsuperscript{b}, four amino acids (Tyr-171, Tyr-159, Tyr-84 and Thr-143) mediate these interactions. Second, atoms from the peptide main chain interact with MHC residues along the length of the groove. Third, specific peptide side chains interact with MHC residues. While the
Figure 1. The structure of the class I MHC molecule H-2D\(^\delta\) complexed with NP366-374 peptide. The heavy chain (\(\alpha_1, \alpha_2\) and \(\alpha_3\) domains) is shown in red, beta 2-microglobulin in green, and the peptide in blue. A. View along the length of the peptide binding groove. B. Molecule rotated 90\(^\circ\), displaying the pronounced arch in the peptide backbone. C. Overhead view of the peptide binding (\(\alpha_1\alpha_2\)) domain. D. Spacefilling model of the peptide binding domain shown in panel C, including the partially buried peptide. Illustrations were generated using RasMol v2.6 software.
first two interactions allow for permissive ligand binding, the latter introduces an element of peptide specificity, as described below.

The surface of the peptide binding groove is irregularly shaped, and forms discrete pockets (designated A through F). Some of these pockets are highly selective, binding only a very limited number of peptide side chains. These restrictive pockets structurally define primary anchor residues which comprise the peptide "motif", a feature of class I epitopes which has been deduced from pool sequencing eluted peptides (55). All D\(^{b}\) binding peptides, for example, contain primary anchor residues at the P5 position (Asn) and C-terminal position (Met). The Asn side chain is buried within the polar C pocket of the D\(^{b}\) cleft, forming three hydrogen bonds with heavy chain residues Tyr-156, Gln-97 and Gln-70. The C-terminal Met is buried in a deep hydrophobic cavity (pocket F) formed in part by Phe-74 and Phe-116. Other pockets in the cleft are less selective in their peptide side chain preferences, and thus account for the presence of secondary anchor residues in the peptide. The P3 position of D\(^{b}\) peptides, a secondary anchor, binds to the D pocket, facing the \(\alpha_2\) helix. The presence of the large residues Tyr-156 and His-155 in this pocket sterically prevent the binding of bulky peptide side chains, and therefore limit the P3 position to small residues.

While the pockets which bind anchor residues and peptide termini impose strict conformational requirements on the peptide ligand, in many other respects the complex shows remarkable structural flexibility. Peptides can often assume variable conformations, particularly in the middle regions of the cleft. Peptides longer than the canonical nine amino acids can be accommodated through a middle bulge (40), or by a zig-zag within the groove (37), or even by protruding from the C-terminal end of the cleft (38). Shorter (octamer) peptides can bind by reducing their central arch (46), or shifting their N-terminus and using a water molecule to mediate a conserved hydrogen bond (44). The binding of different peptides can also have an effect on MHC structure. These changes are often minor displacements of the \(\alpha_2\) helix and likely explain the ability of certain antibodies to distinguish different subsets of MHC/peptide complexes (56, 57).
Perhaps the most striking feature of MHC/peptide structure is the degree to which the peptide ligand is buried within the groove. In D^b, only 24% of the NP366-374 peptide is solvent exposed, with 79% of this exposure focused on the P4, P6 and P7 residues. The mechanism by which TCRs recognize a select group of side chains protruding from the largely inaccessible peptide has been deduced by solving the crystal structures of TCRs contacting MHC/peptide complexes in both human (58) and mouse systems (59, 60). The TCR binds diagonally across the α1/α2 domain, with a deep pocket formed by the α and β CDR3 loops over the central region of the peptide. The diagonal fit allows the TCR to access the peptide by avoiding the two highest points of the MHC molecule, located at the N-terminal regions of the α1 and α2 helices. The TCR makes contact with both the MHC (mostly through conserved residues) and peptide, primarily through the protruding middle section. Interestingly, there appears to be poor surface complementarity between the TCR and MHC/peptide complex, with rather precarious TCR-peptide contacts. As a consequence, the TCR is likely able to easily scan (with relatively low affinity) different peptides within an MHC context.

2. The Proteasome and Peptide Generation

Seminal experiments by Townsend and colleagues established that CTL responses are focused on peptide epitopes (61, 62), a finding which could be explained in structural terms by the emerging crystallographic data on class I molecules. The observation that class I restricted antigens originate from cytosolic proteins (63, 64) implicated a cytosolic proteolytic system in the generation of class I restricted epitopes. As discussed below, a plethora of data (at times controversial) has implicated the proteasome as the responsible protease.

The proteasome is a multisubunit, multicatalytic protease complex which is responsible for the majority of nonlysosomal intracellular protein degradation (65). The 20S catalytic core of
the proteasome consists of seven different α and β subunits, present in two copies each, arranged in four heptameric rings, with the configuration α,β,β,α. The complex is approximately 15 nM long and 11 nM in diameter, and has three internal cavities. The two outer cavities (antechambers) are formed by α and β rings, whereas the inner catalytic chamber is formed by the β rings. The ends of the cavities are constricted such that protein substrates must be unfolded and wind their way through the complex (66).

The proteasome is a member of the Ntn (N-terminal nucleophile) hydrolase family of enzymes, and utilizes an active site threonine residue in the β subunits (67). There are three principal catalytic activities: chymotrypsin-like (cleavage after hydrophobic residues), trypsin-like (cleavage after basic residues) and glutamyl-peptide hydrolyzing activity (cleavage after acidic residues). In addition, mammalian proteasomes possess two other activities: cleavage after branched chain amino acids, and after small, neutral residues (68). One of the principal functions of the complex is to degrade ubiquitin-conjugated proteins, although the digestion of nonubiquitinated substrates is also observed.

The 20S proteasome can interact with different regulatory complexes, including PA700 and PA28 (69). PA700 is a multisubunit 19S complex which can attach itself flexibly to the ends of the 20S core, and allows the proteasome to recognize ubiquitinated proteins and convert them to a degradation-competent form. PA28 is a multisubunit 11S complex, which is also able to cap the ends of the 20S core. Although PA28 has no catalytic activity of its own, it is able to enhance the digestion of peptides.

There are several lines of evidence which support a role for the proteasome in the generation of class I binding peptides. First, the MHC region encodes two proteins which show sequence and serological similarity to proteasome subunits and are upregulated by IFN-γ (70-74), a cytokine which coordinately regulates the expression of other antigen presentation molecules (75). After IFN-γ stimulation, these subunits, termed LMP2 and LMP7, displace two proteasome housekeeping subunits (X and Y, respectively) (76). Mice with engineered deficiencies in LMP2 (77) and LMP7 (78) show a number of antigen processing defects
(including reduced presentation of viral peptides and diminished numbers of CD4\(^{+}\)8\(^{+}\) cells) consistent with proteasome involvement in epitope production. Using purified proteasomes, Driscoll et al. demonstrated that the MHC-encoded LMP subunits alter the peptidase activity of proteasomes, specifically by enhancing the chymotryptic and tryptic-like activities (79). This would allow for the production of peptides ideally suited for MHC binding, since all known class I molecules require either hydrophobic or basic residues as C-terminal peptide anchors (80). A number of groups have subsequently demonstrated a modulation of proteasome activity by the LMP2 and LMP7 subunits, although there is no firm consensus on the nature of these alterations (81-84). More recently, a third IFN-\(\gamma\) inducible subunit, MECL-1 (which is encoded outside the MHC) has been discovered, which replaces the constitutive proteasome subunit Z (85, 86) and is capable of altering proteasome activity (87). IFN-\(\gamma\) also upregulates PA28 subunits (88). The addition of the PA28 regulator to the 20S core has been observed to alter the nature of the peptides produced by proteasomes, and enhance CTL lysis (89, 90). PA28 appears to enhance the efficiency of antigen presentation by promoting coordinated dual cleavages in peptide substrates (91).

Second, a number of studies have implicated the proteasome-dependent ubiquitin pathway in antigen presentation. Cell lines with temperature-sensitive mutations in ubiquitination show impaired ability to present antigens through class I (92, 93). Furthermore, enhancing the proteasome-mediated degradation of proteins through the N-end rule (94) results in more efficient antigen presentation (95-97).

Third, membrane-permeable pharmacological inhibitors of the proteasome, first introduced by Rock et al. (98), have profound effects on antigen presentation. Treatment of cells with these agents results in a peptide-deficient phenotype, where class I assembly and surface transport is impaired, and the presentation of cytosolic antigens is diminished (98-102).

Lastly, in vitro digestions using purified proteasomes have been shown to yield peptides which are identical to naturally processed epitopes (103-105). Additionally, proteasome digestion has been observed to correlate with in vivo epitope hierarchy. Two reports have
demonstrated that immunogenic peptides are efficiently liberated during proteasome digestion, whereas subdominant epitopes are poorly generated due to cleavage within the peptide (106, 107).

While these data argue strongly for the involvement of the proteasome in antigen presentation, there are a number of studies which have generated conflicting results. Cell lines deficient in the MHC-encoded proteasome subunits LMP2 and LMP7 frequently show no obvious defects in antigen presentation (108-111), and a failure of IFN-γ to modulate proteasome activity has been reported (112). A lack of correlation between N-end rule destabilization and enhanced antigen presentation has been observed (113), and data from ubiquitination-defective cell lines have been questioned (114). Furthermore, the presentation of various class I epitopes appears to be insensitive, or only partially sensitive, to proteasome inhibitors (115, 116), and can be influenced by antigen size and mode of expression (117-120). In fact, proteasome inhibitors have been observed to actually increase the presentation of certain epitopes (116, 120), apparently due to proteasome-mediated epitope destruction (120). These results may be further confounded by the fact that proteasome inhibitors can block the activity of other cellular proteases (98, 101, 121). A further consideration, argued by Yewdell and colleagues (116), is that proteasome inhibitors can induce a cellular stress response, which may influence antigen presentation.

Thus, the balance of evidence supports the notion that the proteasome contributes substantially to the pool of class I peptides, but there is sufficient data to indicate other cytosolic proteases also play a role. In accordance, it has been recently demonstrated that prolonged culture in the presence of a proteasome inhibitor leads to the growth of adapted cells utilizing a non-proteasomal cytosolic proteolytic system, which can degrade ubiquitinated proteins and supply peptides to assembling class I molecules (122). As will be described in a subsequent section, it is also possible that non-cytosolic (ER) proteases also contribute to the production of class I peptides.
3. TAP and Peptide Transport

Since class I molecules are assembled in the endoplasmic reticulum (123, 124), a topological question arises: how do peptides, which originate in the cytosol, gain access to the folding compartment? This problem is solved by a peptide transporter, referred to as TAP (transporter-associated with antigen processing) which belongs to the large ABC (ATP binding cassette) family of transporters found in species ranging from bacteria to humans (125).

A role for TAP in the transport of class I binding peptides was originally based on the finding that the MHC region of the mouse, rat and human encode two proteins (TAP1 and TAP2) which bear sequence homology to transport proteins (126-130). This hypothesis was further strengthened by demonstrations that TAP transfection can correct the peptide-deficient phenotype exhibited by a number of antigen presentation-defective cell lines (131-133). Furthermore, mice with a deleted TAPI gene have a defective class I peptide supply, such that they exhibit reduced class I surface expression, defective intracellular class I assembly, diminished numbers of CD4+8+ cells, and are unable to present cytosolic antigen (134). The development of in vitro assays to monitor peptide binding and translocation have firmly established that TAP is capable of transporting peptides, in an ATP dependent fashion, into the ER (135-137).

The TAP complex is a heterodimer of TAP1 and TAP2 (131, 138), and is localized to the ER and cis-Golgi (139). Its transmembrane nature has made structure determination difficult, although recent analysis of bacterially expressed TAP1 has suggested there are eight membrane-spanning segments, with large ER exposed loops and cytosolic N and C termini (140). Using radiolabeled peptides and photoactivatable crosslinkers, several groups have demonstrated that both TAP1 and TAP2 contribute to the peptide binding site (138, 141, 142). Transport appears to be a distinct two-step process, involving ATP-independent peptide binding followed by ATP-dependent translocation (141).
A major issue regarding TAP-mediated transport is selectivity: does TAP discriminate between different peptides? With respect to peptide size, TAP does exhibit clearly defined specificity. In mouse, rat and human systems, TAP is best able to transport substrates 9-12 residues long (143-145), which matches the size preference of class I molecules (80). While there is a strict adherence to the lower limit, the upper limit has been more difficult to define. TAP can transport peptides 30 or 40 amino acids long, albeit with progressively lower efficiency (145, 146).

TAP selectivity with regard to peptide sequence is more complex, and exhibits species specific differences. Mouse TAP appears the most selective, with an obvious preference for peptides which have a hydrophobic C terminal residue (143, 147), which is in concordance with the class I peptide binding motifs found in murine alleles (80). Rat TAP exhibits specificity at the allelic level: the rat TAP2A allele translocates peptides with both hydrophobic and basic C-terminal residues, whereas the rat TAP2B allele shows preference for only hydrophobic C-terminal amino acids (147, 148). This has provided a molecular explanation for the previously documented cim (class I modifier) effect, which found that the behaviour of the rat class I molecule RT1A° differed among haplotypes (149, 150). It is interesting to note that this allele selectivity appears exclusively in the rat, since the different TAP alleles in mice and humans do not appear to have functional significance (151, 152).

The peptide sequence selectivity of human TAP is a controversial issue. Early studies have argued that human TAP is generally non-selective (145, 147), although more recent experiments have demonstrated that the affinity of peptide binding to TAP can vary over a large (3-log) range (153). This discrepancy may be attributable to the nature of the peptide libraries studied: either defined class I epitopes (145) which may be biased towards efficiently translocated peptides (154), or completely randomized libraries (153). Van Endert and colleagues have defined a peptide binding “motif” for human TAP: a preference for hydrophobic or charged residues at the P2 and C-terminal position, and hydrophobic residues at P3 and P7. Acidic residues at P1, P3, and P7, hydrophobic amino acids at P1, or a Pro at
any of the first three positions appear to be deleterious (155). The strongly negative influence of Pro near the N-terminal positions has been confirmed by other groups (153, 156). This "motif", however, is not as strict as a class I peptide binding motif, where peptide affinity is dramatically influenced by a few dominant anchors. Rather, the binding affinity of a peptide for TAP is the sum of positive and negative interactions, with the effect of deleterious residues more easily compensated by favourable interactions. It is also curious that the peptide binding motif of human TAP appears to conflict with the peptide preferences of certain HLA molecules, such as the B7 family, which prefer peptides containing a P2 position proline (157).

4. The Assembly of Class I Complexes in the Endoplasmic Reticulum

The assembly of functional trimeric class I complexes in the endoplasmic reticulum is now recognized to be a highly regulated process, involving a number of chaperones and accessory proteins (summarized in Figure 2). The pathway is thus designed to achieve "quality control", ensuring correctly folded class I molecules are efficiently loaded with peptide ligand for transport to the cell surface for CTL recognition.

Immediately after biosynthesis, class I heavy chains associate with calnexin, the first component of the assembly pathway (158). Calnexin is a type I ER membrane protein which participates in the folding and assembly of a number of different protein complexes (159). The association of calnexin with immature class I molecules, its dissociation upon maturation and transport, as well as its ability to control the cellular location of misfolded complexes has definitively established it as an MHC chaperone (160-162). In mouse cells, calnexin remains associated with assembling class I complexes until both β2m and peptide have bound (160), whereas in human cells it interacts principally with free HLA heavy chains (163). Human heterodimers then interact with calreticulin, a 46 kD soluble ER chaperone which is related to
Figure 2. The conventional assembly of class I complexes in the endoplasmic reticulum. **Step 1**: shortly after biosynthesis, the heavy chain (HC) associates with the chaperone calnexin (Clnx). **Step 2**: upon β₂m binding, calnexin is replaced by calreticulin (Clrt). **Step 3**: the chaperoned heterodimer interacts with TAP via tapasin (Tps). The protein ER-60 may also be present at this point. **Step 4**: peptides, produced by proteasomal degradation of a ubiquitinated (Ub) protein are loaded onto TAP-associated class I molecules. **Step 5**: once assembled, the class I heterotrimer exits the ER to the Golgi, and is ultimately expressed at the cell surface. Note that for simplicity, the assembly pathway for HLA molecules is shown. Murine class I complexes exhibit minor differences in calnexin and calreticulin association (see text).
calnexin (164). Calreticulin has also been observed to bind to mouse heterodimers (165), and it is unclear at present whether these chaperones serve redundant or unique features within the assembly pathway.

Both calnexin and calreticulin exhibit lectin-like activity, binding monoglucosylated (Glc$_1$Man$_9$GlcNAc$_3$) carbohydrate moieties (166). Since folding glycoproteins oscillate between nonglucosylated and monoglucosylated forms due in part to the action of a glucosyl transferase which shows specificity for misfolded proteins (167), the chaperones are capable of specifically sampling immature complexes. It is noteworthy that human and mouse class I molecules differ in their extent of N-linked glycosylation: HLA chains have a single site (Asn 86) whereas mouse heavy chains have one (Asn 176) or two (Asn 256) additional sites (168). This difference may explain the species-related differences in chaperone interactions. Indeed, the addition of a second glycosylation site on human heavy chains enhances the interaction with calnexin and reduces calreticulin association (169). Calnexin is also able to interact with polypeptide segments on its substrate, although the nature of this interaction is currently unresolved (170, 171). Thus, a two-step mechanism for calnexin activity has been proposed: an initial weak interaction with monoglucosylated sugars, which allows binding to protein determinants (166).

The chaperone-assisted formation of heterodimers allows the next stage of class I assembly to occur: the binding of peptide ligand. This process is facilitated by an interaction between empty class I heterodimers and the TAP peptide transporter (172, 173). This physical interaction, which occurs only through the TAP1 subunit (138) presumably allows for more efficient peptide loading onto class I molecules, although this has not been formally demonstrated. The binding of peptide results in the dissociation of class I complexes from TAP and calreticulin, although calnexin dissociation is not induced (174). Interestingly, different class I alleles appear to vary in the extent of their TAP interaction, with the efficiency being controlled by residue 116, which is critical in defining the C-terminal pocket in the MHC binding groove (175).
The interaction between chaperoned class I molecules and TAP is mediated by an accessory protein, termed tapasin (TAP-associated glycoprotein). Tapasin is a 48 kD proline-rich type I ER membrane glycoprotein, which is a member of the Ig superfamily and is encoded by an MHC-linked gene (176). Tapasin appears to form a bridge connecting calreticulin-class I complexes and TAP (164), and has been recently shown to increase the level of TAP and TAP-mediated peptide translocation (177). In its absence, class I molecules do not associate with TAP and are thus expressed at low levels on the cell surface and cannot present antigen (176, 178), although some allelic variation in tapasin dependence has been noted (179). Up to four chaperoned class I-tapasin complexes may interact with a single TAP, achieving a stoichiometry which may favor peptide binding (176). Strikingly, tapasin also appears to be able to promote TAP-independent class I assembly, since mutant cells expressing only a soluble form of tapasin which is unable to associate with TAP can assemble class I molecules and present antigen (177).

There are a number of other ER proteins which have been observed to interact with assembling class I molecules, although their functional significance is unknown. BiP, an ER chaperone belonging to the HSP70 family has been observed to associate with free HLA heavy chains (163). The invariant chain, which performs a number of important chaperone functions for MHC class II molecules (180) can interact with class I molecules and has been speculated to play a role in exogenous antigen presentation (181) or peptide loading (182), although evidence to the contrary has been reported (183). More recently, ER-60 (Erp57), a resident ER protein known to exhibit thiol-dependent reductase and cysteine protease activities and act as a glycoprotein chaperone, has been found in association with class I heterodimers, TAP, tapasin and calreticulin complexes (184-186). It is hypothesized, but as yet unproven, that ER-60 may assist in disulfide bond formation during class I assembly, or even be involved in peptide trimming.
5. Comparison to the Class II Presentation Pathway

Class II MHC molecules present exogenous peptide antigens to CD4+ T cells, which contribute, via cytokines, to the production of humoral and cell-mediated immune responses. The class I and class II presentation pathways have a number of remarkable differences, owing in large part to the invariant chain (Ii) chaperone, a type II transmembrane protein which associates with newly synthesized class II αβ heterodimers in the ER (187). Ii is present as four different isoforms in human cells due to alternative exon and/or initiation codon usage, and forms multimeric complexes with class II with an Ii₃(αβ)₃ stoichiometry. A linear stretch of Ii, termed CLIP (Class II-associated invariant chain peptide), binds to the class II groove in conventional fashion and prevents αβ heterodimers from associating with resident ER peptides (180). Thus, unlike their class I counterparts, class II molecules do not sample the antigenic milieu of the endoplasmic reticulum. Rather, they are targeted to the endosomal system, where they encounter and bind peptides derived from exogenous antigens.

The targeting of class II molecules to the endosomal system occurs via signals in the cytoplasmic tail of Ii (188). Once in the appropriate compartment, the invariant chain is degraded and CLIP exchanged for an antigenic peptide. This compartment, often referred to as MIIC (MHC class II compartment) due to its rich content of class II molecules, shares a number of features with late endosomes or early lysosomes, including low pH and active enzymes (189). In these vesicles, class II molecules encounter peptides derived from exogenous antigens. These antigens are internalized either by receptor mediated processes, phagocytosis, or pinocytosis, and degraded by a poorly understood proteolytic system, which may involve cathepsin D (190). The exchange of CLIP for antigenic peptide is facilitated by HLA-DM (191), a class II-like αβ heterodimer, which itself appears to be selectively regulated by another class II-like molecule, HLA-DO (192). Once Ii is degraded and CLIP exchanged, the class II-peptide complexes traffic to the cell surface, for CD4+ T cell recognition.
The proteolytic removal of Ii is a pivotal event in the class II presentation pathway. The cysteine protease cathepsin S is largely responsible for this process (193), although recent data have suggested that thymic cortical epithelial cells, which positively select T cells, utilize cathepsin L (194). Importantly, the degradation of Ii appears to control the traffic of intracellular class II molecules. In immature dendritic cells, which are actively acquiring antigen, little class II is expressed at the cell surface, and is largely diverted to the lysosome and degraded. Upon maturation, when dendritic cells become potent antigen presenters, almost all of the class II is expressed at the cell surface (195). This change in class II distribution is due to cathepsin S-mediated Ii cleavage, and appears to be regulated by cystatin C, a potent inhibitor of cathepsin S which is downregulated and redistributed upon dendritic cell maturation (196).

Thus, the presentation of class II MHC occurs through a distinct pathway from class I molecules, principally due to the numerous roles of the Ii. While class I molecules acquire their peptide cargo during ER assembly, class II molecules are diverted to the endocytic pathway, and bind peptide only after the regulated removal of CLIP. As will be discussed below, this clear distinction between endogenous (class I) and exogenous (class II) antigen presentation has become blurred, as class I molecules appear increasingly able to present exogenous antigens.
C. Non-Conventional Class I Antigen Presentation

1. The Presentation of Signal Sequence Peptides

The conventional pathway of class I assembly, as described above, relies on the TAP transporter for supplying peptides to class I heterodimers. Thus, TAP deficient cell lines exhibit dramatically reduced expression of class I at the cell surface. One exception, however, is the HLA-A2 molecule, which is expressed at the cell surface at reasonably high (approximately 50% wild type) levels on TAP deficient T2 cells (197). This anomaly was explained by Engelhard and Cresswell, who found that A2 molecules in T2 cells bind a limited set of peptides, principally derived from signal sequences (198, 199). Signal sequences are short (15-30 residue) amino terminal peptides which target newly synthesized secretory proteins to the endoplasmic reticulum (200). Upon entry into the ER, the signal sequence is proteolytically removed by a signal peptidase enzyme, which is physically associated with the protein translocating channel on the lumenal side of the ER membrane (201). Six signal sequence-derived peptides were identified, which appeared to have undergone ER digestion. Four originated from near the C-terminus of the sequence, while one was derived from the immediate N-terminus, suggesting differential ER trimming (one peptide was originally unidentified but has been subsequently matched to the middle portion of a signal sequence (202)). Note, however, that the observed trimming appears incomplete, as five of the peptides are longer than the optimal nine residues. In addition to these observations regarding the class I associated peptide repertoire in TAP deficient cells, TAP-independent presentation has been subsequently demonstrated using an artificial signal sequence containing an N-terminal A2-restricted epitope (203).

The presentation of signal-sequence peptides by class I molecules occurs not only in TAP deficient cell lines, but also in normal cells. The A2-restricted signal peptides found in T2 cells
are also observed in parental T1 cells (198), and four different signal sequence-derived peptides have been isolated from HLA-B7 molecules (157). Furthermore, CTLs reactive against an A2-restricted peptide derived from a tumor antigen signal sequence have been observed in human melanoma patients (204). Since it is not known whether TAP is required for the presentation of these epitopes, it is unclear whether these peptides are generated directly in the ER or whether they originate from a cytosolic antigen reservoir created by aberrant protein synthesis or ER targeting. Such mistargeted or misfolded polypeptides, referred to as defective ribosomal products (DRiPs), have been speculated to contribute to the pool of cytosolic antigens (205). It is also possible that the ER contents (including signal peptides) are recycled back to the cytosol. The possible existence of such a retrograde transport system will be discussed in Section C4.

In contrast, exclusively TAP-dependent signal sequence presentation has been demonstrated. An immunodominant LCMV epitope (gp33), located in the signal sequence of a viral glycoprotein (206), has been shown to require TAP for both in vitro and in vivo presentation (207). In addition, TAP is required for the presentation of signal sequence-derived peptides through the mouse class Ib molecule Qa-1b (208) and the human molecule HLA-E (209). Thus, conflicting evidence exists regarding the TAP-dependence of signal sequence presentation, a point that will be further discussed in Chapter 2.

2. The Fate of Signal Sequences

The observation that signal sequences can provide epitopes for class I molecules (sometimes in a TAP-independent fashion) raises the question: what happens to signal sequences after they are cleaved? In bacteria, the digestion of cleaved signal peptides is well established, and both cytoplasmic and membrane-bound enzymes which mediate this
proteolysis have been identified (210). It was assumed that similar processes degrade signal sequences in eukaryotes, although only scant evidence had been offered (211), and the responsible enzymes were not identified. In fact, the isolation of signal sequence-derived peptides from class I molecules on T2 cells were the first reports identifying eukaryotic signal sequence fragments (198, 199).

Additional data on the fate of cleaved signal sequences have begun to emerge more recently. Studies using the preprolactin signal peptide have observed that following signal peptidase cleavage, the signal sequence is cut by an unknown enzyme near the C-terminal end of the central hydrophobic region. The resulting large N-terminal fragment is initially associated with the membrane, and then is released into the cytosol, whereas the fate of the smaller C-terminal fragment is unknown (212, 213). It is speculated that the C-terminal fragment may fall back into the ER (and thus could associate with class I molecules in TAP-deficient cells) which would be consistent with a loop model of signal sequence insertion, where the signal peptide spans the ER membrane with its C-terminal side, bearing the signal peptidase cleavage site, near the ER lumen (214). These studies, however, must be interpreted with caution, since preprolactin signal peptide possesses a rare property: its N-terminal fragment is able to interact with the cytosolic protein calmodulin (215). Since the release of signal peptide fragment into the cytosol is enhanced by the calmodulin interaction (215), it is unclear whether other signal sequence fragments, which do not interact with cytosolic factors, are similarly released. The fact that class I molecules can associate with some N-terminal signal sequence fragments in the absence of TAP (199, 203) indicates that cytosolic release is not obligatory. It is possible, although unproven, that the ultimate location of signal sequence fragments (cytosol, ER or membrane-retained) may be a function of their physical characteristics, such as hydrophobicity (212). Thus, the dependence on TAP for antigen presentation would be peptide-specific. It is also noteworthy that a different signal peptide (from cecropin A) has been observed to undergo complete digestion in the absence of cytosol (213).
3. ER Degradation in Antigen Presentation

The processing of signal sequences raises the larger issue of the role of ER degradation in the generation of class I peptides. Proteolysis within the ER was first hypothesized by Rammensee's group as an explanation for how the expression of MHC molecules appears to govern the cellular peptide pool (216). At first glance, the notion that ER proteolysis may contribute substantially to the pool of class I peptides may seem fanciful, since TAP deficient cell lines (217) and mice (134) exhibit profound antigen presentation defects. Nevertheless, some circumstantial evidence suggested that ER trimming may occur. Despite the presentation block, TAP1 knockout mice are capable of generating a diverse, peptide-specific CTL repertoire (218), and the in vivo CTL response during certain viral infections can be predominantly directed against membrane proteins (219). Furthermore, TAP is able to efficiently translocate longer than optimal class I binding peptides (143-145), consistent with a final proteolytic step in the ER. In fact, several immunodominant viral epitopes are poorly transported by TAP unless extended by their natural flanking residues (156). In vitro assays using microsomes have even revealed slow trimming of TAP translocated peptides within the ER compartment (220).

More definitive proof of ER degradation in class I presentation has emerged. Elliott et al. have demonstrated that TAP deficient cells are capable of processing and presenting epitopes from long flu NP fragments targeted to the ER (221). In one case, 40 amino acids were trimmed from the N-terminus, while 120 residues were cleaved off the C-terminal end. Similarly, Siliciano has shown that several epitopes in the extracellular domain of the HIV envelope protein can be presented in TAP deficient cells when targeted to or retained in the ER (222, 223). Interestingly, this TAP-independent presentation is selective, as two other epitopes located in the same region require TAP. These peptides both contain N-linked glycosylation sites, although treatment of cells with a glycosylation inhibitor does not confer TAP-independent presentation. It has been suggested that carbohydrates may play a role in
“sheltering” glycoproteins from ER degradation (221). In fact, recent data have indicated that glycosylation can regulate the ER degradation of glycoproteins (224). Possibly, glycoproteins are rescued from ER degradation by their (carbohydrate-dependent) interaction with the ER quality control pathways.

Despite these reports which suggest rather promiscuous cleavage within the ER, the balance of evidence indicates that ER proteolysis is more restrictive, favoring aminopeptidase activity. This was first suggested by Snyder et al., who found that when tandem epitope constructs were targeted to the ER in T2 cells, only the C-terminal peptides were presented (225). Subsequent experiments have demonstrated TAP-independent presentation of epitopes when tagged to the C-terminal end of several soluble and membrane proteins (226, 227). This is in agreement with findings from Rock and colleagues, who observed that TAP-deficient cells could process ER-targeted peptides extended at the N-terminus, but not at the C-terminus (119). In fact, the addition of two residues to the C-terminal end of an optimal epitope can block presentation, unless a carboxypeptidase (angiotensin-converting enzyme) is deliberately expressed in the secretory pathway (228). This implies a natural deficiency in carboxypeptidase activity in the ER. As mentioned previously in Section B3, the finding that unique sets of C-terminal peptides are supplied by the rat TAP transporter (the cim effect) also supports the notion that cleavage at the C-terminus is controlled by cytosolic factors (150).

An ER compartment which possesses amino but not carboxypeptidase activity is consistent with the emphasis the antigen presentation machinery places upon the C-terminal residue. C-terminal interactions, including both the binding of the anchor side chain to its cognate pocket and the conserved interactions at the free terminus, are critical to MHC/peptide stability. In fact, the peptide binding is severely compromised when peptides are extended at the C-terminus, more so than with N-terminal extensions (229, 230). As reviewed previously, there is evidence (albeit controversial) that the proteasome preferentially generates peptides with MHC-matched C-terminal residues, and that TAP (particularly in mouse) exhibits similar C-terminal selectivity. Thus, the antigen processing machinery on the cytosolic side of the ER membrane
is likely responsible for generating the important C-terminus of the peptide. It would seem reasonable, therefore, to allow the ER to specialize in aminopeptidase trimming, which is supported by the data reviewed above. The results described in Chapter 2, in which the presentation of a CTL epitope incorporated into the N-terminus of an ER signal sequence is shown to be TAP dependent, is consistent with a carboxypeptidase deficient ER.

4. Recycling Antigens from the ER to the Cytosol

In the preceding sections, I have described how class I peptides can be generated from cytosolic proteins and transported into the ER by TAP, or under certain circumstances, be generated by a poorly defined proteolytic system in the ER. An alternative, yet more complex pathway, is for class I peptides to be generated from proteins which enter the ER and recycle back to the cytosol. Such a mechanism was postulated by Roelse et al., upon observing that TAP translocated peptides are transported, in an ATP-dependent and TAP-independent fashion from the ER back to the cytosol, where they can re-enter the ER by TAP (220).

While such a mechanism may seem exotic, recent experiments on the fate of misfolded proteins in the ER has made it seem more feasible. Traditionally, the ER was believed to be the site of degradation for membrane and secretory proteins which did not fold or assemble properly (231). Yet this requires the two conflicting processes of protein folding and protein degradation to be carried out within the same compartment. This dichotomy has been resolved by demonstrating that protein degradation within the ER is actually occurring by the ubiquitin-proteasome pathway in the cytosol (232). It appears that misfolded proteins, both membrane and soluble, undergo retrograde transport back to the cytosol, where they are deglycosylated, ubiquitinated, and ultimately degraded by the proteasome. Such a pathway is hypothesized to purge the ER of misfolded proteins and allow their degradation to occur away from the
sensitive processes of protein folding. Class I MHC heavy chains themselves, when misfolded due to a lack of peptide or $\beta_m$, undergo this ER to cytosol transport prior to proteasomal degradation (233). The nature of this retrograde transport is not fully understood, but studies using yeast mutants (234) and a viral protein which causes class I degradation (235) have indicated that Sec61, the major component of the translocon, is involved. Thus, proteins may exit the ER through the same channel they use to enter during translocation.

Is retrograde transport important in class I antigen presentation? Interestingly, an HLA-A2 restricted tyrosinase epitope found on melanoma cells has been shown to undergo a posttranslational modification (conversion of an Asn to Asp) which involves ER to cytosol export (236, 237). In addition, experiments using flu NP with engineered glycosylation sites have demonstrated ER to cytosol transport (238). However, the extent to which a retrograde transport pathway contributes to the production of class I binding peptides is currently unknown. Given the paradigm shift which has occurred regarding “ER” degradation, it is conceivable that the majority of epitopes derived from secreted and membrane proteins may be generated in the cytosol.

5. The Presentation of Exogenous Antigens by Class I MHC

Traditionally, the class I MHC presentation pathway was believed to focus on intracellular antigens, while class II MHC molecules appeared devoted to the presentation of peptides derived from exogenous proteins. This comfortable division of labor seems ideally suited to minimize bystander cell destruction, by restricting CTL mediated lysis to cells which are virus infected, not cells which have simply acquired antigens from the extracellular environment. A plethora of recent experiments have demonstrated, however, that class I MHC molecules are capable of presenting many different types of exogenous antigens. This presentation was
originally described for macrophages (239), but has subsequently been demonstrated to occur in other professional APCs such as dendritic cells (240) and B cells (241), and even mast cells (242). Thus, the conventional paradigm is being challenged, and the distinctions between class I and class II MHC presentation are becoming obscured. This section will review the salient features of class I mediated exogenous antigen presentation.

The presentation of exogenous antigens by class I molecules was originally proposed by Bevan (a phenomenon termed “cross-priming”) to explain how the CTL response to minor histocompatibility antigens from donor cells was controlled by host APCs (243). It is now recognized that in vivo CTL responses can be generated by a vast array of different exogenous proteins, including tumor antigens, peptides, bacterial and parasite antigens, bead-associated antigens, lipid-associated antigens, multimeric protein complexes, and heat shock proteins (HSPs) (244). HSPs, by virtue of their ability to act as peptide chaperones, appear to be of central importance in class I exogenous antigen presentation. Immunization with HSPs have been shown to induce CTL responses against tumor (245), viral (246), and minor histocompatibility antigens (247). Note that exogenous antigen presentation by class I molecules is not only considered with respect to CTL induction, but may be responsible for achieving tolerance to peripheral antigens (“cross-tolerance”) (248).

Mechanistically, there are two general pathways that permit class I molecules to acquire peptides derived from extracellular antigens. The first involves the transfer of external proteins to the cytosol for TAP-dependent presentation. Rock’s group was the first to demonstrate that macrophages are capable of phagocytosing particulate antigen and transferring the phagosome contents to the cytosol, where they can associate with class I in a Brefeldin A (BFA) sensitive pathway which involves both TAP and the proteasome. (239, 249). It is unclear, however, whether this represents a novel antigen transport pathway or simply inefficient leakage of material into the cytosol due to phagocytic overload, or “indigestion” (250). The uptake of exogenous antigens for TAP-dependent class I presentation in macrophages can also be induced through macropinocytosis (251). More recently, this process was found to occur.
constitutively, and more efficiently, in bone marrow-derived dendritic cells (252). Thus, several pathways in APCs may contribute to the unconventional introduction of exogenous material into the conventional class I presentation pathway. In addition, certain intracellular bacteria, such as *Listeria monocytogenes*, have natural mechanisms (pore-forming proteins) which result in the introduction of endosomal material into the cytosol, where it can be processed for class I presentation (253). Such delivery systems can be exploited to efficiently deliver exogenous epitopes to class I molecules (see Section C4 below).

The second general pathway of exogenous class I presentation involves TAP-independent mechanisms (254, 255). Several different processes could be responsible. First, internalized antigens could be processed in a phagosome/lysosome and the resulting peptides secreted at the cell surface for interaction with class I molecules. Such a “regurgitation” pathway has been documented, although it appears to work inefficiently (256-258) (see below for further discussion of exogenous peptide binding to class I). Second, exogenously derived peptides may be loaded internally onto class I molecules which are recycled from the cell membrane. The recycling of cell surface class I molecules is known to occur in some cell types, although its significance is unclear (259). There is recent evidence that an “empty” form of L^d^ expressed at the cell surface (which is an unusual thermostable free heavy chain which is unlike empty molecules found on TAP-deficient cells) is internalized, where it acquires peptides from exogenous antigens and then is expressed back on the cell surface as a properly conformed heterotrimer (260). Curiously, this recycling mode of presentation is critically dependent upon the internalization, but not surface binding, of exogenous β_2m_ (261). Third, nascent class I molecules may be diverted to the endosomal pathway where they can acquire peptide cargo in a manner similar to class II molecules. As previously mentioned, the invariant chain has been found to associate with some class I molecules, and divert them to an endosomal location (181). Thus far, there is only circumstantial evidence for this mechanism of class I presentation (262, 263), although it is interesting to note that class I molecules can bind peptides at the low pH levels which are frequently found in endocytic compartments (264). Lastly, recent data
have indicated that exogenous peptides can traffic, via a vesicular pathway, directly to the ER where they can interact with nascent class I molecules (265). This pathway delivers peptides to the ER in TAP-independent fashion, without traversing the Golgi or cytosol, and may represent a general pathway for delivering small exogenous molecules to the ER.

6. The Exchange of Peptide and β₂m at the Cell Surface

The goal of class I assembly, whether it occurs through endogenous or exogenous pathways, is to produce stable heterotrimeric complexes at the cell surface. At physiological temperature, surface class I molecules exhibit a 5-10 hour functional half-life, which is likely controlled by the intrinsic dissociation of the molecular complex (266). The unfolding of surface class I molecules ultimately results in a free heavy chain, which is internalized and degraded (267), and a released peptide, which undergoes rapid digestion by extracellular proteases (268). Yet the dynamic properties of class I molecules are not restricted to terminal dissociation; they can bind and exchange both peptide ligand and β₂m from the extracellular milieu.

Despite the initial difficulties in demonstrating peptide binding to purified class I molecules (269), peptide binding to cell surface class I molecules has been extensively characterized (270-273) and the addition of exogenous peptides to live cells is a routine method of forming CTL target structures (see Chapters 2-4). Quantitating the fraction of class I complexes which are receptive to exogenous peptides has yielded variable results (10% for Dᵇ (273), 36% for B27 (271), 64% for Kᵇ (274)), and is influenced by a number of factors, including TAP (270) and the presence of exogenous β₂m (see below). In vitro data using purified class I molecules have indicated that the binding of exogenous peptides is limited by the rate of dissociation of the endogenous ligand (275). However, peptide binding to class I molecules on the surface of live
cells can be inhibited by BFA and cycloheximide, suggesting peptides associate with newly emerging "empty" class I molecules (271). It is unclear whether these empty class I molecules are truly devoid of peptide, or have bound low affinity ligands.

Exogenous β2m is also capable of binding to class I molecules at the cell surface, even across species barriers (276, 277). β2m binds stably and with high affinity to class I molecules (278), and associates rapidly with cell surface MHC (t1/2 of 15 minutes at 37°C) (279). The fraction of surface class I molecules which exchange β2m is unclear. One study has demonstrated that 90% of molecules can bind exogenous β2m (280), although more recent data have indicated that only a minority (10-25%) of surface MHC undergo exchange (279). The notion that certain populations of surface class I molecules are involved in peptide and β2m exchange is consistent with results which have demonstrated the existence of an "empty" surface form of Ld which is refractory towards exogenous peptide binding (281).

Interestingly, the ability of cell surface class I molecules to bind exogenous peptides is related to the exchange of β2m. Numerous studies have demonstrated that peptide binding to cells is enhanced by the presence of exogenous β2m, even if this β2m is obtained from bovine serum used in cell culture (282-284). Two distinct mechanisms were postulated: a cooperative model, where exchange of β2m releases the bound peptide allowing an exogenous peptide to bind, or a free heavy chain model, in which the exogenous β2m stabilizes free heavy chains at the cell surface thus creating peptide receptive molecules. Since peptide and β2m exchange do not appear to be kinetically coupled (264, 285), and there is evidence for a free heavy chain intermediate along the β2m exchange pathway (279), the latter model is favoured. Thus, the dissociation of class I molecules at the cell surface can generate free heavy chains, which can be loaded with exogenous peptides after stabilization by β2m binding. Since the in vivo concentration of extracellular β2m is reasonably high (2 μg/ml in normal serum) (286) such an exchange process may occur normally. Furthermore, the β2m-enhanced peptide loading pathway is potentially useful for eliciting peptide specific CTL responses in vivo. Indeed, Rock et al. have shown that β2m can act as an "adjuvant", enhancing CTL responses to otherwise
weak peptide immunogens (287). Exploiting the ability of $\beta_2m$ to promote peptide loading forms the basis for the experiments reported in Chapters 3 and 4 of this thesis. I will describe how tethering class I binding peptides to $\beta_2m$ via a polypeptide linker allows for the efficient creation of CTL target structures and enhances the stability, and thus antigenicity, of cell surface class I complexes.

D. The Application of Class I MHC Antigen Presentation to Vaccine Design

This Introduction has described a number of different pathways which contribute to the production of CTL target structures. Indeed, a wealth of information is now available regarding the generation of class I binding peptides and the mechanisms, both intra- and extracellular, which are responsible for assembling an MHC/peptide complex. This knowledge has not only allowed us to understand the basic immunological phenomenon of antigen presentation, but has at times contributed to a greater understanding of biological processes (for example: experiments regarding viral interference in class I assembly (235) were instrumental in elucidating the mechanism of retrograde protein transport). Yet we must not lose sight of the fact that CTLs are of central importance in antiviral and antitumor immunity (see Section A). Thus, a thorough understanding of how CTL target structures are generated could greatly assist the rational design of CTL-based vaccines and immunotherapies. In this section, I will illustrate how the current knowledge of class I MHC structure and antigen presentation can be applied to this practical endeavour. To begin, the basic process of CTL priming \textit{in vivo} will be reviewed.
I. The Generation of CTL Responses In Vivo

The full activation of naïve T cells is believed to require not only an antigen specific signal (MHC/peptide) but also an antigen nonspecific signal (costimulation) (288). Thus, while CTL targets can be ubiquitously expressed on cells throughout the body, CTL priming requires professional APCs, most notably dendritic cells, which can be induced to express costimulatory properties (289). The best understood costimulatory pathway involves the interaction of B7 molecules on the surface of APCs with their T cell counterreceptors CD28 and CTLA-4 (290). Although there is evidence to indicate that B7 costimulation is important in the in vivo generation of CTLs (291-293), its precise role has been difficult to define (294). Likewise, the role of CD4+ T cell help in CTL responses has been poorly understood, as both T-helper dependent (295, 296) and independent (297, 298) in vivo CTL priming has been observed. Recent data, however, have emerged which have demonstrated that T cell help for CTL responses is mediated by the CD40 dependent activation of dendritic cells (299-301). Ligation of CD40 by its ligand on activated T-helper cells appears to empower the dendritic cell with the capacity to prime naïve CTLs, likely through the upregulation of B7 costimulatory molecules and the secretion of cytokines such as IL-12 (302).

These data and other recent advances in dendritic cell biology (289) lead to the following model to describe in vivo CTL priming in response to a viral infection. First, peripheral immature dendritic cells at the site of infection acquire antigen (either through direct infection or a process such as phagocytosis) and are stimulated to mature. A number of factors can trigger this maturation, including pro-inflammatory cytokines released as a consequence of infection (303). Second, the dendritic cells migrate to lymph nodes, where they can interact with antigen specific T-helper cells, and receive a CD40 signal. Third, the CD40 "conditioned" dendritic cells in the lymph node can now activate naïve CTLs. Finally, the activated CTLs can emigrate from the node to the periphery, and clear the offending infection. Note that this model predicts that the requirement for T-helper cells could be bypassed if the dendritic cells were sufficiently
activated by other means. Such CD4+ independent activation could occur as a result of dendritic cell infection with certain viruses, or through the action of immunostimulatory adjuvants.

2. CTL Target Structure Density and Immunogenicity

Experiments using TAP deficient cell lines, which can be loaded with high levels of exogenous peptide, have demonstrated a strong correlation between high MHC/peptide density and strong CTL priming in vitro and in vivo (304, 305) (see section D6 below). One of the best understood factors which controls the density, and thus immunogenicity, of CTL target structures is the affinity of a peptide ligand for its class I molecule (306-309). More specifically, the rate at which peptides dissociate from class I molecules appears to be the most important binding parameter, with more immunogenic peptides displaying slower off-rates (310). This has been convincingly demonstrated by Melief and colleagues, who showed that the strength of peptide-specific CTL responses in mice correlates more strongly with peptide dissociation than peptide affinity (311). Presumably, peptides which confer high stability to the MHC complex permit more prolonged TCR interactions, a factor which is known to be critical in governing the fate of T cells (312).

Aside from the intrinsic binding relationship between peptides and MHC, various components of the antigen processing machinery could influence the MHC/peptide complex density. The proteasome and TAP, for example, are both known to exhibit substrate selectivity (see Introduction, Section B), which could modulate the level of specific MHC/peptide complexes at the cell surface by controlling the supply of peptides to assembling class I molecules. The role that such factors play in shaping CTL responses has been difficult to assess. Recently, a study which examined the CTL responses to all of the Kd binding peptides from influenza virus found that aside from peptide affinity, immunogenicity is influenced
principally by antigen proteolysis and minimally by TAP transport (309). In addition, epitope-specific CTL responses appear to be modulated by the presence of immunodominant peptides in the same protein (309), although the mechanism of this suppression is currently unresolved.

As a further complication, it is clear that supraoptimal stimulation through the T cell receptor can be detrimental to T cell activation (312), and result in tolerance induction in vivo (313). In fact, Berzofsky and colleagues have observed an inverse relationship between peptide concentration and CTL avidity (314). High avidity CTLs are generated by small numbers of stable MHC/peptide complexes, which are otherwise deleted by high antigen concentrations (315). It should be noted that this unexpected relationship was observed during restimulation of primed CTLs, and thus may not be applicable to the activation of naïve T cells. It nevertheless demonstrates the complexity of issues which must be considered when designing in vivo strategies.

In the discussion which follows, I will illustrate how the knowledge of class I antigen presentation is being applied to the issue of vaccine design, with specific emphasis on the strategies which attempt to augment the level of specific MHC/peptide complexes. It is recognized that such a bias towards the formation of antigen specific signals ignores the costimulatory aspect of CTL priming (see above). It is noteworthy, however, that antigen specific signaling and costimulation are interrelated events, such that increasing the strength of an MHC/peptide signal can lessen the costimulatory requirements during T cell priming (316-318). Thus, manipulating antigen presentation to form large numbers of CTL targets could prove useful in reducing otherwise inhibitory costimulation thresholds.
3. Enhancing Antigen Degradation

The observation that normal cells express unstable "empty" class I molecules at the cell surface (319), and that the addition of peptide ligands can induce the dissociation of nascent class I molecules from TAP (172, 173) suggests that the intracellular production of high affinity peptides may be limiting. Thus, increased expression of MHC/peptide complexes could be achieved by designing methods to accelerate the proteolysis of antigen substrates. This was first reported by Townsend and colleagues, who showed that a virus-induced block in antigen presentation could be overcome by engineering antigens to undergo rapid degradation due to cytosolic localization or N-end rule destabilization (95).

Recently, this approach has been used to promote CTL activity in vivo. Tobery and Siliciano demonstrated that the CTL response to membrane bound HIV env protein could be enhanced by removing its signal sequence, thus localizing the protein to the cytosol, where it presumably undergoes proteasomal digestion. Furthermore, augmented anti-nef CTL responses were demonstrated when the nef protein was destabilized through the N-end rule (320). In a similar fashion, enhancing proteasome-mediated digestion of β-galactosidase through N-end rule destabilization has been shown to enhance specific CTL responses in DNA vaccinated mice (321). Directly conjugating antigens to ubiquitin has also proved effective in augmenting in vivo CTL responses. LCMV nucleoprotein, when fused to ubiquitin, is rapidly degraded and in plasmid immunized mice can induce protective CTL responses which are superior to the responses elicited by unconjugated antigen (322). A comparable improvement in immunogenicity is observed when ubiquitin is appended to an oligopeptide construct (323). Thus, increasing the efficiency of antigen processing by accelerating the rate of protein digestion is a promising strategy in the development of gene-based CTL vaccines. Interestingly, this method allows for the induction of CTL immunity without priming antibody
responses, presumably because the rapid proteolysis prevents the release of large polypeptides (321, 322).

4. Bypassing the Antigen Presentation Machinery using Minigenes

Since the proteasome and TAP are known to introduce elements of epitope selectivity to antigen presentation (see above), bypassing one or both of these steps may enhance the efficiency of class I presentation. This can be accomplished using minigenes: DNA constructs which encode minimal class I determinants. These epitopes can be synthesized in the cytosol using an initiating methionine residue which may be removed by a specific cytosolic protease (324), or can be directed into the ER by fusion to a signal sequence (325). Thus, the minimalist approach of minigenes offers an interesting contrast to strategies (such as ubiquitin conjugation) which attempt to accelerate antigen processing.

Bennink and colleagues have used kinetic analysis, antibody blocking, and peptide quantitation to definitively show that minigene-expressed peptides (either cytosolic or ER-targeted) are presented in vitro more efficiently than epitopes derived from full length proteins (326). In fact, the level of peptide presentation from a vaccinia virus-encoded minigene is quite extraordinary, generating over 85,000 MHC/peptide complexes per cell, thus loading approximately 85% of the available class I (10-100 fold higher than full length antigen) (274). In vivo data using a variety of recombinant vaccinia viruses have demonstrated that ER-targeted peptides consistently elicit strong CTL responses, which always equal or exceed the responses induced by full length proteins (327). Interestingly, minigenes producing cytosolic peptides yield variable results, and for one antigen were shown to be the least efficient means of generating CTL. More recently, the in vivo immunogenicity of a suboptimal SV40 large T antigen epitope was enhanced by expression as an ER-targeted (but not cytosolic) vaccinia
minigene construct (328). Similarly, the immunogenicity of a plasmid-encoded p53 epitope has been augmented by fusion to a signal sequence (329). These data indicate that the direct insertion of class I epitopes into the ER, thus bypassing the proteasome and TAP, allows for efficient induction of CTL responses. Such a strategy is applicable using either recombinant viral vectors or DNA plasmids.

5. Enhancing the Delivery of Exogenous Antigens

The presentation of exogenous antigens by class I MHC molecules is no longer considered an anomaly, but is now suspected to play a major role in the in vivo CTL response (see Section B5). As expected, this has renewed interest in employing exogenous antigens in a vaccine context, particularly in light of the impressive results found using heat shock proteins (330). Expanding our knowledge of the mechanisms responsible for exogenous antigen presentation has greatly assisted the development of novel vaccine delivery systems. One promising approach is to utilize recombinant intracellular bacteria which have innate mechanisms of introducing proteins into the cell cytosol. Recombinant Listeria monocytogenes, which escape from phagosomes to the cytosol via the action of the pore forming protein listeriolysin, have been shown to induce protective, antigen-specific CTL responses in vivo (331, 332). Similar results have been observed using avirulent Salmonella bacteria, which is an attractive delivery vehicle given its ability to induce mucosal immunity via oral immunization (333). Exogenous antigen delivery does not require whole organisms, but has been shown to be effective by coupling antigens to a variety of proteins which can cross cell membranes, including listeriolysin (334), HIV tat (335), and the Antennapedia (a Drosophila transcription factor) homeodomain (336).
6. TAP Deficient Cells as APCs for CTL Priming

Cells which are unable to supply peptides to assembling class I molecules due to a defect in TAP express low levels of class I on the cell surface, although they produce thermolabile MHC molecules which can be stabilized by low temperature and peptides (337). This property has made these mutant cells invaluable tools in dissecting the class I presentation pathway. Additionally, this phenotype allows TAP deficient cells to be used as effective APCs for the in vitro and in vivo generation of CTLs.

Numerous experiments have demonstrated that TAP deficient cells are capable of binding more exogenous peptide than normal cells (270, 304, 305). For example, RMA-S cells bind 5-fold more peptide than dendritic cells, despite the fact that dendritic cells express 30-fold more class I at the cell surface (305). Thus, TAP deficient cells can achieve a higher density of specific MHC/peptide complexes. This property was initially exploited by Melief and colleagues, who demonstrated that peptide pulsed RMA-S, but not wild type RMA cells, can induce CTL responses in vitro (304). In fact, RMA-S cells are as effective as dendritic cells in priming CTLs in culture (305), and their antigen presenting capacity can be enhanced by transfection with the costimulatory molecule B7 (338). In vivo, RMA-S cells can induce specific CTL responses, even when pulsed with unfractionated peptides that are acid extracted from nonimmunogenic tumors (339, 340).

The use of TAP deficient cells as in vivo or in vitro APCs need not be restricted by the availability of the few mutant tumor lines such as RMA-S. Treating normal tumor cells or adherent splenocytes with TAP-2 antisense oligonucleotides can induce an RMA-S like phenotype. These antisense treated cells are able to prime protective anti-tumor CTL responses in vivo (341). Furthermore, several viral gene products, such as ICP47 from herpes simplex virus (342, 343) and US6 from human cytomegalovirus (344, 345) have been shown to specifically interfere with TAP function. Thus, additional reagents are now available for converting normal cells into potent APCs for potential use in vaccines and immunotherapies.
7. Enhancing Immunogenicity Through Epitope Engineering

Many epitopes of clinical importance display suboptimal binding and immunogenicity. This is of particular relevance in tumor immunology, since the CTL response to high affinity self (tumor) antigens may be tolerized (346). In fact, some of the most extensively characterized tumor epitopes from melanoma antigens such as Melan-A and gp100 exhibit only low to intermediate MHC affinity, in some cases due to the lack of consensus anchor residues (347-349). Given the relationship between peptide binding and immunogenicity (see above), this has serious implications for vaccine and immunotherapy design. For example, driving CTL responses in vitro using PBMCs from melanoma patients and suboptimal gp100 peptides often requires multiple rounds of stimulation and is successful only in a fraction of patients (350).

In order to circumvent this problem, numerous groups have undertaken an “epitope engineering” approach. This involves generating immunogens in which one or more of the peptide residues which interact unfavorably with the MHC molecule are replaced by more favorable ones. By retaining the TCR contact residues, a higher affinity peptide can be engineered which will generate CTL responses which cross react with the wild-type epitope. In mice, such a strategy has been successfully employed to induce in vivo CTL responses to suboptimal viral (351, 352) and tumor epitopes (353). In humans, a more MHC stable analogue of a highly conserved, low affinity HIV polymerase epitope has been shown to have enhanced in vitro immunogenicity (354). A modified Melan-A tumor epitope has been demonstrated to promote more efficient tumor infiltrating lymphocyte (TIL) recognition and augmented in vitro CTL responses (355). Similar results have been reported for higher affinity analogues of gp100 tumor epitopes (349). Encouraging clinical trial data have begun to emerge on the epitope engineering approach to human cancer treatment. Rosenberg et al. have recently demonstrated significant clinical responses in melanoma patients immunized with a modified gp100 peptide and IL-2 (356). Unfortunately, it is difficult to accurately predict the effect of specific peptide residue changes on MHC stability (357). Thus, epitope engineering, while
highly promising, is rather labor intensive insofar as it requires analyzing the contribution of individual residues within a given epitope, and screening prospective analogues for high affinity binding. As will be argued more extensively in Chapter 4, an alternative strategy of enhancing MHC stability by tethering epitopes to \( \beta_2m \) may offer a simpler means of engineering immunogenic epitopes.

8. **Covalently Coupling Peptides to Class I Molecules**

Perhaps the most obvious approach to generating high levels of stable, defined class I MHC/peptide complexes is to physically link peptides to the MHC molecule. If this linkage is covalent in nature, the coupled peptide epitope will be unable to dissociate from the class I molecule, thus producing extremely stable complexes. One method of achieving covalent class I/peptide complexes is to employ peptide derivatives which contain photoactivatable cross-linking groups. It has been demonstrated that mice can mount a CTL response when immunized with activated spleen cells which have been pretreated with a photoactivatable peptide analogue and UV light (358, 359). This CTL response is critically dependent upon cross-linking, and the enhanced stability it imparts, as cells simply pulsed with peptides are nonimmunogenic. This approach, however, has clinical limitations, in that it requires that the cross-linking group not adversely affect peptide binding, and can result in a CTL response which is primarily specific for the altered peptide (358).

A more elegant strategy is to couple peptide and MHC at the genetic level. This was first employed successfully using class II molecules, by linking the C-terminus of peptide epitopes to the N-terminus of mouse \( \beta \) chains (360). Since the peptide binding groove is more narrow in class I molecules and the free peptide C-terminus is involved in conserved interactions (see Section B1), such a linkage was predicted to be more difficult for class I MHC. Nevertheless,
Mottez et al. have generated a number of class I/peptide fusions in which the C-terminus of the peptide epitope is coupled to the N-terminus of the heavy chain (361). These structures display surprising flexibility, accommodating polypeptide linkers which range in size from 10 to 30 amino acids. Importantly, the covalent complexes are immunogenic in mice when expressed on the surface of transfected cells. These findings have recently been extended to a human system. Linking peptides from the Melan-A or gp100 melanoma antigens to HLA-A2 allows for the efficient \textit{in vitro} induction of CTLs using PBMCs from melanoma patients (362). Note, however, that these systems have used cell-associated fusion proteins. An alternative strategy, as described in Chapters 3 and 4, is to link epitopes to the \( \beta_2 \)m subunit. This approach allows the construction of soluble immunogens and exploits the ability of \( \beta_2 \)m to promote CTL target structure formation.

\textbf{E. Experimental Objectives}

The experiments described in this thesis were designed to investigate novel approaches for generating CTL target structures using MHC-peptide coupled systems. In this Introductory chapter I have endeavored to review the basic mechanisms which govern the production of class I MHC/peptide complexes. Our knowledge of MHC structure, biochemistry and cell biology has allowed us to comprehend the basic process of antigen presentation, which is of fundamental immunological importance. Additionally, as our knowledge of class I MHC presentation expands, the possibility of designing efficacious CTL vaccines and immunotherapies moves closer to becoming a reality. Indeed, the field of MHC research has entered an exciting phase, one in which the gap between basic and applied knowledge is being bridged. Within this framework, therefore, I have designed two distinct strategies for generating CTL target structures. Both were undertaken with the dual purpose of: 1)
investigating the basic mechanisms underlying class I MHC presentation and 2) assessing the potential of MHC coupled systems in the context of vaccine and immunotherapy design. The first goal is principally addressed in Chapter 2, whereas Chapters 3 and 4 focus on the second goal.

In Chapter 2, I describe a system whereby a CTL epitope is inserted into the signal sequence of a class I heavy chain. The objective was to couple peptide antigen and MHC spatially and temporally, by linking their biosynthesis and ER translocation. This modified heavy chain was successfully targeted to the ER, and the signal sequence incorporated epitope presented to specific CTLs. This presentation, however, was shown to be dependent upon the TAP transporter, indicating that the antigen must be processed in the cytosol, not the ER. This system, therefore, highlights the paucity of processing in the ER compartment, a subject which has generated considerable interest and debate.

Chapter 3 describes a different, and more direct method of coupling antigen and MHC: covalently linking peptides to $\beta_2m$. This approach offers the unique opportunity to exploit the ability of $\beta_2m$ to promote peptide loading and serve as a soluble immunogen. CTL target structures could be efficiently generated by expressing peptide-$\beta_2m$ fusion proteins in transfected cells or by exogenous addition of a peptide-$\beta_2m$ protein produced in bacteria. Evidence is presented which demonstrates that the activity of these fusion proteins is due to a linked epitope, and not a free peptide.

In Chapter 4, the peptide-$\beta_2m$ system is explored further using suboptimal epitopes, in an effort to approach a more realistic clinical situation. Peptide-$\beta_2m$ fusion proteins purified from bacteria show enhanced ability to stabilize class I molecules and sensitize target cells for CTL lysis relative to their uncoupled counterparts. This approach, therefore, offers an attractive system for enhancing CTL responses to non-optimal class I binding peptides.
Chapter 2

An Influenza Nucleoprotein Epitope Incorporated into the H-2D\textsuperscript{b} Signal Sequence Requires TAP for Presentation\textsuperscript{1}

\textsuperscript{1} This chapter was published essentially in this form in Uger, R.A. and B. H. Barber (1997) J. Immunol. 158:685
Introduction

Peptides presented by MHC class I molecules are usually derived from the degradation of cytosolic proteins (363). The transporter associated with antigen processing (TAP) complex, an ATP dependent peptide transporter, is responsible for translocating the cytosolic peptides into the endoplasmic reticulum (ER), where they encounter newly synthesized class I molecules (135-137). Although there is a large body of evidence in support of this pathway of antigen presentation, cell lines deficient in TAP suggest that certain epitopes may be presented by an alternative route. In particular, signal sequence derived peptides demonstrate an ability to be presented by HLA-A2 molecules in T2 cells, which are devoid of TAP function (198, 199). This is presumably a result of signal peptides gaining access to the ER via the normal protein targeting pathway, and being subsequently degraded by ER enzymes. The presentation of signal sequence peptides also occurs in TAP expressing cell lines (157, 198), and signal peptide derived CTL epitopes have been observed in both tumor (204) and viral (206) systems. However, it has been reported that TAP knockout mice exhibit a profound lack of cell surface class I molecules (134), implying that TAP independent signal peptide presentation is not the dominant pathway. Furthermore, conflicting evidence has accumulated as to whether signal peptide presentation is strictly TAP independent. Although TAP deficient cell lines have been observed to present certain signal sequence antigens (198, 199, 203), presentation of signal sequence peptides by H-2D\textsuperscript{b} (207) and the non-classical MHC molecule Qa-1\textsuperscript{b} (208) has been shown to require TAP.

The demonstration that signal sequence derived peptides can be presented in the absence of TAP raises the larger issue of whether ER proteolysis contributes to the production of class I binding peptides. Data are now accumulating which implicate as yet unidentified ER proteases in the generation of class I binding epitopes. Specifically, TAP deficient cell lines have been
shown to a) present multiple epitopes from HIV-1 env protein targeted or retained in the ER (222, 223), b) present a CTL epitope incorporated into the amino terminus of an "artificial" hemagglutinin signal sequence (203), c) liberate COOH-terminal epitopes targeted to the ER by signal peptides (225), d) process large (170 aa) ER targeted protein fragments down to optimal epitopes (221).

In an effort to take advantage of the processing potential of the ER, we designed a recombinant H-2D\(^b\) heavy chain with an optimal (D\(^b\) restricted) influenza nucleoprotein (NP) epitope incorporated into its signal sequence. The rationale was to create a system where the biosynthesis and ER translocation of MHC and peptide antigen are coupled, expecting that this spatial and temporal linkage between heavy chain and epitope would promote the formation of specific MHC-peptide complexes. Such a system could significantly enhance the potential to prime for epitope-specific CTL responses via intramuscular injection of plasmid DNA (364), particularly since muscle cells are limited in their endogenous expression of class I molecules (365).

In this report, we describe the initial characterization of this heavy chain signal sequence/antigen coupled system. We find that the signal peptide epitope can be processed and presented by class I MHC and form appropriate CTL target structures. This presentation, however, is found to be dependent on the presence of TAP transporters, implying that cytosolic degradation, rather than ER proteolysis, is responsible for generating the epitope. These findings help clarify the issue of whether signal peptide presentation requires TAP, and are discussed with regard to antigen processing within the ER.
Materials and Methods

Cell Lines and Antibodies

P815, BW5147, EL4, and COS-7 cells were obtained from the American Type Culture Collection (Rockville, MD); T2 cells were kindly provided by P. Cresswell (Yale University, New Haven, CT). Cells were maintained in complete medium (RPMI 1640 supplemented with 10% FBS (v/v), L-glutamine, antibiotics, non-essential amino acids, and sodium pyruvate). P815 and BW5147 transfectants were cultured in the presence of G418 (600-750 μg/ml). T2 transfectants were grown in 1.5 mg/ml G418. Antibodies B22-249.R1 (366, 367), 28-14-8S (366, 368) and HB-82 (369) have been described. Antibody KH95 (370) was purchased from Pharmingen (San Diego, CA).

Vector Construction

Full length D$^b$ was generated by standard RT-PCR using mRNA isolated from C57BL/6 splenocytes. NP-HC was generated by similar methods, using a PCR primer which encoded the Met-NP366-74 sequence in place of the first 24 nucleotides of the wild type D$^b$ leader sequence. HindIII and XbaI restriction sites were incorporated into the 5' and 3' oligonucleotide primers. The constructs were initially ligated into the TA Cloning Vector (Invitrogen, San Diego, CA) and sequenced with T7 polymerase (Pharmacia, Piscataway, NJ). Appropriate inserts were removed by HindIII/XbaI digestion and ligated into the pRc/CMV eukaryotic expression vector (Invitrogen).

Transfections

For P815 and BW5147 transfection: 20 μg of linearized plasmid was added to 2 x 10$^7$ cells (washed twice in serum-free medium) in a 0.4 cm cuvette, and pulsed at 700 V, 25 μF with a Gene Pulser apparatus (Bio-Rad, Richmond, CA). Cells were then incubated for 10 minutes at
room temperature, 1 ml medium added, and incubated for an additional 20 minutes. Transfectants were cultured overnight in normal medium, and transferred to G418 selection medium. T2 cells were transfected with 25 μg of linearized plasmid essentially as described (371) using G418 resistant, irradiated HeLa feeder cells (kindly provided by P. Cresswell, Yale University, New Haven, CT). For COS transfection: 1 μg of DNA was mixed into 5 ml of serum free medium containing 400 μg/ml DEAE-dextran and 0.1 mM chloroquine, and added to partially confluent COS plates for 2 hours at 37°C. Medium was then aspirated and cells were pulsed for 1 minute in 10% DMSO, washed, and cultured for 3 days in complete medium.

Flow Cytometry

10⁶ cells were incubated for 1 hour on ice with 2-3 μg primary antibody (in PBS containing 1% BSA, 0.1% sodium azide). Cells were washed twice, and incubated for 1 hour on ice with the secondary antibody, goat anti-mouse FITC conjugate (Sigma Chemical Co., St. Louis, MO). Cells were washed twice and resuspended in 0.5 ml PBS (0.1% sodium azide). In some experiments, paraformaldehyde fixative was added to a final concentration of 1% (v/v). Samples were analyzed on a Coulter flow cytometer (Hialeah, FL).

CTL Assays

CTLs were primed by immunizing C57BL/6 mice with 200 hemagglutinin units of the X-31 strain of influenza virus, which possesses the NP gene from the A/PR/8/34 virus (372). At least 4 weeks post infection, spleen cells were harvested and restimulated in vitro for 5 days with flu infected, irradiated syngeneic tumor or spleen cells at a 10:1 responder:stimulator ratio, in complete medium supplemented with 50 μM 2-ME. The restimulated effectors were then used in a standard ⁵¹Cr release assay to assess target cell lysis. Target cells (10⁶) were labeled for 1.5 hours with 100 μCi of Na₂[⁵¹Cr]O₄ (Amersham, Arlington Heights, IL), washed
repeatedly, and $10^4$ cells were dispensed into 96-well round bottom plates. When necessary, targets were incubated with 100 nM NP366-74 peptide (Alberta Peptide Institute, Alberta, Canada) for at least 30 minutes at room temperature prior to plating. Titrated effector cells were added, and plates were incubated for 4 hours at 37°C. Supernatants were harvested using filters (Skatron, Lier, Norway) and radioactivity was measured by gamma-counting (Beckman, Fullerton, CA). Per cent specific lysis was calculated, using the mean of triplicate samples, as: 

$$\text{Percent Specific Lysis} = \frac{100 \times [(\text{experimental cpm} - \text{spontaneous cpm})/((\text{maximum cpm} - \text{spontaneous cpm})].}$$

Spontaneous cpm values were determined by incubating labeled targets in medium alone, and maximum values by lysing targets in 1% Triton X-100.

**Immunoprecipitation**

Immunoprecipitations were done essentially as described (373). After washing and preincubating in methionine free medium, cells ($10^7$) were labeled for 30 minutes with $^{35}$S-methionine (Amersham), washed with cold PBS (0.1% BSA) and subsequently with cold TBS (10 mM Tris, 150 mM NaCl, pH 7.4). Cells were lysed, on ice for 20 minutes, in 1 ml lysis buffer (0.5% NP-40, 10 mM Tris, 50 mM NaCl, 1 mM MgCl$_2$, pH 7.4) supplemented with 1mM phenylmethylsulfonylfluoride and 5 mM iodoacetamide. Nuclei were pelleted, and peptides added where appropriate. Supernatants were precleared overnight with 40 µl of 25% (v/v) Protein-A agarose (Life Technologies, Grand Island, NY). Cleared lysates were adjusted to 0.1% dialyzed BSA, 20 µg monoclonal antibodies were added, and samples were incubated at 4°C for at least 2 hours. Antibodies were precipitated with the addition of 80 µl 25% (v/v) Protein-A agarose, and incubated at 4°C for an additional 2 hours. Beads were washed at least five times with NTSE (0.5% NP-40, 10 mM Tris, 150 mM NaCl, 1 mM EDTA, pH 7.4), and protein eluted by the addition of 60 µl SDS-PAGE sample buffer and heating at 60°C for 5 minutes. For pulse/chase analysis, cells were pulsed with $^{35}$S-methionine for 5 minutes, and chased with 9 volumes of complete medium supplemented with 1 mM L-methionine. At
various time points, aliquots were removed and incubated on ice. Cells were lysed and proteins immunoprecipitated as described above. For Endo H digestion, washed beads were resuspended in 0.1 M sodium citrate/0.2 % SDS, and heated at 70°C for 5 minutes. An equal volume of 0.1 M sodium citrate was added, and samples were split into two aliquots. One sample received 2 mU Endo H (Boehringer Mannheim, Indianapolis, IN), the other was left untreated. Both samples were incubated overnight at 30°C, then diluted with SDS-PAGE sample buffer. In all cases, samples were boiled in the presence of 2-ME and run on SDS-polyacrylamide gels, which were soaked briefly in 1 M sodium salicylate prior to vacuum drying.

Results

Experimental Design

A class I MHC/antigen coupled system was devised in which the optimal D\(^\text{b}\) restricted CTL epitope NP366-74 from influenza nucleoprotein (374) was incorporated into the signal sequence of H-2D\(^\text{b}\). By necessity, the epitope had to be incorporated into the heavy chain signal sequence in such a manner as not to adversely affect signal peptide function. Based on structure-function studies of signal sequences (200, 375), the amino terminal (n) region was judged to be the optimal site for manipulation. Using RT-PCR, a recombinant D\(^\text{b}\) heavy chain containing the NP366-74 sequence in place of its wild type amino terminal (n) region was constructed (designated NP-HC; Figure 3). This n region substitution preserves the densely hydrophobic h region, which is critical for signal sequence interaction with the SRP (376), and leaves the signal peptidase recognition site untouched. Note that a methionine residue was added to the amino terminus of the NP-HC construct, in order to initiate mRNA translation.
Figure 3. The design of D$_b$ signal sequence constructs. The amino acid sequence (single letter code) of the wild type (WT) D$_b$ signal sequence is shown at the top. The characteristic n, h, and c regions are indicated. Signal peptidase cleavage occurs after the C-terminal alanine residue in the c region. To generate NP-HC, the first 8 amino acids of the WT signal peptide were replaced with the nine-amino acid NP366-74 epitope (bold), preceded by a methionine residue. Both WT D$_b$ and NP-HC were cloned into the pRc/CMV eukaryotic expression vector, shown at the bottom. The inserts are under the control of the CMV promoter (pCMV), and utilize the bovine growth hormone polyadenylation signal (BGH pA). The plasmids contain both ampicillin (Amp) and neomycin (Neo) drug resistance genes, the latter under the control of the SV40 promoter (pSV40).
We expect this methionine to be removed shortly after protein synthesis (377), and thus should not extend the NP epitope to a tenth (and potentially destabilizing) residue. In fact, the presence of methionine at the amino terminus of the NP366-74 epitope results in a thousand fold reduction in biological activity, as assessed by CTL target cell sensitization (Figure 4).

NP-HC, as well as a wild type (WT) D^b heavy chain, was cloned, sequenced, and inserted into the pRc/CMV eukaryotic expression vector. The constructs were then evaluated for signal sequence function, and the presentation of NP epitope assessed, with particular emphasis on the role of TAP in signal sequence presentation.

*Signal Sequence Function in NP-HC*

In order to assess whether the presence of the NP epitope within the D^b signal sequence impacts upon the ability of the signal peptide to target nascent chains to the secretory pathway, the NP-HC vector (and WT control) was transfected into two mouse cell lines: P815, an H-2^d mastocytoma, and BW5147, an H-2^k lymphoma. G418 resistant, stably transfected lines were then analyzed for cell surface expression of D^b by flow cytometry (Figure 5 A,B). NP-HC lines stained positive for D^b, using two different monoclonal antibodies. Expression levels were comparable to those obtained with a wild type D^b vector, and were consistent with the levels of D^b at the surface of EL4 cells, an H-2^b T lymphoma. NP-HC was also transiently transfected into COS-7 cells, which demonstrated high surface expression (Figure 6). Together, these results indicate that incorporation of the NP366-74 epitope into the amino terminal region of the D^b signal peptide does not significantly alter the capacity of the signal sequence to deliver heavy chains to the secretory pathway.
Figure 4. NP366-74 peptide extended with an additional N-terminal Met has reduced ability to sensitize target cells for CTL lysis. $^{51}$Cr-labeled EL4 cells were pulsed with titrated amounts of the optimal NP366-74 peptide (ASNENMETM, closed symbols) or the decameric variant (MASNENMETM, open symbols) and incubated with NP366-74-specific CTLs (10:1 effector to target ratio) in a standard 4 hour chromium release assay.
**Figure 5.** $D^b$ is expressed at the surface of BW5147 and P815 transfectants. 

A) BW5147 cells transfected with WT (BW.WT) or NP-HC vectors (BW.NP-HC), untransfected BW5147, and EL4 positive control cells were stained with the $D^b$-specific Ab B22-249.R1 and a FITC-labeled goat anti-mouse conjugate (open histograms). Cells were also stained with an isotype-matched control antibody (shaded histograms).

B) P815 cells transfected with WT (P815.WT) or NP-HC vectors (P815.NP-HC), untransfected P815 and EL4 positive control cells were similarly analyzed for $D^b$ expression using the Ab KH95 (open histograms) or an isotype-matched control Ab (shaded histograms).
Figure 6. $D^b$ is expressed at the surface of COS transfectants. COS cells were transfected with WT vector (bottom panel), NP-HC (middle panel) or mock treated (top panel) and stained for $D^b$ expression using antibody B22-249.R1 and a FITC-conjugated goat anti-mouse antibody.
Presentation of Signal Sequence Antigen to CTL in TAP Expressing and TAP Deficient Cell Lines

P815 and BW5147 cells transfected with the NP-HC vector were analyzed for their ability to form CTL target structures in a standard chromium release assay, using CTLs derived from influenza virus immunized mice (Figure 7 A,B). For both cell lines, NP-HC transfectants were lysed at levels comparable to the positive control, EL4 cells pulsed with NP366-74 peptide. Untransfected P815 and BW5147 cells, and transfectants expressing a wild type D<sup>b</sup> molecule, exhibited only background lysis. Thus, these results clearly indicate that an optimal CTL epitope incorporated into a class I heavy chain signal sequence can be processed correctly, such that an appropriate CTL target structure can be generated.

The NP-HC vector was also transfected into the human B/T hybrid cell line T2 (197), which is deficient in both TAP gene products due to a large deletion in the MHC region, and consequently demonstrates a profound inability to assemble class I molecules and present antigen (217). T2 was chosen in preference to the murine TAP-deficient cell line RMA-S (which endogenously expresses D<sup>b</sup>) in order to facilitate monitoring of the expression and CTL target formation with the transfected D<sup>b</sup>. Since TAP deficient cells can present some endogenous signal sequence epitopes (198, 199, 203), it was expected that T2 transfected with NP-HC would be sensitized for lysis by influenza specific CTLs. In chromium release assays, however, the G418 resistant T2.NP-HC lines were resistant to CTL killing (Figure 8). The absence of lysis is not due to an inability of murine CTLs to recognize human T2 transfectants, since the addition of NP366-74 peptide to T2-NP.HC cells results in their lysis. Thus, D<sup>b</sup> presentation of an optimal epitope located within the D<sup>b</sup> signal sequence requires TAP.
Figure 7. TAP-expressing transfected cell lines are lysed by NP366-74-specific CTL. Standard 4 hour chromium release assays were performed, using CTLs obtained from the spleens of influenza virus-immunized C57BL/6 mice and restimulated in vitro, as described in Materials and Methods. EL4 cells pulsed with NP366-74 peptide served as the positive control, untreated EL4 cells as the negative control. A, Untransfected BW5147 cells (BW), BW5147 transfected with WT vector (BW.WT) or NP-HC vector (BW.NP-HC) as target cells. B, Untransfected P815 cells, P815 cells transfected with WT vector (P815.WT) or NP-HC vector (P815.NP-HC) as target cells.
Figure 8. TAP-deficient T2 cells are not lysed by NP366-74-specific CTL. Standard 4 hour chromium release assay using untransfected T2 cells, T2 cells pulsed with NP366-74 peptide, and T2 cells transfected with the NP-HC vector (T2.NP-HC) as targets. RMA cells pulsed with NP366-74 served as the positive control, unpulsed RMA cells as the negative control. The CTLs were obtained from the spleens of influenza virus-immunized C57BL/6 mice and restimulated in vitro, as described in Materials and Methods.
Analysis of T2.NP-HC Transfectants

The finding that presentation of a signal sequence encoded antigen requires TAP prompted us to investigate further the T2.NP-HC transfectants. Initially, T2.NP-HC cells were analyzed for expression of the transfected gene product. RT-PCR analysis, using an oligonucleotide primer specific for the NP366-74 sequence, indicated that T2.NP-HC cells were synthesizing mRNA from the recombinant NP-D^b construct (data not shown). D^b protein expression in T2.NP-HC cells was assessed by metabolic labeling with ^35^S-methionine and immunoprecipitating D^b heavy chains with specific monoclonal antibodies (Figure 9). Antibody 28-14-8S, which recognizes an epitope in the α3 domain of D^b (366) and thus captures heavy chains from EL4 cells but not untransfected T2, gave a strong SDS-PAGE band, indicating that D^b heavy chain protein is produced in T2.NP-HC. Immunoprecipitation with monoclonal antibody B22-249.R1 (B22), which recognizes D^b in a conformation dependent manner (378), failed to give a strong signal from T2.NP-HC lysates. Upon addition of NP366-74 peptide, B22 reactive heavy chains could be immunoprecipitated from T2.NP-HC cells. The specificity of B22 was demonstrated by its ability to capture only heavy chains from EL4 cells and not human class I molecules from untransfected T2. These results suggest that while T2.NP-HC cells are producing D^b protein, most are in an unfolded or misfolded (i.e. B22 non-reactive) state. Recovery of B22 reactivity with exogenous peptide indicates that D^b heavy chains can be correctly folded with sufficient peptide ligand.

The presence of D^b at the cell surface of T2.NP-HC cells was assessed by flow cytometry (Figure 10). T2.NP-HC transfectants stained with either B22 or 28-14-8S showed only a small increase in surface D^b relative to untransfected T2 cells, suggesting that only a small population of D^b heavy chains reaches the cell surface, the vast majority of which are properly folded (i.e. B22 reactive). This pool of class I molecules is sufficient to render T2.NP-HC cells susceptible to CTL mediated lysis, if the NP366-74 peptide is provided exogenously (see Figure 8). The
Figure 9. T2 transfectants synthesize D\textsuperscript{b} protein. EL4, untransfected T2 cells, and T2 transfected with NP-HC vector (T2.NP-HC) were radiolabeled for 30 min with [\textsuperscript{35}S]methionine, and D\textsuperscript{b} heavy chains were immunoprecipitated with various antibodies, as indicated above each lane. 1, Ab 28-14-8S, which recognizes an epitope in the \(\alpha\)3 domain of D\textsuperscript{b}. 2, conformation dependent Ab B22-249.R1, in the presence of exogenously added NP366-74 peptide. 3, B22-249.R1 without added peptide. 4, a control antibody. Reduced samples were run on a 15\% SDS-polyacrylamide gel. Arrows indicate the position of the D\textsuperscript{b} heavy chains.
Figure 10. T2.NP-HC transfectants express low amounts of surface D\(^b\). Untransfected T2 cells, T2 transfected with NP-HC vector (T2.NP-HC), or T2 transfected with WT D\(^b\) (T2.WT) were stained for D\(^b\) expression using either Ab B22 or 28-14-8S and a FITC-labeled goat anti-mouse conjugate. Antibodies used are indicated on the left of the histograms, and the cells lines on the right. T2 cells were also stained with a negative control Ab (bottom histogram) and HB-82 (top histogram), which recognizes HLA-A2 molecules.
presence of the signal sequence incorporated epitope had little effect on D\(^b\) surface expression, as T2 cells transfected with wild type D\(^b\) (T2-W\(^t\)) had comparable fluorescence to T2-NP-HC cells. Note that T2 and its transfectants exhibit substantial expression of the endogenous human class I molecule HLA-A2, which has been attributed to its ability to bind signal sequence peptides derived from abundant self proteins.

The absence of D\(^b\) heavy chains at the cell surface despite heavy chain synthesis suggested that class I molecules are being retained within the secretory pathway. Heavy chains from TAP deficient cell lines show a profound impairment in their ability to traffic to the medial Golgi, as judged by the failure to acquire resistance to Endo H digestion (379). To assess the state of heavy chain trafficking in T2.NP-HC cells, a pulse chase analysis was performed. T2 transfectants, and EL4 cells (positive control), were pulsed with \(^{35}\)S-methionine for five minutes and then chased with cold methionine for up to three hours. At various time points during the chase, the cells were lysed, and D\(^b\) heavy chains immunoprecipitated with 28-14-8S. Each sample was divided in half, and either digested overnight with Endo H or mock treated, and then analyzed by SDS-PAGE (Figure 11A,B). The positive control EL4 cells began to acquire Endo H resistance by 30 minutes, and at the conclusion of the three hour chase, virtually all heavy chains exhibited resistance to Endo H and migrated with a higher molecular weight, due to the acquisition of complex oligosaccharides. A minority of chains in the Endo H digested samples migrated in an intermediate position, between the fully digested chains and those bearing complex oligosaccharides, and likely represent D\(^b\) molecules bearing both complex and high mannose glycans. In T2.NP-HC cells, resistance to Endo H digestion is acquired much more slowly, and by the end of the chase period, a significant fraction of the D\(^b\) heavy chains still contain high mannose glycans, and only a minority of molecules achieved the mature, highest molecular weight form (note that the faint bands in the Endo H digested samples indicate that enzymatic digestion may have been incomplete). These results indicate that in T2.NP-HC cells, D\(^b\) egress from the ER or cis-Golgi is greatly impaired. This is a
Figure 11. D^b heavy chains in T2.NP-HC transfectants exhibit an immature glycosylation pattern. Control EL4 cells (A) and T2 cells transfected with the NP-HC plasmid (B) were pulsed with [35S]methionine for 5 minutes and chased with cold methionine for the indicated time periods. At each point, cells were lysed and D^b heavy chains immunoprecipitated with Ab 28-14-8S. Isolated heavy chains were incubated at 30°C overnight in the presence (+) or absence (-) of Endo H. Reduced samples were run on 10% SDS-polyacrylamide gel. The position of the Endo H-resistant (Endo H^R) and -sensitive (Endo H^S) heavy chains is indicated. Mature heavy chains, bearing complex oligosaccharides and migrating at a higher molecular weight, are denoted by an arrow on the right.
characteristic of peptide deficiency, which confirms that in the absence of TAP, the signal sequence incorporated peptide fails to reach peptide receptive class I molecules.

Discussion

The TAP complex, by virtue of its ability to translocate cytosolic peptides into the ER, represents an important component of the class I antigen processing pathway. In its absence, cells exhibit poor expression of assembled class I molecules at the cell surface and resistance to CTL mediated lysis (217). The defect imposed by TAP deficiency, however, is not absolute. HLA-A2 molecules, for example, exhibit appreciable (approximately 50% wild type) expression on the surface of T2 cells (197). This anomaly was explained by the discovery that A2 molecules from T2 cells bind a highly restricted set of peptides, some of which are derived from signal sequences (198, 199). These signal peptides are likely directed to the ER via the normal protein targeting pathway, and processed by resident ER enzymes. This alternative pathway of class I presentation is not restricted to TAP deficient cell lines, as signal sequence peptides have also been extracted from class I molecules on the surface of normal cell lines (157, 198).

The presentation of signal sequence-derived peptides addresses the larger issue of antigen processing within the ER. TAP independent presentation implies that proteolytic enzymes within the ER (in addition to signal peptidase) are capable of processing antigen and generating class I binding epitopes. The notion of peptide trimming within the ER was proposed by Rammensee and colleagues, based on observations relating the expression of class I molecules and the composition of the cellular peptide pool (216, 380). Demonstrations that the TAP complex could translocate longer than optimal class I binding peptides (143-145) suggested that a population of peptides requiring further proteolysis may exist in the ER. Recently, more
direct evidence implicating ER proteases in antigen presentation has been obtained. Elliott et al. (221) have demonstrated that T2 cells can process very large (170 aa) ER targeted protein fragments down to optimal epitopes, and present these short peptides to CTLs. T2 cells are also able to present several epitopes from the HIV-1 envelope protein targeted or retained within the ER (222, 223). Snyder and colleagues have argued that T2 cells possess ER aminopeptidases, though they observe a lack of carboxy- and endo-peptidase activity (225). This contrasts with results obtained with signal peptide epitopes, which often appear at least partially trimmed at their carboxy termini (198). Together, these data suggest the ER is a proteolytically active environment, capable of contributing to the pool of class I binding peptides.

In the expectation of this processing potential, we designed a recombinant H-2Db heavy chain with an optimal NP epitope incorporated into its signal sequence (NP-HC). The objective was to engineer an MHC-antigen coupled system which exploits the alternative (TAP independent) pathway of class I presentation. In our system, coupling is achieved by spatially and temporally linking the biosynthesis and ER translocation of heavy chain and antigenic peptide. We anticipated that the co-localization of antigen and heavy chain in the ER would provide a competitive advantage for the NP epitope, and lead to the preferential formation of class I molecules with bound NP peptide. This construct could be particularly useful for eliciting antigen specific CTL responses using a plasmid DNA immunization strategy (364).

Our in vitro experiments with NP-HC yielded three principal results. First, we demonstrated that the incorporation of NP366-74 into the amino terminal region of the D^b signal sequence does not adversely affect signal peptide function. Two different murine cell lines, P815 and BW5147, transfected with NP-HC showed high levels of cell surface D^b, as determined by flow cytometry using two D^b specific antibodies (Figure 5 A,B).

Second, the NP-HC construct renders TAP expressing cell lines susceptible to CTL mediated lysis. P815 and BW5147 cells transfected with NP-HC were lysed by D^b restricted, influenza specific CTLs at levels comparable to the positive control (Figure 7 A,B). Target cell
killing was not simply attributable to D\(^b\) expression, as cells carrying a wild type D\(^b\) heavy chain exhibited only minimal background lysis. Thus, the modified heavy chain (NP-HC) is capable of sensitizing TAP expressing cell lines to CTL specific for the incorporated epitope.

Third, the NP-HC construct failed to render TAP deficient T2 cells susceptible to CTL lysis (Figure 8). The inability of influenza specific CTLs to kill T2.NP-HC target cells was not due to a lack of gene expression. RT-PCR analysis indicated the presence of NP-HC mRNA (data not shown), and immunoprecipitation with an antibody (28-14-83) specific for the \(\alpha3\) domain of D\(^b\) indicated that transfectants were synthesizing D\(^b\) protein (Figure 9). Further analysis of the T2.NP-HC transfectants revealed additional characteristics consistent with a deficiency in peptide loading. Thus, the transfectants exhibited low surface expression of conformed class I molecules, as determined by flow cytometry (Figure 10). Immunoprecipitation showed that the heavy chains produced were mainly B22 unreactive, indicating a failure to fold or assemble into conformationally mature complexes (Figure 9). Finally, pulse/chase analysis indicated that D\(^b\) heavy chains in T2 transfectants were principally retained in an early secretory compartment, and were largely defective in their acquisition of complex oligosaccharides, as assessed by Endo H digestion (Figure 11). Taken together, these data demonstrate that the presentation of the incorporated signal sequence epitope requires the TAP transporters.

Two previous reports have offered conflicting evidence on the TAP dependence of signal peptide presentation by MHC class I molecules. One demonstrated that an HLA-A2 restricted CTL epitope incorporated into the amino terminus of an “artificial” glycoprotein signal sequence could be processed and presented by the TAP deficient cell line 721.174 (203). In contrast, another more recent study demonstrated that cell lines derived from TAP knockout mice are unable to present LCMV gp33, an immunodominant CTL epitope naturally located within a viral glycoprotein signal sequence (207). Our results with NP-HC are consistent with this latter finding, and provide strong evidence that signal peptide presentation generally requires TAP transport. Gueguen et al. (203) utilized vaccinia virus constructs as their expression vectors, and thus it is possible that their finding of TAP independent presentation
might have been influenced by alterations in membrane permeability or proteolytic activity resulting from the active virus infection. There are now several examples of virus encoded proteins which disturb unique steps within the class I presentation pathway, including the recently identified HSV encoded ICP47 gene product, which specifically interferes with TAP function (342, 343).

The TAP dependence of signal sequence presentation suggests that the cytosol, rather than the ER, provides the dominant source of class I binding epitopes. Hence, in our system, a population of cytosolic signal sequence-derived peptides must exist, despite the fact that the NP-HC encoded heavy chains are successfully targeted to the ER. There are three possibilities which could account for cytosol localized signal sequences. One is that a fraction of NP containing heavy chains are mistargeted, and remain in the cytosol, rather than reaching the ER. Since CTL lysis of target cells is exquisitely sensitive, requiring less than 200 peptide specific class I complexes (273), it is possible that this population of mistargeted heavy chains is exceedingly small or rapidly degraded. Alternatively, a cytosolic pool of signal peptides may result from peptide recycling across the ER membrane. It has been previously shown that TAP translocated peptides which fail to bind class I molecules in the ER are released back into the cytosol (220). It is feasible that such an efflux mechanism may operate on signal peptides as well, and thus, in our transfectants, provide a source of cytosol based NP epitopes. Finally, cytosolic signal sequence peptides may simply result from the natural process of signal sequence insertion into the ER membrane. It is possible that the amino terminal portion of the heavy chain signal peptide may be exposed to the cytosol (and to proteases) during translocation (214), although the balance of evidence suggests that signal peptides reside in an aqueous membrane channel which is not cytosol accessible during protein insertion (381). Interestingly, a recent study has demonstrated that signal peptides are cleaved near their hydrophobic core while associated with the membrane, and the resulting amino terminal signal peptide fragment is slowly released into the cytosol (212). This mechanism could, potentially, account for a population of cytosol-localized NP epitopes in our NP-HC system.
In summary, we have demonstrated that the presentation of an optimal CTL epitope incorporated into a class I MHC heavy chain signal sequence requires TAP transport. Thus, in contrast to our expectations, we find it is not possible to spatially and temporally couple peptide antigen and MHC using the signal sequence incorporation strategy we have devised. Our results imply that the ER is unable to effectively process a targeted signal sequence. It should be noted, however, that this conclusion is based on results obtained with only one signal sequence construct. The expansion of the NP-HC system to include other epitopes in different signal peptide configurations, in addition to the continually emerging data regarding peptide processing within the ER, will help to better assess this conclusion. These issues will be discussed more fully in Chapter 5.
Chapter 3

Creating Cytotoxic T lymphocyte Targets with
Epitope-linked $\beta_2$-microglobulin Constructs$^2$

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$^2$This chapter was published essentially in this form in Uger, R. A. and B. H. Barber (1998) J. Immunol. 160:1598
Introduction

MHC class I molecules bind intracellular peptide antigens, both foreign and self, and display them at the cell surface for scrutiny by CD8+ CTLs. A single cell may display thousands of different class I bound peptides, the vast majority of which are present in extremely low amounts (<0.1% of total) (80). Since the density of specific peptide-MHC complexes on the cell surface can determine the degree of T cell responsiveness (382-384), the ability to generate high numbers of a particular class I complex could be of great value for eliciting strong CTL responses in the context of vaccination or immunotherapy. Unfortunately, many peptides of clinical importance (e.g. tumor antigens) have relatively low MHC binding affinity and sub-optimal immunogenicity (347, 348, 350, 385).

One potential approach to augmenting the surface display and immunogenicity of an epitope is to physically couple it to its presenting MHC molecule. The peptide binding class I dimer is comprised of a polymorphic, 44 kDa membrane bound heavy chain interacting with an invariant, 12 kDa soluble light chain, β2-microglobulin (β2m) (386). In previous reports, peptide antigens have been tethered, via flexible polypeptide linkers, to the heavy chain of the mouse class I molecule Kd (361) and the human heavy chain from HLA-A2 (362). The resulting fusion proteins have been shown to elicit CTL responses when expressed in transfected cells. In addition, CTLs could be induced in vivo using a chemically modified, photoreactive peptide crosslinked to Kd complexes (358). However, there has been no attempt, to the best of our knowledge, to exploit the potential of the β2m subunit for coupling peptide antigen.

Structurally, tethering a peptide to β2m is less demanding than coupling antigen to the heavy chain, as the carboxyl end of the peptide and amino terminus of β2m are positioned relatively close together (49). Since β2m is a soluble molecule, it is amenable for use as a protein immunogen, unlike peptide-heavy chain fusions which must be cell surface bound.
Additionally, β₂m protein has been observed to act as an “adjuvant” for enhancing peptide-specific CTL responses in vivo (287), presumably by assisting in the MHC loading of peptides, a phenomenon which has been extensively investigated in vitro (282-284). Therefore, an epitope-linked β₂m molecule could provide a simple and more efficient means to enhance the formation of defined peptide-MHC complexes.

In this report, we describe two different peptide-β₂m fusion proteins. One molecule, a human β₂m (hβ₂m) with a tethered D^9 restricted influenza nucleoprotein epitope, could form CTL target structures endogenously through expression in transfected murine cell lines. A second protein, hβ₂m with a linked K^d restricted influenza nucleoprotein epitope, was expressed in E. coli and could sensitize cells for peptide-specific lysis when added to target cells exogenously. In both scenarios, target cell killing was attributable to the intact epitope-linked β₂m, and was not due to a free, uncoupled peptide. Thus, peptide-linked β₂m molecules, whether produced endogenously through the normal biosynthetic pathway or added as exogenous protein, can form specific peptide-MHC complexes which trigger CTL mediated killing. These molecules therefore offer an attractive strategy for designing new CTL priming vaccines.

Materials and Methods

Cell lines, Antibodies, and hβ₂m

P815 (H-2^d) is a murine mastocytoma (TIB-64, American Type Culture Collection, Rockville, MD). RMA-S (H-2^b) is a mutagenized and immunoselected variant of the mouse T lymphoma cell line RBL-5, and is defective in class I surface expression due to a defect in the TAP-2 gene (387, 388). RMA (H-2^b) is a mutagenized but non-selected control. RMA and RMA-S were cultured in complete medium (RPMI 1640 supplemented with 10% (v/v) FBS,
L-glutamine, antibiotics, non-essential amino acids, and sodium pyruvate). P815 cells were cultured in defined serum-free conditions (10% FBS replaced by 2% Ultroser-HY (Life Technologies, Grand Island, NY)). RMA and RMA-S transfectants were cultured in the presence of G418 (500 μg/ml). The anti-H-2D<sup>b</sup> antibodies B22-249.R1 (366, 367) and 28-14-8S (366, 368) and the anti-hβ<sub>2m</sub> antibody BBM.1 (389) have been described. Purified hβ<sub>2m</sub> was purchased from Calbiochem (San Diego, CA).

Construction of Eukaryotic Expression Plasmids

Full length hβ<sub>2m</sub> (including signal sequence) was cloned by RT-PCR from mRNA isolated from the human lymphoblastoid cell line T2 (197). Amplification was performed with the 5' primer 5'TCTAAGCTTGCCACCATGTCTCGCTCCGTG<sup>3</sup> (which includes a HindIII restriction site and Kozak sequence) and a 3' primer 5'TATTCTAGATTACATGTCTCGATCCACCA<sup>3</sup> (encoding an XbaI site). The PCR product was cloned into the HindIII and XbaI sites of the pcDNA3 plasmid (Invitrogen, San Diego, CA) and sequenced to confirm its identity. A unique XhoI site was created by site-directed mutagenesis (Transformer Site-Directed Mutagenesis Kit, Clontech, Palo Alto, CA) in order to facilitate NP(366-74)-L8-hβ<sub>2m</sub> creation.

NP(366-74)-L8-hβ<sub>2m</sub> was generated by a two-step PCR process. Initially, wild-type hβ<sub>2m</sub> was amplified using the 5' primer 5'GGAGGAGGATCCGGAGGTGGCAGCATCCAGCGTACTCCAAAGAATCAGGG<sup>3</sup>, which hybridizes to hβ<sub>2m</sub> immediately adjacent to the signal sequence, and encodes the eight amino acid linker sequence GGGSGGGS (single letter amino acid code). This PCR product was then used as a template for a subsequent PCR reaction, using the 5' primer 5'GTACTCGAGGCTGTCTCCATGAAAATATGGAGACTATGGGAGGAGGATCCCGAGGTGCC<sup>3</sup>, which hybridizes to the region encoding the glycine-serine linker, and contains, as an overhang, the sequence encoding the NP366-74 epitope and an XhoI site. Both rounds of amplification used the abovementioned 3' primer, which encodes an
Xbal site. The Xhol/Xbal fragment of wild type h\(\beta_2\)m was removed and replaced with the similarly digested PCR product, producing NP(366-74)-L8-h\(\beta_2\)m (see Figure 12A).

**Construction of Bacterial Expression Plasmids**

Wild type h\(\beta_2\)m was generated by PCR using the \(\beta_2\)m.phn1 plasmid (a kind gift from Dr. D. Wiley) as a template, using oligonucleotide primers \(^5\)TATCATATGATCCAGCGTACTCTCCA\(^3\) (encoding an Ndel site) and \(^5\)TATGGATCCITACATGTCTCGATCCCCA\(^3\) (encoding a BamHI site). The PCR product was initially cloned into the pNoTA/T7 vector (5prime-3prime, Boulder, CO), sequenced, and cloned, by Ndel/BamHI digest, into the pET-12a vector (Novagen, Madison, WI).

NP(147-155)-L12-h\(\beta_2\)m was constructed in a two-step PCR process. Initially, the \(\beta_2\)m.phn1 plasmid was amplified with the oligonucleotides \(^5\)GGAGGAGGAGGATCTGGA-GGAGGAGGATCTGGAGGAATCCAGCTCCAAAGATTCAUGTT\(^3\) (encoding the glycine/serine linker and hybridizing to the first 27 bases of h\(\beta_2\)m) and \(^5\)TATGGATCCITACATGTCTCGATCCCCA\(^3\) (encoding a BamHI site). This PCR product was then amplified in a second reaction, using the forward primer \(^5\)CATATGACCTACCCGATCCCGTGCTGTTGGAGGAGGAGGATCTGGAGGA GGAGGATCT\(^3\) (encoding the NP147-55 epitope and an Ndel site as an overhang) and the reverse primer from the first amplification. The final PCR product was cloned into the pNoTA/T7 vector (5prime-3prime), sequenced, and cloned, by Ndel/BamHI digest, into the pET-12a vector (Novagen).
**Transfections**

20 μg of hβ2m or NP(366-74)-L8-hβ2m were linearized with *PvuI* and added to 1.5 x 10⁷ cells (washed twice in serum-free medium) in a 0.4 cm cuvette and pulsed at either 300 V, 900 μF (for RMA) or 250 V, 500 μF (for RMA-S) with a Gene Pulser apparatus (Bio-Rad, Richmond, CA). Cells were then incubated for 10 min at room temperature, 1 ml of complete medium was added, and cells were incubated for an additional 20 min. Transfectants were cultured for two days in normal medium before being transferred to G418 selection medium.

**Flow Cytometry**

10⁶ cells were incubated on ice for 1 hour with 2 μg of primary antibody (in PBS containing 1% BSA and 0.1% sodium azide). Cells were then washed twice and incubated for 1 hour with a goat anti-mouse FITC conjugate (Sigma Chemical Co., St. Louis, MO). Cells were washed three times and resuspended in 0.5 ml PBS (0.1% sodium azide) and paraformaldehyde fixative was added to a final concentration of 1% (v/v). Samples were analyzed on a Coulter flow cytometer (Hialeah, FL).

**Immunoprecipitation**

Cells (2.5 x 10⁷) were preincubated for 30 minutes in methionine-free medium and then pulsed for 15 minutes with [35S]methionine. Cells were lysed and immunoprecipitations performed as described previously (390). Immunoisolated proteins were run on a 15% reduced SDS-polyacrylamide gel.
CTL Assays on Transfectants

CTLs were primed by immunizing mice with 200 hemagglutinin units of the X-31 strain of influenza virus, which possesses the nucleoprotein gene from the A/PR/8/34 virus (372). At least 4 weeks post infection, spleen cells were harvested and either restimulated for five days with virus infected, irradiated spleen cells (at a 10:1 responder to stimulator ratio) or restimulated for seven days in the presence of 2 μg/ml NP366-74 peptide (Alberta Peptide Institute, Alberta, Canada). The restimulated effectors were then used in a standard $^{51}$Cr release assay. $10^6$ target cells were labeled for 1.5 h with 100 μCi of Na$_2$$^{51}$CrO$_4$ (Amersham, Arlington Heights, IL) and washed repeatedly. When necessary, targets were incubated with peptide (>100 nM) for at least 30 minutes at room temperature. $10^4$ cells were dispensed into 96 well plates, titrated effectors added, and the plates incubated at 37°C for 4 hours. Supernatants were harvested using a filter system (Skatron, Lier, Norway) and radioactivity measure by gamma-counting (Beckman, Fullerton, CA). Percent specific lysis was calculated, using the mean of triplicate samples, as: 100 X [(experimental cpm - spontaneous cpm)/[(maximum cpm - spontaneous cpm)]. Spontaneous cpm values were determined by incubating target cells alone in medium, and maximum values by lysis of targets in 1% Triton X-100 (v/v).

Peptide Elution

$10^7$ cells were incubated in a peptide stripping buffer (0.13 M Citric Acid, 66 mM Na$_2$HPO$_4$, 150 mM NaCl, 17 μg/ml phenol red, pH 3.2) (391, 392) for one min at room temperature. Cells were then pelleted, supernatants recovered, and neutralized by dropwise addition of NaOH. Peptides were filtered and stored at -20°C. The acid treatment had no effect on cell viability, as assessed by trypan blue staining. As a positive control, RMA cells were first cultured overnight at 37°C with 1 μg/ml NP366-74, then washed extensively in PBS, prior to extraction.
Acid-eluted fractions were tested in a standard CTL assay, essentially as described above, with the following modifications. After $^{51}$Cr labeling and washing, target cells (RMA) were resuspended at 2 x $10^5$/ml and h$\beta_2$m was added to a final concentration of 5 $\mu$g/ml. The addition of exogenous h$\beta_2$m was intended to compete out any peptide-linked h$\beta_2$m molecules which may have been acid stripped. 750 $\mu$l of cells were transferred to 750 $\mu$l of medium containing 10-fold serial dilutions of the acid-eluted material (starting at 1:8 dilution), and incubated at room temperature for 20 min. 100 $\mu$l ($10^4$) cells were then plated out in 96-well plates which contained 4 x $10^5$ effector cells/well, thus achieving a 40:1 effector to target ratio.

**Preparation of Bacterial Lysates**

Wild type h$\beta_2$m and NP(147-55)-L12-h$\beta_2$m plasmids (in pET vectors) were transformed into the bacterial strain BL21(DE3)plysS (Novagen). Protein expression and lysate preparation was performed essentially as described (393). Briefly, bacterial cultures were grown in the presence of ampicillin (100 $\mu$g/ml) and chloramphenicol (34 $\mu$g/ml) until $OD_{600}$=0.4 to 0.8. Cultures were then induced by addition of isopropyl $\beta$-D-thiogalactopyranoside (IPTG) to a final concentration of 0.4 mM, and grown for 2-3 hours. Bacteria were harvested by centrifugation and the cell pellet resuspended in 10 mM Tris, pH 8, supplemented with 1 mM EDTA, 20 $\mu$g/ml DNase, 20 $\mu$g/ml RNase, and 50 $\mu$g/ml phenylmethylsulfonylfluoride. Cells were lysed by repeated cycles of freeze-thaw, with or without sonication. Lysates were centrifuged (10,000 x g) for 20 min, and the pellet washed with 10 mM Tris, pH 8. The pellet was then solubilized in 1/20 vol 8M urea/100 mM Tris, pH 8 and centrifuged at 100,000 x g for 1 hour at 4°C. The h$\beta_2$m protein in the supernatant was then refolded by dialysis against 10 mM Tris, pH 7, and stored at -70°C.
Western Blotting

Lysates were run on 10-20% gradient SDS-PAGE gels and transferred to nitrocellulose (Trans-Blot, Bio-Rad). Filters were probed with an anti-\(h\beta_2\)m rabbit serum (1:500 dilution) and detected with a goat anti-rabbit HRP conjugate (Sigma, 1:1000 dilution) using the ECL Western Blotting Detection Reagents kit (Amersham) according to the manufacturer's protocols.

Quantitating \(h\beta_2\)m by Competitive ELISA

96-well plates were coated with 3 \(\mu\)g/ml \(h\beta_2\)m in PBS for at least 1 hour at 37°C, and then blocked with 5% milk/PBS for 2 hours at 37°C. In parallel, a competitor plate was set up, in which 75 \(\mu\)l of 10 \(\mu\)g/ml BBM1 antibody was incubated with 75 \(\mu\)l of diluted inhibitors (either purified \(h\beta_2\)m or bacterial lysates, in PBS/1% BSA) at 37°C. Both plates were subsequently washed in PBS. 100 \(\mu\)l/well was transferred from the competitor plate to the blocked, \(h\beta_2\)m plate and incubated for 1 hour at 37°C. After washing, 100 \(\mu\)l/well of 1:1000 diluted alkaline phosphatase goat anti-mouse conjugate (Cedarlane, Hornby, Ontario) was added, and the plate incubated for an additional 1 hour at 37°C. The reaction was developed by adding 100 \(\mu\)l/well of 1 mg/ml p-nitrophenolphosphate substrate (Sigma). Absorbance was read at 405 nM.

CTL Assays Using Bacterial Lysates

CTLs from X-31 immunized BALB/c mice were restimulated in vitro for 7 days by culturing cells in the presence of 2 \(\mu\)g/ml NP147-55 peptide (Alberta Peptide Institute). Target cells (P815) were labeled with \(^{51}\)Cr as described above, except labeling was done using a defined serum replacement (Ultroser-Hy, Life Technologies) which is free of bovine \(\beta_2\)m. Targets were incubated with various dilutions of the NP(147-55)-L12-\(h\beta_2\)m lysate for 1 hour.
at 37°C, washed once, plated and assayed as described above. For hβ₂m inhibition assays, target cells were incubated with wild type hβ₂m bacterial lysate (1:20 final concentration) for 45 min at room temperature prior to treatment with the NP(147-55)-L12-hβ₂m lysate.

Results

Design of Mammalian Expression Constructs

A mammalian expression vector was constructed which encodes a recombinant hβ₂m with covalently attached peptide antigen (Figure 12A). The hβ₂m linked antigen, NP366-74, is an optimal H-2Dβ restricted immunodominant epitope from influenza virus nucleoprotein (374). It was connected to the amino terminus of the mature domain of hβ₂m via an eight amino acid polypeptide linker (GGGSGGGS, single letter amino acid code). The epitope and linker were inserted immediately following the hβ₂m signal sequence, and thus the recombinant molecule should be targeted to the endoplasmic reticulum for cell surface expression. Note that this peptide-hβ₂m fusion, designated NP(366-74)-L8-hβ₂m, is heterologous in nature, utilizing a mouse class I binding peptide and β₂m of human origin. The mouse influenza virus model offers a convenient, well characterized system in which CTL target structure formation can be readily assessed, while the human β₂m allows for the recombinant fusion protein to be monitored amidst a background of mouse β₂m. In addition, the hβ₂m subunit is known to interact with mouse class I heavy chains with slightly higher affinity than mouse β₂m (278), potentially enhancing the effectiveness of NP(366-74)-L8-hβ₂m.

NP(366-74)-L8-hβ₂m, and a control construct, wild type hβ₂m (no tethered peptide), were cloned into a mammalian expression vector (pcDNA3) which utilizes the cytomegalovirus (CMV) promoter for gene expression. The vectors were transfected into murine cell lines, and
Figure 12. Design of peptide-hβ2m constructs. A, NP(366-74)-L8-hβ2m. The H-2D\(^b\) restricted influenza NP epitope NP366-74 and an eight amino acid glycine-serine linker were inserted between the hβ2m signal sequence and the mature domain. The construct was cloned into the mammalian expression vector pcDNA3. B, NP(147-55)-L12-hβ2m. The H-2D\(^d\) restricted influenza NP epitope NP147-55 and a 12 amino acid glycine-serine linker were inserted upstream of the hβ2m mature domain. Note that an initiating methionine residue was added to the amino terminus of the epitope. The DNA construct was cloned into the pET-12a bacterial expression vector.
the ability of NP(366-74)-L8-\(h\beta_2m\) molecule to form appropriate CTL target structures was assessed.

**Expression and Target Structure Formation in NP(366-74)-L8-\(h\beta_2m\) Transfectants**

NP(366-74)-L8-\(h\beta_2m\), and the control wild type \(h\beta_2m\) vector were initially transfected into the murine cell line RMA (H-2\(^b\)). Stable, drug resistant lines were analyzed for expression of the transfected \(h\beta_2m\) gene product at the cell surface by flow cytometry, staining with a monoclonal antibody (BBM.1) specific for \(h\beta_2m\). As shown in Figure 13A, NP(366-74)-L8-\(h\beta_2m\) transfectants showed only a modest increase in surface staining (2-3 fold higher mean fluorescence) than untransfected RMA cells. This lower than expected staining cannot be attributed to negative effects from the presence of the epitope and/or linker, since RMA cells transfected with a wild type \(h\beta_2m\) encoding vector showed only a similar slight increase in mean fluorescence. Indeed, the low expression of transfected \(h\beta_2m\) at the cell surface is likely attributable to an overall level of low protein production, as determined by metabolic labeling and immunoprecipitation (Figure 14).

Vectors were also transfected into the cell line RMA-S (387), an immunoselected relative of RMA, which is defective in the TAP transporter, and thus cannot supply cytosolic peptides to assembling class I molecules in the endoplasmic reticulum (136, 388). Since the NP366-74 epitope and linked \(h\beta_2m\) should be translocated into the ER by virtue of its signal sequence in a TAP independent manner, one would expect RMA-S transfectants to appear similar to the TAP expressing RMA cells. Indeed, flow cytometry analysis with the BBM.1 antibody reveals the presence of small amounts of \(h\beta_2m\) on the surface of RMA-S.NP(366-74)-L8-\(h\beta_2m\) transfectants, although at a level somewhat lower than seen on the RMA transfectants (Figure 13B).
Figure 13. RMA and RMA-S transfectants express low levels of hβ₂m at the cell surface. A, untransfected RMA, RMA transfected with NP(366-74)-L8-hβ₂m (RMA.NP(366-74)-L8-hβ₂m) and RMA transfected with hβ₂m (RMA.hβ₂m) were stained for hβ₂m expression using the Ab BBM1. B, RMA-S, RMA-S transfected with NP(366-74)-L8-hβ₂m (RMA-S.NP(366-74)-L8-hβ₂m) and RMA-S transfected with hβ₂m were similarly stained with BBM1. C, cells from B were stained with B22, an Ab specific for folded H-2D<sup>b</sup> molecules.
Figure 14. hβ₂m protein is not detectable by immunoprecipitation in RMA transfectants. Cell lines (indicated below the gel) were radiolabeled with [³⁵S]methionine for 15 minutes, lysed, and immunoprecipitated with the following antibodies (indicated at the top of each lane): BBM1 (anti-hβ₂m), control antibody (anti-HLA-A2), W6/32 (anti-HLA), and 28-14-8S (anti-Dᵇ). C1R-B27 is a human B lymphoblastoid cell line transfected with the HLA-B27 gene which expresses hβ₂m and thus serves as a positive control. Immunoisolated proteins were separated on a 15% reduced SDS-polyacrylamide gel. The arrow on the left indicates the position of a 14.4 kD molecular weight marker. The 28-14-8S bands which comigrate with the BBM1 band likely represent mouse β₂m, which is precipitated as part of the Dᵇ complex.
Since RMA-S cells cannot supply peptides to nascent class I molecules, only low amounts of properly folded class I molecules are expressed at the cell surface (394). Thus, expression of transfected NP(366-74)-L8-hβ2m can also be assessed by monitoring the level of H-2D\textsuperscript{b} cell surface expression with the conformation sensitive antibody B22.249.R1 (B22). As shown in Figure 13C, RMA-S cells transfected with NP(366-74)-L8-hβ2m showed only a very slight increase in B22 staining. Thus, the transfectants appear to be producing only small amounts of the NP epitope. This is consistent with the above results, and suggests that there is low-level expression of the transfected gene product.

Despite low cell surface expression of NP(366-74)-L8-hβ2m, RMA and RMA-S transfectants are capable of efficiently generating a CTL target structure recognizable by NP366-74 specific CTLs. In a standard cytotoxicity assay using CTL effectors from influenza virus immunized mice, RMA and RMA-S cells transfected with NP(366-74)-L8-hβ2m were lysed at levels comparable to the positive control, untransfected cells pulsed with free peptide (Figure 15 A,B). The killing was specific for the tethered epitope, as RMA and RMA-S cells transfected with wild type hβ2m exhibited only background lysis, similar to untreated, non-transfected RMA and RMA-S. Thus, transfection of mouse cell lines with a vector encoding hβ2m with covalently attached class I binding peptide results in target structure generation and cell lysis. The observation that RMA-S transfectants can generate recognizable CTL target structures indicates that the presentation of the tethered NP antigen does not require TAP transport, which is consistent with the antigen being covalently linked to the β2m subunit (see below). The high level of specific lysis in the context of low transfected gene product expression suggests a potent effect from the NP(366-74)-L8-hβ2m molecule.
Figure 15. RMA and RMA-S cells transfected with NP(366-74)-L8-hβ2m are lysed by NP366-74-specific CTL. Standard 4 hour chromium release assays were performed using CTLs derived from influenza virus-immunized C57BL/6 mice and restimulated in vitro as described in Materials and Methods. A, RMA cells pulsed with NP366-74 peptide (positive control), untreated RMA (negative control), RMA transfected with wt hβ2m (RMA.hβ2m), and RMA transfected with NP(366-74)-L8-hβ2m (RMA.NP(366-74)-L8-hβ2m) are used as target cells. B, RMA-S cells pulsed with NP366-74 peptide (positive control), untreated RMA-S (negative control), RMA-S transfected with wt hβ2m (RMA-S.hβ2m), and RMA-S transfected with NP(366-74)-L8-hβ2m (RMA-S.NP(366-74)-L8-hβ2m) are used as target cells.
Assessing the Integrity of the Polypeptide Linker by Acid Elution of Free Peptides

While the observation that RMA-S cells transfected with NP(366-74)-L8-h\(\beta\)\(_2\)m are sensitized for CTL lysis indicates that the NP epitope cannot originate from the cytosol, it does not conclusively demonstrate that the peptide antigen is remaining covalently tethered to the h\(\beta\)\(_2\)m subunit throughout the presentation pathway. Indeed, it is possible that the polypeptide linker is cleaved, perhaps during assembly of the class I complex in the endoplasmic reticulum or at the cell surface, and the resulting free peptide is mediating the observed CTL killing. To address this issue, we attempted to isolate free NP peptide from the surface of RMA.NP(366-74)-L8-h\(\beta\)\(_2\)m transfectants by acid elution. In this experiment, transfectants were incubated briefly in a low pH buffer which is sufficient to cause dissociation of surface class I molecules, yet gentle enough not to affect cell integrity. Free NP peptide was detected by titrating the acid eluted fraction in a standard CTL assay. As a positive control, NP peptide was eluted from RMA cells which had been pre-incubated with NP366-74 (and washed extensively) prior to elution. The results are shown in Figure 16. There was no detectable NP peptide in the material acid eluted from NP(366-74)-L8-h\(\beta\)\(_2\)m transfectants, or from the negative control, untransfected RMA cells. In contrast, peptides acid eluted from the positive control cells, RMA pre-pulsed with NP366-74, were capable of sensitizing target cells for lysis over an extremely large dilution range, titrating out near \(10^5\). Thus, there is almost 100,000 fold less elutable free peptide on RMA.NP(366-74)-L8-h\(\beta\)\(_2\)m transfectants compared to peptide-pulsed RMA cells. Since we expect approximately \(10^4\) NP peptides on each peptide-pulsed RMA cell (10\% occupancy of roughly \(10^5\) surface class I molecules (273)), there must be less than 1 free NP peptide per NP(366-74)-L8-h\(\beta\)\(_2\)m transfectant. Hence, it is reasonable to conclude that target cell lysis is being mediated through peptide-linked h\(\beta\)\(_2\)m molecules, and not through the action of a free, uncoupled peptide.
Figure 16. Free NP peptide is not detected in acid extracts from RMA.NP(366-74)-L8-hβ2m cells. Peptides bound to surface class I MHC molecules were eluted from $10^7$ RMA.NP(366-74)-L8-hβ2m transfectants using gentle acid extraction. The acid eluted material was then titrated for CTL sensitization on $^{51}$Cr labeled RMA targets cells, using bulk cultures of NP366-74-specific CTLs, in a standard 4 hour release assay, at a 40:1 E:T ratio. hβ2m (5 μg/ml) was included in the assay to compete out any peptide-linked hβ2m molecules that may have been coincidentally acid extracted. Peptides were similarly extracted from untreated RMA cells and RMA cells cultured overnight with 1 μg/ml NP366-74 peptide (RMA + NP). The data shown represent one of four independent experiments.
Bacterial Expression of a Peptide Tethered \( h\beta_2m \) Molecule

In a related strategy, we have engineered a recombinant \( h\beta_2m \) molecule with covalently attached class I binding peptide for expression in \( E. \ coli \). This molecule, \( \text{NP}(147-55)-\text{L12-}h\beta_2m \), has the \( K^d \) restricted influenza nucleoprotein epitope (NP147-55) (374) tethered to \( h\beta_2m \) via a 12 amino acid glycine/serine linker (Figure 12B). Note that this molecule does not possess a signal sequence, and thus should be localized to the bacterial cytoplasm. The epitope is preceded by a methionine residue, in order to initiate mRNA translation. This methionine will likely be removed shortly after protein synthesis (377), and therefore is not expected to be incorporated into the NP epitope.

\( \text{NP}(147-55)-\text{L12-}h\beta_2m \) and a wild type \( h\beta_2m \) constructs were cloned into the pET-12a bacterial expression vector (Novagen), which utilizes an inducible T7 RNA polymerase promoter. \( E. \ coli \) transformants were grown, induced, and lysates prepared as described in the Materials and Methods section. Western Blot analysis of lysates using an anti-\( h\beta_2m \) rabbit serum revealed a single band from the wild type \( h\beta_2m \) transformants which comigrates with a standard \( h\beta_2m \) (Figure 17). The \( \text{NP}(147-55)-\text{L12-}h\beta_2m \) transformant lysate shows a single reactive band approximately 2 kDa larger, consistent with the additional mass from the linker and epitope. These bands are not visible in a control lysate, prepared from bacteria harboring an irrelevant gene (\( \beta \)-galactosidase). There are no smaller bands detected by the anti-\( h\beta_2m \) serum in the \( \text{NP}(147-55)-\text{L12-}h\beta_2m \) lysate, indicating that the epitope tethered \( h\beta_2m \) molecule is not being significantly degraded. Typically, 0.5 - 1 mg of \( \text{NP}(147-55)-\text{L12-}h\beta_2m \) protein is obtained per liter of bacteria culture, as determined by competitive ELISA analysis (Figure 18). The yield of wild type \( h\beta_2m \) is dramatically higher (approximately 100 mg per liter) for reasons that are currently unknown.
Figure 17. Western blot analysis of bacterial lysates. Lysates were prepared from BL21(DE3)plysS bacteria transformed with pET vectors encoding wt hβ₂m, NP(147-55)-L12-hβ₂m, or a control (β-galactosidase) gene as described in Materials and Methods. Samples were run on a 10-20% gradient SDS-PAGE gel, transferred to nitrocellulose, and probed with an anti-hβ₂m rabbit serum. Purified, commercially available hβ₂m (10 μg) (standard) was run as a comparison. The NP(147-55)-L12-hβ₂m and control lysates were run undiluted; the wt hβ₂m sample was diluted 1:50 before loading. The arrow to the left indicates the migration of a 14.4 kDa standard.
Figure 18. Quantitation of hβ₂m in bacterial lysates by competitive ELISA. The anti-hβ₂m Ab BBM1 was pre-incubated with various concentrations of soluble inhibitors, and subsequent Ab binding to purified, plate immobilized hβ₂m was quantitated using an alkaline phosphatase-conjugated goat anti-mouse reagent. A, standard curve using purified hβ₂m as the inhibitor. The dotted lines indicate the concentration of soluble hβ₂m (0.5 μg/ml) which results in 50% inhibition of BBM1 binding. B, inhibition by WT and NP(147-55)-L12-hβ₂m lysates. The WT lysate exhibits 50% inhibition at a dilution factor of 5000, whereas the NP(147-55)-L12-hβ₂m shows half-maximal inhibition at a dilution of 40. From the standard curve in A, this corresponds to actual hβ₂m concentrations of 2.5 mg/ml in the WT lysate and 20 μg/ml in the NP(147-55)-L12-hβ₂m lysate. Since each liter of culture produces 50 ml of lysate, the yields of WT and NP(147-55)-L12-hβ₂m are 125 mg/l and 1 mg/l, respectively.
CTL Sensitization by Bacterially Produced NP(147-55)-L12-hβ2,m

The ability of recombinant NP(147-55)-L12-hβ2,m produced in E. coli to form recognizable CTL target structures was assessed by a simple cytotoxicity assay. P815 cells (H-2d) grown in defined serum free medium (to exclude any competition from bovine β2,m) were pre-treated with unpurified lysate from NP(147-55)-L12-hβ2,m transformants prior to incubation with CTL effectors derived from influenza infected BALB/c mice. As shown in Figure 19, exposing P815 cells to the NP(147-55)-L12-hβ2,m lysate resulted in a high level of target cell lysis, comparable to the killing obtained by pulsing target cells with free NP147-55 peptide. This cytotoxic effect was specific for the epitope, as incubation with lysates from wild type hβ2,m or control transformants failed to sensitize targets. The NP(147-55)-L12-hβ2,m lysate was remarkably potent. Less than 1 ng/ml of NP(147-55)-L12-hβ2,m protein was capable of sensitizing target cells for CTL killing (Figure 20). Thus, a bacterially expressed, recombinant hβ2,m with covalently attached peptide is capable of efficiently sensitizing target cells for lysis when added exogenously as a component of an unpurified lysate.

Inhibition of NP(147-55)-L12-hβ2,m Activity with Wild Type hβ2,m

As in the case with the mammalian expressed NP(366-74)-L8-hβ2,m construct, we wished to determine whether the biological activity observed with the NP(147-55)-L12-hβ2,m protein was truly due to a peptide-linked hβ2,m structure, or was a result of a free peptide generated through cleavage of the glycine/serine linker. Although the NP(147-55)-L12-hβ2,m lysate was extensively dialyzed during preparation, thus removing any contaminating free peptide, it is possible that the recombinant protein was being degraded during the course of the in vitro CTL assay. To address this issue, we attempted to inhibit the activity of the NP(147-55)-L12-hβ2,m
Figure 19. NP(147-55)-L12-hβ2m protein from bacterial lysates sensitizes cells for CTL mediated lysis. 

Figure 19. NP(147-55)-L12-hβ2m protein from bacterial lysates sensitizes cells for CTL mediated lysis. $^{51}$Cr-labeled P815 cells were incubated at 37°C for 1 hour with either 100 nM NP147-55 peptide (positive control), NP(147-55)-L12-hβ2m bacterial transformant lysate (1:250 final concentration) or wt hβ2m transformant lysate (WT hβ2m, 1:50 final concentration), or a control lysate (from bacteria carrying the β-galactosidase gene, 1:50 final concentration). The lysate dilutions were adjusted such that equivalent volumes of induced bacteria culture (= 1 μl) were added. After pulsing, target cells were washed once and then incubated in a standard 4 hour chromium release assay using CTLs derived from influenza virus-immunized BALB/c mice. The entire assay was performed using a defined serum substitute devoid of any bovine β2m.
Figure 20. Target cells are sensitized for CTL lysis by less than 1 ng/ml NP(147-55)-L12-hβ2m. 

\[ ^{31}\text{Cr-labeled P815 cells (grown in β2m-free medium) were pulsed with titrated amounts of NP(147-55)-L12-hβ2m lysate, or 200 nM NP147-55 peptide (positive control) or a control lysate (1:50 final concentration, negative control). Target cells were then incubated in a standard 4 hour chromium release assay using NP147-55-specific CTLs. Note that the most dilute NP(147-55)-L12-hβ2m sample (0.6 ng/ml) corresponds to a concentration of approximately 50 pM.} \]
lysate with wild type hβ2m protein. If target structure formation is a result of an NP tethered hβ2m molecule interacting with cell surface Kd heavy chains, then the biological activity of NP(147-55)-L12-hβ2m should be diminished by providing an excess of competitor wild type hβ2m. Alternatively, if a free peptide is mediating CTL sensitization, then the presence of additional wild type hβ2m should have no inhibitory effect. In the experiment shown in Figure 21, P815 target cells were pre-incubated with wild type hβ2m lysate, as a source of competitor hβ2m, prior to exposure to low concentrations of the NP(147-55)-L12-hβ2m lysate. Pre-treatment of target cells with the wild type lysate had a dramatic inhibitory effect on the activity of NP(147-55)-L12-hβ2m protein. In contrast, when P815 cells were pre-treated with wild type lysate and then pulsed with free NP147-55 peptide, no inhibitory effect was observed (the "inhibitory" hβ2m actually resulted in slightly higher lysis of peptide pulsed targets). Similarly, inhibition of low concentrations (< 1 ng/ml) of NP(147-55)-L12-hβ2m could also be accomplished using 20 μg/ml purified, commercially available hβ2m (data not shown). Note that the inhibition of NP(147-55)-L12-hβ2m protein by wild type hβ2m can only be observed at very low concentrations of the epitope tethered hβ2m, presumably because of the potent activity of this recombinant molecule.

The inhibition of NP(147-55)-L12-hβ2m by wild type hβ2m thus demonstrates that CTL target structure formation is mediated by a peptide linked hβ2m structure, and is not simply the result of a free peptide arising from cleavage of the linker or degradation of the recombinant protein. This finding is consistent with our inability to detect free NP peptide at the surface of NP(366-74)-L8-hβ2m transfectants (Figure 16), and suggests that the peptide-linked hβ2m molecules are relatively resistant to degradation.
Figure 21. NP(147-55)-L12-hβ2m mediated lysis is inhibited by wild type hβ2m. 51Cr-labeled P815 cells were left untreated or pulsed with lysate (1:20 final concentration) from wild type hβ2m bacteria transformants (+ WT) for 45 minutes at room temperature. Cells were then incubated with either 100 nM NP147-55 peptide or NP(147-55)-L12-hβ2m lysate (1:156,250 final concentration, corresponding to <0.002 µl of bacteria culture) for 1 hour at 37°C. Targets were washed and incubated with NP147-55-specific CTLs in a standard 4 hour chromium release assay.
Discussion

The ability to generate a robust, peptide-specific CTL response is a central issue in designing successful vaccine or immunotherapy strategies to combat many viral and neoplastic diseases. Since the degree of CTL responsiveness can be modulated by the number of peptide-MHC complexes displayed at the cell surface (382-384), it is desirable to express optimally high levels of a given CTL target structure. This, however, may be difficult to accomplish with many naturally occurring, clinically relevant epitopes, which can display low MHC binding affinity and sub-optimal immunogenicity (347, 348, 350, 385). Hence, a mechanism to enhance the cell surface expression and immunogenicity of a peptide would be of great value.

Physically coupling a peptide to its presenting class I MHC molecule is one such mechanism that has been investigated. The structural and biochemical data available on peptide-MHC interactions have demonstrated the importance of free peptide-termini in binding to class I molecules (40, 42, 46, 395), and have suggested that a linked peptide may not bind stably to MHC. Nevertheless, optimal binding class I peptides have been successfully tethered, via their carboxyl termini, to the amino termini of both mouse and human class I heavy chains (361, 362). These recombinant molecules were capable of presenting their linked epitope and inducing CTLs when expressed in transfected cell lines. While these studies demonstrate the feasibility of such an approach, their applications may be limited by the requirement to express the peptide-MHC fusions in transfectants. Alternatively, covalent class I-peptide complexes have been formed using a modified peptide which contains a photoreactive chemical crosslinker (358). This strategy, however, requires that the chemical modifications not disturb peptide binding to MHC, and can result in a CTL response which is primarily specific for the altered peptide.

In this report, we describe an alternative, and as yet unexplored approach: tethering peptides to the β2m subunit. Structurally, this linkage should prove less demanding than a
peptide-heavy chain fusion, since the carboxyl terminus of a class I bound peptide is located significantly closer to the amino terminus of β₄m than to the amino terminus of the heavy chain (46, 49). As β₄m is a small soluble protein, a peptide-β₄m fusion molecule could be utilized as a soluble protein immunogen. In this context, it is noteworthy that β₄m protein has been observed to augment epitope specific CTL responses in vivo when coinjected with peptide (287). This adjuvant effect was attributed to the ability of β₄m to enhance peptide loading of surface class I molecules, a well defined in vitro phenomenon (282-284). We anticipated that a more potent immunogen could be generated if the peptide was covalently linked to β₄m, thereby restricting its diffusion and creating a molecule which contains two high affinity binding sites for class I heavy chains.

Towards this goal, we have created two unique peptide-linked β₄m molecules, which utilize different presentation pathways. The first, NP(366-74)-L8-hβ₂m, is a recombinant hβ₂m which possesses the H-2Dᵇ restricted influenza nucleoprotein epitope NP366-74 tethered to its amino terminus via an 8 amino acid polypeptide linker. This molecule was expressed in transfected murine cell lines, and would presumably function within the normal, endogenous class I biosynthetic pathway. Despite only modest cell surface expression (Figure 13), which we attribute to a generally low level of protein production (Figure 14), NP(366-74)-L8-hβ₂m transfectants, but not wild type hβ₂m transfectants, were efficiently lysed by NP366-74 specific CTLs (Figure 15). In this regard, it is important to note that other studies have indicated that only small numbers of peptide-class I MHC complexes (ranging from several hundred to less than ten) are required to sensitize a cell for lysis (273, 396, 397).

In order to demonstrate that the observed lysis was due to a peptide-linked β₄m structure, and not a free peptide resulting from protein degradation, acid elution experiments were performed (Figure 16). We were unable to liberate any detectable NP peptide from the surface of NP(366-74)-L8-hβ₂m transfectants, in contrast to NP peptide-pulsed control cells, from which the acid eluted material could sensitize targets for CTL lysis over a 10² fold dilution.
range. Hence, the biological activity of NP(366-74)-L8-hβ2m molecules in the mouse transfectants is attributable to an antigenic peptide physically coupled to the hβ2m subunit.

The second recombinant molecule, NP(147-55)-L12-hβ2m, couples the H-2Kd restricted influenza nucleoprotein epitope NP147-55 to the amino terminus of hβ2m using a 12 residue glycine-serine linker (Figure 12B). Rather than expressing this peptide-β2m fusion molecule endogenously in transfected cell lines, the recombinant protein was produced in E. coli to permit CTL target structure formation through an exogenous pathway. Using an IPTG inducible expression system, NP(147-55)-L12-hβ2m protein was produced in BL21(DE3)plysS transformants. This NP(147-55)-L12-hβ2m molecule could be detected as a single band when bacterial lysates were analyzed by Western blot using an anti-hβ2m rabbit serum (Figure 17). When added to H-2d expressing P815 target cells exogenously, the unpurified NP(147-55)-L12-hβ2m lysate resulted in CTL mediated lysis (Figure 19). Target cell sensitization by the NP(147-55)-L12-hβ2m lysate could be inhibited by pre-treating cells with an excess of competitor wild type hβ2m lysate (Figure 21), indicating that the activity of the NP(147-55)-L12-hβ2m lysate is a result of a β2m-linked epitope, not a free peptide. This is consistent with the abovementioned observation that the mammalian expressed NP(366-74)-L8-hβ2m molecule also mediates CTL target structure formation via a linked peptide-hβ2m structure.

Our results have indicated that target cells can be rendered susceptible to CTL mediated killing by exposure to a peptide-hβ2m linked molecule. This strategy for forming defined class I MHC complexes demonstrates versatility in two respects. First, the route of presentation can be either endogenous, through expression of a peptide-hβ2m fusion in transfected cells, or exogenous, via treatment of cells with bacterially-derived peptide-hβ2m protein. It is noteworthy that both routes of presentation appear quite potent. Transfected cell lines are lysed despite low levels of transfected gene expression (as judged by flow cytometry) and lysate treated target cells are sensitized by extremely small quantities (sub-nanomolar) of recombinant protein. Second, the strategy has proven successful for two different epitopes (influenza
NP366-74 and NP147-55), restricted through different class I molecules (D\text{b} and K\text{d}), utilizing two different sizes of polypeptide linkers (8 and 12 residues). This suggests that a general peptide-\(\beta_2m\) fusion strategy could be extended to a variety of class I binding peptides.

In summary, we have demonstrated that CTL target structure formation can be accomplished \textit{in vitro} by physically coupling peptide antigen to the \(\beta_2m\) subunit, either in the context of DNA transfection, or as an exogenous, bacterially-derived protein. We are currently investigating the ability of peptide-\(\beta_2m\) molecules to elicit primary CTL responses \textit{in vivo}. Since \(\beta_2m\) has been observed to augment otherwise weak CTL responses to peptide immunogens (287), a peptide-linked \(\beta_2m\) strategy may offer a safe, convenient and effective method of inducing CTL immunity to desired class I restricted epitopes. This may be achieved by plasmid DNA immunization (398) with an appropriate epitope-linked \(\beta_2m\) expression vector, or alternatively by saline injection of the purified epitope-linked \(\beta_2m\) protein. Success with either approach would offer a new alternative with respect to the induction of adjuvant-independent, epitope-specific CTL responses.
Chapter 4

Covalent Linkage to $\beta_2m$ Enhances the MHC stability and Antigenicity of Suboptimal CTL Epitopes

Steven M. Chan assisted with the work described in this chapter.
Introduction

The identification of MHC class I epitopes of both viral and tumor origin, and the ability to routinely generate *ex vivo* dendritic cells, has increased the prospects of developing effective peptide based vaccines and immunotherapies. The design of such strategies requires careful selection of peptide epitopes, in order to maximize *in vivo* efficacy. Numerous experiments have demonstrated that the degree of immunogenicity of a peptide is related to its binding affinity for class I molecules (306-308). More specifically, the rate of dissociation of MHC/peptide complexes appears to be the most important binding parameter, with immunogenic peptides displaying slower off-rates (310, 311). Thus, rational peptide vaccine design should focus on class I epitopes which display a high MHC/peptide complex stability. Unfortunately, many epitopes which are clinically relevant, including HIV and melanoma-derived peptides, display relatively poor MHC binding and suboptimal immunogenicity (347-350, 355, 399, 400). This is of particular concern in tumor immunology, since many tumor-specific CTL are directed against suboptimal self epitopes, due to the tolerance of CTLs to high affinity self peptides.

A potential solution to this problem is to convert suboptimal epitopes into more effective immunogens by enhancing their MHC binding and stability. Numerous groups have taken such an approach, synthesizing peptide analogues in which deleterious MHC binding residues are replaced with more favourable ones, while preserving TCR contact amino acids. This strategy has been employed successfully in mouse viral (351) and tumor models (353). In human systems, modifications made to an HIV epitope (354) and numerous melanoma peptides (355, 385, 401) have resulted in enhanced MHC binding and immunogenicity. Furthermore, Rosenberg *et al.* recently reported that immunization of melanoma patients with a modified, higher-affinity melanoma gp100 peptide analogue can result in positive clinical outcome (356). Thus, engineering class I epitopes to have increased MHC binding capacity
appears to be a promising approach for the in vivo induction of specific CTL responses. This technique, however, can be laborious, in so far as it requires analyzing the binding contribution of individual residues within a given epitope and selection of appropriate high affinity analogues.

In this report, we describe an alternative strategy for enhancing suboptimal class I peptides: tethering epitopes to human β2-microglobulin (hβ2m). Previously, we demonstrated that CTL target structures could be effectively generated by covalently linking optimal Db or Kd restricted influenza virus nucleoprotein (NP) peptides to hβ2m (402). Interestingly, we observe that the ability of the optimal Kd restricted epitope to sensitize target cells for CTL lysis is enhanced when linked to hβ2m. To further examine the enhancing role of hβ2m linkage, we generated bacterially expressed fusion proteins in which suboptimal variants of the Db-restricted NP366-374 peptide are tethered to hβ2m. Covalently linking these peptides results in greater MHC/peptide stability, and increased antigenicity over uncoupled peptides. This approach, therefore, offers a simple method of enhancing the biological activity of suboptimal epitopes, and could be useful for the design of peptide-based vaccines and immunotherapeutics.

Materials and Methods

Cell Lines, Ab, and hβ2m

EL4 (H-2b) is a mouse T lymphoma cell line; RMA-S (H-2b) is a mutagenized and immunoselected variant of the mouse T lymphoma line RBL-5, and is defective in class I surface expression due to a defect in the TAP-2 gene (387, 388). Cells were grown in AIM-V serum-free medium (Life Technologies, Grand Island, NY). The anti-Db antibody B22.249R1 (B22) has been previously described (366, 367). Purified hβ2m was purchased from Fluka (Ronkonkoma, NY).
Construction and Purification of Peptide-hβ2m Fusion Proteins

Peptide-hβ2m fusion proteins have a nine amino acid NP epitope (K<sup>d</sup> or D<sup>b</sup> restricted) connected to hβ2m via a 12 amino acid linker (GGGSIGSGSGLS, single letter amino acid code). NP(147-155)-hβ2m was generated by PCR using NP(147-55)-L12-hβ2m plasmid (402) as a template and the 5' primer 5’GACGACGACAAGACCTACCAGCGTACCCGGTGC” (the underlined portion hybridizes to the NP147-155 epitope) and the 3’ primer LIC.2 (5’GGAACAAGACCGTACCTGTCTCGATCCCCTT”). NP(366-374)-hβ2m and variants were constructed by two-step PCR reactions. First, wild type hβ2m was amplified using the 5' primer 5’GGTGGCGTATCTGGTACCGGAAGTGGCAGCGGTTCAATCCAGCGTACTCCAAAGATT” which encodes the 12 residue linker (underlined) and the 3’ primer LIC.2. This PCR product was subsequently amplified using primers LIC.2 and one of three 5’ primers: 5’GACGACGACAAGCTTCCAATGAAAAATATGGGATCTATGGGTGGCGGATCTGGTACCCGA” (to generate NP(366-374)-hβ2m), or 5’GACGACGACAAGGCTCGTAATGAAAAATATGGGAAGACTATGGGTGGCGGATCTGGTACCCGA” (to generate R2-hβ2m), or 5’GACGACGACAAGGCTGATATGAAAAATATGGGAAGACTATGGGTGGCGGATCTGGTACCCGA” (to generate D2-hβ2m). The underlined codons indicate the changes made to the second amino acid of the NP366-374 epitope. All of the final PCR products listed above were inserted into the pCAL-n-EK bacterial expression vector (Stratagene, La Jolla, CA) using a ligation-independent cloning procedure, according to the manufacturer’s instructions. The pCAL-n-EK vector encodes an N-terminal calmodulin binding protein (CBP) tag and adjacent enterokinase (EK) cleavage site. Digestion with EK liberates peptide-hβ2m fusion proteins without any additional amino acids.

Vectors were transformed into BL21(DE3)plysS bacteria (Stratagene), IPTG induced, and lysates prepared essentially as described previously (402). Refolded fusion protein was adjusted to calmodulin binding buffer (50 mM Tris, pH 8.0, 300 mM NaCl, 2 mM CaCl<sub>2</sub>) and purified over a calmodulin affinity column (Stratagene), eluting in 50 mM Tris (pH 8.0), 300 mM NaCl, 2 mM EDTA. Eluted fusion protein was then exchanged into EK cleavage buffer.
(20 mM Tris, pH8.0, 50 mM NaCl, 2 mM CaCl$_2$) using a G-25 Sephadex column (PD-10, Pharmacia, Uppsala, Sweden) and digested overnight at room temperature with EK. Enzyme and free CBP were removed by addition of STI-agarose and calmodulin affinity resin, and the digested protein was concentrated (Centriprep, Amicon, Beverly, MA) and exchanged into PBS.

**MHC Stability Determination**

The stability of folded D$^b$ complexes was determined using a cell panning enzyme immunoassay (CPEIA) described previously (403). RMA-S cells were grown overnight in serum-free (AIM-V) medium at low temperature (26°C) to favor the formation of “empty” class I molecules and pulsed with 10 μM free peptide and 10 μM hβ$_2$m or 10 μM fusion protein for 1 hour at 26°C, in the presence of 5 μg/ml Brefeldin A (BFA). Cells were washed in PBS, resuspended at 2x10$^6$ cells/ml in serum-free medium with BFA, and incubated at 37°C. At various times during the 37°C incubation, 2x10$^5$ cells were removed and dispensed into a 96-well plate which had been coated with 10 μg/ml purified B22 Ab (2 hours at 37°C) and blocked with 5% milk (2 hours at 37°C). Cells were incubated on the plate for 1 hour at room temperature, and then the wells were washed gently six times with PBS. Bound cells were lysed and quantified by adding 100 μl of lactate dehydrogenase (LDH) substrate (0.167 mg/ml p-iodonitrotetrazolium violet, 0.043 mg/ml phenazine methosulfate, 2.435 mg/ml lactic acid, 0.431 mg/ml β-NAD, 1% Triton X-100 in 0.2M Tris, pH8.2), which undergoes an LDH catalyzed yellow-red color change. The reaction was stopped by adding 100 μl of 3M HCl, and absorbance at 492 nM read. All samples were analyzed in triplicate, and the percent stability determined by normalizing the LDH values relative to the initial (no 37°C incubation) point.
**CTL Assays**

Bulk CTL cultures were generated by immunizing C57BL/6 or BALB/c mice with influenza virus and restimulating spleen cells with 1 μM optimal peptide, essentially as described (390). EL4 (H-2b) or P815 (H-2d) target cells were labeled with 100 μCi of Na$_2$[${}^{51}$Cr]O$_4$ (Amersham, Arlington Heights, IL), washed, and pulsed with titrated amounts of purified fusion protein or equivalent amounts of free peptide and hβ$_2$m at a concentration of $10^3$ cells/ml for 1 hour at room temperature. $10^4$ targets were dispensed into 96-well plates, effectors added, and the plates incubated at 37°C for 4 hours. Plates were centrifuged, and supernatant harvested and quantitated using a TopCount scintillation counter (Canberra Packard, Mississauga, Ontario). Percent specific lysis was calculated, using the mean of triplicate samples, as: 100 × [(experimental cpm - spontaneous cpm)/[(maximum cpm - spontaneous cpm)]. Spontaneous cpm values were determined by incubating target cells alone in medium, and maximum values by lysis of targets in 1% Triton X-100 (v/v).

**Results and Discussion**

Covalently coupling a peptide epitope to its presenting MHC class I molecule, through either the heavy chain or β$_2$m subunit, has recently emerged as a potentially useful method of generating defined CTL target structures (361, 362, 402). The physical association between epitope and MHC could increase the stability of the MHC/peptide complex by restricting peptide diffusion and slowing dissociation, which can be critical for immunogenicity (310, 311). From the perspective of peptide vaccine design, the β$_2$m subunit appears ideally suited, since it is a small, soluble molecule which is known to promote peptide binding *in vitro* (282-284) and augment peptide specific CTL responses *in vivo* (287). It was previously demonstrated that D$^b$ and K$d$ binding peptides could be genetically fused to hβ$_2$m, and CTL target structures formed by both an endogenous (expression in transfected cell lines) and
exogenous pathway (pulsing target cells with fusion protein) (402). Interestingly, we observed that when NP147-155, an optimal K\textsuperscript{d} restricted epitope, is tethered to h\textbeta\textsubscript{2}m there is enhanced target cell sensitization compared to the free peptide mixed with h\textbeta\textsubscript{2}m (Figure 22). To further explore this phenomenon, we have generated recombinant fusion proteins in which D\textsuperscript{b} binding class I peptides are tethered to h\textbeta\textsubscript{2}m via a flexible polypeptide linker (Figure 23). The optimal peptide NP(366-374) and two variant peptides (designated R2 and D2) were chosen as a model system. These variant peptides have a single amino acid substitution at the P2 position (Ser changed to Arg and Asp, respectively). This peptide position, although not a dominant anchor, is buried within the MHC groove (49) and is thus expected to affect MHC binding but not TCR recognition. Previous measurements indicate that the R2 and D2 variant peptides show significantly reduced D\textsuperscript{b} binding (404, 405) and we observe a dramatic (3 log) decrease in the ability of the mutant peptides to sensitize target cells for CTL lysis (Figure 25).

Initially, we compared the ability of the purified peptide-h\textbeta\textsubscript{2}m fusion proteins and their corresponding free peptides to stabilize D\textsuperscript{b} complexes on RMA-S cells using a cell panning immunoassay (403). Incubation of RMA-S cells at low temperature (26°C) promotes the formation of thermolabile empty class I molecules, which can be stabilized with exogenously added peptides (337). The rate of decay of these complexes at 37°C gives an indication of stability. In the assays performed, RMA-S cells were grown at 26°C overnight, pulsed with 10 μM of fusion protein or free peptide and h\textbeta\textsubscript{2}m, washed, and transferred to 37°C for various time periods. The extent of class I stabilization was determined by exposing the cells to a plate coated with a conformation sensitive Ab (B22) followed by the measurement of lactate dehydrogenase activity to quantitate the number of cells bound. Brefeldin A was included in the assay to prevent the emergence of new class I molecules at the cell surface, increasing the likelihood that any observed loss of class I stabilization will reflect peptide off-rates. As shown in Figure 24A, both free NP(366-374) peptide and the corresponding fusion protein, NP(366-374)-h\textbeta\textsubscript{2}m, stabilized D\textsuperscript{b} molecules equally well. In contrast, when the suboptimal R2 variant peptide was tethered to h\textbeta\textsubscript{2}m (NP(366-374)-h\textbeta\textsubscript{2}m protein) there was a marked increase in
Figure 22. Linking NP(147-155) peptide to h\(\beta_2\)m enhances the formation of CTL target structures. \(^{51}\text{Cr}-\)labeled P815 cells (H-2\(^d\)) were pulsed with 1 nM, 0.1 nM, or 0.01 nM NP(147-55)-h\(\beta_2\)m fusion protein (A) or equal amounts of free NP(147-155) peptide and h\(\beta_2\)m (B) and incubated in a standard chromium release assay using NP(147-155)-specific CTLs. The NP(147-55)-h\(\beta_2\)m fusion protein has the NP(147-155) nonamer epitope connected to the amino terminus of h\(\beta_2\)m via a 12 residue linker (GGGSGTGSGSGS, single letter amino acid code).
Figure 23. Epitope-linked h\(\beta_2\)m fusion proteins. The D\(^b\) restricted epitope NP(366-374) (ASNENMETM, single letter amino acid code) and two suboptimal variants (designated R2 and D2) were linked to the amino terminus of h\(\beta_\)m via a 12 residue linker (GGGSGTGSGSGS). The altered residues in the P2 position are underlined.
Figure 24. Suboptimal peptides tethered to hβ₂m have enhanced ability to stabilize class I molecules. RMA-S cells cultured overnight at low temperature were pulsed with 10 μM fusion protein or 10 μM free peptide and 10 μM hβ₂m for 1 hour at 26°C, and then shifted to 37°C for the indicated time periods. The decay of Dᵇ complexes was measured using a cell panning LDH assay as described in Materials and Methods. A, comparison of uncoupled NP366-374 peptide and hβ₂m and the corresponding fusion protein. B, comparison of free R2 peptide and R2-hβ₂m fusion protein. C, comparison of D2 peptide and D2-hβ₂m fusion protein. The dotted lines indicate the time required for 50% decay.
stability over the free peptide simply mixed with hβ2m in equal amounts (Figure 24B). Pulsing with uncoupled R2 peptide and hβ2m results in complexes which are 50% dissociated by 30 min, while the tethered fusion protein extends this time to nearly 1.5 h. This effect was even more pronounced with the D2 variant (Figure 24C). Like the R2 peptide, free D2 peptide and hβ2m result in class I complexes which dissociate rapidly (50% loss by 30 min). The D2-hβ2m fusion protein, however, stabilized complexes such that the half life was extended beyond 1.5 hours. Thus, tethering suboptimal peptides to hβ2m results in a measurable increase in MHC complex stability over uncoupled peptide and hβ2m alone.

To determine if this increase in stability translates into an increase in antigenicity, free peptides and fusion proteins were compared for their ability to sensitize EL4 (H-2b) target cells for lysis by flu specific CTLs in standard chromium release assays. As shown in Figure 25A, the free optimal peptide and NP(366-374)-hβ2m fusion protein are generally comparable in their ability to sensitize for CTL lysis, although a difference is observed at the 1 pM concentration. Note that tethering an optimal Kd peptide to hβ2m actually augments antigenicity compared to the uncoupled counterparts (Figure 22). The reason for this discrepancy is not clear, although it could relate to the relatively weak binding of β2m to Dβ heavy chains (406). In the case of the R2 variant epitope, covalent coupling to hβ2m dramatically increases antigenicity (Figure 25B). Free R2 peptide and hβ2m sensitizes target cells for lysis out to 10^{-10} M, whereas the corresponding fusion protein titers out near 10^{-13} M, an approximately 1,000 fold improvement. To a lesser extent, D2-hβ2m also outperforms its uncoupled counterpart (Figure 25C). While free D2 peptide and hβ2m titer out at 10^{-10} M, the hβ2m fusion protein is active at a log lower concentration. Thus, tethering an optimal peptide to hβ2m does not significantly impair its ability to sensitize target cells for lysis, and most importantly, coupling suboptimal epitopes to hβ2m improves antigenicity. It is curious that the D2-hβ2m fusion protein, which induces slightly more Dβ stability than R2-hβ2m, actually shows a smaller improvement in antigenicity. This may be due to suboptimal TCR recognition of the D2 variant epitope by CTLs restimulated on the optimal NP(366-374) peptide.
Figure 25. Peptides linked to hβ₂m exhibit increased antigenicity over unlinked peptides. 

$^{51}$Cr-labeled EL4 (H-2b) cells were pulsed with titrated equal amounts of free peptide and hβ₂m or peptide-hβ₂m fusion protein and incubated in a standard 4 hour chromium release assay using NP366-374-specific CTLs at an effector to target ratio of 40:1. A, comparison of uncoupled NP366-374 peptide and hβ₂m and the corresponding fusion protein, NP(366-374)-hβ₂m. B, comparison of free R2 peptide and R2-hβ₂m fusion protein. C, comparison of D2 peptide and D2-hβ₂m fusion protein.
The ability to enhance the stability and antigenicity of suboptimal epitopes by covalent linkage to β2m, as described above, may offer new approaches for the development of peptide-based vaccines and immunotherapies. It would allow the repertoire of class I epitopes to be expanded to include low affinity peptides, which is of clinical relevance for viral and neoplastic disease treatments (347-350, 355, 400). A β2m coupling approach does not require analyzing the contribution that individual amino acids make towards the binding of a particular peptide, and may therefore represent a simple, global strategy for converting suboptimal peptides into optimal epitopes. It could be amenable for use in protein immunizations, in an analogous fashion to the peptide immunization experiments conducted by Rosenberg et al. (356), as a DNA immunogen, or used in combination with ex vivo generated dendritic cells for adoptive immunotherapy. In considering the latter strategy, it is noteworthy that Berzofsky and colleagues have shown that the avidity and thus in vivo efficacy of CTLs are inversely related to the concentration of restimulating peptide (314, 315). Thus, maximally effective CTL responses may require exceedingly low amounts of high affinity peptides, which may only be attainable by modifying suboptimal epitopes, such as by a β2m coupling approach. Furthermore, recent MHC tetramer technology has provided new opportunities to assess epitope-specific CD8+ T cell responses (407). Using an epitope-linked β2m approach, the repertoire of MHC tetramer reagents could be increased to include low affinity peptides which would otherwise form unstable complexes.
Chapter 5

Discussion
A. Summary of Results

This thesis describes two unique strategies for generating CTL target structures by coupling MHC and peptide antigen. The first approach integrates the biosynthesis and ER translocation of a class I heavy chain and CTL epitope by inserting a D\(^b\) restricted peptide into the D\(^b\) signal sequence. In the second approach, a more permanent linkage between MHC and peptide is accomplished by tethering class I epitopes to the \(\beta_2m\) subunit. From these systems, three conclusions can be drawn:

1. The presentation of a D\(^b\) restricted CTL epitope incorporated into the amino terminus of the D\(^b\) signal sequence requires TAP.

2. CTL target structures can be generated using epitope-linked \(\beta_2m\) constructs, through both endogenous and exogenous presentation pathways.

3. Covalent linkage to \(\beta_2m\) enhances the MHC stability and antigenicity of suboptimal class I binding peptides.

In the discussion that follows, I will elaborate upon these conclusions. In particular, I will detail the mechanisms responsible for TAP-dependent signal sequence presentation, and its relevance to basic issues in antigen processing. Peptide-linked \(\beta_2m\) molecules will be discussed with specific emphasis on future applications.
B. Signal Sequence Presentation: Mechanisms and Future Considerations

1. Different Signal Sequence Peptides Exhibit Different TAP Requirements

The example of TAP-dependent signal sequence presentation described in Chapter 2 is part of a growing body of literature regarding the presentation pathway of signal peptides. As summarized in Table I, there is conflicting evidence in favor of both TAP-dependent and independent signal sequence processing. In addition, there are a number of class I associated signal sequence peptides whose TAP dependence has not been established. Despite the fact that these data are limited, several generalizations can be made which merit further discussion.

First, the epitopes which require TAP for presentation appear to have longer C-terminal flanking regions within the signal peptide. In the NP-HC molecule described in Chapter 2, and the naturally occurring LCMV gp33 signal sequence studied by Zinkernagel and colleagues (207), 16 amino acids lie between the signal peptidase cleavage site and the C-terminal end of the epitope. Similarly, the peptides presented by the class Ib molecules Qa-1b and HLA-E both have 13 residues at the C terminal end. In contrast, the seven signal peptides whose presentation is documented in TAP deficient cells have notably shorter C-terminal regions (the longest is eight amino acids). In one instance, the signal peptidase cleavage site actually generates the C-terminal end of the epitope. These data suggest that signal peptides which require extensive trimming at the C-terminal end must utilize a cytosolic processing system (and thus require TAP to enter the ER), which is consistent with the concept of a carboxypeptidase deficient ER (see Section B4, below).

Second, signal sequence-derived peptides which do not require TAP for presentation appear to be longer in length than those which require TAP transport. Of the six signal sequence-derived peptides which were eluted from class I molecules on TAP deficient cells, five were longer than the canonical nine residues. This suggests that the ER is a relatively poor
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</tr>
<tr>
<td>Calreticulin</td>
<td>10*</td>
<td>A2</td>
<td>0 7C</td>
<td>No</td>
<td>(198)</td>
</tr>
<tr>
<td>HLA-DP</td>
<td>9*</td>
<td>B7</td>
<td>8N 12C</td>
<td>?</td>
<td>(157)</td>
</tr>
<tr>
<td>HLA-DP</td>
<td>10*</td>
<td>B7</td>
<td>8N 11C</td>
<td>?</td>
<td>(157)</td>
</tr>
<tr>
<td>HLA-A2</td>
<td>9*</td>
<td>B7</td>
<td>4N 11C</td>
<td>?</td>
<td>(157)</td>
</tr>
<tr>
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<td>9*</td>
<td>B7</td>
<td>1N 14C</td>
<td>?</td>
<td>(157)</td>
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<tr>
<td>Tyrosinase</td>
<td>9</td>
<td>A2</td>
<td>0 9C</td>
<td>?</td>
<td>(204)</td>
</tr>
</tbody>
</table>

**Table I.** Signal sequence-derived peptides presented by class I molecules. The asterisks (*) indicate peptides whose size has been definitively established by mass spectrometry. Flanking residues refers to the number of amino acids adjacent to the epitope within the signal sequence. Thus “1N-16C” indicates the presence of one N-terminal and 16 C-terminal residues within the signal peptide which must be removed to produce the peptide of indicated size. Question marks denote signal peptides whose TAP dependence has not been established.
proteolytic environment, and that full cleavage requires access to the cytosol, although there is evidence to the contrary (see Section B4). Note, however, that the length of signal sequence-derived peptides which require TAP for presentation has not been definitively established. Although it is likely that the NP-HC transfectants described in Chapter 2 are presenting nonamer NP366-374 peptide to CTLs, it is possible that the peptide may be incompletely trimmed. It would be necessary to sequence peptides eluted from surface class I molecules in order to resolve this issue. Since such peptide elution/sequencing experiments have not been performed by other groups demonstrating TAP-dependent signal peptide presentation, only indirect evidence for complete peptide trimming exists.

Finally, it is apparent that the only two examples of TAP-dependent signal sequence presentation by class Ia molecules involve H-2D^b. This raises the possibility that this phenomenon is unique to the D^b molecule. Possibly, D^b may be particularly dependent upon TAP for folding and/or assembly. Although allele specific differences in TAP interactions have been observed for human class I molecules (175), similar data for mouse alleles have not been reported. Yet in the absence of functional TAP, D^b behaves similarly to K^b with respect to surface expression and low temperature induction (337). In fact, there is no evidence to support the notion that D^b has a unique TAP dependence which could explain a specific inability to present signal sequence-derived peptides in a TAP-independent fashion. This issue could be definitively resolved by demonstrating D^b presentation of ER-targeted peptides in RMA-S (TAP deficient) cells. While this experiment has not been reported, a recent publication has demonstrated that a mutant D^b molecule which associates poorly with TAP can still present peptides targeted to the ER by signal sequences (408). Thus, it is highly improbable that D^b possesses a TAP dependence which is unique among mouse class I molecules.
2. Further Analysis of TAP Dependence Using Systematically Altered Signal Peptides

While the previous discussion has highlighted several trends which are appearing from the signal sequence peptide data, it underscores the need for further experimentation. The identification of naturally occurring signal sequence peptides is limited, and the NP-HC system described in Chapter 2 is one of only three systems which have been established to investigate the role of TAP in signal sequence presentation. It is clear that additional useful data could be obtained by expanding the NP-HC system, as described below.

One interesting approach would be to shift the NP366-374 epitope to the C-terminal end of the D\(^b\) signal sequence. Given the sequence flexibility of signal peptides in this region (409) (notwithstanding the signal peptidase cleavage site, which could be maintained by retaining the last three residues) it is likely that this could be accomplished without adversely affecting D\(^b\) targeting to the ER. Determining the TAP dependence in T2 cells would provide crucial insight into the role of a C-terminal position in signal sequence presentation. If the trend discussed above is legitimate, one would expect a C-terminal epitope to be presented in the absence of TAP.

Another useful experiment would involve altering the identity of various residues within the D\(^b\) signal peptide and assessing the consequences with respect to TAP dependence. Any changes which allow for TAP-independent presentation would provide insights into the role of amino acid sequence on ER processing. Such experiments could rely upon rational signal sequence mutagenesis (i.e. increasing the hydrophobicity). Alternatively, a more ambitious (and potentially rewarding) approach would be to generate random signal sequence constructs containing the NP epitope and identify TAP deficient transfectants which are lysed by specific CTLs. Since generating numerous stable T2 transfectants can be labor intensive, this system could be potentially simplified by using a transient transfection system (i.e. COS) in which TAP is blocked by the viral gene product ICP47. By recovering the sequence of D\(^b\) from
transfectants which exhibit TAP-independent presentation, one could decipher the general signal peptide features which control TAP dependence.

In addition, the NP-HC system could be expanded to include other class I molecules, such as K\textsuperscript{d}. By similarly incorporating the K\textsuperscript{d} restricted NP147-155 epitope into the K\textsuperscript{d} signal sequence, the potential influence that the MHC molecule may have on TAP-dependent signal sequence presentation could be easily assessed. Collectively, these experiments highlight the utility of the NP-HC system. While the TAP-dependence of NP presentation may preclude NP-HC from serious consideration as a vaccine strategy, it could be useful for expanding our limited knowledge of signal sequence presentation.

3. How Are Signal Sequence Peptides Localized to the Cytosol?

A requirement for TAP in signal peptide presentation indicates that signal peptides (or some fragment thereof) must, at some point in time, reside in the cytosol. How is this cytosolic localization of ER-targeted signal sequences accomplished? There are three distinct (but not mutually exclusive) mechanisms which could be responsible, which are explored below.

First, signal sequences may be diverted to the cytosol as a result of aberrant protein synthesis. Such “defective ribosomal products” may result from premature termination of protein synthesis or a failure to properly engage the machinery (i.e. signal recognition particle and its receptor) responsible for delivering emerging protein chains to the ER (205). Although the fidelity of protein biosynthesis and translocation is unknown, it is likely that a low frequency of defective products are stochastically produced. A report from Ferris et al. has suggested that such a mechanism may be responsible for generating an epitope located in the ER lumenal domain of HIV gp41 (410). The issue of defective ribosomal products was not addressed experimentally in Chapter 2. Since CTLs are capable of lysing target cells expressing
very small (<100) numbers of MHC/peptide complexes (273, 397), it is possible that a
defective ribosomal product could contribute to the production of cytosolic signal peptides yet
remain biochemically undetectable.

Secondly, signal peptides may be recycled back to the cytosol from the endoplasmic
reticulum. The retrograde transport of membrane proteins has now been convincingly
established, and appears to function as a mechanism of removing misfolded proteins from the
ER for cytosolic degradation (232). Class I molecules themselves have been observed to
undergo such displacement when improperly folded due to a lack of peptide or B2m (233).
Although not completely understood, this ER to cytosol transport involves protein movement
back through the translocon, the Sec61p membrane channel through which secretory proteins
are originally inserted (234, 235). In addition, there is evidence that peptides can recycle from
the ER to the cytosol (220). The pathway responsible for this translocation has yet to be
defined, and may be distinct from the Sec61p channel that large proteins utilize. Importantly,
ER to cytosol antigen transport has been demonstrated to occur in experimental systems (238)
and for naturally occurring CTL epitopes (236, 237). In the latter case, a naturally presented
epitope in the melanoma antigen tyrosinase was shown to undergo transport from the ER to the
cytosol prior to its TAP dependent reentry into the ER. The ability to decipher the movement of
the peptide is facilitated by an N-linked glycosylation sequence: it is glycosylated in the ER and
upon deglycosylation by a cytosolic enzyme, the Asn residue is converted into an Asp. This
strategy could be similarly employed in the NP-HC system to determine if the NP containing
signal peptide recycles from the ER to the cytosol. An N-linked glycosylation site (Asn-X-
Ser/Thr) could be introduced by converting the P6 Met residue in NP366-374 (sequence
ASNENMETM) to an Asn. Since the P6 side chain points away from the binding groove for T
cell recognition (49), the presence of an Asn (or Asp) should not disrupt D\(^b\) binding. Thus,
peptides bound to D\(^b\) molecules on the surface of cells transfected with the modified NP-HC
construct could be eluted and sequenced. The presence of an Asp at P6 would signify
deglycosylation and thus indicate ER to cytosol recycling.
Finally, signal sequence-derived peptides may simply gain access to the cytosol as a result of the natural process of protein translocation. Signal peptide insertion into the ER membrane has been traditionally described by a loop model, where the N-terminal region is on the cytosolic side and C-terminal region is lumenal (214). This would predict that epitopes localized to the N-terminal region of signal peptides could be processed by cytosolic enzymes, whereas C-terminal epitopes could undergo ER proteolysis. Note that this prediction is generally consistent with the observation that TAP-dependent (i.e. cytosolic) signal peptides tend to have large C-terminal flanking regions (see above). It is now recognized, however, that signal sequence insertion is a more complex event than a simple looping mechanism. During early translocation, the signal sequence is found in the aqueous translocon channel and is not accessible to the cytosol due to a tight seal between the ribosome and the ER membrane (381). Furthermore, the signal peptide is likely bound at the protein-lipid interface while in the Sec61p channel (411). Nevertheless, recent experiments have indicated that membrane associated signal peptides are cleaved near the C-terminal end of the hydrophobic region by a lumenal enzyme, resulting in the release of an N-terminal fragment into the cytosol (212, 213). The location of the C-terminal fragment could not be determined, but would be predicted on the basis of the loop model to be lumenal. These results reinforce the notion that epitopes located near the N-terminus of signal sequences (as in the NP-HC example) would require TAP to enter the ER. It is noteworthy, however, that a subsequent study has demonstrated that the cytosolic release of the N-terminal fragment of the signal sequence in question (from preprolactin) is enhanced by its unique ability to bind the cytosolic protein calmodulin (215). One must also consider that there are two conflicting examples of TAP-independent presentation of N-terminal signal sequence epitopes (see Table I). Thus, although there is reason to suspect that the natural translocation process may contribute to the cytosolic localization of signal peptides, further investigation is required. The previously mentioned experiments in which the NP366-374 epitope is shifted to the C-terminus of the D\(^{b}\) signal peptide would be helpful in resolving this issue.
4. Peptide Generation in the ER

Determining the TAP dependence in signal sequence presentation has provided information regarding the ability of the endoplasmic reticulum to generate class I binding peptides. On its own, the lack of consensus concerning TAP involvement (see Table I) suggests that the protease environment of the ER is limited. However, the relatively small sample size regarding signal peptide presentation has made it difficult to draw more precise conclusions about the nature of ER proteolysis, although the balance of data suggest only partial N-terminal trimming occurs. How do these data fit with the general conclusions regarding the ability of the ER to generate peptides?

Yewdell and colleagues have proposed that ER proteolysis is governed by a “C-end rule”: peptides are preferentially generated from the C-terminal end of ER substrates (226). This conclusion was based on two observations: epitopes can be preferentially liberated from the C-terminus of several proteins (Jaw1, CD23, and flu NP) which are targeted to the ER (226, 227); and only C-terminal peptides are presented when tandem epitope constructs are inserted into the ER (225). The C-end rule is supported by the finding that TAP deficient cells can present an ER targeted optimal peptide which is extended by five amino acids at the N-terminus but not if the extension is C-terminal (119). In fact, extending an optimal peptide by only two amino acids at its C-terminus can abrogate presentation unless a specific carboxypeptidase enzyme is purposely expressed (228). Furthermore, the rat cim effect (see Chapter 1, Section B3) suggests that the cytosol, rather than the ER, is responsible for generating the C-terminal residue of the peptide (150). Collectively, these results argue that the ER has the capacity to trim peptides at the amino terminus, but is deficient in C-terminal activity.

There are, however, several studies which contradict this conclusion. Most notably, Townsend’s group has shown that large flu NP fragments expressed in the ER of TAP deficient cells can be extensively degraded (at both termini) to form optimal peptides (221). There are also several epitopes in the ER lumenal domain of the HIV envelope protein which
can be processed and presented in TAP deficient cells (222, 223). At present, it is difficult to reconcile these results with the studies which demonstrate a paucity of ER processing. As discussed below (Section B5), the key factor may lie in the ability of ER proteins to interact with additional components in the secretory pathway.

Although the extent of ER trimming can be disputed, it is clear that some degree of proteolysis is occurring in this compartment. This raises the question: what are the responsible enzymes? To date, only signal peptidase has been identified as contributing to the production of peptides in the ER, and its limited substrate specificity (201) suggests that other peptidases must also be involved. The TAP-independent generation of A2 restricted signal sequence peptides is blocked by two agents known to inhibit a wide range of proteases, including the proteasome and cathepsin B (101). Interestingly, the cleavage of the preprolactin signal peptide is suggested to occur by an ER lumenal component, which is not inhibited by reagents which block classical serine, cysteine, and metalloproteases (including ER-60 and protease IV, an enzyme implicated in the digestion of bacterial signal sequences) (213). The cleavage is, however, inhibited by the immunosuppressant cyclosporin A, which is known to bind proline isomerase enzymes in the ER. Clearly, further studies are required to decipher the mechanism of cyclosporin A inhibition.

5. Peptides in the ER: A Myriad of Fates

Throughout this discussion, I have attempted to convey the controversy which surrounds the issues of signal sequence presentation and ER degradation. The inability to reach firm conclusions is in part due to a paucity of data (i.e. the lack of identification of ER enzymes), but is also a general reflection on the complexity of class I antigen presentation. In 1993, when the work in this thesis was begun, there was a comfortably simple view regarding the fate of
peptides in the ER: they could bind MHC molecules or be destroyed. It has since become apparent that additional fates await peptides in the endoplasmic reticulum (see Figure 26). For example, peptides may be exported back to the cytosol, possibly by the Sec61p channel through which secretory proteins are known to recycle through (see above). Once in the cytosol, peptides may be fully degraded or trimmed such that they can re-enter the ER and bind to class I molecules. Alternatively, ER peptides may be released from cells. Gabathuler et al. have recently demonstrated that antigenic peptides which enter the ER can be secreted from cells by a mechanism which is BFA-sensitive but does not involve the expression or transport of MHC molecules (412). This peptide release, therefore, is not simply due to the dissociation of cell surface class I molecules, but may be part of the natural “bulk flow” of material along the secretory pathway. As noted previously, there is evidence to indicate that extracellular peptides can traffic directly to the ER without traversing the cytosol or Golgi (265). Thus, it is possible that there is a dynamic exchange of peptides between the ER and extracellular environment.

The fate of antigenic peptides in the ER is likely to be inextricably linked with the ER proteins to which they bind. The identified repertoire of ER components which are capable of binding peptides is currently expanding. The heat shock protein gp96, for example, is known to bind peptides which enter the ER by TAP and TAP-independent mechanisms (413). Since HSPs can elicit CTL responses in vivo, they must be capable of transferring their peptide cargo into the class I presentation pathway. It is hypothesized, although unproven, that HSPs play a role in normal peptide loading and class I assembly (414). Protein disulfide isomerase, which acts as an ER chaperone in protein folding (415), has been observed to efficiently bind TAP translocated peptides in the ER, even in competition with class I molecules (416). Furthermore, several well defined components of the class I assembly pathway are also known to bind ER peptides, including calreticulin (which binds only glycopeptides) (417) and tapasin (418). In addition, cross-linking studies using radiolabeled peptides have found a number of unidentified proteins which can bind TAP translocated peptides with varying specificities (417, 419). It is
Figure 26. The fate of peptides in the ER. Peptides in the ER could bind to MHC class I molecules, be secreted into the extracellular environment, be exported to the cytosol (possibly for destruction) or be degraded. Some or all of these fates may be influenced (dotted lines) by the interaction of peptides with various ER proteins, including heat shock proteins (HSPs) and protein disulfide isomerase (PDI).
tempting to speculate that the ultimate fate of peptides in the ER (MHC binding, degradation or export) is decided by the accessory proteins with which they interact (see Figure 26).

If this holds true, then the question becomes: what determines which ER proteins a particular peptide will interact with? Obviously, there are a multitude of important parameters which deserve consideration, including the quantity of peptide in the ER, which itself may be controlled by susceptibility to (or availability of) proteases, and the binding capacity of a peptide with respect to MHC or other proteins. An intriguing argument can be made for considering protein glycosylation as a crucial factor in determining peptide fate in the ER. In their experiments demonstrating ER trimming of flu NP fragments, Townsend and colleagues noted that TAP-independent presentation could not occur when the NP substrate contained glycosylation sites (221). Indeed, a subsequent study has demonstrated that TAP-independent presentation of ER-targeted NP can occur only if glycosylation is disrupted either through pharmacological treatment or site-directed mutagenesis (224). It should be noted that the carbohydrate attachment sites in question do not overlap the class I epitopes, and thus the inhibitory effect of glycosylation is not simply due to sugar residues disrupting MHC binding or T cell recognition. Rather, the glycosylation sites on the protein may divert it into a quality control system which prevents the ER processing of distal epitopes. As described previously, many ER chaperones (such as calnexin and calreticulin) bind the carbohydrate moieties on their substrates. Glycosylation, therefore, may represent a key control point which diverts substrates to various accessory components, which in turn ultimately control protein (and thus peptide) fate.
C. Manipulating *In Vivo* Immune Responses with Epitope-Linked $\beta_2 m$ Constructs

The prospects of using epitope-linked $\beta_2 m$ molecules to manipulate *in vivo* CTL responses is based upon two principles: enhancing peptide loading and enhancing MHC stability. As reviewed in Chapter 1, exogenous $\beta_2 m$ is known to augment peptide binding to surface class I MHC, presumably by stabilizing free heavy chains. In Chapter 4, data were presented which demonstrate that peptides linked to $\beta_2 m$ are better able to stabilize surface class I molecules and sensitize target cells for CTL lysis than uncoupled peptides mixed with $\beta_2 m$. Collectively, these data argue that peptide-$\beta_2 m$ molecules should increase the density and lifespan of specific MHC/peptide complexes at the cell surface, parameters which are known to be critical in controlling primary T cell responses (304, 312). The following discussion explores the potential applications of peptide-linked $\beta_2 m$ constructs in clinical medicine.

1. Vaccine and Immunotherapy Strategies

The induction of CTLs is an important consideration in designing vaccines and immunotherapies for a number of viral pathogens, such as HIV, and for generating anti-tumor responses *in vivo*. For the latter, the identification of numerous epitopes from human cancers has made the prospect of mobilizing effective, tumor-specific CTL immunity a realistic possibility. Towards this goal, peptide-linked $\beta_2 m$ molecules could be utilized under a number of different conditions, including protein immunization (with or without adjuvant), gene immunization, and adoptive transfer of pulsed APCs or *in vitro* expanded CTLs.

The possibility of using $\beta_2 m$ as a protein immunogen is based upon experiments by Rock and colleagues, who showed that CTL responses against nonimmunogenic peptides could be generated if the peptides were mixed with h$\beta_2 m$ and injected (in saline) subcutaneously (287).
This \( \beta_2m \) "adjuvant" effect was postulated to be the result of enhanced cell surface loading in vivo. A logical extension of these findings is to covalently couple the peptide and h\( \beta_2m \), thus restricting peptide diffusion and exploiting the ability of a \( \beta_2m \)-linkage to create more stable class I complexes. Unfortunately, preliminary experiments using the purified peptide-linked \( \beta_2m \) fusion proteins described in Chapter 4 or even peptide mixed with h\( \beta_2m \) have failed to show CTL priming. The reason for this is unclear, although it should be noted that only two other published reports (420, 421) have attempted priming CTL with peptides and \( \beta_2m \), with one reporting failure (420). Possibly, the system is suboptimal due a lack of T-helper activity. Although h\( \beta_2m \) is xenogeneic in a murine system, experiments by Rock et al. demonstrated that its effect is not simply due to the contribution of T-helper epitopes (287). Thus, immunogenicity could be augmented by incorporating a T-helper peptide, as is commonly done when mice are immunized with class I binding peptides emulsified in incomplete Freund's adjuvant (IFA) (307).

Even the inclusion of T-helper epitopes may be insufficient to prime CTL responses with peptide-\( \beta_2m \) fusion proteins in saline solution due to a lack of T cell costimulation. Thus, the use of an adjuvant may be required to recruit professional APCs and/or activate their expression of costimulatory molecules. Obviously, any requirement for an adjuvant would diminish the prospect of utilizing peptide-linked \( \beta_2m \) molecules in widespread prophylaxis (except for alum, which is notably poor at inducing CTL (422)). Nevertheless, adjuvant-based immunogens are more accepted for immunotherapy within patient groups. For example, immunizing cancer patients with tumor antigen-derived peptides emulsified in IFA is a generally well tolerated, clinically acceptable procedure (356, 423).

DNA immunization offers an alternative approach for inducing specific CTL responses in vivo using peptide-\( \beta_2m \) fusions. Administering DNA plasmids, either through intramuscular injection or intradermal gene bombardment, is a safe, simple, and cost-effective method of generating antigen-specific CTL responses (398). Results presented in Chapter 3 demonstrated that transfected tumor cell lines expressing peptide-\( \beta_2m \) fusion proteins could form appropriate
CTL target structures. It would be reasonable to extend this observation in vivo, and assess the ability of plasmids encoding peptide-β2m constructs to generate CTL immunity. One of the advantages of a DNA immunization system is the ease with which costimulation and T cell help can be manipulated. Immunogenicity can be augmented by engineering plasmids to encode costimulatory molecules such as B7-2 (424), cytokines such as IL-12 (424), or additional immunostimulatory sequences (425). Note, however, that peptide-β2m proteins produced in transfected cells must compete with endogenous wild type β2m, and thus the level of gene expression may be critical. Viral vectors (such as canarypox) could be useful in obtaining high levels of gene expression, although there are associated disadvantages, such as unwanted immune responses to viral components (426).

Recent advances in the ex vivo generation of dendritic cells have made adoptive transfer of autologous professional APCs a realistic immunotherapeutic possibility. Using cytokines such as GM-CSF, large numbers of dendritic cells can be expanded in culture from peripheral blood of patients, loaded with peptide, and returned in vivo to elicit CTL responses (427). Although this approach is more time consuming and labor intensive than the previously described strategies, it has proved successful in animal tumor models (428, 429) and has entered into human clinical trials (430, 431). This approach may be limited, however, by the relatively poor MHC binding capacity of many important tumor epitopes (347-349). Furthermore, class I MHC molecules on the surface of dendritic cells (unlike class II) exhibit a relatively short half-life (432). This suggests that the performance of adoptively transferred dendritic cells could be enhanced by utilizing peptide-β2m fusion proteins, which would permit the surface expression of high levels of stable class I complexes.

Finally, peptide-linked β2m molecules could be applied to the ex vivo expansion of epitope-specific CTLs. Generating large numbers of specific autologous CTLs in culture for infusion into patients has emerged as a potentially useful strategy in controlling malignancies and certain viral infections (433). Typically, CTLs are generated from patient peripheral blood cells by culture in the presence of autologous peptide-pulsed APCs and cytokines. Unfortunately, the
low MHC affinity that many epitopes display can limit CTL generation. In fact, the in vitro expansion of specific CTLs from melanoma patients using peptides often requires multiple stimulation cycles and is successful in only a fraction of patients (350). The ability to enhance MHC stability (as demonstrated in Chapter 4) through covalent $\beta_2m$ linkage could prove invaluable for in vitro CTL generation. Although one could attempt to overcome the low affinity limitation by pulsing APCs with extremely high concentrations of peptides, there is evidence that high peptide concentrations generate low avidity CTLs with poor in vivo efficacy (314, 315). Thus, successful expansion of CTLs for effective adoptive transfer may require the display of low levels of very stable MHC peptide complexes on the surface of APCs. For many clinically relevant epitopes, this may only be possible by a covalent linkage to $\beta_2m$.

2. Tolerance Induction

While the discussion thus far has focused on augmenting immunogenicity using epitope-linked $\beta_2m$ molecules, the ability to induce antigen-specific tolerance is an additional point worthy of consideration. Therapies which can selectively downregulate T cell responses in vivo would be of great benefit in the clinical treatment of autoimmune diseases. While tolerance is a complex immunological subject beyond the scope of this thesis, one of the most widely used and reproducible methods of its induction involves the systemic administration of high dose antigens (434). This has been explored extensively for CD4$^+$ T cells, where immunization with class II restricted peptides is known to prevent murine experimental autoimmune encephalomyelitis, a model for human multiple sclerosis (435). The administration of CTL epitopes has also been demonstrated to induce tolerance and prevent the induction of autoimmune disease (436). Presumably, the delivery of large peptide doses results in the formation of immense numbers of MHC/peptide complexes. When this supraoptimal antigenic
signal occurs in the absence of costimulation, tolerance will result. This hypothesis is supported by in vitro observations from Iezzi et al., who have demonstrated that effector T cells undergo deletion when given a prolonged antigenic signal in the absence of costimulation (312). As discussed above, CTL avidity is known to correlate with dose of stimulating peptide, such that high peptide doses produce low avidity CTLs due to the deletion of high avidity clones (314, 315).

If the formation of large numbers of specific MHC/peptide complexes is a key factor in the induction of tolerance, then epitope-linked β₂m molecules could prove useful. By augmenting peptide loading through β₂m exchange and stabilizing the resulting class I trimer through the covalent linkage, peptide-β₂m fusion proteins can achieve a supraoptimal antigenic stimulation. The ability of the peptide-β₂m molecules described in Chapters 3 to induce tolerance could be readily assessed by monitoring flu specific CTL responses in immunized mice which are subsequently challenged with virus. Although it may seem somewhat fanciful to discuss peptide-β₂m fusions as both immunogens and tolerogens, in reality the line between these two extremes is often thin and ill defined. For example, peptide immunizations which induce CTLs for certain epitopes can result in tolerance to others, despite identical protocols (437, 438). One must consider many parameters, such as adjuvants and injection route, as relevant factors in determining the course of specific T cell responses. Under this philosophy, it is worthwhile to explore the use of peptide-β₂m molecules as both inducers and negative regulators of in vivo CTL responses.

D. Further Applications of Peptide-β₂m Constructs

While the primary consideration in undertaking research on epitope-linked β₂m molecules was the design of practical immunogens, the utility of these fusion proteins may extend into
other areas of immunology. Two such examples are discussed below: T cell selection and MHC tetramers. These will serve to highlight the potential utility of peptide-\(\beta_2\)m fusion proteins in important, yet diverse areas of research.

1. Studying T Cell Selection

Recent investigations in T cell selection have focused on the uncertainties regarding the role of self peptides in the selection process. Are individual T cell receptors selected by a single peptide or by a diverse group of peptides? What is the structural relationship between selecting peptides and antigenic peptides? For CD8\(^+\) T cells, these issues are being addressed using \textit{in vitro} fetal thymic organ culture systems. Typically, these “add back” experiments monitor selection by adding the desired peptides to thymic cultures derived from TCR transgenic, TAP knockout mice (346). This approach has proved fruitful, and has suggested that each T cell receptor does not require a single peptide, but can be selected by a mixture of ligands (439). Nevertheless, the \textit{in vitro} nature of such systems has limitations, and obviously does not permit the analysis of the selected T cells \textit{in vivo}.

In contrast, the study of CD4\(^+\) T cells has exploited animals which predominantly express single MHC/peptide complexes. These have been generated by expressing transgenes (on a class II knockout background) encoding class II molecules with covalently bound peptides (440-442). An analogous class I MHC system could be achieved using epitope-liked \(\beta_2\)m constructs. As detailed in Chapter 3, the expression of peptide-coupled \(\beta_2\)m molecules in transfected mammalian cells results in the efficient formation of appropriate CTL target structures. The production of mice expressing a similar construct as a transgene would allow this condition to be reproduced \textit{in vivo}. High levels of peptide loading and surface expression could be achieved by using mice with disrupted \(\beta_2\)m and TAP genes, thus reducing
competition from unlinked \( \beta_2 \text{m} \) and uncoupled, naturally occurring peptides. Generating such mice with a \( \beta_2 \text{m} \) coupled to a self peptide would allow for a number of questions to be addressed. What level of positive selection is achieved by the single MHC/peptide complex? Are the transgenic mice capable of mounting CTL responses to unrelated (i.e. viral) antigens? Are their allogeneic responses affected? Answers to these questions could provide valuable insights into the role peptide diversity plays in positive selection and the generation of the CTL repertoire.

2. \( \beta_2 \text{m} \)-Linked Epitopes in MHC Tetramers

In 1996, Altman \textit{et al.} published the first report describing the quantitation of epitope-specific CD8\( ^+ \) T cells using MHC tetramers (443). Class I tetramers are complexes in which four refolded class I molecules are aggregated using a biotin-avidin interaction. Typically, the heavy chain and \( \beta_2 \text{m} \) are expressed in bacteria, and refolded in the presence of a desired peptide ligand. Biotinylation can occur by incorporating a specific enzyme recognition sequence in the C-terminus of the heavy chain, or alternatively, through chemical modification of the \( \beta_2 \text{m} \) subunit (444). By using avidin conjugated to specific fluorochromes, a highly specific and sensitive staining reagent is created for use in flow cytometry analysis. Stable interactions with the cognate T cell receptor, which are normally low affinity (445), are made possible through the tetramer’s high avidity nature.

Tetramer analysis and quantitation of specific CD8\( ^+ \) cells has been applied to a number of viral infection models, including HIV (18), SIV (446), LCMV (444, 447), EBV (448) and influenza (449). The results have generally indicated that virus-specific CD8\( ^+ \)s comprise a greater proportion of the expanding T cells than previously believed. For example, tetramer analysis has indicated that 50-70\% of activated CD8s during LCMV infection are virus-
specific, whereas traditional measurements using limiting dilution analysis (LDA) indicated a much lower (1-5%) frequency (447). This discrepancy highlights the limitations of the widely used LDA, which underestimates T cell frequency by selecting only cells which can undergo sustained proliferation (407).

While tetramers are proving invaluable tools for understanding the basic parameters of the cellular immune response, their application in clinical research is also becoming apparent. By replacing labor intensive (and inaccurate) assays such as LDA with simple flow cytometry, the monitoring of peptide-specific CD8+ responses in patients may be revolutionized in terms of efficiency and accuracy. For example, tetramer staining of peripheral blood from HIV infected patients (or vaccinated subjects) would provide a rapid, simple and precise method of monitoring peptide-specific T cell responses and cellular immune status. Such experiments have begun to emerge in animal vaccine studies (450).

A major limitation, however, in MHC tetramer technology is the requirement for peptides which can bind stably to the refolded class I molecules. As discussed previously, many epitopes of clinical importance, in both viral and neoplastic diseases, do not display high affinity. Consider, for example, the HIV pol(346-354) peptide, an A2 restricted epitope which overlaps the catalytic site of the reverse transcriptase enzyme, and is highly conserved among all HIV strains (and all lentiviruses). As the presence of pol(346-354) specific CTLs has been correlated with non-progressor status (451), it has become an attractive vaccine candidate, and the ability to monitor pol(346-354) specific T cells could be of clinical importance. Unfortunately, tetramer staining of pol(346-354) specific CTLs by tetramers is not possible due to the low affinity of the peptide for A2 (S. Rowland-Jones, unpublished). Since MHC stability can be augmented through covalent linkage to β2m (Chapter 4), a pol(346-354)-hβ2m fusion protein could facilitate the production of a stable tetramer. Thus, epitope-linked β2m molecules could significantly expand the repertoire of MHC tetramers, to allow the visualization and quantitation of clinically relevant populations of CD8+ T cells.
E. Final Remarks

Over time, my thinking with respect to both MHC/peptide coupling approaches described in this thesis has undergone change and evolution. For signal sequence presentation, the shift has been from an intriguing vaccine possibility to a useful tool for probing ER events in class I presentation. Initial enthusiasm over the discovery of a “second” (signal sequence) pathway of antigen presentation (198) and the first report of TAP-independent presentation using a contrived signal sequence system (203), led to the belief that a distinct advantage could be endowed upon a CTL epitope by coupling its biosynthesis and ER translocation to a heavy chain via signal sequence incorporation. Of course, the subsequent demonstration that TAP was required for NP-HC presentation has tempered this enthusiasm, and led to a strategic reevaluation. Although the mechanism responsible for presentation in the NP-HC system is unresolved, the reliance upon the cytosol for processing negates the apparent advantages the coupling process was originally intended to achieve. But rather than be judged a failure, the NP-HC system has yielded an interesting result which is relevant for understanding the nature of ER processing in class I presentation, a nebulous area which currently suffers from conflicting data. Admittedly, the finding of TAP-dependent presentation in NP-HC is just one small result. Yet progress in science often occurs in small increments. The gratification must come from the knowledge that even minor contributions can impact upon diverse fields of study. It is thus noteworthy that signal sequence epitope presentation, apart from addressing basic issues in antigen presentation, has just recently been recognized as being of particular relevance for the understanding of NK cell recognition (452).

The peptide-β2m strategy has also undergone a change in thinking, although different in nature: from impossibility to possibility. When I began my work, it was almost heretical to believe that peptides could be tethered to class I molecules. The available structural and biochemical data had indicated the importance of free termini in peptide binding to class I, and it was presumed that little could be gained by losing such a stabilizing force. The 1995
publication of peptides tethered to a class I heavy chain (361) was therefore greeted with much interest. My enthusiasm only grew stronger when a cursory examination of an MHC crystal structure revealed that a peptide-β₂m linkage would be an even simpler matter to achieve than a peptide-heavy chain coupling. Thus, peptide-β₂m fusions grew from an impossibility to a realistic strategy. I believe the data reported in this thesis have significantly strengthened the argument that peptide-β₂m constructs are worthy of investigation. It is regrettable that there was insufficient time to explore the vast (especially in vivo) applications of these molecules.

Throughout this thesis, I have attempted to convey the dual purpose of my research: to explore basic mechanisms of class I presentation and assess potential strategies for vaccine or immunotherapy design. These two goals are by no means mutually exclusive, and in fact are closely interrelated. As our knowledge of class I presentation continues to expand, our ability to design effective treatments for human diseases becomes even further developed. We have entered an enlightened phase in immunology, one in which the extensive understanding of antigen presentation can be applied to the growing identification of viral and tumor-specific epitopes. This close relationship between basic and applied research offers tremendous promise for the development of new disease prevention and treatment strategies.


